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Kristína Szmicseková

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Non-neuronal cholinergic system

Système cholinergique non-neuronale

Neneuronálny cholínnergický system

Par Kristína Szmicseková, PharmD.

Thèse de doctorat de Neurosciences

Dirigée par Eric Krejci, PhD.,
par Anna Paul Hrabovská, Assoc. prof., PharmD., PhD.
et co-encadré par Peter Křenek, Assoc. prof., PharmD., PhD.

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Non-neuronal cholinergic system

Neneuronálny cholínergický systém

Système cholinergique non-neuronale

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Declaration on Word of Honour

I hereby declare that I prepared this dissertation thesis independently, under the leadership of my supervisors and using the cited literature.

.....

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ABSTRACT

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Introduction: Despite the lack of cholinergic innervation, vessels are highly reactive to the presence of acetylcholine (ACh). Moreover, this neurotransmitter is commonly used to assess the endothelial function of vessels. However, information about vascular cholinesterases (ChE), the enzymes that terminate ACh action, is sparse. **The main aim** of this dissertation thesis was to characterize vascular ChE and overall non-neuronal cholinergic system in the aorta under physiological and pathological conditions. **Methods:** Adult male Wistar rats and spontaneously hypertensive rats (SHR) were used in the project, fed either with regular or high-fat diet. Relative expression of studied enzymes and transporters were determined by RT-qPCR method. ChE activities in tissue extracts were measured by Ellman's assay, activity staining was performed by Tsuji's method and proteins localizations were done by dual immunohistochemistry. Molecular forms of ChE were studied by sucrose gradients. **Results and conclusion:** The enzymes and transporters necessary for ACh synthesis, storage, release, and degradation were detected in the rat aorta at mRNA and at protein levels. This confirms that aorta is a non-neuronal cholinergic tissue, capable to fully support the ACh life cycle. ChE are present mainly as PRiMA-anchored forms in each part of the aorta, while butyrylcholinesterase (BChE) is the dominant ChE, localized primarily in the smooth muscle. In SHR, lower levels of BChE were detected, accompanied by decreased relative expressions of carnitine acetyltransferase and organic cation transporters. This suggests lower cholinergic signaling in SHR aorta as compared to normotensive rats. In the pharmacological experiment, both inhibition of BChE and high-fat diet resulted in significant weight gain and increased serum TAG levels. Moreover, a high-fat diet induced mRNA expression of BChE. Our data suggest BChE involvement in lipid metabolism.

Keywords: non-neuronal cholinergic system, cholinesterases, aorta, spontaneously hypertensive rats, lipid metabolism

ABSTRAKT

SZMICSEKOVÁ, Kristína: Neneuronálny cholinergický systém. (Dizertačná práca). Univerzita Komenského v Bratislave. Farmaceutická fakulta, Katedra farmakológie a toxikológie. Školiteľ: Doc. PharmDr. Anna Paul Hrabovská, PhD. a Université de Paris, Centre Borelli, Cognac G. Školiteľ: Eric Krejci, PhD.

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Úvod: Napriek absencii cholinergickej inervácie, cievy sú vysoko reaktívne na prítomnosť acetylcholínu (ACh). Okrem toho, práve tento neurotransmitter sa využíva vo fyziologických experimentoch na sledovanie funkčnosti endotelu. Napriek tomu však chýbajú informácie o prítomnosti cholinesteráz (ChE), enzýmov, ktoré ukončujú jeho účinok v cievach. **Cieľom** tejto dizertačnej práce bola detailne charakterizovať tieto enzýmy a kompletne preštudovať prítomnosť komponentov neneuronálneho cholinergického systému v aorte normotenzných a spontánne hypertenzných potkanov (SHR). **Metódy:** V experimentoch boli použité 12-týždňové potkany rodu Wistar a SHR, ktoré boli kŕmené buď štandardnou alebo vysoko-tukovou stravou. Relatívna expresia študovaných enzýmov a transportérov bola stanovená metódou RT-qPCR. Aktivity ChE boli stanovené v tkanivových extraktach pomocou Ellmanovej metódy, aktivitné farbenie bolo prevedené podľa Tsujihio metódy. Na vizualizáciu a lokalizáciu bola využitá metóda dvojfarebnej imunohistochemie. Molekulové formy ChE boli charakterizované pomocou metódy sacharózového gradientu. **Výsledky a diskusia:** Všetky enzýmy a transportéry, ktoré sú potrebné na syntézu, uchovávanie, vylučovanie a degradáciu ACh boli potvrdené nielen na úrovni mRNA, ale aj na úrovni proteínov. Tieto zistenia potvrdzujú, že aorta patrí medzi neneuronálne cholinergické tkanivá. ChE sú prítomné primárne v PRiMA-kotvenej forme v každej časti aorty, pričom butyrylcholinesteráza (BChE) je dominantná a je prítomná hlavne v hladkom svalstve. V spontánne hypertenznom modeli bola detegovaná nižšia BChE aktivita a tiež nižšia relatívna expresia karnitínacetyltransferázy a organických kationových transportérov. Vo farmakologickom experimente, aj inhibícia BChE, aj vysoko-tuková strava spôsobila signifikantný prírastok hmotnosti a zvýšenie sérových hladín triacylglycerolov. Okrem toho, vysoko-tuková strava indukovala zvýšenie hladín mRNA pre BChE, čo naznačuje dôležitú úlohu tohto enzýmu nielen vo fyziológii ciev, ale aj v metabolizme lipidov.

Klíčové slova: neneuronální cholinergický systém, cholinesterázy, aorta, spontánně hypertenzní potkany, lipidový metabolismus

RÉSUMÉ

SZMICSEKOVÁ, Kristína: Système cholinergique non neuronal. (Thèse de doctorat). Université Comenius de Bratislava, Faculté de pharmacie, Département de pharmacologie et de toxicologie. Superviseur : Anna Paul Hrabovská, Prof. assoc., PharmD., PhD., Docteur en pharmacie et Université de Paris, Centre Borelli, Cognac G. Directeur de thèse : Eric Krejci, PhD.

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Introduction: Malgré l'absence d'innervation cholinergique, les vaisseaux sont très réactifs à la présence d'acétylcholine (ACh). De plus, ce neurotransmetteur est couramment utilisé pour évaluer la fonction endothéliale des vaisseaux. Cependant, les informations sur les cholinestérases vasculaires (ChE), les enzymes qui mettent fin à l'action de l'ACh, sont rares. **Le principal objectif** de cette thèse de doctorat était de caractériser les ChE vasculaires et l'ensemble du système cholinergique de l'aorte dans des conditions normales et pathologiques. **Méthodes:** Des rats Wistar mâles adultes et des rats spontanément hypertendus (SHR) nourris avec une alimentation régulière ou riche en graisses ont été utilisés dans le cadre du projet. L'expression relative des enzymes et des transporteurs étudiés a été déterminée par la méthode RT-qPCR. Les activités de ChE dans les extraits de tissus ont été mesurées par la méthode d'Ellman, la coloration de l'activité a été réalisée par la méthode de Tsuji et la localisation des protéines a été faite par double immunohistochimie. Les formes moléculaires de ChE ont été séparées par des gradients de saccharose. **Résultats et conclusion:** Toutes les enzymes et tous les transporteurs impliqués dans la synthèse, le stockage, la libération et la dégradation de l'ACh ont été détectés dans l'aorte du rat au niveau de l'ARNm et au niveau des protéines. Cela confirme que l'aorte est un tissu cholinergique non neuronal, capable de soutenir pleinement le cycle de vie des ACh. Les ChE sont présents principalement sous forme de formes ancrées dans la PRiMA dans chaque partie de l'aorte, tandis que la butyrylcholinestérase (BChE) est l'enzyme dominante, localisée principalement sur le muscle lisse. Dans les rat SHR, des niveaux plus faibles de BChE ont été détectés, accompagnés d'une diminution des expressions relatives de la carnitine acétyltransférase et des transporteurs de cations organiques. Cela suggère une signalisation cholinergique plus faible dans l'aorte de la SHR par rapport aux rats normotendus. Dans l'expérience pharmacologique, l'inhibition du BChE et l'alimentation riche en graisses ont

toutes deux entraîné une prise de poids significative et une augmentation des taux sériques de TAG. De plus, un régime riche en graisses a induit une augmentation des niveaux d'ARNm du BChE, indiquant son implication dans le métabolisme des lipides.

Mots clés: système cholinergique non neuronal, cholinestérases, aorte, rats spontanément hypertendus, métabolisme des lipides

PREFACE

The primary concept that cholinergic transmission is strictly linked to the nervous system has been reconsidered after the introduction of so-called non-neuronal cholinergic system. Till now, its active participation in numerous physiological and pathological processes have been described, indicating the possibility of development of new and more effective therapeutic strategies.

The vast majority of vessels are not innervated by cholinergic nerves, however, are highly sensitive to acetylcholine, which is considered to be the main neurotransmitter of the cholinergic system. Moreover, this substance is frequently used in physiological experiments to assess endothelial function. Despite the fact, that the action of acetylcholine is terminated by two cholinesterases, information about their presence and activity in vessels is still lacking. Here, we aim to examine these enzymes and characterize them in detail in physiological conditions, and also in pathological conditions, using the aorta as a model vessel. Additionally, the hypothesis that the aorta is one of the non-neuronal cholinergic tissues, capable to synthesize, store, and release acetylcholine is also tested.

We truly believe that our results will make a noteworthy contribution to our understanding of the function of the cholinesterases and also of the non-neuronal cholinergic system in vessel physiology and pathology.

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ABBREVIATIONS

Ab	antibody
ACh	acetylcholine
AChE	acetylcholinesterase
AD	Alzheimer's disease
ATC	acetylthiocholine
BChE	butyrylcholinesterase
BTC	butyrylthiocholine
ChAT	choline acetyltransferase
ChE	cholinesterases
ChT-1	choline transporter
ColQ	collagen Q
CrAT	carnitine acetyltransferase
CTL	choline transporter like proteins
CVS	cardiovascular system
DAG	diacylglycerol
DMPTB	2, 3-dimercapto-1-propanol tributyrat
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
eNOS	endothelial nitric oxide synthase
FFA	free fatty acid
HDL	high-density lipoprotein
HF	high-fat
G ₁ ^a	amphiphilic monomers

G ₄ ^a	amphiphilic AChE/BChE tetramers (PRiMA anchored)
IDL	intermediate-density lipoprotein
iso-OMPA	tetraisopropyl pyrophosphoramidate
LDL	low-density lipoprotein
MAG	monoacylglycerol
mAChR	muscarinic acetylcholine receptor
MLCK	myosin light-chain kinase
nAChR	nicotinic acetylcholine receptor
NChS	neuronal cholinergic system
NMJ	neuromuscular junction
NN	non-neuronal
NN-ACh	non-neuronal acetylcholine
NNChS	non-neuronal cholinergic system
NO	nitric oxide
OCT	organic cation transporter
OP	organophosphates
PBS	phosphate buffered saline
PRiMA	proline-rich membrane anchor
SD	standard diet
SHR	spontaneously hypertensive rat
SLC	solute carrier family
TAG	triacylglycerol
VACht	vesicular acetylcholine transporter
VLDL	very low-density lipoprotein

INTRODUCTION

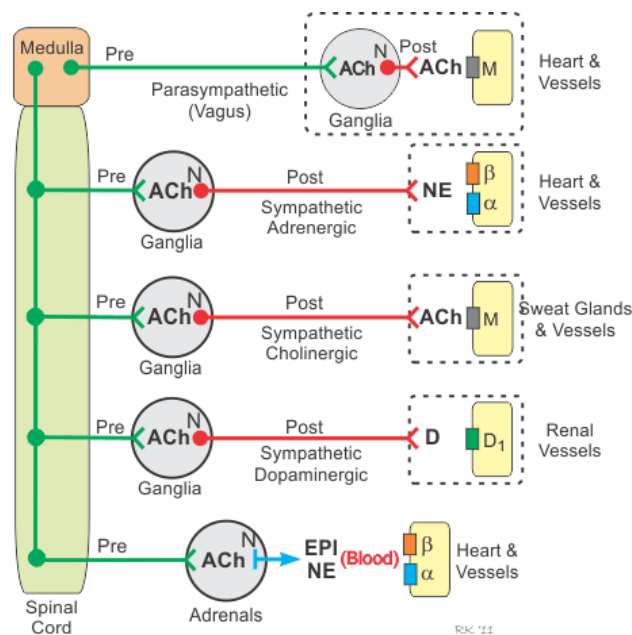
The fact that cholinergic system is not related solely to cholinergic innervation has opened a new chapter in cholinergic science. There is a lot of evidence, that the non-neuronal cholinergic system can due to its auto-, para-, and endocrine mechanisms also have an influence on physiological and pathological processes in the human body. Many recent studies have focused on the connection between dysregulation of this system and the occurrence of various cardiovascular diseases. The most common accompanying symptom of cardiovascular comorbidities is endothelial dysfunction, which can be characterized as impaired endothelium-dependent vasodilation in response to acetylcholine. There is a lack of information about the origin of acetylcholine in vessels and also about the enzymes, which terminate its action, namely cholinesterases. Furthermore, several studies have described a significant correlation between the levels of butyrylcholinesterase and plasma lipids. Additionally, atherosclerosis, the main inflammatory disease of arteries is rising from an imbalance in lipid metabolism. So, the possible participation of cholinesterases on vessel pathologies development is a very attractive topic. The complex study of cholinesterases activity, localization, and molecular forms would give a greater understanding of how these enzymes participate in vascular physiology and pathology.

LITERATURE REVIEW

1.1 ACETYLCHOLINE AND THE CHOLINERGIC SYSTEM

Cholinergic system can be defined as a network of neurons that employs acetylcholine (ACh) as a signalling molecule (Figure 1) (Ritter M. et al., 2015; Sarter and Parikh, 2005). The crucial role of this system, both in central and peripheral nervous system has been well documented. Alterations of the cholinergic neurotransmission may have pathological, pharmacological and toxicological significance (Deiana et al., 2011; Ritter M. et al., 2015).

ACh is a quaternary amine with low molecular weight (Todman, 2008). It was first proposed in 1900 that this molecule has pharmacological action, when Reid Hunt discovered its blood pressure reducing effect. Sir Henry Dale and Otto Loewi were awarded the Nobel prize in 1936 for the discovery of ACh and the establishment of chemical synaptic transmission (Picciotto et al., 2012; Taylor and Brown, 1999; Todman, 2008). ACh acts as a key signalling molecule not only at the periphery (at all autonomic preganglionic nerve terminals, all postganglionic parasympathetic junctions, and few postganglionic sympathetic junctions) but at all cholinergic synapses in central nervous system. Moreover, motoneurons can release ACh at the neuromuscular junctions (Castro et al., 2009).



CNS = central nervous system; Pre = preganglionic; Post = postganglionic;
 ACh = acetylcholine; N = nicotinic receptor; NE = norepinephrine; EPI = epinephrine;
 D = dopamine; M = muscarinic receptor; β = β -adrenoceptor; α = α -adrenoceptor;
 D₁ = dopaminergic receptor

Figure 1: Cholinergic synapses in the peripheral nervous system (Ritter M. et al., 2015)

In the last century, the existence of non-neuronal ACh (NN-ACh), produced by other cells than neurons, was described (Wessler and Kirkpatrick, 2008). Since then, numerous non-neuronal (NN) cells have been described as a source of this neurotransmitter. Nowadays, the function of the NN-ACh within both neuronal and non-neuronal transmission are in the spotlight, because it appears to be involved both, in physiological and pathological processes of body (Beckmann and Lips, 2013; Grando et al., 2015).

ACh is synthesized from acetyl-coenzyme A and choline (Figure 2) (Prado et al., 2002). Acetyl-coenzyme A is a pivotal molecule in biological systems (Taylor and Brown, 1999), which participates on several biochemical reaction in metabolism of proteins fats and carbohydrates (e.g. in oxidation of free fatty acids, on post-translational modifications of protein synthesis, etc. (Pietrocola et al., 2015)). The sources of acetyl-coenzyme A for ACh - synthesis are mainly the products of glycolysis, for example in mammalian brain, liver, kidney or smooth muscle glucose and pyruvate (Pietrocola et al., 2015). The other cofactor for ACh-synthesis is choline (Blusztajn et al., 1987), which can be endogenously produced by liver, but it is not enough to meet metabolic needs. Therefore, the intake of phosphatidylcholine and sphingomyelin in diet is required (Taylor and Brown, 1999). Moreover, choline is recycled after ACh breakdown. This choline is the main source of choline for ACh synthesis, and its reuptake is the rate-limiting step in ACh synthesis (Ribeiro et al., 2006).

Neuronal ACh is synthesized almost exclusively by the enzyme choline acetyltransferase (ChAT) (Oda, 1999). ACh is then transported by vesicular ACh transporter (VAChT) into vesicles (Castro et al., 2009), where it is stored until nerve impulse arrives. As a response to the increased intracellular Ca^{2+} levels, ACh is released from vesicles into the synaptic cleft (Picciotto et al., 2012; Sarter and Parikh, 2005). In NN-cells, ChAT action can be substituted by carnitine acetyltransferase (CrAT) and VAChT may not be present (Beckmann and Lips, 2013). Instead, ACh may be found directly in the cytoplasm and is released *via* non-specific transporters of the following groups: organic cation transporters (OCT or OCTN), and choline transporter-like protein (CTL) or mediato-phores (Beckmann and Lips, 2013; Grando et al., 2015; Wessler and Kirkpatrick, 2008). In NNChS, it has not been elucidated yet, how the release of ACh is controlled and which other molecules are involved in this process. ACh released into the extracellular environment acts *via* muscarinic ACh receptors (mAChR, (Caulfield, 1993)) or nicotinic ACh receptors (nAChR, (Dani,

2015)) in both the NChS and NNChS. Within NChS, ACh is a short-acting molecule, while within NNChS its action seems to be continual (Wessler et al., 2003). The degradation of ACh is ensured by cholinesterases (ChE) (Chatonnet and Lockridge, 1989). One product of the hydrolysis is choline, which is taken up into nerve cells *via* the high-affinity choline transporter (ChT-1) (Ribeiro et al., 2006). In NN-cells, the choline-reuptake can be ensured by non-specific transporters from the OCT, OCTN, or CTL groups (Beckmann and Lips, 2013).

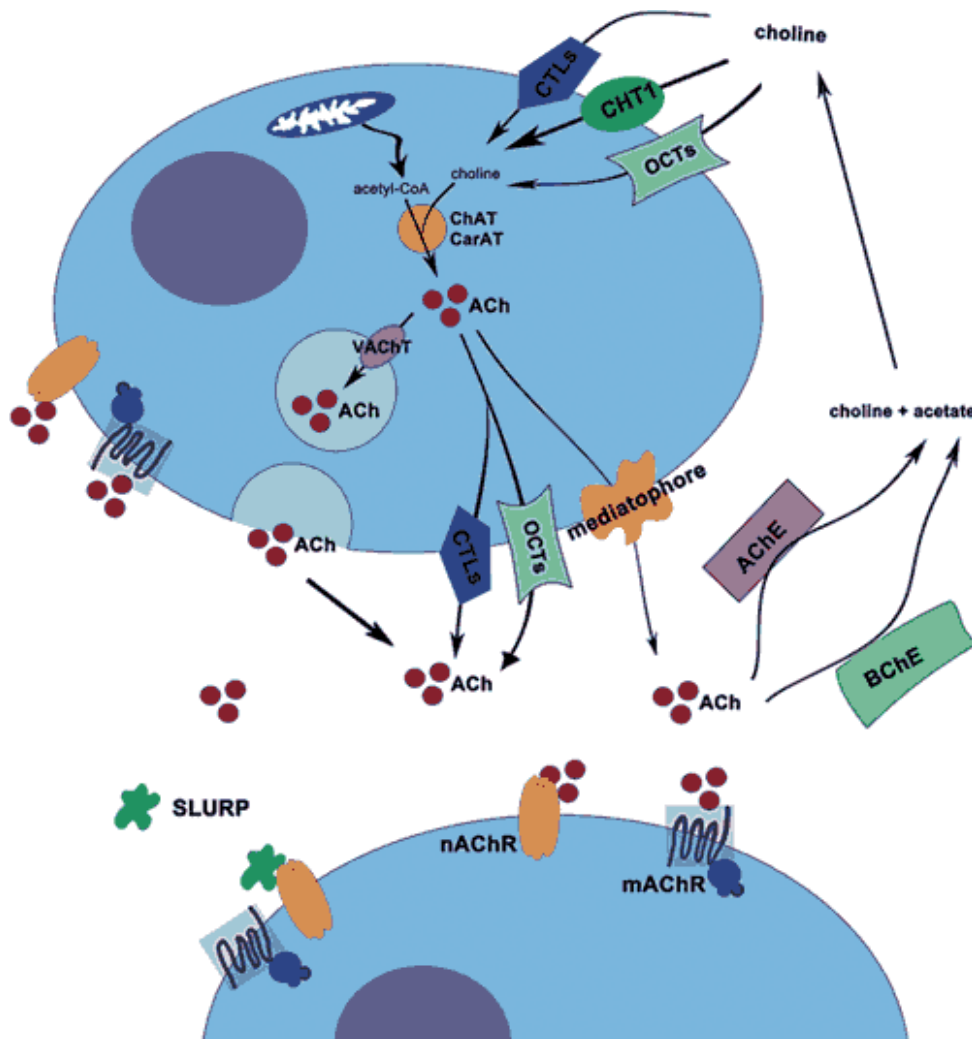


Figure 2: Schematic figure of all components involved in neuronal and NN-ACh life cycle (Beckmann and Lips, 2013)

1.1.1 Synthesis, transport, and release of acetylcholine

Neuronal ACh is synthesized in the cell while its action is on receptors localized on the cell surface (Taylor and Brown, 1999). Therefore, multiple molecules, including catalytic

enzymes and transporters need to participate in the ACh life cycle. Nevertheless, main differences between NChS and NNChS were described for the synthesis, transport and release of ACh, which is based on different need of ACh in response to the stimuli and character of the signaling (Beckmann and Lips, 2013; Wessler et al., 2003). This reflects in the specific protein makeup – while some of the proteins are preserved in both cholinergic systems (ChAT, VAcHT, ChT-1) and others are specific for the NNChS (OCT, OCTN, CTL, mediato-phores).

1.1.1.1 Cholinacetyltransferase

ChAT, the primary enzyme responsible for ACh synthesis in the cytoplasm of the nerve endings (Oda, 1999), is a single-stranded globular protein synthesized in the perikaryon of cholinergic neurons transported by both rapid and slow axoplasmic flows, while the slow flow rate is more frequent (Misawa et al., 1997; Oda, 1999). In nerve terminals two types of ChAT can be found: the soluble and the non-ionically membrane-bound form (Oda, 1999). In humans, it is encoded by a single gene found on chromosome 10 and is encoded by 4 exons (Wessler et al., 1998). As a result of several alternative splicings of non-coding exons, several types of mRNA transcripts have been described (Bejanin et al., 1994). In humans, we distinguish M, S, R, N1 and N2 isoforms (Misawa et al., 1997; Oda, 1999). It consists of a binding and catalytic domain with a tunnel in the middle, where the active site is localized. Each domain consists of a six-stranded β - sheet which is surrounded by several α -helices (Govindasamy et al., 2004). The genetic organization of this enzyme is very specific, as the first intron serves as the only exon of VAcHT, so the expression of these two proteins is usually very closely related (Oda, 1999). The highest activities have been found in brain, where its low activity correlates with Alzheimer's disease (AD) (Baskin et al., 1999). Abnormalities of this enzyme are associated also with diseases such as schizophrenia, congenital myasthenic syndrome or amyotrophic lateral sclerosis (Cai et al., 2004).

1.1.1.2 Carnitine-O-acetyltransferase

CrAT is an enzyme from the group of carnitine acyltransferases (Hunt et al., 2014; White and Wu, 1973) and is a key enzyme in the metabolism of higher monocarboxylic acids as this enzyme is responsible for fatty acids transportation for β -oxidation (Hunt et al., 2014; McGarry and Brown, 1997). CrAT is a soluble protein and is present in the peroxisomes (Jogl and Tong, 2003), the endoplasmic reticulum and the mitochondrial matrix, all of which

are encoded in humans by one gene localized on chromosome 9q34 (Corti et al., 1994). Mitochondrial CrAT, a longer variant, is 626 amino acids long and consists of 14 exons (Chung et al., 1991). It is expressed in the human liver and high levels of its mRNA have been detected also in the skeletal muscle (Chung et al., 1991). The second variant is the peroxisome CrAT and contains 605 amino acids (Robic et al., 2009). This enzyme catalyzes the reversible transfer of acyl groups between acyl coenzyme-A and carnitine to form acylcarnitine (Hynes et al., 2011; Ramsay et al., 2001). By this process, CrAT regulates the level of coenzyme A, which serves as a carrier of activated acetyl groups in the oxidation processes of energy metabolism and substrates in the synthesis of fatty acids and lipids (Hynes et al., 2011). Moreover, it serves as an alternative enzyme in the process of ACh synthesis (Beckmann and Lips, 2013). Till now, CrAT activity has been confirmed in several tissues with no cholinergic innervation, and it was suggested that this enzyme is responsible for the ACh synthesis (Beckmann and Lips, 2013; Wessler and Kirkpatrick, 2008). Activity of this enzyme is correlated with metabolic disorders, as diabetes mellitus or obesity (Seiler et al., 2014). Moreover, CrAT inhibition seems to be interesting pharmacological intervention for treatment of refractory angina (Jaudzems et al., 2009; Sharma and Black, 2009).

1.1.1.3 Vesicular acetylcholine transporter

ACh is a cationic substance in nature, it cannot penetrate the phospholipid membrane into vesicles by passive transport (Taylor and Brown, 1999). VACHT (*SLC18A3- Solute Carrier Family 18 Member A3*)(Prado et al., 2002), which is a co-transporter and antiporter (integrated membrane protein), ensures the entry of ACh into the vesicle in cholinergic tissues (Nguyen et al., 1998). This process, similar to the accumulation processes of other biogenic amine neurotransmitters, is connected with proton efflux (Nguyen et al., 1998). VACHT is a polypeptide of approximately 500 amino acids that consists of 12 transmembrane domains with a hydrophilic N- and C-terminus with intracellular localization and shows homology to the vesicular monoamine transporter 1 and vesicular monoamine transporter 2 from the “major facilitator superfamily” (Castro et al., 2009; Prado et al., 2002). As it was mentioned before, VACHT has a very unique genetic structure, as the gene encoding VACHT is localized in the first intron of the ChAT gene and together with ChAT forms the so-called cholinergic locus (Erickson et al., 1994; Prado et al., 2002). It is coded by a single-exon gene, and in humans is localized on chromosome 10q11 (Bejanin et al.,

1994; Erickson et al., 1994). Total absence of VACHT is not compatible with life, VACHT knockout mice die immediately after birth (Prado et al., 2006). Slightly reduced activity of this enzyme results in impaired cognitive abilities and muscle weakness (Castro et al., 2009; Prado et al., 2006). Significant inhibition of VACHT activity causes severe symptoms, e.g., myasthenic syndrome (Castro et al., 2009; Prado et al., 2006). Each vesicle contains up to 10.000 of ACh molecules, adenosine-triphosphate, and a vasoactive intestinal polypeptide, which serve also as a co-transporter (Westfall, 2009). ACh is released as a response to depolarization, which results in Ca^{2+} entry into the nerve ending and allows the vesicle membrane to fuse with the plasma membrane (Nguyen et al., 1998). Release of ACh by vesicle exocytosis is the only mechanism of neuronal ACh release into the extracellular environment (Fisher and Wonnacott, 2012; Nguyen et al., 1998). Nevertheless, ACh release independent of VACHT was demonstrated as evolutionarily older (Castro et al., 2009). This means that ACh is not stored into vesicles and therefore no VACHT is required in NN-cells. However, VACHT-mediated vesicular storage and release of ACh has been described also in some NN-cells, such as pancreatic cells, endothelial cells and cardiomyocytes (Beckmann and Lips, 2013; Haberberger et al., 2000; Rodriguez-Diaz et al., 2011; Roy et al., 2016).

1.1.1.4 Choline transporter-1

The high affinity choline transporter (ChT-1, *SLC5A7*) is localized on the presynaptic membrane (Choudhary et al., 2017; Ribeiro et al., 2006). Its encoding gene is composed of 9 exons and is localized in the position 2q12 (English et al., 2009; Ribeiro et al., 2006). Human ChT-1 is coded by 580 amino acids and shows 93% homology with the rat cDNA (Okuda and Haga, 2003; Okuda et al., 2000). It is a Cl^- and Na^+ dependent cotransporter, which use the electrochemical gradient of Na^+ for choline transport (Okuda and Haga, 2003; Okuda et al., 2000). ChT-1 does not show homology to other neurotransmitter transporters, but rather to sodium-dependent glucose transporters and thus belongs to the family of sodium-glucose linked transporters-1 (Okuda et al., 2000). There is no evidence about the existence of other transporters mediating choline reuptake in neurons. The choline-reuptake is the rate-limiting step in ACh synthesis (Brandon et al., 2004). Moreover, the specific ChT-1 inhibitor, hemicholin-3 causes a dramatic decrease in activity cholinergic functions confirming that ChT-1 activity and ACh synthesis is closely related to each other (Brandon et al., 2004; Inazu, 2014; Ribeiro et al., 2006). For human ChT-1, the so-called “single nucleotide polymorphism” has been described, meaning one amino acid is replaced in

sequence by another resulting in reduced choline uptake at the nerve ending with no change of ChT-1 expression on the cell surface and consequent negative effect on ACh synthesis (Okuda and Haga, 2003). This transporter is essential for surviving, as mutant mice with gene deletion for this receptor which are able to synthesize and release just a very small quantity of ACh, which is not enough for surviving. These mutant mice are immobile, have breathing complications and die within one hour after birth (Ferguson and Blakely, 2004; Inazu, 2014; Ribeiro et al., 2006).

1.1.1.5 Organic cation transporters

In several NN-cholinergic cells, ACh is not stored in vesicles but is directly released by transporters (Wessler and Kirkpatrick, 2008). The most likely candidates for mediating the release of NN-ACh and choline reuptake for ACh synthesis within NN-cells are OCT (Beckmann and Lips, 2013; Wessler et al., 2001). OCT are Na⁺ and Cl⁻ independent, by hemocholine-3 inhibitable, corticosterone sensitive proteins involved in the absorption, distribution, and elimination of endogenous compounds, as well as exogenous xenobiotics that have a positive charge at physiological pH (Inazu, 2014; Koepsell, 2015; Koepsell and Endou, 2004; Koepsell et al., 2007). OCTs can carry various cations across the cell membrane in both directions and are also characterized by a low affinity for choline, thanks to which they are able to mediate its reuptake (Koepsell, 2015; Koepsell and Endou, 2004).

OCT belongs to the solute carrier family 22 (*SLC22*)(Gründemann et al., 1994; Koepsell et al., 2007), which contains six subtypes, namely OCT-1 (*SLC22A1*), OCT-2 (*SLC22A2*), OCT-3 (*SLC22A3*), OCTN-1 (*SLC22A4*), OCTN-2 (*SLC22A5*) and OCTN-3 (*SLC22A9*). Each OCT possesses different substrate specificity and interspecific animal differences have also been described. The genes encoding these transporters in humans are localized in a cluster on chromosome 6.q26-7, each contains 11 exons and 10 introns (Jonker and Schinkel, 2004; Koepsell and Endou, 2004; Koepsell et al., 2007). Like most members of the *SLC22* family, organic cationic and carnitine transporters have a putative membrane topology that contains 12 α -helical transmembrane domains an intracellular N-terminus, a large glycosylated extracellular loop between transmembrane domain 1 and 2, a large intracellular loop with a phosphorylation site between transmembrane domain 6 and 7 and intracellular C-terminus (Figure 3)(Jonker and Schinkel, 2004; Koepsell, 2015; Koepsell et al., 2007).

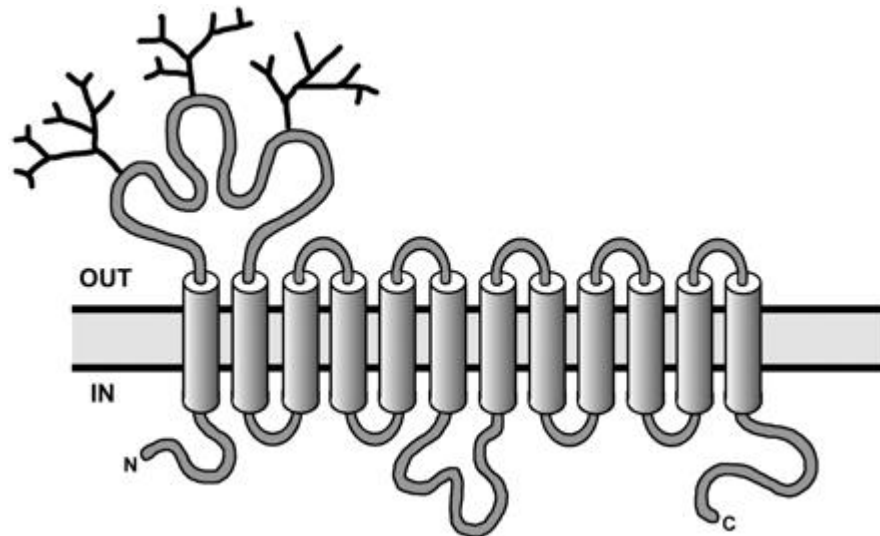


Figure 3: Predicted secondary structure of OCT-1 (Jonker and Schinkel, 2004)

OCT-1 is most ubiquitous in hepatocytes but is also expressed in some neurons, stomach, intestines, spleen, kidney, heart, skeletal muscle, tumor cells (Jonker and Schinkel, 2004; Koepsell et al., 2007). Like OCT-1, OCT-2 is also present in epithelial cells and neurons. It can be found in the largest amount in the kidneys, but also in other organs such as the small intestine, lungs, skin, and brain (Jonker and Schinkel, 2004; Koepsell and Endou, 2004; Koepsell et al., 2007). Substrates of OCT, in particular OCT-1, OCT-2 and OCT-3, such as e.g. amiloride, noradrenaline, verapamil, or cimetidine inhibit the release of ACh (Jonker and Schinkel, 2004). OCT-3 is widely expressed in humans in skeletal muscle, liver, placenta, and heart, as well as in the brain or some lines of cancer cells (Jonker and Schinkel, 2004; Koepsell et al., 2007). One of the most recently described transporters responsible for ACh release is OCTN-1, which is able to translocate ACh in both directions and is expressed in epithelial and muscle cells of many tissues. It is most expressed in the kidney, skeletal muscle, trachea, and bone marrow (Pochini, 2016; Pochini et al., 2012a, 2013, 2019). OCTN-2 is carnitine and cationic transporter that has a relatively ubiquitous distribution, found in epithelial, muscle cells, glia, macrophages, lymphocytes, and sperm. It is most present in the liver, kidney, skeletal muscle, heart, and placenta. The presence of OCTN-2 has been detected in some cancer cell lines (Pochini, 2016; Pochini et al., 2012a, 2013, 2019). OCTN-3 was cloned from mice and rats, where it is found mainly in the testis, and was also detected in other organs and neurons. The OCTN-3 gene has been described first in laboratory mice, but later the gene in mammalian was also identified (Pochini, 2016; Pochini et al., 2019).

An example of NN-cholinergic tissue, which uses OCT to release ACh is the placenta. As the placenta is not innervated by cholinergic neurons, it is a suitable model for studying NNChS (Wessler et al., 2001). OCT are widely expressed in this organ. OCT also plays a role in the respiratory system, where airway epithelial cells use OCT-1 and OCT-2 transporters to release ACh (Kummer et al., 2008). Application of corticosterone (an inhibitor of OCT-1,2,3) reduces the concentration of released ACh (Haberberger et al., 2000; Lips et al., 2007).

1.1.1.6 Choline transporter like proteins

CTL proteins are Na⁺-independent transporters with medium affinity to choline (Song et al., 2013). The CTL family is characterized by 10 transmembrane domains, 11 highly conserved cysteines and no signal peptide (O'Regan et al., 2000). We distinguish 5 different proteins belonging to this family, CTL-1-5. (*SLC44A1-5*) (Song et al., 2013; Traiffort et al., 2005). CTLs have been extensively studied on various types of tumor cells since there is a strong correlation between abnormal choline metabolism and cancer cells proliferation. Cell line NG108-15 (neuroblastoma glial cells) despite the fact, that does not express VAChT or ChT-1 is able to scavenge choline, probably *via* CTL-1. Suppression of CTL-1 by siRNA caused impaired growth of these cells, so it can be concluded that choline reuptake by CTL-1 serves mainly to the membrane phospholipids synthesis (Machová et al., 2009; Song et al., 2013). CTL-1 is the predominant transporter of choline reuptake also in the airways (Kummer et al., 2008) and keratinocytes (Song et al., 2013). Reduced choline reuptake into the cells was observed by suppression of CTL-1, CTL-2 and CTL-5 transporters, but it has no effect on ACh synthesis. Inhibition of CTL-4 in tumor cells led to a significant decrease of ACh concentrations, increasing CTL-4 expression caused increased ACh secretion, indicating that in tumor cells, CTL-4 is not only mediating choline reuptake, but is involved also into the synthesis and secretion of ACh (Song et al., 2013).

1.1.1.7 Mediatophores

Mediatophores have been proposed as other proteins mediating ACh release as they possess the capability of ACh translocation (Falk-Vairant et al., 1996; Israël and Dunant, 1998, 1999). They are homooligomeric membrane proteolipids of 220 kDa, that show homogeneity with the proteolipid subunit *c* of vacuolar H⁺-ATPase (Fujii et al., 2012). They have the ability to translocate ACh in response to increased intracellular calcium levels (Israël and Dunant, 1998, 1999). The involvement of mediatophores in the transport of NN-

ACh has been described in the plasma membrane of human microvascular endothelial cells (Dunant and Gisiger, 2017) and in the cytoplasm and plasma membrane of human T-lymphocytes which do not express OCT (Fujii et al., 2012). The reduction in mediatoaphore expression by siRNA resulted in a significant reduction in the concentration of leached ACh. This result confirms that mediatoaphores are involved in ACh release in NN-cholinergic tissues (Fujii et al., 2012).

1.1.2 Acetylcholine activates different receptors

As it was mentioned above, ACh is essential for correct function of cholinergic system in human body. This molecule acts on 2 types of cholinergic receptors (Figure 4), nicotinic (ionotropic) cholinergic receptors (nAChR) and muscarinic (metabotropic) cholinergic receptors (mAChR) (Caulfield, 1993; Dani, 2015; Eglen, 2006; Haga, 2013; Kruse et al., 2012; Saternos et al., 2017).

Cholinergic receptors can be found also in NNChS. Their expression, function and significance in various physiological and pathological conditions is still monitored (Beckmann and Lips, 2013; Wessler and Kirkpatrick, 2008, 2001; Wessler et al., 2003).

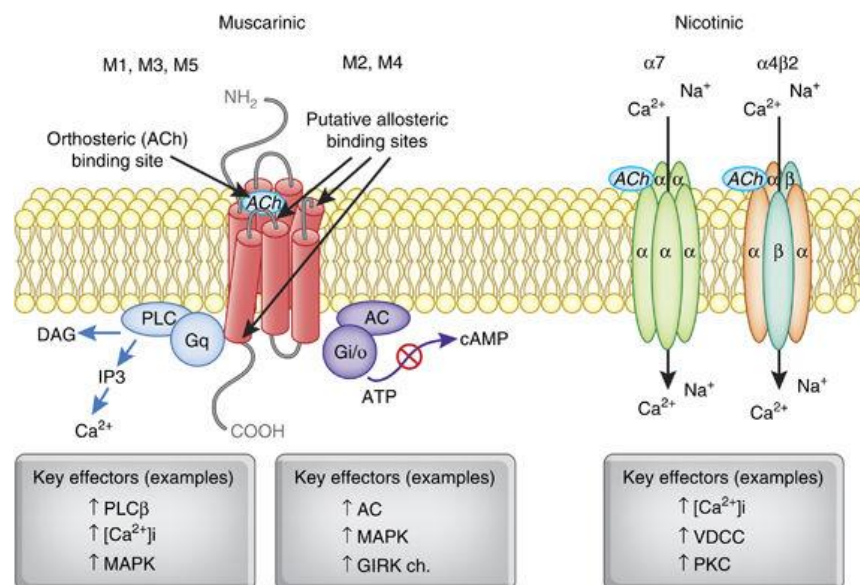


Figure 4: Schematic structure and signaling pathways of mAChRs and nAChRs

(Jones et al., 2012)

1.1.2.1 Muscarinic acetylcholine receptors

We distinguish five mAChR subtypes, which differ in their molecular structure, ligand specificity and location (Kruse et al., 2014; Wess et al., 2007). Their study was limited by the lack of highly selective agonists and antagonists caused by their high sequence

homology within the ACh-binding site, but now, thanks to the genetic approaches (total deletion or localized deletion of the gene) a huge progress has been made. All mAChR are G-protein-coupled receptors but mediate various cellular responses (Felder, 1995). m1AChR, m3AChR and m5AChR receptors are coupled with pertussis-insensitive $G_{q/11}$ protein (Caulfield, 1993; Caulfield and Birdsall, 1998; Felder, 1995). The activation of these receptors causes the stimulation of phospholipase C, which hydrolyses membrane phosphatidylinositol-4,5-diphosphate. The first product, inositol-triphosphate immediately induces release of intracellular Ca^{2+} (from endoplasmic reticulum) and induces the contraction of smooth muscle and secretion. The second product, diacylglycerol activates phosphokinase C, which is responsible for phosphorylation or dephosphorylation of numerous proteins, while the physiological response depends on the type of protein (Caulfield, 1993; Caulfield and Birdsall, 1998; Felder, 1995). The second enzyme, which can be stimulated by activation of m1AChR, m3AChR and m5AChR receptors is phospholipase A2 and it results in arachidonic acid release from synaptic membrane phospholipid (Basselin et al., 2009). Activation of m2AChR and m4AChR, which are coupled with $G_{i/o}$ protein cause the inhibition of adenylyl cyclase. This leads to the decrease in cyclic adenosine-monophosphate concentration, activation of K^+ and inhibition of Ca^{2+} channels. The consequence of these mechanism is hyperpolarization and inhibition of excitable membranes (Caulfield, 1993; Caulfield and Birdsall, 1998; Felder, 1995; Jones et al., 2012; Spindel, 2012).

m1AChR (neuronal) are widely expressed in the central nervous system and peripheral neurons (Kruse et al., 2014). They are partially responsible for the regulation of both, cognitive function and locomotor activity (Kruse et al., 2014, 2014), so selective activation of these receptors may have therapeutic significance in the treatment of schizophrenia or AD (Anagnostaras et al., 2003; Carruthers et al., 2015; Cropley et al., 2015; Jiang et al., 2014).

m2AChR (cardiac) are localized mainly in the heart and their activity is regulated by *n. vagus* (Brodde and Michel, 1999; Dhein et al., 2001). ACh modifies not only the contractility, excitability, and the frequency of the cardiac musculature, but decreases the rate of neurotransmission and the auto-excitation of sinoatrial-nodes (Brodde and Michel, 1999; Kruse et al., 2014). These receptors can be found also in various areas of the brain,

where they modulate the release of other neurotransmitters (Brodde and Michel, 1999; Gomeza et al., 1999; Levey, 1993).

m3AChR are present in smooth muscles, e.g. in lungs, endocrine and exocrine glands, ileus, bladder, where they are responsible for the contraction of smooth muscle (Kruse et al., 2012). Some authors believe that these receptors are involved in the regulation of food intake, learning and also in the pathophysiology of type 2 diabetes mellitus (Caulfield, 1993; Caulfield and Birdsall, 1998; Gautam et al., 2006; Kruse et al., 2012).

m4AChR can be found mostly in striatal areas of the brain (Kruse et al., 2014). It seems, that they are involved in regulation of locomotor activity and social behavior (Koshimizu et al., 2012). The selective targeting of these receptors may be beneficial for treatment of the motoric symptoms of Parkinson's disease (Dencker et al., 2012). In addition, the latest research shows, that pharmacological stimulation of these and m1AChR is prosperous for the treatment of AD (Foster et al., 2014).

In contrary to other types of mAChR, m5AChR receptors have been described very poorly. They probably collaborate with m1AChR and m4AChR receptors on controlling of cognitive functions (Dencker et al., 2012; Kruse et al., 2014). Their key role in the development of drug addiction, e.g. cocaine or morphine addiction is confirmed (Gunter et al., 2018; Thomsen et al., 2005).

1.1.2.2 Nicotinic acetylcholine receptors

nAChRs represent a well-characterized family of allosteric transmembrane proteins, which are widely expressed in almost all tissues. Each nAChR are composed of 5 subunits, which are arranged in a barrel-like configuration in the cell membrane (Albuquerque et al., 2009). Till now, 16 isoforms of subunits have been described: of $\alpha 1$ - $\alpha 10$ ($\alpha 8$ has not been found in mammals), $\beta 1$ - $\beta 4$, γ , δ , ϵ subunits (Albuquerque et al., 2009; Dani, 2015; Hendrickson et al., 2013). We distinguish homopentameric and heteropentameric forms (Figure 5), which differ in their ion permeability, open time, ligand affinity, kinetics, location, pharmacological properties and their sensitivity to α -bungarotoxin (Albuquerque et al., 2009; Dani, 2015; Gwilt et al., 2007; Hendrickson et al., 2013; Kalamida et al., 2007; Young et al., 2003).

These receptors belong to the *cys*-loop superfamily of ligand gated ion channels (Chang et al., 2009; Changeux and Paas, 2009). The binding of endogenous (ACh) or

exogenous agonists (e.g. nicotine) to the binding site of the receptors leads to the opening of ion channel, which is permeable for Na^+ , K^+ , Ca^{2+} ions (Albuquerque et al., 2009; Changeux and Paas, 2009).

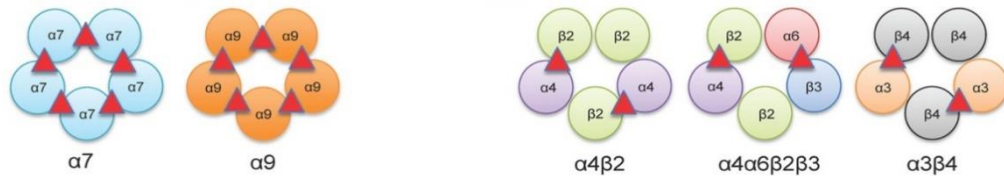


Figure 5: Models of homomeric and heteromeric forms of nAChRs
(Hendrickson et al., 2013)

nAChRs are broadly classified in three classes based on their primary sites of their expression:

- *The muscle type* is made up from $\alpha 1_{(2)}$, $\beta 1$, δ , and ϵ subunits (in the fetus, a γ subunit is replaced by the ϵ subunit). This type can be found at the periphery, more specifically at neuromuscular junctions, where it is responsible for muscle contraction and muscle tone (Albuquerque et al., 2009; Engel et al., 2015). Impaired neuromuscular transmission is characteristic of diseases like Myasthenia gravis or congenital myasthenic syndromes (Engel et al., 2015; Wu et al., 2015).
- *The neuronal-type* may consist as various combinations of α subunits ($\alpha 2$ - $\alpha 10$) and β subunits ($\beta 1$ - $\beta 4$) (Albuquerque et al., 2009). Their high diversity has been described primarily in brain, indicating their different roles in brain functions, especially cognitive functions (Hendrickson et al., 2013; Kalamida et al., 2007). Deficit or impairment of these receptors was observed in AD, Parkinson's disease or schizophrenia confirming their critical role in memory function (Dani, 2015; Hendrickson et al., 2013). The most abundant subtypes in CNS are $\alpha 4$ and $\beta 2$ subunits, after $\alpha 6$ and $\beta 2$, while from the homopentameric form, $\alpha 7$. Receptors containing $\alpha 4\beta 2$ subtype are linked to abuse and addiction (Sharma and Vijayaraghavan, 2008), while subtype $\alpha 6\beta 2$ in sensory system (Quik and Wonnacott, 2011). Moreover, the expression of $\alpha 6$ subunit in neurons indicates their nociceptor character (Hone and McIntosh, 2018). Homopentameric $\alpha 7$ receptors are linked to several pathologies, as pain, inflammation, or immune response (Hendrickson et al., 2013; Kalamida et al., 2007). However, several principal questions about the exact mechanism of their participation on pathophysiology of these diseases remain unanswered (Dani, 2015; Hendrickson et al., 2013; Kalamida et al., 2007).

- *The ganglionic type* mediates the transmission at sympathetic, parasympathetic, and enteric ganglia. Despite the fact, that autonomic ganglia neurons express several subunits, the nAChR at mammalian ganglionic synapses is mainly formed by $\alpha 3$ and $\beta 4$ subunits (Ritter M. et al., 2015; Vernino et al., 2009).

1.1.3 Degradation of acetylcholine

The level of ACh at cholinergic synapses is controlled by cholinesterases (ChE), namely acetylcholinesterase (AChE; EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8). They both are able to cleave ACh and belong to the family of serine hydrolases, but are distinguishable by substrate specificity, sensitivity to ChE inhibitors, structure, location and function in organism (Chatonnet and Lockridge, 1989; Pohanka, 2011).

1.1.3.1 Basic characterization of cholinesterases

There is on average 4,35 mg of AChE and 58 mg of BChE in human body (Lockridge, 2015a). Even though in different concentrations, both of ChE were detected in all tissues (Lockridge, 2015b). AChE can be found at the highest concentration in brain, NMJ and erythrocyte membrane, while BChE is mainly found in organs of first contact, such as plasma, liver and skin (Chatonnet and Lockridge, 1989; Lockridge, 2015b). AChE can hydrolyze more likely small substrates (e.g. ACh), while BChE is characterized by broad substrate specificity (Fang et al., 2011). It hydrolyses not only small substrates, but also larger substrates (e.g. cholines with longer aliphatic or aromatic chain) (Hrabovská et al., 2006). Ghrelin was described as the largest substrate which BChE is able to hydrolyze (Schopfer et al., 2015). ChE differ also in their behavior in presence of high concentration of some substrates: AChE shows substrate inhibition, BChE exhibits substrate activation (Chatonnet and Lockridge, 1989; Lockridge, 2015b; Massoulié et al., 1993a).

1.1.3.2 Structure of cholinesterases

ChE can be structurally characterized as glycoprotein, occurring in homomeric (monomeric, dimeric, or tetrameric forms consisting from catalytic subunits) and heteromeric forms (consisting from catalytic subunits and anchoring proteins). The disulphide bonds among them have no effect on activity but ensure the thermostability of the enzyme (Çokuğraş, 2003; Lockridge, 2015b).

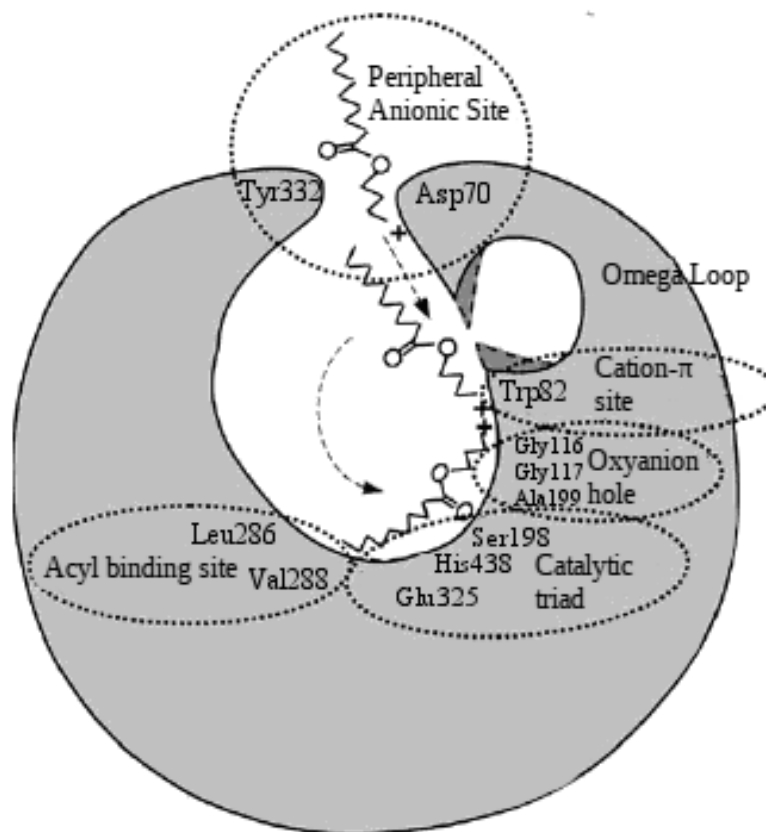


Figure 6: Schematic structure of the active site of BChE (Çokuğraş, 2003)

The ACh hydrolysis takes place in the *active site* of ChE (Figure 6), which lies at the bottom of 20 Å-deep gorge padded with aromatic residues. The first step of hydrolysis is the interaction between substrate and present amino acids - Tyr and Asp-, at the active center gorge entry, called *peripheral anionic site*. As a consequence of this action, conformational changes occur in the monomer. After that, the two flexible arms of Ω - loop get closer, what enables substrate to slide down to Trp of the *choline binding site*. The *oxyanion hole* is responsible for the rotation of substrate from vertical to horizontal position. At the same time, the acyl group of the substrate binds the *acyl binding site* (AChE: Phe and Phe; BChE: Leu and Val). When the substrate is stabilized by these processes, the enzymatic hydrolysis by the *catalytic triade* (human AChE: Ser²⁰³, His⁴⁴⁷, and Glu³³⁴; human BChE: Ser¹⁹⁸, His⁴³⁸, Glu³²⁵) can begin (Chatonnet and Lockridge, 1989; Çokuğraş, 2003).

As it has already been mentioned, ChE differ in their substrate specificity. It may be explained based on the differences in the sequence of amino acids in the acyl pocket (Çokuğraş, 2003; Nicolet et al., 2003).

1.1.3.3 Molecular biology and protein organization of cholinesterases

Human BChE gene is localized on chromosome 3q26.1 and comprises four exons (Lockridge, 2015b). The most important is the second exon since it codes 83% of the amino acid sequence of the catalytic domain. The second part of the catalytic domain is coded by the third exon. The fourth exon is responsible for coding of the C-terminal peptide. Even though BChE exists just in form BChE_T, there are several allelic variants, like atypical, silent, fluoride-resistant type or the K-variant, which correlates with the incidence of the AD (Lockridge, 2015b; Lockridge et al., 2016; Massoulié, 2002).

AChE has the encoding gene on human chromosome 7q22.1 and contains six exons (Ehrlich et al., 1992; Heilbronn, 1993). The first exon of both AChE and BChE is non-coding, while exons 2, 3, 4 code the catalytic domain (Figure 7). Exons 5 and 6 code the C-terminal peptide. Alternative splicing at these positions during the transcription of AChE gene results in existence of various splice variants. Their molecular forms differ in the C-end, but the catalytic domain is identical (Massoulié, 2002; Taylor et al., 1993). The AChE_S structure can be characterized as soluble, amphipathic monomers. Another type is AChE_H, which generates non-soluble, amphipathic, glycolipid-anchored dimers. This type is expressed mainly on mammal erythrocytes, but also in the large intestine (Massoulié, 2002). Even though the AChE_R type occurs in a human body in small amounts, it has got a signification function. It seems that non-amphipathic, soluble monomers play a role also in the process of embryogenesis, since the occurrence has been described in embryonic cells. Studies point to an increased expression of mRNA of this AChE_R in stressful situations, but also after intoxication, e.g., with soman (Grisaru et al., 1999; Perrier et al., 2005). The last type, AChE_T secures the enzyme degradation of ACh in synapses. The presence of C-terminal peptide, which comprises forty AA, determines diversity of molecular forms, which are depicted in the Figure 7. In general, subunits are divided into homo-oligomer and hetero-oligomer forms of AChE_T (Massoulié, 2002; Massoulié and Bon, 1982).

ChE are enzymes constituted of glycoprotein subunits forming different monomers or oligomers (Massoulié, 2002; Massoulié and Bon, 1982). We distinguish two main groups based on the structure of ChE: homo-oligomeric forms and hetero-oligomeric forms, which possess similar catalytic, but not hydrodynamic properties (Massoulié et al., 1993a). The hetero-oligomeric forms possess higher stability and resistance than homo-oligomer forms (Giles, 1997).

Except for AChE_T, the other splice variants do not have the ability to create hetero-oligomer forms, since the C-terminal peptide, containing the Trp amphiphilic tetramerization domain (WAT domain) and Cys which secures dimerization, are missing (Simon et al., 1998). To create tetrameric forms, another domain is needed, the proline-rich attachment domain (PRAD)(Simon et al., 1998), which is localized at the N-terminal end of the anchoring proteins. PRAD was first identified in anchoring protein Collagen-Q (ColQ) (Bon et al., 1997). One PRAD interacts with four WAT domains that are in the tetramer form and the resulting character of the interaction is polar, hydrophobic (Dvir et al., 2004, 2010; Massoulié et al., 1993b). We distinguish 3 types of anchoring proteins, ColQ, proline rich membrane anchor (PRiMA) and lamellipodin (RAPH1). These anchoring proteins differ in the length of PRAD (Noureddine et al., 2008). ColQ possess two Cys and these are linked to two AChE_T subunits by disulphide bond creating a “heavy dimer”, the other two subunits create “light dimer” also by disulphide bond (Noureddine et al., 2008). The second anchoring protein, PRiMA has 4 Cys in its PRAD and creates almost exclusively “light dimers” and just a small amount of heavy dimers. The ColQ-anchored forms are present in largest amount at NMJ on the basal lamina (Krejci et al., 1997), while PRiMA creates amphipathic, membrane-bound form, which is the dominant form of ChE in mammalian brain (Perrier et al., 2002). For long time it was thought that AChE and BChE are able to form similar heteromeric tetramers with longer PRAD subunits coming from bigger proteins (ColQ or PRiMA) or just with short PRAD-peptides forming soluble tetramers (Krejci et al., 1997; Perrier et al., 2002). However, lately a new type of BChE tetramer, the C5 type was described. In this type the PRAD comes from the protein named lamellipodin (RAPH1) (Schopfer et al., 2017). This type of BChE is controlled by both genes encoding BChE (3q26.1–q26) and lamellipodin (Chr 2q33) (Schopfer et al., 2017)

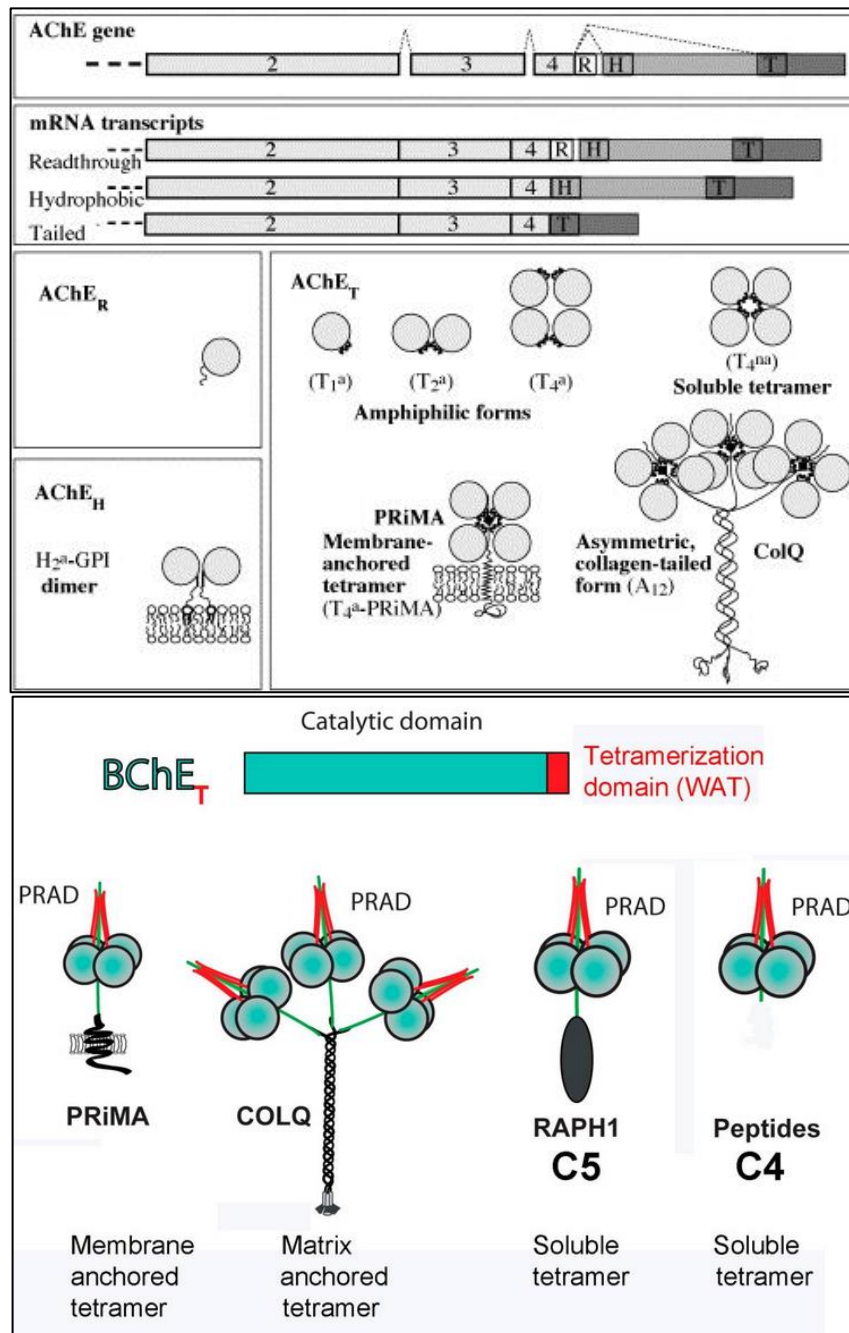


Figure 7: Schematic representation of the gene structure, transcripts and molecular forms of mammalian A)AChE (Massoulié et al., 2005) and B) BChE (Schopfer et al., 2017)

1.1.3.4 Function of cholinesterases

As mentioned above, AChE plays a critical role in terminating ACh-mediated neurotransmission. AChE, one of the fastest enzymes in the human body (Dvir et al., 2010) inactivates ACh by rapid hydrolysis into free choline and acetic acid. This rapid hydrolysis protects the cholinergic receptors from overstimulation by accumulated ACh (Duysen et al., 2002, 2001). The presence of AChE is crucial for life - knockout mice with no AChE (AChE $-/-$) and normal BChE activity are undistinguishable from their littermates at birth, however, they show growth retardation, impaired thermoregulation, motor tremor, suffer from bacterial infection in digestive tract and cannot survive without special care (Duysen et al., 2002). Double mutant AChE, BChE $-/-$ die at birth (Spieker et al., 2017). Surprisingly, the presence of AChE is not the most critical in brain, as mutant mice with almost no AChE activity in brain – PRiMA knockout mice - have no clear phenotype (Dobbertin et al., 2009; Farar et al., 2012). In contrast, mice with no AChE at NMJ (ColQ $-/-$ or del AChE1iRR $-/-$ mice) (Camp et al., 2010), have not only muscle weakness, but also breathing (Nervo et al., 2019) and digestive problems (E. Krejci, personal communication, 2020), indicating that the control of ACh release at NMJ is crucial and if it is missing, ACh may be toxic for several organs (Nervo et al., 2019).

Even if the concentration of BChE in human body is about 10-times higher than AChE, its physiological role is still unknown. Because this enzyme is synthesized in liver, it was originally considered as liver function marker (Lockridge, 2015b; Santarpia et al., 2013). Since then a lot of correlation between BChE activity and the occurrence of various pathological conditions has been described, such as hypertension, diabetes mellitus, low-grade systemic inflammation, hyperlipidemia or insulin resistance (Das, 2007; Iwasaki et al., 2007; Lockridge, 2015b; Santarpia et al., 2013). BChE is able to metabolize a lot of xenobiotics, such as acetylsalicylic acid, succinylcholine, bambuterol or cocaine (Çokuğraş, 2003; Manoharan et al., 2007; Masson et al., 2007), as it possess with esterase, aryl-acyl amidase and proteolytic activity. Furthermore, BChE is in the registration process as an orphan drug (Research, 2020) in case of cocaine overdose thanks to its capability of reduction and clearance of toxic blood levels (Mattes et al., 1998). In general, this enzyme is considered as a detoxification enzyme. No obvious pathology was observed in BChE knockout mice, but for AChE $-/-$ mice, the presence of this enzyme is crucial for survival, indicating that BChE may backup the function of its sister enzyme (Li et al., 2002; Mesulam

et al., 2002). Its crucial function of succinylcholine metabolism was also described (Lockridge, 2015b). Thanks to its ability of scavenging subclinical doses of organophosphates (OP) and saving AChE activity, BChE has an important role in protection against OP intoxication. (Lockridge, 2015b; Lockridge et al., 2016; Masson and Lockridge, 2010). In last decades, several NNCh functions of BChE were confirmed as well. Recently, papers suggest its active participation in lipid metabolism.

1.1.3.4.1 Butyrylcholinesterase in lipid metabolism

As it was mentioned in before, the activity of BChE correlates with occurrence of disorders like metabolic syndrome, atherosclerosis, hypertension, diabetes mellitus, insulin resistance, obesity or lipid disbalance and it seems to be a prognostic marker for these pathologies (Annapurna et al., 1991; Boberg et al., 2010; Chen et al., 2016; Iwasaki et al., 2007; Santarpia et al., 2013).

Thanks to genetic engineering, it was possible to produce BChE knockout mice (BChE $-/-$). They have no detectable enzyme activity in tissues and plasma and are used in science as models for human BChE deficiency (Li et al., 2006, 2008a). Feeding $-/-$ BChE mice with a high fat (11%) diet has resulted in obesity. This kind of increase in weight has not been observed in BChE $-/-$ mice fed with a standard fat (5%) diet as their weight was comparable to the wild-type strain. This study has overturned the hypothesis that BChE $-/-$ mice became obese due to increased caloric intake or reduced exercise. It has been suggested that BChE is involved in low density lipoprotein metabolism (Li et al., 2008a).

In BChE $-/-$ mice on a long-term high-fat diet, accumulation of triacylglycerols (TAGs) in liver has been observed (Li et al., 2008b). The liver serves as a spare fat reservoir when the fat tissue approaches saturation, at the same time the increase of amount of free fatty acids (FFAs) promotes TAG synthesis and very-low-density lipoprotein production. This imbalance between TAG synthesis and very-low-density lipoprotein leads to accumulation of intrahepatic TAGs, which can lead to cytotoxicity and liver dysfunction. However, the resistance of these BChE $-/-$ mice to obesity is renewable by transfection of gene for BChE into liver cells (Chen et al., 2017a). With exception of hyperinsulinemia, all other parameters characterizing lipid metabolism were comparable to wild-type strain. This observation justified the fact that the transfected BChE does not cross the blood-brain barrier and the elevated insulin level is caused by the absence of BChE in the brain or spinal cord.

The vector-treated mice possessed significantly higher BChE activity compared to control wild type, but no differences in weight has been observed (Chen et al., 2016).

ChE may also serve as a potential marker of synthetic function of liver or fat metabolism. The combination of conjugated linoleic acid in food with physical activity had a positive effect on the insulin profile and obesity parameters. There has been described an increase of free fatty acids and lipoprotein lipase activity together with a decrease in hepatic lipoprotein and BChE synthesis (Bulut et al., 2013).

It has been shown that BChE plays a key role in the inactivation of ghrelin (Chen et al., 2015, 2017b; Schopfer et al., 2015). Ghrelin is a peptide composed of 28 AA whose active form is esterified with octanoic acid (De Vriese et al., 2004). Active octanoyl-ghrelin stimulates appetite and reduces fat utilization, helping to increase weight (Tschöp et al., 2000).

Potential involvement of BChE in lipid metabolism may affect also the systemic insulin resistance and lipid profile of patients (Iwasaki et al., 2007). This hypothesis has been confirmed by experiment, in which selective inhibitor of BChE (iso-OMPA) was administrated to an obese rat with diabetic mellitus type 2. Significant decrease in serum TAG, total cholesterol and LDL concentration has been observed (Annapurna et al., 1991). Currently, most of the evidence supporting participation of BChE in lipid metabolism, are based on correlations and clinical observations (Johnson and Moore, 2012). The association of altered BChE activity with obesity, lipid metabolism disorder, diabetes mellitus type 2 and CVS risks has been demonstrated in several clinical trials on human participants (Boberg et al., 2010; Chen et al., 2016; Inácio Lunkes et al., 2006; Iwasaki et al., 2007; Mushtaq et al., 2014; Sridhar et al., 2005).

Various genetic variations of BChE also point to the association with obesity and other associated diseases. People with CHE2 C5 + allele (about 10% of the population) have on average 34% higher BChE plasma activity, lower weight and BMI in comparison to control group (Alcântara et al., 2001). In contrast, people with the "K variant of BChE" show 40% lower plasma BChE activity and have a higher tendency to obesity, diabetes mellitus and coronary artery diseases (Chen et al., 2016).

In our laboratory, a medium correlation has been observed between plasma BChE (activity and protein) and low-density lipoprotein levels in patients with CVS disorders.

More significant correlation was found between plasma BChE and TAG levels in the same group of CVS patients (Ondříková, 2011).

1.1.3.5 Inhibition of cholinesterases in pharmacology

As mentioned above, concentration of ACh at cholinergic synapses is controlled by ChE. Inhibition of the hydrolysis leads to prolonged action of this neurotransmitter, what may have not only pharmacological, but also toxicological importance (Hrabovska and Krejci, 2013; Pohanka, 2011).

ChE inhibitors fall into 3 main categories, according to their duration of action, which is associated with the type of interaction between the active site and inhibitor. We distinguish short-acting, medium-duration and irreversible inhibitors (Pohanka, 2011). Another classification is based on their substrate specificity – selective AChE inhibitors (e.g., BW284C51, Figure 8), selective BChE inhibitors (e.g., iso-OMPA, Figure 9), non-selective inhibitors (e.g., rivastigmine) (Pohanka, 2011, 2014).

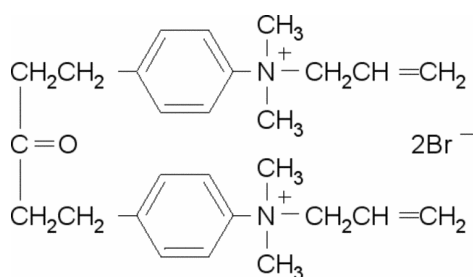


Figure 8: Structure of BW284C51

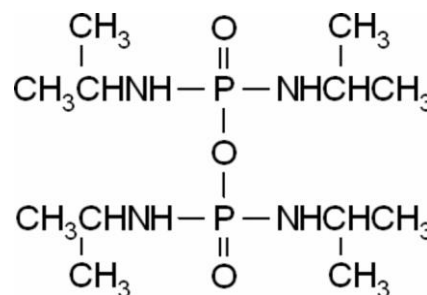


Figure 9: Structure of iso-OMPA

Inhibitors of ChE are frequently used drugs in therapy of several pathologies, which are associated with cholinergic system dysfunction. Mainly, they are accompanied with cognitive, behavioral and/or motor disabilities, like AD or Myasthenia gravis (Čolović et al., 2013; Tata et al., 2014). Their ability to cross the brain blood barrier determines the action on periphery or in the CNS (Pohanka, 2011, 2014).

AD is a very common neurodegenerative disease, characterized by formation of neurofibrillary tangles (formed by hyperphosphorylated tau protein) and senile plaques (formed by beta-amyloid protein), which results in reduced number of mAChR and nAChR and neuron loss mainly in basal forebrain and cerebral cortex (Cacabelos, 2007; Hrabovska and Krejci, 2013; Tata et al., 2014). Moreover, there are four drugs registered for the treatment of AD, three of them (donepezil, rivastigmine and galantamine) enhance

cholinergic transmission by inhibition of ACh-hydrolysis in the synaptic (Čolović et al., 2013; Mushtaq et al., 2014; Pohanka, 2011; Tabet, 2006). Increased level of ACh seems to be beneficial for improvement of cognitive functions, but this topic is still disputable and not completely understood. As inflammatory processes have crucial role in pathogenesis of AD and NN-ACh is considered as inflammation modulator, the pharmacological targeting of “cholinergic anti-inflammatory pathway” may be beneficial (Andersson and Tracey, 2012; Rosas-Ballina and Tracey, 2009; Tracey, 2016). Involvement of cholinergic transmission by increasing the level of ACh is also used in the therapy Huntington’s disease or various psychiatric disorders (e.g., schizophrenia) (Hrabovska and Krejci, 2013; Tata et al., 2014). Enhancement of cholinergic transmission is needed at the NMJs of patients suffering from Myasthenia gravis, which is a rare autoimmune disease (Jayam Truth et al., 2012). Edrophonium, a short-acting ChE inhibitor is used in the diagnostic (Pascuzzi, 2003), while neostigmine, pyridostigmine with steroids and immunosuppressive drugs are the most useful drugs in the therapy (Pohanka, 2011).

ChE inhibitors seem to be promising drugs for other disorders, too. Studies showed that donepezil has positive effect on cognitive functions of patients with Down-syndrome (clinicaltrials.gov NCT00570128). Moreover, this drug is in phase 1 of clinical trials for testing for treating amblyopia in young population, because high levels of ACh in brain improved vision (clinicaltrials.gov NCT01584076). Another drug from this group, pyridostigmine is tested for its anti-inflammatory effect as supplementary drug in therapy of HIV-infected patients (clinicaltrials.gov NCT03312244). It is also tested for the treatment of neuropathic pain and the study is already in phase 4 of clinical trials (clinicaltrials.gov NCT01743976).

1.1.3.6 Inhibition of cholinesterases in toxicology

Long-term or permanent inhibition of the termination of ACh-hydrolysis causes accumulation of ACh and desensitization of cholinergic receptors (Kalamida et al., 2007). This injury manifests itself by respiratory failure, muscle paralysis, seizures, which consequences are mostly fatal. The two main group, which can cause cholinergic crisis are OP and carbamates. They covalently bind the esteratic part of the active site by carbamylating, phosphorylation or phosphonylation (Gonçalves et al., 2010; Pohanka, 2011).

Some carbamate compounds and OP are commonly used pesticides and insecticides (Bajgar, 2004). Some other OP with high level of toxicity (e.g., soman, cyclosarin, sarin, tabun) were misused as lethal chemical warfare during World War II. Structurally, they are pentavalent phosphorus compounds often with fluoride or labile organic group (Allon et al., 1998; Masson and Lockridge, 2010; Pohanka, 2011). The main problem with the treatment of intoxication is the especially rapid absorption of these compounds and the fast AChE deactivation caused by conformational changes in the binding site of enzyme. BChE is endogenous scavenger of OP due to its ability to inactivate OP by making covalent bond with them, so the prompt administration of pure enzyme in acute intoxication is life-saving. Therefore, the enzyme was registered as an orphan drug for the treatment of OP intoxication (Lockridge, 2015b; Lockridge et al., 2016; Marsillach et al., 2011; Wieseler et al., 2006).

There is another group of chemical compounds, which can be used as reactivators of ChE in OP intoxication, oximes. Their ability to displace the phosphoryl group from active site of AChE is conditioned by the present of nucleophile group in their structure (Figure 10). The most important limiting factor is the time because the enzyme undergoes irreversible chemical changes (dealkylation) with time. This mechanism is called “ageing”. The most well-known oximes are: pralidoxime, obidoxime, 2-PAM, TMB-4 (Bajgar, 2004; Guimarães et al., 2011; Lockridge, 2015b; Masson and Lockridge, 2010).

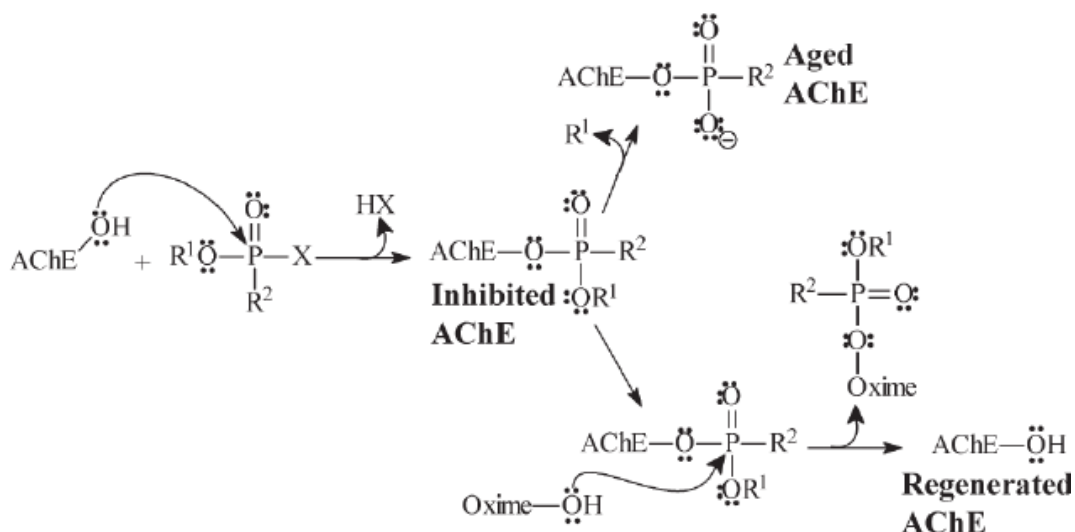


Figure 10: Inhibition, regeneration and ageing of AChE (Guimarães et al., 2011)

1.2 Non-neuronal cholinergic system in cardiovascular system

NNChS is a term used to describe the ubiquitous presence of all components of cholinergic system, meaning all components participating in synthesis, storage, secretion, and degradation of ACh, and also cholinergic receptors in tissues – independently of the presence of neurons. (Grando et al., 2015). Prior discovery of NN-ACh, it had been assumed that the heart is controlled solely *via* sympathetic and parasympathetic innervation. Proven synthesis of ACh in other cells, than neurons led to exploration of NN-ACh also in the heart (Rana et al., 2010). Based on recent research, it can be expected that NNChS in heart would be a promising pharmacological target for the treatment of cardiovascular system (CVS) diseases.

1.2.1 Cardiomyocytes produce and release ACh

Cardiomyocytes, according to the results of RT-PCR analysis, immunohistochemistry and also Western Blot, show an ability to synthesize, transport and secrete ACh (Kakinuma, 2015; Rana et al., 2010; Rocha-Resende et al., 2012). Substantial role of the cardiomyocytes in regulation of cell energy metabolism have been suggested (Oikawa et al., 2015). ACh plays an important role in cell energy metabolism and protects myocardial cells by controlling the level of adenosine-triphosphate and reducing the oxygen consumption. This hypothesis was proven by studies in ChAT *-/-* cells (cells not able to synthesize ACh), where an increased activity of mitochondria together with increased oxygen consumption have been described in knockout cells (Kakinuma et al., 2009). The importance of ACh coming from cardiomyocytes has been confirmed in other study, where mutant mice with no VACHT in cardiomyocytes were used (Roy et al., 2013). The mice showed no differences in hemodynamic parameters under normal conditions. However, under stress, higher heart rate has been observed. In these mutant mice not only an imbalance in adrenergic and cholinergic signalization, but also cardiomyocytes remodeling, hypertrophy, impairment of calcium-signaling and increased oxidative stress have been observed (Roy et al., 2013). Furthermore, overexpression of VACHT in heart has no effect on heart rate and blood pressure of these mutant mice. Moreover, the overexpression of VACHT seems to be beneficial in heart failure, as it helps to delay the progression of the pathology and to offset cardiac remodeling (Roy et al., 2016). Taken together ACh coming from the neurons and ACh coming from the cardiomyocytes cooperates on maintaining the

cardiac homeostasis and is essential for the normal heart function (Kakinuma et al., 2009, 2012; Oikawa et al., 2015; Rocha-Resende et al., 2012; Roy et al., 2013).

Effect of ACh in the heart is also terminated by ChE, whose presence and precise localization in heart has been described by Dingová et al. (Dingova, 2014), and the physiology of ChE by Kučera et al. (Kučera, 2015). The activity of BChE in the whole mouse heart was 2.5 times higher, than the AChE activity. While the AChE activity is localized mainly in the atria, the BChE activity is significant in the ventricles (Dingova, 2014).

1.2.2 Some types of lymphocytes produce and release ACh

Lately, the occurrence of NNChS in cells of the immune system and its possible participation in immune response is becoming a very attractive topic. Research of the NNChS focuses mainly on T- and B-lymphocytes (Fujii and Kawashima, 2001). The occurrence of ACh in blood has been described first by the research team of Kawashima (Kawashima et al., 1998). Radiometrical studies revealed, that about 60% of ACh is localized in mononuclear leucocytes (Kawashima et al., 1998; Neumann et al., 2007). The presence of ACh and ChAT in human T-lymphocytes has been also confirmed (Kawashima et al., 1998; Olofsson et al., 2016). ACh produced by lymphocytes has several functions. Mice lacking ChAT in CD4+ T-lymphocytes have higher arterial blood pressure, while infusion of Jurkat T-cells with overexpression of ChAT decreased the blood pressure in these mice (Olofsson et al., 2016). Furthermore, these ACh-producing T-cells are crucial also in the inhibition of cytokine production as a consequence of *n. vagus* stimulation and this way control the innate immune responses (Reardon et al., 2013; Rosas-Ballina and Tracey, 2009). The innate immune response is regulated also by ACh produced by B-cells, as they control the local recruitment of neutrophils. In T-lymphocytes, all subtypes of mAChR and several types of nAChR have been described. There is a theory that immune response is modulated by ACh synthesized directly in T- cells, which acts *via* mAChRs (Fujii and Kawashima, 2001; Kawashima et al., 2015). Some studies claim that the activation of mAChR has got a pro-inflammatory effect (Beckmann and Lips, 2013). On the other hand, it is likely that ACh working *via* $\alpha 7$ nAChR regulates production of cytokines in immune cells, and this way influences the growth and differentiation of T cells (Kawashima et al., 2015; Olofsson et al., 2016; Reardon et al., 2013; Rosas-Ballina et al., 2011). Expression of AChE is proved in mononuclear cells, dendritic cells and also in macrophages, it is presupposed that its function is the control of level of released ACh (Kawashima et al., 2007).

1.2.3 Non-neuronal cholinergic system in blood

ChE also occur in blood, while their amount and activity are species-specific. The highest BChE serum levels were detected in monkeys (7.71 U/ml), mice (2.40 U/ml) and human (2.44 U/ml), while rats have several times lower serum BChE activity (0.09 U/ml). In contrast, humans have low level of AChE activity in serum (0.05 U/ml). Mice possesses high serum levels of AChE (0.49 U/ml), while rats have also lower levels (0.22 U/ml) (Li et al., 2002). Changes in the serum activity of ChE have been observed in various pathologies such as neurodegenerative disease (Holzgrabe et al., 2007), liver pathologies (Meng et al., 2013), diabetes mellitus (Inácio Lunkes et al., 2006), chronic obstructive pulmonary disease (Liggett et al., 1988) and others.

1.3 VESSELS

1.3.1 Anatomical and physiological properties of aorta

The aorta is the largest blood vessel in the body and is responsible for supplying the oxygenated blood from the left ventricle to the whole body (Komutrattananont et al., 2019; Shahoud et al., 2020; White et al., 2020). The mean pressure in the aorta is high (100 mm/Hg) due to the fact, that heart pumps blood continually (Shahoud et al., 2020; Tortora and Derrickson, 2018). Aorta begins at the left ventricle by aortic root, where the aortic valve is localized (Figure 11). It is one of the two semilunar valves and prevents the blood leak into the heart. Ascending aorta connects the aortic root with aortic arch. The three branches coming from this part (brachiocephalic artery, left common carotid artery, left subclavian artery) supply the head and upper limbs. The descending aorta has two parts: the thoracic aorta (localized between the aortic arch and the diaphragm), which supplies the blood to all the muscles of the thoracic part. The second part of descending aorta is the abdominal aorta localized between diaphragm and pelvis. It has 5 different branches (superior and inferior mesenteric artery, celiac artery, renal and iliac arteries), which provide blood to the organs of the abdominal cavity (Komutrattananont et al., 2019; Shahoud et al., 2020; Tortora and Derrickson, 2018; White et al., 2020).

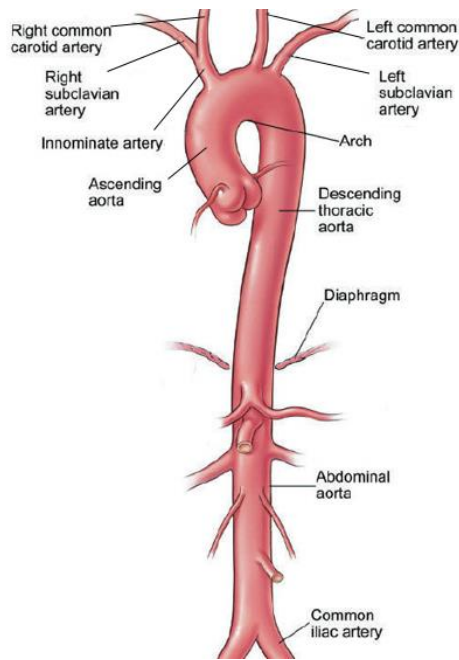


Figure 11: Aortic segments

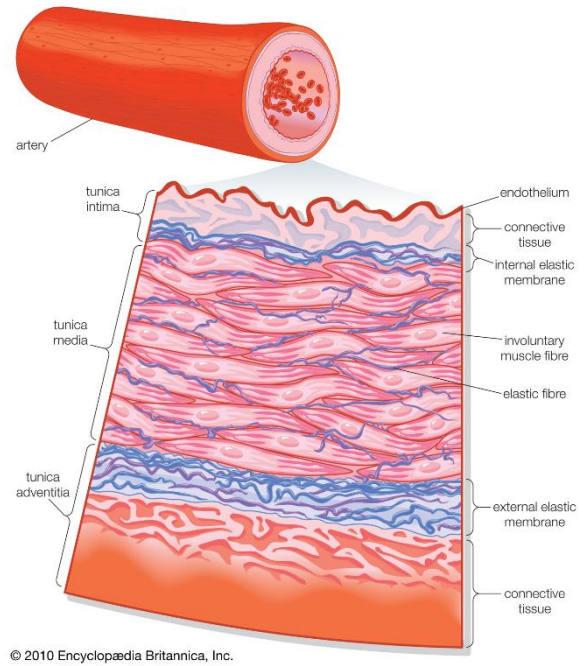


Figure 12: Cross-section of the aorta

Aorta belongs to arteries and its wall consists of three different layers (Figure 12). Tunica intima (innermost layer) is made up of connective tissue and elastic fibers (Shahoud et al., 2020; Tortora and Derrickson, 2018). Endothelium, a layer of simple squamous epithelium, belongs also to this part of the aorta (Gimbrone and García-Cardena, 2016; Shahoud et al., 2020; Tortora and Derrickson, 2018). Endothelium, at the time of the discovery was thought just as a physical barrier between blood and tissues. Nowadays, it is reconsidered as a highly dynamic tissue capable to react to the presence of a huge amount of substances and also is considered also as the major regulator in cardiovascular system function, including the control of vascular tone (Furchgott, 1991; Furchgott and Vanhoutte, 1989). The middle layer is tunica media and consists of smooth muscle cells organized in spiral layers. The third layer is tunica adventitia and is made up of collagen fibres which ensure the elasticity of the aorta (Tortora and Derrickson, 2018).

1.3.2 The endothelium and the effect of acetylcholine on vessels

As it was mentioned before, endothelium is a highly dynamic tissue regulating, among others, the vascular tone (Busse et al., 1985), as it releases numerous vasoactive factors. Some of them have vasodilatory effect (NO, prostacyclin-PGI₂ and endothelium derived hyperpolarizing factor) or vasoconstrictive effect (thromboxane and endothelin-1) (Favero et al., 2014; Persson, 2015).

The mechanism that ACh cause vasodilation by the production of nitric-oxide (NO) just in the presence of intact endothelium (Busse et al., 1985; Furchgott and Zawadzki, 1980; Norel et al., 1996) was firstly described in 1980 by research team of Furchgott and Zawadzki (Furchgott and Zawadzki, 1980). Now, we know that the production is catalyzed by NO synthase (NOS), which exists in form of 3 isoforms (endothelial (e-NOS), neuronal NOS, inflammatory NOS) and each of them were reported to be involved on vascular tone regulation (Laspar et al., 2014). The neuronal NOS has an impact on vascular tone not only by endothelium-dependent but also independent mechanism (Laspar et al., 2014). Induction of inducible NOS is connected with impairment of vascular response, while the relative expression of this type of NOS is regulated by several cytokines (Laspar et al., 2014). The most widely described NOS is eNOS, which is the main isoform involved in endothelium-dependent regulation of both blood flow and vascular tone (Gericke et al., 2019). ACh can cause vasodilation also by prostaglandin production (Haberberger et al., 2000; Thomas et al., 2002). Moreover, it is known, that the lack of eNOS can be functionally compensated by neuronal NOS and cyclooxygenase-metabolites (Gericke et al., 2019).

The production of NO is strongly associated with the action of ACh. ACh is able to activate mAChRs located both on the endothelium and smooth muscle, while its direct action on these receptors results in activation of completely different pathways (Furchgott, 1991; Furchgott and Vanhoutte, 1989).

In vessels, the most important isoform is eNOS (Busse et al., 1985; Wood et al., 2013). All the NO agonists (besides ACh also bradykinin, substance P, thrombin, adenosine-diphosphate, and adenosine-triphosphate) causes eNOS activation by Ca^{2+} increase from endoplasmic reticulum (Gericke et al., 2019). Ca^{2+} is necessary for the cleavage of eNOS from caveolin, protein, to which is inactive eNOS bound. Subsequently, the Ca^{2+} channels open and the intracellular levels of Ca^{2+} increases and activate calmodulin (Chen et al., 2018). After structural changes, it binds to the eNOS, which in consequence converts L-arginine into NO (Palmer et al., 1988). However, the increased levels of Ca^{2+} is not sufficient for long-term NO-synthesis. The most important additional mechanism is the protein kinase A and cyclic guanosine monophosphate protein kinase dependent II-mediated eNOS phosphorylation (Francis et al., 2010; Weinmeister et al., 2008).

Subsequently, NO diffuses to the smooth muscle, where it activates the enzyme soluble guanylyl cyclase, which causes an increase in the rate of conversion of guanosine triphosphate to cyclic guanosine monophosphate (Friebe and Koesling, 2003; Roy and Garthwaite, 2006). This molecule not only significantly inhibits the release of Ca^{2+} from sarcoplasmic reticulum, but also promotes to restore calcium to the sarcoplasmic reticulum (Friebe and Koesling, 2003; Roy and Garthwaite, 2006; Russwurm and Koesling, 2004). These actions prevent the contraction of smooth muscle cells. When the activity of NO was totally inhibited by methylarginine, a dose-dependent increase in blood pressure was observed confirming that the continuously produced NO has an important role in maintaining the basal vasodilator tone (Friebe and Koesling, 2003; Roy and Garthwaite, 2006; Russwurm and Koesling, 2004). Moreover, NO induces vascular relaxation, not only through its direct effect on endothelium, but also by inhibiting endothelin-1 and thromboxane (Cardillo et al., 2000), which are potent vasoconstrictors and also by stimulating production of a vasodilatory prostacyclin (Mitchell et al., 2008). As well as causing vasodilation, NO has a protective effect on vessels from atherosclerosis and thrombosis. It inhibits the proliferation of smooth muscle cells, the platelet adhesion and also the adhesion and migration of monocytes (Wang et al., 1998).

On the other hand, direct activation of mAChR on smooth muscle causes vasoconstriction (Brozovich et al., 2016; Szadujkis-Szadurska et al., 2012). ACh activates present G_q -coupled mAChRs, which results in the activation of phospholipase C and production of inositol-triphosphate (IP_3) and diacylglycerol (DAG) production from membrane (Brozovich et al., 2016; Brunton, 2017; Szadujkis-Szadurska et al., 2012). DAG activates protein kinase C and, which is responsible for vasoconstriction, while IP_3 causes a release of Ca^{2+} from sarcoplasmic reticulum and promotes vasoconstriction. If endothelium is not impaired, the production of NO by endothelium overrides the vasoconstriction of smooth muscle (Brozovich et al., 2016; Szadujkis-Szadurska et al., 2012).

The action of ACh is contingent also on the vascular tone. While under normal vascular tone, an increase of lobar arterial pressure has been observed, under increased vascular tone the exact opposite has been reported (Furchgott and Vanhoutte, 1989). In case of precontracted aorta, the response to ACh is dependent upon the presence of intact endothelium (Furchgott, 1991; Furchgott and Zawadzki, 1980; Norel et al., 1996).

1.3.2.1 Endothelial dysfunction

Endothelial dysfunction is principally characterized as an impairment of NO bioavailability, which is an important pathophysiological feature of diseases like hypertension (Bernatova, 2014; Dharmashankar and Widlansky, 2010), atherosclerosis (Alvarez and Ganz, 2004; Gimbrone and García-Cardena, 2016), obesity (Engin, 2017; Iantorno et al., 2014), metabolic syndrome (Paoletti et al., 2006), etc. Evidence suggests that NO plays an important role in regulation of blood pressure (Gimbrone and García-Cardena, 2016; Steyers and Miller, 2014). As it was mentioned before, the imbalance in the production of vasoactive substances has a negative consequence on vessel tone.

The main consequence of endothelial dysfunction is reduced NO bioavailability (Bonetti et al., 2003; Quyyumi et al., 1997). Impaired biosynthesis of NO is accompanied by facilitated secretion of reactive oxygen species, pro-oxidant hem-molecules, nicotinamide adenine dinucleotide phosphate. Moreover, the activation of lipoxygenases, cyclooxygenases, xanthine oxidase occurs (Bonetti et al., 2003; Esper et al., 2006; Sitia et al., 2010). All of them promotes the progression of endothelial function and facilitate the formation of atherosclerosis (Gimbrone and García-Cardena, 2016). Among other CVS disorders, endothelial dysfunction is associated with hypertension (Bernatova, 2014; Gimbrone and García-Cardena, 2016; Steyers and Miller, 2014). In general, vessels from various animal models with hypertension showed decreased endothelium-dependent relaxation and increases endothelium-dependent contraction (Bernatova, 2014; Leung et al., 2016). The connection between endothelial dysfunction and hypertension is proven also by the fact that long-term use of antihypertensive drugs increases the relaxation response to ACh from spontaneously hypertensive rats (SHR), however, the exact mechanism is still unknown (Leung et al., 2016).

Other pathologies are associated with endothelial dysfunction and are characterized by changed response to present ACh. One of them is atherosclerosis. It has been shown by quantitative angiography, that vessels even with minimal impairment are constricted when ACh is applied. It means, that changed response is present even in very early phases of atherosclerosis and minimal damage of endothelium has an impact on vessel physiology (Ludmer et al., 1986). In other study, authors claim that there is reduced basal activity of NO activity due to the presence of atherosclerotic plaques (Quyyumi et al., 1997). On the other hand, other groups results indicate, that the response of coronary arteries to ACh was

independent of the presence of plaques, the greater vasoconstrictive response was associated rather with the presence of other risk factors for coronary artery disease (e.g. higher blood pressure, cholesterol, obesity or diabetes mellitus) (Nishimura et al., 1995). The results of Egashira et al. (Egashira et al., 1993) are in line with these results, as they showed that hypertension, hypercholesterolemia, age, and the total number of coronary risk factors, but not the presence of atherosclerotic plaques are associated with the impaired increase of coronary blood flow response to ACh (Egashira et al., 1993).

1.3.2.2 Atherosclerosis and endothelial dysfunction

As it was mentioned before, endothelial dysfunction is one of the main predisposing pathological factor for the development of atherosclerosis (Alvarez and Ganz, 2004; Gimbrone and García-Cardena, 2016). Atherosclerosis, an inflammatory and degenerative vascular disease, can be characterized as an inability of the vascular endothelium to cope with a high content of low-density lipoproteins and its penetration into the subendothelial space (Alvarez and Ganz, 2004; Gimbrone and García-Cardena, 2016; Ross, 1993). They are proven significant correlations between ischemic heart disease (myocardial infarction or angina pectoris) with a spectrum of lipoproteins and cholesterol (Alvarez and Ganz, 2004; Gimbrone and García-Cardena, 2016). The reduction of their serum concentrations is a primary goal in the prevention of CVS diseases. Atherosclerosis is further associated with metabolic syndrome (Paoletti et al., 2006). The characteristic features of metabolic syndrome are dyslipidemia (elevated triacylglycerol (TAG) low density lipoproteins (LDL) levels, and reduced levels of high density lipoproteins (HDL) (Paoletti et al., 2006). Long-term hypertension impairs the structure of vessels and its transport properties and thus facilitates the penetration of transport forms of cholesterol or LDL into the vessel wall. Subsequently, the oxidation of LDL occurs and the free radicals reaching the vessel wall induce immune response. Oxidized LDL are taken up by macrophages and T-lymphocytes. However, these blood cells are not able to process the oxidized form of LDL and they form “foam cells”, which have a high content of lipid droplets. These aggregation of foam cells participates in the formation of atherosclerotic plaque and tissue necrosis occurs. Necrosis occurs and toxic substances are released which promote plaque rupture and blood clot formation (Alvarez and Ganz, 2004; Gimbrone and García-Cardena, 2016; Paoletti et al., 2006).

1.3.2.3 Obesity and endothelial dysfunction

Obesity is a chronic condition associated with various metabolic syndromes and can be characterized as an imbalance between energy intake and expenditure (Engin, 2017). In addition to genetic predisposition, excessive calorie intake and lack of physical activity belong to the primary cause of obesity (Caballero, 2003; Iantorno et al., 2014). Moreover, obesity can be the side effect of some drugs or can be caused by several endocrine disease (Kokkoris and Pi-Sunyer, 2003) or mental disorders (Rajan and Menon, 2017). We divide obesity into visceral type and gynoid type. Abdominal obesity, characterized by the accumulation of fat tissue in the abdominal cavity, is important in the etiopathogenesis of the metabolic syndrome (Caballero, 2003; Iantorno et al., 2014). It is associated with pathologies as arterial hypertension, dyslipidemia, diabetes mellitus type 2 or coagulation disorders (Caballero, 2003; Iantorno et al., 2014). The second type, gynoid obesity, is not considered as a risk factor for the metabolic syndrome but is associated with gynecological pathologies of women or increased risk of cancer (Dieli-Conwright et al., 2019; Dorgan et al., 2012).

The excessive deposition of fat, what is one of the main characteristics of the obesity interfere with all the physiological metabolic processes in the body. The changes in the adipose tissue induces the secretion and release of several inflammatory mediators, such as IL-6, IL- β , TNF- α or leptin (Caballero, 2003; Engin, 2017; Iantorno et al., 2014). Another consequence is the stimulation of monocyte chemoattractant protein-1, which results in lower adiponectin levels and initiation of proinflammatory state. Moreover, this monocyte chemoattractant protein-1 has a negative effect on endothelial cells as it activates adhesion molecules and the proliferative processes of leukocytes (Engin, 2017; Iantorno et al., 2014). These processes (i.e. overexpression of proinflammatory cytokines and reduction of anti-inflammatory markers in obesity) are considered to be the link between obesity and endothelial dysfunction as they increase the atherogenic and thromboembolic potentials of vessels (Caballero, 2003; Engin, 2017; Iantorno et al., 2014). As it was explained before, endothelial dysfunction is characterized, among others, by reduced NO bioavailability and imbalance in vasodilatory and vasoconstrictor mechanisms of vessels (Gimbrone and García-Cardena, 2016; Persson, 2015). In addition, alterations of adipose tissue and inflammation may activate the renin-angiotensin-aldosterone-system, which results in

elevated production of reactive oxygen species (Engin, 2017; Iantorno et al., 2014; Steyers and Miller, 2014).

1.3.2.4 High blood pressure and endothelial dysfunction

Endothelial dysfunction is strongly associated with hypertension, independently on the type of hypertension (primary essential, or secondary – as a concomitant syndrome of endocrine or renal pathologies) (Bolad and Delafontaine, 2005; Dharmashankar and Widlansky, 2010; Oishi et al., 2018). Moreover, the severity of the impairment of endothelial dysfunction is correlated with the severity of the hypertension. We talk about a complex connection between these two pathologies, as it has not been established, whether endothelial dysfunction is the cause or the consequence of hypertension (Bolad and Delafontaine, 2005; Dharmashankar and Widlansky, 2010). Application of eNOS inhibitors results in hypertension (Li et al., 2015; Wood et al., 2013), human with endothelial dysfunction have several times higher risk for developing hypertension. On the other hand, higher systolic blood pressure was also associated with increased incidence of endothelial dysfunction (Bernatova, 2014; Bolad and Delafontaine, 2005; Dharmashankar and Widlansky, 2010). Moreover, the excess of aldosterone is also associated with impaired endothelial function in patients with resistant hypertension (independent of high blood pressure) (Chen et al., 2019).

1.3.2.5 Spontaneously hypertensive rats as a model to explore cardiovascular diseases

The strain of spontaneously hypertensive rats (SHR) was produced by team of Okamoto (Okamoto and Aoki, 1963) by selective inbreeding of Wistar-Kyoto rats with high blood pressure. This strain is therefore primarily used for the study of hypertension and other cardiovascular diseases (Okamoto and Aoki, 1963). Similar to humans, the hypertension is a result of modification of multiple genes (Mohammed-Ali et al., 2017). Hypertension starts to develop in SHR around the fifth week and in this phase increased cardiac output and normal total peripheral resistance is characteristic. However, these parameters normalize when hypertension stabilizes (Dickhout and Lee, 1997). It means that these rats possess normal cardiac output and higher total peripheral resistance due to the hypertrophied blood vessels (Dickhout and Lee, 1997). Around the fourteenth week of age renal pathologies begin to develop, which results around 30-32 weeks of age in a significant decrease in glomerular filtration rate in male, but not in female rats (Mohammed-Ali et al., 2017).

Thanks to their relatively long life spans (2-3 years) they are suitable model also for the study of aging process in hypertension (Mohammed-Ali et al., 2017). SHR are characterized by progressive deterioration of the anatomical, morphological, and physiological characteristics of vessels. During aging, SHR display decreased lipolytic esterase and ChE activities and increased lysosomal enzyme activity, collagen, glycosaminoglycan, and elastin content, tunica media thickness, and modified smooth muscle cell behavior (migration, secretion, proliferation) (Hadjiisky et al., 1987). Moreover, SHR develop endothelial dysfunction and subsequently, the vasodilatory response to ACh is modified. Indeed, isolated thoracic aorta rings from SHR showed significantly lower relaxation response to ACh application when compared to normotensive rats (Berenyiova A et al. 2018; Félétou, Verbeuren, and Vanhoutte 2009). This model is further used for cognitive-related disorders (learning disorders) (Grünblatt et al., 2015) and also diabetes mellitus (when SHR are treated with streptozocin – (Costa and Vianna, 2008)).

1.3.3 Non-neuronal cholinergic system in vessels

The presence of the NNChS in blood vessels, also the distribution of its components in smooth muscle, resp. in vascular endothelium is a very attractive topic for scientists as CVS diseases are highly frequent in the population.

Kirkpatrick et al. described first the presence of the NNChS in cultures of human umbilical vein endothelial cells and cultures of human angiosarcoma endothelial cells (Kirkpatrick et al., 2003). They successfully localized ChAT and VAcHT in the cytoplasm of human umbilical vein endothelial cells. (Kirkpatrick et al., 2003). After, the presence of ChAT in vessels of blood vessels of the human skin was detected at protein levels by confocal microscopy (Kurzen et al., 2007). Haberberger et al. investigated the presence of the "cholinergic gene locus" in the pulmonary vasculature (Haberberger et al., 2000). Positive immunoreactivity to ChAT and VAcHT was described not only close to the interface of media-adventitia of the pulmonary artery, but after also in human, pig and guinea pig endothelia (Kirkpatrick et al., 2003).

As mentioned above, the rate-limiting step in the process of ACh synthesis is the ChT-1 mediated reuptake of choline. Its presence has been demonstrated in endothelium cell cultures of brain, lung, and aorta and also in carotid artery smooth muscle cell at both mRNA and protein levels. *In vivo*, immunohistochemistry was positive in all smooth muscle cells

of various arteries and endothelial cells in the lung strain and inconsistently in other vessels. The highest immunoreactivity was observed in the smooth muscle of the arterial wall. Moreover, autoradiographic studies have shown that the detected reuptake of [³H]-choline is primarily localized in tunica media (Lips et al., 2003). On the other hand, we cannot forget that choline also contributes to the construction of the cell membranes and high dynamic cell expansion (Inazu, 2014).

Presence of muscarinic receptors was also documented in several species. In bovine aortic endothelial cells, m1AChR, m2AChR and m3AChR were detected (Tracey and Peach, 1992). m1AChR was found in human pulmonary endothelium (Walch et al., 2000). m1AChR and m2AChR were present in the rabbit saphenous artery (Komori and Suzuki, 1987), while the mRNA expression for m3AChR and m5AChR in other rat arteries was confirmed (Phillips et al., 1997). In the endothelium of the pulmonary arteries, on the other hand, relaxation was mediated by m1AChR and m3AChR (Norel et al., 1996). In the smooth muscle of the pulmonary arteries, the m3AChR receptor is thought to be involved in constriction (Norel et al., 1996).

It is known that NNChS system greatly affects angiogenesis, especially in terms of regulation. nAChRs, mainly $\alpha 7$ type, play an important role in proliferation, migration, vascular wall cell survival, and also in vessel formation, both *in vivo* and *in vitro* (Cooke, 2007; Cucina et al., 2008; Egleton et al., 2008; Neumann et al., 2007; Wu et al., 2009). Moreover, nAChR antagonists reduce neovascularization in response to inflammation, ischemia, and neoplasia (Neumann et al., 2007). In addition, nicotine stimulates interactions between endothelial cells and monocytes, thereby facilitating arteriogenesis (Cucina et al., 2008).

In some vessels, the presence of ChE was already confirmed. In human endothelial cell culture, the activity of AChE was detected (Wang et al., 2001). Moreover, in human veins, the activity of ChE is roughly twice as high as in the arteries. In human arteries, BChE was described as the dominant ChE and it seems to be involved in smooth muscle contraction, not endothelium-dependent smooth muscle relaxation (Kotelevets et al., 2005; Walch et al., 1997).

Recently, several studies have demonstrated impairment of ChE in various diseases, but the nature of the link between these enzymes and CVS disease has not been clearly

established. Our previous results (Piváčková and Křenek, 2012; Szmicseková, 2015; Vetešková, 2014) have shown that also ChE may serve as regulators of the vascular tone, but to understand their exact function in physiology, further experiments need to be performed.

■ AIMS OF THE PROJECT

It is known, that ACh is highly involved in physiological but also in pathological processes, while CVS diseases are no exceptions. Despite sparse cholinergic innervation, vessels are highly responsive to ACh. Nevertheless, little is known about the present and possible functions of enzymes terminating its action in vessels. Moreover, several publications link the activity of ChE, enzymes terminating ACh, with hypertension, hypercholesterolemia, and atherosclerosis, which belong among main CVS risk factors accompanied by pathophysiological changes in vessels. Therefore, this project focuses on the characterization of ChE and NNChS in vessels of healthy and pathologically changed models.

The effect of ACh on vessels is well described but little is known about mechanisms responsible for its degradation in vessels. Therefore, **the first aim of this project was to detailly characterize ChE in model vessel, aorta, of healthy rats.** To meet this aim, various molecular, biochemical and microscopic methods were used to determine the presence of ChE mRNA, ChE proteins and to quantify ChE activities at the longitudinal level (full length of the aorta divided into *arcus aortae*, *aorta thoracalis*, *aorta abdominalis*) and transversal level (endothelium vs. smooth muscle). Additionally, we examined the presence of all the enzymes and transporters needed to synthesize, transport and release ACh in all studied parts of aorta. We aimed to study these proteins involved in ACh life cycle at mRNA and protein levels.

Endothelial dysfunction, which can be characterized as altered response to ACh, is often associated with pathological conditions, such as hypertension, hypertriglyceridemia, or metabolic syndrome. Lately, BChE activity was suggested as a marker of these comorbidities. Nevertheless, despite the fact that ACh is being commonly used to examine vessel reactivity and thus intactness and proper function of endothelium *ex vivo*, information about NNChS in vessels with impaired endothelial function are sparse. **The second aim was therefore to characterize the aortic NNChS in spontaneously hypertensive rats, in which endothelial dysfunction has been well described before.** We focused on all cholinergic markers involved in the turnover of ACh at mRNA, protein, and activity levels.

The physiological function of BChE remains unknown, but recently published data provide evidence about its important role in lipid metabolism and obesity. Based on the high level of BChE in vessels that we observed, **our third aim was to examine the possible**

relationship between aortic BChE and increased lipid intake. We studied the impact of high-fat diet on clinical profiles of normotensive rats and SHR under selective BChE inhibition and without it. Additionally, the lipase activity of BChE hypothesized based on the described correlation between plasma BChE activities and TAG levels, was also examined.

METHODS

3.1 Materials

3.1.1 Chemicals

Unless specified, all the chemical reagents used in experiments were obtained from Sigma Aldrich or Thermo Fisher Scientific. The reference numbers are given in the text.

3.1.2 Purified enzyme

Purified human recombinant BChE enzyme - rhBChE 4Sugoff (7000U/ml, 10mg/ml) (Nachon et al., 2002) was used in the experiments. This enzyme was a gift from Prof. Oksana Lockridge, PhD. from Eppley Institute, University of Nebraska Medical Centre in Omaha, NE, USA. Dilutions of the enzyme were prepared in 0.1 M phosphate buffer pH=7.5 and stored at 4°C.

3.1.3 Antibodies

All primary antibodies (Ab) and their binding partners, which were used in experiments are listed in Table 1. The optimal antibody dilution was individually determined for each experiment to achieve the best staining with minimal non-specific background. Dilutions were performed in 1% animal serum/0.01 M phosphate buffer saline (PBS). Binding partners for primary antibodies were chosen depending on the host of primary antibodies, for example, if primary antibodies were raised in mouse, anti-mouse secondary antibodies were used.

Table 1: Primary antibodies and their binding partners used in the experiments

Type of antibody	Host	Producer	Dilution
Polyclonal anti-AChE	Rabbit	Gift from prof. Palmer Taylor	1:1000
Monoclonal anti-BChE (4H1)	Mouse	(Hrabovska et al., 2010)	1:100
Monoclonal anti-MLCK	Mouse	Abcam (ab203292)	1:100
Monoclonal anti-eNOS	Mouse	BD Biosciences (612393)	1 :100
Polyclonal anti-ChAT	Rabbit	Millipore (AB143)	1 :1000
Polyclonal anti-CrAT	Rabbit	Abcam (ab153699)	1 :1000
Polyclonal anti-ChT-1	Rabbit	ProteinTech (20747-1-AP)	1 :1000
Polyclonal anti-VACHT	Sheep	Abcam (ab31544)	1 :1000

Alexa Fluor® Anti-rabbit 488 (pre-adsorbed)	Goat	Abcam (ab150081)	1 :500
Alexa Fluor® Anti-rabbit 594 (pre-adsorbed)	Goat	Abcam (ab150088)	1 :500
Alexa Fluor® Anti-mouse 488 (pre-adsorbed)	Goat	Abcam (ab150117)	1 :500
Alexa Fluor® Anti-mouse 594 (pre-adsorbed)	Goat	Abcam (ab150120)	1 :500
DyLight Anti-rabbit 594	Donkey	Jackson ImmunoResearch (711-585-152)	1 :500
Alexa Fluor® Anti-sheep 488	Rabbit	Abcam (ab150181)	1 :500
Alexa Fluor® 488-conjugated streptavidin	n.a.	Jackson ImmunoResearch (016-540-084)	1 :500

3.1.4 Primers for RT-qPCR

All primers used in RT-qPCR analysis (Table 2.) were designed manually *de novo* using bioinformatic software Primer Blast (www.ncbi.com) or designed and characterized before in our laboratory (Krajčovičová, 2016; Piváčková and Křenek, 2012; Vetešková, 2014). The main criteria were: melting temperature 59-61°C, length of 18-24 base pairs, 40-60% GC-content and primer pairs should not have complementary regions. All of the primers were characterized in a positive control reaction (diluted 1:20, 1:40, 1:80, 1: 160 and 1: 320 in series) to give a single PCR product of defined size as verified with electrophoresis on 2% agarose gel and melting point. They were also tested for the absence of PCR product in the negative control reaction (RNase- and DNase-free water; RNA).

Table 2: Primer pairs used in experiments

Gene	Accession number (RefSeq)		Sequence	Length (bp)
AChE	NM_172009.1	forward	CAATTTTGCCCGCACAGGGGAC	110
		reverse	AAGGCTTCAGGTTTCAGGCTCACG	
BChE	NM_022942.1	forward	GGGCTGAGGAAATCTTTAGTCGA	95
		reverse	GGAGCCCGGAGTTTAGAGTTTA-3	
ChAT	NM_001170593.1	forward	AAATGGCGTCCAACGAGGAT	114
		reverse	CCCGGTTGGTGGAGTCTTTT	
CrAT	NM_017348.2	forward	ATTGTCGCTCTGTGGACC	145
		reverse	TCTGTTTGGCCTTCTCTATGTC	

ChT-1	NM_053521.1	forward	TCATCGTCAGCGACCTATGC	117
		reverse	TTGGTTCCAGTCTGTGGAGG	
VACHT	NM_031663.2	forward	CGACAAGTATCCCGAGGAGC	113
		reverse	CGAACTCGTAGAGGATGCC	
OCT-1	NM_012697.1	forward	GTATACGACACTCCCGGCTC	71
		reverse	TCCAATTTCCAGGCGTCTC	
OCT-2	NM_031584.2	forward	GAAAGTCGGTGCCGGTCTC	151
		reverse	AGAACAGAGCTCGTGAACCAAT	
OCT-3	NM_019230.1	forward	CATTACGGAACCTTTGGGGTTTCA	149
		reverse	AAGGCCACCACAGACAGATG	
OCTN-1	NM_022270.1	forward	GTCTGTGATGCTGTGGATGCTA	85
		reverse	CAGGTAGACATCTCCGTGCAA	
OCTN-2	NM_019269.1	forward	ATGGTCTATGTGTACACGGCC	167
		reverse	ATGGTCAGACTTCCCATGAGG	
ActB	NM_031144.2	forward	CCGCGAGTACAACCTTCTTG	81
		reverse	GCAGCGATATCGTCATCCA	
B2M	NM_012512.1	forward	ATGGAGCTCTGAATCATCTGG	105
		reverse	AGAAGATGGTGTGCTCATTGC	
e-NOS	NC_000071.6	forward	GGGTGATGAGCTCTGCGGCC	147
		reverse	TGGCGTTTCCAGCTGCGCTT	
PRiMA	NM_001108721.2	forward	GCGGTGGTCTGTGCCTCTCTG	116
		reverse	GGGGTACTCAGCTACGCTGGC	
L-Ca ²⁺ channel	NC_000072.6	forward	GTTGCCCTGGGTGTATTTTG	109
		reverse	GGCTTTCTCCCTCTCTTTGG	
m1AChR	NM_080773	forward	TGGCCTTCATCGGGATCACCACA	132
		reverse	AGGCCAGGCTCAGCAGGAAGTA	
m2AChR	NM_031016	forward	TGTGGTCGAGTGTGTCTTGGGGA	136
		reverse	ACGGAGCATGGGCGCAATGA	
m3AChR	NM_012527	forward	TCGGCAGACAAGACCACGGCTA	136
		reverse	TGGGCGGCCTTCTTCTCCTTGA	
m4AChR	NM_031547	forward	ACCAGCAGAGAAAGACAGAAGC	87
		reverse	TGCCATTGACAGGCGTGAA	
m5AChR	NM_017362	forward	ACAGCAAAGTCGATGAGGCA	109
		reverse	TGCTCTATGTTTGGCCTCCC	
nAChR α 1	NM_024485.1	forward	CCAGGAGTCTAACAAATGCGTC	128
		reverse	CAATGAGCCGACCTGCAAA	
nAChR α 2	NM_133420.1	forward	TCAATGTACACCACCGCTC	97
		reverse	CGGTTTCATCATCAGCCACC	
nAChR α 3	NM_052805.2	forward	GCTGGTGAAGGTGGATGAAG	129
		reverse	GCAGGAACACGCATGAACT	
nAChR α 4	NM_024354.1	forward	CTGACACGGGCAGTAGAAGG	84
		reverse	CCAGTCCTCCTTACCGAGA	
nAChR α 5	NM_017078.2	forward	TGTTTCGATAATGCAGATGGACG	71
		reverse	GACAGTGCCGTTGTACCTG	
nAChR α 6	NM_057184.1	forward	TCCTGGACTTACGACAAGGC	103
		reverse	AGGCATCGACAATTTCCAC	
nAChR α 7	NM_012832.3	forward	TGGATGTGGATGAGAAGAACCA	101
		reverse	TCCGGGGTACTCAGACATGT	
nAChR α 9	NM_022930.1	forward	GGAATCAGAGCCGTAGAGACA	147
		reverse	TATCTGGGAGAGCGTGACC	

nAChR α 10	NM_022639.1	forward	ACTTTACAACAAGGCGGACA	139
		reverse	AAAAGGGAAGGCAGACACGT	
nAChR β 1	NM_012528.1	forward	GCGCACTTGGATTTTGTTCCTTG	132
		reverse	GGACCTTACAGTAGCCAACAG	
nAChR β 2	NM_019297.1	forward	GACCAGAGTGTGAGGGAGG	149
		reverse	TGGAGGAAGGTAGTGGCAG	
nAChR β 3	NM_133597.1	forward	CTTTGAAAATGCTGACGGACG	107
		reverse	AGGAACTCTTGTAGCTGGCG	
nAChR β 4	NM_052806.2	forward	CCTCCTGAACAAAACCCGGT	120
		reverse	TGTTCTCGCTCATTCACTGA	
nAChR γ	NM_019145.1	forward	TGTGGTCGTGCTCAATGTGT	81
		reverse	GGAGTCTAACAATGCGTCTGAGG	
nAChR δ	NM_019298.1	forward	CTCTGAGCAGGTCCAACAGG	121
		reverse	GTTCCAGTTGTCCTTCTCCTCA	
nAChR ϵ	NM_017194.1	forward	ACCGCAGCTTTTACCGAGAA	128
		reverse	CGACGGATGATGAGCGTGTA	

3.2 Experimental animals

All experiments and methods were performed in accordance with the guidelines approved by State Veterinary and Food Administration of the Slovak Republic and the French Republic. Animals were purchased from Department of Toxicology and Laboratory Animals Breeding IEPT, CEM SAS (Dobrá Voda, SK CH 24011), placed to an enriched environment and kept under controlled environmental conditions (22–24°C and 12 h light/dark cycles), for at least 1 week before starting the experiments. Rats were given *ad libitum* access to food and water.

3.2.1 Analysis of cholinergic system in aorta

For analysis of the cholinergic system in aorta, 10-12 weeks old male mice (with B6D2 genetic background) or normotensive Wistar rats and SHR on standard diet (SD) were used. After 1 week of compulsory quarantine, the euthanasia of experimental rats was performed in CO₂ chamber, while mice were decapitated. The whole aorta was dissected, cleaned from blood and the fat tissue and further processed for biochemical analysis (see [3.3. Biochemical methods](#)), microscopy (see [3.4. Microscopy](#)) or RT-qPCR (see [3.5. RT-qPCR](#)).

3.2.2 Analysis of the effect of HF diet on cholinergic system

In pharmacological experiment, 24 male Wistar rats and 24 male SHR, 12 weeks of age were used. Animals in each strain were randomly divided into 4 groups (6 animals/group, 2 animals/cage): SD or high-fat (HF) diet group and treated or non-treated group. The composition (% energy) of the SD was 11% fat, 24% proteins, 65% carbs (3227 kcal/kg), and the composition of HF diet was 45% fat, 18% protein, 37% carbs (4497

kcal/kg). Animals were subjected to the experimental conditions for 13 weeks. Body weight was monitored weekly, food consumption each 3 days and water consumption every day. Animals were placed in pairs into the cages, while accurate individual records of consumed food and water were ensured by adding “buddy barriers” into cages enabling sufficient social contact between animals. This separation grid enabled social stimulation (visual, auditory, tactile and olfactory contact between the animals in cage), ensuring no stress was induced (Boggiano et al., 2008).

3.2.2.1 Pharmacological treatment

To inhibit the BChE activity in studied models, a selective inhibitor of this enzyme, tetraisopropyl pyrophosphoramidate (iso-OMPA) was used. This substance belongs to covalent inhibitors of ChE and it blocks the active site of ChE (Pohanka, 2011). To avoid stress caused by the application, iso-OMPA was administered in water. The administered concentration of this compound was set based on literature as 3 mg/kg/day (Gupta and Kadel, 1990; Gupta et al., 2000). To ensure that each animal received the right dosage, the water intake had been followed one week before the experiment to determine the habit of water drinking and controlled each 24 hours. The concentration of iso-OMPA was adjusted based on animal weight and on changed drinking habit caused by iso-OMPA. All the treated animals received each day **2,96 ± 0,0052 mg/kg/day**.

3.2.2.2 Studied end-points

At the end of the experiment, the animals were euthanized in CO₂ chamber. Biometrical parameters (weight, total body length, abdominal circumference) were determined. Subsequently, blood was collected from *vena cava inferior* in appropriate collection tubes to obtain serum and plasma (Monovette® K3-EDTA, Serum-Gel, Li-Heparin). Serum AChE and BChE activities were determined by Ellman's assay (see 3.3.2. Ellman's assay). Other biochemical parameters (alkaline phosphatase, alanine aminotransferase, aspartate transaminase, albumin, Ca, P, Cl, Mg, Na, K, glucose, Fe, creatinine, urea, creatine kinase, C-reactive protein) were determined in serum of the animals at the Department of Clinical and Experimental Pharmacotherapy of Slovak Medical School in Bratislava.

Collected tissue samples for mRNA analysis were flash-frozen in liquid nitrogen and stored at -80°C until RNA extraction (see 3.5. RT-qPCR). Samples for microscopy were

frozen in TissueTek® frozen section medium slowly in liquid nitrogen and stored at -80°C until further analysis as described below (see [3.4. Microscopy](#)).

3.3 Biochemical methods

3.3.1 Extract preparation

Tissue extractions were prepared in the presence of 5 volumes of ice-cold extraction buffer (10 mM HEPES buffer pH=7.4, 10 mM EDTA, 0.8M NaCl and 1% CHAPS) in pre-chilled 2-ml microfuge tubes. After tissue agitation (2.5 min, RT, 25 Hz), homogenates were centrifuged (10 min, 4°C, 14 000 x g). Supernatants containing the tissue extracts were immediately used in further experiments. The total protein quantification was done by using commercially available Pierce™ BCA Protein Assay Kit strictly following the manufacturer instructions.

3.3.2 Ellman's assay

Due to its simplicity and rapidity, Ellman's assay is a widely used method for determination of free sulfhydryl groups in samples in the presence of Ellman's reagent (5,5'-dithio-bis-(2-nitrobenzoic acid), DTNB) (Dingova et al., 2014). A solution of this reagent produces a yellow-colored product when it reacts with free sulfhydryl groups. This yellow-colored product can be quantified colorimetrically at 412 nm. Previous modifications enabled to use this method also for the determination of ChE activity. In this case, the hydrolytic cleavage of specific thio substrates of ChE, more precisely acetylthiocholine (ATC) and BTC generate the intermediate product with free thiol group, which can interact with DTNB. The conditions for detection of ChE activity in biological samples were optimized previously in our lab, allowing higher sensitivity and lower background (Dingova et al., 2014).

Samples were preincubated with 0.5 mM DTNB to block the free sulfhydryl groups for 20 minutes at RT. For determination of AChE activity, samples were further preincubated with a selective inhibitor of BChE activity, 1 mM iso-OMPA, for BChE activity with a selective inhibitor of AChE activity, 1 μM BW284C51 for 30 minutes. The standard composition of the reaction mixture in each well was: 0.5 mM DTNB, 5 mM HEPES (pH=7.4), 1 mM ATC or BTC. Enzyme activity in tissue extracts was detected each 30 second for 30 minutes in tissue extracts, while in gradients each 30 minutes for 5 hours in gradients (BioTek Synergy™ H4 Hybrid Multi-Mode Microplate Reader). All experiments were performed in triplicates and repeated at least three times.

3.3.3 Sucrose gradients

The method of sucrose gradient enables us to separate the macromolecules with different sizes and molecular weight. During the long-term, high-speed centrifugation, the macromolecules migrate through the sucrose gradient (which remains stable) at different rates depending on their molecular weight (M. Carr, 2000).

In our experiment, sedimentation standards (beta-galactosidase (16S) and alkaline phosphatase (6.1S)) were added to the extract (see [3.3.1. Extract preparation](#)) and applied on the top of 5-20% sucrose gradient (50 mM phosphate buffer pH 7.4, 0.8 M NaCl, 10 mM EDTA, 0.2% BRIJ 97 or 1% CHAPS). The change of the detergent enabled us to distinguish between the amphiphilic forms from non-amphiphilic forms of ChE. Gradients were centrifuged for 18 hours at 7 °C and 38 000 rpm in pre-chilled SW41 rotor (Beckman Coulter Optima LE-80K). Afterwards, they were distributed to 48 wells of the microtiter plate using an automatic pump (GILSON, MINIPULS 3) and fraction collector (GILSON, FC 2013B), while one fraction represented about 250 µl. Subsequently, the activities of internal standards were measured. To determine beta-galactosidase activity, 3.15% 2-nitrophenyl beta-D-galactopyranoside, and to determine alkaline phosphatase activity, 1.35% p-nitrophenyl disodium phosphate hexahydrate (in the presence of 50 mM phosphate buffer, pH 7.4) were used. ChE activities were measured using Ellman's method (see [3.3.2. Ellman's assay](#)). Results were corrected to the amount of protein in the sample. ChE and internal standard activities were converted to Svedberg units, and the present molecular forms of ChE were identified according to (Bernard et al., 2011) based on peak positions (Table 3). All the experiments were repeated three times.

Table 3: Molecular forms of ChE in detergent BRIJ

ChE	Position (S)	Molecular form
AChE	16.1	A ₁₂ - 3 tetramers AChE anchored by ColQ
	12.5	A ₈ - 2 tetramers AChE anchored by ColQ
	10.5	G ₄ ^{na} - non-amphiphilic AChE tetramers
	9.2	G ₄ ^a - amphiphilic AChE tetramers (PRiMA anchored AChE)
	4.5	G ₂ ^a or G ₁ ^{na} - amphiphilic dimers or non-amphiphilic monomers
	2.4	G ₁ ^a - amphiphilic monomers
BChE	12.5	G ₄ ^{na} - non-amphiphilic BChE tetramers
	12.5	G ₄ ^a - amphiphilic BChE tetramers (PRiMA anchored BChE)
	4.5	G ₁ ^a - amphiphilic monomers

3.4 Microscopy

3.4.1 Preparation of cryosections

At animal sacrifice, the whole aorta was dissected, perivascular fat tissue removed, and the aorta was divided into three parts (*arcus aortae*, *aorta thoracalis* and *aorta abdominalis*), embedded in Richard-Allan Scientific™ Neg-50™ Frozen Section Medium. Rings of aorta, 10 μm-thick, were prepared and stored at -80°C until use.

3.4.2 Tsuji's activity staining

Tsuji's staining protocol is one of the most frequently used methods for ChE activity visualization in various tissues. It belongs to the so-called “thiocholine methods”, which use different thiocholine esters as substrate (Tsuji and Larabi, 1983). It is based on the histochemical method originally introduced by Koelle & Friedenwald (Koelle and Friedenwald, 1949), optimized later by Karnovsky (Karnovsky and Roots, 1964). Up to date, there are different modifications of this protocol, where the type of buffer and the concentration of Cu²⁺ ions is optimized depending on the studied tissue. The main principle is the same in all modifications though: ChE mediate the hydrolysis of ester bonds in the thio substrates and the final product (Cu²⁺ salt) of this multi-step mechanism is detectable with a light microscope (the mechanism of this reaction is schematically illustrated in Figure 13.). Stability of the final product is ensured by the presence of buffer. The incubation time,

type and pH of the buffer should be individually optimized for each type of experiments. This method has several advantages, such as ability to detect low enzyme activities are detectable, free thiol groups of tissues do increase the background, a direct proportionality of the product amount of product is directly proportional to the enzyme activity (Tsuji and Larabi, 1983).

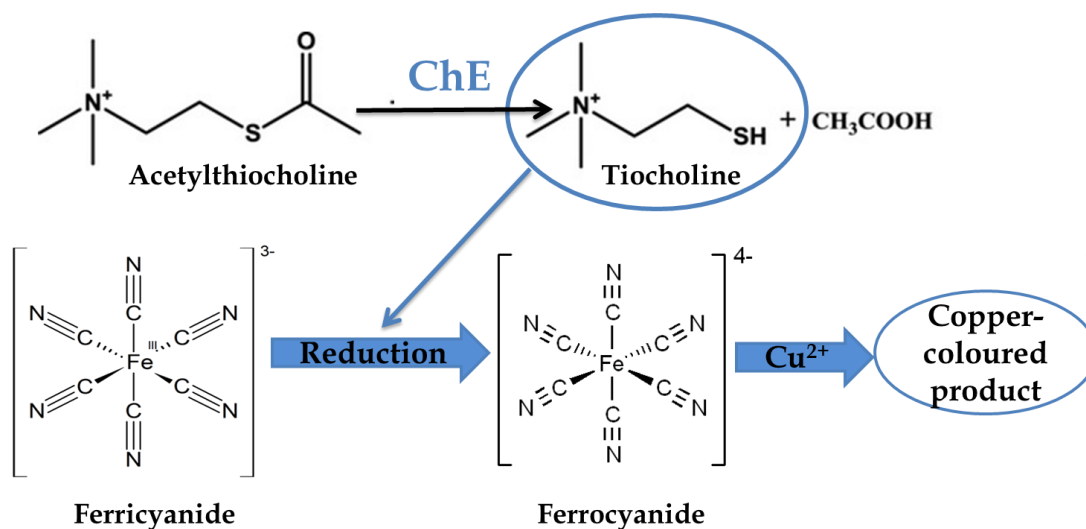


Figure 13: Schematic figure illustrating the mechanism of detection ChE activity by Tsuji's staining protocol (according to Tsuji & Larabi, 1983)

In our experiments, tissue sections were fixed in 4% paraformaldehyde for 15 min at room temperature. Subsequently, slides were pre-incubated for 30 min with inhibitors of ChE (1 mM iso-OMPA for determination of AChE activity, 1 μ M BW284C51 for determination of BChE activity). The optimal concentration of inhibitors was determined by Ellman's assay in our previous experiments (Szmicseková, 2015). In this experiment, the positive controls were incubated without any inhibitors, while the negative controls were stained with solution containing inhibitors for both ChE. This step was followed by incubation of slides in the staining solution for 3 hours (BChE activity) and for 6 hours (AChE activity). The content of the incubation solution is summarized in Table 4. The staining solution incubation medium was prepared freshly before use and the incubation was performed at room temperature in the dark. After incubation, slides were washed in 15 mM citrate buffer (3x5 min), dehydrated in graded alcohol (with 50 % - 70 % - 100 % ethanol) (2x5 min), washed in xylene substitute (2x5 min) and mounted with coverslips with Eukitt[®] medium. To compare the ChE activity in the aorta of normotensive rats and SHR, the

same procedure was performed simultaneously for both strains. Images were captured by using a light microscope (Olympus BX61). All experiments were repeated at least three times.

Table 4: Composition of the Tsuji's staining solution

	AChE activity	BChE activity	Positive control	Negative control
Citrate buffer pH=5.5	15 mM	15 mM	15 mM	15 mM
CuSO₄	2.8 mM	2.8 mM	2.8 mM	2.8 mM
K₃[Fe (CN)₆]	0.47 mM	0.47 mM	0.47 mM	0.47 mM
Iso-OMPA	10 ⁻³ M	-	-	10 ⁻³ M
BW284C51	-	10 ⁻⁶ M	-	10 ⁻⁶ M
ATC	1.7 mM	1.7 mM	1.7 mM	1.7 mM

3.4.3 Immunohistochemistry

Dual immunohistochemistry staining is a quite frequently used detection method for protein visualization. It is based on immunological reaction antigen ↔ Ab. The conditions (such as fixation time, permeabilization method, incubation time with Ab, etc.) should be optimized for all types of experiments (Chen et al., 2010).

The tissue sections were fixed with 4 % paraformaldehyde for 20 min. Then, the sections were washed in 50 mM solution of glycine, which binds free aldehyde groups. Aldehyde groups can bind both, primary and secondary Ab, leading to false-positive results. To avoid non-specific binding and high background, further blocking step was needed. Solution of 5 % normal serum of the animal, in which the secondary Ab were raised was used (Chen et al., 2010; Hockfield, 1993). After that, slides were washed in PBS three times for 5 min. This step was followed by incubation with primary Ab for 2 hours at RT. In double staining immunohistochemistry, it is necessary to use primary Ab from different species (Chen et al., 2010). This step was followed by several washings with PBS. Subsequently, slides were incubated with secondary Ab or streptavidin conjugated with fluorescent dyes, which should have minimal cross-reactivity with other species for 30 minutes at RT and protected from the light. All the binding partners used in experiment were made in the same animal to avoid cross-reacting and false-positive results (Chen et al., 2010; Hockfield, 1993). Lastly, slides were washed in PBS and coverslipped with Fluoromount™ Aqueous

Mounting Medium or Fluoroshield™ with 4, 6-diamidino-2-phenylindole. Images were captured by using a fluorescent microscope. All experiments were repeated at least three times.

3.5 RT-qPCR

One of the most frequently used method for the gene expression analysis is the method of real-time quantitative reverse transcription (RT-qPCR) (Bustin et al., 2009; Holland et al., 1991; Kubista et al., 2006). Using species-, and gene-specific primers one can simply assess the mRNA levels of different enzymes, transporters, receptors in various types of tissues (Bustin et al., 2009; Holland et al., 1991). Despite the fact, that mRNA levels do not predict accurately the protein levels in studied tissues all the time (Fortelny et al., 2017), the importance of this technique in molecular biology is not questionable.

3.5.1 Preparation of the endothelial and smooth muscle fractions by Tri-Reagent Perfusion

In order to distinguish between expression of a particular mRNA in vascular endothelium and smooth muscle, a specific method enabling tissue fractionation was developed at our department (Krenek et al., 2006). Briefly, 3 mm ring of aorta thoracalis with intact endothelium was immediately frozen in liquid nitrogen (AoE+). The rest of the aorta was tied by one end to a pipette tip, fitted to a syringe fixed vertically on a stand. After a flushing with heparinized-Krebs solution (1U/ml, pH=7.4), the aorta was filled *via* the syringe with 200 µl of Tri-Reagent and the solution was kept inside the aorta for 20 seconds. The aorta was then flushed twice with 200 µl of Tri-Reagent. The total volume collected in the first tube (E1) was thus 600 µl. Second and third fractions (E2, E3) were obtained the same way. Subsequently, the aorta was quickly opened longitudinally, the rest of endothelium form the luminal face was gently wiped with a cotton swab and the tissue (AoE-) was immediately frozen in liquid nitrogen until RNA isolation. The origin and purity of the collected fractions were verified by the determination of relative expression of endothelium-specific gene (e-NOS) and smooth muscle-specific gene (L-type calcium channel).

3.5.2 RNA isolation from other tissues

Total RNA from all tissues or fractions was isolated by the method of phenol-chloroform extraction. A frozen tissue sample (100 mg) was homogenized in liquid nitrogen. Subsequently, 500 µl of Tri-Reagent was added and the sample was thoroughly homogenized with Tri-Reagent until became a fine powder. The fine powder was carefully

transferred to a 2 ml Eppendorf tube, 1000 μ l of Tri-Reagent was added, and the open tube was placed in a refrigerator, till the remaining samples got processed in a similar manner. After that, the samples were vortexed, and the remaining pieces of tissue disrupted by repeated pipetting (20-30 times). Samples were centrifuged for 10 minutes at 12 500 x g and 4°C, (Hettich Micro 200R, Tuttlingen, Germany). The supernatant was transferred into another 2 ml tube, and 300 μ l of chloroform was added (Centralchem, Bratislava, Slovak Republic) and the samples were shaken dynamically for 30 seconds. The samples were incubated for another 15 minutes at RT and then centrifuged for 20 minutes at 12 500 x g and 4° C. After removal from the centrifuge, the aqueous phase with dissolved RNA was carefully collected into a clean tube on ice. To precipitate the dissolved RNA, isopropanol was added in a 1: 1 ratio. Subsequently, the samples were mixed, incubated for 15 minutes at RT and centrifuged for 30 minutes at 12 500 x g, 4 ° C. The supernatant was decanted, and the pellet was washed twice with 75-80% ethanol (Merck, Darmstadt, Germany). After that, the pellet was dried and dissolved in RNase- and DNase-free water (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA) by repeated pipetting. Dissolved RNA was stored at -80°C.

As aorta belongs among the tissues with lower yields, so the protocol needed to be modified: the first volume of Tri-Reagent (300 μ l) was added to the liquid nitrogen directly with the sample to prevent evaporation of the sample together with nitrogen, the second volume was 700 μ l. Also, 2 μ l of glycogen (5 mg/ml, Roche, Basel, Switzerland) was added to the collected aqueous phase with dissolved RNA on ice for better visibility of the pellet formed by isopropanol precipitation.

3.5.3 Determination of concentration and quality verification of isolated RNA

The concentration of isolated RNA was determined spectrophotometrically (BioTek Synergy™ H4 Hybrid Multi-Mode Microplate Reader). After adjusting the blank value by measuring RNase- and DNase-free water (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA), 2 μ l of the measured sample were applied. The software automatically calculated the sample concentration (ng/ μ l) as well as both absorbance ratio values 260 nm/280 nm and 260 nm/230 nm, which indicate the RNA purity. The quality of isolated RNA was also verified by the method of electrophoresis on 2% agarose gel.

3.5.4 Transcription to cDNA

The transcription of the samples was performed using commercially available kit (High-Capacity cDNA R transcription kit, Thermo Fisher Scientific, Carlsbad, CA). The same concentration of RNA was used in each reaction (aorta fraction 45 ng/μl, other samples of 100 ng/μl). First, we prepared the RT-Master Mix from the individual components following the manufacturer's instructions. Subsequently, 10 μl of RT-Master Mix and 10 μl of diluted sample was mixed for transcription. Samples were placed in thermocycler (Biometra Personal Cycler, Biometra, Göttingen, Germany) under reaction conditions: 10 minutes at 25°C, 120 minutes at 37°C and 5 seconds at 85°C. After completion of the reaction, samples of the resulting cDNA were diluted with RNase- and DNase-free water (control organs 1:50, whole aorta and fractions 1:20) and stored at -20° C. RT-qPCR was performed using commercially available kits (SYBR® Select Master Mix, Applied Biosystems, Thermo Fisher Scientific, Carlsbad, CA).

3.5.5 RT-qPCR reaction

Five μl of the diluted cDNA sample and 10 μl of the Master Mix (F and R primers were used at concentration of 0.2 μM) were pipetted into clean tubes. The reaction was performed in a StepOnePlus Real-Time PCR System and Quant Studio 3 (Thermo Fisher Scientific, Carlsbad, CA) under the conditions described in Table 5.

Table 5: Conditions for RT-qPCR reactions

	Temperature	Duration	Number of cycles
UDG activation	50°C	2 min	1x
DNA polymerase activation	95°C	2 min	1x
Denaturation	95°C	15 sec	40x
Annealing	60°C	15 sec	
Polymerization	72°C	45 sec	

3.5.6 Data analysis

Quantification of Cq values and amplification efficiencies (Ruijter et al., 2009) for each RT-qPCR reaction were determined using LinRegPCR software (version 2017.1). The expression of target genes was normalized to the geometric mean of expression housekeeping genes β2-microglobulin (B2M) and β-actin (ActB).

3.6 Lipase activity of BChE

In order to examine hypothetical lipase activity of BChE, commercially available kits were used. Procedures were performed according to the manufacturer instructions but optimized for BChE in our laboratory. The reactions were optimized in regard to the buffer, pH, temperature, and substrate and enzyme concentrations (see below). Two synthetic TAG-like substrates were used to examine BChE lipase activity, a short thioester and a longer ester resembling endogenous TAG. All experiments were performed in duplicates and repeated at least three times.

3.6.1 Lipase activity assay kit (MAK047)

The lipid activity was determined based on the principle of Ellman's assay (Ellman, 1959). The reaction was stoichiometric, thus the concentration of final product was directly proportional to the lipase activity. The kit utilized a short synthetic TAG with two thioester moieties - 2, 3-dimercapto-1-propanol tributyrate (DMPTB) (a concentration not provided by the manufacturer) as a substrate (Figure 14). The intermediate product of its cleavage (based on the free SH groups) reacted with DTNB (a concentration not provided by the manufacturer), and the final product was detected colorimetrically at wavelength 412 nm (BioTek SynergyTM H4 Hybrid Multi-Mode Microplate Reader).

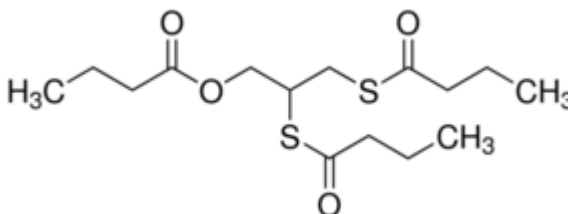


Figure 14: Chemical structure of DMPTB (MAK047)

BChE was tested in approximate human plasmatic concentration (3 µg/ml; (Browne et al., 1998)) and higher concentrations (5, 10, 15 µg/ml). As for the substrate, a concentration recommended by the producer and a double amount of substrate was used in the study.

3.6.2 Lipase activity assay kit (MAK048)

The determination of lipase activity in this kit was based on the cleavage of ester bonds of a larger synthetic substrate, chemical structure of which was close to naturally occurring endogenous TAG (Figure 15). The final product – methylresorufin was detected

fluorometrically at the wavelength λ_{ex} 529 nm/ λ_{em} 600 nm (BioTek Synergy™ H4 Hybrid Multi-Mode Microplate Reader). The standard curve was generated from standard solution of methylresorufin. The determined fluorescent intensity was directly proportional to the enzymatic activity. Lipase activity of BChE was tested at several concentration (3, 5, 10, 15 $\mu\text{g/ml}$) with substrate concentrations recommended by the manufacturer as well as with a half and double amount of the substrate. The changes in reaction included the type of buffer (HEPES/PBS), pH of the buffer (pH=3,5-11,5), temperature of the reaction (RT, 37°C).

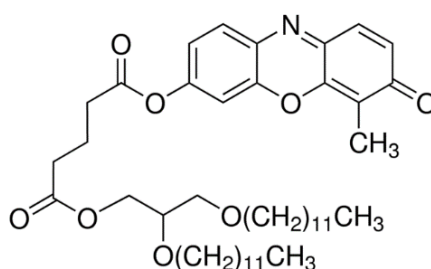


Figure 15: Chemical structure of synthetic TAG (MAK048)

3.7 Statistical analysis

All results are expressed as mean values \pm SEM. Shapiro-Wilk test was used for testing the normality of data. Two-group comparisons were conducted with a two-tailed t-test (normal distribution of data) or Mann-Whitney test (not normal distribution of data), and $P < 0.05$ was considered significant. Difference among three or more groups were tested using one-way or two-way analysis of variance ANOVA (normal distribution of data) with Tukey post-hoc test or Kruskal-Wallis test (not normal distribution of data) with Dunn post-hoc test. $P < 0.05$ values were considered significant. Outliers were identified by Grubb's test. All statistical analyses were performed in GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

4.1 Cholinergic system in rat aorta

We aimed to study the cholinergic system in rat aorta. We examined the presence of the cholinergic markers throughout the whole tissue, divided into *arcus aortae*, *aorta thoracalis* and *aorta abdominalis*. Transversally, we studied their localization in the endothelium and the smooth muscle. Our main aim was a throughout analysis of the ACh degradation enzymes - AChE, and BChE. Further, we addressed our hypothesis about the existence of aortic NNChS, thus we assessed the presence of the transporter and enzymes of ACh life cycle.

4.1.1 Cholinesterases in aorta

4.1.1.1 Detail analysis of cholinesterases in different parts of aorta

First, we assessed the relative expression of ChE in different parts of the aorta by RT-qPCR method. Based on the Cq values, BChE expression level exceeded AChE in each part of rat aorta. The mRNA level of AChE was the highest in *arcus aortae* (Figure 16A). Relative expressions in two descending parts of aorta were comparable, however, trend of higher mRNA levels in *aorta abdominalis* was observed. Relative expression of BChE was the lowest in *aorta thoracalis*, representing about half of the expression levels observed in other two parts of the aorta (Figure 16B).

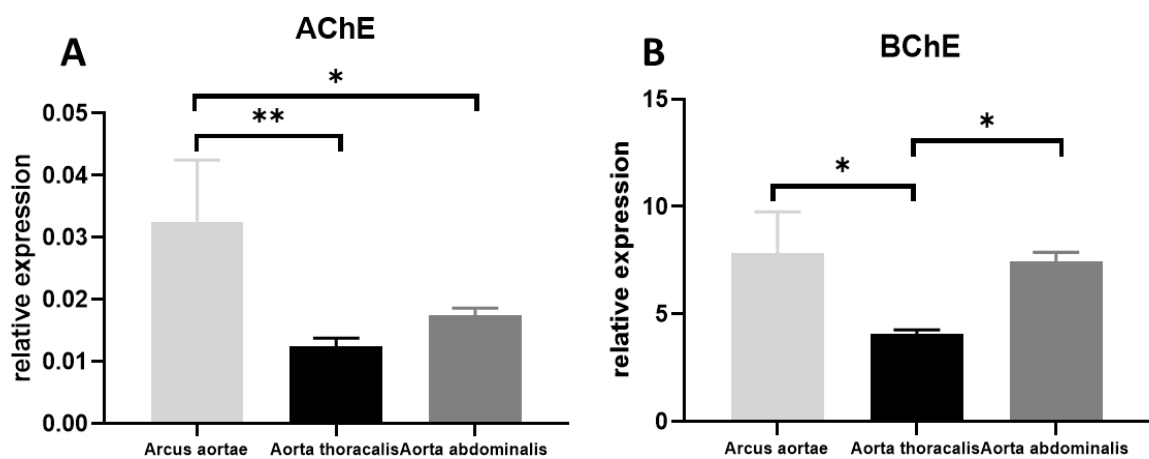


Figure 16: Relative expression of ChE in different parts of aorta. The relative expression of BChE exceeds AChE. The mRNA level for AChE was the highest in *arcus aortae* (A). The relative expression of BChE was comparable in *arcus aortae* and *aorta abdominalis* and significantly lower in *aorta thoracalis* (B). Data are presented as mean \pm SEM, $n = 4-6$.

Subsequently, ChE activities in tissue extracts (containing the same amount of the total protein) were determined by Ellman's assay. In agreement with results from RT-qPCR, rat aorta showed higher BChE activity than AChE activity. AChE activities were decreasing in the following order: *arcus aortae* > *aorta abdominalis* > *aorta thoracalis* (Figure 17A). BChE activity in *arcus aortae* was approximately twice the activity in other studied parts (Figure 17B).

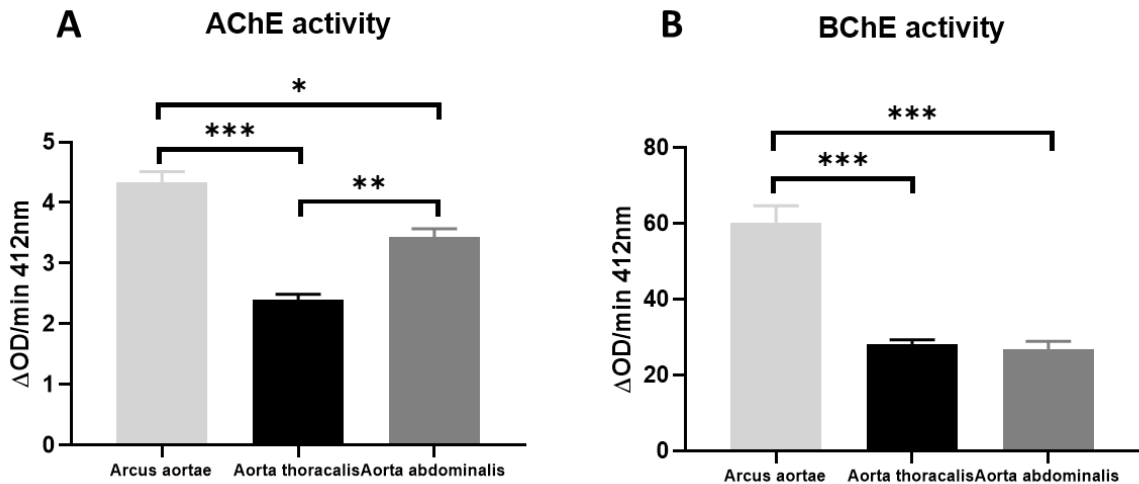


Figure 17: ChE activities in different parts of aorta measured by Ellman's assay. BChE activity was several times higher than AChE in each part of rat aorta (B). The highest AChE activities were determined in *arcus aortae*, while the lowest in *aorta thoracalis* (A). BChE activity was comparable in *aorta thoracalis* and *aorta abdominalis* (B). Data are presented as mean \pm SEM, $n = 4-6$.

Further on we separated the molecular forms of ChE in different parts of aorta, using the method of sucrose gradients. Amphiphilic molecular forms were distinguished from the non-amphiphilic forms by changing the detergent. There was an interaction of these forms with BRIJ detergent, which resulted in a shift in the position of peak compared to detergent CHAPS.

In each part of the aorta, AChE activity was distributed into 2 peaks (Figure 18A-C). The first peak was detected around 2.5S (BRIJ) indicated the presence of amphiphilic monomers. Another peak detected around 9.2S (BRIJ) corresponded to the PRiMA anchored AChE. Two types of molecular forms of BChE were detected (Figure 18D-E). First, the

position around 4.5S indicated the presence of amphiphilic monomers, while the second peak around 9S the presence of G4a form, which is known as PRiMA anchored BChE.

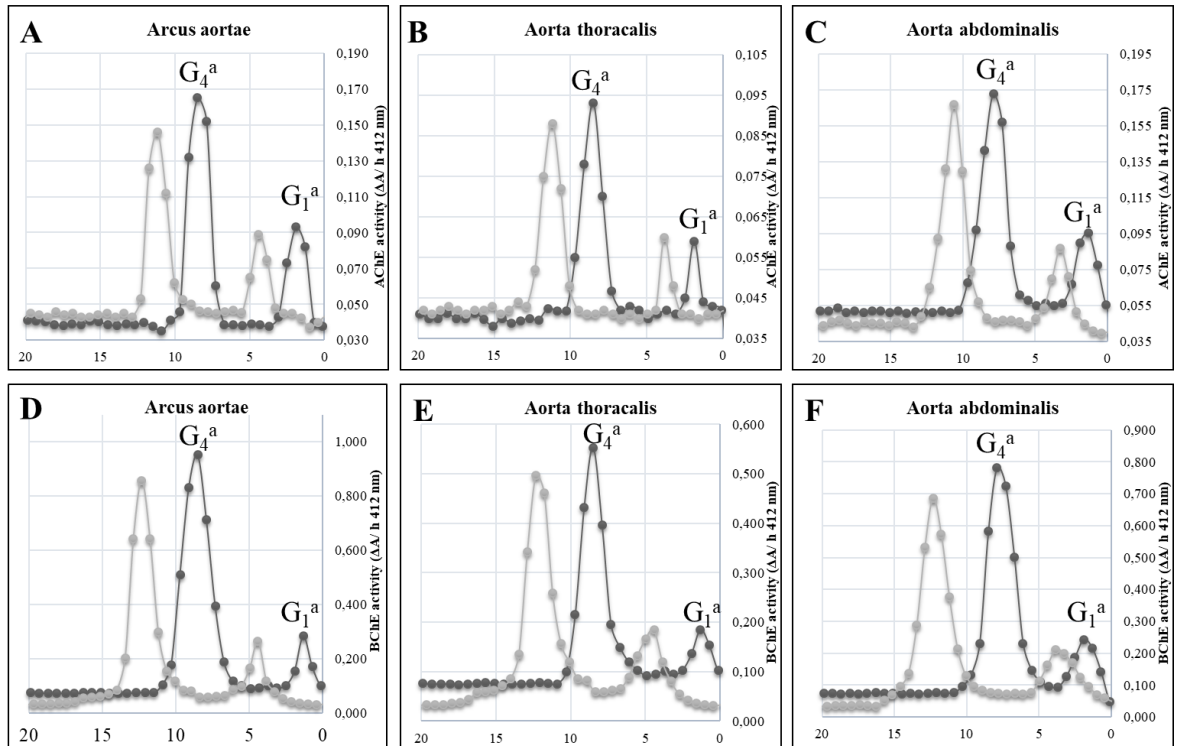


Figure 18: Molecular forms of AChE and BChE in different parts of aorta of normotensive rat. In each part of aorta, we detected PRiMA anchored forms of both AChE (A-C) and BChE (D-F). The minor part of ChE was present in form of amphiphilic monomers. The amphiphilic forms were distinguished from non-amphiphilic form by changing the detergent (\bullet BRIJ, \circ CHAPS).

4.1.1.2 Localization of cholinesterases within aortic tissue

ChE proteins were visualized in tissue sections from thoracic aorta using immunohistochemistry. The presence of AChE was undetectable by immunohistochemical visualization (data not shown), while BChE provided a strong fluorescent signal (Figure 19A, D). In agreement with our previously presented results, BChE exceeded AChE in rat aorta. For precise localization within aorta, co-localization with the marker of endothelium (e-NOS, Figure 19E) and the marker of smooth muscle (anti-MLCK, Figure 19B) was performed. Results revealed that BChE is present in the smooth muscle (Figure 19C) and partially in endothelium (Figure 19F). However, the fluorescent signal localized to the smooth muscle was much stronger, than endothelium.

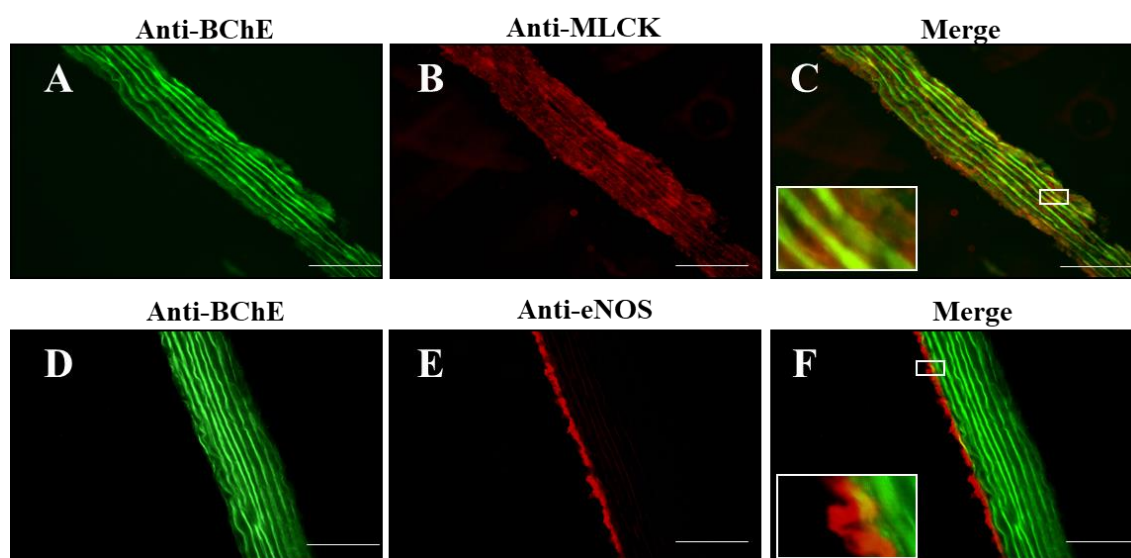


Figure 19: Distribution of BChE in aorta. The protein of BChE (A, D) was colocalized with the marker of smooth muscle (C) and partially with the marker of endothelium (F) confirming the presence of the enzyme along the whole aorta. Scale bar 50 μ m.

Presence of both ChE in rat aorta was also confirmed by activity staining performed according to Tsuji (Tsuji and Larabi, 1983). A series of optimization experiments were conducted to determine the optimal staining conditions. The incubation times and the pH of the citrate buffer seemed to be the most crucial steps, with optimum at pH=5.5 and 2h incubation for BChE and 6 h incubation for AChE activity detection. Detected AChE activities (Figure 20A-C) were much lower, than BChE activities (Figure 20D-F) confirming the previous observations, that the dominant ChE in all parts of the aorta is BChE (Figure

20D-F). Surprisingly, the activity of BChE did not seem to be consistent, rather it was gradually increasing from the endothelium towards the smooth muscle (Figure 20D-F).

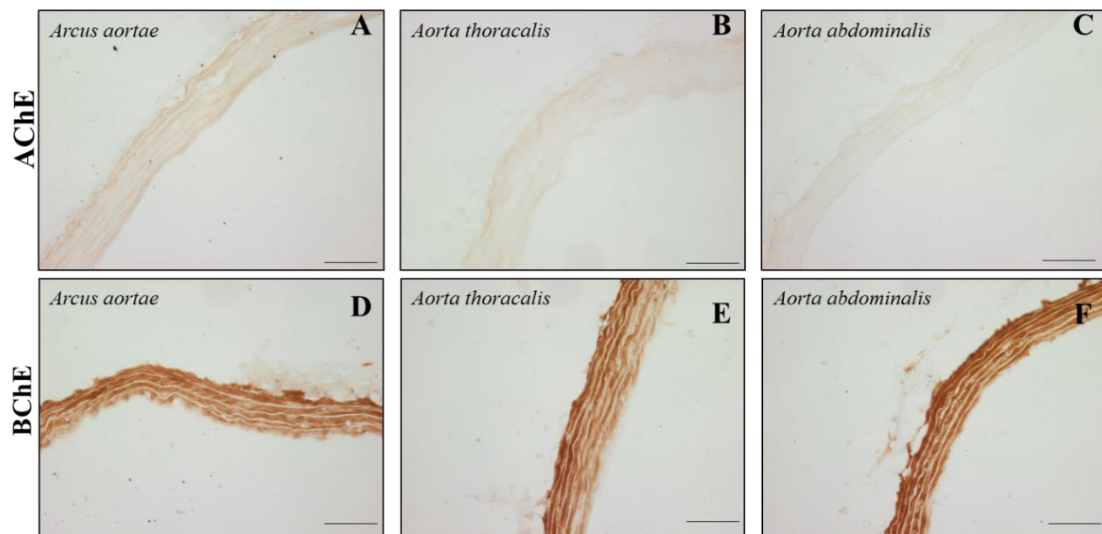


Figure 20: *Tsuji's activity staining in different parts of Wistar rat. Coloration in samples for AChE activity was several times weaker (A-C), than for BChE (D-F) indicating BChE is the dominant enzyme in each part of aorta. Scale bar 50 μ m.*

To confirm this observation, we examined BChE expression within the tissue, employing a technique developed at our department, by which we obtained the fractions of aorta corresponding to the endothelium (E1-3) and to the smooth muscle (AoE-). Indeed, the relative expression of BChE was showing increasing tendency from E1 to E3, while in agreement with our previous observation, the highest mRNA level of BChE was detected in AoE-, exceeding BChE expression in E3 more than 8-times (Figure 21).

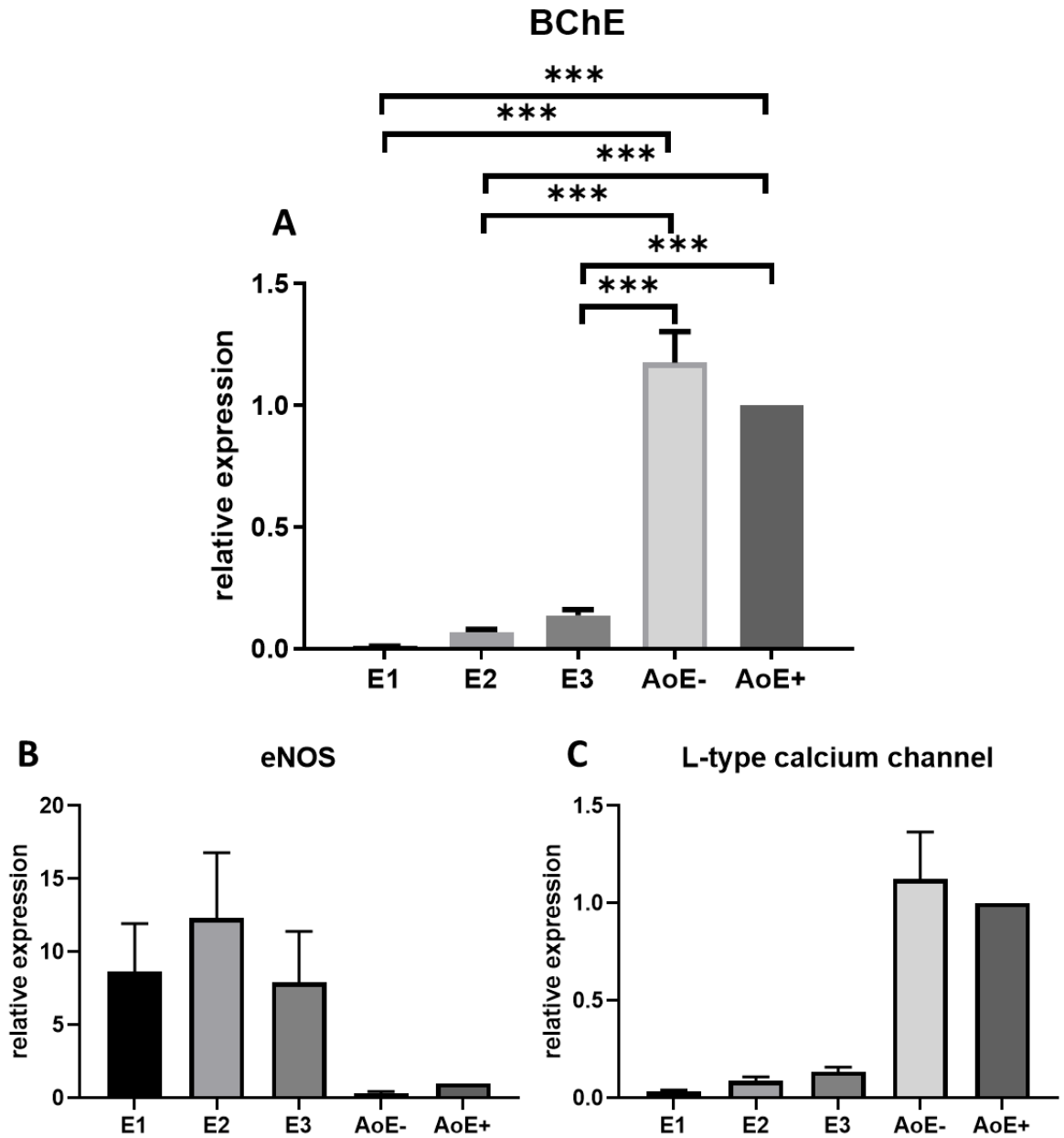


Figure 21: Relative expressions of BChE in fractions of aorta. The mRNA level of BChE (A) was several times higher in smooth muscle (AoE-), than in endothelium (E1-E3). The relative expression of endothelial marker, eNOS (B) and the relative expression of smooth muscle marker, L-type calcium channel (C) confirmed the purity of aortic fractions. Data are presented as mean \pm SEM, $n = 7-8$.

4.1.1.3 Cholinesterases in mouse aorta

Our results from the rat aorta have led us to a hypothesis that high BChE activity in rat vessels may compensate for negligible BChE activity in blood. In contrary to rat, the serum BChE activity in mice is considerably higher than in rats, while there is a lack of information about its levels in the aorta. Thus, in line with our hypothesis we expected BChE activity in mouse aorta to be low. We performed the Tsuji's activity staining protocol to determine the ChE activity in *aorta thoracalis* of mice, while the conditions and the complete staining protocol were the same as for the rat aorta.

Indeed, in contrast to rats, where we detected low AChE and substantial BChE activities, *aorta thoracalis* of mice showed very low levels of both, AChE (Figure 22A) and BChE (Figure 22B) activities.

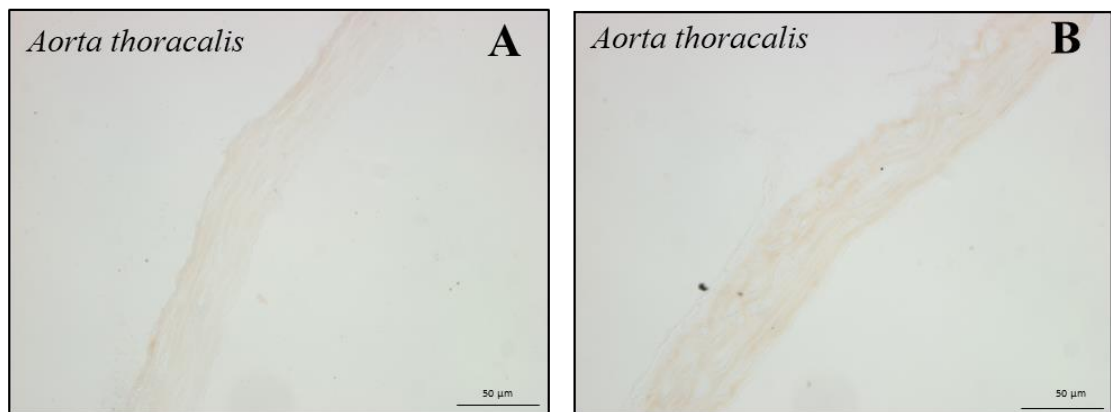


Figure 22: Cholinesterases in mouse aorta. Tsuji's activity staining revealed low activity of both AChE (A) and BChE (B). Scale bar 50µm.

4.2 Other proteins and transporters involved in ACh life cycle

First, we tested the presence of ChAT, enzyme primarily responsible for ACh synthesis. We did not detect ChAT at mRNA level in whole aorta (AoE+), endothelial fractions (E1-E3), or residual smooth muscle (AoE-) (data not shown). Also, in immunohistochemistry, we did not detect any fluorescent signal for ChAT in endothelium (Figure 23A, C) or smooth muscle (Figure 23D, F). These results indicate that ChAT is completely missing in the aorta and ACh synthesis is not catalyzed by ChAT.

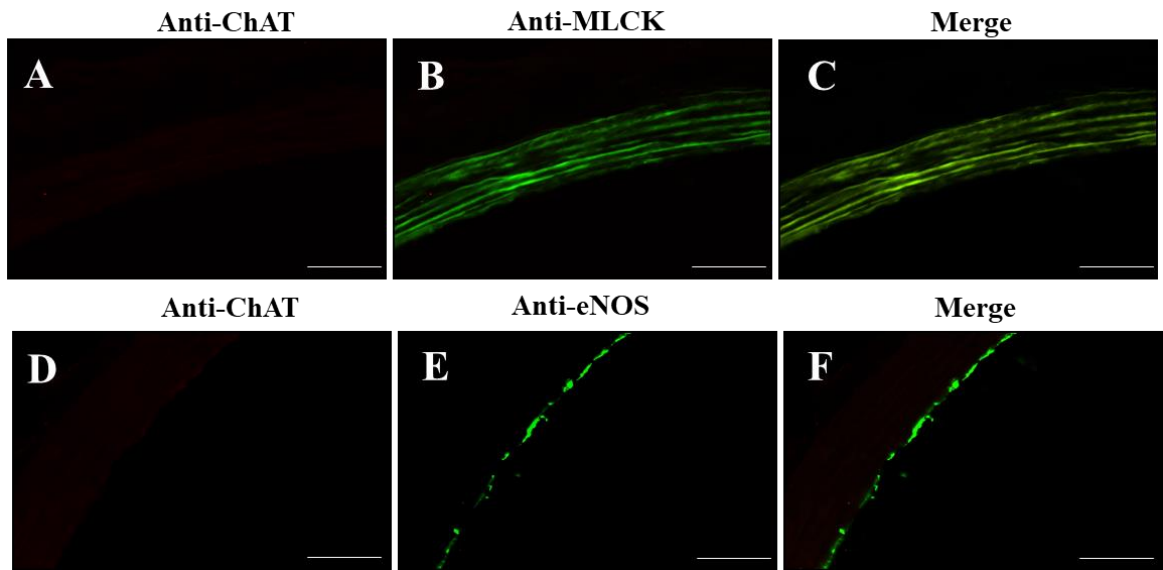


Figure 23: Immunohistochemical detection of ChAT. No signal was detected for the protein ChAT (A, D) while the marker of smooth muscle (B) and the marker of endothelium (E) gave the correct signal. Scale bar 50 μ m.

However, we confirmed the presence of the alternative ACh synthesizing enzyme – CrAT in all studied fractions (Figure 24). In endothelial fractions, the mRNA relative expressions were increasing from E1 to E3, and the highest value was reached in smooth muscle (where the relative expression was almost 2.5 times higher).

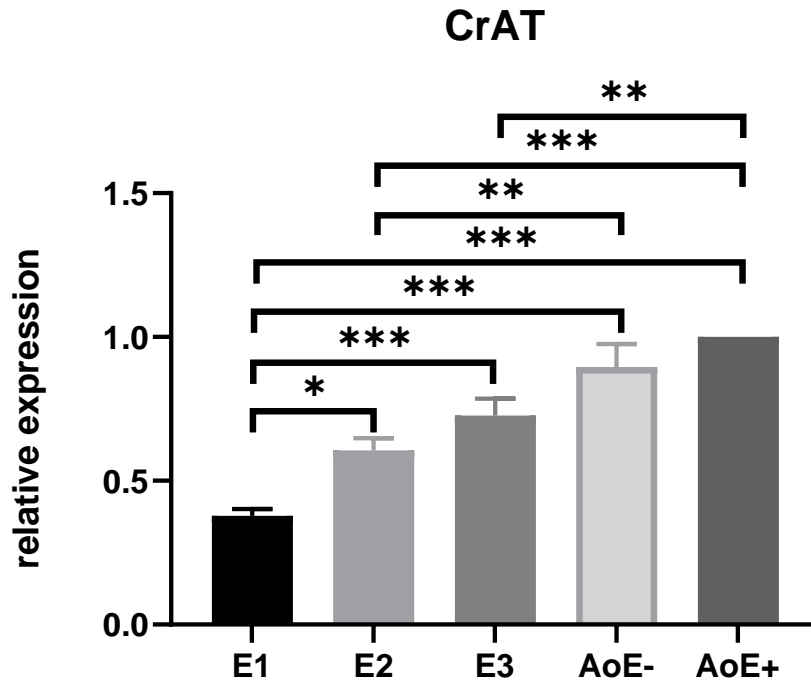


Figure 24: Relative expressions of CrAT in fractions of aorta. The presence of CrAT was confirmed by RT-qPCR both in endothelium and smooth muscle. Data are presented as mean \pm SEM, $n = 7-8$.

Immunohistochemistry confirmed the results from RT-qPCR. The protein CrAT (Figure 25A, D) was present throughout the whole tissue as documented by colocalization with the marker of smooth muscle (anti-MLCK) (Figure 25A-C) and with the marker of endothelium (anti-eNOS) (Figure 25D-F).

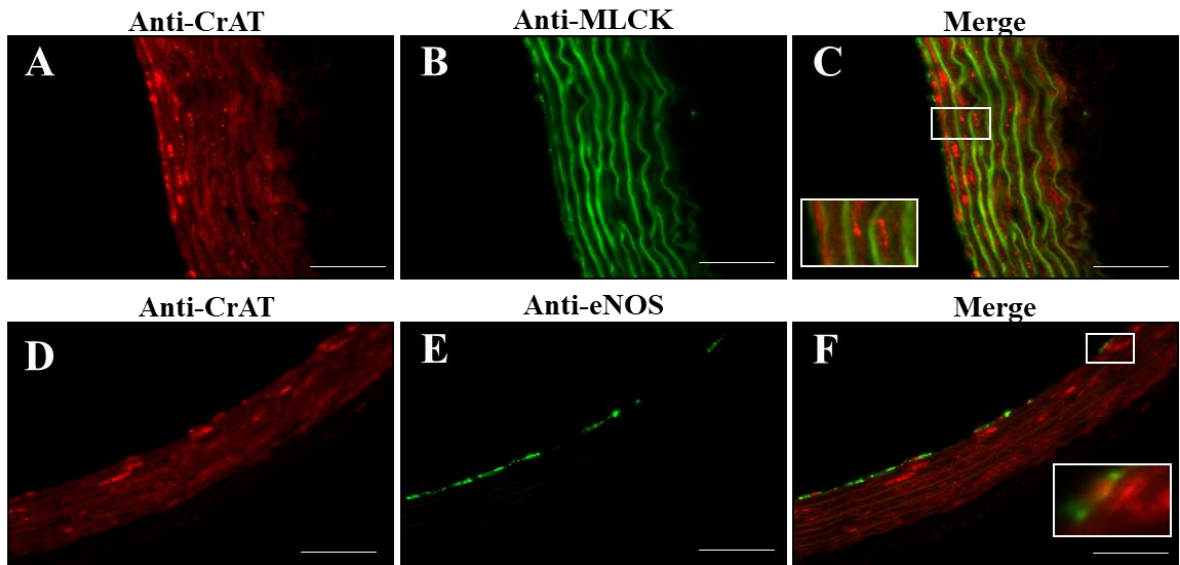


Figure 25: Immunohistochemical detection of CrAT in aorta. The protein was colocalized with the marker of smooth muscle (A-C) and also with the marker of endothelium (D-F). Scale bar 50 μ m.

The relative expression of VACHT was low in E1 and E2 fractions, but significantly higher in E3 fraction and AoE- fraction (Figure 26), meaning that VACHT was expressed both in endothelium and smooth muscle (Figure 26).

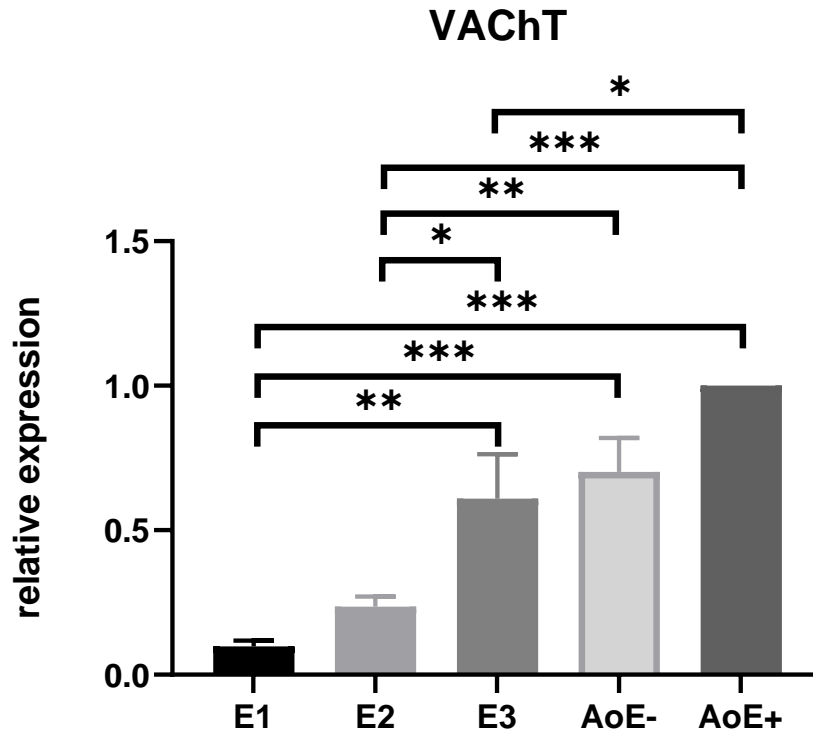


Figure 26: Relative expression of VACHT in fractions of aorta. mRNA levels of VACHT were detected in smooth muscle and also endothelium. Data are presented as mean \pm SEM, $n = 7-8$.

The presence of VACHT in the aorta, was also confirmed at protein levels (Figure 27A, D). The protein was present both in smooth muscle (Figure 27A-C) and endothelium (Figure 27D-F).

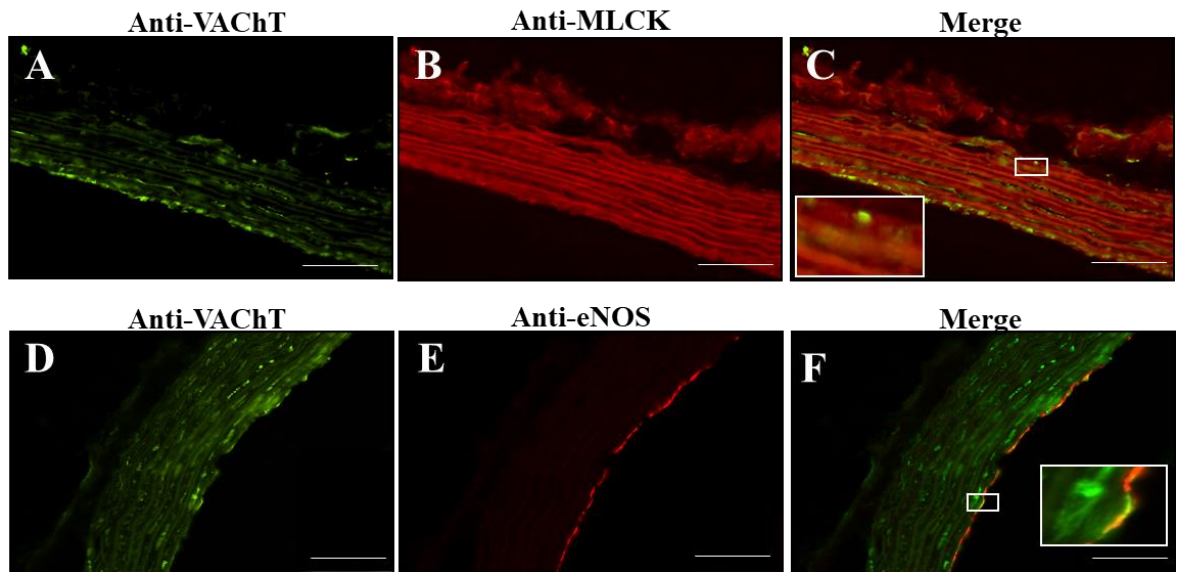


Figure 27: Immunohistochemical detection of VACHT. The protein was co-localized with the marker of endothelium (D-F) and also smooth-muscle (A-C). Scale bar 50 μ m.

The mRNA levels of ChT-1 were significantly higher in AoE- comparing to any of the endothelial fractions, while no significant differences were observed in relative expressions of ChT-1 among E1-E3 (Figure 28). Based on these observations, we can conclude, that this protein was ubiquitously present in the whole aorta, with dominant localization in the smooth muscle.

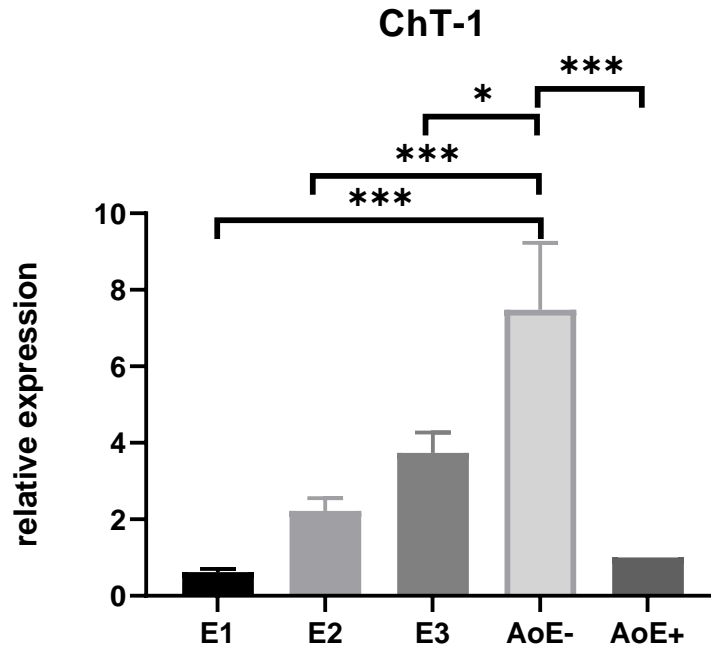


Figure 28: Relative expressions of ChT-1 in fractions of aorta. Endothelium and smooth muscle expressed this transporter. Data are presented as mean \pm SEM, $n = 7-8$.

Immunohistochemistry confirmed the results obtained by RT-qPCR, the protein (Figure 29A, D) was colocalized with the marker of smooth muscle (Figure 29A-C) and endothelium (Figure 29D-F).

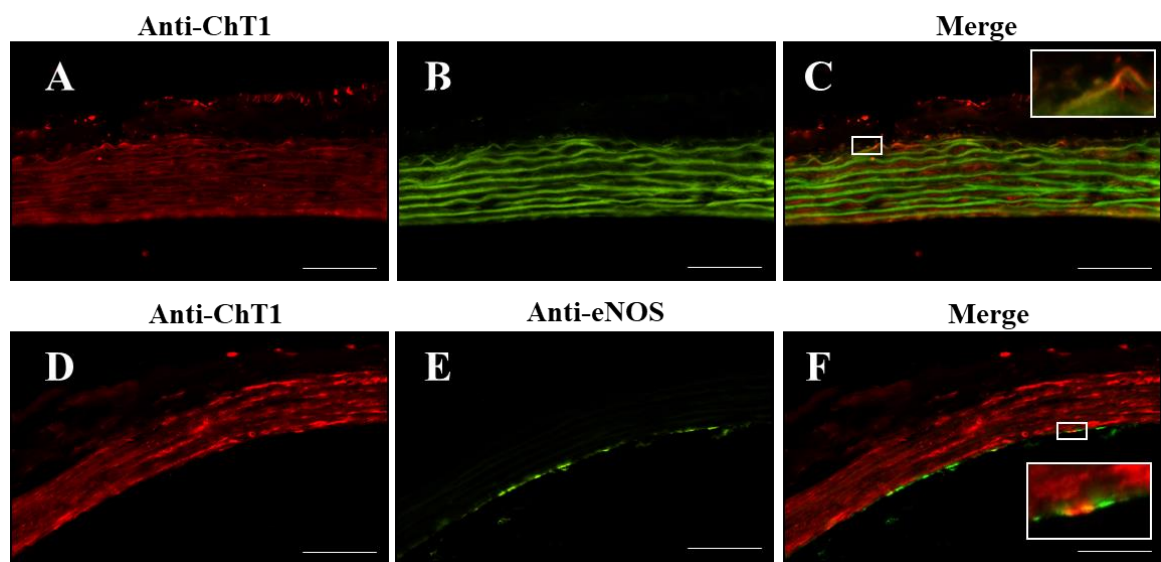


Figure 29: Immunohistochemical detection of ChT-1. The fluorescent signal of ChT-1 was co-localized with the smooth muscle (A-C) and endothelium (D-F). Scale bar 50 μ m.

No relative expression of OCT-1 was detected in any of the studied fractions indicating that this transporter is not present in rat aorta (Figure 30A). The relative expression of OCT-2 and OCT-3 showed similar expression patterns (Figure 30B, C). The highest mRNA levels were observed in AoE-, while the relative expression levels were roughly 3-fold lower in E1 and about 10-times lower in E3 as compared to the AoE-. Relative expressions of OCTN-1 in E1-3 and AoE- were similar (Figure 30D). OCTN-2 showed some differences in the expression levels between the fractions, nevertheless, it is expressed ubiquitously (Figure 30E).

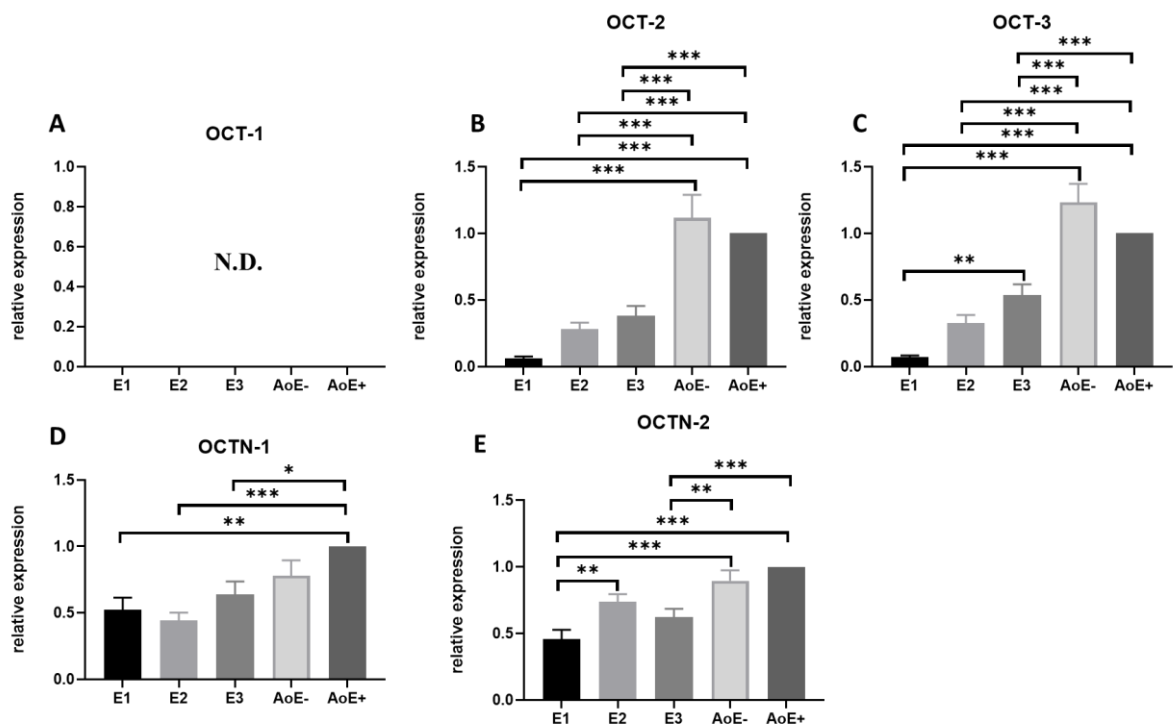


Figure 30: Relative expressions of OCT and OCTN receptors in aorta fractions. The relative expressions were determined for OCT-2 (B), OCT-3 (C), OCTN-1 (D) and OCTN-2 (E), but the relative expression for OCT-1 (A) was not detectable (N.D.) Data are presented as mean \pm SEM, $n = 7-8$.

Immunohistochemical visualization of different OCT and OCTN transporters was impossible to perform due to unavailability of specific antibodies.

4.2.1 Cholinergic receptors

4.2.1.1 mAChR

We detected all of the mAChRs in *aorta thoracalis* of Wistar rat (Figure 31A-E), with exception for m4AChR (Figure 31D). The highest relative expression was observed for m3AChR (Figure 31C). Majority of its detected mRNA belonged to the endothelial fractions (E1-E3), but low mRNA levels were also seen in the smooth muscle. m1AChR (Figure 31A) and m2AChR (Figure 31B) mRNA was present throughout the whole aortic tissue, while we detected relatively low expressions in the endothelial fractions and somewhat higher expression levels in the smooth muscle. There was no m4AChR mRNA detected in any of the fractions, endothelial E1-E3 or smooth muscle AoE- (Figure 31D). The relative expression of m5AChR was low, strictly localized to the endothelium (Figure 31E).

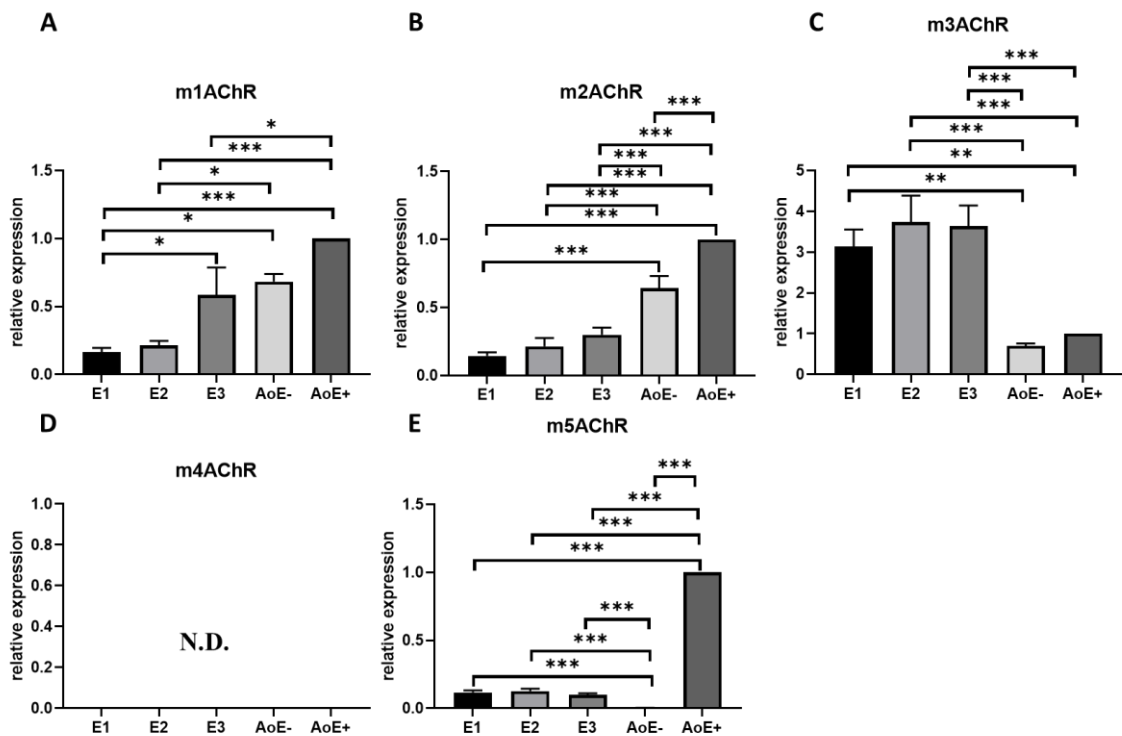


Figure 31: Relative expressions of mAChRs in aorta fractions. Low levels of relative expression for m1AChR (A) and m2AChR (B) were detected in E1-E3, and in AoE-. The most abundant mAChR was the m3AChR (C), which was primarily detected in E1-E3. mRNA of m4AChR (D) was not detectable (N.D.). A small amount of m5AChR was detected also in E1-E3, but not in AoE- (E). Data are presented as mean \pm SEM, $n = 7-8$.

4.2.1.2 nAChR

The relative expressions of all nAChR subunits were also determined (Figure 32) in the whole *aorta thoracalis*. Except for nAChR δ , all the subunits were detected in studied tissue indicating the presence of several nAChR with heterogenous composition. The highest expressions were observed for nAChR α 7 and nAChR β 2 subunits.

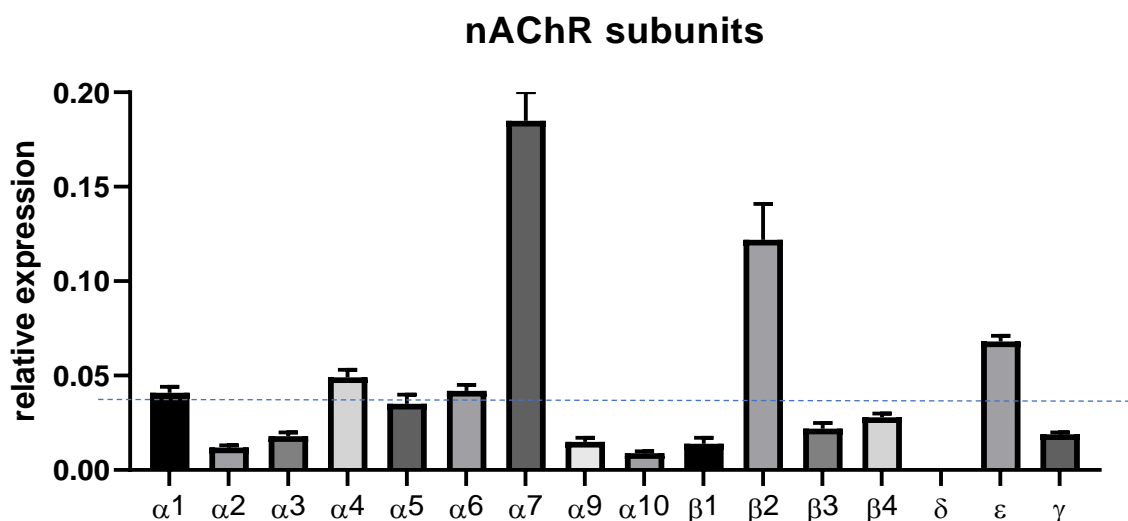


Figure 32: Relative expression of nAChR subunits in aorta thoracalis of Wistar rat. All the nAChR subunit, except for nAChR δ were expressed in aorta, with the highest expressions of nAChR α 7 and nAChR β 2. ---- indicates mRNA detection limit in aortic fractions. Data are presented as mean \pm SEM, $n = 8$.

In the next step, we focused on the relative expressions of different nicotinic subunits in aortic fractions. However, the mRNA levels of some subunits were undetectable (see Figure 32, where the blue line indicates the detection limit). We were able to determine the relative expression in the aortic fractions for the following subunits: α 1, α 4, α 6, α 7, β 2 and ϵ . The α 1 subunit was primarily present in smooth muscle (Figure 33A), while its mRNA level in endothelial fractions was at the detection limit. The α 4 subunit was detected in all the fractions, with increasing tendency from E1 to AoE-, where it reached the highest mRNA level (Figure 33B). The α 6 subunit was abundantly expressed at comparable levels in all studied fractions (Figure 33C). The presence of α 7 subunit was also documented in all the fractions, while the highest mRNA levels were in AoE- (Figure 33D). The relative expressions of β 2 subunit was rather comparable in each fraction, with tendency to increase

towards AoE- (Figure 33E). The ϵ subunit was abundantly expressed at comparable levels in all studied fractions (Figure 33F).

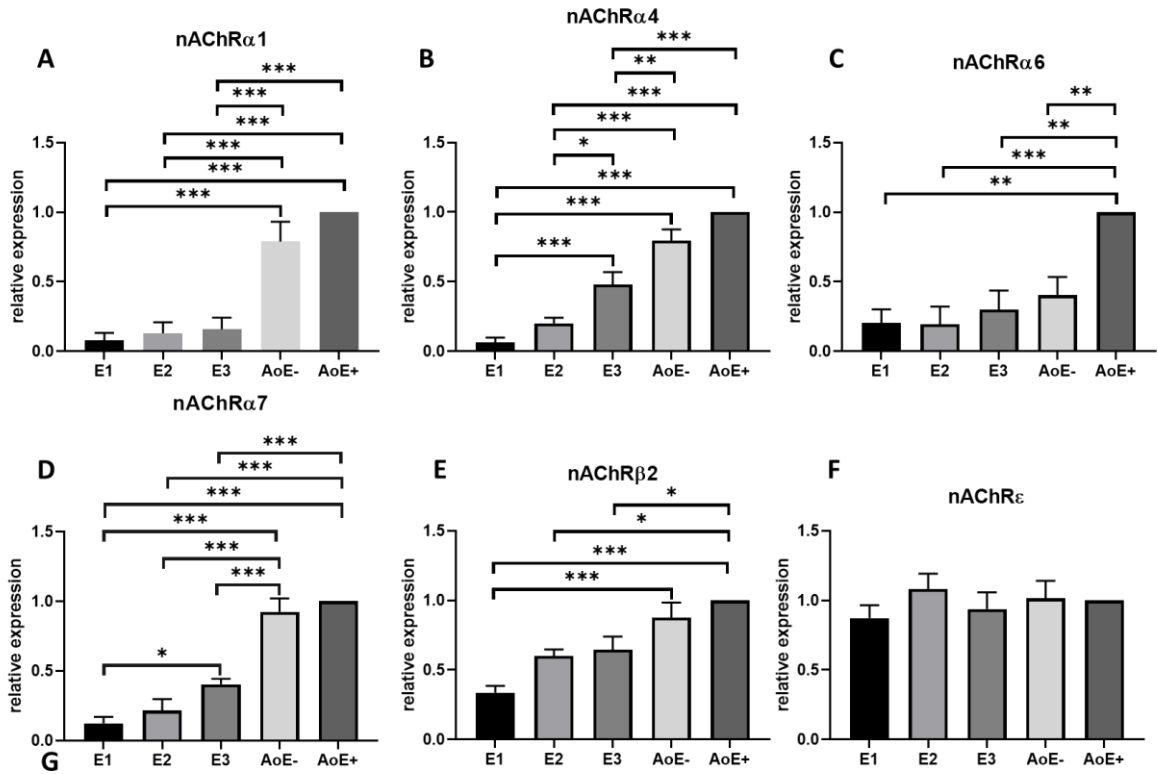


Figure 33: Relative expressions of different nicotinic subunits in aorta fractions. All the studied subunits were detected in all aortic fractions, while $\alpha 1$ (A), $\alpha 4$ (B) and $\alpha 7$ (D) were primarily present in smooth muscle. The mRNA levels of $\alpha 6$ (C), $\beta 2$ (E) and ϵ (F) were comparable in all the fractions. Data are presented as mean \pm SEM, $n = 8$.

4.3 Cholinergic system in pathologically changed aorta of SHR

As it has been described, the SHR model reacts to the presence of ACh differently than the normotensive model. We wanted to test, whether the changes in this response can be caused by the differences in the NNChS between the two studied models.

4.3.1 Cholinesterases in different parts of aorta

Similarly to the Wistar rats, the mRNA expression of both ChE was detected in all segments of the aorta also in SHR model (Figure 34). Moreover, the relative expression of BChE was several times higher than AChE (Figure 34B). The relative expression of AChE in SHR was comparable to Wistar in each part, except for *aorta abdominalis*, where SHR showed significantly higher mRNA levels, reaching the value comparable to the arcus aortae (Figure 34A). With the exception of *arcus aortae*, no differences were observed in BChE mRNA levels between Wistar and SHR. The relative expression of BChE in *arcus aortae* was significantly lower in SHR, thus no significant differences in the relative expression of BChE in SHR model were detected between *arcus aortae* and *aorta thoracalis* (Figure 34B). The mRNA level of BChE remained (similarly to the Wistar) significantly higher in *aorta abdominalis* than in *aorta thoracalis*. The relative expression of BChE in *aorta thoracalis* was comparable in both strains, showing a trend towards lower mRNA level in SHR.

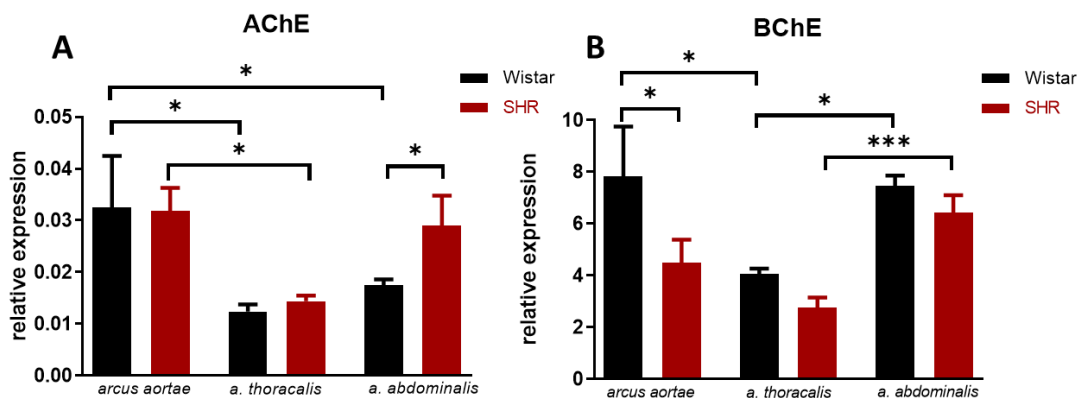


Figure 34: Comparison of relative expressions of ChE in different parts of aorta of Wistar and SHR. The relative expression of AChE in *arcus aortae* and *aorta thoracalis* was comparable in both studied models, but higher in *aorta abdominalis* of SHR. In case of BChE, the mRNA levels were similar in *aorta thoracalis* and *aorta abdominalis* of Wistar and SHR, but significantly lower in *arcus aortae* of SHR. Data are presented as mean \pm SEM, $n = 4-6$.

Results from the Ellman's assay were overall in line with the results from RT-qPCR. Both ChE activities were detected in all segments of aorta in both studied models, with BChE activity markedly exceeding AChE activity (Figure 35). Increased AChE mRNA level observed in *aorta abdominalis* of SHR as compared to Wistar was confirmed in activity assay (Figure 35A). AChE activity in *arcus aortae* and *aorta thoracalis* was (similarly to mRNA levels) comparable between both studied strains. As for BChE, the Ellman's assay confirmed decreased BChE level in SHR *arcus aortae* and *aorta thoracalis*, and preserved BChE levels in *aorta abdominalis* (Figure 35B).

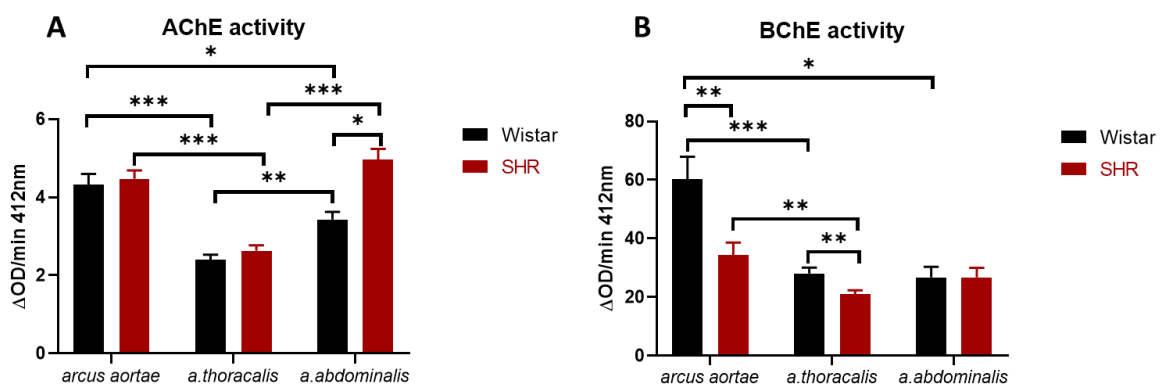


Figure 35: Comparison of activities of ChE in different parts of aorta of Wistar and SHR. The AChE activity in *arcus aortae* and *aorta thoracalis* was comparable in both studied models, but higher in *aorta abdominalis* of SHR (A). In case of BChE, the activities were significantly lower in *arcus aortae* and *aorta thoracalis* in SHR, but similar in *aorta abdominalis* (B). Data are presented as mean \pm SEM, $n = 4-6$.

Next, we examined the present molecular forms in different parts of aorta of SHR model. In all studied parts of the aorta, we saw no change in the ChE molecular form composition in comparison to Wistar. In the case of AChE, the peak detected around 2.4S indicated the presence of G_1^a amphiphilic forms and the peak around 9.2S the presence of PRiMA anchored AChE (Figure 36A-C).

In each part of the aorta, we detected a peak around 12.5S, while a shift was observed between gradients in the presence of detergents CHAPS and BRIJ, indicating the presence of PRiMA anchored BChE (Figure 36D-F). The second observed peak around 4.5S represents the presence of G_1^a forms (Figure 36D-F).

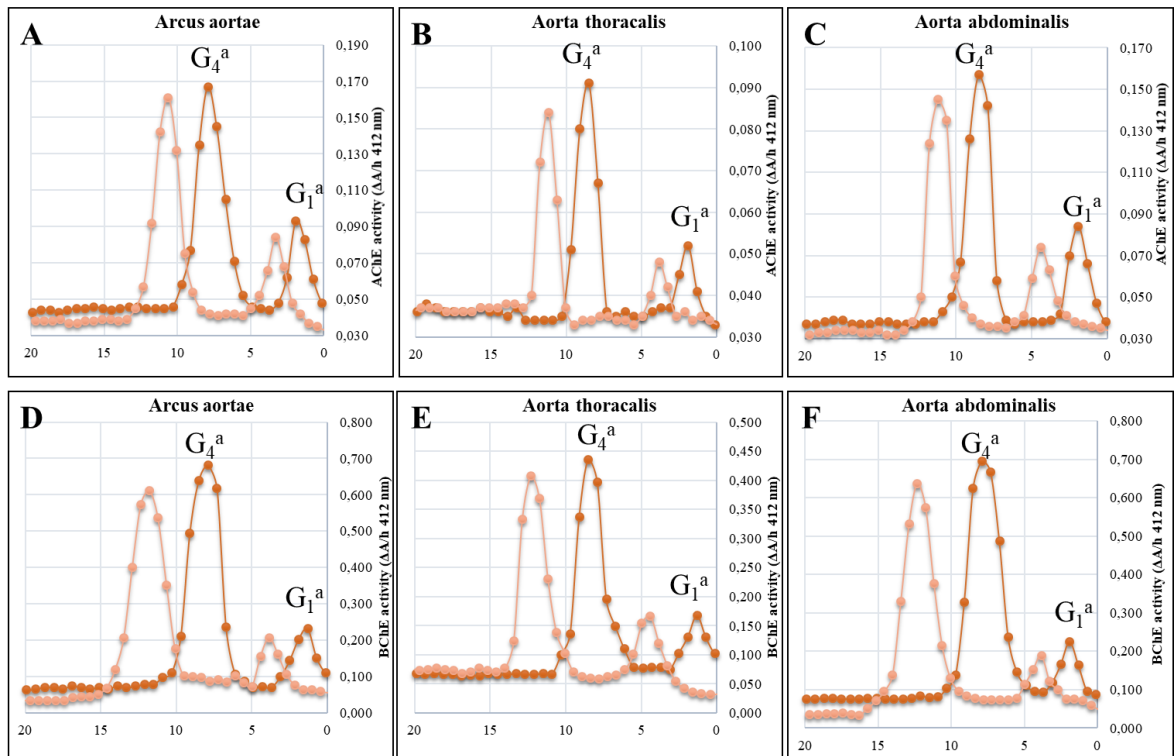


Figure 36: Molecular forms of AChE and BChE in different parts of aorta of SHR. In each part of SHR aorta, we detected the same molecular forms as in Wistar, i.e. PRiMA anchored forms of both AChE (A-C) and BChE (D-F). The minor part of ChE were present also in form of amphiphilic monomers. The amphiphilic forms were distinguished from non-amphiphilic forms by changing the detergent (• BRIJ, • CHAPS).

The presence of ChE was confirmed also by activity staining, while the results are overall in line with the results presented above. AChE activities in SHR were comparable in all parts of aorta (Figure 37A-C). The staining for BChE activity (proportional to the enzyme activity) was several times stronger in each part of aorta in SHR (Figure 37D-F), than the AChE activity, so this enzyme was dominant not only in normotensive, but also in pathological model. The staining for BChE was stronger in *arcus aortae* and *aorta abdominalis* (Figure 37F).

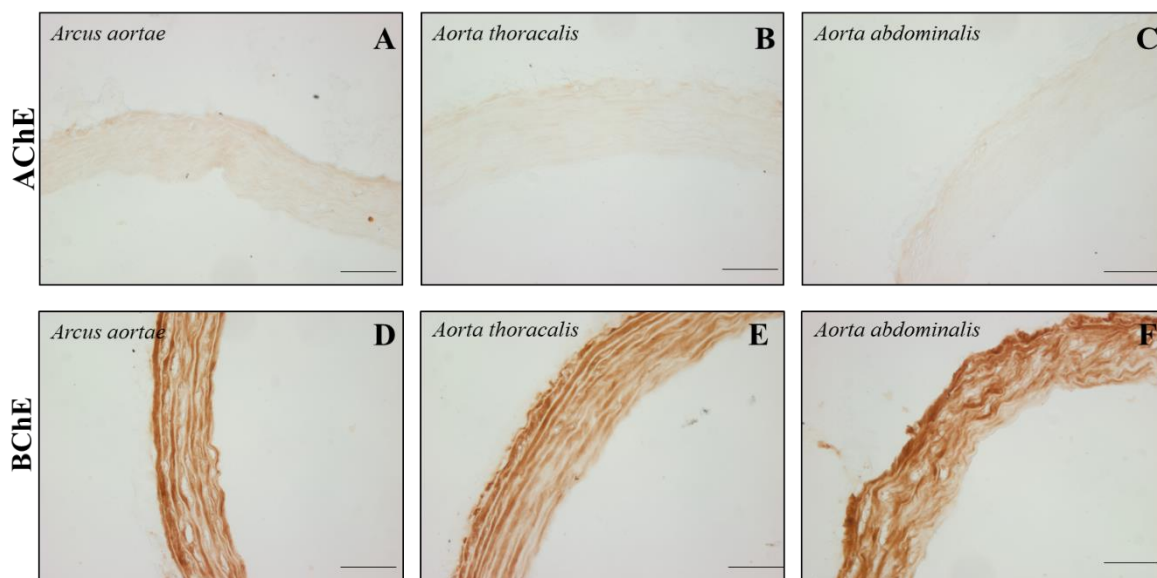


Figure 37: Tsuji's activity staining in different parts of SHR. Similarly, to Wistar, the coloration for AChE activity was several times weaker (A-C), than for BChE (D-F) indicating BChE is the dominant enzyme in each part of aorta also in SHR. Scale bar 50 μ m.

4.3.2 Other proteins and transporters involved in ACh life cycle

Relative expression of other proteins and transporters involved in ACh synthesis, transport and release were compared in *aorta thoracalis* of normotensive and SHR model by RT-qPCR method.

Similarly to Wistar rats, no mRNA levels were detected for ChAT (Figure 38C) and OCT-1 (Figure 38G) in SHR. No significant differences were observed in the levels of ChT-1 (Figure 38F). Surprisingly, SHR possessed higher mRNA levels of VAcHT (Figure 38E) and m3AChR (Figure 38L) than normotensive model. In addition to the lower CrAT levels in SHR aorta (Figure 38D) as compared to the normotensive rats, lower relative expression of OTC-2 and OCT-3 were detected in SHR rats (Figure 38H-I). The most significant decreases were observed in the relative expression levels of OCT-2 (Figure 38H), OCTN-1 (Figure 38J) and OCTN-2 (Figure 38K).

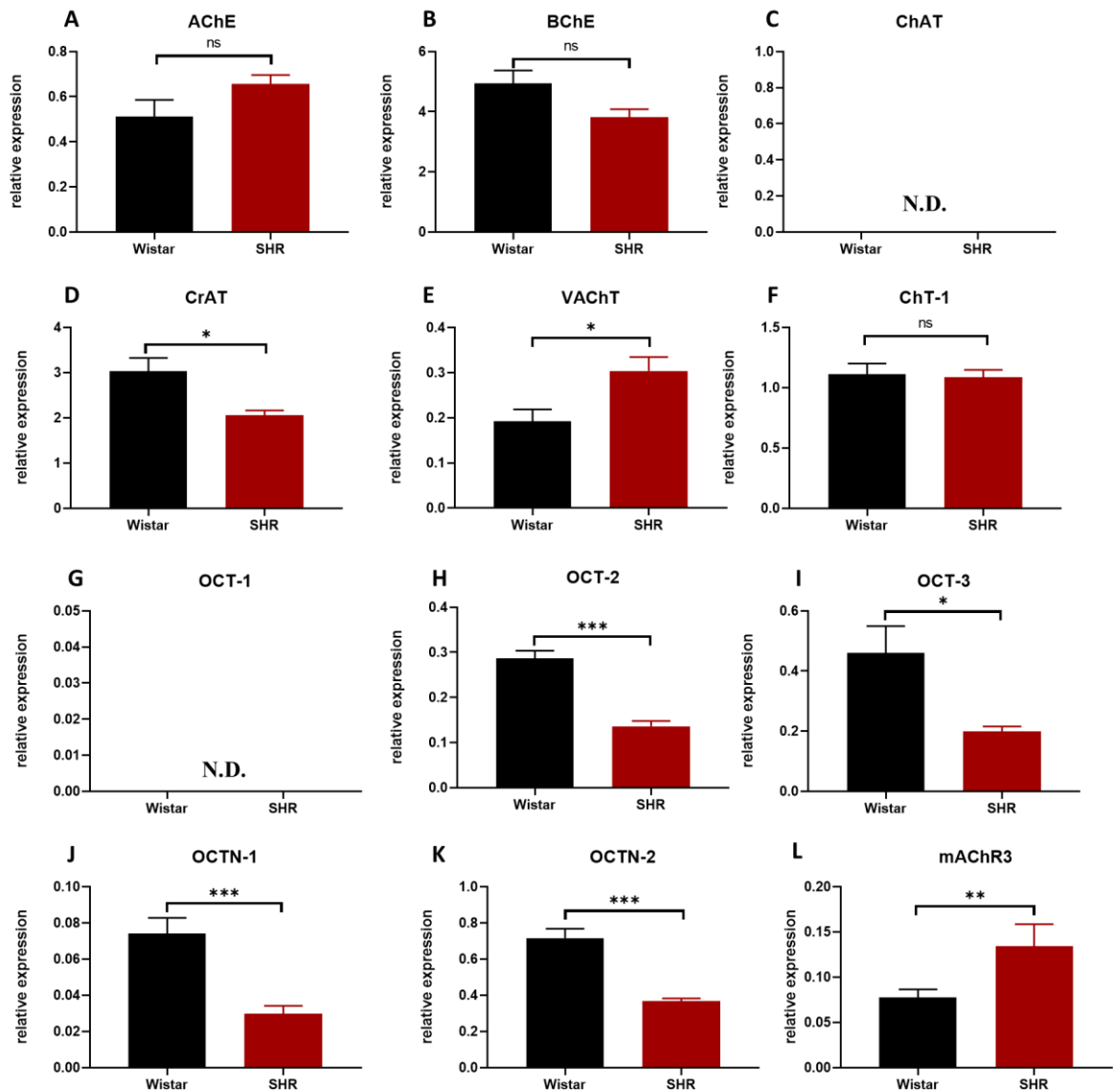


Figure 38: Comparison of relative expressions of different enzymes and transporters involved in ACh life cycle in aorta thoracalis of Wistar and SHR rats. No differences in relative expression of AChE (A), BChE (B) and ChT-1 (F) were observed. The mRNA levels of ChAT (C) and OCT-1 (G) were not detectable (N.D.) in both strains. Aorta thoracalis of SHR expressed less CrAT (D), OCT-2 (H), OCT-3 (I), OCTN-1 (J) and also OCTN-2 (K), but more VAcHT (E) and m3AChR (L). Data are presented as mean \pm SEM, n = 6.

4.4 Effect of the inhibition of BChE and/or high fat diet on vessels in normotensive rats and SHR

As described above, plasma BChE is involved in lipid metabolism and seems to play a role in pathogenesis of numerous cardiovascular diseases. These observations have, however, been made in mice and humans. Based on our results, rat cardiovascular cholinergic system differs from those in mouse and human. Here we therefore decided to examine an impact of the high-fat diet on rat in which, as we showed above, BChE activity is low in plasma but high in vessels. We also included SHR in the experiments, the animals in which we confirmed lower BChE activity in vessels as compared to the Wistar normotensive rats. For selective inhibition of BChE, iso-OMPA was administered to the animals. We examined impact of the HF diet on the overall state of the animals with special interest on vascular cholinergic system.

4.4.1 Energy ingestion, weight gain and body fat composition

Wistar rats consumed overall more food than SHR. Nevertheless, the weight of consumed food normalized to the weight of the animal (SHR are of significantly smaller size and weight comparing to Wistar rats) showed no differences between two studied strains. Moreover, there were no statistically significant differences observed in the weight of food consumed by different groups within one strain (Table 6). With this in line, energy intake (kcal/day) was higher in the groups fed by HF. In general, the water consumption of rats fed with HF was significantly decreased. Interestingly, iso-OMPA administration increased water intake in Wistar rats but not in SHR. Similarly, iso-OMPA normalized water consumption of Wistar rats on HF diet but not of HF-fed SHR.

Table 6: Food intake and water consumption of studied groups. Wistar rats generally had higher food intake (g/day), than SHR rats. HF diet ensured higher caloric intake in both studied strains. Administration of iso-OMPA caused an increase, while HF diet caused a decrease in water consumption. Data are presented as mean \pm SEM, n = 6. Statistical significance is in reference to Wistar control group (*) or to SHR control group as (x).

	Wistar				SHR			
	control	iso-OMPA	HF	iso-OMPA	control	iso-OMPA	HF	iso-OMPA
Food intake (g/day)	44.94 \pm 0.49	47.46 \pm 1.495	43.79 \pm 0.591	42.06 \pm 0.45	36.80 \pm 0.613	36.59 \pm 2.508	33.86 \pm 0.823	33.81 \pm 1.066
Food intake (kcal/day)	145.0 \pm 1.593	153.1 \pm 4.824	196.9 \pm 2.660*	189.2 \pm 2.050*	118.7 \pm 1.988*	118.1 \pm 3.304	152.3 \pm 3.702^x	152.0 \pm 4.794^x
Water consumption (ml)	33.86 \pm 0.893	39.47 \pm 0.95*	27.56 \pm 1.091*	33.88 \pm 0.868	35.61 \pm 0.431	34.30 \pm 0.77	31.00 \pm 0.727^x	31.64 \pm 1.011^x

HF diet caused significant increase in weight gain in Wistar rats, but not in SHR. Surprisingly, administration of iso-OMPA to Wistar rats caused comparable weight increase, while it did not lead to additional increase in weight gain of Wistar rats fed with HF diet. Differences observed in changes of abdominal circumferences are in line with these observations, as comparable significant increase was observed in all the group of Wistar rats (Table 7), but not in SHR.

BMI calculated both for total length or body length of animals were significantly increased as a result to the iso-OMPA administration and/or HF diet in Wistar rats (Table 7). However, Lee index was increased only in the groups on HF diet. No changes in BMI or Lee index were observed in SHR (Table 7).

Differences in fat masses and fat storage in different parts of the body has also been observed (Table 7). Wistar rats on HF diet had more epididymal fat, retroperitoneal fat and perirenal fat than control animals. Interestingly, in Wistar rats on SD diet with iso-OMPA administration, only epididymal adipose tissue was increased (Table 7), while the weight of

the epididymal fat was comparable to the HF-fed groups (with or without iso-OMPA treatment). No differences were detected between individual SHR groups (Table 7).

Table 7: Weight gain, BMI and fat tissue composition of studied groups. In Wistar rats not only HF diet, but also administration of iso-OMPA caused a significant increase in weight gain, which was reflected in increase of BMI and abdominal circumference. Among SHR rat, we did not detect a significant increase in any of the studied parameters. Data are presented as mean \pm SEM, n = 6. Statistical significance is in reference to Wistar control group (*) or to SHR control group as (*).

	Wistar				SHR			
	control	iso-OMPA	HF	iso-OMPA +HF	control	iso-OMPA	HF	iso-OMPA +HF
Weight gain (Δ (g))	184.5 \pm 8.732	231.5 \pm 7.751*	241.5 \pm 7.429*	219.8 \pm 8.518*	133 \pm 3.838	127.5 \pm 6.697	131.3 \pm 6.637	133.3 \pm 6.707
Weight gain (%)	58.40 \pm 3.544	78.50 \pm 2.533*	84.50 \pm 3.294*	76.00 \pm 1.683*	58.50 \pm 1.979	53.83 \pm 3.516	57.17 \pm 2.257	57.33 \pm 2.894
Abdominal circumference (cm)	20.67 \pm 0.358	22.00 \pm 0.316*	22.00 \pm 0.516	22.00 \pm 0.447*	18.67 \pm 0.211	18.50 \pm 0.428	18.08 \pm 0.201	18.17 \pm 0.307
BMI (g/cm ² - total length)	0.225 \pm 0.006	0.227 \pm 0.004	0.252 \pm 0.004*	0.250 \pm 0.005*	0.237 \pm 0.008	0.235 \pm 0.002	0.227 \pm 0.004	0.233 \pm 0.006
BMI (g/cm ² - body length)	0.693 \pm 0.022	0.743 \pm 0.012*	0.782 \pm 0.015*	0.773 \pm 0.001*	0.660 \pm 0.012	0.682 \pm 0.020	0.652 \pm 0.013	0.675 \pm 0.023
Lee index (g/cm ³)	0.297 \pm 0.003	0.303 \pm 0.005	0.310 \pm 0.003*	0.308 \pm 0.002*	0.305 \pm 0.002	0.307 \pm 0.004	0.302 \pm 0.003	0.307 \pm 0.004

Epididymal fat (g)	3.789 ± 0.350	6.466 ± 0.357*	8.494 ± 1.368*	7.115 ± 0.834*	2.119 ± 0.834 *	2.116 ± 0.048	2.184 ± 0.116	2.812 ± 0.220
Retroperitoneal fat (g)	2.394 ± 0.233	4.082 ± 0.314	7.098 ± 1.566*	4.738 ± 1.034	1.818 ± 0.130	1.908 ± 0.210	2.029 ± 0.118	1.845 ± 0.143
Perirenal fat (g)	1.314 ± 0.338	1.769 ± 0.344	3.176 ± 0.495*	2.726 ± 0.320*	0.776 ± 0.097	0.950 ± 0.092	0.605 ± 0.101	1.065 ± 0.228

4.4.2 Biochemical parameters

4.4.2.1 Cholinesterase activities in serum

We used Ellman's assay to detect the ChE activities in the serum of the animals. iso-OMPA treatment and/or HF diet did not change serum AChE activity in the studied rat models (Figure 39A, B). Nevertheless, in Wistar rats, the HF diet caused an increase in serum BChE activity (Figure 39C), which was restored by concomitant iso-OMPA treatment. Remaining BChE activity was possible to inhibit *in vitro* by high concentration of iso-OMPA (10^{-3} M). The dose that normalized the increased BChE activity in HF-fed Wistar rats fully inhibited the serum BChE activity in rats on SD and had no effect on serum AChE activity (confirming complete but selective inhibition of BChE) (Figure 39C). No such observations were made in SHR, where HF diet had no impact on ChE activity in serum (Figure 39D).

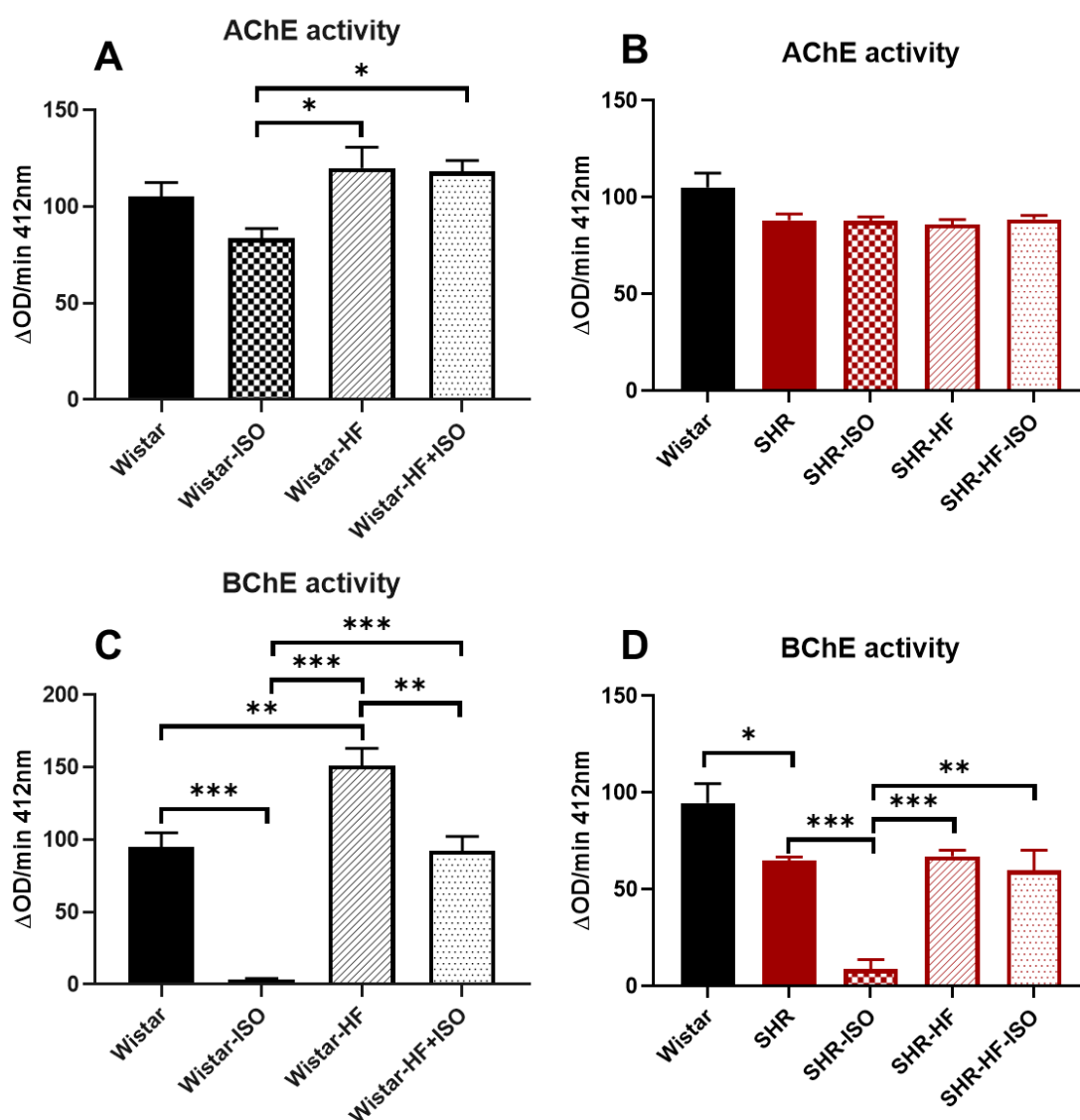


Figure 39: Serum levels of AChE and BChE of studied groups. Neither HF diet, nor iso-OMPA administration had any effect on AChE activity of animals (A, B). BChE activity was completely inhibited by iso-OMPA, while HF diet caused an increase in BChE activity in Wistar rats (C), but not in SHR (D). Data are presented as mean \pm SEM, $n = 6$.

4.4.2.2 Lipid metabolism

As BChE activities are frequently correlated with the parameters of lipid metabolism, we wanted to determine the effect of its long-term inhibition on lipid metabolism. HF diet caused an increase in serum cholesterol (Figure 40A) and TAG (Figure 40E) levels in Wistar rats, while serum HDL levels were not significantly changed (Figure 40C). Interestingly, iso-OMPA treatment led also to an increased serum TAG levels (Figure 40E), while the

increase was comparable to the HF diet. There was no additive effect on serum TAG levels upon iso-OMPA administration in HF-diet-fed animals though (Figure 40E). No changes were observed in remaining studied lipid markers (Figure 40C). With exception for lower TAG levels (Figure 40F), there were no other significances observed for SHR controls in comparison to the Wistar controls or among the SHR groups (Figure 40B, D).

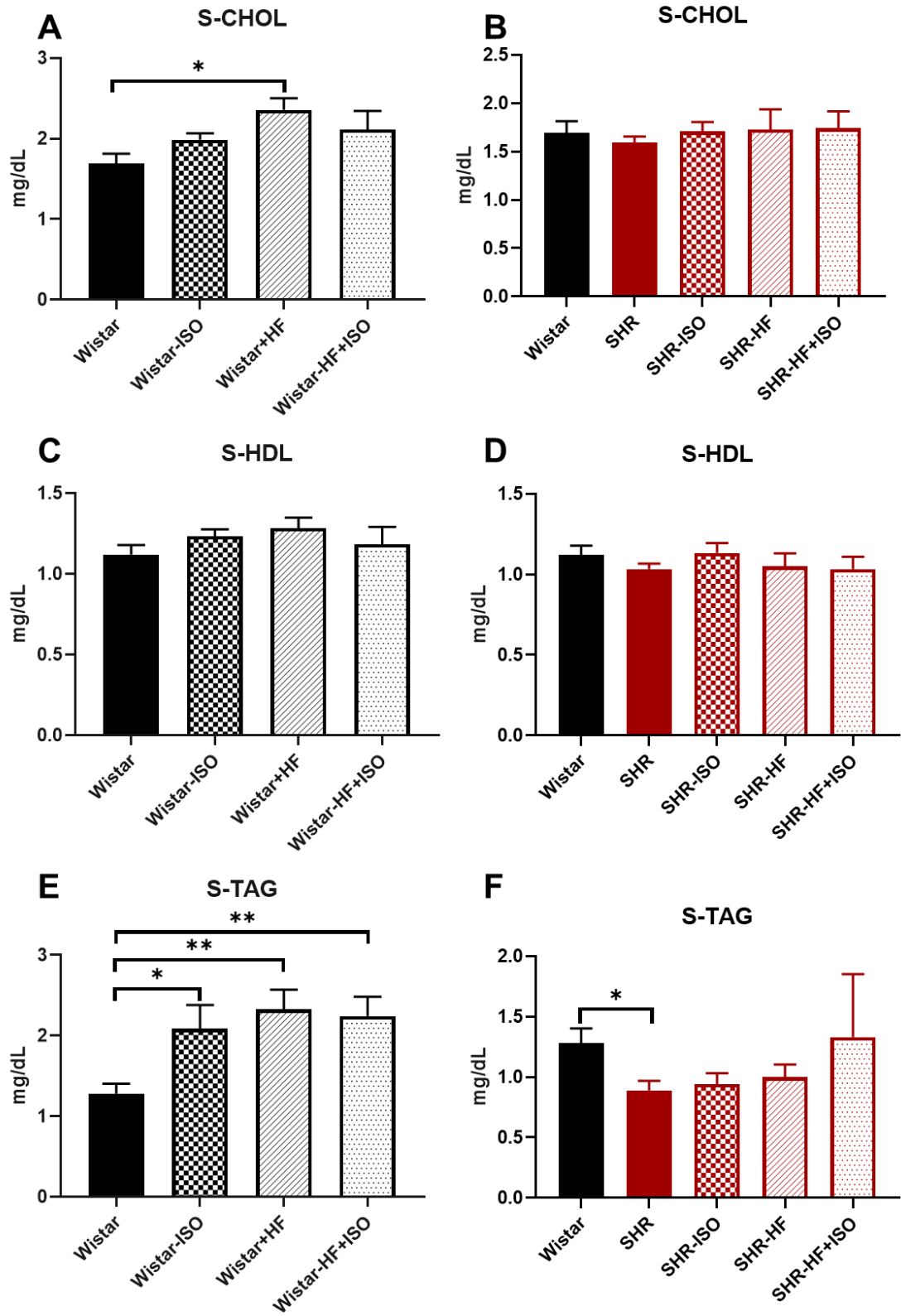


Figure 40: Serum levels of total cholesterol (S-CHOL), HDL-cholesterol (S-HDL) and triacylglycerols (S-TAG) of studied groups. HF diet caused an increase in serum total cholesterol levels in Wistar rats (A), but not in SHR (B). Neither HF diet, nor iso-OMPA

had effect on serum HDL levels (C, D). Both iso-OMPA and also HF diet caused a significant increase in serum TAG levels in Wistar rats (E), but not in SHR (F). Data are presented as mean \pm SEM, n = 6.

4.4.2.3 Other biochemical parameters

Values for the different biochemical parameters evaluated in serum of Wistar and SHR rats, with or without iso-OMPA administration, fed either with SD or HF diet are shown in Table 8. In general, iso-OMPA administration of the HF diet did not have any profound effect on the serum biochemical parameters, independently of the studied animal strain (Table 8). HF diet caused an increase in serum alanine aminotransferase (Table 8) and serum calcium levels (Table 8) of Wistar rats and the serum phosphor levels in SHR (Table 8). Administration of iso-OMPA caused a decrease in serum magnesium levels in Wistar rats on SD diet (Table 8). Nevertheless, iso-OMPA also caused a decrease in creatinine levels of SHR both in groups on SD and HF diet (Table 8).

Table 8: Other biochemical parameters of studied groups. In Wistar rats. HF diet caused an increase in S-ALT and S-Ca levels, while administration of iso-OMPA a decrease S-Mg. In SHR rats, HF diet caused an increase in S-P levels while treatment with iso-OMPA a decrease in S-Crea levels. Data are presented as mean \pm SEM, n = 6. Statistical significance is in reference to Wistar control group (*) or to SHR control group as (*).

	Wistar				SHR			
	Control	iso-OMPA	HF	iso-OMPA +HF	Control	iso-OMPA	HF	iso-OMPA +HF
S-ALP (U/L)	3.530 \pm 0.390	2.952 \pm 0.444	3.458 \pm 40.454	3.940 \pm 0.315	4.830 \pm 0.730	5.085 \pm 0.351	5.082 \pm 0.903	6.697 \pm 1.045
S-ALT (U/L)	1.128 \pm 0.084	1.173 \pm 0.108	1.646 \pm 0.198*	1.600 \pm 0.177	1.580 \pm 0.068*	1.647 \pm 0.096	1.627 \pm 0.123	1.778 \pm 0.070
S-AST (U/L)	1.646 \pm 0.114	1.478 \pm 0.029	1.703 \pm 0.150	1.882 \pm 0.076	2.228 \pm 0.181*	2.135 \pm 0.192	2.033 \pm 0.236	2.130 \pm 0.146

S-ALB (g/dL)	34.90 ± 0.874	34.95 ± 1.060	36.32 ± 0.799	34.73 ± 0.956	39.38 ± 1.107*	37.92 ± 0.487	36.70 ± 0.895	36.88 ± 0.565
S-Ca (mg/dL)	2.900 ± 0.039	2.907 ± 0.061	3.092 ± 0.062*	3.066 ± 0.038	2.940 ± 0.059	2.912 ± 0.047	2.988 ± 0.038	2.985 ± 0.075
S-P (mg/dL)	3.240 ± 0.153	3.343 ± 0.180	3.425 ± 0.257	3.42 ± 0.177	2.527 ± 0.084*	2.405 ± 0.106	3.28 ± 0.145 [×]	3.008 ± 0.097 [×]
S-Cl (mg/dL)	96.20 ± 1.497	97.67 ± 1.585	97.00 ± 1.528	98.83 ± 0.792	97.50 ± 0.992	98.67 ± 1.202	96.83 ± 0.946	98.67 ± 1.453
S-Mg (mg/dL)	1.526 ± 0.056	1.320 ± 0.060*	1.545 ± 0.039	1.405 ± 0.065	1.512 ± 0.086	1.480 ± 0.062	1.517 ± 0.067	1.535 ± 0.090
S-K (mg/dL)	8.940 ± 0.49	8.367 ± 0.374	8.667 ± 0.484	9.333 ± 0.327	6.933 ± 0.382*	6.700 ± 0.157	7.700 ± 0.601	6.683 ± 0.311
S-Fe (mg/dL)	44.27 ± 1.694	42.88 ± 1.734	47.49 ± 2.143	43.28 ± 2.366	42.08 ± 2.201	37.52 ± 4.844	50.27 ± 1.964	38.87 ± 6.230
S-Glu (mg/dL)	16.20 ± 1.709	14.48 ± 1.815	18.47 ± 1.340	14.73 ± 1.437	15.04 ± 1.658	14.00 ± 1.082	15.62 ± 1.626	14.87 ± 1.045
S-Na (mg/dL)	141.2 ± 0.374	143.00 ± 1.155	142.8 ± 1.682	142.7 ± 1.085	144.3 ± 0.667	144.00 ± 1.713	141.5 ± 0.7188	144.2 ± 1.662
S-Crea (mg/dL)	32.40 ± 6.125	27.7 ± 2.271	27.37 ± 1.473	31.23 ± 3.541	39.68 ± 6.513	26.17 ± 2.463 [×]	34.65 ± 2.524	26.32 ± 0.429 [×]

S-Urea (mg/dL)	7.310 ± 0.711	7.613 ± 0.758	7.120 ± 0.533	6.648 ± 0.696	8.110 ± 0.774	7.587 ± 0.444	6.968 ± 1.040	6.542 ± 0.356 ^x
S-CK (g/dL)	11.66 ± 4.428	10.29 ± 2.925	10.62 ± 2.371	17.36 ± 3.786	17.00 ± 7.676	9.252 ± 3.538	16.48 ± 5.482	11.29 ± 1.560
S-CRP (g/dL)	2.60 ± 0.245	3.167 ± 0.477	1.833 ± 0.401	2.333 ± 0.333	1.50 ± 0.500	2.500 ± 0.619	2.00 ± 0.447	2.40 ± 0.245

4.4.3 Changes in cholinergic system of aorta

To examine the effect of iso-OMPA administration and/or HF diet on the cholinergic system of *aorta thoracalis* of both normotensive rats and SHR, we determined the relative expression of all the enzymes and transporters which can be involved in the ACh life cycle.

4.4.4 ACh synthesis, transport, and release

In Wistar groups, the HF-diet did not affect the levels of enzymes and transporters involved in ACh life cycle (Figure 41). BChE inhibition with iso-OMPA caused – independently of the type of diet- a decrease in relative expression levels of OCT-2 (Figure 41D). The relative expression of other transporters from the group of OCT or OCTN was not affected (Figure 40D-G). We did not detect any significant differences in relative expression levels of CrAT (Figure 40A), ChT-1 (Figure 40B) or VAcHT (Figure 40C) upon iso-OMPA administration.

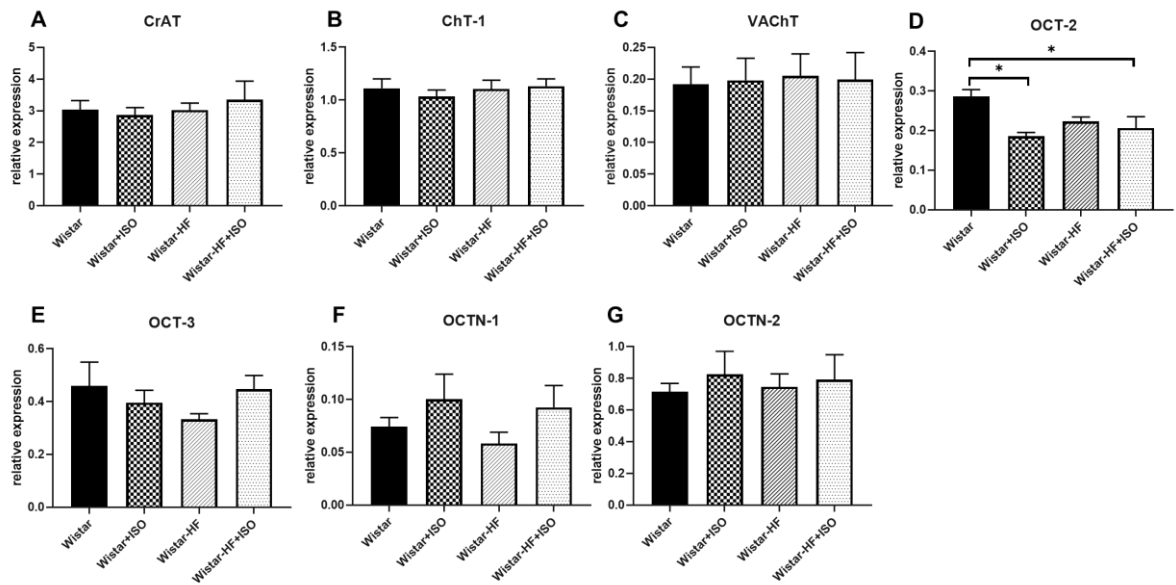


Figure 41: Relative expressions of transporters involved in life cycle of acetylcholine in aorta thoracalis of Wistar groups. Neither HF diet, nor administration of iso-OMPA had an effect on relative expressions of all studied transporters (A-C, E-G), except for OCT-2 (D), where iso-OMPA caused a significant decrease in mRNA levels. Data are presented as mean \pm SEM, $n = 6$.

Surprisingly, SHR model acted differently to the HF-diet and iso-OMPA administration. In contrary to normotensive rats, iso-OMPA did not cause a significant decrease in OCT-2 relative expression levels (Figure 42D), but instead, an increase in OCT-3 (Figure 42E) and OCTN-2 (Figure 42G) relative expressions in group on standard diet was observed. Additionally, iso-OMPA induced an increase of CrAT (Figure 42A) in SHR on standard diet. There were no changes in relative expression levels of ChT-1 (Figure 42B) and VAcHT (Figure 42C).

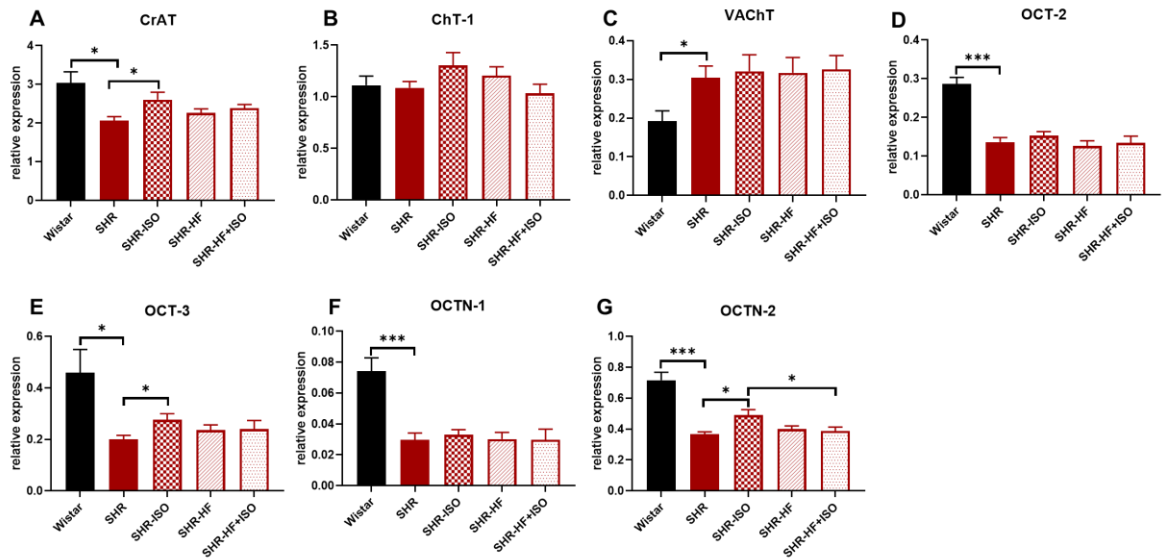


Figure 42: Relative expressions of transporters involved in the life cycle of the acetylcholine in aorta thoracalis of SHR groups. Administration of iso-OMPA caused a significant increase in CrAT (A), OCT-3 (E) and OCTN-2 (G) mRNA levels in SHR. Data are presented as mean \pm SEM, $n = 6$.

4.4.5 Degradation of ACh

Previously (see [chapter: Cholinergic system in pathologically changed aorta of SHR](#)) we detected lower BChE activity in *aorta thoracalis* of SHR than of Wistar rats indicating that their activity and function is impaired in pathological models. Therefore, we wanted to test, whether the HF diet (which is associated with higher risk of CVS pathologies) have an impact on them.

Neither iso-OMPA administration nor HF diet had an effect on the relative expression of AChE in both strains (Figure 43A, B). Surprisingly, a significant increase in the relative expression of BChE was observed in Wistars fed with HF diet (Figure 43C), but not in SHR (Figure 43D). Surprisingly, relative expression of ChE anchoring protein PRiMA did not change as a consequence of the HF diet or iso-OMPA application (Figure 43E, F).

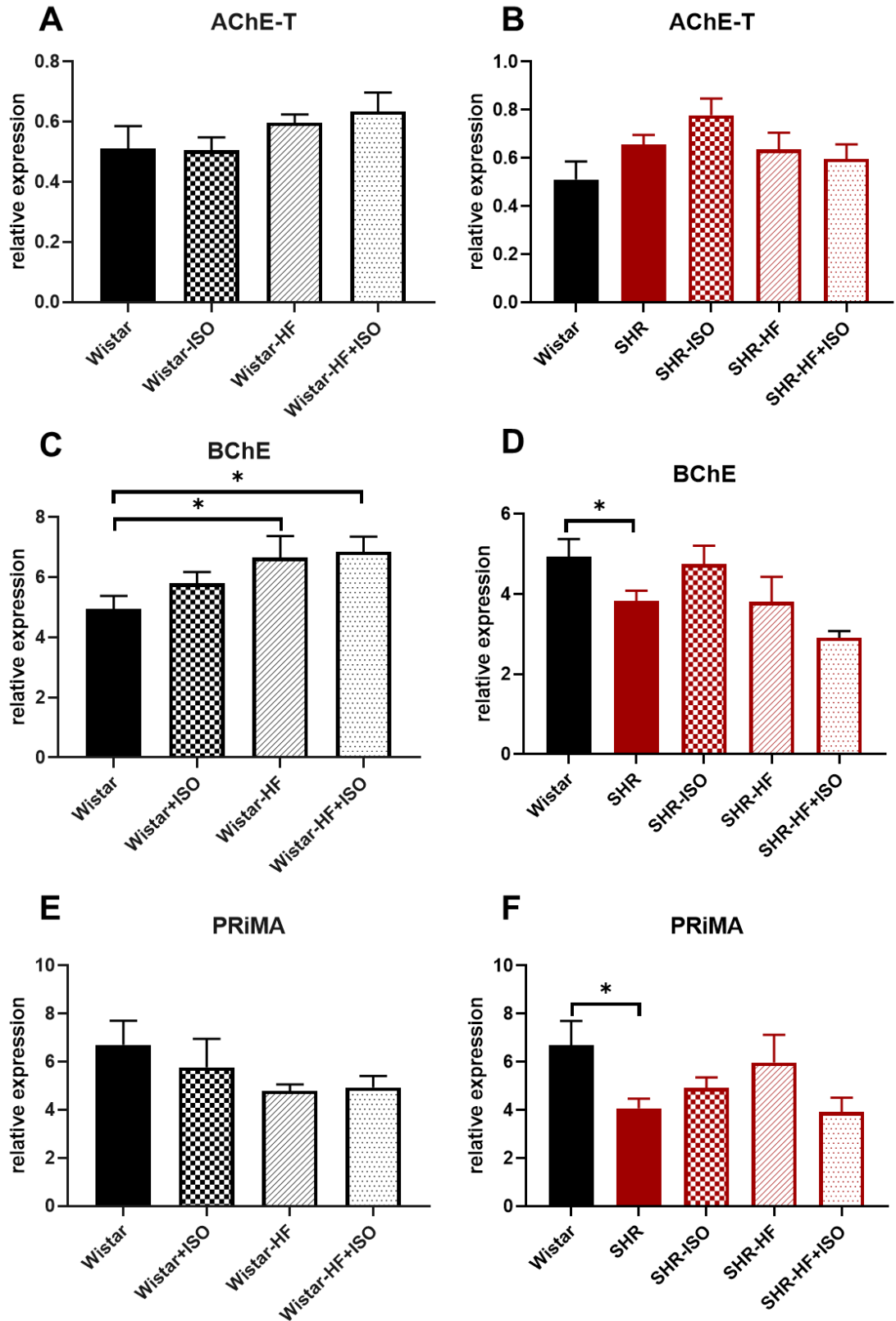


Figure 43: Relative expressions of AChE-T, BChE, and PRiMA in aorta thoracalis of studied groups. Relative expression of AChE was not affected either by HF diet or iso-

OMPA administration in both studied strains (A, B). A significant increase in relative expressions of BChE was detected in Wistar rats (C), but not SHR (D) when the HF diet was applied. The total mRNA levels of PRiMA gene were not affected in any of the studied groups (E, F). Data are presented as mean \pm SEM, $n = 6$.

4.4.6 Muscarinic receptors

A significant increase of m3AChR was observed in Wistar rats when iso-OMPA was administered, independently of the type of diet (Figure 44A). No significant change in mRNA levels of m3AChR3 was observed within the SHR strain group (Figure 44B).

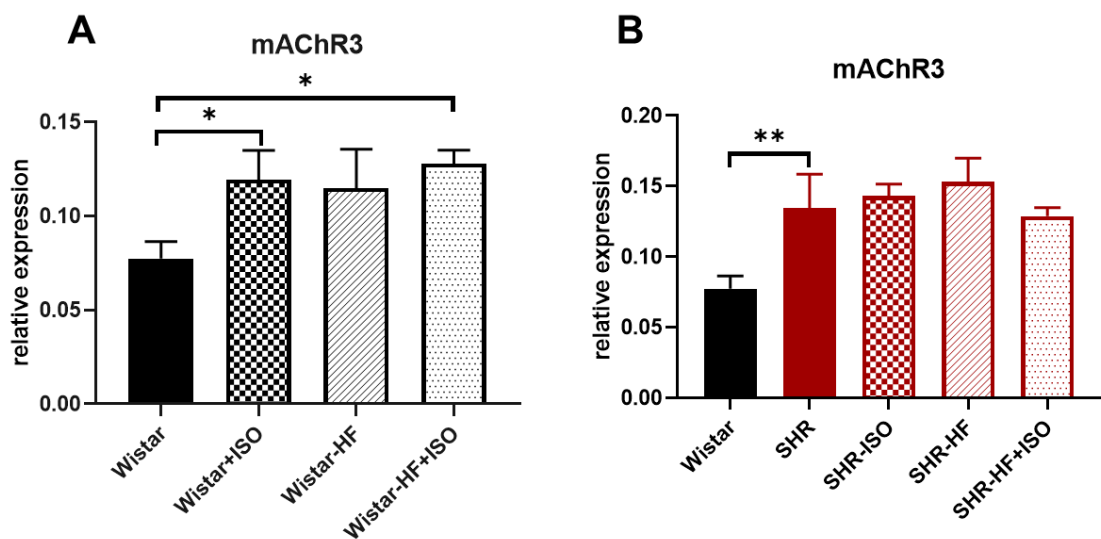


Figure 44: Relative expression of m3AChR in aorta thoracalis of studied groups. Administration of iso-OMPA caused a significant increase in the relative expression of mAChR3 in Wistar (A), but not in SHR (B). Data are presented as mean \pm SEM, $n = 6$.

4.4.7 Lipase activity of BChE

Our results uncovered a link between plasma BChE activity and TAG in rat (Figure 39,40), which could be hypothetically explained by the involvement of BChE in TAG degradation. We therefore examined a capability of BChE to cleave TAG.

4.4.7.1 Lipase activity of BChE in the presence of thioester analogue of TAG

First, the lipase activity of BChE was tested with the commercially available kit described in [chapter: Lipase activity assay kit \(MAK047\)](#)

Several concentrations of BChE were tested in the presence of TAG-like substrate, DMPTB (Figure 45). The results suggest that human plasma concentration of BChE possesses lipase activity towards TAG-like substrate. We did not observe any activation or inhibition of the hydrolysis at higher BChE concentrations (Figure 45).

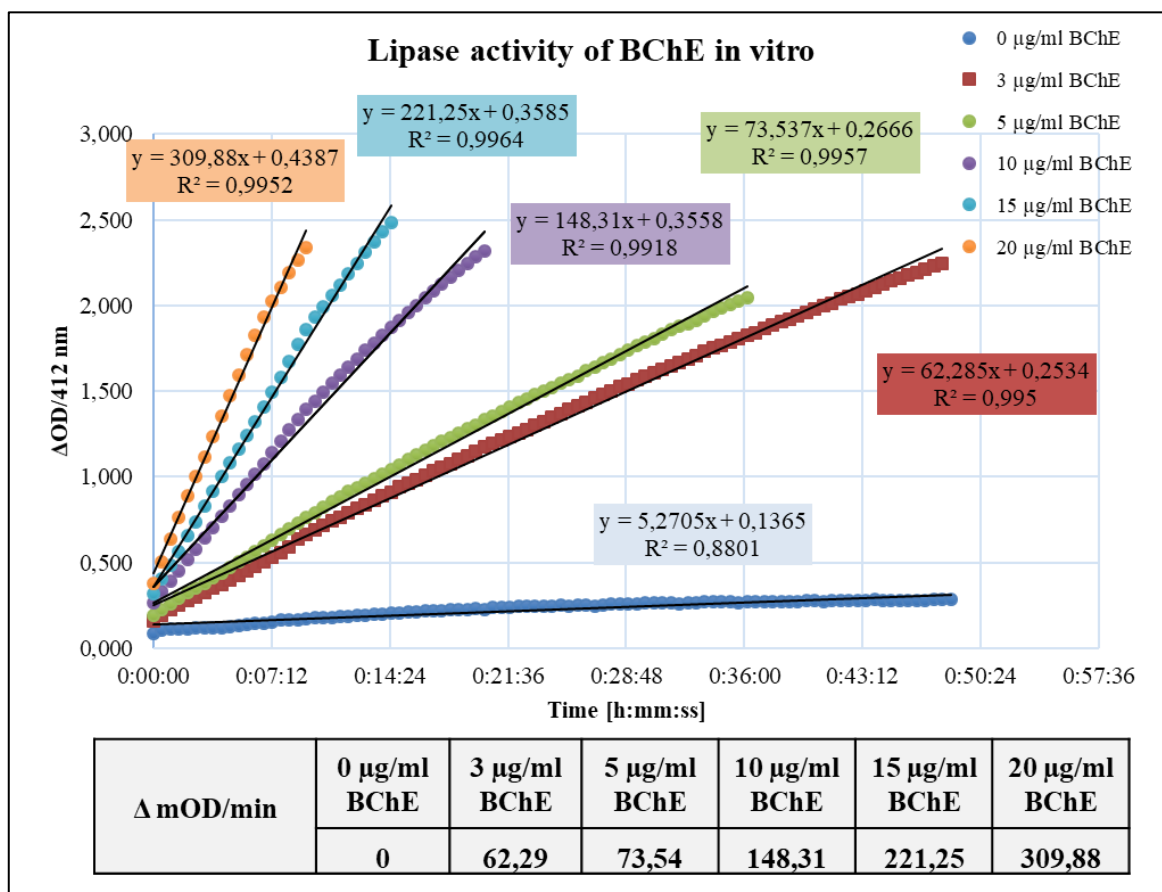


Figure 45: Lipase activity of BChE in the present of DMPTB. Human plasmatic level of BChE (3 μg/ml •) was able to cleave the substrate. Several concentrations of BChE were tested, but no changes in the kinetic behavior were observed.

Detected lipase activity can be clearly assigned to BChE as it was inhibited by iso-OMPA (Figure 46). Lipase activity of BChE was, however, significantly lower than its esterase activity (Figure 47). Inhibitory effect of the buffer on BChE activity was ruled out in preliminary experiments during which the reaction conditions were optimized.

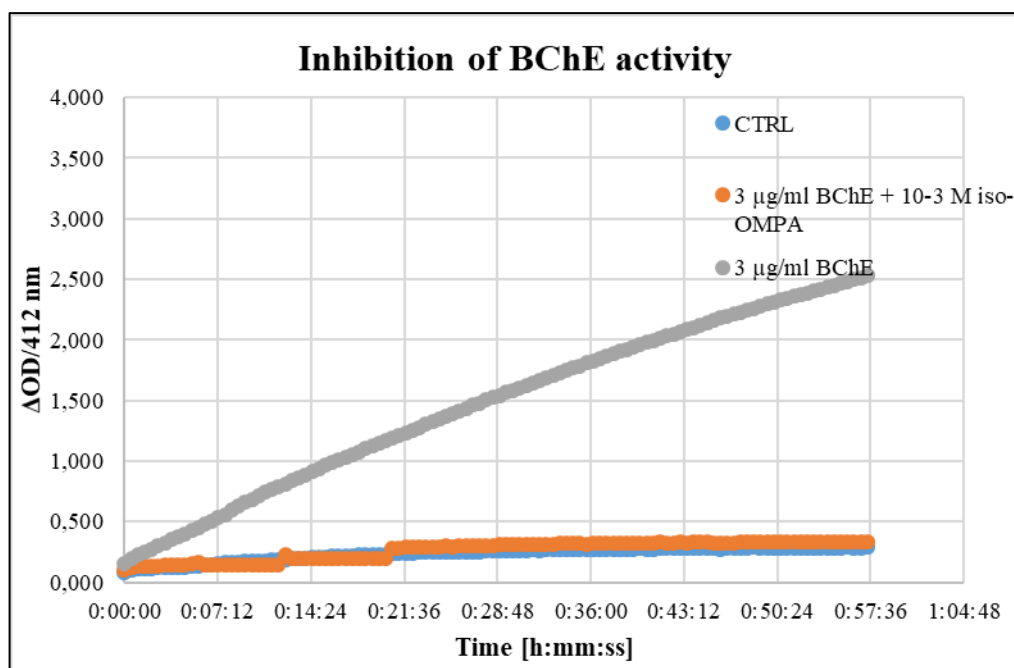


Figure 46: Inhibition of BChE activity with iso-OMPA. Human plasmatic concentration of BChE was able to cleave DMPTB (•). When BChE was inhibited (•), the activity was comparable to control (•), indicating that lipase activity can be assigned to BChE.

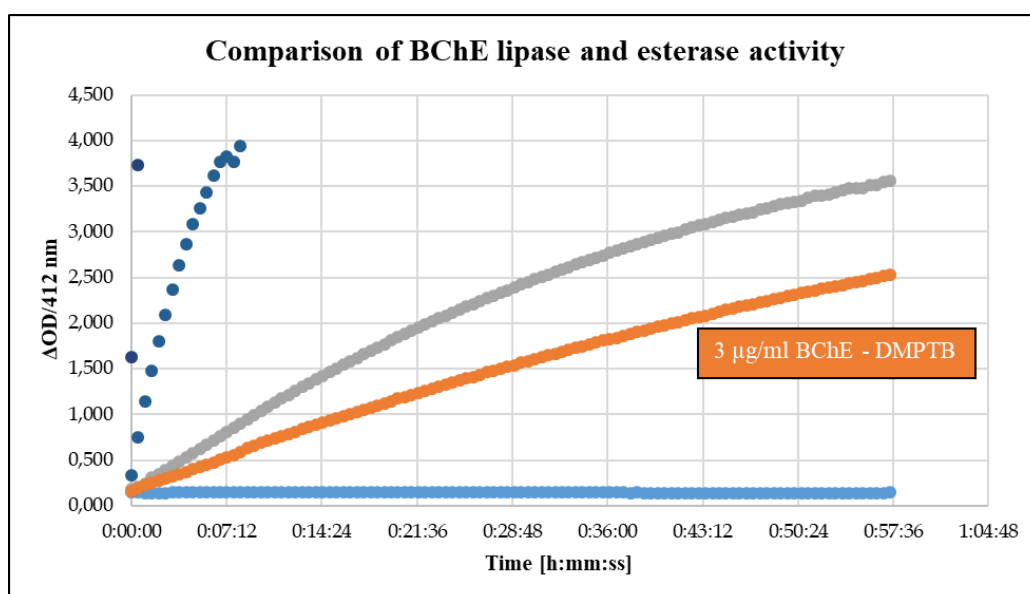


Figure 47: Comparison of BChE lipase and esterase activity. The hydrolysis of BTC by BChE was more efficient not only at human plasmatic levels (•), but also 10x (•) and 100x (•) lower amount of enzyme, as the hydrolysis of DMPTB (•).

4.4.7.2 Lipase activity of BChE in the presence of larger synthetic substrate

The next step was to confirm the lipase activity of BChE with a larger substrate, which is structurally closer to the endogenous TAGs. We used the kit described in [chapter: Lipase activity assay kit \(MAK048\)](#)

Similarly to previous experiment, we tested the inhibitory effect of the buffer from the kit, while no inhibition was observed (data not shown). Several concentrations of BChE were used to test the hypothesis, that BChE is able to cleave bulkier substrates similar to endogenous TAG (Figure 48). Despite different reaction conditions (pH or type of the buffer, temperature), we failed to detect any lipolytic activity of BChE with this substrate. Likewise, changes in concentration of substrate or enzyme did not result in lipolysis catalyzed by BChE, indicating this enzyme was not able to cleave this larger synthetic TAG.

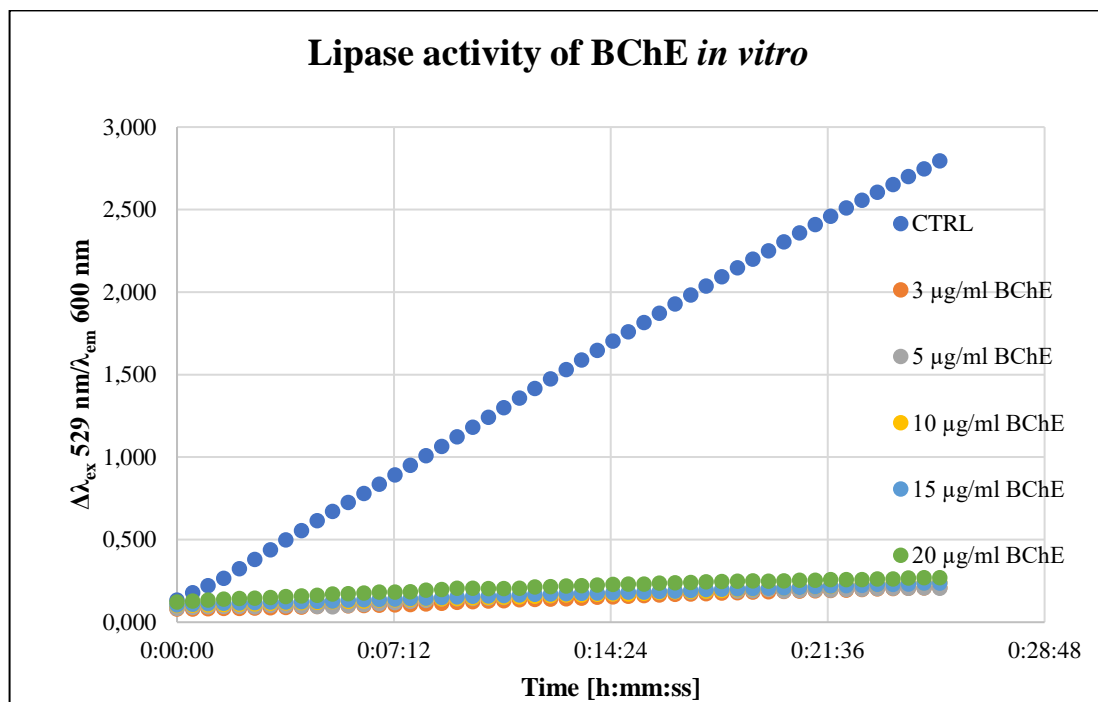


Figure 48: Lipase activity of BChE in vitro. BChE did not cleave the synthetic TAG-like substrate even at higher concentrations. The positive control reaction (CTRL •) was performed with the lipase provided in the kit.

DISCUSSION

CVS diseases belong not only to the leading causes of death in the whole population but also to the costliest diseases. The symptoms and the risk factors are strongly associated, so the diagnostic and the pharmacotherapy are a complex process. Surprisingly, a lot of diseases of this group (hypertension, endothelial dysfunction, atherosclerosis, etc.) and risk factors (obesity, impaired lipid metabolism, high BMI, insulin resistance, etc.) are associated with the serum levels of ChE. Therefore, in this work, we focused on the complex study of the connection ChE-vessels-lipid metabolism.

Cholinesterases are present in rat aorta

Vessels are one of the tissues with no cholinergic innervation. However, their physiological properties are usually tested by the main cholinergic mediator, ACh. It is known that there is a difference in response to ACh when endothelial dysfunction is present. As information about ChE, enzymes terminating the action of ACh, in vessels are sparse, we focused on their detail study.

We confirmed the presence of AChE and BChE along the whole rat aorta, while the relative expression (mRNA), protein level and activity of BChE were several times higher than of AChE, in each part of aorta. BChE activity exceeds AChE in majority of the tissues except for brain and muscle in human (Lockridge, 2015b) mouse (Li et al., 2002) and rat (Boeck et al., 2002; Li et al., 2002).

As described in the introduction, ChE exist in the body in multiple molecular forms. According to our results, the main molecular form of both AChE and BChE in rat aorta is tetramer anchored by PRiMA. PRiMA is a small transmembrane protein that organizes AChE or BChE into tetramer and anchors the tetramer to the plasma membrane (Perrier et al., 2002). In the process of AChE tetramerization, the crucial step is the interaction of AChE with the PRAD domain of PRiMA, as PRAD carries the tetramer through the whole secretory cascade and anchors it on the cell surface (Dobbertin et al., 2009). Lack of PRAD domain of the PRiMA protein leads to the complete lack of AChE on the cell surface, whilst the AChE monomers are retained in the endoplasmic reticulum (Dobbertin et al., 2009). The absence of ChE on the membrane surface was accompanied by several times increased extracellular levels of ACh in brain (Farar et al., 2012), confirming heterooligomers to be the functional forms of the ChE. Based on these facts, we can assume that the functional

form of ChE in the rat aorta are the PRiMA-anchored ChE with a small amount of detected monomers represent intracellular intermediate products of the tetramerization.

We hypothesize that the high level of BChE in rat vessels compensate for its low level in rat serum and *vice versa*. Our hypothesis is supported also by the fact, that mice possesses high BChE levels in their serum (Li et al., 2002), while almost no BChE activity is present in their aorta. One could therefore expect an important role of BChE in vascular system. Nevertheless, function of BChE in the vascular physiology or pathophysiology and even overall in the body remains unclear. The higher concentration of BChE was also confirmed in organs of first contact, where it might serve as a detoxification enzyme (Ashani, 2000). Indeed, BChE possess wide substrate specificity, plus, it is able to cleave other, than ester bonds (Çokuğraş, 2003). It was therefore proposed, that BChE serves as a protective enzyme that cleaves toxic xenobiotics (Lockridge, 2015b). On the other hand, the different kinetic behavior of ChEs brings a very interesting aspect to the whole issue. It is well known, that ChEs act differently in excess of selected substrates, including ACh (Massoulié et al., 1993a). Nevertheless, in excess of ACh, BChE is activated and becomes even more efficient than AChE (Massoulié et al., 1993a; Norel et al., 1993). Additionally, results from experiments with ChE knock-out mice suggest that BChE may serve as a back-up enzyme to maintain ACh homeostasis in the absence of AChE (Li et al., 2002). Thus, we can speculate, that ChE in rat aorta serve as a regulators of the ACh levels – AChE hydrolyses the physiological levels of ACh and BChE may play a protective role against excess of ACh or when AChE is inhibited or not present to avoid the overstimulation of present cholinergic receptors.

The origin of acetylcholine affecting blood vessels

ACh in physiology is considered as a vasorelaxant causing decrease in blood vessel resistance and blood pressure. However, the origin of ACh that would affect vascular ACh receptors is presumably not limited to the cholinergic innervation as it is sparse in vessels. We tested whether ACh may arise directly from the vascular tissue. Our results suggest that aorta belongs to the group of the organs that are capable of ACh synthesis independently on the presence of neurons. (Wessler and Kirkpatrick, 2008). We confirmed the presence of all proteins essential for the life-cycle of ACh in aorta. As described in the introduction (see chapter: Synthesis, transport, and release of acetylcholine), two enzymes could participate

on ACh synthesis. ChAT is an exclusive ACh-synthesizing enzyme in neurons (Tuček, 1982) but its presence was confirmed also in some NNChS, e.g. cardiomyocytes (Kakinuma, 2015; Rocha-Resende et al., 2012), and T-lymphocytes (Koichiro Kawashima et al., 2015) (or see review (I. Wessler & Kirkpatrick, 2008)). Participation of CrAT in ACh synthesis was suggested in some non-neuronal cells, including urothelium (Lips et al., 2007), rat maxilla (Guo et al., 2014) or skeletal muscle (Tuček, 1982) or see review (Beckmann and Lips, 2013). We did not detect any mRNA or protein for ChAT in aorta, which was in line with observations previously made by Krajčovičová et al. (Krajčovičová, 2016). We confirmed the presence of CrAT in all aortic fractions and colocalized its protein with markers of both endothelium and smooth muscle, indicating that ACh can be synthesized in both cell types of aorta. However, CrAT may have a dual function, as its higher amount in smooth muscle can be associated also with its function in FFA metabolism (Longo et al., 2016). This dual function of CrAT may be determined by the presence of two splicing variants (Robic et al., 2009). It would be therefore useful to characterize more precisely which splicing variant is present in rat aorta.

VACHT is a protein responsible for the translocation of ACh from the cytoplasm into vesicles. It is an irreplaceable function, which was confirmed by mutant mice with no VACHT, who died immediately after birth, because the neurons cannot trigger the contractions of the skeletal muscle and the mice were not able to breathe (Prado et al., 2006). We detected VACHT mRNA in each fraction of aorta, while the relative expression was higher in smooth muscle fraction. This observation was confirmed by immunohistochemistry. Nevertheless, Krajčovičová et al. (Krajčovičová, 2016) compared the relative expression of VACHT in whole aorta with relative expression of VACHT in brain and observed that VACHT mRNA in aorta is several times lower. The presence of this transporter may indicate that ACh is also stored in vesicles in aorta.

In some NN-cells, VACHT is not crucial and ACh can stay freely in the cytoplasm or can be directly released from the cytoplasm to the extracellular space via different transporters (Beckmann and Lips, 2013; Fujii et al., 2017; Kummer and Krasteva-Christ, 2014; Wessler et al., 2001), OCTN (Pochini, 2016; Pochini et al., 2012b, 2019), CTL (Inazu, 2014; Song et al., 2013; Traiffort et al., 2005) or mediato-phores (Kawashima et al., 2015), while OCT and OCTN are involved more frequently in this process. We did not detect any mRNA for OCT-1 in endothelial or smooth muscle fractions. On the other hand, we detected

high relative expressions for both OCT-2 and OCT-3 in endothelial and smooth muscle cells, with the highest levels for both transporters observed in smooth muscle fraction. So, these transporters may participate in ACh release. However, we cannot include that OCT present in rat aorta have another function and carries other cationic substances (Jonker and Schinkel, 2004). In pharmacological view, several drugs inhibit or block precisely these transporters (Belzer et al., 2013; Boxberger et al., 2014; Grube et al., 2011; Koepsell, 2015; Panfen et al., 2019). We therefore propose that these drugs could affect the vessel physiology *via* changed ACh release. The relative expression of both OCTN was detected in all the studied fractions, but in contrast to OCT, these transporters were expressed evenly along the endothelium and smooth muscle. OCTN-2 has a high affinity to carnitine (Iwata et al., 2008) so may be involved in other processes of aorta, not only ACh life cycle. Furthermore, its impaired function causes systemic carnitine deficiency, which usually results in cardiomyopathies (Iwata et al., 2008; Wu et al., 2000). So, it is obvious that their correct function is necessary also for CVS physiology. Mutations of these transporters were also described in Crohn's disease (Pochini et al., 2012a) which belongs to the inflammatory diseases and thus is linked to impaired ACh signaling (Goverse et al., 2016).

The rate-limiting step of the ACh synthesis in neurons is the choline reuptake, mediated by ChT-1 (Okuda and Haga, 2003). We confirmed the presence of ChT-1 in each fraction of rat aorta, with the highest relative expression being observed in the fraction of smooth muscle. The RT-qPCR results were confirmed by immunohistochemistry, where the protein was colocalized with markers of endothelium and smooth muscle. Again, the results of (Krajčovičová, 2016) showed that in whole aorta the relative expression was much lower, than in brain.

ACh is believed to act in both, endothelium, and smooth muscle, while employing different molecular mechanisms resulting in opposite vascular effects (see chapter [1.2.2.3. Effect of acetylcholine on vessels](#)). The main mAChR in vascular physiology is m3AChR, which is responsible for endothelium dependent vasodilation in vessels (see [chapter: Effect of acetylcholine on vessels](#)). We observed the highest mRNA levels for this receptor, while the expression was mostly localized in the endothelium. In addition, we detected all known mAChR with exception for m4AChR. The presence of different mAChR were documented by other authors as well, while the distribution of these receptors was species-specific. In rabbits, endothelium of *arteria saphena magna* expressed m1AChR and m2AChR (Komori

and Suzuki, 1987), endothelium of human pulmonary veins expressed m1AChR (Walch et al., 2000), m1AChR and m3AChR were expressed in human pulmonary arteries (Norel et al., 1996), in rat arteries m1AChR and m5AChR were detected (Phillips et al., 1997), in mesenteric arteries of rat m1AChR and m3AChR were confirmed. (Tangsucharit et al., 2016). In our set of experiments, the second most abundant mAChR in rat aorta was m1AChR, while it was detected both in endothelium and smooth muscle. Stimulation of this receptor in human pulmonary arteries is associated with endothelium-dependent vasodilatation (Norel et al., 1996; Tangsucharit et al., 2016). However, in cutaneous and renal interlobar arteries, involvement of m1AChR in ACh – facilitated vasodilatation has not been confirmed (Gericke et al., 2011). The m5AChR was detected only in endothelium. However, its function in vessel is not yet established as the lack of this receptor in vessels did not affect the physiological processes in vessels (Gericke et al., 2009, 2011; Radu et al., 2017).

The relative expression of nAChR subunits were also determined. The presence of all the subunits (neuronal or muscular type), except for $\alpha 9$ was confirmed, with highest relative expression of $\alpha 7$ in each aortic fraction. These results are in line with the results obtained by other teams (Brüggmann et al., 2002). It has been shown, that these $\alpha 7$ nAChRs are involved in vascular tone regulation in selected CVS pathologies. Under blockade of mAChRs, ACh induced relaxation in the aortae of SHR, but not in Wistar-Kyoto rats (Zou et al., 2012). In SHR, several changes were described in $\alpha 7$ nAChR-mediated cholinergic anti-inflammatory pathway (Chen et al., 2012). The role in angiogenesis was also confirmed (Heeschen et al., 2002; Ni et al., 2010). The upregulation of $\alpha 7$ nAChR was observed during proliferation, and also due to hypoxia *in vitro* or ischemia *in vivo*. Pharmacological inhibition of these receptors significantly inhibits inflammatory angiogenesis, tumor growth or reduces ischemia-induced angiogenesis (Heeschen et al., 2002). Nevertheless, these receptors were proposed as therapeutic targets not only in angiotensin II- associated vascular aging disorders (Li Dong-Jie et al., 2016), but also in several physiological and pathological angiogenesis, e.g. inflammation or hypoxia-induced angiogenesis, as these conditions upregulate these receptors in endothelial cells (Carmeliet and Jain, 2000; Egleton et al., 2008; Heeschen et al., 2002). In other study, nicotine - induced nAChR-mediated inhibition of apoptosis and stimulation of proliferation in aortic smooth muscle cells were observed (Cucina et al., 2008). The pro-atherogenic effect of nicotine and other nAChR ligands on

vessels was documented in other study, which focused on plaque growth and neovascularization (Santanam et al., 2012). Furthermore, it seems, that these receptors are associated also with insulin resistance, as insulin is involved in progression of atherosclerosis by promoting the proliferation of the vascular smooth cells (Ross, 1993).

On the other hand, we cannot forget about other sources of ACh, that may affect vessels. Moreover, it was shown, that ACh secreted by non-neuronal cells has equally important function in physiology of CVS, as ACh produced by neurons (Rana et al., 2010; Roy et al., 2013) indicating that ACh with different origins participate on vessel physiology. For example, CD4⁺ T-lymphocytes are able to produce ACh *via* present ChAT, while the lack of ChAT in these cells caused elevated blood pressure and the overexpression of this transporter (higher ACh production) caused decreased blood pressure (Olofsson et al., 2016). Furthermore, ACh synthesized by cardiomyocytes is responsible for heart rate regulation (Roy et al., 2013). Other origin of ACh may be also the neuromuscular junction (where the possibility of ACh spillover was described, Nervo et al., 2019). Nevertheless, one cannot rule out a possible “cholinergic contamination” arising from parasympathetic innervation at the base of the heart. We can speculate, that ACh from these sources also impact vessels, but there is a lack of information about the exact mechanism. We expect, this ACh coming from different sources by blood activates the m3AChR at the luminal side of endothelium and activates the pathway responsible for NO-production and vasodilation. The ACh produced by the smooth muscle of aorta may activate mAChR located on the surface of smooth muscle and causes vasoconstriction. Under physiological conditions we expect to have these mechanisms in equilibrium. On the other hand, we can speculate, that the ACh produced by smooth muscle is released to the extracellular area and has an important, till now not described function. One cannot include the possibility, that ACh is synthesized and released as a response to a particular condition or stimuli.

Lower BChE levels in aortic wall of SHR

The action of exogenous ACh and the subsequent response depends on several factors, one of the main ones being whether it is a precontracted artery or an artery with resting tone. It has long been known to pharmacologists that in the case of a resting tone artery, dose-dependent vasoconstriction occurs after ACh stimulation, and this is independent of the presence or absence of endothelium (Furchgott and Zawadzki, 1980;

Norel et al., 1996). In contrary, ACh-stimulation of the precontracted artery with an intact endothelium causes vasodilation. However, in case of impaired endothelium (or its absence), the artery responds to ACh stimulation by a dose-dependent vasoconstriction (Furchgott and Vanhoutte, 1989; Norel et al., 1996). Thus, endothelial cells play a key role in the regulation of vascular tone. Besides that, their role in angiogenesis, and in the vascular inflammatory response has been described (Cooke, 2007; Ni et al., 2010), as they synthesize prostaglandins, EDRF but also various enzymes that are able to activate or degrade vasoactive hormones. Therefore, endothelial targeting is used in the treatment of several CVS diseases (Hadi et al., 2005). According to recent studies, NN- ACh also regulates these mechanisms through the modulation of the production and release of endothelial autacoids, such as e.g. NO or various prostaglandins (Hadi et al., 2005; de Wit et al., 2000). Recently, endothelial dysfunction has been described in SHR, represented by impaired anatomical, morphological and pharmacological characteristics and altered vessels reaction to various vasodilatory or vasoconstrictor substances (including ACh) (Berenyiova et al., 2018; Cacanyiova et al., 2016; Cacányiová et al., 2008; Gerová et al., 2005; Gluais et al., 2005; Puzserova et al., 2014; Rapoport and Williams, 1996). (Hadjiisky et al., 1987).

There were several differences observed between the mRNA levels of different proteins and enzymes involved in ACh life cycle. First, we determined the mRNA levels in different parts of aorta of SHR and compared to the aorta of Wistar. Significant difference in AChE mRNA levels was only detected in *aorta abdominalis*, where the expressions were higher in SHR. BChE mRNA expression and activity pattern of SHR aorta followed those in Wistar while reached overall lower levels. Lowered BChE expression (and thus activity as well) may represent compensatory adaptation to the insufficient ACh signaling. However, SHR model is characterized by endothelial dysfunction, which is characterized not only by impairment of NO-production (and consequent impaired vasodilatory mechanism) and also by higher permeability for chemical substances present in blood (including ACh). Decreased BChE levels may not be sufficient to control the levels of ACh in smooth muscle, strengthen the vasoconstriction and this way may cause pathological conditions.

In the SHR model, we observed decreased level of CrAT which could be unrelated to the cholinergic system, taken in consideration multiple roles of this enzyme in the cell biology and the complexity of SHR pathophysiology. Lower level of CrAT, however, decreases ACh level, which can be explained as an adaptative response to lower BChE

activity and the not-sufficient control of ACh signaling. On the other hand, lower cholinergic signaling may have also negative consequences. Therefore, compensatory mechanisms are activated - increased uptake to the vesicles (upregulated VACHT) and up-regulation of the m3AChR. Taken in consideration the different response of endothelium and smooth muscle to the presence of ACh, we can hypothesize, that the enhancement of cholinergic signaling is beneficial only in endothelium, where ACh induce a pathway resulting in dilation of vessels. One cannot exclude that increased VACHT is caused by adaptative mechanism of vessel to decrease the effect of ACh on smooth vessels to prevent vasoconstriction. On the other hand, VACHT expression was shown to directly regulate ACh level, as an increased VACHT expression led to heightened cholinergic transmission (Roy et al., 2016; Sugita et al., 2016). Though, in mice, the long-term overexpression of VACHT induced structural changes in muscle fibers at NMJ (Sugita 2016). VACHT overexpression induced morphological changes also in other systems (Janickova et al., 2017), so we can include that long-term overexpression of this transporter may have negative consequences on smooth muscle of aorta. Moreover, increased levels of VACHT seemed to be beneficial in heart failure (Roy et al., 2016). However, the impact of the long-term increase in VACHT expression on vessels has not been established yet. Another observation was related to the OCT2, OCT3, OCTN1 and OCTN2. Despite the fact that those transporters were linked to NN-ACh transport (see above), due to their promiscuity in transported molecules, we cannot conclude that their decreased mRNA levels in SHR are a direct consequence to impaired ACh levels. Interestingly, L-carnitine transport in rat endothelial cells is mediated through the OCTN1 and OCTN2 and is impaired in SHR (Salsoso et al., 2014). Thus, changes in the OCTNs levels that we observed in SHR could be a consequence to the impaired CrAT levels and by extension to the L-carnitine synthesis.

Our results confirmed the impairment of the cholinergic system in SHR aorta. As AChE levels in rat aorta were preserved, the lower activity of BChE suggests involvement in the regulation of the ACh signaling. Additionally, participation of BChE in vascular pathogenesis may be suggested based on the above-described non-cholinergic catalytic properties and supported with clinical evidence. Endothelial dysfunction is associated with essential hypertension and CVS risk factors such as aging, hypercholesterolemia or diabetes mellitus (Bonetti et al., 2003; Esper et al., 2006; Gimbrone and García-Cardena, 2016). The literature describes a link between these condition and ChE, primarily BChE levels in related

tissues (Bradamante et al., 2004; Duchnowicz et al., 2018; Iwasaki et al., 2007; Lucić Vrdoljak et al., 2005; Ondříková, 2011; Santarpia et al., 2013; Sridhar et al., 2005). Endothelial dysfunction, also characterized by a reduction in the bioavailability of NO (Gimbrone and García-Cardena, 2016), increases the excess of oxidants and the expression of adhesion molecules involved in the initiation and progression of atherosclerotic plaque formation and the incidence of CVS events (Alvarez and Ganz, 2004; Bonetti et al., 2003; Gimbrone and García-Cardena, 2016).

Butyrylcholinesterase is involved in lipid metabolism

As it was mentioned before, the exact physiological role of BChE has not yet been established. There are numerous functions that has been proposed for this enzyme, while recently BChE participation in lipid metabolism and in pathophysiology of obesity has been gaining a lot of interest (see chapter: Butyrylcholinesterase and lipid metabolism). Indeed, mice with inactivated gene for BChE fed on HF diet showed significant increase in weight (Chen et al., 2017b; Li et al., 2008a). Moreover, experiments with BChE gene transfer showed that recovery of BChE activity blocked weight gain in HF-fed BChE $-/-$ mice (Chen et al., 2017b). In contrary to BChE knock-out mice, we observed significant weight gain, and an increase in abdominal circumference and BMI in Wistar rats on standardized diet when BChE was inhibited by iso-OMPA. This discrepancy could be explained by the lack of adaptation mechanisms that are most likely present when BChE is missing from the birth. In line with this, SHR rats, which possess significantly lower serum BChE activity, had BMI comparable to Wistar rats. The weight gain of Wistar rats after iso-OMPA was comparable to the rats on HF diet and surprisingly, was not further increased when both, HF and iso-OMPA were combined. It may indicate that the mechanism of both HF and BChE-inhibition affect the same pathway, while saturation of the process of weight gain may occur. SHR rats did not respond to the HF diet, neither inhibition of BChE. In general, the impact of HF diet on SHR is controversial, and resistance to the weight gain was reported before (Bosse et al., 2013; Pereira et al., 2019; Shiou et al., 2018; Yuan et al., 1998).

It was proposed in literature that stabilization of the weight after BChE gene transfer is achieved by suppression of the appetite-promoting signals. BChE was previously described as an enzyme hydrolyzing ghrelin, the "hunger hormone" (Chen et al., 2015, 2017b; De Vriese et al., 2004) and by this action to strongly effect ghrelin circulating levels

and also its action in the control of hunger, body temperature and also emotions like anxiety or aggression (Chen et al., 2015, 2017b; Schopfer et al., 2015). However, to better understand the BChE-ghrelin axis in this context, we have to complete the information about ghrelin levels in serums of our experimental animals. On the other hand, the connection between BChE and ghrelin was documented in a mouse with high serum BChE level (Chen et al., 2015, 2017b), not in rats, so we can hypothesize that in different strains different mechanisms are employed and the low serum level of BChE is not sufficient to control the levels of ghrelin. Another two very important hormones, which regulates among others, the energy reserves and obesity are leptin (Kelesidis et al., 2010) and adiponectin (Forny-Germano et al., 2019). The serum leptin levels strongly correlate not only with serum BChE levels, but also with mean arterial blood pressure, total cholesterol, and TAG, while the serum concentrations of adiponectin were observed to negatively correlate with BChE activity (Iwasaki et al., 2007). In continuation of this project, we are therefore planning to complete the story with this information and to focus more in detail on these indicators of obesity and systemic inflammation.

As lower BChE activities were previously associated with higher food intake (Chen et al., 2017b), we expected increased food consumption in rats, to which iso-OMPA was administered, but this hypothesis was not confirmed. The food intake (consumed grams) was not affected by BChE inhibition, nor observed in SHR, which as described above, possess lower BChE activities. The observed weight gain is therefore more likely to be caused by changes in the metabolism. On the other hand, while HF-fed Wistar rats drank less water (which is in line with other authors (Kaunitz et al., 1956; Ramalho et al., 2017; Volcko et al., 2020)), Wistar rats but not SHR receiving iso-OMPA drank statistically more. Similar observation was reported before when ChE were inhibited by OP (Hazarika, 2014). We cannot rule out that the change in weight in these animals is partially caused by water retention. Indeed, morphological analysis uncovered differences in the weight gain of adipose tissues. While in Wistar rats on HF diet, all collected adipose tissues increased in weight, in Wistar rats on SD and iso-OMPA this increase was less dramatic, reaching significance only in the epididymal tissue.

Our hypothesis about changed metabolism is supported by the parameters of the lipid metabolism. As expected, HF diet increased cholesterol and TAG levels. Moreover, similarly increased TAG levels were observed in Wistar rats treated with iso-OMPA,

regardless the diet. The association between BChE activity and serum TAG levels was previously described (Iwasaki et al., 2007; Ondříková, 2011). We hypothesize that BChE participates in the regulation of TAG levels and lack of BChE activity leads to the increased plasmatic TAGs in Wistar rats treated with iso-OMPA. This hypothesis is further supported by the fact that the increase in the plasmatic levels of TAG in Wistar rats on HF diet are accompanied by increased plasmatic BChE activities. Similar observation was made in humans as well, as it has been observed that the serum concentration of BChE positively correlates with levels of TAG (Vicky Ping Chen et al., 2016; Iwasaki et al., 2007; Lucić Vrdoljak et al., 2005; Ondříková, 2011).

In rat model, this compensatory BChE increase to HF-diet –induced TAG in plasma was also observed in vessels. BChE was the only impaired cholinergic marker in affected aorta. The relative expressions of AChE and of enzymes and transporters of the ACh life cycle, except for OCT-2 remained unchanged. We can speculate that this BChE increase is a compensatory mechanism to the higher levels of TAG and vascular BChE acts as a detoxification enzyme for these molecules and this way is involved in the prevention of atherosclerosis. This hypothesis is in line with the fact that plasma BChE activities correlate with higher LDL and TAG levels (Bradamante et al., 2004; Duchnowicz et al., 2018; Iwasaki et al., 2007; Lucić Vrdoljak et al., 2005; Ondříková, 2011; Santarpia et al., 2013; Sridhar et al., 2005), known risk factors for atherosclerosis (Goldberg et al., 2011; Peng et al., 2017; Talayero and Sacks, 2011).

BChE possesses esterase, but also peptidase or acyl-aryl-amidase activity (Lockridge, 2015b). TAG is made from FFA, which are attached to glycerol by ester bond, so hypothetically this bond may be cleaved by BChE. Moreover, the catalytic triade of BChE and TAG-hydrolase contains the same amino acids (Kumar et al., 2016; Lockridge, 2015b). Results from our *in vitro* experiments with a synthetic TAG-like substrates suggests that BChE may indeed possess lipolytic activity. We observed that human plasmatic concentration of BChE (3,02 µg/ml – Browne et al., 1998) is able to degrade small thio-TAG-like substrate (DMPTB), while this activity was completely inhibited by iso-OMPA, confirming that the hydrolysis was done exclusively by BChE. The rate of hydrolysis of DMPTB was however significantly slower than the rate of the hydrolysis of BTC. This observation was in line with previous observations, as the hydrolysis of bulkier substrates is much slower, than the hydrolysis of smaller substances (Chen et al., 2015, 2017b). Further,

a structurally closer substrate to native TAG for BChE lipase activity was tested as well, but despite various tested experimental condition, we failed to demonstrate capability of BChE to cleave this substrate. Even if BChE shows lower substrate specificity (Massoulié et al., 1993a) and is able to cleave bigger substrates (Brimijoin et al., 2018; Chen et al., 2015; Zheng et al., 2014), we cannot claim that this synthetic substrate is suitable for BChE. As lipid metabolism is a complicate, multi-step reaction and we can find several other enzymes in serum involved in fat metabolism, e.g. paraoxonases (Kudchodkar et al., 2000; Meneses et al., 2019) or carboxylesterases (Di, 2018; Lian et al., 2018), we cannot rule out that BChE is involved in this complicated process. Possibly it works as a co-enzyme and for correct functioning needs the presence of another enzyme. As we did not have completely physiological conditions for the experiment, we can speculate, that BChE may have different behavior *in vivo*. It is also possible, that *in vivo*, there is a trigger signal which induce BChE to cleave the substrates with TAG character.

**■ CONCLUSIONS AND
CLINICAL
IMPLEMENTATIONS OF
THE RESULTS**

ChE are irreplaceable enzymes in the whole organism, including CVS. Their thorough study in heart was done before, but detail information about vessels were missing. In this thesis, their complete study in rat aorta was done. We confirmed the presence of both ChE at mRNA and protein levels, determined their activities and described their molecular forms, too. All the results indicated that BChE was the dominant enzyme in each part of aorta, while its activity was the highest *in arcus aortae* and comparable in other two parts, *aorta thoracalis* and *aorta abdominalis*. Our results confirmed the presence of this enzyme mainly in PRiMA-anchored form and localized on the surface of smooth muscle indicating that BChE has an important function in vascular physiology. One of the role of vascular physiology that we propose is hydrolysis of ACh. ACh could originate from blood cells (e.g., T-lymphocytes) or more distant sources (e.g., cardiomyocytes) or, as our results suggests, it can be synthesized and released by vessels.

Indeed, we confirmed all the transporters and enzymes needed for ACh synthesis, storage, and release, indicating that aorta is able to produce ACh (Figure 49A). We detected low levels of VAcHT suggesting the possibility of the storage of synthesized ACh, however, the stimuli that may activate the release of ACh from vesicles needs to be further examined. On the other hand, we determined high relative expressions for different OCT, so one cannot exclude that produced ACh is released *via* OCT receptors to extracellular space and has an autocrine/paracrine function, which has not been described yet. Therefore, further experiments are needed. Nevertheless, as it is well known, direct activation of mAChR on endothelium causes vasodilation, while direct activation of these receptors on smooth muscle results in vasoconstriction, we can hypothesize that under physiological condition, the vasodilatory and vasoconstrictor mechanisms are in equilibrium. We further hypothesize that the effect of ACh distributed by blood on endothelial m3AChRs is regulated by serum ChE levels (which activates the vasodilatory pathway of vessel) and the action of ACh synthesized by smooth muscle (which causes vasoconstriction by activation of mAChRs) is regulated by present BChE. For a schematic representation of our main findings see Figure 49.

To better understand the function of ChE and the ACh produced by aorta, we performed the detail analysis of the SHR model, in which endothelial dysfunction has been described. We observed several differences between these two models: SHR had lower BChE and CrAT levels, but higher relative expressions of VAcHT and m3AChRs. All these

enhance of vasoconstrictor mechanism, which are further enhanced by lower BChE activities (Figure 49B). The rat aorta has all the equipment needed for ACh synthesis, while in SHR model we expect an impairment of ACh life cycle.

Besides a role in ACh hydrolysis, our results suggest also other vascular function of BChE. We confirmed the association between BChE serum levels and lipid metabolism. The effect of HF-diet and BChE inhibition were tested on rat model. Our results are summarized in Table 9. We observed an impact of BChE on water intake and weight gain. Rats with inhibited BChE drank significantly more water and gained significantly more weight (comparable to the rats fed on HF-diet), than their non-treated littermates on SD. This weight gain was reflected in changed BMI, Lee index and fat tissue distribution. Moreover, a significant association between serum BChE levels and serum TAG levels was observed in experimental animals. Rats with inhibited BChE in their body had an increase in TAG levels without higher fat intake in their diet. In the serum of rats fed by HF we observed an increase in serum BChE activities indicating the effort of organism to deal with higher fat intake and resulting increased serum TAG levels. In aorta, we had similar observation, HF diet induced an increase in mRNA levels of BChE. On the other hand, inhibition of BChE activity by isoOMPA lead to increased TAG levels. These two observations together indicate that BChE participates in control of the plasmatic TAG levels. We are however uncertain about the mechanisms by which this is achieved so far. While we recorded the lipase activity of BChE with small thioester analogue of TAG, we failed to confirm that with bigger substrate *in vitro*. Nevertheless, taken in consideration methodological limitation of the assay, direct BChE hydrolytic activity of TAG is not ruled out. Taken together, our results confirmed the presence of BChE in vessels and also its participation on lipid metabolism.

Clinical implication of our observations is in the field of vascular physiology, pathophysiology and pharmacology. The confirmed presence of ChE and the NNChS in vessels provides new insight in vessel physiology. During the years, the use of ACh to test the integrity of vessels became a commonly used experimental method, however, the influence of present ChE were never taken into account. Based on our results, we hypothesize, that the activity of these enzymes in vessels may highly affect the vessels response to ACh and thus this method does not necessary reflects selectively intactness of endothelium but also impaired BChE activity. Thus, results obtained from the pathological models with impairment of endothelium depend on changes in BChE vascular levels. The

changes in BChE activity, or in the NNChS overall may be responsible for altered response to exogenous ACh. We therefore recommend assessing ChE activities when the vessel physiology is tested.

As it was mentioned before, inhibitors of ChE are widely used in pharmacotherapy of patients with myasthenia gravis, AD, or other dementias. Moreover, several of them are already in clinical trials as a potential drug in the treatment of different types of CVS pathologies. So, the strong association between ChE activity and CVS pathologies is obvious. However, till now, the main focus was on the study of ChE within the heart. Here, we provide important information about the connection of ChE, more precisely BChE and vessels. Based on our results, we can hypothesize that BChE has an important function in vessel physiology as a regulator of ACh levels and this way have an impact on vasodilatory/vasoconstrictor mechanism of the vessels. It is well described that the imbalance in these mechanisms are associated with pathologies. We can conclude that the changed levels of BChE may be responsible for the changes in vessel response to ACh. However, the question what causes the decrease of BChE levels remains unanswered and to answer it, further experiments are needed. The main observation, that BChE is present in vessels and we propose that it has an important function in vessel physiology, is a new information and is applicable universally in clinical pharmacology.

Moreover, therapy with ChE inhibitors does not selectively affects an organ but activities of ChE within whole body are inhibited. Thus for instance inhibitors of ChE indicated for demencia in AD do not selectively affect just brain but also cholinergic (neuronal and non-neuronal) systems at periphery. As we confirmed the presence of BChE in vessels, its involvement in obesity, and lipid metabolism, one has to keep in mind that all the non-selective ChE inhibitors used in pharmacotherapy may affect other processes of the body, included the ones associated with obesity and lipid metabolism. Our results suggest that approach to the pharmacotherapy of patients on ChE inhibitors should be revisited. As in clinical practice not only selective AChE inhibitors (donepezil, galantamine), but also non-selective inhibitors are used (neostigmine, pyridostigmine, rivastigmine), one has to focus on the other comorbidities of patients in the selection of appropriate drug. For example, for patients with hypertension and/or endothelial dysfunction may be a better choice a selective AChE inhibitor to avoid the negative consequences of BChE inhibition on vessels. Moreover, our results may help to choose more personalized treatment for patients with

metabolic problems as obesity or lipid imbalance. One should take in consideration these pathologies in the process of individualization of the pharmacotherapy, as these patients should avoid the further inhibition of BChE. Furthermore, as it was mentioned before, different inhibitors of ChE are in clinical trials for the treatment of several CVS diseases, their impact on vessel physiology and lipid metabolism should be examined.

Biometric parameters of experimental animals	Wistar CTRL	Wistar iso-OMPA	Wistar HF	Wistar HF+iso-OMPA	SHR CTRL	SHR CTRL	SHR iso-OMPA	SHR HF	SHR HF+iso-OMPA
	REF	↑***	↑***	↑***	REF	REF	REF	REF	REF
Weight gain (Δ)	REF	↑***	↑***	↑***	REF	-	-	-	-
Water consumption (ml/day)	REF	↑***	↓****	-	REF	-	-	↓***	↓**
Food consumption (g/day)	REF	-	-	-	REF	-	-	-	-
Abdominal circumference (cm)	REF	↑*	↑*	↑*	REF	↓***	-	-	-
BMI (total length of animals)	REF	↑*	↑***	↑***	REF	-	-	-	-
Lee index	REF	-	↑**	↑**	REF	-	-	-	-
ChE activities and parameters of lipid metabolism	Wistar CTRL	Wistar iso-OMPA	Wistar HF	Wistar HF+iso-OMPA	SHR CTRL	SHR CTRL	SHR iso-OMPA	SHR HF	SHR HF+iso-OMPA
Epididymal fat (g)	REF	↑**	↑****	↑***	REF	-	-	-	-
Retropitoneal fat (g)	REF	-	↑*	-	REF	-	-	-	-
Perirenal fat (g)	REF	-	↑**	↑*	REF	-	-	-	-
Serum BChE activity	REF	↓***	↑***	-	REF	↓*	↓***	-	-
Serum AChE activity	REF	-	-	-	REF	-	-	-	-
S-CHOL	REF	-	↑*	-	REF	-	-	-	-
S-TAG	REF	↑*	↑**	↑**	REF	↓*	-	-	-
S-dHDL	REF	-	-	-	REF	-	-	-	-
Relat.expr. of the components of NNChS in a.thoracalis	Wistar CTRL	Wistar iso-OMPA	Wistar HF	Wistar HF+iso-OMPA	SHR CTRL	SHR CTRL	SHR iso-OMPA	SHR HF	SHR HF+iso-OMPA
AChE-T	REF	-	-	-	REF	-	-	-	-
BChE	REF	-	↑*	↑*	REF	↓*	-	-	-
PRiMA	REF	-	-	-	REF	↓*	-	-	-
ChAT	REF	-	-	-	REF	-	-	-	-
CrAT	REF	-	-	-	REF	↓*	↑*	-	-
ChT-1	REF	-	-	-	REF	-	-	-	-
VAcHT	REF	-	-	-	REF	↑*	-	-	-
OCT-1	REF	-	-	-	REF	-	-	-	-
OCT-2	REF	↓*	-	↓*	REF	-	-	-	-
OCT-3	REF	-	-	-	REF	↓*	↑*	-	-
OCTN-1	REF	-	-	-	REF	↓***	-	-	-
OCTN-2	REF	-	-	-	REF	↓***	↑*	-	-
m3AChR	REF	↑*	-	↑*	REF	↑**	-	-	-

Table 9: Summarization of results obtained in the pharmacological experiment
AChE – Acetylcholinesterase, BChE – Butyrylcholinesterase, PRiMA – proline rich membrane anchor, ChAT – Cholinacetyltransferase, CrAT – Carnitine-O-acetyltransferase, ChT-1 – choline transporter-1, VAcHT – vesicular acetylcholine transporter, OCT-organic cationic transporter, m3AChR- muscarinic acetylcholine receptor type 3

RESUMÉ

Introduction:

Depuis que la présence d'acétylcholine (ACh) dans des tissus sans innervation nerveuse a été décrite, l'ACh n'est plus seulement considérée comme un neurotransmetteur classique, mais plutôt comme un médiateur omniprésent, qui peut être synthétisé par de nombreux types cellulaires. Le système cholinergique non neuronal est un terme utilisé pour décrire la présence des composants du système cholinergique dans les tissus non nerveux, c'est-à-dire les protéines et les enzymes participant à la synthèse, au stockage, à la sécrétion et à la dégradation de l'ACh, ainsi que les récepteurs cholinergiques. Jusqu'à présent, sa participation active dans de nombreux processus physiologiques et pathologiques a été décrite, indiquant la possibilité de développer de nouvelles stratégies thérapeutiques plus efficaces.

Plusieurs différences entre l'ACh neuronale et non neuronale ont été décrites. Comme on le sait, l'ACh neuronale est synthétisée presque exclusivement par la choline acétyltransférase, puis est transportée par le transporteur vésiculaire d'ACh dans des vésicules, où elle est stockée jusqu'à l'arrivée de l'influx nerveux. En réponse à l'augmentation des niveaux de calcium intracellulaire, l'ACh est libérée par fusion des vésicules dans la fente synaptique. Dans les cellules cholinergiques non neuronales, l'action de la choline acétyltransférase peut être remplacée par la carnitine acétyltransférase et le transporteur vésiculaire d'ACh peut ne pas être présent. À la place, l'ACh peut se trouver dans le cytoplasme et être libérée par des transporteurs non spécifiques comme : transporteurs de cations organiques, protéine de type transporteur de choline ou médiatophores. Dans le système cholinergique non neuronal, on n'a pas encore élucidé comment la libération d'ACh est contrôlée et quelles autres molécules sont impliquées dans ce processus. L'ACh libérée dans l'environnement extracellulaire agit *via* les récepteurs muscariniques à l'ACh ou les récepteurs nicotiques à l'ACh tant dans le système cholinergique neuronal que dans le système cholinergique non neuronal. Dans le système cholinergique neuronal, l'ACh est une molécule à courte durée d'action, tandis que dans le système cholinergique non neuronal, son action semble être continue. La dégradation de l'ACh est assurée par les cholinestérases (ChE). L'un des produits de l'hydrolyse est la choline, qui est absorbée dans les cellules nerveuses par le transporteur de choline de haute affinité. Dans les cellules non neuronales, la recapture de la choline peut être assurée par des transporteurs non spécifiques (transporteurs de cations organiques, protéine de type transporteur de choline ou médiatophores).

Les ChE sont des enzymes du groupe des sérine hydrolases capables de cliver l'ACh, mais elles se distinguent par la spécificité de leur substrat, leur sensibilité aux inhibiteurs des ChE, leur structure, leur localisation et leur fonction dans l'organisme. Bien qu'à des concentrations différentes, les deux ChE ont été détectées dans tous les tissus. L'AChE est présente à la plus forte concentration dans le cerveau, la jonction neuromusculaire et la membrane des érythrocytes, tandis que la butyrylcholinestérase (BChE) est principalement présente dans les organes de premier contact, tels que le plasma, le foie et la peau. L'AChE peut hydrolyser plus probablement de petits substrats, tandis que la BChE se caractérise par une large spécificité de substrat. Les ChE sont des enzymes constituées de sous-unités glycoprotéiques formant différents monomères ou oligomères. Nous distinguons deux groupes principaux basés sur la structure des ChE : les formes homo-oligomères et les formes hétéro-oligomères, qui possèdent des propriétés catalytiques, mais pas hydrodynamiques, similaires. Parmi plusieurs variantes d'épissage, seule l'AChE-T possède la capacité de créer des formes hétéro-oligomères grâce à la présence d'un domaine de tétramérisation amphiphile et de Cys. Un autre domaine nécessaire à la tétramérisation est le domaine d'attachement riche en proline, qui est localisé à l'extrémité N-terminale des protéines d'ancrage. On distingue 3 types de protéines d'ancrage, le collagène Q, l'ancre membranaire riche en proline et la lamellipodine.

Malgré l'absence d'innervation cholinergique, les vaisseaux sont très réactifs à l'ACh, qui est couramment utilisée pour évaluer la fonction endothéliale des vaisseaux. L'effet de l'ACh sur les vaisseaux a fait l'objet d'un examen approfondi ; son action sur les récepteurs muscariniques endothéliaux entraîne une vasodilatation, tandis que son activation directe des récepteurs muscariniques sur les muscles lisses provoque une vasoconstriction. Le mécanisme supposé de l'action de l'ACh est le suivant : l'ACh active les récepteurs muscariniques endothéliaux, ce qui, à son tour, entraîne une relaxation du muscle lisse vasculaire. L'effet de l'ACh dépend également du tonus vasculaire et de la présence et de l'intégrité de l'endothélium. Bien que l'action de l'ACh soit interrompue par deux ChE, les informations sur leur présence et leur activité dans les vaisseaux font encore défaut. De plus, plusieurs publications relient l'activité des ChE, enzymes stoppant l'ACh, à l'hypertension, l'hypercholestérolémie et l'athérosclérose, qui font partie des principaux facteurs de risque cardiovasculaire accompagnés de changements physiopathologiques dans les vaisseaux. Par

conséquent, ce projet se concentre sur la caractérisation des ChE et du système cholinergique non-neuronal dans les vaisseaux de modèles sains et pathologiquement modifiés.

Objectifs et Méthodes :

Le premier objectif de ce projet était de caractériser en détail les ChE dans un vaisseau modèle, l'aorte, de rats sains. Pour atteindre cet objectif, diverses méthodes moléculaires, biochimiques et microscopiques ont été utilisées pour déterminer la présence de ChE (ARNm et protéines) et pour quantifier les activités des ChE au niveau longitudinal (longueur totale de l'aorte divisée en *arcus aortae*, *aorta thoracalis*, *aorta abdominalis*) et transversal (endothélium vs muscle lisse). De plus, nous avons examiné la présence de toutes les enzymes et transporteurs nécessaires à la synthèse, au transport et à la libération de l'ACh dans toutes les parties étudiées de l'aorte. Nous avons cherché à étudier ces protéines impliquées dans le cycle de vie de l'ACh au niveau de l'ARNm et des protéines.

Le dysfonctionnement endothélial, qui peut être caractérisé comme une réponse altérée à l'ACh, est souvent associé à des conditions pathologiques, telles que l'hypertension, l'hypertriglycémie ou le syndrome métabolique. Récemment, l'activité BChE a été suggérée comme un marqueur de ces comorbidités. Néanmoins, malgré le fait que l'ACh soit couramment utilisée pour examiner la réactivité des vaisseaux et donc l'intégrité et le bon fonctionnement de l'endothélium ex vivo, les informations sur le système cholinergique non neuronal dans les vaisseaux dont la fonction endothéliale est altérée sont rares. Le deuxième objectif était donc de caractériser le système cholinergique non neuronal aortique chez des rats spontanément hypertendus, chez qui le dysfonctionnement endothélial a été bien décrit auparavant. Nous nous sommes concentrés sur tous les marqueurs cholinergiques impliqués dans le turnover de l'ACh au niveau de l'ARNm, des protéines et de l'activité.

La fonction physiologique de la BChE reste inconnue, mais des données récemment publiées apportent des preuves de son rôle important dans le métabolisme des lipides et l'obésité. Sur la base du niveau élevé de BChE dans les vaisseaux que nous avons observé, notre troisième objectif était d'examiner la relation possible entre la BChE aortique et l'augmentation de l'apport lipidique. Nous avons étudié l'impact d'un régime riche en graisses sur les profils cliniques de rats normo-tendus et de rats spontanément hypertendus sous inhibition sélective de la BChE et sans inhibition. En outre, nous avons examiné l'activité lipasique de la BChE,

hypothèse basée sur la corrélation décrite entre les activités butyrylcholinestérasiques plasmatiques et les niveaux de triacylglycérol.

Résultats et conclusion:

Les ChE sont présentes dans l'aorte du rat

Nous avons confirmé la présence d'AChE et de BChE le long de toute l'aorte du rat, tandis que l'expression relative (ARNm), le niveau de protéine et l'activité de la BChE étaient plusieurs fois plus élevés que ceux de l'AChE, dans chaque partie de l'aorte. L'activité de la BChE dépasse celle de l'AChE dans la majorité des tissus, à l'exception du cerveau et du muscle chez l'homme (Lockridge, 2015), la souris (Li et al... 2002) et le rat (Boeck et al... 2002), 2002) et du rat (Boeck et al., 2002 ; Li et al., 2002). La concentration plus élevée de BChE a également été confirmée dans les organes de premier contact, où elle pourrait servir d'enzyme de détoxification (Ashani, 2000). En effet, la BChE possède une large spécificité de substrat, de plus, elle est capable de couper d'autres liaisons que les liaisons ester (Çokuğraş, 2003). Il a donc été proposé que la BChE serve d'enzyme protectrice qui clive les xénobiotiques toxiques (Lockridge, 2015). Le comportement cinétique différent des ChE apporte un aspect très intéressant à l'ensemble de la question. Il est bien connu que les ChE agissent différemment en cas d'excès de certains substrats, notamment l'ACh (Massoulié et al., 1993a). En excès d'ACh, la BChE est activée et devient encore plus efficace que l'AChE (Massoulié et al., 1993a ; Norel et al., 1993). De plus, les résultats d'expériences menées sur des souris knock-out pour les ChE suggèrent que la BChE pourrait servir d'enzyme de secours pour maintenir l'homéostasie de l'ACh en l'absence d'AChE (Li et al., 2002).

Comme décrit dans l'introduction, les ChE existent dans l'organisme sous de multiples formes moléculaires. D'après nos résultats, la principale forme moléculaire de l'AChE et de la BChE dans l'aorte de rat est un tétramère ancré par PRiMA. PRiMA est une petite protéine transmembranaire qui organise l'AChE ou la BChE en tétramère et ancre le tétramère à la membrane plasmique (Perrier et al., 2002). Dans le processus de tétramérisation de l'AChE, l'étape cruciale est l'interaction de l'AChE avec le domaine PRAD de PRiMA, car PRAD transporte le tétramère à travers toute la cascade sécrétoire et l'ancre à la surface cellulaire (Dobbertin et al., 2009). L'absence du domaine PRAD de la protéine PRiMA entraîne l'absence totale d'AChE à la surface des cellules, tandis que les monomères d'AChE sont retenus dans le réticulum endoplasmique (Dobbertin et al., 2009). L'absence de ChE à la

surface de la membrane s'est accompagnée de niveaux extracellulaires d'ACh très élevés dans le cerveau (Farar et al., 2012), confirmant que les hétéro-oligomères sont les formes fonctionnelles des ChE. Sur la base de ces faits, nous pouvons supposer que la forme fonctionnelle des ChE dans l'aorte de rat sont les ChE ancrées à PRiMA avec une petite quantité de monomères détectés représentant des produits intermédiaires intracellulaires de la tétramérisation.

L'origine de l'acétylcholine affectant les vaisseaux sanguins

En physiologie, l'ACh est considéré comme un vasorelaxant entraînant une diminution de la résistance des vaisseaux sanguins et de la pression artérielle. Cependant, l'origine de l'ACh qui affecterait les récepteurs vasculaires de l'ACh n'est vraisemblablement pas limitée à l'innervation cholinergique car elle est rare dans les vaisseaux. Nous avons testé si l'ACh pouvait provenir directement du tissu vasculaire. Nos résultats suggèrent que l'aorte appartient au groupe des organes qui sont capables de synthétiser de l'ACh indépendamment de la présence de neurones. (Wessler et Kirkpatrick, 2008). Nous avons confirmé la présence de toutes les protéines essentielles au cycle de vie de l'ACh dans l'aorte. Comme décrit dans l'introduction, deux enzymes pourraient participer à la synthèse de l'ACh. La ChAT est une enzyme qui synthétise exclusivement l'ACh dans les neurones (Tuček, 1982) mais sa présence a été confirmée également dans certains NNChS, par exemple les cardiomyocytes (Kakinuma, 2015 ; Rocha-Resende et al., 2012), et les lymphocytes T (Koichiro Kawashima et al., 2015) (ou voir la revue (I. Wessler & Kirkpatrick, 2008)). La participation de la CrAT à la synthèse de l'ACh a été suggérée dans certaines cellules non neuronales, notamment l'urothélium (Lips et al., 2007), le maxillaire de rat (Guo et al., 2014) ou le muscle squelettique (Tuček, 1982) ou voir la revue (Beckmann et Lips, 2013). Nous avons confirmé la présence de la CrAT dans toutes les fractions aortiques et avons colocalisé sa protéine avec les marqueurs de l'endothélium et du muscle lisse, ce qui indique que l'ACh peut être synthétisée dans les deux types de cellules de l'aorte.

VACHT est une protéine responsable de la translocation de l'ACh du cytoplasme vers les vésicules. C'est une fonction irremplaçable, qui a été confirmée par des souris mutantes sans VACHT, qui sont mortes immédiatement après la naissance, car les neurones ne peuvent pas déclencher les contractions du muscle squelettique et les souris ne sont pas capables de respirer (Prado et al., 2006). Nous avons détecté l'ARNm de VACHT dans chaque fraction

de l'aorte, tandis que l'expression relative était plus élevée dans la fraction musculaire lisse. Cette observation a été confirmée par immunohistochimie. Néanmoins, Krajčovičová et al. (Krajčovičová, 2016) ont comparé l'expression relative de VACHT dans l'aorte entière avec l'expression relative de VACHT dans le cerveau et ont observé que la quantité d'ARNm codant VACHT dans l'aorte est bien inférieure à celle du cerveau.

Dans certaines cellules non-neuronales, VACHT n'est pas crucial et l'ACh peut rester libre dans le cytoplasme et être directement libéré du cytoplasme vers l'espace extracellulaire *via* différents transporteurs (Beckmann et Lips, 2013 ; Fujii et al., 2017 ; Kummer et Krasteva-Christ, 2014 ; Wessler et al, 2001), OCTN (Pochini, 2016 ; Pochini et al., 2012b, 2019), CTL (Inazu, 2014 ; Song et al., 2013 ; Traiffort et al., 2005) ou médiatophores (Kawashima et al., 2015), tandis que OCT et OCTN sont plus fréquemment impliqués dans ce processus. Nous n'avons pas détecté d'ARNm pour OCT-1 dans les fractions endothéliales ou musculaires lisses. En revanche, nous avons détecté des expressions relatives élevées pour OCT-2 et OCT-3 dans les cellules endothéliales et musculaires lisses, les niveaux les plus élevés pour les deux transporteurs étant observés dans la fraction musculaire lisse. Ainsi, ces transporteurs pourraient participer à la libération d'ACh. Cependant, nous ne pouvons pas exclure que les OCT présents dans l'aorte de rat aient une autre fonction et transportent d'autres substances cationiques (Jonker et Schinkel, 2004). Du point de vue pharmacologique, plusieurs médicaments inhibent ou bloquent précisément ces transporteurs (Belzer et al., 2013 ; Boxberger et al., 2014 ; Grube et al., 2011 ; Koepsell, 2015 ; Panfen et al., 2019). Nous proposons donc que ces médicaments puissent affecter la physiologie des vaisseaux *via* une libération modifiée d'ACh. L'expression relative des deux OCTN a été détectée dans toutes les fractions étudiées, mais contrairement aux OCT, ces transporteurs étaient exprimés uniformément le long de l'endothélium et du muscle lisse. L'OCTN-2 a une grande affinité pour la carnitine (Iwata et al., 2008) et peut donc être impliqué dans d'autres processus de l'aorte, et pas seulement dans le cycle de vie de l'ACh. En outre, sa fonction altérée entraîne une carence systémique en carnitine, qui se traduit généralement par des cardiomyopathies (Iwata et al., 2008 ; Wu et al., 2000). Il est donc évident que leur fonction correcte est également nécessaire à la physiologie du SVC. Des mutations de ces transporteurs ont également été décrites dans la maladie de Crohn (Pochini et al., 2012a), qui fait partie des maladies inflammatoires et est donc liée à une altération de la signalisation de l'ACh (Goverse et al., 2016).

L'étape limitant le taux de synthèse de l'ACh dans les neurones est la recapture de la choline, médiée par ChT-1 (Okuda et Haga, 2003). Nous avons confirmé la présence de ChT-1 dans chaque fraction de l'aorte de rat, l'expression relative la plus élevée étant observée dans la fraction du muscle lisse. Les résultats de la RT-qPCR ont été confirmés par immunohistochimie, où la protéine était colocalisée avec les marqueurs de l'endothélium et du muscle lisse. Encore une fois, les résultats de (Krajčovičová, 2016) ont montré que dans l'aorte entière l'expression relative était beaucoup plus faible, que dans le cerveau.

On pense que l'ACh agit à la fois sur l'endothélium et sur les muscles lisses, tout en utilisant des mécanismes moléculaires différents qui entraînent des effets vasculaires opposés. Le principal mAChR dans la physiologie vasculaire est le récepteur muscarinique m3, qui est responsable de la vasodilatation dépendante de l'endothélium dans les vaisseaux. Nous avons observé les niveaux d'ARNm les plus élevés pour ce récepteur, tandis que l'expression était principalement localisée dans l'endothélium. De plus, nous avons détecté tous les récepteurs muscariniques connus à l'exception du récepteur m4. La présence de différents mAChR a été documentée par d'autres auteurs également, alors que la distribution de ces récepteurs était spécifique à l'espèce. Chez les lapins, l'endothélium de l'artère saphena magna exprime les récepteurs muscariniques m1 et m2 (Komori et Suzuki, 1987), l'endothélium des veines pulmonaires humaines exprime le récepteur muscarinique m1 (Walch et al., 2000), les récepteurs muscariniques m1 et m3 sont exprimés dans les artères pulmonaires humaines (Norel et al., 1996), dans les artères de rat les récepteurs muscariniques m1 et m5 ont été détectés (Phillips et al., 1997), dans les artères mésentériques de rat les récepteurs muscariniques m1 et m3 ont été confirmés. (Tangsucharit et al., 2016).

L'expression relative des sous-unités nAChR a également été déterminée. La présence de toutes les sous-unités (de type neuronal ou musculaire), à l'exception de $\alpha 9$ a été confirmée, avec une expression relative la plus élevée de $\alpha 7$ dans chaque fraction aortique. Ces résultats sont en accord avec les résultats obtenus par d'autres équipes (Brüggmann et al., 2002). Il a été montré, que ces $\alpha 7$ nAChRs sont impliqués dans la régulation du tonus vasculaire dans certaines pathologies du CVS. Sous blocage des mAChRs, l'ACh induit une relaxation dans les aortes de SHR, mais pas chez les rats Wistar-Kyoto (Zou et al., 2012). Chez le SHR, plusieurs modifications ont été décrites dans la voie anti-inflammatoire cholinergique médiée par $\alpha 7$ nAChR (Chen et al., 2012). Le rôle dans l'angiogenèse a également été confirmé (Heeschen et al., 2002 ; Ni et al., 2010). L'upregulation des $\alpha 7$ nAChR a été

observée lors de la prolifération, mais aussi en raison de l'hypoxie *in vitro* ou de l'ischémie *in vivo*. L'inhibition pharmacologique de ces récepteurs inhibe significativement l'angiogenèse inflammatoire, la croissance tumorale ou réduit l'angiogenèse induite par l'ischémie (Heeschen et al., 2002). Néanmoins, ces récepteurs ont été proposés comme cibles thérapeutiques non seulement dans les troubles du vieillissement vasculaire associés à l'angiotensine II (Li Dong-Jie et al., 2016), mais aussi dans plusieurs angiogenèses physiologiques et pathologiques, par exemple l'angiogenèse induite par l'inflammation ou l'hypoxie, car ces conditions régulent à la hausse ces récepteurs dans les cellules endothéliales (Carmeliet et Jain, 2000 ; Egleton et al., 2008 ; Heeschen et al., 2002). Dans une autre étude, on a observé une inhibition de l'apoptose et une stimulation de la prolifération des cellules musculaires lisses aortiques médiées par la nicotine (Cucina et al., 2008). L'effet pro-athérogène de la nicotine et d'autres ligands nAChR sur les vaisseaux a été documenté dans d'autres études, qui portaient sur la croissance des plaques et la néovascularisation (Santanam et al., 2012). De plus, il semble que ces récepteurs soient également associés à l'insulino-résistance, l'insuline étant impliquée dans la progression de l'athérosclérose en favorisant la prolifération des cellules lisses vasculaires (Ross, 1993).

D'autre part, nous ne pouvons pas oublier d'autres sources d'ACh, qui peuvent affecter les vaisseaux. De plus, il a été démontré que l'ACh sécrétée par des cellules non neuronales a une fonction aussi importante dans la physiologie du SVC que l'ACh produite par les neurones (Rana et al., 2010 ; Roy et al., 2013), ce qui indique que des ACh d'origines différentes participent à la physiologie des vaisseaux. Par exemple, les lymphocytes T CD4+ sont capables de produire de l'ACh *via* le ChAT présent, alors que l'absence de ChAT dans ces cellules provoquait une pression artérielle élevée et que la surexpression de ce transporteur (production plus importante d'ACh) provoquait une diminution de la pression artérielle (Olofsson et al., 2016). Par ailleurs, l'ACh synthétisé par les cardiomyocytes est responsable de la régulation du rythme cardiaque (Roy et al., 2013). Une autre origine de l'ACh peut également être la jonction neuromusculaire (où la possibilité d'un débordement d'ACh a été décrite, Nervo et al., 2019). Néanmoins, on ne peut pas exclure une possible "contamination cholinergique" provenant de l'innervation parasympathique à la base du cœur. Nous pouvons spéculer que l'ACh provenant de ces sources a également un impact sur les vaisseaux, mais il y a un manque d'informations sur le mécanisme exact. Nous pensons que l'ACh provenant de différentes sources par le sang active le m3AChR du côté luminal

de l'endothélium et active la voie responsable de la production de NO et de la vasodilatation. L'ACh produit par le muscle lisse de l'aorte peut activer le mAChR situé à la surface du muscle lisse et provoque une vasoconstriction. Dans des conditions physiologiques, nous nous attendons à ce que ces mécanismes soient en équilibre. D'autre part, nous pouvons spéculer que l'ACh produite par le muscle lisse est libérée dans la zone extracellulaire et a une fonction importante, jusqu'à présent non décrite. On ne peut pas exclure la possibilité que l'ACh soit synthétisée et libérée en réponse à une condition ou à un stimulus particulier.

Des niveaux plus bas de butyrylcholinestérase dans la paroi aortique de SHR

On sait depuis longtemps que dans le cas d'une artère au repos, une vasoconstriction dépendant de la dose se produit après une stimulation par l'ACh, et ceci indépendamment de la présence ou de l'absence d'endothélium (Furchgott et Zawadzki, 1980 ; Norel et al., 1996). Au contraire, la stimulation par l'ACh de l'artère précontractée avec un endothélium intact provoque une vasodilatation. Cependant, en cas d'altération de l'endothélium (ou de son absence), l'artère répond à la stimulation par l'ACh par une vasoconstriction dépendante de la dose (Furchgott et Vanhoutte, 1989 ; Norel et al., 1996). Ainsi, les cellules endothéliales jouent un rôle clé dans la régulation du tonus vasculaire. De plus, leur rôle dans l'angiogenèse et dans la réponse inflammatoire vasculaire a été décrit (Cooke, 2007 ; Ni et al., 2010), car elles synthétisent les prostaglandines, l'EDRF mais aussi diverses enzymes capables d'activer ou de dégrader les hormones vasoactives. Par conséquent, le ciblage endothélial est utilisé dans le traitement de plusieurs maladies CVS (Hadi et al., 2005). Selon des études récentes, l'ACh non-neuronale régule également ces mécanismes par la modulation de la production et de la libération d'autacoïdes endothéliaux, comme par exemple le NO ou diverses prostaglandines (Hadi et al., 2005 ; de Wit et al., 2000). Récemment, un dysfonctionnement endothélial a été décrit chez le SHR, représenté par une altération des caractéristiques anatomiques, morphologiques et pharmacologiques et une modification de la réaction des vaisseaux à diverses substances vasodilatatrices ou vasoconstrictrices (y compris l'ACh) (Berenyiova et al, 2018 ; Cacanyiova et al., 2016 ; Cacányiová et al., 2008 ; Gerová et al., 2005 ; Gluais et al., 2005 ; Puzserova et al., 2014 ; Rapoport et Williams, 1996). (Hadjiisky et al., 1987).

Une différence significative dans les niveaux d'ARNm codant l'AChE a été détectée uniquement dans *l'aorte abdominale*, où les expressions étaient plus élevées chez les rats de

type SHR. L'expression de l'ARNm codant la BChE et l'activité BChE dans l'aorte des rats de type SHR ont suivi celles des rat Wistar tout en atteignant des niveaux globalement plus faibles. La diminution de l'expression de la BChE (et donc de son activité) peut représenter une adaptation compensatoire à l'insuffisance de la signalisation de l'ACh. Cependant, le modèle rat de type SHR est caractérisé par un dysfonctionnement endothélial, qui se caractérise non seulement par une altération de la production de NO (et par conséquent du mécanisme vasodilatateur), mais aussi par une plus grande perméabilité aux substances chimiques présentes dans le sang (y compris l'ACh). La diminution de BChE peut ne pas être suffisante pour contrôler le niveau d'ACh dans les muscles lisses, renforcer une vasoconstriction déclenchée par l'ACh.

Dans le modèle SHR, nous avons observé une diminution du niveau de CrAT qui pourrait être sans rapport avec le système cholinergique, compte tenu des rôles multiples de cette enzyme dans la biologie cellulaire et de la complexité de la physiopathologie du SHR. Un niveau plus faible de CrAT, cependant, diminue le niveau d'ACh, ce qui peut être expliqué comme une réponse adaptative à une activité BChE plus faible et à un contrôle insuffisant de la signalisation par l'ACh. D'autre part, une baisse de la signalisation cholinergique peut également avoir des conséquences négatives. Par conséquent, des mécanismes compensatoires sont activés - augmentation de l'absorption dans les vésicules (VAcHT régulé) et régulation positive du récepteur muscarinique m3. Compte tenu de la réponse différente de l'endothélium et des muscles lisses à la présence d'ACh, on peut supposer que l'augmentation de la signalisation cholinergique n'est bénéfique que dans l'endothélium, où l'ACh induit une voie entraînant la dilatation des vaisseaux. On ne peut exclure que l'augmentation de VAcHT soit causée par un mécanisme adaptatif du vaisseau pour diminuer l'effet de l'ACh sur les vaisseaux lisses afin de prévenir la vasoconstriction. D'autre part, il a été démontré que l'expression de VAcHT régule directement le niveau d'ACh, car une expression accrue de VAcHT entraîne une augmentation de la transmission cholinergique (Roy et al., 2016 ; Sugita et al., 2016). Cependant, chez la souris, la surexpression à long terme de VAcHT induit des changements structurels dans les fibres musculaires au niveau de la NMJ (Sugita 2016). La surexpression de VAcHT a induit des changements morphologiques également dans d'autres systèmes (Janickova et al., 2017), donc nous pouvons inclure que la surexpression à long terme de ce transporteur peut avoir des conséquences négatives sur le muscle lisse de l'aorte. En outre, l'augmentation des niveaux

de VAcHT semble être bénéfique dans l'insuffisance cardiaque (Roy et al., 2016). Cependant, l'impact de l'augmentation à long terme de l'expression de VAcHT sur les vaisseaux n'a pas encore été établi. Une autre observation concerne les transporteurs OCT2, OCT3, OCTN1 et OCTN2. Malgré le fait que ces transporteurs étaient liés au transport de l'ACh non neuronale en raison de leur promiscuité dans les molécules transportées, nous ne pouvons pas conclure que leurs niveaux d'ARNm diminués chez le SHR sont une conséquence directe de l'altération des niveaux d'ACh.

La butyrylcholinestérase intervient dans le métabolisme des lipides.

Récemment, la participation du BChE dans le métabolisme des lipides et dans la physiopathologie de l'obésité a suscité beaucoup d'intérêt. En effet, des souris dont le gène de la BChE est inactivé et qui sont nourries avec un régime riche en graisses ont montré une augmentation significative de leur poids (Chen et al., 2017b ; Li et al., 2008a). De plus, des expériences de transfert de gène du BChE ont montré que la récupération de l'activité du BChE bloquait la prise de poids chez les souris BChE *-/-* nourries avec des graisses élevées (Chen et al., 2017b). Contrairement aux souris knock-out BChE, nous avons observé une prise de poids significative, ainsi qu'une augmentation de la circonférence *abdominale* et de l'IMC chez les rats Wistar soumis à un régime standardisé lorsque le BChE était inhibé par l'iso-OMPA. Cette divergence pourrait s'expliquer par l'absence des mécanismes d'adaptation qui sont très probablement présents lorsque la BChE est absente à la naissance. Dans le même ordre d'idées, les rats SHR, qui possèdent une activité BChE sérique significativement plus faible, avaient un IMC comparable à celui des rats Wistar. Le gain de poids des rats Wistar après l'iso-OMPA était comparable à celui des rats soumis à un régime riche en graisses et, étonnamment, il n'a pas augmenté davantage lorsque les deux régimes, riche en graisses et iso-OMPA, ont été combinés. Cela peut indiquer que le mécanisme d'inhibition de l'hyperglycémie et de la BChE affecte la même voie, alors qu'une saturation du processus de prise de poids peut se produire. Les rats SHR n'ont pas répondu au régime riche en graisses, ni à l'inhibition de la BChE. En général, l'impact du régime riche en graisses sur le SHR est controversé, et une résistance à la prise de poids a déjà été signalée (Bosse et al., 2013 ; Pereira et al., 2019 ; Shiou et al., 2018 ; Yuan et al., 1998).

Il a été proposé dans la littérature que la stabilisation du poids après le transfert de gène du BChE est obtenue par la suppression des signaux favorisant l'appétit. Le BChE a été

précédemment décrit comme une enzyme hydrolysant la ghréline, " l'hormone de la faim " (Chen et al., 2015, 2017b ; De Vriese et al., 2004) et par cette action d'affecter fortement les niveaux circulants de ghréline et aussi son action dans le contrôle de la faim, de la température corporelle et aussi des émotions comme l'anxiété ou l'agression (Chen et al., 2015, 2017b ; Schopfer et al., 2015). Cependant, pour mieux comprendre l'axe BChE-ghréline dans ce contexte, nous devons compléter les informations sur les niveaux de ghréline dans les sérums de nos animaux expérimentaux. D'autre part, le lien entre le BChE et la ghréline a été documenté chez une souris avec un taux sérique élevé de BChE (Chen et al., 2015, 2017b), et non chez les rats, nous pouvons donc émettre l'hypothèse que dans différentes souches, différents mécanismes sont employés et que le faible taux sérique de BChE n'est pas suffisant pour contrôler les niveaux de ghréline.

Deux autres hormones très importantes, qui régulent entre autres les réserves énergétiques et l'obésité, sont la leptine (Kelesidis et al., 2010) et l'adiponectine (Forny-Germano et al., 2019). Les taux sériques de leptine sont fortement corrélés non seulement avec les taux sériques de BChE, mais aussi avec la pression artérielle moyenne, le cholestérol total et le TAG, tandis qu'on a observé que les concentrations sériques d'adiponectine étaient négativement corrélées avec l'activité du BChE (Iwasaki et al., 2007). Dans la continuité de ce projet, nous envisageons donc de compléter ces informations et de nous intéresser plus en détail à ces indicateurs d'obésité et d'inflammation systémique.

Comme des activités BChE plus faibles ont été précédemment associées à une consommation alimentaire plus élevée (Chen et al., 2017b), nous nous attendions à une augmentation de la consommation alimentaire chez les rats, auxquels l'iso-OMPA a été administré, mais cette hypothèse n'a pas été confirmée. La prise alimentaire (grammes consommés) n'a pas été affectée par l'inhibition du BChE, ni observée chez les rats SHR qui, comme décrit ci-dessus, possèdent des activités BChE plus faibles. La prise de poids observée est donc plus probablement due à des modifications du métabolisme. D'autre part, alors que les rats Wistar nourris avec des graisses élevées buvaient moins d'eau (ce qui est en accord avec d'autres auteurs (Kaunitz et al., 1956 ; Ramalho et al., 2017 ; Volcko et al., 2020)), les rats Wistar, mais pas les rats SHR, recevant de l'iso-OMPA buvaient statistiquement plus. D'autre part, alors que les rats Wistar nourris avec des graisses élevées buvaient moins d'eau (ce qui est en accord avec d'autres auteurs (Kaunitz et al., 1956 ; Ramalho et al., 2017 ; Volcko et al., 2020)), les rats Wistar mais pas les SHR recevant l'iso-

OMPA buvaient statistiquement plus. Une observation similaire avait été rapportée auparavant lorsque les ChE étaient inhibées par les OP (Hazarika, 2014). Nous ne pouvons pas exclure que le changement de poids chez ces animaux soit partiellement causé par la rétention d'eau. En effet, l'analyse morphologique a mis en évidence des différences dans la prise de poids des tissus adipeux. Alors que chez les rats Wistar soumis à un régime riche en graisses, tous les tissus adipeux collectés ont augmenté en poids, chez les rats Wistar soumis à un régime standard et à l'iso-OMPA, cette augmentation a été moins spectaculaire, n'atteignant un niveau significatif que dans le tissu épидидymaire.

Notre hypothèse sur la modification du métabolisme est confirmée par les paramètres du métabolisme lipidique. Comme prévu, un régime riche en graisses a augmenté les taux de cholestérol et de TAG. De plus, une augmentation similaire des taux de TAG a été observée chez les rats Wistar traités par l'iso-OMPA, quel que soit le régime alimentaire. L'association entre l'activité du BChE et les taux sériques de TAG a été décrite précédemment (Iwasaki et al., 2007 ; Ondříková, 2011). Nous supposons que le BChE participe à la régulation des taux de TAG et que le manque d'activité du BChE entraîne une augmentation des TAG plasmatiques chez les rats Wistar traités par l'iso-OMPA. Cette hypothèse est étayée par le fait que l'augmentation des taux plasmatiques de TAG chez les rats Wistar soumis à un régime riche en graisses s'accompagne d'une augmentation de l'activité plasmatique du BChE. Une observation similaire a été faite chez l'homme également, puisqu'il a été observé que la concentration sérique de BChE est positivement corrélée aux niveaux de TAG (Vicky Ping Chen et al., 2016 ; Iwasaki et al., 2007 ; Lucić Vrdoljak et al., 2005 ; Ondříková, 2011).

Dans le modèle de rat, cette augmentation compensatoire de la BChE par rapport au TAG plasmatique induit par un régime riche en graisses a également été observée dans les vaisseaux. La BChE était le seul marqueur cholinergique altéré dans l'aorte affectée. Les expressions relatives de l'AChE et des enzymes et transporteurs du cycle de vie de l'ACh, à l'exception de l'OCT-2, sont restées inchangées. Nous pouvons supposer que cette augmentation de la BChE est un mécanisme compensatoire aux niveaux plus élevés de TAG et que la BChE vasculaire agit comme une enzyme de détoxification de ces molécules et participe ainsi à la prévention de l'athérosclérose. Cette hypothèse est en accord avec le fait que les activités BChE plasmatiques sont corrélées avec des niveaux plus élevés de LDL et de TAG (Bradamante et al., 2004 ; Duchnowicz et al., 2018 ; Iwasaki et al., 2007 ; Lucić Vrdoljak et al., 2005 ; Ondříková, 2011 ; Santarpia et al., 2013 ; Sridhar et al., 2005), facteurs

de risque connus d'athérosclérose (Goldberg et al., 2011 ; Peng et al., 2017 ; Talayero et Sacks, 2011). La BChE possède une activité estérase, mais aussi peptidase ou acyl-aryl-amidase (Lockridge, 2015b). Le TAG est fabriqué à partir des AGF, qui sont attachés au glycérol par une liaison ester, donc hypothétiquement cette liaison peut être clivée par le BChE. De plus, la triade catalytique de la BChE et de la TAG-hydrolase contient les mêmes acides aminés (Kumar et al., 2016 ; Lockridge, 2015b). Les résultats de nos expériences in vitro avec un substrat synthétique de type TAG suggèrent que le BChE pourrait effectivement posséder une activité lipolytique.

Nous avons observé que la concentration plasmatique humaine de BChE (3,02 µg/ml - Browne et al., 1998) est capable de dégrader un petit substrat de type thio-TAG (DMPTB), alors que cette activité était complètement inhibée par l'iso-OMPA, confirmant que l'hydrolyse était faite exclusivement par la BChE. La vitesse d'hydrolyse du DMPTB était cependant significativement plus lente que celle de l'hydrolyse de la CTB. Cette observation est conforme aux observations précédentes, car l'hydrolyse de substrats plus volumineux est beaucoup plus lente que l'hydrolyse de substances plus petites (Chen et al., 2015, 2017b). De plus, un substrat structurellement plus proche du TAG natif pour l'activité lipasique du BChE a également été testé, mais malgré les diverses conditions expérimentales testées, nous n'avons pas réussi à démontrer la capacité du BChE à cliver ce substrat. Même si la BChE présente une spécificité de substrat plus faible (Massoulié et al., 1993a) et est capable de cliver des substrats plus grands (Brimijoin et al., 2018 ; Chen et al., 2015 ; Zheng et al., 2014), nous ne pouvons pas affirmer que ce substrat synthétique convient à la BChE. Comme le métabolisme des lipides est une réaction compliquée, à plusieurs étapes, et que nous pouvons trouver plusieurs autres enzymes dans le sérum impliquées dans le métabolisme des graisses, par exemple les paraoxonases (Kudchodkar et al., 2000; Meneses et al., 2019) ou les carboxylestérases (Di, 2018 ; Lian et al., 2018), nous ne pouvons pas exclure que la BChE soit impliquée dans ce processus compliqué. Il est possible qu'elle fonctionne comme une coenzyme et que son fonctionnement correct nécessite la présence d'une autre enzyme. Comme nous ne disposons pas de conditions totalement physiologiques pour l'expérience, nous pouvons spéculer que le BChE peut avoir un comportement différent in vivo. Il est également possible qu'in vivo, il existe un signal de déclenchement qui incite la BChE à cliver les substrats à caractère TAG.

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