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INVESTIGATION OF NOVEL THERAPEUTIC OPTIONS FOR THE TREATMENT OF ISCHEMIC AND METABOLIC CARDIOVASCULAR DISEASES

Ph.D thesis

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List of Abbreviations

ACC	- acetyl-CoA carboxylase
ACE	- angiotensin-converting-enzyme
ADSCs	- adipose-derived stem cells
AKT	- protein kinase B
AMPK	- AMP-activated Protein Kinase
ARBS	- angiotensin II Receptor Blockers
ATG	- autophagy related
AUC	- area under curve
BAT	- brown adipose tissue
BCA	- bicinchoninic Acid Protein Assay Kit
BMI	- body mass index
BSA	- bovine serum albumin
cAMP	- cyclic adenosine monophosphate
CAP	- chloramphenicol
CAPS	- chloramphenicol succinate
CB_1 receptor	- cannabinoid receptor type 1
CCL2	- monocyte chemoattractant protein 1
CCL3	- macrophage inflammatory protein 1-alpha
CD36	- fatty acid translocase

CK - creatine kinase

CMA - chaperone-mediated autophagy

CNS - central nervous system

CON - control diet

CQ - chloroquine

CT - computed tomography

CVD - cardiovascular disease

DA - dopamine

DGAT - diglyceride acyltransferase

DI - discrimination index

DMEM - Dulbecco's Modified Eagle Medium

DPA - dynamic plantar aesthesiometer

EMA - European Medicines Agency

ERK1/2 - extracellular signal–regulated kinases 1/2

FAD - flavin-adenin-dinukleotid

FBS - fetal bovine serum

FDA - Food and Drug Administration

GAPDH - glyceraldehyde 3-phosphate dehydrogenase

GLP-1 - glucagon-like peptide-1

GLUT1 - glucose transporter 1

GLUT4 - glucose transporter 4

GSK3b - glycogen synthase kinase 3 beta

5-HT	- serotonin
HDL	- low density lipoprotein
H&E	- hematoxylin and eosin
HFS	- high-fat, high-sucrose diet
HPRT1	- hypoxanthine phosphoribosyltransferase 1
ITT	- insulin tolerance test
K130R	- TAT-HA- Atg5K130R
KH	- Krebs-Henseleit solution
LAD	- left anterior descending coronary artery
LC3	- microtubule-associated protein light chain 3
LDL	- low density lipoprotein
MAO	- monoamine oxidases
MAO-A	- monoamine oxidase A
MAO-B	- monoamine oxidase B
MI	- myocardial infarction
mTORC1	- mammalian target of rapamycin complex 1
NDUFA1	- NADH dehydrogenase 1 alpha
NE	- norepinephrine
NIH	- National Institutes of Health
NOR	- novel objection recognition assay
NRCMs	- neonatal rat cardiomyocytes
OGTT	- oral glucose tolerance test

PBS	- phosphate buffered saline
PI3K	- phosphoinositide 3-kinase
PNPLA2	- adipose triglyceride lipase
RIPA	- radio-Immunoprecipitation assay buffer
S	- selegiline
SREBP-1c	- sterol regulatory element-binding protein 1c
TBST	- tris-buffered saline with 0.05% Tween 20
T2DM	- type 2 diabetes mellitus
t2familiar	- exploration time of familiar object
t2novel	- exploration time of novel object
ULK1	- unc-51 Like Autophagy Activating Kinase 1
VAT	- visceral adipose tissue
WAT	- white adipose tissue
WHO	- World Health Organization

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1 Introduction

1.1 Obesity: diagnosis and treatment

In recent decades, obesity and its metabolic complications have become one of the biggest public health issues worldwide. According to the data of World Health Organization (WHO), the prevalence of obesity has nearly tripled between 1975 and 2016. In 2016, more than 1.9 billion adults were overweight, of whom over 650 million were obese (WHO 2018). Furthermore, obesity is a serious problem not just for adults but children also. The prevalence of obesity in children has dramatically increased in the last few years (Sahoo, Sahoo et al. 2015). In 2016, 41 million children under the age of 5 were overweight or obese in 2016 and over 340 million children and adolescents aged 5-19 were overweight or obese. Furthermore, obesity increases the risk of developing several comorbidities such as Type 2 diabetes mellitus (T2DM), high blood pressure, high blood cholesterol and high triglyceride levels, thereby reduces life quality and expectancy. Overweight and obesity are most common in developed and developing countries (Hruby and Hu 2015, Hales, Carroll et al. 2017). The cost of medical treatment of obesity and of the obesity-related diseases put a large economic burden on the individual and nations also (Levy, Levy et al. 1995, Birmingham, Muller et al. 1999, Tremmel, Gerdtham et al. 2017). Besides increased health care expenditure, obesity also imposes further costs in the form reduced ability to work, increased morbidity and mortality.

1.1.1 Obesity and its metabolic complications

Obesity is most commonly caused by a combination of excessive food intake, lack of physical activity, and genetic susceptibility (Yazdi, Clee et al. 2015). Occasionally it can be triggered by medications (Bernstein 1987, Ness-Abramof and Apovian 2005), endocrine disorders (Pujanek, Bronisz et al. 2013) or mental disorder (Bleich, Cutler et al. 2008). Obesity is a condition in which excess fat has accumulated in the body, such that it might have adverse effect on health (Van Itallie 1979). Usually it is defined and

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classified by body mass index (BMI, calculated by a person's weight in kilograms divided by the square of his height in meters), and further evaluated in terms of fat distribution via the waist circumference and body fat percentage. A person with a BMI of 18.5-24.9 kg/m² has a normal weight. A BMI of 25 to <30 kg/m² is defined as overweight and BMI ≥ 30 kg/m² is classified as obese, BMI ≥40 kg/m² defined as extreme obese (NIH 1998) (Table 1).

Table 1 Classification of overweight and obesity by BMI, waist circumference and associated disease risk.

Table was adopted from reference (NIH 1998).

**Disease risk for Type 2 diabetes, hypertension, and cardiovascular disease.*

†Increased waist circumference can also be a marker for increased risk even in persons of normal weight.

Classification	**BMI (kg/m^2)**	**Obesity Class**	**Disease Risk* (Relative to Normal Weight and Waist Circumference)**	
			Men ≤102 cm Women ≤ 88 cm	> 102 cm > 88 cm
Underweight	< 18.5		-	-
Normal†	18.5–24.9		-	-
Overweight	25.0–29.9		Increased	High
Obesity	30.0-34.9 35.0–39.9	I II	High Very High	Very High Very High
Extreme Obesity	≥ 40	III	Extremely High	Extremely High

Adipose tissue is one of the main types of a connective tissue which mainly comprises adipocytes. Besides adipocytes, adipose tissue contains the stromal vascular fraction (preadipocytes, fibroblasts, vascular endothelial cells) and a variety of immune cells (Frayn, Karpe et al. 2003). Adipose tissue is a complex, essential and highly active metabolic and endocrine organ and a main energy store of the body (Kershaw and Flier 2004). Beside the two main function of white adipose tissue (WAT) which are lipogenesis (fatty acid synthesis and storage) and lipolysis (mobilization or hydrolysis of triglycerides), WAT secretes also biologically active substances known as adipokines

(Proença, Sertié et al. 2014). Adipokines play a critical role in many biological functions, for example regulation of carbohydrate and lipid metabolism, regulation of food intake and insulin sensitivity (Fasshauer and Bluher 2015). Besides adipokines, monoamines (epinephrine, norepinephrine (NE), dopamine (DA) and serotonin (5-HT)) via dopaminergic and noradrenergic pathways play a key role in regulating carbohydrate and lipid metabolism (D'Souza and Abraham 2016).

Excess adiposity and adipocyte dysfunction result in dysregulation of adipokines, which may contribute to impaired glucose (Antuna-Puente, Feve et al. 2008) and lipid metabolism (Cao 2014, Tang 2016) as well as inflammatory responses (Balistreri, Caruso et al. 2010). Previous studies suggest that chronic inflammation in adipose tissue may play a significant role in the development of obesity-related metabolic dysfunction (Hotamisligil 2006, Lumeng and Saltiel 2011). Adipocyte dysfunction is also commonly associated with vascular diseases, like hypertension (Zhou and Qin 2012) and atherosclerotic vascular disease (Hajer, van Haeften et al. 2008, Lee, Wu et al. 2010).

The detrimental effects of obesity on health are due to the enlargement of adipose tissue, particularly visceral adipose tissue. In mammals, there are two main types of adipose tissue, the WAT, which stores energy, and brown adipose tissue (BAT), which generates body heat. Moreover, researchers recently discovered another type of adipose tissue. After thermogenic stimuli brown adipocytes may appear in WAT, derived from precursor cells which are different from the classical BAT and are closer to the white adipocyte cell lineage. This process is called "browning". These adipocytes are often referred as "inducible, beige, or brite adipocytes" (Giralt and Villarroya 2013). The adipose tissue divided into different regional depots with variation in biological function, structural organization and cellular size (Bjørndal, Burri et al. 2011). The main WATs are subcutaneous adipose tissue and visceral adipose tissue. Visceral fat, also known as organ fat or intra-abdominal fat is located between internal organs and torso, inside the peritoneal cavity. Visceral fat is composed of several adipose depots including mesenteric, epididymal white adipose tissue (omental) and perirenal fat (Figure 1.). The change in the volume of fat depots alters a number of physiological processes.

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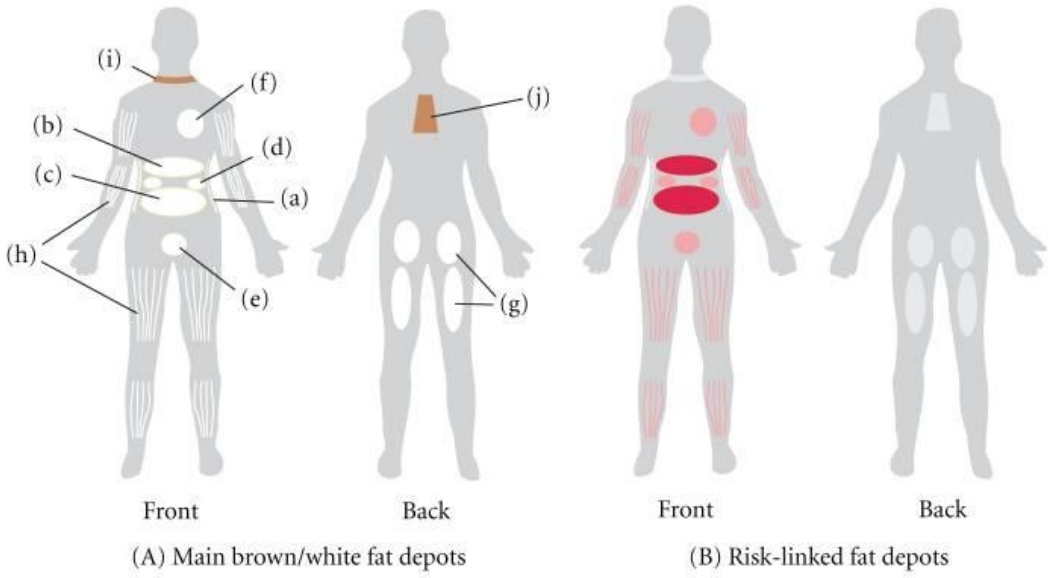

Figure 1. *(A) The main white adipose tissues (WATs) are abdominal subcutaneous adipose tissue (SAT, (a)), and visceral adipose tissue (VAT). VAT surrounds the inner organs and can be divided in omental (b), mesenteric (c), retroperitoneal ((d): surrounding the kidney), gonadal ((e): attached to the uterus and ovaries in females and epididymis and testis in men), and pericardial (f). The omental depot stars near the stomach and spleen and can expand into the ventral abdomen, while the deeper mesenteric depot is attached in a web-form to the intestine. The gluteofemoral adipose tissue (g) is the SAT located to the lower-body parts and is measured by hip, thigh, and leg circumference. WAT can also be found intramuscularly (h). Brown adipose tissue is found above the clavicle ((i): supraclavicular) and in the subscapular region (j). Although the mentioned subcutaneous and visceral adipose tissues are found in humans, depots (d) and (e) are mostly studied in rodents. (B) The adipose tissue depots that have been linked to risk of developing obesity-related diseases are indicated in red. The best-documented link to risk is found for the omental and mesenteric VAT. Figure was adopted from reference (Frayn, Karpe et al. 2003).*

Researchers first started to focus on visceral obesity in the 1980s when they realized that the distribution between the fat depots is more important than the total adipose tissue mass for the risk of developing obesity-associated diseases. Visceral obesity greatly increases the risk of several diseases, such as T2DM (Golay and Ybarra 2005, Wells 2017), non-alcoholic fatty liver disease (Lopez-Velazquez, Silva-Vidal et al. 2014), dyslipidemia (Jung and Choi 2014), cardiovascular disease (Eckel 1997, Poirier, Giles et al. 2006), certain types of cancers (Gallagher and LeRoith 2015), and depression (Luppino, de Wit et al. 2010). Obesity and its metabolic complications are linked to more deaths worldwide than underweight. Therefore, pharmacological tools,

which can prevent or treat obesity and the co-morbidities associated with advanced obesity are intensively studied (Ferdinandy, Hausenloy et al. 2014) (Figure 2).

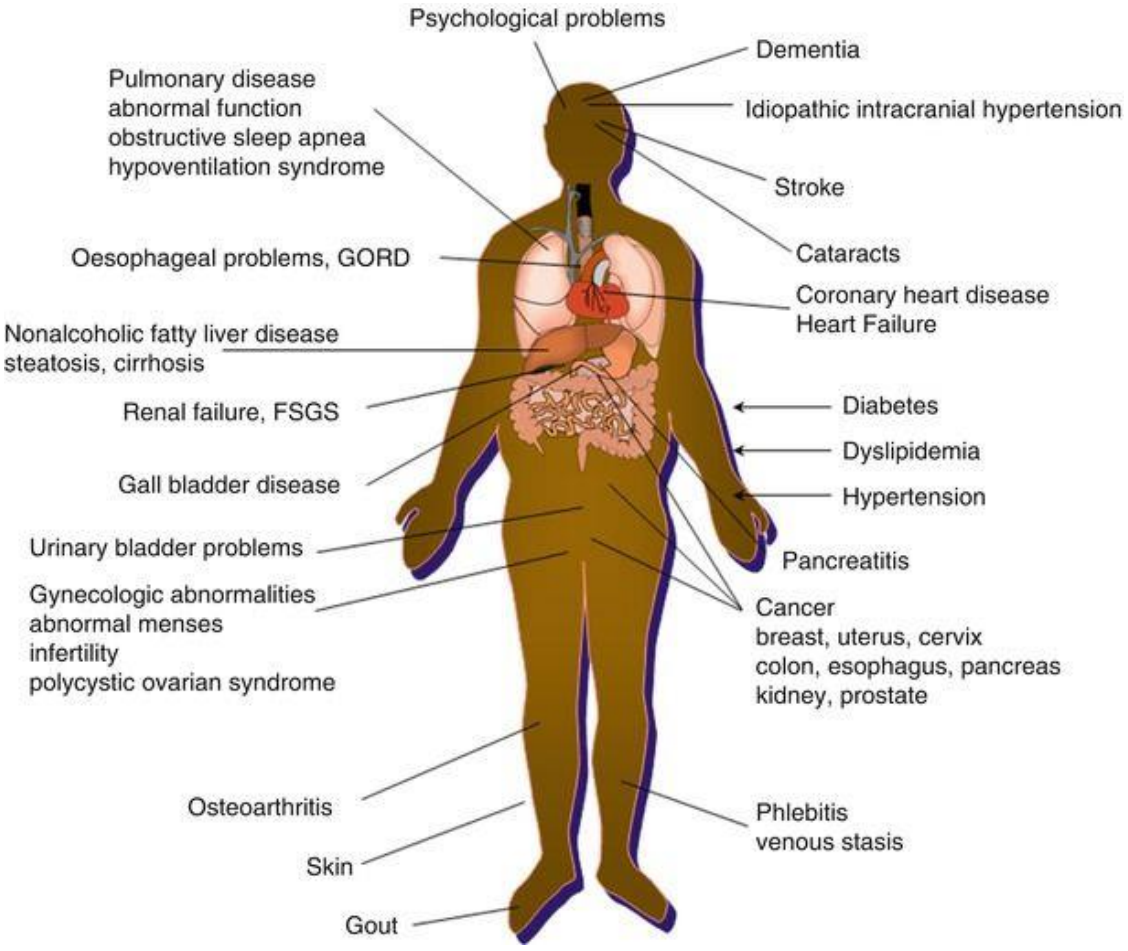

Figure 2. Medical complications of obesity.

GORD- gastro-oesophageal reflux disease, FSGS- Focal Segmental Glomerulosclerosis.

Figure was adopted from: Emmanuel J.J., Coppack S.W. (2016) Health Consequences–Obesity Associated Comorbidities. In Agrawal S. (eds) Obesity, Bariatric and Metabolic Surgery. Springer, Cham).

1.1.2 Therapies for treatment of obesity

The purpose of treatment of overweight and obesity is to reduce body weight and prevention of weight regain and thus to reduce the consequent health risks. Weight reduction can be achieved by a combination of the following: lifestyle interventions (diet, physical activity), surgery (bariatric surgery) and pharmacotherapy (appetite

suppressants, metabolism inducers, absorption inhibitors) (Cannon and Kumar 2009). All of these interventions are very important in the treatment of obesity but in this thesis we only focus on pharmacological therapy.

Nowadays, beside the pharmacotherapeutic treatment of obesity, cell-based therapies are getting more attention. Numerous studies suggest that, therapies using adipose-derived stem cells (ADSCs) (Illouz, Sterodimas et al. 2011, Payab, Goodarzi et al. 2018) or ADSCs-derived exosomes (Zhao, Shang et al. 2018) seems to be a potential treatment strategy to manage obesity and related metabolic disorders in the near future.

At the beginning of anti-obesity pharmacotherapy investigation, in the 70s, centrally-targeted and sympathetic-like agents showed very promising effects on decreasing body weight. These drugs activated the sympathetic nervous system inducing the release of catecholamines, meanwhile promoted satiety and reduced appetite (Motycka, St Onge et al. 2011). However, despite of these encouraging findings, numerous serious side effects occurred during the application of anti-obesity drugs. Due to the lack of sufficient efficacy and/or due to safety issues several drugs have been withdrawn from the market in the last 10 years. In 1973, US Food and Drug Administration (FDA) approved the combined pharmacological therapy fenfluramine-phentermine, and it was pulled out from the market in 1997 following life-threatening side effects such as pulmonary hypertension, and heart diseases (Connolly, Crary et al. 1997). Furthermore, rimonabant, a synthetic CB_1 receptor inverse agonist was withdrawn in 2009 because patients exhibited increased depression and suicide risks (Moreira and Crippa 2009). Sibutramine, a serotonin (5-HT) or 5-hydroxytryptamine-norepinephrine reuptake inhibitor, was withdrawn from the market in 2010 due to increased risk of cardiovascular events (Krentz, Fujioka et al. 2016).

At present there are only few available anti-obesity drugs on the market (Srivastava and Apovian 2018) (Table 2). One of them is lorcaserin, with higher selectivity for the 5-HT_{2C} receptors and less side effects than previous 5-HT agonists (fenfluramine and dexfenfluramine). In 2013, Fleming *et al.* showed that chronic injections of lorcaserin significantly decreasing food intake and body weight in high-fat diet obese-induced rats (Fleming, McClendon et al. 2013). Furthermore, Phase III clinical trial data show (BLOOM, BLOSSOM and BLOOM-DM) that lorcaserin is safe

and well tolerated in Type 2 diabetic patients and in non-diabetic patients, respectively (Greenway, Shanahan et al. 2016). Another anti-obesity drug in the market is Qnexa, which is the combination of phentermine and topiramate. Qnexa significantly decreases body weight (Garvey, Ryan et al. 2012) and improves obesity-related metabolic dysfunctions such as waist circumference, blood pressure, triglycerides and cholesterol levels, however, topiramate has teratogenic potential and increases the heart rate (Shin and Gadde 2013). Orlistat is the only anti-obesity drug which is driven by a peripheral approach and it has been on the market for more than 10 years. Orlistat reduces dietary fat absorption in the small intestines by inhibiting gastrointestinal lipase, however, may occur as a side effect the malabsorption of fat-soluble vitamins (Cahill and Lean 1999), severe liver injury (Sall, Wang et al. 2014) and kidney injury (Dietrich and Horvath 2012).

However, despite all the significant achievements, the current pharmacological treatments for obesity are only modestly effective and have several side effects. Therefore, further research is needed to develop a novel strategy to enhance the effectiveness of obesity treatment and to reduce side effects, including repurposing of drugs already in use or development of novel agents.

Table 2 ***Anti-obesity drugs.***

Table was adopted from reference (Srivastava and Apovian 2018) and (Dietrich and Horvath 2012). CNS- central nervous system, CV- cardiovascular; EMA- European Medicines Agency, FDA- US Food and Drug Administration, GLP-1- Glucagon-like peptide-1

**If applicable. ‡Never approved by the FDA owing to concerns related to adverse psychiatric side effects.*

Drug	Mechanism of action	Side effects	Comments
Approved			
Phentermine	• An amphetamine that increases the release of noradrenaline, dopamine and serotonin	• CV elevation in blood pressure, tachycardia • CNS: insomnia, restlessness, alters sexual behaviour, hormonal secretion and mood	• Approved by the FDA in 1959 • Recommended for short-term use (less than 3 months)

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Table 2 (continued)			
Drug	**Mechanism of action**	**Side effects**	**Comments**
Orlistat	• Pancreatic lipase inhibitor	• malabsorption of fat-soluble vitamins, steatorrhoea, fecal incontinence, flatulence • Rare cases of severe liver injury	• Approved by the FDA in 1999
Lorcaserin	• 5-HT receptor agonist that is more specific than previous compounds on the market, for example, fenfluramine	• Headache, dizziness, nausea, valvulopathy • Possible carcinogenic effects in rodents	• Approved by the FDA in June 2012 • Under evaluation by the EMA • Post-marketing, long-term CV outcomes trial required
Phentermine + topiramate (Qnexa)	• Phentermine: mechanism of action as above • Topiramate: anticonvulsant	• Possible teratogenic effects with topiramate • Can increase heart rate	• Approved by the FDA in July 2012 • Post-marketing, long-term CV outcomes trial required
Naltrexone/ bupropion sustained-release	• Bupropion: inhibitor of dopamine and noradrenalin uptake • Naltrexone: μ-opioid receptor antagonist	• Nausea, constipation, vomiting, dizziness, insomnia, dry mouth, and diarrhea	•Approved by the FDA in 2014 •Approved by the EMA in 2015
Liraglutide 3.0 mg	• GLP-1 analogue, binding to the same receptors as does the endogenous hormone GLP-1 that stimulates insulin secretion	•Nausea, hypoglycemia, diarrhea, constipation, vomiting, headache, decreased appetite, dyspepsia, fatigue, dizziness, abdominal pain, and increased lipase	• Approved by the FDA in 2014
Withdrawn			
Fenfluramine	• Increases the release of serotonin • Serotonin re-uptake inhibitor	• Hallucinations, valvulopathy, pulmonary hypertension	• Approved by the FDA in 1973 • Withdrawn in 1997
Sibutramine	• Noradrenalin and serotonin re-uptake inhibitor	• Increased risk of heart attack and stroke in patients with high risk of CV disorders	• Approved by the FDA in 1997 • Withdrawn in 2010
Rimonabant	• Cannabinoid 1 receptor antagonist	• Risk of suicide	• Approved by the EMA in 2006‡ • Withdrawn in 2009

1.1.3 Monoamine oxidase (MAO) inhibitors

Monoamines (epinephrine, NE, DA and 5-HT) participate in many physiological activities of the body. Monoamines are released from the synapse and exert their actions by activating dopaminergic, serotonergic and noradrenergic pathways. Monoaminergic signaling plays a critical role in regulating cognitive processes, as well as involved in the regulation of carbohydrate and lipid metabolism. MAOs are ubiquitous enzymes and they are responsible for the oxidative deamination of monoamines and they are found bound to the outer membrane of mitochondria in most cell types of the body. MAOs contain the covalently bound cofactor FAD, therefore, they are classified as flavoproteins. In mammals there are two subtypes of MAO: MAO-A and MAO-B. Both enzymes found in neurons and astroglia. Nevertheless, there are differences between their tissue distribution outside the central nervous system and the substrate selectivity of the two enzymes (Adeghate and Parvez 2004). MAO-B is mostly found in platelets and generally metabolizes DA, benzylamine and phenylethylamine. In contrast, MAO-A is found in the liver, pulmonary vascular endothelium, gastrointestinal tract and shows greater affinity for NE, 5-HT, DA and tyramine.

MAO inhibitors have two main groups: selective and non-selective, within these we distinguish reversible and irreversible inhibitors. At present, MAO inhibitors are mostly used for psychiatric and neurological disorders (Fiedorowicz and Swartz 2004, Finberg and Rabey 2016). MAO-B inhibitors (selegiline, rasagiline) are primarily used alone or in combination to treat Alzheimer's and Parkinson's diseases (Cai 2014, Dezsi and Vecsei 2017). MAO-A inhibitors (clorgyline, moclobemide) generally used as antidepressant (Meyer, Ginovart et al. 2006, Corbineau, Breton et al. 2017) and antianxiety drugs (Lader 1976).

However, in recent decades the use of MAO inhibitors by psychiatrists is significantly decreased; this decline is in context with the increase in the number of available novel antidepressants and with the concern about food- (Berlin and Lecrubier 1996, Flockhart 2012) and drug interactions (Sjöqvist 1965, Aboukarr and Giudice 2018). Nevertheless, since researchers showed that MAOs are highly expressed in human WAT, likely being involved in noradrenaline clearance and catecholamine-

dependent regulation of lipid metabolism in adipocytes (Pizzinat, Marti et al. 1999), thus, they may play an important role in obesity and lipid metabolism disorders and it may be an useful drug target in metabolic diseases.

Previous studies have shown that semicarbazide sensitive amine oxidase inhibitors administered in combination with certain non-selective and/or irreversible MAO inhibitors can reduce body weight and fat deposition in animal models of diet-induced obesity (Carpene, Iffiu-Soltesz et al. 2007, Carpene, Abello et al. 2008). Mattila *et al.* showed that administration of pargyline (30 mg kg^{-1}) increased lipolysis and levels of plasma free fatty acid in rats (Mattila and Torsti 1966). Furthermore, diet-induced obesity increased MAO activity in the enlarged omental WAT of dogs (Wanecq, Bour et al. 2006). Selegiline is a clinically widely used, irreversible and selective inhibitor of MAO-B, which is primarily used to treat Parkinson's disease (Jankovic and Poewe 2012, Zhao, Cai et al. 2013, Chiu, Carlsson et al. 2014, Cereda, Cilia et al. 2017), Alzheimer's disease (Riederer, Lachenmayer et al. 2004) and depression (Fiedorowicz and Swartz 2004, Pae, Patkar et al. 2014, Thomas, Shin et al. 2015). However, it has also been shown to have pleiotropic effects not related to the MAO-B inhibition (Tatton, Ju et al. 1994, Tatton, Chalmers-Redman et al. 2002). Békési *et al.* showed that selegiline (5–10 mg kg^{-1}) significantly decreased liver fat but not body weight in rats fed with a lipid-rich diet (Bekesi, Tulassay et al. 2012) (cholesterol 1%, olive oil 10%).

Therefore, we hypothesized that MAO inhibitors may have favourable metabolic effects in obesity.

1.2 Cardiovascular diseases (CVDs)

CVDs such as heart failure, hypertensive heart disease, cardiomyopathy, cardiac arrhythmia and coronary artery diseases (angina and myocardial infarction) are leading causes of morbidity and mortality worldwide (Pagidipati and Gaziano 2013, Roth, Johnson et al. 2017). Only in 2015, 17.7 million people died from CVDs in the world. Most of these deaths occurred in low-income and middle-income countries (WHO 2018). Nearly 300 risk factors play a role in the development of CVDs. These include high blood pressure, high cholesterol, diabetes, obesity, smoking, excessive alcohol

consumption, inherited factors, age and psychosocial stress, etc. (Balakumar, Maung-U et al. 2016).

The most common type of CVD is myocardial infarction (MI). Only in 2015, about 15.9 million MI occurred, worldwide (Vos, Allen et al. 2016). MI occurs when the blood flow reduces or stops for a part of the heart that causes an oxygen and nutrient depletion in the heart muscle, which leads to myocardial necrosis (Boateng and Sanborn 2013). Most myocardial infarction in the world is due to atherosclerosis, when a coronary artery becomes occluded following the rupture of an atherosclerotic plaque, which leads to the formation of a blood clot. Besides atherosclerosis, many other factors may also cause myocardial infarction such as hyperthyroidism, low red blood cell count, or low blood pressure.

1.2.1 Therapies of CVDs

In the management of patients with heart disease, besides pharmacotherapy, cardioprotective interventions (Bousselmi, Lebbi et al. 2014, Thuret, Saint Yves et al. 2014, Aimo, Borrelli et al. 2015) and cell therapies (Reed, Foldes et al. 2013, Goradel, Hour et al. 2018) are becoming more and more important. In this thesis we focus on pharmacotherapy which is a major treatment modality in CVDs. The purpose of pharmacological therapies is to prevent CVDs and to improve patient outcomes. The use of current cardiovascular drugs such as antiplatelet drugs, anticoagulants, nitrates, beta-blockers, renin-angiotensin inhibitors, and statins depends on the heart condition and symptoms (Table 3). However, despite all the achievements in this field, there are still certain types of CVDs that do not have appropriate therapies. Furthermore, a major problem is that there are no adequate targets for treating several diseases, such as ischemic heart disease. Therefore, a further major objective of drug development is finding new cardiovascular agents (Stern and Lebowitz 2010, Huffman and Bhatnagar 2012) and searching for new pharmacological targets (Olson 2014).

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Table 3 Classes of drug used in cardiovascular diseases

ACE- angiotensin-converting-enzyme; ARBS- Angiotensin II Receptor Blockers. Table was adopted from: http://www.secondscount.org/treatments/treatments-detail-2/medications-cardiovascular-disease#.W7MQhHszbDf.

Medication type	Purpose	Possible Side effects, Interactions, and Special Instructions
ACE Inhibitors and ARBS	To lower blood pressure and allow blood flow more easily from the heart	Dizziness, cough, low blood pressure. Kidneys and potassium levels should be monitored with blood tests.
Antiarrythmics	To control irregular heartbeat	Depends on the class of drugs. Channel blockers can cause headaches, ankle swelling. Amiodarone can increase sensitivity to sunlight and affect eyesight.
Antiplatelet Medications	To thin the blood and help prevent and dissolve clots in arteries and stents	Stomach pain, headache, dizziness, and breathing difficulties. Side effects more severe in patients with asthma and allergies. Take with food.
Aspirin	To prevent and dissolve clots in the arteries	Stomach upset, headaches, and drowsiness. An allergic reaction could cause breathing difficulties. Other severe side effects include blood in the stool or coughing blood. Take with food to reduce risk of upset stomach.
Beta-Blockers	To lower blood pressure and heart rate	Dizziness, fatigue, dry mouth, slow heart rate, weight gain, cold hands and feet. May reduce side effects if taken with food.
Thrombolytics	To restore blood flow during a heart attack or stroke and to break up blood clots in the legs (deep vein thrombosis)	Bleeding, abnormal heart beat, new clotting.
Anticoagulants	To prevent blood clots from forming in the arteries and heart	Bleeding, vomiting or coughing blood, blood in stool, headaches, and dizziness. Do not take with aspirin unless directed by doctor.
Digoxin	To improve your heart's ability to pump blood and helps to slow down an irregular heartbeat	Side effects are more common if too much is taken and may include nausea, vomiting, diarrhea, stomach pain, loss of appetite, unusual tiredness, and slow heartbeat. Take on an empty stomach, high-fiber foods can decrease its absorption.
Smoking Cessation Medication	To make it easier to stop smoking	See "SecondsCount Guide to Medications That Help you Quit Smoking"

Table 3 (continued)		
Medication type	**Purpose**	**Possible Side effects, Interactions, and Special Instructions**
Statins	To lower your cholesterol level, reduce the risk of heart attacks, strokes	Muscle pain, liver damage, memory loss, nausea, gas, diarrhea, constipation, rash.
Diuretics	To lower blood pressure	Frequent urination, dehydration, blurred vision, fatigue, rash, loss of appetite. Monitor blood pressure and kidney function.
Vasodilators	To widen the blood vessels to increase the flow of blood and lower blood pressure	Headaches, nausea, and dizziness (especially older people). Do not drink grapefruit juice. May interact negatively with cold medicine.

A number of cardioprotective interventions have been shown in the last decades, which reduced ischemic injury in animal models but failed to induce cardioprotection in human studies (Bolli, Becker et al. 2004). Then, numerous studies demonstrated that the efficacy of cardioprotective therapies is significantly affected by comorbidities such as obesity, diabetes or dyslipidaemia (Paulson 1997, Yi, Sun et al. 2011). For example, patients with T2DM have increased sensitivity to ischemia-reperfusion (IR) injury and that endogenous cardioprotective mechanisms are also inhibited (Ferdinandy, Schulz et al. 2007). Moreover, it has been shown that not only chronic diseases but also acute changes, such as acute hyperglycemia, can negatively affect cardioprotective interventions (Baranyai, Nagy et al. 2015). Furthermore, several publications reported that hypercholesterolemia changes cellular signalling and metabolism, thereby attenuates pharmacological and non-pharmacological cardioprotective interventions (Andreadou, Iliodromitis et al. 2017). In 2016, Ma *et al.* reported that remote ischemic preconditioning-induced cardioprotection was abolished in hypercholesterolemic rats by alteration of PI3K-Akt-GSK3b signaling pathway. In another study, Koncsos *et al.* showed that hypercholesterolemia-induced decrease in basal autophagy and increase in apoptosis leads to loss of cardioprotection (Giricz, Koncsos et al. 2017).

Therefore, a growing amount of evidence suggests that increased autophagy plays a critical role in cardioprotective interventions (Huang, Yitzhaki et al. 2010, Giricz, Mentzer et al. 2012) and that besides MAO inhibition, autophagy could be a potential therapeutic target for the treatment of metabolic- and cardiovascular diseases.

1.2.2 Autophagy

Autophagy is an intracellular degradation process which eliminates unnecessary or dysfunctional organelles and long-lived proteins through lysosomal breakdown for recycling intracellular components (Gustafsson and Gottlieb 2008). Accordingly, autophagy is primarily considered as a cytoprotective process. However, excessive self-degradation has detrimental effects. Therefore, autophagic dysfunction is related with different human pathologies, such as liver, and heart disease, neurodegeneration, cancer and metabolic diseases (Rubinsztein, Codogno et al. 2012). In mammalian cells, three main types of autophagy are commonly described: microautophagy, chaperone-mediated autophagy (CMA) and macroautophagy.

Microautophagy is a process which involves the direct engulfment of cytoplasmic material into the lysosome. The cytoplasmic contents enter the lysosome through an invagination or deformation of the lysosomal membrane (Li, Li et al. 2012). The second type of autophagy, CMA is a very complex and extremely selective for a subset of cytosolic soluble proteins (Kaushik and Cuervo 2008). Macroautophagy, (herein referred to as autophagy) eradicate damaged cell organelles or unused proteins. Autophagy consists of sequential steps: the initiation phase, which includes formation of the autophagosomal membrane also known as a phagophore that is usually derived from lipid bilayer contributed by the endoplasmic reticulum or the trans-Golgi. During sequestration phase, the phagophore expands and engulf protein aggregates or dysfunctional organelles and finally constitute a spherical double-membraned autophagosome. This is regulated by the autophagy proteins Atg4, Atg7, LC3, and the complex of Atg12-Atg5-Atg16L. In the degradation phase, autophagosome matures via fusion with lysosome, this structure is called autophagolysosome. This step initiates the degradation of the inner membrane of the autophagosome and cytoplasm-derived materials by lysosomal hydrolases. Lysosomal permeases and transporters export amino acids and other products of degradation to the cytosol for use by the cell in biosynthetic processes or to generate energy (Mizushima 2007, He and Klionsky 2009, Parzych and Klionsky 2014). Mitophagy is a specific type of autophagy, which means the selective degradation of injured or dysfunctional mitochondria (Ding and Yin 2012) (Figure 3).

Under normal circumstances autophagy has a low activity in cells but metabolic stress such as nutrient starvation or hypoxia can increase the activity of autophagy. During metabolic stress degradation of intracellular components promotes cell survival by maintaining cellular energy levels. Furthermore, the role of autophagy in the development of metabolic disorders (insulin resistance, diabetes mellitus, obesity, atherosclerosis) has been studied extensively using different genetic animal models (Kim and Lee 2014). Therefore, the use of autophagy-inducing agents could be an effective therapy in cardiovascular diseases and metabolic diseases.

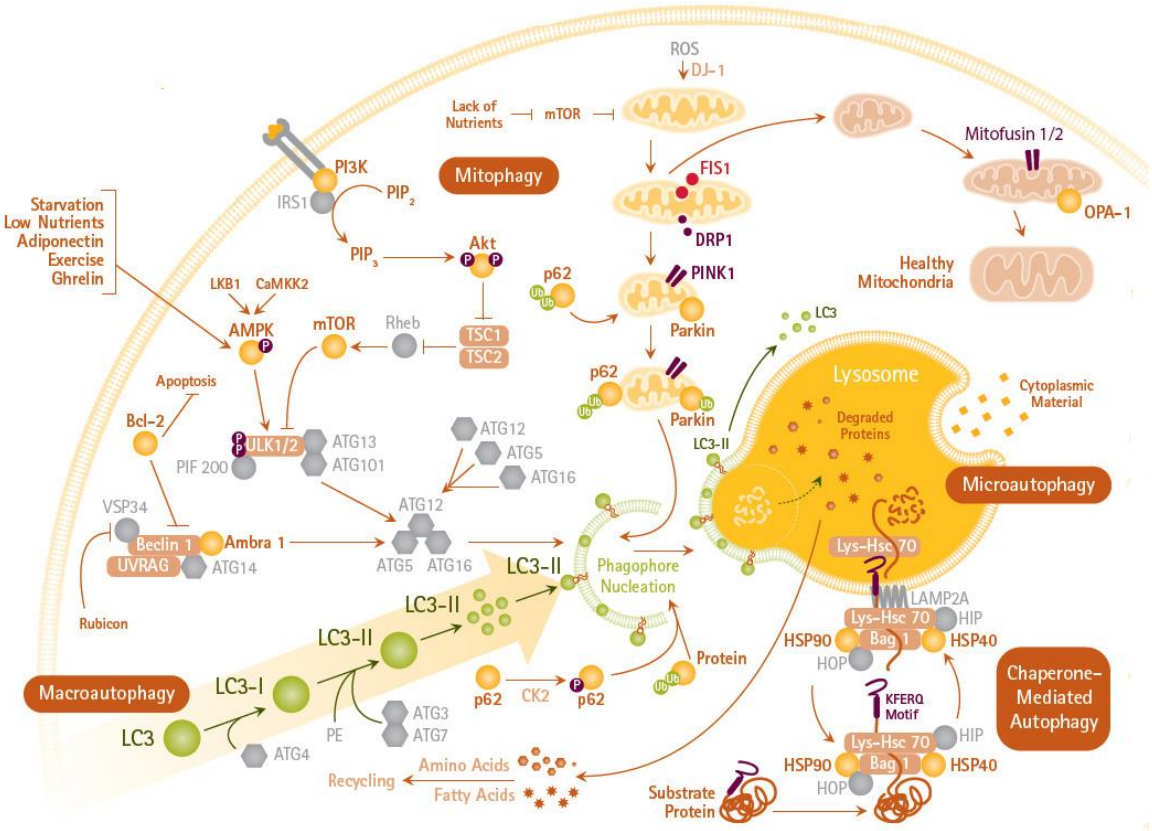

Figure 3. Autophagy mechanisms and signaling pathways. (macroautophagy, mitophagy and microautophagy).

Figure was adopted from: https://www.sigmaaldrich.com/technical-documents/articles/biology/cell-culture/cellular-assays/autophagy-assays.html

1.2.3 Compounds influencing autophagy

Previous studies reported that several therapeutic agents which are already in clinical use, for example hydrophobic statins (Andres, Hernandez et al. 2014), sevoflurane (Shiomi, Miyamae et al. 2014), sulfaphenazole (Huang, Liu et al. 2010) and certain antibiotics, such as chloramphenicol (CAP) (Prigione and Cortopassi 2007), may also induce autophagy in addition to their primary effects (Levine, Packer et al. 2015, Schiattarella and Hill 2016) (Table 4).

Table 4 Compounds that induce autophagy.

AMPK- AMP-activated Protein Kinase, cAMP- Cyclic adenosine monophosphate, mTORC1- mammalian target of rapamycin complex 1, ULK1- Unc-51 like autophagy activating kinase. Table was adopted from reference (Levine, Packer et al. 2015).

Compound	**Mechanism of autophagy induction**
FDA approved drugs	
Carbamazepine	Lowers inositol and Ins(1,4,5)P_3 levels
Clonidine	Lowers cAMP levels
Lithium	Lowers inositol and Ins(1,4,5)P_3 levels
Metformin	Upregulates AMPK, which phosphorylates ULK1 and beclin 1
Rapamycin (and rapalogs)	Inhibits mTORC1
Rilmenidine	Lowers cAMP levels
Sodium valproate	Lowers inositol and Ins(1,4,5)P_3 levels
Verapamil	Inhibits L-type Ca^{2+} channel, lowering intracytosolic Ca^{2+}
Statins	Depletion of geranylgeranyl diphosphate activates AMPK
Tyrosine kinase inhibitors	Inhibit Akt-mTOR signaling and beclin 1 tyrosine phosphorylation, increase beclin 1/Parkin interaction
Investigational drug	
BH3 mimetics	Disrupt binding between beclin 1 and Bcl-2 family members
Chloramphenicol	Increase in formation of Atg12-Atg5 conjugates, and in beclin-1 and LC3-II levels
Nutritional supplements	
Caffeine	Inhibits mTOR signaling
Omega-3 polyunsaturated fatty acids	Inhibit Akt-mTOR signaling; disrupt beclin 1 and Bcl-2 binding
Resveratrol	Activates sirtuin 1
Spermidine	Acetylase inhibitor
Vitamin D	Calcium signaling, hCAP18/LL37-dependent transcription of autophagy genes

Since previous studies reported that CAP protects the heart against ischemia/reperfusion injury and upregulates autophagy markers (He, Chen et al. 2001, Granville, Tashakkor et al. 2004, Sala-Mercado, Wider et al. 2010). It has been shown that the induction of autophagy is required for cardioprotective mechanisms but no detailed investigation has been performed on which stage of autophagy is necessary for cardioprotection. Since autophagy is a multi-step process, it can be inhibited at different steps. Previous studies have shown that inhibition of early-stage autophagy can be achieved by 3-methyladenine (Li, Liu et al. 2015) and wortmannin which are inhibit the class III phosphatidylinositol-3 kinase, or TAT-HA-Atg5K130R, a dominant negative mutant fusion protein of a key mediator of autophagy, Atg5 (Pyo, Jang et al. 2005, Hamacher-Brady, Brady et al. 2006, Hamacher-Brady, Brady et al. 2007). The late phase of autophagy, lysosomal fusion and degradation, can be arrested by elevating lysosomal pH, e.g., by the use of an antimalarial drug, chloroquine (CQ, see for review: (Kimura, Takabatake et al. 2013)). Although a few studies utilized these substances to investigate the mechanism of cardioprotective stimuli, it is still unknown which phase of CAP-induced autophagy is necessary for cardioprotection.

Therefore, here we hypothesized that CAP induces cardioprotection, via increasing the level of autophagy in cardiomyocytes.

DOI:10.14753/SE.2020.2308

2 Objectives

Therefore, we had the following aims:

- To investigate the effect of selegiline treatment on metabolic parameters in high-fat, high-sucrose diet-induced moderate obesity, together with alterations in hemodynamic parameters and markers of neuropathy and behavioural patterns.
- To investigate whether CAP-induced autophagy is necessary for cardioprotection.
- To assess whether sequestration and/or degradation phases of autophagy are necessary for the cardioprotective effect of CAP.

DOI:10.14753/SE.2020.2308

3 Methods

This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and was approved by the animal ethics committee of the San Diego State University, San Diego, California and Semmelweis University, Budapest, Hungary. Furthermore, conforms to Directive 2010/63/EU and was authorized by the regional animal health authority in Hungary (registration numbers: XIV-I-001/450-6/2012). Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny, Browne et al. 2010, McGrath and Lilley 2015).

3.1 Study designs

3.1.1 Study design: diet-induced experimental obesity

5-7 weeks old male Long-Evans rats (RRID: RGD_2308852) were purchased from Charles River Laboratories (Wilmington, MA, USA). We chose Long-Evans rats for our study which are commonly used for experiments on diet-induced obesity (Li, Zhang et al. 2008, Estridge, Dey et al. 2017). Furthermore, this strain is leaner as compared to Sprague-Dawley and Wistar rats, therefore, the effects of mild obesity could be studied with higher reliability in this strain. Furthermore, our laboratory has already experience with this strain in metabolic studies (Koncsos, Varga et al. 2016). Animals were housed in conventional animal house, 4 animals were housed in a polyethylene EUROSTANDARD TYPE IV S cages (480 x 375 x 210 mm) containing corncob bedding from J. Rettenmaier & Söhne GmbH & Co and housed in a room maintained in a 12/12 h light/dark cycle and constant temperature of 21 °C. Animals were allowed access to food and water ad libitum. After 1 week of acclimatization, rats were randomly divided into two groups: control (CON; n=20) and high-fat, high-sucrose group (HFS; n=20). The CON group was fed control rat chow, whereas the HFS group was fed a chow supplemented with 20% lard and 15% sucrose as a HFS diet (Table 5).

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Table 5 Composition and nutritional data of chows.
Data are expressed as g/100g or otherwise noted. CON- control, HFS- high-fat, high-sucrose.

	CON	HFS
Corn	58	18
Gluten/corn	25	30
Sugar	0	15
Lard	0	20
Fasermix (straw pellet)	6	6
Sodium chloride	0.4	0.4
Zeolite, universal	6.7	6.7
Mono calcium phosphate (MCP)	1.5	1.5
Feed lime	1.3	1.3
Adhesive	0.6	0.6
Vitamin and trace element supplement	0.5	0.5
Gross energy (Mj/kg)	15.52	20.31

From week 16, groups of animals were further randomly divided and received subcutaneous injections of 0.25 mg kg^{-1} selegiline (CON+S and HFS+S) or vehicle (CON, HFS) once daily (n=10 in each group). Previous articles showed that rats treated with daily subcutaneous dose of 0.25 mg kg^{-1} selegiline selectively inhibited the B form of the enzyme, leaving the activity of the A form practically unchanged. With a higher (1 mg kg^{-1}) dose the selectivity was nearly lost (Knoll 1978, Heinonen, Anttila et al. 1994).

Body weights were measured monthly. Blood was taken, and fasting blood glucose levels were measured from the saphenous vein every month (Accu-Check; Roche, Basel, Switzerland). At week 24, oral glucose tolerance test (OGTT) was performed on conscious overnight fasted rats with per os administration of 1.5 g kg^{-1} glucose and measurements of plasma glucose levels at 15, 30, 60, and 120 min. Insulin tolerance test (ITT) was also performed at week 24 in overnight fasted rats. Insulin (0.5 IU/kg, Humulin R; Ely Lilly, Utrecht, The Netherlands) was injected intraperitoneally (i.p.), and plasma glucose levels were checked at 15, 30, 45, 60, 90, and 120 min. At week 24 after 1-day acclimatization, food intake was observed for 24 hours in a

metabolic cage. At week 25 of the diet animals were sacrificed, anesthetized with pentobarbital (60 mg kg^{-1}, intraperitoneally; Euthasol; Produlab Pharma, Raamsdonksveer, The Netherlands). After hemodynamic measurements, epididymal and interscapular brown fat tissue, markers of adiposity, were isolated, and their weights were measured. Blood and tissue samples were collected and stored at −80 °C (Figure 4.). To exclude the natural variability between weights, organ weights were normalized to tibia length (Tsujita, Muraski et al. 2006, Nemeth, Matyas et al. 2016).

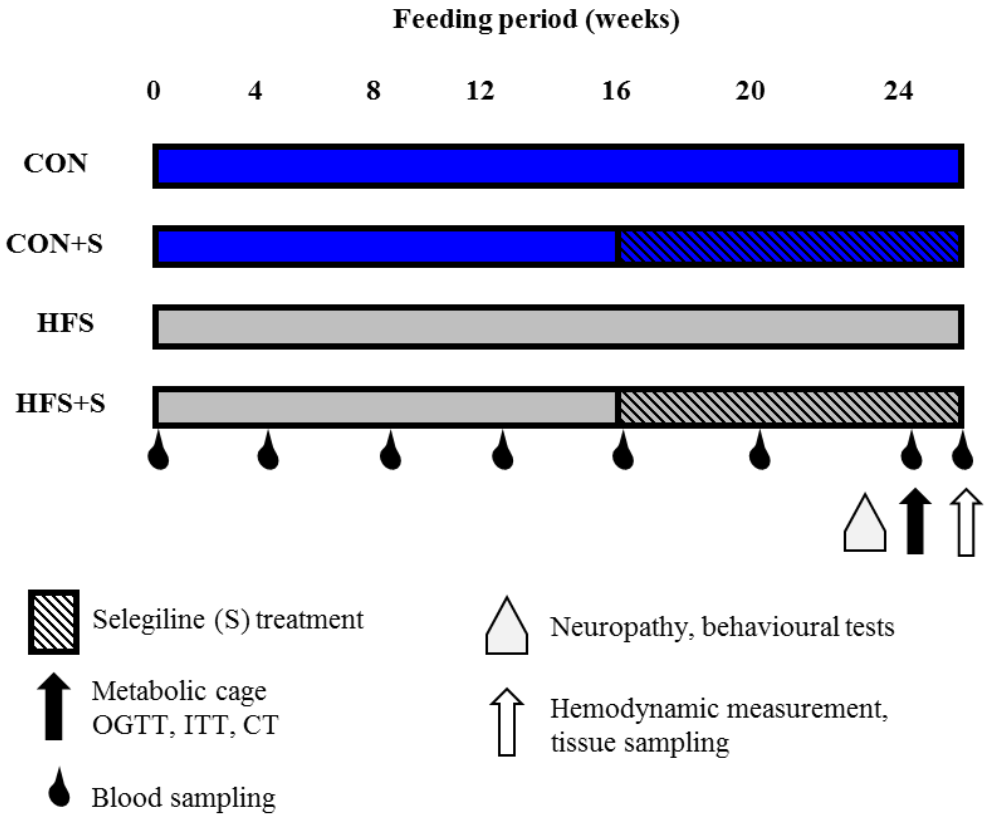

Figure 4. Experimental protocol.

Male Long-Evans rats were fed with control (CON, n=10) diet or with high-fat, high sucrose (HFS, n=10) diet for 25 weeks; CON+S (n=10) and HFS+S (n=10) groups were treated with 0.25 mg kg-1 selegiline (S) from week 16 to 24. Body weights and blood glucose were measured monthly. Neuropathy, behavioural tests were measured at weeks 22-23. Oral glucose tolerance test (OGTT), insulin tolerance test (ITT), metabolic cage and CT (computed tomography) were performed at week 24. Hemodynamic analysis was performed at week 25. Tissue sampling was performed after terminal procedures.

3.1.2 Study design: effect of CAP on autophagy in different cardiac cells

To identify the most suitable model system, in a pilot study we examined the effect of CAP on autophagy in neonatal rat cardiomyocytes (NRCMs), in H9c2 cardiac myoblast cells and in isolated hearts. We found that CAP induced autophagy in isolated hearts but not in NRCMs or in H9c2 cells (see Figure 14A–C). The efficacy of CAP and TAT-HAAtg5K130R was also assessed in a pilot experiment where we observed that LC3-II/I ratio was decreased after 15 min of administration of 200 nM TAT-HA-Atg^{5K130R} in isolated hearts as compared to vehicle controls (Figure 14D).

Therefore, in the main series of experiments, we used an ex vivo model of acute cardiac ischemia/reperfusion injury to assess the effects of autophagy inhibitors (TAT-HA-$Atg5^{K130R}$ and CQ) on CAP-induced cardioprotection. Since the availability of TAT-HA-$Atg5^{K130R}$ was limited due to technical limitations in production and purification of the protein in quantities necessary for ex vivo heart perfusion experiments, we had to reduce the number of isolated hearts treated with TAT-HA-$Atg5^{K130R}$.

3.2 Evaluation of whole body fat

To assess obesity, computed tomography (CT) measurements were performed on NanoSPECT/CT PLUS (Mediso, Budapest, Hungary) at week 24. The semicircular CT scanning was acquired with a 55 kV tube voltage, 500 msec exposure time, 1:4 binning, and 360 projections in 18 min. During the acquisitions, rats were placed in a prone position in a dedicated rat bed and were anesthetized with 2% isoflurane in oxygen. Temperature of the animals was kept at 37.2 ± 0.3 °C during imaging. In the reconstruction, 0.24 mm in-plane resolution and slice thickness were set, and Butterworth filter was applied (volume size: 76.8×76.8×190 mm). Images were further analysed with VivoQuant (inviCRO, Boston, MA) dedicated image analysis software products by placing appropriate volumes of interest on the whole body fat of animals. The aim of segmentation was to separate the fat from other tissues. The connected threshold method helped to choose the adequate attenuated pixels for fat tissue analysis,

and then the isolated points were detected by erode 4 voxel and dilate 4 voxel steps. After the measurements, animals recovered from anaesthesia. Adiposity index was calculated by the following formula: (CT whole body adipose tissue volume/body weight) ×100. Subcutaneous and total visceral fat volumes were evaluated on CT images by CTan software (Bruker microCT, Kontich, Belgium).

3.3 Immunohistochemistry

For immunohistochemistry, deparaffinized sections underwent antigen retrieval (pH=6 citrate buffer, at 95 °C for 15 min). After blocking endogenous peroxidase activity (Bloxall, Vector Laboratories, Burlingame, CA, USA), the sections were blocked in appropriate sera (2.5% goat serum in PBS). The primary antibody recognizing the monocyte/macrophage-specific protein Iba-1 (Wako Pure Chemical Industries, Chuo-Ku, Osaka, Japan), was incubated with the sections overnight in blocking solution at 4°C. After the primary antibody incubation, the sections were washed three times in PBS and incubated for an hour with an anti-rabbit IgG peroxidase polymer (ImmPress reagents, Vector Laboratories, Burlingame, CA, USA). Secondary antibodies were washed 3 times for 10 min and the specific signal was developed with diaminobenzidine (ImmPACT DAB EqV Peroxidase (HRP) Substrate, Vector Laboratories, Burlingame, CA, USA). The specific staining was visualized and images were acquired using Leica DM3000 LED microscope and MC 190 HD camera (Leica Microsystems, Wetzlar, Germany).

After routine formalin-fixed paraffin-embedded specimen processing, 4 μm thick tissue sections were prepared and stained with hematoxylin and eosin (H&E), for histological evaluation of adipocyte morphology. Adipocyte cross sectional area was measured with the ImageJ based software, Adiposoft (Galarraga, Campion et al. 2012).

3.4 Adipocyte cross sectional area

Adipocyte cross sectional area was measured with the ImageJ-based software, Adiposoft (Galarraga, Campion et al. 2012). Images of adipocyte cross sectional area were subtracted using ImageJ and then analyzed with Adiposoft without using automated method. Cells which were smaller than 25 μm in diameter were excluded. Analyzed images were edited using manual method in Adiposoft.

Cells on the borders of image were excluded, separated sections that clearly belonged to one cell were merged together, and cells which were not recognized by software were added and analyzed manually. Adipocyte tissue areas in μm² were then further used for analysis.

3.5 Total RNA isolation

Total RNA from white adipose tissue (epididymal) was extracted with a precipitation method. Briefly, RNAzol® RT (Sigma, USA) was added to each sample and homogenized with TissueLyser (Qiagen, Germany). Homogenates were centrifuged, and DNA and protein were precipitated with nuclease-free water. Furthermore, 4-bromoanisole (Sigma), phase separation step was incorporated to maximize the DNA elimination. Total RNA was precipitated with isopropanol (vWR, USA), and pellet was washed twice with ethanol (vWR, USA). Finally, total RNA was resuspended in nuclease-free water. RNA concentrations were measured with NanoDrop (Thermo Scientific, Waltham, USA).

3.6 cDNA synthesis and qRT-PCR

Total RNA was used as a template for cDNA synthesis, using Sensifast cDNA synthesis kit (Bioline; BLI 65053) according to the manufacturer's protocol. qRT-PCR reactions were performed with a LightCycler® 480 Real-Time PCR System (Roche Diagnostics, Germany) in the presence LightCycler® RNA Master SYBR Green I (Roche; 03064760001) according to the manufacturers protocol. Forward and reverse primers for glucose transporter 1 (*Glut1*), glucose transporter 4 (*Glut4*), acetyl-CoA

carboxylase (*Acc*), adipose triglyceride lipase (*Pnpla2*), diglyceride acyltransferase (*Dgat*), fatty acid translocase (*Cd36*), sterol regulatory element-binding protein 1c (*Srebp-1c*), NADH dehydrogenase 1 alpha (*Ndufa1*), monocyte chemoattractant protein 1 (*Ccl2*) and macrophage inflammatory protein 1-alpha (*Ccl3*), glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) were used for analysis. *Hprt* was used as reference gene. Results were calculated with 2log-ΔΔCp evaluation method. Sequences of primers are indicated in Table 6.

Table 6 Sequences of primers

Primer	Forward	Reverse
Acc	TGAGGAGGACCGCATTTATC	GCATGGAATGGCAGTAAGGT
Cd36	CCAGAACCCAGACAACCACT	CACAGGCTTTCCTTCTTTGC
Dgat	TGCTCTTTTTCACCCAGCTT	TTGAAGGGCTTCATGGAGTT
Gapdh	TACCAGGGCTGCCTTCTCTTG	GGATCTCGCTCCTGGAAGATG
Glut1	CGGGCCAAGAGTGTGCTGAA	TGACGATACCCGAGCCGATG
Glut4	AGAGTGCCTGAAACC	CCCTAAGTATTCAAGTTCTG
Hprt	GTCCTGTTGATGTGGCCAGT	TGCAAATCAAAAGGGACGCA
Ccl2	TGATCCCAATGAGTCGGCTG	GCTTGGTGACAAATACTACAGCTT
Ccl3	GCTGCTTCTCCTATGGACGG	TCTCTTGGTCAGGAAAATGACACC
Ndufa1	GGTTGGAGTGTGAGTAACGGT	TCCAGGCCCTTGGACACATAG
Pnpla2	CACCATCCGTTTGTTGGAGTG	GCAGATGCTACCTGTCTGCT
Srebp-1c	CGTTAACGTGGGTCTCCTCC	AGCATGTCTTCGATGTCGGTC

3.7 Measurement of plasma lipid, insulin and leptin levels

Low density lipoprotein (Beckman Coulter LDL-Cholesterol, Ref.: OSR6183), high density lipoprotein (Beckman Coulter HDL-Cholesterol, Ref.: OSR6187) and triglyceride (Beckman Coulter Triglyceride, Ref.: OSR60118) were measured from plasma samples according to the manufacturers protocol by Beckman Coulter AU 5800 Clinical Chemistry System.

Plasma samples and pulverized pancreas samples were used to determine pancreatic insulin content. Analysis was performed with insulin (I-125) IRMA Kit (Izotop Kft, Budapest, Hungary) according to the manufacturer's instructions.

Plasma leptin was measured by ELISA (KRC2281, Invitrogen, Camarillo, CA, USA) according to manufacturer's instructions. Technical triplicates were used to ensure the reliability of single values.

3.8 Measurement of liver lipid content

Approximately 50 mg of frozen liver tissue was homogenized with Tissuelyser LT (Quiagen, Germany) (50 s-1; 2×3 min) in a closed tube with 1.0-mm metal beads and 1.0 mL SET buffer (sucrose 250 mM, EDTA 2 mM, and Tris 10 mM). Complete cell destruction was done by 2 freeze-thaw cycles and 3 times passing through a 26-gauge syringe needle and a final freeze-thaw cycle. Total cholesterol (Beckman Coulter Cholesterol, Ref.: OSR6116), and triglyceride (Beckman Coulter Triglyceride, Ref.: OSR60118) were measured from homogenized liver samples according to the manufacturers protocol by Beckman Coulter AU 5800 Clinical Chemistry System. Hepatic cholesterol and triglyceride levels were normalized to protein content, measured with the bicinchoninic acid (BCA) method (Thermo Fisher Scientific, Waltham, USA) as did in previous article (Houben, Oligschlaeger et al. 2017).

3.9 Hemodynamic measurements

Arterial blood pressure measurements were performed using a 2F microtip pressure microcatheter (SPR-838, Millar Instruments, Houston, USA). Rats were anesthetized with pentobarbital (60 mg kg-1, intraperitoneally, Produlab Pharma, Raamsdonksveer, The Netherlands), tracheotomized, intubated and artificially ventilated, while core temperature was maintained at 37 °C. Thereafter the catheter was inserted into the right carotid artery and advanced into the ascending aorta. After stabilization for 5 min, arterial blood pressure curve was recorded by the PowerLab data

acquisition system (AD Instruments, Colorado Springs, USA), stored, displayed and analyzed on a personal computer by the LabChart Software System (AD Instruments) to calculate heart rate, systolic and diastolic blood pressure values.

3.10 Behaviour and nociceptive tests

To test if altered caloric intake and obesity may influence motor activity, motility was measured with a 3 channel CONDUCTA system (EXPERIMETRIA Ltd. Hungary). Animals were placed to one of the 48×48×40 cm observation boxes for 30 min. Their movements were detected with infrared sensors. The time spent with four mutually exclusive movement types were analyzed: rearing (vertical activity), immobility (complete motionlessness), ambulation (horizontal activity) and local movements (non-locomotor activity).

Novel object recognition assay (NOR) was performed to assess the effects of selegiline on HFS diet-induced cognitive dysfunctions. This assay is a model for the investigation of visual recognition memory in rodents. The task procedure consists of three phases: Day 1: habituation to the test box for 3 min; Day 2: familiarization with two identical objects, (trial 1, t1); Day 3: test phase: after 24 h intertrial delay one of the familiar objects was replaced by a novel object and the exploration time of each object was measured for 3 min (trial 2, t2). The animals were observed through a video camera system. Recognition memory was characterised by the discrimination index (DI): (t2novel-t2fam)/ (t2novel+t2fam) ×100, where t2novel and t2_fam are exploration time of novel and familiar object, respectively, in the test phase in seconds. Therefore, higher DI indicates better recognition memory.

To investigate sensory changes in prediabetes and obesity related to potential neuropathic complications, we performed two mechano-nociceptive tests. The dynamic plantar aesthesiometer (DPA; Ugo Basile Model No. 37450, Comerio, Italy) is a modified method of the classic Von Frey assay to assess touch sensitivity. The animals were put in mesh bottom plastic boxes. A blunt-end thin metal filament was targeted to the middle region of the plantar surface of the hind paws with an increasing force up to

the maximum of 50 g with 10 g/sec ramp. Each paw was tested three times and the touch sensitivity thresholds were determined. Since this touch stimulus is basically non-painful for the rat under normal conditions, decreased sensitivity threshold is considered as mechanical allodynia. The Randall-Selitto test (Ugo Basile Model No. 18476, Comerio, Italy) measures the pressure sensitivity of the paws. The cone-shaped pusher of the instrument applied a continuously increasing pressure on the hind paw of the loosely held, restrained rat and the pressure threshold of the withdrawal reaction was registered. Each hind paw was tested in this assay once. Unlike the touch stimulus, this pressure represents a mild painful stimulus under healthy conditions, therefore, decreased pressure withdrawal threshold shows mechanical hyperalgesia. The Randall-Selitto test was performed after the DPA measurement to avoid the possible influence of the compression on touch sensitivity.

3.11 Isolation and treatment of NRCMs

Preparation of NRCM culture used in the pilot study was described before in details (Gorbe, Giricz et al. 2010). Briefly, neonatal Wistar rats were sacrificed by cervical dislocation and hearts were removed and placed into ice-cold phosphate buffered saline (PBS) solution. Ventricles were minced and resuspended in 0.25% trypsin (Thermo Fisher Scientific, USA) solution. Digested tissue was centrifuged at 400 ×g, for 15 min at 4 °C. Cell pellet was resuspended in Dulbecco's Modified Eagle Medium (DMEM) supplemented with L-glutamine, Antibiotic-Antimycotic solution and 10% fetal bovine serum (FBS, Thermo Fisher Scientific, USA). Cells were counted in a hemocytometer, and seeded into 75 cm^2 flasks at a density of 4 $\times 10^6$ cell/flask. After 24 h, the growth medium was replaced with differentiation medium containing 1% FBS. Cardiomyocytes were kept under normoxic conditions (37 °C, in 95% air and 5% CO2 gas mixture) for three days prior to treatment and cell collection. On the 3rd day of culturing, cell medium was supplemented with vehicle (saline, 5% v/v) or 300 μM CAP for 1 h. Then cell cultures were washed with ice cold PBS and lysed for 5 min in 500 μL homogenization buffer (1× Radio-Immunoprecipitation Assay Buffer, RIPA, supplemented with a protease and phosphatase inhibitor cocktail). Lysed cells were scraped, collected and sonicated with an ultrasonic homogenizer (Ultrasonic Processor

UP200H, Hielscher, USA) for 10 s on ice. The homogenate was centrifuged at 10,000 ×g, 4 °C for 10 min; and the supernatant was collected and stored at −80 °C. Protein concentration was assessed by Bicinchoninic Acid Protein Assay Kit (BCA Protein Assay kit, Pierce, USA).

3.12 Treatment of H9c2 cells

H9c2 cells were seeded in T25 flasks (10^6 cells/flask) in 6 mL DMEM supplemented with 10% FBS, Antibiotic-Antimycotic solution, glucose, MEM Non-Essential Amino Acid Solution and L-glutamine. Then incubated at 37 °C in 5 % CO_2. After 24 h cells were treated with 300 μM CAP or vehicle (CON). Cells were incubated for 1 h, then scraped in 200 μL RIPA lysis buffer and sonicated (Ultrasonic Processor UP200H, Hielscher, USA) for 3 ×15 s at 50% power. Homogenates were centrifuged for 10 min at 10,000 ×g, 4 °C. Supernatants were aliquoted and stored at −80 °C. Protein concentration was assessed with BCA kit.

3.13 Preparation of K130R

TAT-HA-Atg5^{K130R} protein was purified from BL21(DE3)pLysS E. coli bacteria transformed with pTAT-HA-Atg5^{K130R} plasmid as described elsewhere [8]. Briefly, crude cellular extract was purified on a Ni-NTA column (Thermo Fisher Scientific, USA) followed by desalting on a PD- 10 column (GE Healthcare, United Kingdom) into PBS. Purified protein was used immediately after assessing its concentration by the BCA method.

3.14 Ex vivo heart perfusion

Sprague-Dawley rats (250–300 g) were anesthetized with i.p. pentobarbital (30 mg/kg) and anticoagulated with i.v. heparin (100 U/kg). Hearts were excised and perfused in Langendorff mode with Krebs-Henseleit solution (KH) for 15 min(Bell,

Mocanu et al. 2011). From the 15th min, a group of heart received KH containing 300 μM CAP. To inhibit CAP-induced autophagosome formation a group of hearts were perfused with KH containing 300 μM CAP and 200 nM cell-permeable recombinant TAT-HA- Atg5K130R (CAP + K130R) for 15 min at the beginning of the protocol, then received CAP alone. Another group of hearts received 300 μM CAP and 10 μM CQ (CAP + CQ) throughout the protocol to inhibit lysosomal degradation of CAP-induced autophagosomes. Further groups of hearts were perfused with 10 μM CQ or 200 nM TAT-HA-Atg5^{K130R} alone (CQ and K130R, respectively). At the 35 minutes of the experimental protocol global ischemia was applied. After the 30 min global ischemia, reperfusion was restored. At the end of the 120 min reperfusion, hearts were harvested for infarct size evaluation (Figure 5).

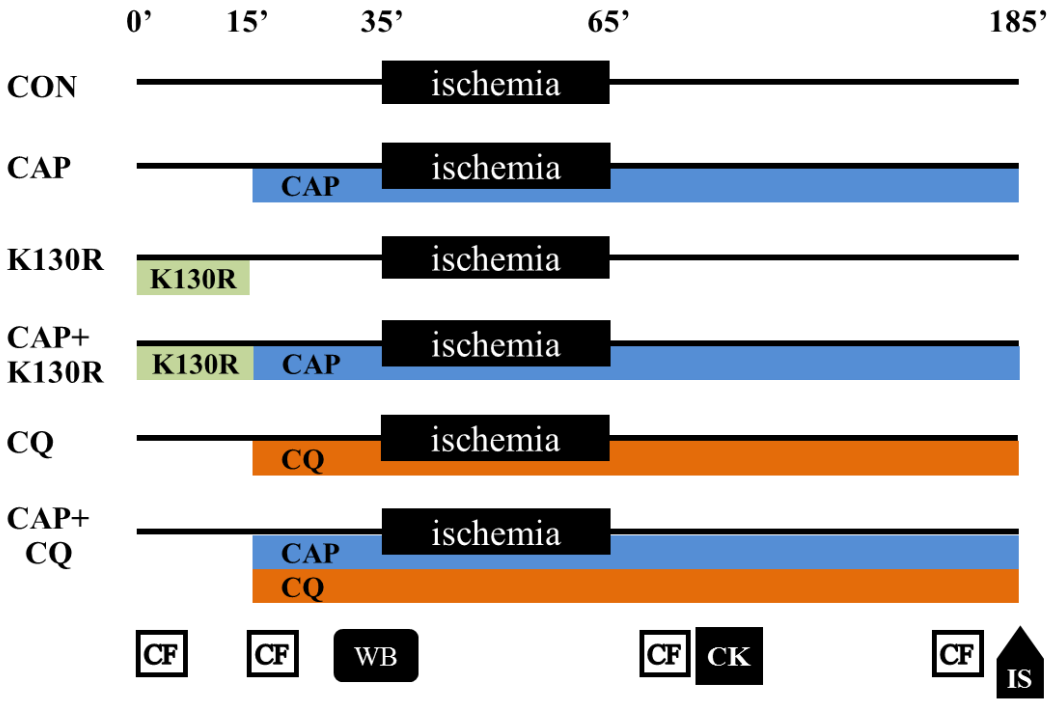

Figure 5. Experimental protocol.

CAP- Chloramphenicol, CQ – Chloroquine; CON- Control, K130R- TAT-HA-Atg5K130R, CK- Creatine kinase, CF- Coronary flow measurement, WB- Western blot, IS- Infarct size.

3.15 Measurement of infarct size, coronary flow and creatine kinase release

At the end of perfusion, hearts were sliced into 2 mm-thick slices, and right ventricles were removed. Slices were immersed in 1% triphenyltetrazolium- chloride for 20 min, then in 4% formalin for 24 h and scanned. Weight of slices was measured. Necrotic area was evaluated by planimetry (ImageJ). Data is expressed as a percentage of left ventricular mass. Coronary flow rate was measured throughout the protocol by timed collection of coronary effluent. Creatine kinase (CK) release was measured in the coronary effluent collected from 10 min to 20 min after the onset of the reperfusion by a colorimetric assay (Stanbio™ CK Liqui-UV™ Test, Stanbio Laboratory, USA).

3.16 Western blotting

After 35 min aerobic perfusion, left atria or whole hearts were snap frozen. Samples were lysed in 500 μL RIPA buffer with Tissuelyser LT (Quiagen, Germany) at 4 °C. Lysates were centrifuged at 10,000 ×g at 4 °C for 15 min. Supernatants were aliquoted and stored at −80 °C. Protein concentration was assessed by BCA kit, then samples containing 40 μg protein were prepared in Laemmli-buffer and electrophoresed on 4–12% polyacrylamide gels (Thermo Fisher Scientific, USA), then transferred to polyvinylidene difluoride membranes. Even loading was assessed with Ponceau staining. Membranes were blocked with 5% non-fat milk or 5% bovine serum albumin (BSA) in Tris-buffered saline with 0.05% Tween 20 (TBST) for 2 h. Antibodies against LC3 A/B, p-Akt, Akt, p-Erk1/2, Erk1/2 (Cell Signaling, USA) were diluted 1:1000 in 5% milk or 5% BSA in TBST and added to membranes and incubated for overnight at 4 °C. After washing in TBST for 3× 5 min, secondary antibody conjugated to horseradish peroxidase diluted 1:2000 in 5% milk or 5% BSA in TBST was added for 1 h at room temperature. Signals were detected by enhanced chemiluminescence kit (Thermo Fisher Scientific, USA) and quantified with ImageQuant software (Bio-Rad, USA).

3.17 Data and statistical analysis

Data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis, Bond et al. 2015). Data analysis was performed in a blinded fashion. Group sizes were estimated based on previous experiments (Koncsos, Varga et al. 2016). Data are expressed as mean ± standard error of mean (SEM). Statistical analysis was performed by two-way ANOVA using Fisher's LSD as post hoc test or One-way ANOVA using Fisher's LSD as post-hoc test or Student's t-test or non-parametric Kruskal-Wallis test (coronary flow data, 14–20 min timepoint) only if F achieved $p<0.05$ and there was no significant variance in homogeneity by Prism 6 (GraphPad, GraphPad Software, USA). For motor activity multivariate analysis of variance was performed as statistical evaluation. Differences were deemed significant in case $p < 0.05$.

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4 Results

4.1 HFS diet elevated body weight despite a similar caloric intake

To investigate the effect of chronic HFS diet and selegiline treatment in rats, we measured body weight, food and caloric intake. We found that body weight was slightly but significantly elevated in HFS and HFS+S groups from week 16 with a 14% increase at the end of the study showing moderate obesity (Figure 6A, B). Observations in metabolic cages showed that food intake was lower in HFS and HFS+S groups resulting in similar caloric intake in all groups (Figure 6C, D). Chronic selegiline had no influence on body weight or food intake. In HFS group one animal died due to procedural error.

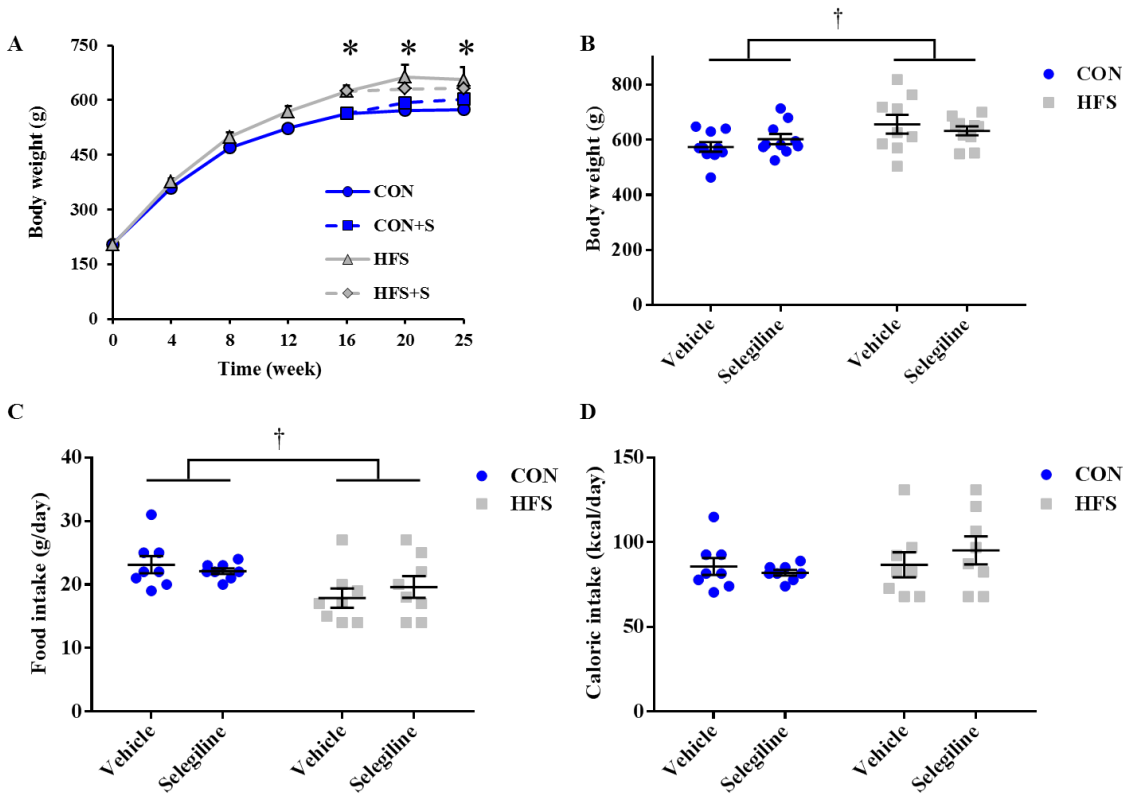

Figure 6. High-fat, high-sucrose diet increased body weight despite the similar caloric intake from week 16.

*(A) Body weight changes during the experiment (n=10) (B) Mean body weights at week 25 (n=10) (C) Daily food intake (n=8) (D) Daily caloric intake (n=8). Data are presented as mean ± SEM, *p<0.05 vs. CON, † vs. control diet groups. CON- control; CON+S- control+selegiline; HFS- high-fat, high-sucrose; HFS+S- high-fat, high-sucrose+selegiline.*

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4.2 Selegiline reduced HFS diet-induced adiposity

To determine the effects of HFS diet and selegiline treatment on obesity, we evaluated adiposity and measured weights of epididymal- and brown adipose tissues and organ weights. Selegiline treatment significantly decreased HFS diet-induced adiposity (Figure 7A, B). Here we found that total visceral and subcutaneous fat volumes increased significantly due to HFS diet and that selegiline treatment reduced these elevations in total visceral and subcutaneous fat depots (Figure 7C, D). For histological evaluation of adipocyte morphology epididymal adipose tissue sections were prepared and stained with H&E (Figure 8).

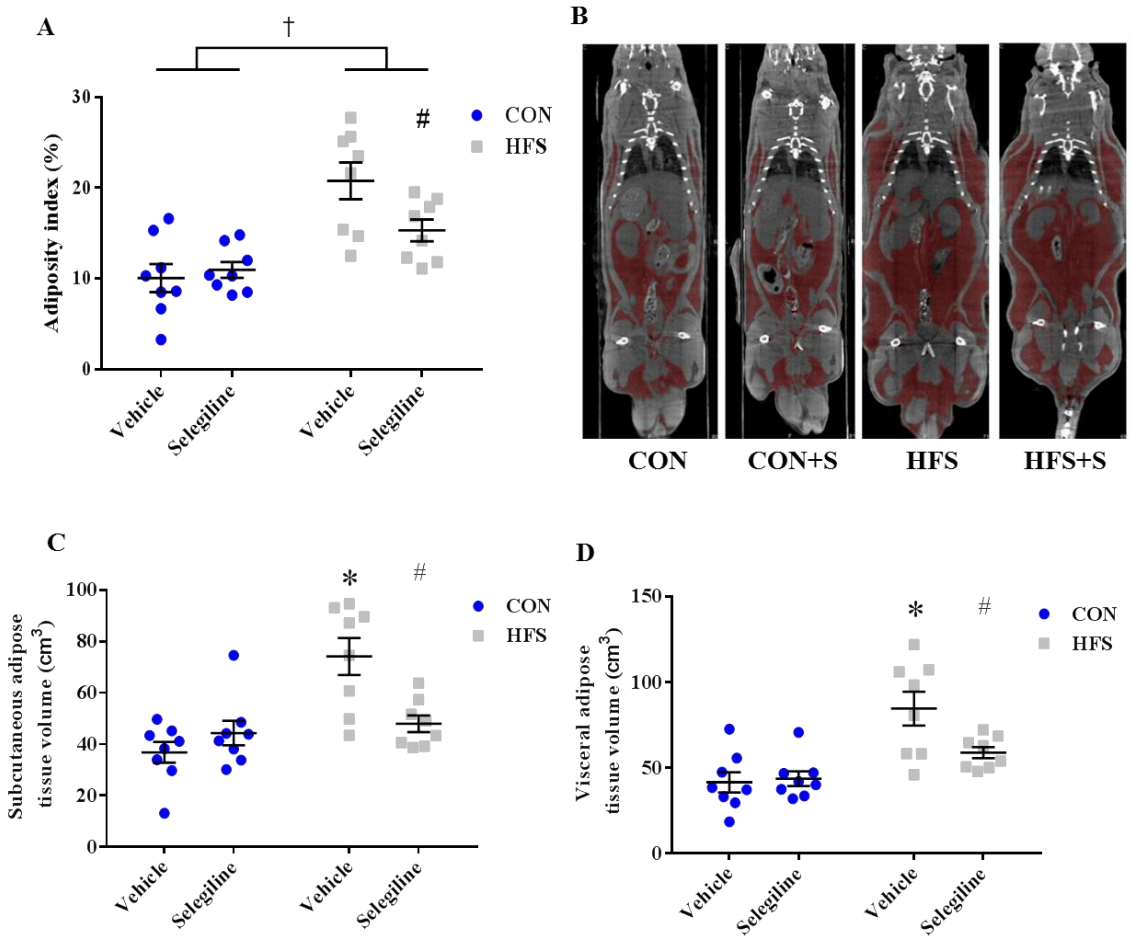

Figure 7. Selegiline reduced high-fat, high-sucrose diet-induced adiposity.

*(A) Adiposity index at week 24; (B) Representative CT scan (coronal plane). Red shows fat tissue. Visceral (C) and subcutaneous (D) adipose tissue volume. p <0.05 * vs CON, # vs HFS, † vs. control diet groups. Data are mean ± SEM, CON- control (n=8); CON+S- control+selegiline (n=8); HFS- high-fat, high-sucrose (n=8); HFS+S- high-fat, high-sucrose+selegiline (n=8).*

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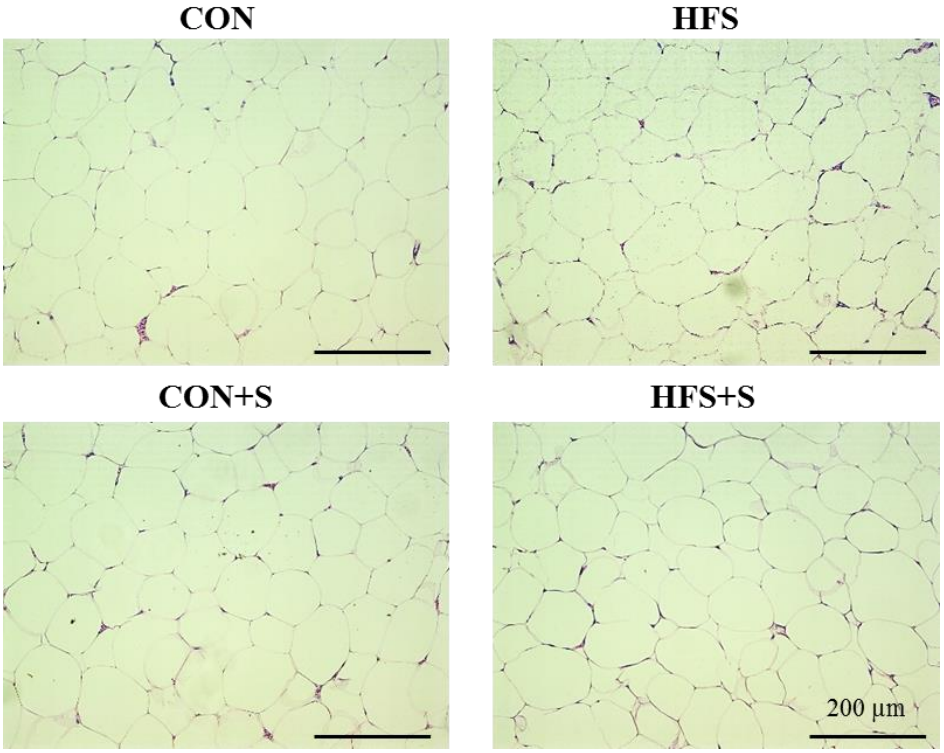

Figure 8. Adipocyte morphology.

Hematoxylin-eosin staining of epididymal adipose tissue. CON- control (n=10); CON+S- control+selegiline (n=10); HFS- high-fat, high-sucrose (n=9); HFS+S- high-fat, high-sucrose+selegiline (n=10).

Furthermore, we measured the size of adipocytes in epididymal and inguinal white adipose tissues but neither diet nor selegiline had any influence on adipocyte area (Table 7).

Table 7 Adipocyte area.

CON- control (n=10); CON+S- control+selegiline (n=10); HFS- high-fat, high-sucrose (n=9); HFS+S- high-fat, high-sucrose+selegiline (n=10).

Adipocyte area (µm^2)	**CON**	**CON+S**	**HFS**	**HFS+S**
Epididymal adipose tissue	3867.6±612.6	4310.1±424.8	4320.5±259.4	3362.7±271.8
Inguinal adipose tissue	4464.1±636.8	4673.3±614.6	3201.0±325.8	4112.3±755.2

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Weight of epididymal adipose tissue but not perirenal or brown adipose tissue was elevated by HFS diet compared to the control group. Furthermore, selegiline treatment significantly decreased epididymal adipose tissue weight in HFS diet as normalized to tibia length (Table 8).

Table 8 Organs weight normalized to tibia length.

*p <0.05 *vs CON, # vs HFS Data are mean ± SEM. CON- control (n=8); CON+S- control+selegiline (n=8); HFS- high-fat, high-sucrose (n=8); HFS+S- high-fat, high-sucrose+selegiline (n=8).*

Organs weight/ Tibia length (mg/mm)	CON	CON+S	HFS	HFS+S
Brown adipose tissue	23±1.1	21.5±2.9	25.6±3.6	21.7±2.4
Epididymal adipose tissue	230.9±27	296.9±22.6#	443.3±47.9*	347.7±21.3*,#
Heart	41.3±1.7	40.4±1.8	42.4±1.7	43.4±1.1
Kidney	89.9±4.6	77.8±12.2	93.8±6.2	85.1±12.1
Liver	445.9±26.5	478.3±32.6	505.3±56.2	496.3±23.1
Pancreas	34.1±3.4	33.9±4.1	35.3±2.4	33.5±3

We found no significant differences in organ weights, plasma leptin and lipid levels. We assessed the blood level of thyroid hormone T3 and T4, and we found no difference in their levels due to diet or selegiline treatment (Table 9). To assess effect of selegiline on lipid metabolism, we measured free fatty acid levels in the blood but we found no difference due to diet or selegiline treatment (Table 9).

To investigate whether selegiline affects liver lipid content in animals on HFS diet, we measured hepatic total cholesterol and triglyceride levels. We found that hepatic total cholesterol and triglyceride levels were significantly higher in HFS groups compared to CON groups. However, selegiline had no effect on hepatic lipid levels in our model of obesity (Table 9).

Table 9 Plasma parameters and pancreas insulin content.

**p <0.05 vs control diet groups Data are mean ± SEM. CON- control (n=8); CON+S- control+ selegiline (n=10); HFS- high-fat, high-sucrose (n=8); HFS+S- high-fat, high-sucrose+ selegiline (n=7); HDL- high-density lipoprotein, LDL- low-density lipoprotein, T4- thyroxine, T3- triiodothyronine.*

	CON	CON+S	HFS	HFS+S
Plasma triglyceride (mmol/L)	1.26±0.23	1.17±0.17	1.04±0.15	1.28±0.28
HDL cholesterol (mmol/L)	1.03±0.14	0.96±0.11	0.91±0.15	0.91±0.14
LDL cholesterol (mmol/L)	0.45±0.06	0.39±0.05	0.55±0.08	0.48±0.08
Plasma leptin (ng/L)	4.72±0.7	5.29±0.5	5.38±0.6	5.81±0.6
Plasma insulin (µIU/mL)	113.43±19.7	88.7±6.5	101.7±15.6	109.2±28.9
Pancreas insulin (µIU/mL)	341.1±45.6	567.1±109.9	597.9±123.4	572.5±118.8
Hepatic total cholesterol (mmol/mg protein)	0.02±0.002	0.02±0.001	0.03±0.005*	0.03±0.002*
Hepatic triglyceride (mmol/mg protein)	0.1±0.00	0.1±0.00	0.25±0.09*	0.22±0.03*
Free fatty acid (pmol/mL)	81.7±17.4	64.9±9.9	66.8±14.9	92.8±15.0
T3 (pg/mL)	253.0±17.3	293.3±38.0	281.3±19.9	300.6±23.0
T4 (ng/mL)	51.0±3.0	52.4±3.4	47.6±3.2	54.3±3.2

4.3 Selegiline had no effect on HFS diet-impaired glucose homeostasis

In our study, we aimed to characterize the changes in glucose homeostasis induced by chronic HFS diet. OGTT and ITT tests showed that insulin tolerance developed due to HFS diet on week 24 (Figure 9A, B, C, D), although fasting blood glucose level (Figure 9E), or plasma and pancreas insulin levels (Table 9) were not affected by diet or selegiline treatment. These results suggest that chronic HFS diet led to the development of prediabetic state.

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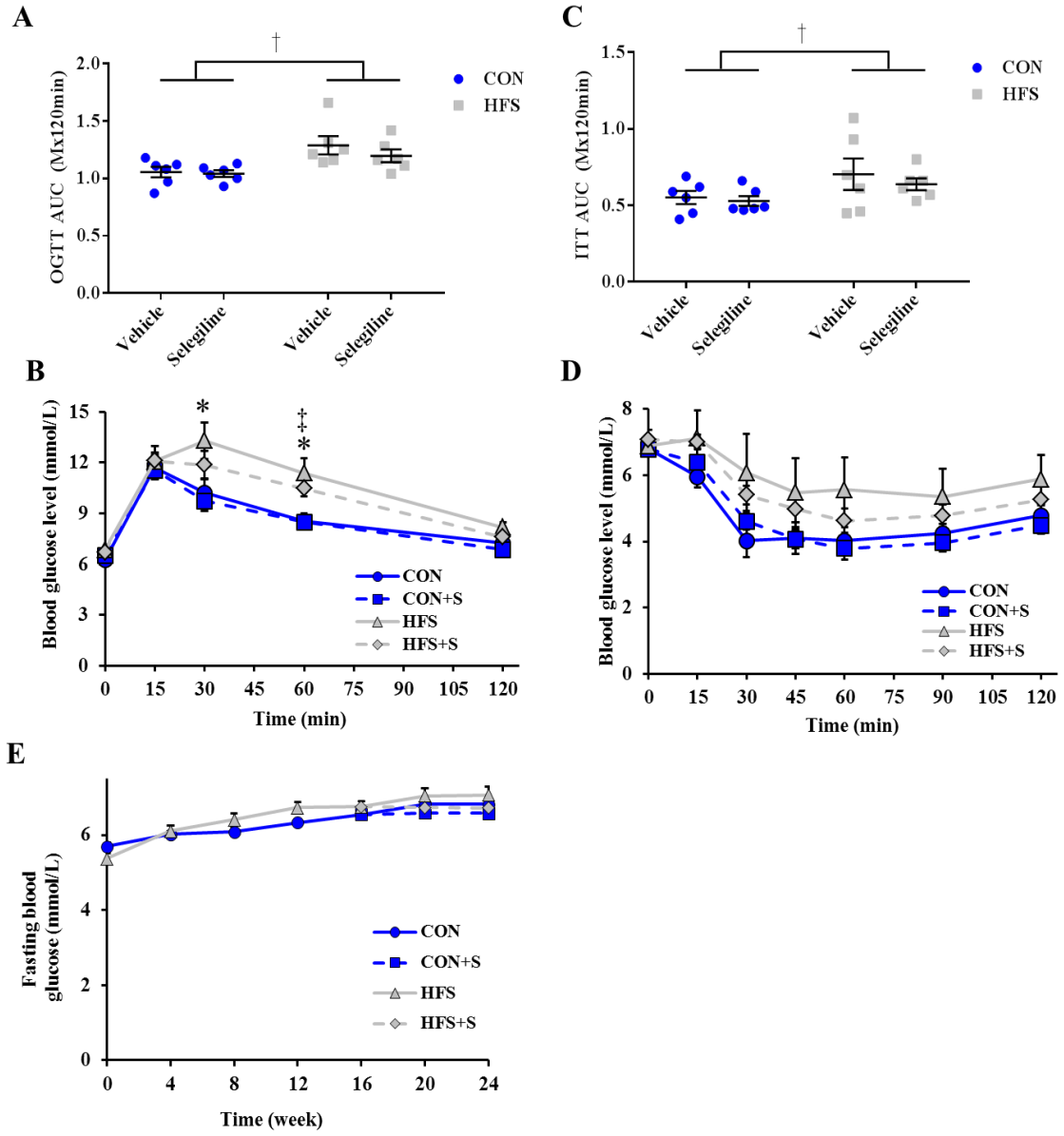

Figure 9. High-fat, high-sucrose diet impaired glucose homeostasis.

*(A) Oral glucose tolerance test (OGTT) results at week 24 and (B) Fasting blood glucose levels during OGTT (C) Insulin tolerance test (ITT) at week 24 (D) Fasting blood glucose levels during ITT (E) Fasting blood glucose levels during feeding period. A-D: n=6, E: n=9-10. Data are presented as mean ± SEM. *p <0.05 vs. CON, ‡ vs. CON+S, † vs. control diet groups. AUC- area under curve; CON- control; CON+S- control+selegiline; HFS- high-fat, high-sucrose; HFS+S- high-fat, high-sucrose+selegiline.*

4.4 Selegiline interferes with glucose uptake and lipid metabolism via modulating expression of Glut1, Srebp-1c and Ndufa1 in HFS diet

To examine the glucose uptake in visceral white adipose tissue, we measured *Glut1*, *Glut4* expression. In our study, HFS diet significantly increased gene expression of Glut1 but not of Glut4 (Figure 10). HFS diet induced the expression of *Gapdh* in adipose tissue, but selegiline did not affect the expression of *Gapdh*.

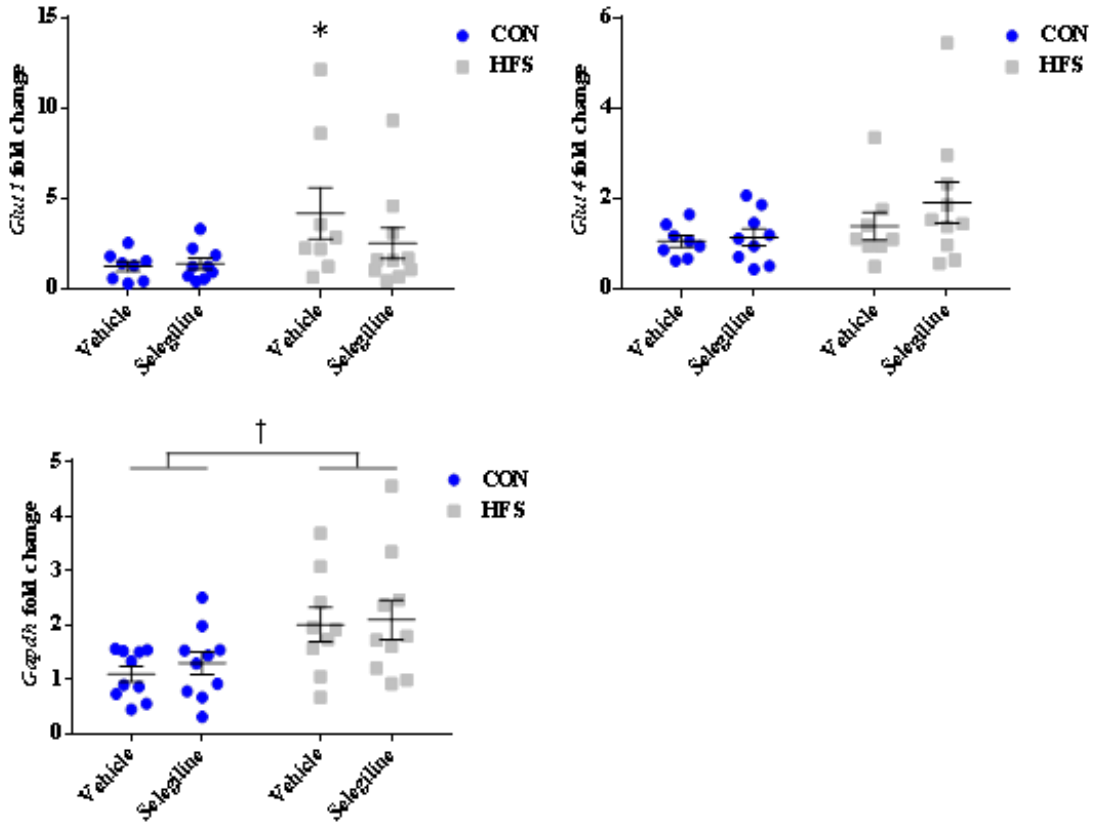

Figure 10. Gene expression of carbohydrate metabolism markers.

*Data are presented as mean ± SEM. *p <0.05 vs. CON, # vs HFS, † vs. control diet groups. CON- control (n=10); CON+S- control+selegiline (n=10); HFS- high-fat, high-sucrose (n=9); HFS+S- high-fat, high-sucrose+selegiline (n=10).*

Furthermore, we found that HFS diet induced the expression of *Dgat* in adipose tissue, but selegiline treatment had no effect on this parameter. We also found that neither diet nor selegiline had any influence on gene expression of *Acc*, *Pnpla2*, and *Cd36* in white adipose tissue. However, we found that HFS diet induced *Srebp-1c*

expression and selegiline treatment reduced *Srebp-1c* gene expression in white adipose tissue in HFS diet. Furthermore, selegiline may have prevented the HFS diet-induced elevation in *Ndufa1* in white adipose tissue (Figure 11).

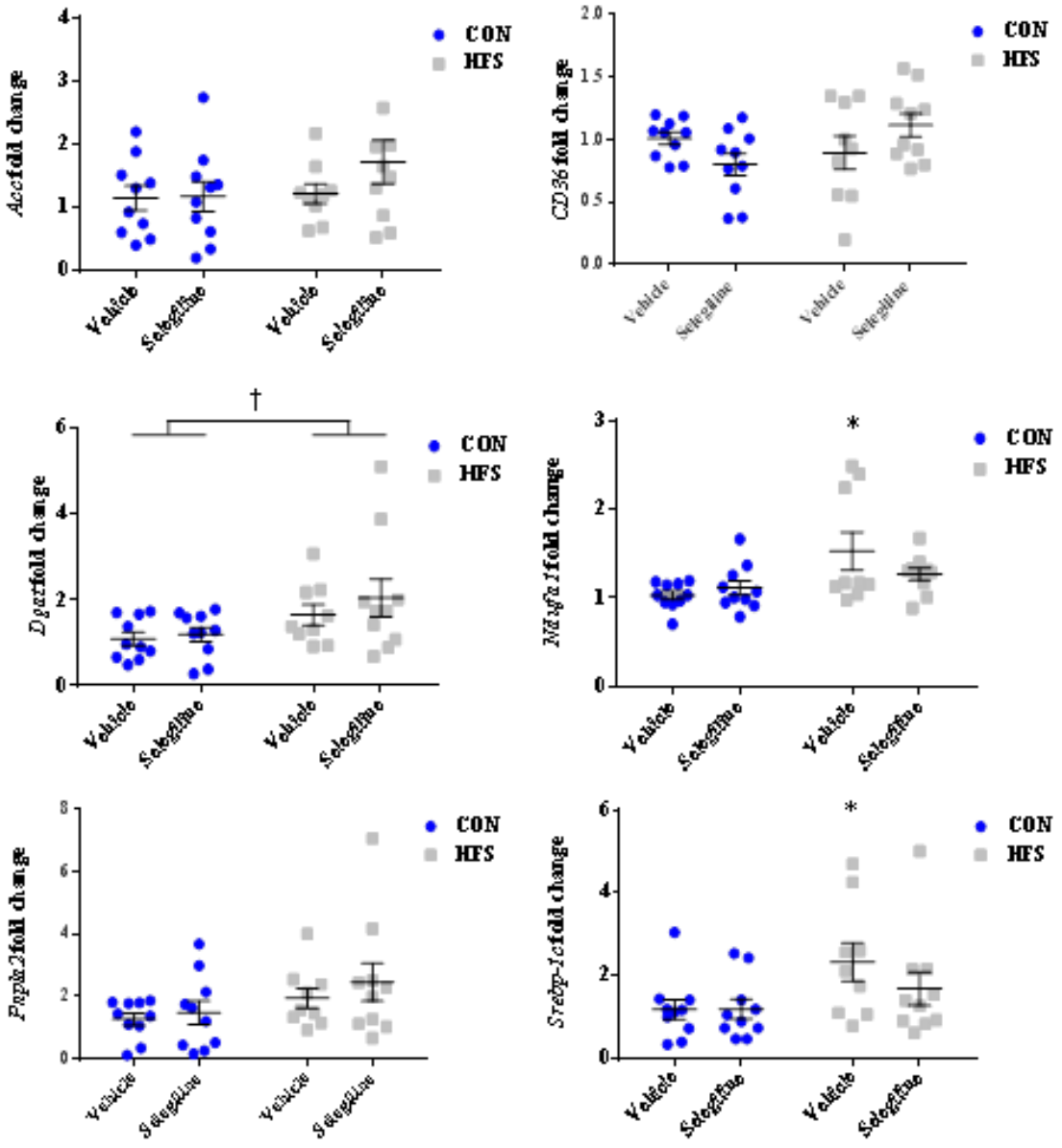

Figure 11. Gene expression of lipid metabolism markers.

*Data are presented as mean ± SEM. *p <0.05 vs. CON, # vs HFS, † vs. control diet groups. CON- control (n=10); CON+S- control+selegiline (n=10); HFS- high-fat, high-sucrose (n=9); HFS+S- high-fat, high-sucrose+selegiline (n=10).*

4.5 Selegiline reduces HFS diet-induced inflammation in white adipose tissue by modulating expression of *Ccl3*

To investigate whether selegiline has effect on inflammation in the white adipose tissue, we measured gene expression of markers of adipose tissue inflammation. We found no difference in expression of *Ccl2* (Figure 12), in the number of macrophages in the epididymal and inguinal white adipose tissue (Table 10) and in monocyte/macrophage-specific protein Iba-1 staining (Figure 13).

Table 10 Number of macrophages in adipose tissue samples.

CON- control (n=10); CON+S- control+selegiline (n=10); HFS- high-fat, high-sucrose (n=9); HFS+S- high-fat, high-sucrose+selegiline (n=10).

Number of adipocytes (cells/view field)		**CON**	**CON+S**	**HFS**	**HFS+S**
Epididymal adipose tissue	total	2.8±0.9	3.1±1.1	2.3±1.0	3.4±1.3
	activated	1.1±0.3	1.7±0.6	0.8±0.3	1.9±0.8
Inguinal adipose tissue	total	2.3±0.4	1.3±0.6	2.1±0.8	2.7±0.4
	activated	1.1±0.3	0.6±0.3	1.3±0.4	0.6±0.2

However, HFS diet induced gene expression of *Ccl3* which was reduced by selegiline treatment (Figure 12).

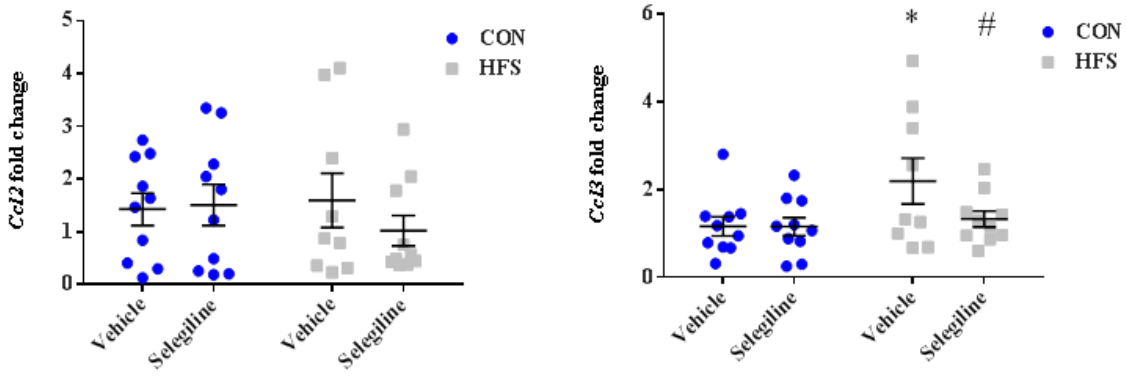

Figure 12. Selegiline reduces HFS diet-induced adipose tissue inflammation.

*Gene expression of inflammatory markers. Data are presented as mean ± SEM. p <0.05 * vs CON, # vs HFS. → 'crown-like' structures CON- control (n=10); CON+S- control+selegiline (n=10); HFS- high-fat, high-sucrose (n=9); HFS+S- high-fat, high-sucrose+selegiline (n=10).*

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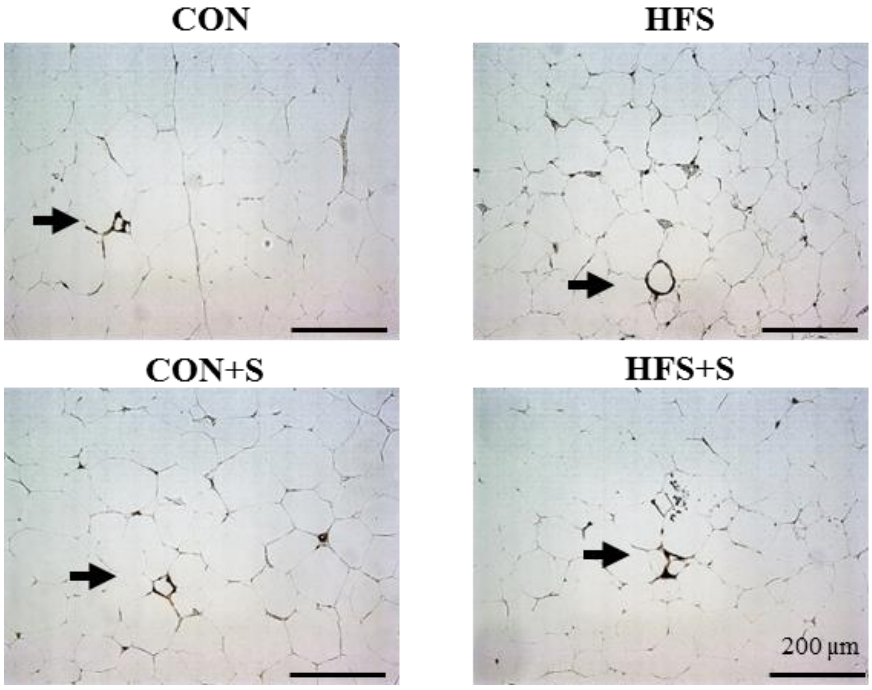

Figure 13. Immunohistochemistry of Iba1 in epididymal adipose tissue.

CON- control (n=10); CON+S- control+selegiline (n=10); HFS- high-fat, high-sucrose (n=9); HFS+S- high-fat, high-sucrose+selegiline (n=10).

4.6 Selegiline decreased arterial systolic pressure in control diet

To examine whether HFS diet or selegiline influences hemodynamic parameters, we measured heart rate, arterial systolic and diastolic pressures. We found that selegiline significantly decreased systolic pressure in rats fed control diet, however this effect was abolished upon HFS diet feeding (Table 11).

Table 11 Hemodynamic parameters.

***p <0.05 vs CON Data are mean ± SEM. CON- control (n=6); CON+S- control+selegiline (n=5); HFS- high-fat, high-sucrose (n=5); HFS+S- high-fat, high-sucrose+selegiline (n=5).*

	CON	CON+S	HFS	HFS+S
Heart rate (bpm)	408±8	387±13	412±14	393±13
Arterial systolic pressure (mmHg)	174±2	146±11*	175±8	159±8
Arterial diastolic pressure (mmHg)	138±2	119±8	135±6	130±7

4.7 HFS diet and selegiline did not induce behavioural alterations or sensory neuropathy

Since selegiline improves cognitive functions in aged animals (Brandeis, Sapir et al. 1991) and since high-fat diet has been shown to induce cognitive decline (Noble, Mavanji et al. 2014), we aimed to assess the effect of selegiline on cognition in our model. To investigate the movement pattern and recognition memory, we performed spontaneous motor activity test and novel object recognition test at the end of our study. Neither HFS diet nor selegiline treatment significantly affected the motor activity (Table 12).

Table 12 Measurement of spontaneous locomotor activity.

Data are mean ± SEM CON- control (n=10); CON+S- control+selegiline (n=10); HFS- high-fat, high-sucrose (n=9); HFS+S- high-fat, high-sucrose+selegiline (n=10).

Time (min)	CON	CON+S	HFS	HFS+S
Ambulation	3.6±0.3	3.9±0.3	3.2±0.2	3.4±0.3
Local movement	9.3±0.2	8.7±0.3	9.2±0.2	9.4±0.3
Immobility	14.6±0.6	13.8±0.6	14.5±0.4	14.7±0.4
Rearing	2.5±0.3	3.5±0.3	3.1±0.3	2.5±0.2

Furthermore, all groups showed a moderate level of recognition memory: rats explored the novel object for 60-80% longer than the familiar one. Neither selegiline treatment nor HFS diet caused statistically significant alterations in the performance of the animals (Table 13).

Table 13. Novel object recognition assay.

Data are mean ± SEM. $t2_{novel}$- exploration time of novel object; $t2_{familiar}$- exploration time of familiar object; CON- control (n=7); CON+S- control+selegiline (n=9); HFS- high-fat, high-sucrose (n=8); HFS+S- high-fat, high-sucrose+selegiline (n=8).

	CON	CON+S	HFS	HFS+S
T2 novel (sec)	16.9±3.5	13.5±2.3	11.0±1.5	12.0±1.2
T2 familiar (sec)	10.0±2.1	7.8±0.9	6.1±0.8	6.5±0.7
Discrimination index	0.2±0.1	0.2±0.1	0.3±0.1	0.3±0.1

For detecting the presence of neuropathy and neuropathic pain in HFS diet we performed two nociceptive tests. For testing pressure hyperalgesia, we performed Randall-Selitto paw pressure test and for testing mechanical allodynia we used DPA. Results showed no difference in pain thresholds between groups neither for DPA nor Randall-Selitto experiments (Table 14). These results indicate that disturbed glucose homeostasis did not cause neuropathy.

Table 14. Assessment of neuropathy.

Data are mean ± SEM. CON- control (n=10); CON+S- control+selegiline (n=10); HFS- high-fat, high-sucrose (n=9); HFS+S- high-fat, high-sucrose+selegiline (n=10).

	CON	**CON+S**	**HFS**	**HFS+S**
Pressure sensitivity threshold (g)	195±5	207.5±5	201.9±12	207.8±10
Touch sensitivity threshold (g)	47.4±2	47.8±1	47.9±2	50±0

4.8 CAP induces autophagy in ex vivo perfused hearts but not in NRCMs and H9c2 cells

In a pilot study we aimed to compare the effect of CAP on autophagy in neonatal cardiomyocytes, in H9c2 cells and in ex vivo-treated hearts. Therefore, we used the most common autophagy marker LC3 to measure changes in autophagy. In isolated rat neonatal cardiomyocytes and in H9c2 cells there was no significant difference in LC3-II to I ratio between corresponding CON and CAP groups (Figure 14A, B). Since CAP failed to induce autophagy in NRCMs and H9c2 cells, in our further experiments we used the isolated rat heart model. In the left atrium of hearts perfused with CAP, the ratio of LC3-II to LC3-I was significantly increased after 35 min compared to CON samples indicating that CAP induced cardiac autophagy (Figure 14C). We also tested the effect of CAP and TAT-HA-Atg5^{K130R} on autophagy-related protein LC3 levels in whole heart tissue (Figure 14D).

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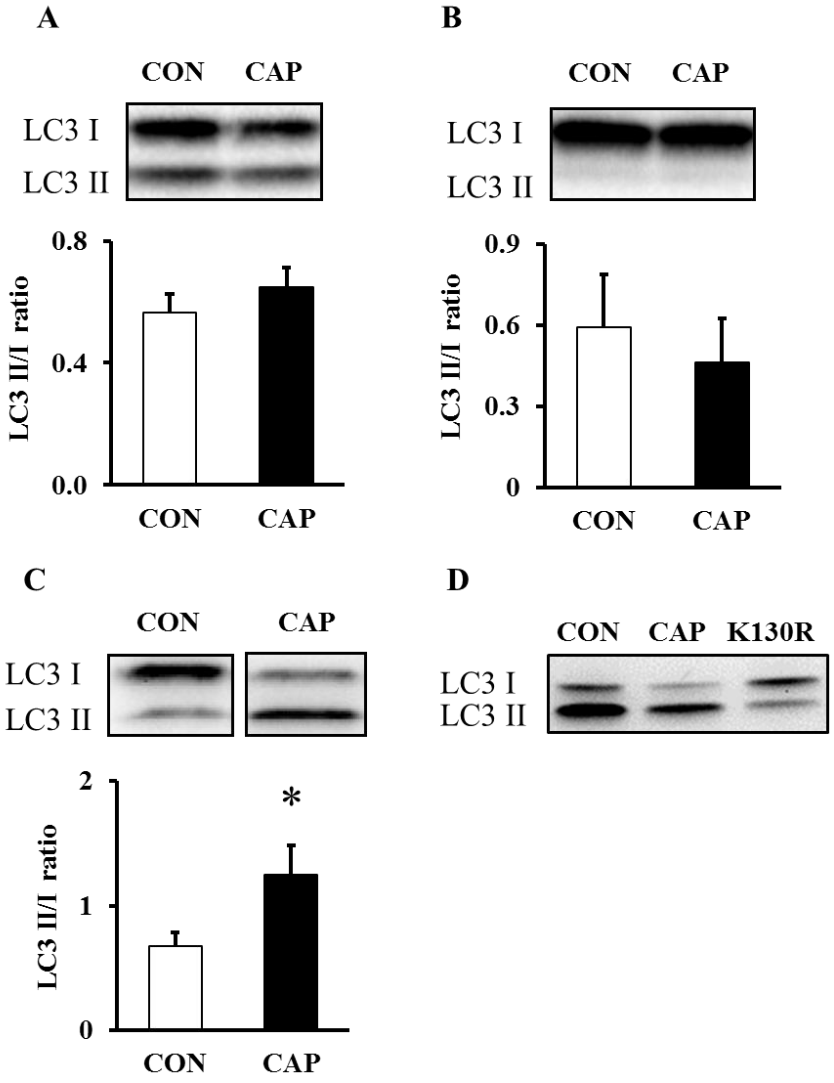

Figure 14. The effect of CAP on autophagy-related protein LC3 levels in (A) NRCMs (n=8) (B) in H9c2 cells (n=8) and (C) ex vivo heart lysates (n=6) *p < 0.05 vs. CON (D) Pilot study: effect of CAP and TAT-HA-Atg5^{K130R} on autophagy-related protein LC3 levels in whole heart (n=2).

Data are presented as means ± SEM. Chloramphenicol - CAP, Control - CON, Microtubule-associated protein 1A/1B-light chain 3 - LC3, Neonatal rat cardiac myocytes – NRCMs.

The discrepancy might be attributed to the difference in metabolic status of cultured cells and the ex vivo perfused hearts. Since insulin, fatty acids and other factors are present in cell culture medium due to the use of fetal calf serum, and since these factors are not included in the heart perfusion buffer, signaling pathways related to

autophagy might be differentially modulated by CAP. Furthermore, the presence of cell types other than cardiomyocytes (endothelial cells, fibroblasts) in the heart might also contribute to the cardioprotective effects of CAP (see for a recent review: (Hausenloy, Garcia-Dorado et al. 2017)).

4.9 TAT-HA-Atg5^{K130R} blocks CAP-induced cardioprotection in isolated hearts

In order to assess whether the sequestration and/or degradation phases of autophagy are necessary for the cardioprotective effect of CAP, we used ex vivo Langendorff heart perfusion experiments. CAP treatment significantly reduced infarct size and CK release as compared to CON hearts. Pretreatment with TAT-HA-Atg5^{K130R} abolished the infarct size limiting effect of CAP, while pretreatment with CQ did not interfere with CAP-induced cardioprotection (Figure 15A, B).

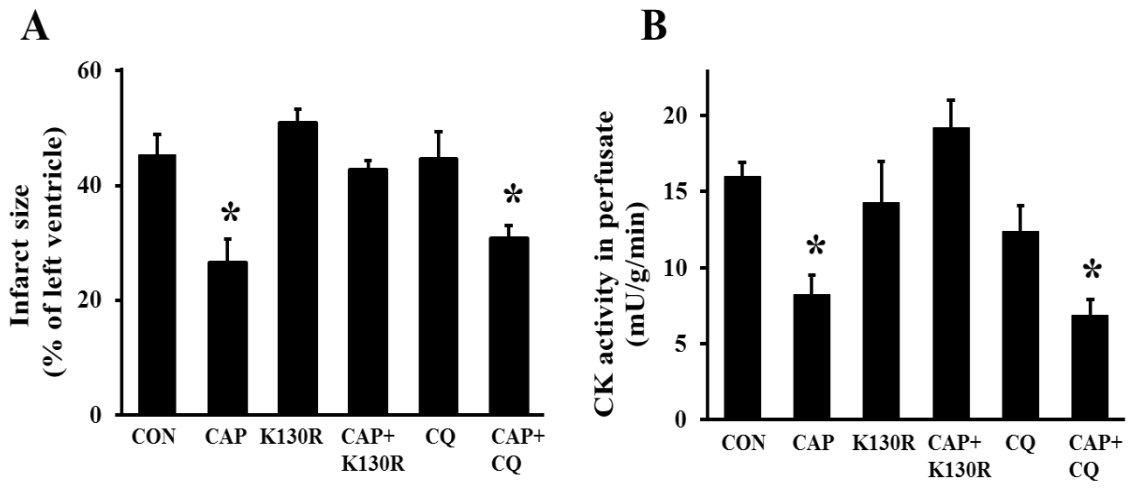

Figure 15. (A) Inhibition of autophagosome formation by TAT-HA-Atg5^{K130R} abolishes CAP-induced cardioprotection (B) Inhibition of autophagosome formation (K130R) abolishes the CAP induced CK activity reduction in perfused rat hearts.

*n=3–8 *p < 0.05 vs. CON Data are presented as means ±SEM. CAP- Chloramphenicol; CQ- Chloroquine; CON- Control; K130R- TAT-HA-Atg5^{K130R}; CK- Creatine kinase.*

We also measured LC3 expression in isolated heart samples and the western blot results showed that CAP increased LC3-II/I ratio. Meanwhile, administration of TAT-

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HA-Atg5^{K130R} but not CQ reduced the increase of LC3-II due to CAP administration (Figure 16).

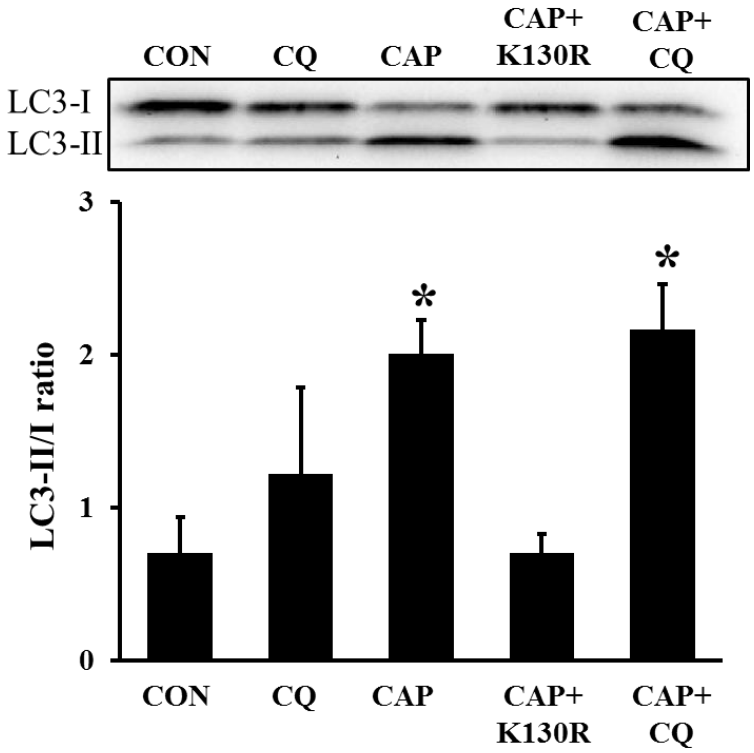

Figure 16. The effect of CAP on autophagy-related protein LC3 levels in isolated hearts.

*n=3 *p < 0.05 vs. CON Data are presented as means ±SEM. CAP- Chloramphenicol; CQ-Chloroquine; CON- Control; K130R- TAT-HA-Atg5^{K130R}.*

Since cardioprotection, as assessed by infarct size and autophagy, as assessed by LC3-II to LC3-I ratio, was lower in CAP +K130R group than in CAP group, these results indicate that CAP-induced cardioprotection requires the process of autophagy sequestrationbut not autophagosomal clearance. To characterize cardioprotective signaling mechanisms modulated by CAP, we assessed activation of proteins involved in the RISK/SAFE pathway. We found that the phosphorylation of Erk1/2 but not of Akt increased significantly due to CAP treatment (Figure 17).

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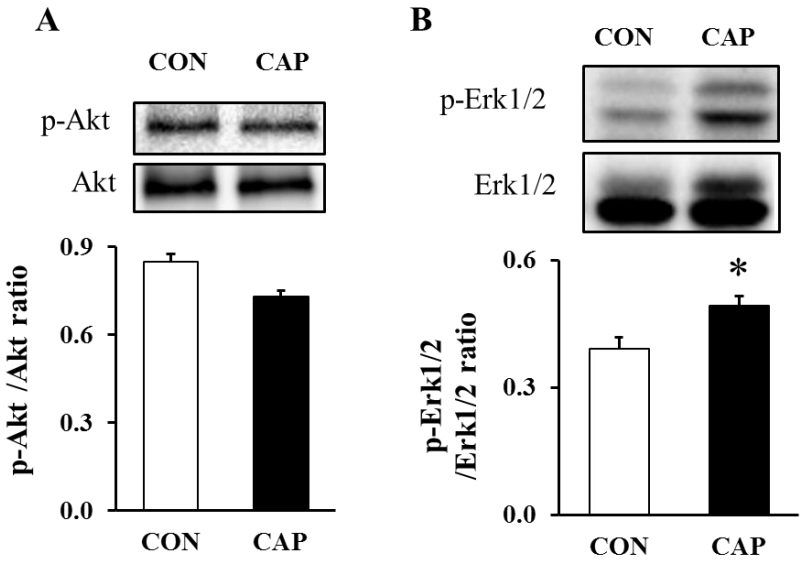

Figure 17. Assessment of cardioprotective pathways from ex vivo whole heart lysates.

(A) Ratio of p-Akt to Akt signals (B) ratio of p-Erk1/2 to Erk1/2. n =5. CAP- Chloramphenicol; CON- Control.

Coronary flow was significantly lower at the beginning of reperfusion (4–5 min) in CAP, CQ and CAP +CQ groups compared to CON group. Prior to ischemia (14–20 min) the coronary flow was lower in CQ and CAP +CQ. After the initiation of reperfusion (65–79 min) the coronary flow was decreased in K130R and CQ groups. At the end of the reperfusion time (169–185 min) the coronary flow was lower in CAP + CQ group compared to CON (Table 15).

Table 15. Measurement of coronary flow in ex vivo perfused hearts.

**p < 0.05, vs. CON n=3–8 data are presented as means ± SEM. CAP- Chloramphenicol; CQ- Chloroquine; CON- Control; K130R- TAT-HA-Atg5^{K130R}.*

Groups	Perfusion time (min)			
	4-5	14-20	65-79	169-185
CON	14±0.6	14±0.9	8.3±0.7	6.7±0.6
CAP	12±0.5*****	10±1.1	9.8±0.7	6.6±0.4
K130R	14.3±0.9	9.5±2.5	5.7±0.9*****	5.7±0.7
CAP+K130R	14.9±0.6	10.6±0.3	7.8±0.4	5.3±0.2
CQ	11±0.3*****	9.2±0.4*****	5±1.1*****	5.7±2.2
CAP+CQ	10.7±1.0*****	8.5±0.7*****	7.8±0.7	4±0.4*****

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5 Discussion

5. 1 Selegiline moderates adiposity induced by HFS diet

5.1.1 Selegiline reduced subcutaneous and visceral fat depots

In this thesis, we demonstrated that selegiline, an irreversible MAO-B inhibitor, significantly decreased adiposity but not body weight in HFS diet by reducing visceral and subcutaneous fat depositions. The effect of amine oxidases inhibition has been previously investigated in models of metabolic disorders, which presented somewhat divergent results. Carpéné *et al.* demonstrated that combined administration of semicarbazide (300 μmol kg^{-1} day), an inhibitor of semicarbazide-sensitive amine oxidase, and of another non-selective MAO-B inhibitor, pargyline (10 mg kg^{-1} day) to young male obese Zucker rats (Carpene, Iffiu-Soltesz et al. 2007) or non-obese Wistar rats (Carpene, Abello et al. 2008) significantly reduced body weight gain and fat deposition by reducing energy intake. Furthermore, pargyline alone was shown to reduce body weight to a minor extent in obese Zucker rats (Carpene, Iffiu-Soltesz et al. 2007), and in control rats (Mattila and Torsti 1966) at doses of 10 and 30 mg kg^{-1} day, respectively. These findings are partly in contrast with our current findings, since although we found that selegiline at human-equivalent therapeutic doses reduced HFS diet-induced adiposity, it neither reduced body weight in control rats, nor affected caloric- or food intake. These discrepancies might be attributed to the different models of metabolic disorders, to the different doses and type of MAO-B inhibitors used, or to the combined inhibition of amine oxidases in the previous study.

Moreover, here we found that selegiline treatment did not cause significant weight gain either in control or HFS diet and that selegiline treatment significantly decreased HFS diet-induced subcutaneous and visceral adiposity. These findings highlight the advantage of selegiline as an antidepressant medication, since first-line antidepressants are known to induce weight gain as a side effect (Abosi, Lopes et al. 2018), whereas, our results show that selegiline is devoid of such effect, moreover, it may reduce body fat content.

Therefore, MAO-B inhibition by therapeutic doses of selegiline in the present study has beneficial effect only in obesity by reducing adiposity. The effect of selegiline on adiposity, especially on visceral adiposity, is important, since visceral adiposity is a major risk factor for cardiovascular disease and for diabetes (Gonzalez, Moreno-Villegas et al. 2017).

5.1.2 Hypothesized mechanisms of the adiposity-lowering effect of selegiline

We also found that selegiline may have affected energy metabolism in the adipose tissue. The exact mechanism behind the adiposity-lowering effect of MAO-B inhibitors is unclear. Here we found that HFS diet significantly increased gene expression of *Glut1* but not of *Glut4.* Furthermore, selegiline treatment slightly, but not significantly reduced gene expression of *Glut1* in HFS diet. A previous study showed that supra-micromolar concentration of selegiline prevented the stimulation of glucose uptake induced by serotonin in cardiomyocytes, indicating a role of MAO in this process (Fischer, Thomas et al. 1995). This data indicate that selegiline might modulate insulin-independent glucose uptake in the adipose tissue. Furthermore, we found that HFS diet induced the expression of *Dgat* and *Gapdh* in adipose tissue, but selegiline treatment had no effect on these parameters. We also found that neither diet nor selegiline had any influence on gene expression of *Acc*, *Pnpla2*, and *Cd36* in white adipose tissue. However, we found that HFS diet induced *Srebp-1c* expression and selegiline treatment tended to reduce *Srebp-1c* gene expression in white adipose tissue in HFS diet, suggesting that selegiline might modulate certain aspects of lipid metabolism in the adipose tissue. Effects of MAO-B inhibition on metabolism have been previously studied. For example, pargyline reduced lipoprotein lipase activity in adipose tissue (Mattila and Torsti 1966). Elsewhere, phenelzine, an antidepressant with a potent MAO- and SSAO-inhibitory activity, prevented cell triglyceride accumulation and adipose conversion, and also reduced expression of several key adipogenic transcription factors, such *Srebp-1c* (Chiche, Le Guillou et al. 2009), which is in line with our current findings.

We also assessed parameters of metabolism from the circulation. In our study, selegiline treatment did not affect plasma leptin, free fatty acid level or lipid levels both in control and in HFS animals. These results conform with data reported by Carpéné *et al.* where treatment with pargyline did not alter lipid metabolism in obese rats (Carpene, Iffiu-Soltesz et al. 2007, Carpene, Abello et al. 2008), however, elsewhere, an increase in lipolytic rate of adipose tissue and elevation of plasma free-fatty acids were reported in non-obese rats treated with pargyline, or non-selective MAO inhibitors iproniazid or pheniprazine (Mattila and Torsti 1966).

We also investigated the effect of selegiline on hormonal regulation of metabolism. Here we found that selegiline had no effect on the level of T3 or T4 either in control or obese rats which is in line with a previous publication where MAO-B inhibition did not modify thyroid hormones in lean rats (Cabanillas, Masini-Repiso et al. 1994).

In summary, these results indicate that selegiline may influence glucose and lipid metabolism of visceral white adipose tissue via non-hormonal regulation, by modulating expression of *Glut1*, *Srebp-1c* and *Ndufa1*. However, measurement of further parameters might be necessary to describe the exact mechanism.

5.1.3 Selegiline alleviates WAT inflammation induced by HFS diet

Browning of white adipose tissue indicates a metabolic activation. We hypothesized that selegiline may influence this phenomenon, however, selegiline treatment seems not to affect the browning process per se. Adipose tissue inflammation is a severe consequence of obesity. MAO-A was recently proposed to play a role regulating alternative macrophage activation via the IL4/IL13 signaling (Cathcart and Bhattacharjee 2014). Moreover, several studies have shown that macrophages are capable of catecholamine synthesis, which play essential role in lipolysis in the adipose tissue (Nedergaard, Bengtsson et al. 2011). Nguyen *et al.* found that macrophages secrete catecholamines to induce lipolytic/thermogenic gene expression changes in brown and white adipose tissues (Nguyen, Qiu et al. 2011). In contrast with these findings, a recently published study proved that adipose tissue macrophages do not

participate in catecholamine synthesis (Fischer, Ruiz et al. 2017). Nevertheless, a recently published articles showed that NE is transported into neuron-associated macrophages and degraded by the activity of MAO-A (Pirzgalska, Seixas et al. 2017), thus proposing MAO inhibition as a tool to promote lipolysis. Although we have growing evidence that MAO-A activity may have important role in inflammatory macrophages, there is no information on the role of MAO-B in macrophages as well as in adipocytes. Here we found no difference in expression of Ccl2 and in the number of macrophages in the epididymal and inguinal white adipose tissue, however, HFS diet induced gene expression of Ccl3 which was reduced by selegiline treatment.

Therefore, inflammation of white adipose tissue might be limited by selegiline, however, measurement of other parameters e.g. cytokines might be necessary to further elaborate on this aspect.

5.1.4 Selegiline had no influence on HFS diet-induced prediabetes

Previously it has been shown that MAOs can be found in both the exocrine and the endocrine parts of the pancreas of different mammalian species (Feldman and Chapman 1975, Feldman, Castleberry et al. 1983, Adeghate and Donath 1990). In 1975, Aleyassine *et al.* demonstrated that MAO inhibitors are able to increase as well as inhibit insulin release in vitro, in a dose dependent manner (Aleyassine and Gardiner 1975), however, their effect on carbohydrate metabolism was not assessed by then.

In this study, insulin resistance developed in rats due the HFS diet, but neither plasma nor pancreas insulin levels nor insulin resistance were affected by selegiline treatment. These results suggest that chronic HFS diet-induced prediabetes is not alleviated by MAO-B inhibition. In contrast to our results, previous articles demonstrated that a high dose of pargyline, reduced blood glucose level in obese and non-obese rats (Mattila and Torsti 1966, Carpene, Iffiu-Soltesz et al. 2007). Similarly, several studies have reported that competitive inhibition of MAO by high doses of benzylamine or tyramine might also alleviate glucose tolerance in animal models of Type 1 (Marti, Abella et al. 2001) or Type 2 diabetes (Visentin, Marq et al. 2003).

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Although several publications report that although MAO inhibition might have beneficial effects on glucose homeostasis in metabolic derangements, therapeutic and safe dose of selegiline might not alleviate glucose tolerance or insulin resistance in diet-induced obesity.

5.1.5 Behavioural alterations or sensory neuropathy was not observed due to HFS diet or selegiline

Neither selegiline treatment nor HFS diet-induced moderate obesity had significant effects on motor activity or recognition memory. Although, previous articles showed that extreme obesity induce cognitive dysfunction (Wang, Huang et al. 2016, Zanini, Arbo et al. 2017) these changes could not be observed in our model of moderate obesity.

The results of mechano-nociceptive tests indicate that HFS diet did not induce peripheral prediabetic neuropathy, and that selegiline did not have any effect on the pain threshold either in HFS diet or in control diet. However, previous articles showed that metabolic syndrome, including prediabetes can induce peripheral prediabetic neuropathy before progression to clinical Type 2 diabetes (Novella, Inzucchi et al. 2001, Sumner, Sheth et al. 2003). Relatively little is known about the effect of MAO-B inhibitors on pain or analgesia. Hozumi *et al.* investigated the relationship between obesity and neuropathic pain, and they found that obesity and moderate overweight can negatively affect neuropathic pain intensity and nerve damage, however, they could not find the pathophysiological mechanisms behind these findings (Hozumi, Sumitani et al. 2016). In this thesis, we showed that moderate obesity induced by HFS diet does not influence sensory functions in rats.

5.2 The process of autophagosome formation is necessary for the infarct size limiting effect of CAP

5.2.1 CAP induces cardioprotection via autophagy in ex vivo hearts

Here we demonstrate that CAP significantly increased the LC3-II/I ratio in ex vivo hearts which indicates the induction of autophagy. Furthermore, CAP treatment reduced infarct size and creatine kinase activity in isolated rat hearts. These results are in agreement with those of Granville *et al.* (Granville, Tashakkor et al. 2004) who showed that CAP reduced infarct size in an open chest rabbit model of regional ischemia. Moreover, similar results were obtained in a previous study with a more water soluble derivative of CAP, chloramphenicol succinate (CAPS), in a swine model of myocardial ischemia reperfusion injury by Sala-Mercado *et al.* (Sala-Mercado, Wider et al. 2010). They found that CAPS, administered as a pre-treatment or before reperfusion, rendered the porcine heart resistant to ischemia-reperfusion injury, however, they did not investigate which stage of autophagy is necessary for cardioprotection. Interestingly, in our present ex vivo rat heart experiment, CAPS did not reduce infarct size (data not shown). Since CAPS is a prodrug activated by esterases in the blood (Ambrose 1984) which are not present in isolated heart perfusion, our data suggests that in vivo metabolism of CAPS into CAP plays a significant role in its cardioprotective effect. In 2014 Shiomi *et al.* (Shiomi, Miyamae et al. 2014) demonstrated that in isolated guinea pig hearts sevoflurane reduced infarct size after 30 min, but not 45 min of cardiac ischemia. In this experiment, CAP pretreatment extended the time of ischemia after which sevoflurane preconditioning reduced infarct size.

These results well demonstrate that CAP or its derivatives are able to induce or facilitate cardioprotection and that autophagy play a significant role in these effects.

5.2.2 Inhibition of autophagosome formation abolish the infarct size limiting effect of CAP

We further investigated whether CAP exerts cardioprotection via autophagy. Therefore, in the present study we determined whether autophagy is required for CAP-

induced cardioprotection and which stage of autophagy is essential for the cardioprotective effect of pharmacologically induced autophagy. We used TAT-HA-Atg5^{K130R} to inhibit autophagosome formation and CQ to investigate the role of lysosomal degradation. Atg5 is not known to participate in other pathways besides autophagy, and therefore TAT-HA-Atg5^{K130R} is the most specific inhibitor available.

In this thesis, we showed that pretreatment with an inhibitor of autophagosome formation (TAT-HA-Atg5^{K130R}, a functional null-mutant protein) abolished the infarct size limiting effect of CAP, while pretreatment with an inhibitor of autophagosome-lysosome fusion (CQ, a blocker of autophagosomal clearance) did not interfere with CAP-induced cardioprotection. Similarly, Shiomi *et al*. found that a non-specific autophagy inhibitor, 3-methyladenine, abolished the CAP-induced restoration of sevoflurane preconditioning after prolonged ischemic insult (Shiomi, Miyamae et al. 2014).

These results clearly show that the sequestration phase of autophagy is essential for CAP-induced cardioprotection against acute ischemia/reperfusion injury (Figure 18).

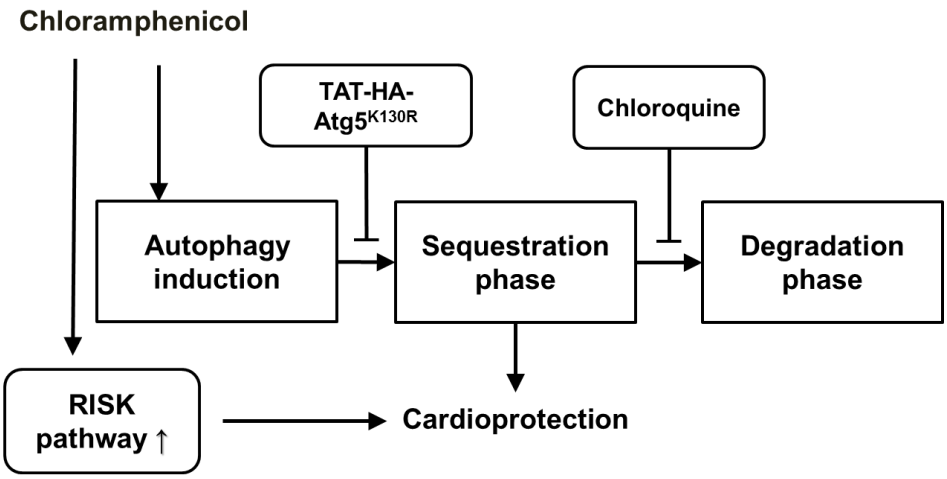

Figure 18. Chloramphenicol-induced cardioprotection requires the sequestration phase of autophagy, not the degradation phase.

The role of autophagosomal clearance in cardioprotection has been investigated in several models. Guo *et al.* (Guo, Xu et al. 2015) showed that 1 h after cardiac ischemia LC3-II/I ratio was significantly elevated, and that ischemic postconditioning limited the elevation in the LC3-II/I ratio evidencing that cardioprotective interventions

might inhibit cardiac autophagy. However, the same study indicated that inhibition of autophagosomal clearance by CQ abolished cardioprotection by ischemic postconditioning, which suggests that inhibition of cardiac autophagy might be controversial in ischemic conditioning. Similarly, Wang *et al.* investigated the mechanisms underlying resveratrol-mediated cardioprotection, and found that chronic inhibition of autophagic clearance with bafilomycin A1 abrogated resveratrol-mediated cardiac protection in diabetic mice (Wang, Yang et al. 2014). On the contrary, in our present study, acute treatment with CQ did not abolish CAP-induced cardioprotection in isolated healthy hearts.

These findings suggest that although in vivo inhibition of autophagic clearance interferes with several types of cardioprotective stimuli, autophagic clearance is not likely to be necessary for cardioprotection triggered by pharmacologically-induced autophagy in an ex vivo setting, the basis of which is unclear.

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6 Conclusions

6.1 Selegiline moderates HFS diet-induced adiposity

This is the first demonstration that selegiline reduces adiposity, modulates adipose tissue energy metabolism and alleviates adipose inflammation induced by HFS diet. Here we have shown that HFS-induced expression of *Srebp-1c*, *Glut1*, and *Ccl3* in adipose tissue was alleviated by selegiline treatment. However, in our experiment selegiline treatment did not affect the increase in body weight, prevent impairment of glucose homeostasis, or affect behaviour. Our results demonstrate that selegiline may influence glucose and lipid metabolism of white adipose tissue and may also alleviate inflammation in white adipose tissue.

These results suggest that specific inhibition of MAO-B by selegiline may mitigate harmful effects of obesity, and reduce the risk of cardiovascular diseases, thereby selegiline may serve as an adjuvant to anti-obesity therapy.

6.2 CAP reduces infarct size via induction of autophagy sequestration

Here we have shown for the first time in the literature that the sequestration, but not the clearance phase of autophagy is necessary for the cardioprotective effect of CAP. In our study, CAP administration reduced myocardial infarction in ex vivo rat hearts. Due to CAP treatment an increase in the LC3-II/I ratio and in the phosphorylation of Erk1/2 could be observed in the heart. The CAP-induced cardioprotection was abolished with an inhibitor of autophagosome formation.

Therefore, therapeutic tools developed on the basis of induction of autophagic sequestration might lead to novel therapeutic options against acute myocardial ischemia/reperfusion injury.

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7 Summary

Obesity and cardiovascular diseases are the leading causes of death worldwide. However, despite numerous experimental and clinical trials in this field, there is still no safe and effective form of pharmacotherapy for these diseases.

Therefore, our aim was to investigate novel approaches to alleviate cardiac effects of metabolic derangements. We assessed the effect of selective MAO-B inhibition on energy metabolism in HFS diet-induced moderate obesity model. We also aimed to clarify the role of autophagy in CAP-induced cardioprotection and to investigate that sequestration and/or degradation phases of autophagy are necessary to induce cardioprotection.

The MAO-B inhibition by selegiline decreased whole body fat, subcutaneous- and visceral adiposity induced by HFS diet. Furthermore, selegiline treatment modulated HFS diet-induced expression of lipid and carbohydrate metabolism markers, such as *Srebp-1c*, *Glut1* and inflammatory marker, such as *Ccl3* in adipose tissue. The oral glucose tolerance and insulin tolerance tests showed impaired glucose homeostasis in HFS diet groups. We found that selegiline decreased systolic pressure in rats fed control diet, however, this effect was abolished upon HFS diet feeding. We also demonstrated that CAP induces cardioprotection via increasing cardiac autophagy and phosphorylation of Erk1/2 in the myocardium. Furthermore, infarct size and CK release were significantly decreased due to CAP treatment. The pretreatment with TAT-HA-Atg5^{K130R} abolished cardioprotection by CAP, while in CAP+ CQ hearts infarct size and CK release were reduced similarly to as seen in the CAP-treated group, showing that autophagosome formation is necessary for the CAP mediated cardioprotection.

Our results help us to get a more comprehensive picture of the molecular changes in mild obesity and the effect of MAO-B inhibition on these changes. Furthermore, based on the roles of phases of autophagy in triggering cardioprotection, it may be possible to develop more targeted therapies for treating acute ischemia/reperfusion injury.

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8 Összefoglalás

Az elhízás és a kardiovaszkuláris megbetegedések világszerte a vezető halálokok közé tartoznak. Azonban, a számos kísérletes és klinikai vizsgálat ellenére, jelenleg sincs még biztonságos és hatékony kezelés ezeknek a betegségeknek a kezelésére.

Ezért célunk az volt, hogy új farmakológiás lehetőségeket vizsgáljunk meg a metabolikus elváltozások szívhatásainak kiküszöbölésére. Vizsgáltuk a szelektív MAO-B gátlás hatását az energia metabolizmusra a magas zsír- és cukor tartalmú étrend által kiváltott enyhe elhízásos modellben. Továbbá, célul tűztük ki az autofágia szerepének tisztázását a klóramfenikol indukálta kardioprotekcióban. Megvizsgáltuk, hogy az autofágia megkötési és/vagy degradációs fázisa szükséges a kardioprotektív hatáshoz.

A szelegilinnel történő MAO-B gátlás csökkentette a testzsírt, valamint a szubkután és a zsigeri adipozitást, melyet a magas zsír- és cukor diéta okozott. Továbbá, a szelegilin kezelés csökkentette a zsírszövetben a magas zsír-és cukor diéta által indukált lipid-és szénhidrát-metabolizmus markerek expresszióját, mint például a *Srebp-1c*, és a *Glut1*, valamint a *Ccl3* gyulladásos markert. Az orális glükóz tolerancia és az inzulin tolerancia teszt károsodott glükóz háztartást mutatott a magas zsír-és cukor tartalmú táppal etetett csoportokban. A szelegilin csökkentette a szisztolés nyomást a normál táppal etetett patkányokban, azonban ez a hatás eltűnt a magas zsír- és cukor diéta következtében. Továbbá kimutattuk, hogy a klóramfenikol kardioprotekciót indukál az autofágia és az Erk1/2 foszforilációjának fokozása révén a szívizomban. A klóramfenikolos kezelés jelentősen csökkentette az infarktusméretet és a kreatin-kináz felszabadulást. A TAT-HA-Atg5^{K130R} előkezelés a klóramfenikol indukálta kardioprotekciót megszüntette, míg a klóramfenikollal és klorokinnal kezelt szívekben az infarktusméret és a kreatin-kináz felszabadulása hasonló mértékben csökkent, mint a klóramfenikollal kezelt csoportban. Ez azt mutatja, hogy a klóramfenikol által közvetített kardioprotekcióhoz az autofagoszóma képződése szükséges.

Eredményeink hozzásegítenek egy átfogóbb kép megalkotásához az enyhe elhízásban végbemenő molekuláris változásokról, valamint arról, hogy a MAO-B gátlás milyen hatással van ezekre a változásokra. Továbbá az autofágia szakaszainak a kardioprotekció kiváltásában betöltött szerepének megismerése lehetővé teszi célzottabb terápiák fejlesztését az akut iszkémia/reperfúziós károsodás kezelésére.

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10 Bibliography of the candidate's publications

10.1 Candidate's publications involved in the current thesis

1. **Nagy CT**, Koncsos G, Varga ZV, Baranyai T, Tuza S, Kassai F, Ernyey AJ, Gyertyán I, Király K, Oláh A, Radovits T, Merkely B, Bukosza N, Szénási G, Hamar P, Mathé D, Szigeti K, Pelyhe C, Jelemenský M, Onódi Z, Helyes Z, Schulz R, Giricz Z, Ferdinandy P.

Selegiline reduces adiposity induced by high-fat, high-sucrose diet in male rats
BRITISH JOURNAL OF PHARMACOLOGY 2018 Sep;175(18):3713-3726.
IF:6.81 (2017)

2. Giricz Z, Varga ZV, Koncsos G, **Nagy CT**, Gorbe A, Mentzer RM Jr, Gottlieb RA, Ferdinandy P

Autophagosome formation is required for cardioprotection by chloramphenicol
LIFE SCIENCES 2017 Oct 1;186:11-16.
IF: 3.234

10.2 Candidate's publications not involved in the current thesis

1. Onódi Z, Pelyhe C, **Nagy CT**, Brenner GB, Almási L, Kittel A, Manček-Keber M, Ferdinandy P, Buzás EI, Giricz Z

Isolation of high-purity extracellular vesicles by the combination of iodixanol density gradient ultracentrifugation and bind-elute chromatography from blood plasma
FRONTIERS IN PHYSIOLOGY 2018 Oct 23;9:1479.
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