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NEUROPEPTIDES IN CARDIOPROTECTION AND CARDIOVASCULAR DISEASES

PhD thesis

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List of abbreviations

ARCM	adult rat cardiomyocytes
BSA	bovine serum albumin
CCR	C-C Motif Chemokine Receptor
CD68	cluster of differentiation 68
CGRP	calcitonin gene-related peptide
CON	control
Cy3	cyanine 3
DapB	*Bacillus subtilis* dihydrodipicolinate reductase
DAPI	4′,6-diamidino-2-phenylindole
DCM	dilated cardiomyopathy
DMSO	dimethyl sulfoxide
D-PBS	Dulbecco's phosphate-buffered saline
DPP4	dipeptidyl-peptidase-4
ELISA	enzyme-linked immunosorbent assay
e-NOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FPKM	fragments per kilobase of transcript per million mapped reads
GAL	galanin
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GLP-1	glucagon-like peptide-1
GO	Gene Ontology
HF	heart failure
ICM	ischemic cardiomyopathy
IPostC	ischemic postconditioning
IPreC	ischemic preconditioning
LAD	left anterior descending coronary artery
LV	left ventricle/left ventricular
LVEF	left ventricular ejection fraction

MAPK	mitogen-activated protein kinase
MCP	monocyte chemotactic protein
MDC	macrophage-derived chemokine
MMP	matrix metalloproteinase
mRNA	messenger RNA
NPY	neuropeptide Y
NTC	no template control
NYHA	New York Heart Association
PBS	phosphate-buffered saline
PECAM-1	platelet endothelial cell adhesion molecule 1
Rac	Ras-related C3 botulinum toxin substrate
RANTES	Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted
RFU	relative fluorescent unit
Rho	Ras homolog family member
RIA	radioimmunoassay
RIC	remote ischemic conditioning
RT-qPCR	real-time quantitative polymerase chain reaction
RYR2	ryanodine receptor 2
SEM	standard error of the mean
SI/R	simulated ischemia/reperfusion
SP	substance P
SST	somatostatin
SSTR1-5	somatostatin receptors 1–5
T2DM	type 2 diabetes (mellitus)
TAGLN	transgelin
TNFα	tumor necrosis factor alpha
TRH	thyrotropin-releasing hormone
TRPV1	Transient Receptor Potential Vanilloid 1
VEH	vehicle
VIM	vimentin
VIP	vasoactive intestinal peptide

1. Introduction

1.1.Neuropeptides

Neuropeptides are chemical messengers made of amino acids and they are synthesized by neurons and often co-stored and co-released with the regular neurotransmitters (1, 2). It is important that most of the neuropeptides can act both as traditional neurotransmitters and as neurohormones (exerting e.g. autocrine/paracrine and endocrine effects) (2, 3). Neurotransmitters are in general synthesized in the nerve endings and released by neurons and exert stimulatory or inhibitory effects on post-synaptic neurons. There is usually a reuptake mechanism carried out by specific transporters, which makes possible reutilization of these substances in transmitter synthesis (4). These effects are in general rapid, short, and rather limited to a smaller area. In contrast to the characteristics of neurotransmitters, neurohormones are produced in the ribosomes of the cell bodies and their transit to the nerve endings occurs through axonal transport. After their release at the synapses they can behave as long range signaling substances (4). Neuropeptides often utilize both types of these effects (e.g. vasopressin, in the collecting ducts of the kidney vs. vasopressin within the central nervous system) (4). Neuropeptide families have extremely long-standing origins developed from primordial genes into the presently known peptides (5). These substances can be found in various organisms from animals (e.g. frog skin (4)) to plants and expressed in various tissues e.g. central/peripheral nervous system (brain, spinal cord, autonomic and sensory ganglia, hypothalamus etc.) gastrointestinal tract. Neuropeptides are synthetized through complex processes first producing larger, inactive precursor peptides those will be transferred afterwards from the endoplasmic reticulum to the Golgi apparatus (4). These precursors are stored in large dense core vesicles with several types of the processing enzymes called convertases (6, 7). The enzymes cleave the precursor molecules creating biologically active peptides. In addition to the enzymatic cleavage, posttranslational modifications may occur afterwards e.g. glycosylation, C-terminal amidation, acetylation, phosphorylation and sulfation. The precursor peptides often contain several copies of the same peptide but they also can contain different types of peptides (e.g. precursor for Thyrotropin-releasing hormone (TRH) contains five copies of the TRH in contrast, from the proopiomelanocortin precursor functional adrenocorticotropin, melanotropins or the opioid peptide β-

endorphin can be processed (8). Neuropeptides can be categorized according to their expressional properties (9). Some neuropeptides are usually expressed at higher levels even on normal circumstances, therefore those are available at any time (e.g. SP and Calcitonin gene-related peptide (CGRP) in primary sensory neurons, or NPY in cortical neurons) (4). Other neuropeptides are normally expressed at quite low levels but their synthesis will be induced/enhanced by certain conditions/stimuli (e.g. vasoactive intestinal peptide, galanin and NPY in sensory neurons) (4). There is also a type of peptides showing significant expression early during development, and they are usually downregulated postnatally (e.g. somatostatin (SST) in many central systems, CGRP in chicken motor neurons, SP in spinal guiding neurons, galanin in primary sensory neurons) (4). Numerous neuropeptide molecules exist and they are classified into several neuropeptide families e.g.

- hypothalamic hormones: oxytocin, and vasopressin,
- hypothalamic releasing and inhibiting hormones: corticotropin releasing hormone, growth hormone releasing hormone, luteinizing hormone releasing hormone, somatostatin, thyrotropin releasing hormone,
- tachykinins: neurokinin α, neurokinin β, neuropeptide K, substance P,
- dynorphin, met- and leu-enkephalin, nociceptin/orphanin FQ,NPY and related peptides: neuropeptide Y, pancreatic polypeptide, peptide tyrosine-tyrosine,
- VIP-glucagon family: glucogen-like peptide-1, Peptide histidine isoleucine, pituitary adenylate cyclase activating peptide, vasoactive intestinal polypeptide,
- other neuropeptides: brain natriuretic peptide, calcitonin gene-related peptide, cholecystokinin, glucagon-like peptide 1, galanin, islet amyloid polypeptide or amylin, melanin concentrating hormone, melanocortins, neuropeptide FF, neurotensin, parathyroid hormone related protein,
- novel neuropeptides: agouti gene-related protein, cocaine and amphetamine regulated transcript/peptide, corticostatin, endomorphin-1 and -2, 5-hydroxytryptamine-moduline, hypocretins/orexins, nocistatin, prolactin releasing peptide, secretoneurin, urocortin (4).

Neuropeptide receptors are usually G protein-coupled receptors those are able to modulate various physiological and neuronal activities such as pituitary control, reproductive functions, water and electrolyte balance, regulation of feeding behaviors,

pain sensation, sleep modulation, anxiety, and stress etc. (4). In addition to these "classical" effects of neuropeptides in last 2-3 decades, the focus of the neuropeptide research was extended with the investigation of their potential role in cardiovascular diseases and cardioprotection.

1.2.Neuropeptides and the cardiovascular system

The heart contains various neuropeptides that are able not only to modulate the sympathetic/parasympathetic neurotransmissions, but also to have effects on several cardiac functions (Table 1) (2).

Table 1. – Various effects of neuropeptides on the heart

Beneficial effects	Neuropeptide	Harmful effects
Anti-inflammatory effects Antinociceptive effects Antisecretory effects Antiproliferative effects Improves cardiac structural and functional abnormalities in acromegaly Protective effects against ischemia-reperfusion injury	**SST**	
Coronary vasodilation Improves contractility Cardioprotective effects against ischemia-reperfusion injury	**SP**	Promotes: Inflammation Apoptosis Matrix metalloproteinase activation Adverse myocardial remodeling Cardiac fibrosis Proliferation of cardiac fibroblasts

Improves contractility of cardiomyocytes	**NPY**	Vasoconstriction/ischemia Promotes: Fibrosis Hypertrophy Cell death
Improves: Perfusion Angiogenesis Apoptosis Fibrosis Oxidative stress	**NPY_{3-36}**	

These neuropeptides (e.g. CGRP, SP, VIP, NPY) often originate from parasympathetic, sympathetic, or sensory nerves of the heart (10). During the long and extensive research of these substances it was discovered that they exert various effects from cardioprotection even to the promotion of adverse cardiac remodeling and heart failure (2), although, their roles in various cardiac pathologies are still not fully clarified (2). It was discovered that the sensory nerves innervating the heart take part in the cardiac adaptation mechanisms to ischemic injury (11, 12). Upon activation the transient receptor potential vanilloid 1-(TRPV1-) expressing capsaicin-sensitive chemosensitive peptidergic afferents release sensory neuropeptides (13) e.g. CGRP or SP. Their cardioprotective effects were proved in various experimental models (14-20).

1.3.Somatostatin

SST is a small cyclic peptide consisting of 14 amino acids and it is released from the central nervous system besides the sensory nerves (21), from inflammatory and immune cells, pancreas, retinal neurons, and epithelial cells (22). SST is expressed in both the atrial and ventricular nerve fibers of muscle bundles (23), in the atria, and the atrioventricular node (24). SST has five G_i-protein-coupled transmembrane receptor subtypes (SSTR1-5) (22). These receptors are widely expressed both in the central nervous system and in the periphery. The most expressed subtypes in endocrine tissues

are SSTR2 and SSTR5 (22), although they can be found both in the atria and ventricles except for SSTR3 (25). While cardiac fibroblasts express SSTR1, 2, 4, and 5, cardiomyocytes express rather only SSTR1 and 2 (25). Although it is rather known for its inhibitory effect on the actions of growth hormone SST also has anti-inflammatory, antinociceptive (21, 26), antisecretory, and antiproliferative effects (22). One quite important and well researched effect of octreotide (SST analogue substance) that it is able to improve the cardiac structural and functional abnormalities in acromegaly (27-30). The effect of SST on the electrophysiology of the heart is sometimes controversial. It was shown that SST can exert negative inotropic effects possibly due to decreasing the inward Ca^{2+} current (31) or the inwardly rectifying K^{+} channel (32) but others have shown a positive inotropic effect in the guinea pig heart (33). Interestingly, synthetic SST analogs were found to exert protective effects against ischemia reperfusion injury in various species and organs e.g. rat and mouse retina (34, 35), rabbit liver (36), rat pancreas (37), and rat heart (38). In addition, SST released from capsaicin-sensitive nerves could improve retinal ischemia/reperfusion injury of the mouse retina (39). However, the SST expression in the human heart and its potential function and involvement in clinically relevant large animal models of cardiac ischemia/reperfusion injury is not known.

1.4.Substance P

SP is a member of the tachykinin neuropeptide family and it was first discovered and isolated in 1931 (40). SP consist of 11 amino acids and it is derived from the *Tac1* gene which encodes not only SP but also neurokinin A, neurokinin B, neuropeptide K and neuropeptide-γ (41). The alternative splicing of the pre-mRNA will result in four different mRNA isoforms (α, β, γ, δ) and the production of SP and the other proteins. SP is mainly expressed by C-fiber sensory nerves innervating the coronary arterial system, intrinsic nerve bundles, interganglionic nerves, cardiomyocytes, and coronary endothelial cells (42-48). Tachykinins can exert their effects through the neurokinin receptors belonging to the G protein coupled receptor family (49). There are three identified neurokinin receptors: neurokinin 1 (NK-1R), neurokinin 2 (NK-2R), and neurokinin 3 receptor (NK-3R). SP binds to the NK-1R with greater affinity and acts primarily through that one (50). NK-1R exert its' effects through G_q and G_s signaling pathways (51). (52)NK-1R is expressed mainly in the central nervous system, lungs, placenta, kidney, T and B cells of

the spleen (52). (51, 52)The NK-1R has two isoforms: a full length receptor (406 amino acids), and a truncated one (41, 53) which has 10 times lower affinity for SP, interestingly it does not affected by rapid desensitization and internalization (54). The truncated isoform is the dominant in the heart (55). SP was found to have a potent, nitric oxide mediated coronary vasodilator effect (56). SP is also able to regulate heart rate (induce bradycardia), and force of contractions (56). SP was found to exert acute cardioprotective effects in ischemia-reperfusion injury probably because SP improves the reperfusion due to its vasodilator effect (57). Capsaicin-induced sensory nerve depletion deteriorated cardiac functions in the reperfusion period after global ischemia in the *ex vivo* rat heart (58). The administration of SP to the capsaicin pretreated heart improved the contractile function and coronary flow showing the protective effect of SP against ischemia-reperfusion injury (58). Several studies used various ischemia reperfusion as well as pre- and post-conditioning models to test the cardioprotective role for SP in the heart. They have found that TRPV1 knockout/inhibition increased the ischemic damage partially due to the decreased SP and CGRP levels. Exogenous SP or CGRP decreased ischemia-reperfusion injury, and their release is increased due to pre-, and postconditioning, suggesting their role in the adaptive mechanism against ischemic injury (16, 17, 59). Despite the acute cardioprotective effects SP often exerts detrimental effects chronically e.g. promoting inflammation, apoptosis, MMP activation, changes to the extracellular matrix, leading to adverse remodeling (57). In a chronic volume overload-induced heart failure model the deletion of the tachykinin precursor 1 gene prevented the development of left ventricular hypertrophy, increased right ventricle mass or lung weight in mice (60). The results of an aortocaval fistula induced volume overload model in mice suggest that SP may play an important role in mediating adverse myocardial remodeling through the activation of cardiac mast cells, increased tumor necrosis factor-α and MMP activation, and degradation of the extracellular matrix (60). SP is able to mediate cardiac fibrosis in the heart of spontaneously hypertensive rats probably through the neurokinin-1 receptor mediated regulation of various genes related to cell adhesion and regulation of the extracellular matrix in cardiac fibroblasts (61). Interestingly, others have found that SP probably serves as a 'primer' for rat fibroblasts rather than exerting direct effects (62).

1.5.Neuropeptide Y

NPY is a neuropeptide consisting of 36 amino acids and it belongs to the same family as peptide YY and pancreatic polypeptide (63). NPY is the most highly expressed neuropeptide in the heart and it has 5 known receptors (Y_{1-5}) from those Y_1, Y_2, and Y_5 are the most abundant in the heart (63-66). All of the Y receptors mainly acts through G_i (67) and they are expressed in the central nervous system, peripheral nervous system, heart, blood vessels, pancreatic β cells, spleen, liver, gastrointestinal tract, in the visceral adipose tissues, adrenal gland, kidney, and the placenta (68). In opposite to the other receptors Y2 is localized primarily presynaptically in neuronal tissues (68). NPY is mainly expressed in the intracardiac ganglia, sympathetic nerves projecting to blood vessels, the endocardium, intrinsic parasympathetic cardiac neurons, and cardiomyocytes (63). NPY is more abundant in the atria than ventricles measured in human autopsy samples (69). NPY and norepinephrine in the cardiac sympathetic nerves are stored and released together upon activation of the nerves (70). Regarding to the cardiovascular effects of NPY it was found to exert a potent vasoconstrictor effect on coronary arteries through smooth muscle cells (63) and to increase cardiomyocyte contractility (70, 71). This was probably caused by increased the Ca^{2+} release after Y_1 receptor and subsequent phospholipase C activation in isolated rat cardiomyocytes (71) or by electrical stimulation induced Ca^{2+}-dependent co-release of noradrenaline and NPY in guinea pig heart (70). It has also been demonstrated that administration of small NPY amounts into human coronary arteries can induce vasoconstriction and ischemia (72). Although, there is some evidence that NPY is also able to decrease contractility of isolated adult rat cardiomyocytes (73). Interestingly, NPY level shows increase in the plasma of various patients with heart failure (74, 75) and in other studies, elevated plasma NPY level was found to be related to the increased severity of chronic HF in patients (76) and an indicator of mortality in patients with non-acute myocardial infarct (77). In line with the human data, plasma NPY concentration also increased in a rat model of cardiac volume overload (65), although, atrial and ventricular NPY levels decreased especially in case of cardiac decompensation. Moreover, Y_1 receptor mRNA expression decreased but Y_2 receptor mRNA expression increased in volume-overloaded hearts (65). Interestingly, in patients with type II diabetes the serum levels of NPY also increase (66, 78), meanwhile both Y_1 and Y_2 receptor expression increased in human atrial biopsies (66). Although, others have

found (79) no change in NPY protein levels, and reduced expression of Y_2 and Y_5 receptors in atrial biopsy samples of patients with diabetes, probably due to the higher number of male diabetic patients, different medication regimes and other underlying cardiac co-morbidities. Surprisingly, Y_1 receptor deletion in mice induced fibrosis in the heart (80). Administration of NPY (10 and 100 nM) in isolated adult rat cardiomyocytes caused hypertrophy (81), probably mediated by the Y_5 receptor (82). Surprisingly, NPY administration induces concentration-dependent cell death in isolated rat neonatal cardiomyocytes (83). Despite the mainly harmful effects of NPY in the swine model of high fat diet and left circumflex coronary artery occlusion-induced myocardial infarction $NPY_{3–36}$ administration to the infarcted region for improved LV relaxation, perfusion to the ischemic area, angiogenesis, apoptosis, fibrosis, and oxidative stress (84). Interestingly, administration of NPY increased the expression of Y_1 and Y_5 receptors, but not Y_2 (85). It is important to note that NPY concentration was lower than the range causes vasoconstriction and the route of administration reduces the chance for systemic effects (85). In summary, it is relatively difficult to judge the cardiac function of NPY because it depends not only on the concentration but also on the etiology, disease progression etc. According to the currently available literature data, NPY shows rather protective effects at low concentrations, but pathological in higher amounts.

1.6. DPP4 and its substrates

Dipeptidyl peptidase-4 (DPP4/ adenosine deaminase complexing protein 2/ cluster of differentiation 26) is an enzyme and a binding protein that was discovered in rat liver homogenates (86). DPP4 shows the highest expression in the kidney but it is also expressed in the lung, adrenal gland, jejunum, liver, spleen, heart, pancreas, brain, endothelial cells of the blood vessels (87), activated T-helper lymphocytes (88), macrophages (89), and often at sites of physiological barriers (e.g. blood–brain barrier) (90). DPP4 is an integral membrane protein and it belongs to the serine proteases, however, a soluble form of DDP4 exist in body fluids which could be either solubilized forms of the membrane counterparts or a specially coded soluble variants with potentially a different physiological role (90). The cleavage of the DPP4 subtrates mainly results in their inactivation e.g. in case of endomorphin-2, enterostatin, β-casomorphin, glucagon-like peptide 1 and 2 (GLP-1, GLP-2), gastric inhibitory peptide, growth hormone-

releasing hormone and monocyte chemoattractant protein etc. (90). In contrast it was also discovered that proteolytic cleavage of a biologically active peptide at the N-terminal site, does not necessarily result in in the complete or partial inactivation but sometimes activation or changes in receptor binding affinity may happen (90). One important exception is SP where the cleavage creates the SP_{5-11} fragment which has lower binding affinity for the NK-1 receptor (primarily preferred receptor of SP). Further cleavage of the truncated SP_{5-11} by DDP4 or other peptidases belongs to the most important degradation pathway in the plasma, vascular endothelium, fibroblasts and myocytes (91-94). Another important example is NPY which is a regulatory peptide consists of 36-amino acids and it shows higher abundance in the central and peripheral nervous system and it is well known for the control of feeding, energy homeostatis and blood pressure (90). DPP4-mediated N-terminal truncation produces $NPY_{3–36}$ which has reduced activity at the Y_1 receptor, but the activities at the Y_2 and Y_5 receptors remain similar (95). DDP4-mediated cleavage also causes changes in the affinity of certain chemokines to their receptors. $RANTES_{3–68}$ (Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted) shows reduced activity to the C-C Motif Chemokine Receptor 1 (CCR1) (96) or C-C Motif Chemokine Receptor 3 (CCR3) (97) receptors, but similar (96) or even increased (98) ability to stimulate the C-C Motif Chemokine Receptor 5 (CCR5) receptors. Interestingly, $RANTES_{3–68}$ was found to be an antagonist for the chemotactic effect of RANTES, macrophage inflammatory protein-1α or β or monocyte chemotactic protein (MCP)-3 on human monocytes (97). $RANTES_{3–68}$ has been isolated from culture supernatants of stimulated human fibroblasts (skin, platelet preparations or sarcoma cells) (99, 100). Eotaxin is a substance known to be an important mediator in allergic reactions because it attracts eosinophils, basophiles and TH2 lymphocytes (90). The *in vitro* chemotactic activity $eotaxin_{3–74}$ (truncated form) is decreased and also the binding to the CCR3 receptor (101). In addition, various N-terminally cleaved eotaxins (desGP, desGPA, desGPAS) were successfully isolated from supernantants of cultured skin fibroblasts (102). Macrophage-derived chemokine (MDC) also behaves as a chemoattractant substance of monocytes, dendritic cells, activated lymphocytes and NK cells. Cleaved products are $MDC_{3–69}$ and $MDC_{5–69}$ (103). Both processed products have reduced activity on target cells (103, 104), although, they retained their activities to attract monocytes probably through different a receptor subtype. MDC is also a chemokine that

is only partially inactivated. SDF-1 (also called as pre-B-cell growth-stimulating factor) is similarly a chemoattractant subbstance for T-lymphocytes and monocytes. Two splice forms (SDF-1α and SDF-1β) exist. Truncation by DPP4 results in the loss of their *in vitro* chemotactic and antiviral activities (104-106).

1.7.Ischemic heart disease

Ischemic heart disease is a special pathological condition in which cardiac coronary blood flow is significantly reduced leading to chronic ischemic damage of the myocardium (107). The various types of cardiovascular diseases with ischemic origin are still among the most common causes of death worldwide (108, 109). The problem of the insufficient coronary perfusion will soon disturb the balance between myocardial oxygen supply and demand inducing further problems and symptoms (107, 110). Various pathological processes such as microvascular dysfunctions, atherosclerotic obstruction, spasm of the coronary arteries, or acute coronary thrombosis can cause cardiac ischemia (107). Several risk factors e.g. smoking, hypertension, diabetes mellitus, and inflammation are able to accelerate or make worse the progress of the atherosclerotic plaque development (111). Despite that ischemic heart disease shows typically chronic and progressive characteristics (107) rather the short term effects of various neuropeptides were investigated in myocardial ischemia. Calcitonin gene-related peptide (CGRP) is one of the most thoroughly researched neuropeptide in cardiac ischemia. It is capable to exert cardioprotective effects against the ischemic damage of the rat heart (20, 112). CGRP shows an increased level in plasma samples of patients with myocardial infarction (113) and the gene expression of calcitonin receptor-like receptor is increased during the reperfusion in the mouse left ventricle (114). Exogenous CGRP administration improves cardiac functional parameters in rats subjected to myocardial ischemia and reperfusion (115). Vasoactive intestinal peptide (VIP) is a neuropeptide also providing an improved blood flow during myocardial ischemia and a cardioprotective effect against the ischemic damage of isolated rat hearts (116). Neuropeptide Y (NPY) has some beneficial effects in myocardial ischemia, but different mechanisms are responsible for those e.g. modulation of angiogenesis, fibrosis, and remodeling (117). Exogenously administered NPY_{3-36} improves myocardial function and increases angiogenesis by upregulated

vascular endothelial growth factor and fibroblast growth factor expression in the pig ischemic myocardium, but NPY_{3-36} did not alter the blood flow (118).
In the long term, one important of the many consequences is that on the basis of cardiac ischemia new onset of heart failure or left ventricular dysfunction may appear.

1.8.Heart failure

Heart failure (HF) is considered as a chronic, progressive condition in which case the heart muscle is not able anymore to provide the necessary volume of blood in order to cover the required amount of oxygen and nutrients. HF is a complex clinical syndrome characterized by various symptoms (e.g. breathlessness, ankle swelling, fatigue, elevated jugular venous pressure, pulmonary crackles, and peripheral edema etc.) (119). During the relatively early period of HF the body will perform several compensatory mechanisms (e.g. increased heart rate, blood vessel constrictions, hypertrophy, volume redistribution from less important organs etc.) in order to keep up the necessary cardiac output (119). These temporary processes are able usually to cover the problem even for a couple of years but as the condition deteriorates and compensation fails to further adapt, more severe symptoms will appear (e.g. fatigue, and dyspnea and pulmonary oedema especially during exercise) (119). The traditional way to classify HF is based on the measurement of the left ventricular ejection fraction (LVEF) because the treatments and the clinical outcomes are different in various LVEF ranges (119). Accordingly, HF has been divided into three phenotypes based on LVEF (119). The first category is called HF with reduced ejection fraction (HFrEF) in which LVEF is defined as $\leq$ 40% (119). Patients with a LVEF between 41% and 49% belong to the category called HF with mildly reduced ejection fraction (HFmrEF) and to the category of HF with preserved ejection fraction (HFpEF) if LVEF is $\geq$ 50% in addition with symptoms and signs of HF, structural and/or functional cardiac abnormalities and/or raised natriuretic peptides (119). The pathomechanism of HF has several important key points. Regarding to the role of neuropeptides in these processes it is essential to highlight at least SP and NPY. According to the literature, SP has harmful effects in myocardial remodeling and heart failure (2). It was revealed in a model of magnesium deficiency in rats and mice, that SP levels increase and the inhibition of the neurokinin 1 receptor decreases the levels of tumor necrosis factor alpha (TNFα) and interleukin-1, and improves diastolic and systolic

function (120-122). In chronic volume overload-induced heart failure of mice the deletion of the tachykinin precursor 1 gene exerts protection from developing left ventricular (LV) hypertrophy possibly by the prevention of matrix metalloproteinase activation and collagen degradation (60). It was also reported that neurokinin 1 receptor inhibition hinders cardiac fibrosis in the hearts of spontaneously hypertensive rats (61). In addition, SP is able to induce proliferation of the adult rat cardiac fibroblasts (123). Various studies suggest that NPY promotes hypertrophy, angiogenesis, cell death of cardiomyocytes, and modulation of protein synthesis in cardiomyocytes contributing to the pathomechanism of HF (63, 81, 82).

Although both non-pharmacological (e.g. device therapy) and pharmacological options are available (such as angiotensin-converting enzyme inhibitors, angiotensin-receptor blockers, angiotensin receptor-neprilysin inhibitor, beta-blockers, mineralocorticoid receptor antagonists, sodium-glucose co-transporter 2 inhibitors, loop diuretics, ivabradine, hydralazine, isosorbide dinitrate, or digoxin), the treatment of HF is still a challenging task for the clinical experts. In addition to these challenges, the presence of cardiovascular comorbidities such as type 2 diabetes makes the odds much worse.

1.9.Type 2 diabetes and diabetic cardiomyopathy

Diabetes can be classified into four major categories. Here, only type 2 diabetes (T2DM) is discussed since the other categories are less relevant in our current work. T2DM (~90–95% of the patients with diabetes, previously referred to as non–insulin-dependent diabetes, or adult-onset diabetes) is caused by the relative loss of β-cell insulin secretion often on the background of insulin resistance. Deficiency of insulin secretion can be related to e.g. genetic causes, inflammation, or metabolic stress (124). Individuals with T2DM are often obese or have an increased percentage of body fat distributed commonly around the abdomen (125). Weight reduction and pharmacotherapy of hyperglycemia usually improve insulin resistance (125). Patients often do not need any insulin therapy in order to manage the state of their diabetes. Patients are at risk of developing macro- and microvascular complications due to hyperglycemia. According to the literature data, diabetes induces several morphological and functional changes to the myocardium including abnormalities of cardiac neural innervation. Damage of the nerve fibers innervating the heart causes parasympathetic and sympathetic dysfunction (126, 127).

Neuropeptides released by damaged nerve fibers may further impair cardiac function; therefore, they potentially play a significant role in the pathomechanism of diabetic cardiomyopathy (117). Diabetic cardiomyopathy is a clinical condition in patients with diabetes that is characterized by the development of ventricular dysfunction without other cardiac risk factors, such as coronary atherosclerosis and hypertension (128, 129). Diabetic cardiomyopathy has often an early, hidden subclinical period with various structural and functional anomalies e.g. left ventricular hypertrophy, fibrosis, and abnormalities of cell signaling. These pathophysiological changes often cause the development of heart failure (HF with normal ejection fraction or systolic dysfunction with HFrEF). NPY is a neuropeptide that potentially plays a role in the pathomechanism of diabetic cardiomyopathy. Various studies revealed that the plasma levels of NPY show an increase in patients with diabetes (66, 78, 130). Interestingly, cardiac level of NPY is decreased in patients with type II diabetes (66, 79). In the rat model of streptozotocin-induced type-I diabetes cardiac expression of NPY both at the gene and the protein level decreases, and the partial, spontaneous improvement of diabetes is associated with increased atrial levels of NPY (131, 132). Atrial NPY receptor Y1 (Y_1) gene expression shows up-regulation in long-term in rats with diabetes (131). Altered cardiac regulation of NPY decreases angiogenesis, increases both apoptosis, and vascular smooth muscle proliferation (117). These changes potentially contribute to the development of diabetic cardiomyopathy. SP and its receptors show alterations in diabetic patients. Expression of SP both at the gene and at the protein level are reduced in the right atrium of patients with diabetes compared to patients without diabetes (79). It was also found that the serum level of SP decreases in diabetic patients (133). In addition, neurokinin 1 receptor is downregulated in the right atrium of streptozotocin-treated rats (134). These data suggest that reductions of cardiac SP levels and its receptors' expression in diabetes could be the part of the pathogenesis of the diabetic cardiomyopathy. VIP is also an important neuropeptide in diabetic cardiomyopathy. It is known that loss of function mutations in VIP are associated with biventricular heart failure (135). Cardiac VIP signaling showed significant changes in the rat model of streptozotocin-induced diabetes. Both preproVIP mRNA expression and the peptide level of VIP are decreased in the rat heart (136). VIP receptor 2 showed an initial decrease in the expression at the mRNA level in the atria, which showed up-regulation in the long term, meanwhile its initial up-regulation returned

to control level in the ventricles in the long term (136). Despite the continuous loss of VIP both VIP receptor 1 and VIP receptor 2 expression returned to the control levels after initial changes (136). CGRP is a neuropeptide that shows changes in diabetes-associated cardiomyopathy but it is rather part of the beneficial/protective processes (137-139).

1.10. DPP4 inhibitors in diabetes

Inhibiting the activity of the enzyme dipeptidyl peptidase 4 (DPP4) is a novel way to treat T2DM (140). Since the marketing of DPP4 inhibitors they have become well established part of managing T2DM because these substances are not typically characterized by an increased risk of hypoglycemia, in addition they have good tolerability, safety profiles and they are small molecules (140). Many different DPP4 inhibitors are currently available as therapeutic options for T2DM. These medications are part of the second- or third-line drugs used in the treatment of T2DM (after metformin and sulphonylureas). The basis of their action is that DPP4 inhibitors increases the endogenous level of the incretin hormone called glucagon-like peptide 1 through the inhibition of its clearance by DPP4 enzyme. GLP-1 is an intestinal peptide responsible for the facilitation of glucose-induced insulin secretion and the suppression of glucagon secretion (141). Increased GLP-1 levels are associated with improved glucose homeostasis (142, 143). DPP4 works as an exopeptidase enzyme cleaving dipeptide substrates from their N-terminal site (90, 144, 145). This process will result usually the inactivation of the substrates, although in certain cases shifts in the receptor affinity can also occur (145). The enzyme is able to cleave a broad range of substrates, e.g., not only incretins (glucagon-like peptide-1, glucose-dependent insulinotropic polypeptide), but also chemokines (e.g., stromal cell-derived factor 1), and various neuropeptides (neuropeptide Y, substance P, peptide YY, and pancreatic polypeptide) (144, 145). The potential off-target effects of other DPP4 substrates (e.g. neuropeptides) contributing to the therapeutic-/side effects of DPP4 inhibitors are not fully elucidated.

1.11. Saxagliptin in HF patients with diabetes

Saxagliptin is a DPP4 inhibitor molecule containing a cyanopyrrolidine structure similarly to vildagliptin and it is metabolized (146) by the cytochrome P450 3A4 or P450 3A5 enzymes of the liver. These processes generate a metabolite called 5-hydroxy-

saxagliptin which is also capable of the inhibition of DPP4 activity but with a lower potency. Saxagliptin has a half-life of 2.5 h while its primary metabolite has a bit longer one with 3 h (147, 148). Saxagliptin is mainly eliminated via liver metabolism and its renal clearance is limited, however its metabolite is rather cleared by filtration in the kidneys (140, 149). Hepatic impairment has only slight effect on drug exposure, but some dose reduction can be necessary when the renal function is decreased (140). The SAVOR-TIMI 53 phase 4 clinical trial (randomized, and placebo-controlled) which aimed to investigate the cardiovascular safety and efficacy of saxagliptin surprisingly revealed that in the saxagliptin-treated patients the rate of hospitalization for heart failure increased (150). This potentially dangerous cardiovascular side effect of saxagliptin is noted in the latest ESC heart failure guideline (119). Accordingly, the administration of saxagliptin in diabetic patients with HF should be avoided (119). Although this is a potentially dangerous side effect, which could restrict the clinical use, the exact mechanism behind this harmful effect of saxagliptin and its other substrates (such as neuropeptides) besides incretins is still unclear (150).

2. Objectives

Although, ischemic heart diseases belong to the leading causes of death worldwide, there are still no effective marketed medications exerting cardioprotective effects against ischemia-reperfusion injury. Therefore, there is an unmet need for developing novel, cardiac tissue protective drug candidates. In order to test the potential cardioprotective/citoprotective effect of SST a relatively quick and efficient first step is the utilization of the *in vitro*, cell culture-based simulated ischemia-reperfusion injury model. Next, to provide further evidence supporting the protective role of SST we used tissue and plasma samples obtained from our previous, translationally relevant pig myocardial infarction and cardioprotection study where we thoroughly tested the protective effects of different ischemic conditioning interventions (151). Briefly, in this model we have found that myocardial necrosis is significantly decreased by ischemic preconditioning, meanwhile myocardial edema is reduced by ischemic postconditioning and remote ischemic conditioning, and microvascular obstruction is rather decreased by ischemic preconditiong and ischemic postconditioning. To further improve the translational relevance of the current results we performed investigations on cardiac tissue samples of healthy patients or patients with chronic heart failure.

The results of the neuropeptide research of the last 2-3 decades highlighted that various neuropeptides may play a role both in protective cardiovascular mechanisms and pathological processes of cardiovascular diseases and their co-morbidities. The SAVOR TIMI 53 clinical trial revealed that DPP4 inhibition by saxagliptin-treatment increased HF-associated hospitalization of patients with chronic HF. We assume that this harmful effect is at least partially mediated by neuropeptide substrates of DPP4 (NPY and SP).

Therefore, the major objectives of this work were:

I. First, to investigate the direct cardiocytoprotective effect of SST against simulated ischemia/reperfusion injury in cardiac cell cultures.
II. Next, to investigate the expression of SST and its receptors in pig and human hearts.

III. In addition, to determine the expression of DPP4 and its substrates in the human heart and cell culture samples both at protein and mRNA levels.

IV. Finally, to set up a relevant cell culture platform to investigate the potentially detrimental effect of DPP4 inhibition by saxagliptin and the role of two important neuropeptide substrates of the DPP4 enzyme such as SP and NPY.

3. Methods

3.1. Cardiac cell cultures

3.1.1. AC16 cell line

Human cardiac myocyte cell line was obtained from Merck (AC16, SCC109). Cells were plated on 6-well or 24-well plates (Thermo Fisher Scientific, Waltham, MA, USA) and maintained in DMEM/F12 (Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient Mixture; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; EuroClone, Pero MI, Italy), 100 U/mL penicillin, and 100 μg/mL streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) in a humidified atmosphere of 5% CO_2 at 37 °C (152). The experiments were started when the cell culture reached 80–90% confluence. During the experimental protocol cells were kept in FBS-free DMEM/F12 medium.

3.1.2. H9C2 cell line

H9C2 cell line (European Collection of Authenticated Cell Cultures) was purchased from Sigma-Aldrich (St. Louis, MO, United States). Cells ($2*10^4$ cells/well) were plated on 96-well plates and incubated for 24 h in DMEM (Corning, NY, United States) containing 10% FBS (Euroclone SpA. Milan/Stockholm) (153).

3.1.3. Primary culture of adult rat cardiomyocytes

The primary culture of adult rat cardiomyocytes was prepared according to the previously published protocol (154). Briefly, male Wistar rats (150-200 g) were anesthetized with 60 mg/kg of pentobarbital and 500 IU/kg of heparin was administered intravenously. Afterwards, hearts were excised and perfused with Krebs-Henseleit solution, next digested by 8000 U/mL of collagenase II for 30–45 min. Ventricles were cut and digestion continued for additional 10 min. Cells were filtered and pelleted under gravity. Ca^{2+} concentration was increased carefully up to 1 mM. Cells (7,500 cells/well) were plated on laminin-coated glass coverslips in a 24-well plate (Thermo Fisher Scientific, Waltham, MA, United States) and maintained in 5% FBS containing M199 medium for 3 h and in serum-free M199 medium for 24 h (153).

3.1.4. Co-culture of neonatal rat cardiac fibroblasts and myocytes

Neonatal rat hearts were isolated from Wistar rats (1–3-day-old, ~3–4 hearts/24-well plate) as specified previously (152, 155). Rats were disinfected with 70% ethanol and sacrificed by cervical dislocation. Hearts were rapidly removed through an abdominal access and placed in ice-cold phosphate-buffered saline (PBS) then washed three times with fresh volumes of ice-cold PBS. In a Petri dish containing ice-cold PBS the ventricles were separated from the atria. Ventricles were minced with fine forceps and collected in Falcon tubes containing 0.25% trypsin (5 mL/heart). Tissue fragments were digested by trypsin for 25 min in a 37 °C water bath. Cells were resuspended every 5 min using a 5-mL pipette. After digestion, the cell suspension was centrifuged at 250 rcf for 15 min at 4 °C, and the supernatant was carefully removed. Cell pellets were resuspended in 10 mL DMEM supplemented with 20% FBS. Cells were manually counted by hemocytometer then resuspended, and then evenly distributed into fibronectin-coated wells. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air in a CO_2 incubator until the confluence reached approximately 100%. On the following day the medium was changed to DMEM containing 10% FBS. The previous medium was changed to DMEM supplemented with 1% FBS 6–8 h after the previous one. Afterwards cells were quality scored. The 1% FBS supplemented medium was refreshed every 2–3 days until the cell culture reached the desired confluence and was used for scratch assay experiments.

3.2. *In vitro* simulated ischemia/reperfusion injury study protocol and cell viability measurement

The study protocols are shown in Figures 2A, and 3A (153). SST was purchased from Sigma-Aldrich (cat.no.: S1763) and it was dissolved in cell culture-grade water. The SST concentrations for the *in vitro* experiments were selected based on the results of competition binding and G-protein activation experiments in CHO cells (26). Cells were treated with either SST (1, 10, 100, and 300 nM and 1 μM) or its solvent containing growth media in a CO_2 incubator for 1 h. Subsequently H9C2 cells and adult rat cardiomyocytes were treated either with normoxic solution (in mM: NaCl 125, KCl 5.4,

NaH_2PO_4 1.2, $MgCl_2$ 0.5, HEPES 20, $CaCl_2$ 1, glucose 15, taurine 5, creatine-monohydrate 2.5, bovine serum albumin (BSA) 0.1%, and pH 7.4) in CO_2 incubator (normoxia groups) or with hypoxic solution (in mM NaCl 119, KCl 5.4, $NaH_2PO_4 \times 1H_2O$ 1.2, $MgCl_2 \times 6H_2O$ 0.5, HEPES 5, $MgSO_4 \times 7H_2O$ 1.3, $CaCl_2 \times 2H_2O$ 0.9, Na-lactate 20, BSA 0.1%, and pH 6.4) in a three-gas incubator (mixture of 95% N_2 and 5% CO_2, simulated ischemia/reperfusion groups), both solutions containing SST or its vehicle for 16 h and 3 h, respectively. During the simulated reperfusion phase of the protocol, cells were kept in media containing vehicle or SST in a CO_2 incubator for 2 h. Cell viability was measured using calcein assay (154). After rinsing the cells with Dulbecco's phosphate-buffered saline (D-PBS), they were incubated with calcein solution (1 μM) for 30 min at room temperature. The fluorescence intensity of the wells was measured by Varioskan Lux plate reader (Thermo Fisher Scientific, Waltham, MA, United States) at room temperature; excitation wavelength: 490 nm; emission wavelength: 520 nm. Four independent experiments were performed (n = 4) in cell cultures with average confluence of 70%. A 96-well format was used for H9C2 cells and an average of six-well relative fluorescence units (RFU) was used in one treatment group. For adult rat cardiomyocytes, a 24-well format was used and an average of four wells RFU was used in one treatment group. Results are expressed as RFU as described previously (156).

3.3. Treatment of AC16 cells with DPP4 inhibitors and neuropeptides

To test the effect of DDP4 inhibitors (alogliptin, linagliptin, saxagliptin, or vildagliptin) on cell viability, AC16 cells were treated with 500 nM of each substances in separate series for 24 h (152). DPP4 inhibitors were dissolved DMSO and further diluted in cell-culture grade water (>1000x). Control group received vehicle containing dimethyl sulfoxide (DMSO).

In another experiment, cells were treated with 500 nM saxagliptin in the presence of various doses (5, 20, 50, and 100 nM) of NPY or SP. We have received SP and NPY from our collaborators at the University of Pécs. These neuropeptides were dissolved in cell culture-grade water and diluted further in cell culture medium. To achieve sufficient inhibition of the DPP4 enzyme, saxagliptin was administered 1 h before the neuropeptide treatment. DMSO containing cell-culture-grade water and pure cell-culture-grade water

were used as solvents for saxagliptin and neuropeptides, respectively. Cell viability was assessed by calcein viability assay (154) as it was described previously.

3.4. Scratch assay

Scratch assays were carried out as it was reported previously (152, 155). Cells were seeded on 24-well plates, and they were pretreated with 500 nM saxagliptin or its solvent for 1 h. Afterwards the cell monolayer was scratched in a 30° angle with a 200 μL pipette tip. The cell debris along with the medium was removed from the wells, and cells were washed twice with Hank's Balanced Salt Solution (Corning Inc., Somerville, MA, USA). Cells were then treated with a combination of saxagliptin and NPY or SP for 24 h in the same way as it is described above. Cells were incubated for 24 h with the treatment solution in a CO_2 incubator at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Images were taken at the start of the treatment (0 h) and 24 h after the scratching process. The wound area (area unoccupied by cells) of the images was measured by ImageJ software (157) and expressed as percentage of baseline value (0 h). Data were collected from three-five independent experiments.

3.5. Closed-chest pig model of acute myocardial infarction and tissue sampling

The porcine left ventricular and plasma samples for the present study were obtained from one of our previous studies in a closed-chest porcine model of acute myocardial infarction and cardioprotection by ischemic preconditioning, postconditioning, and remote conditioning (151). The study was conducted in accordance with 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 the EU Directive (2010/63/EU) and the study was reported according to the ARRIVE guidelines (158). The study was approved by the Animal Ethics Committee of the Hungarian National Food Chain Safety Office (SOI/31/26–11/2014). Pigs were randomly divided into five groups: ischemia/reperfusion, ischemic preconditioning, ischemic postconditioning, remote ischemic conditioning, and sham-operated groups. The ischemia/reperfusion group underwent 90 minutes of myocardial ischemia induced by left anterior descending

coronary artery (LAD) occlusion. The ischemic preconditioning group was subjected to 3 × 5 minutes of myocardial ischemia before to 90-minute LAD occlusion. The ischemic postconditioning group was subjected to 6 × 30 seconds of myocardial ischemia after 90-minute LAD occlusion at the beginning of reperfusion. The remote ischemic conditioning group was subjected to 4 × 5 minutes of hindlimb ischemia during the 90-minute LAD occlusion. In the sham group, the balloon catheter was inserted into the LAD coronary artery, but not inflated (Figure 10A). After 3 hours of reperfusion, plasma samples and myocardial tissue samples were obtained from the ischemic region of the left ventricular myocardium. Group sizes were as follows: sham (n = 6-7), ischemia/reperfusion (n = 5-7), ischemic preconditioning (n = 4-5), ischemic postconditioning (n = 4-5), and remote ischemic conditioning (n = 4-5). Samples were immediately frozen and stored at −80°C. Infarct size measurements confirmed that ischemic preconditioning reduces significantly myocardial necrosis compared to ischemia/reperfusion after 3 hours of reperfusion. There was no difference between groups in terms of area at risk. Ischemic postconditioning and remote ischemic conditioning significantly reduce myocardial edema compared with ischemia/reperfusion after 3 days of reperfusion, and ischemic preconditioning shows only a tendency of decrease. Details of the phenotype of the pig model were published previously (151).

3.6. Human heart tissue collection

Experiments were designed and performed according to the ethical standards of the Declaration of Helsinki (1975). Patients gave their written informed consent to be participate in the study. The protocol was approved by the Polish Local Ethics Committee of the National Institute of Cardiology in Warsaw under the identification number IK-NPIA-0021-14/1426/18. Human tissue samples were collected at the Department of Heart Failure and Transplantology, Cardinal Stefan Wyszyński National Institute of Cardiology, Warsaw, Poland, as previously reported (159). Human hearts obtained from organ donors excluded from transplantation for various reasons served as control samples (CON). Donors with any relevant cardiovascular history or abnormalities were excluded from the current study. Hearts were also obtained from patients suffering from end-stage heart failure of ischemic cardiomyopathy (ICM) or dilated cardiomyopathy (DCM). The clinical parameters of the human tissue samples are summarized in the Table 1, Table 2,

and Figure 12. Samples of the left ventricle and interventricular septum were collected during cardiac explantation, avoiding the inclusion of non-cardiac tissues, e.g., scar, adipose tissue, endocardium, epicardium, or coronary vessels. Samples were immediately rinsed in saline solution, blotted dry, frozen in liquid nitrogen, and stored at −80 °C until processing for further molecular experiments. An additional series of cardiac samples were fixed in neutral buffered formalin and embedded in paraffin for histological studies.

3.7.RNAscope *in situ* hybridization

In situ hybridization of DPP4 enzyme or SSTR1-2 mRNA was performed using RNA Scope® Multiplex Fluorescent Kit v2 according to the manufacturer's instructions (Advanced Cell Diagnostics, Newark, CA, USA) on tissue slides of interventricular septum/left ventricles of human control hearts (152, 153). Accordingly, 4 μm formalin-fixed paraffin-embedded tissue sections were pretreated with heat, H_2O_2, and protease before hybridization with the various target oligo probes: 3plex-Hs-Positive Control Probe (catalog number: 320861), 3plex-Negative Control Probe (catalog number: 320871), Hs-DPP4 (catalog number: 477541, accession no.: NM_001935.3), Hs-SSTR1-C1 (catalog number: 310581, accession no.: NM_001049.2), Hs-SSTR2-C1 (catalog number: 310571, accession no.: NM_001050.2), Hs-TAGLN-C3 (catalog number: 498961-C3, accession no.: NM_003186.3), Hs-VIM-C2 (catalog number: 310441-C2, accession no.: NM_003380.3), Hs-CD68-C2 (catalog number: 560591-C2, accession no.: NM_001040059.1), Hs-PECAM1-O1-C3 (catalog number: 487381-C3, accession no.: NM_000442.4), and Hs-RYR2-C2 (catalog number: 415831-C2, accession no.: NM_001035.2). Cell type-specific markers were utilized to identify cardiomyocytes with a probe recognizing the mRNA of ryanodine receptor 2 (RYR2) (160), endothelial cells by the mRNA of platelet endothelial cell adhesion molecule 1 (PECAM-1) (161), fibroblast cells by the mRNA of vimentin (VIM) (162, 163), macrophages by the mRNA of cluster of differentiation 68 (CD68) (164), and vascular smooth muscle cells by the mRNA of transgelin (*TAGLN*) (165). Preamplifier, amplifier, and HRP-labeled oligo probes were then sequentially hybridized, after which signal development was performed with TSA fluorophores (TSA-Cy3, TSA-FITC, Akoya Biosciences, Marlborough, MA, USA). Each sample was quality-controlled with a positive control probe specific to housekeeping genes and with a negative control probe in order to test the RNA integrity.

The specific RNA labeling signals were identified as red/green dots and cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Imaging was performed with a Leica DMI8 Confocal microscope (Leica, Wetzlar, Germany).

3.8. Western blot

To investigate whether DPP4 expression was altered at the protein level in human hearts, Western blot was performed (152). Frozen tissue samples from the interventricular septum were homogenized with a TissueLyser (Hilden, Germany) in 1× radioimmunoprecipitation assay buffer (RIPA; Cell Signaling Technology, Danvers, MA, USA) containing 1× HALT Protease and Phosphatase Inhibitor (Thermo Scientific, Waltham, MA, USA). Protein concentration of the samples was measured using the bicinchoninic acid assay kit (Thermo Scientific, Waltham, MA, USA). Equal protein amounts (25 μg) from each sample were mixed with 1/4 volume of Laemmli buffer with β-mercaptoethanol (Thermo Scientific, Waltham, MA, USA) and were loaded on Tris-glycine sodium dodecyl sulfate-polyacrylamide (4–20%) gels (Bio-Rad, Hercules, CA, USA). After electrophoresis-based separation, proteins were transferred onto polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA) with Trans-Blot® TurboTM Transfer System (Bio-Rad, Hercules, CA, USA). The membranes were blocked for 2 h at room temperature in 5% BSA (Bio-Rad, Hercules, CA, USA) in Tris-buffered saline with 0.05% Tween-20 (0.05% TBS-T; Sigma, St. Louis, MO, USA). Next, the membranes were incubated overnight at 4 °C with anti-DPP-4 primary antibody (1:1000 dilution). Next, three washes were performed in TBS-T, and then membranes were incubated with HRP-conjugated anti-rabbit secondary antibodies (Cell Signaling, Danvers, MA, USA) for 2 h and washed in TBS-T. Signals were detected after the incubation with enhanced chemiluminescence kit (Bio-Rad, Hercules, CA, USA) by Chemidoc XRS+ (Bio-Rad, Hercules, CA, USA). Image analysis was performed with the Image Lab™ 6.0 software (Bio-Rad, Hercules, CA, USA). Determination of (glyceraldehyde 3-phosphate dehydrogenase) GAPDH was used as loading control. Membranes were incubated with Restore Stripping Buffer for 15 min at room temperature (Thermo Scientific, Waltham, MA, USA), and then incubation with anti-GAPDH primary antibody (1:5000 dilution, overnight at 4 °C), and HRP-conjugated anti-rabbit secondary

antibody (1:2000 dilution, 2 h at room temperature) were performed. Afterwards signals were detected, as described previously.

3.9. Radioimmunoassay

Human interventricular septum samples were homogenized in 1 mL of 20 mM phosphate buffer (K_2HPO_4 and KH_2PO_4, pH: 7.2) and 10 μL protease inhibitor (Gordox, 10,000 KIE/mL, Gedeon Richter Plc, Budapest, Hungary) with tissue homogenizer device (IKA T25 Digital ULTRA TURRAX) (152). Next, centrifugation was performed at 10,000 rpm at 4 °C for 15 min. The supernatant was collected and pooled at −80 °C. SP-like immunoreactivity was measured by a specific and sensitive radioimmunoassay method (described previously (166)) using the substance P competitive radioimmunoassay kit (cat. no. RK-061-05, Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA). Here, the protocol is describe only briefly. Reconstituted positive controls and standards, 100 mL of tissue homogenates in duplicates, and 100 μL antiserum were incubated overnight at 4 °C in test tubes. Next, 100 μL ^{125}I-labelled SP tracer was added to the tubes on the following day and another overnight incubation was performed at 4 °C. Goat anti-rabbit IgG serum and normal rabbit serum were added to the designated tubes on the next day incubation period of 90 min. Centrifugation at 3000 rpm for at least 20 min at 4 °C was performed to collect immunocomplexes, supernatant was carefully discarded, and pellet cpm was determined by g-counter (Gamma NZ-310, Budapest, Hungary). The results were expressed as fmol SP-like immunoreactivity per mg total protein weight.

Determination of the SST-like immunoreactivity in the cardiac and plasma samples was performed similarly as it is described in this paragraph (153). Only the differences in the methodology are highlighted here briefly (153). Tissue samples were homogenized in 8x volumes (in μL) of distilled water containing 10 μL protease inhibitor (Gordox, 10,000 KIE/mL, Gedeon Richter Plc). Protein extraction was performed from 1 ml of the plasma samples with the mixture of 3 ml absolute ethanol and 10 μL 96% acetic acid. Tubes were incubated at room temperature for 30 min, followed by centrifugation at 3,000 rpm for 20 min at 4 °C. Afterwards, the supernatant was collected into a reaction tube and it was dried under a nitrogen flow for 6 h at room temperature. Next, it was resuspended in 300 μl of the assay buffer and centrifuged at 10,000 rpm at 4 °C for 15 min before radioimmunoassay. According to the aim of the measurement, ^{125}I-labeled

somatostatin-14 tracer (3,000 cpm/tube) was used and the antiserum was raised in sheep against somatostatin-14-bovine thyroglobulin. The results were expressed as fmol SST-like immunoreactivity per mg total protein weight in the tissue and per mL in the plasma samples.

3.10. ELISA

Determination of NPY levels in human interventricular septum samples was performed using RayBio® Human/Mouse/Rat Neuropeptide Y competitive Enzyme Immunoassay Kit (cat.nr.: EIA-NPY, RayBiotech Life Inc., Peachtree Corners, GA, USA) following the manufacturer's instructions (152). We only briefly describe here the summary of the protocol. First, 100 µL of the anti-NPY antibody solution was added to each well and then incubated for 1.5 h with gentle shaking at room temperature. Next, the solution was removed and the wells were washed with 200–300 µL of 1× wash buffer solution, which was repeated four times. After the last wash, the wash buffer was removed and the plate was inverted and blotted against clean paper towels. Afterwards, 100 µL of standard reagents, positive control, and samples were added to the appropriate wells. The wells were covered and incubated overnight at 4 °C with gentle shaking. Next, the solutions were removed, and the wells were washed four times with 1× wash buffer solution. 100 µL of the prepared HRP-streptavidin solution was added and the plate was incubated for 45 min at room temperature with gentle shaking. After the following washing step, 100 µL of TMB One-Step Substrate Reagent was added and it was incubated for 30 min at room temperature with gentle shaking, while protecting the assay from light. At the end of the incubation, 50 µL of stop solution was added and color intensity measurement was performed at 450 nm with Labsystems DC plate reader. Results were expressed as ng of NPY/mg of total protein content. Total protein concentration was measured with a bicinchoninic acid assay kit (Thermo Scientific Pierce Protein Research Products, Rockford, IL, USA) using bovine serum albumin as a standard.

3.11. Total RNA isolation and real-time quantitative PCR

Total RNA from porcine left ventricle samples (ischemic zone) was isolated with Direct-Zol RNA Mini Prep (Zymo Research, Irvine, CA, United States) following the manufacturer's instructions (153). RNA was treated with DNase I (Zymo Research,

Irvine, CA, United States), and RNA concentration was measured by spectrophotometer (NanoDrop ND-1000, NanoDrop Technologies Inc., Wilmington, DE, United States). 1 μg of total RNA was reverse transcribed into complementary DNA with Maxima™ First Strand cDNA Synthesis Kit for real-time quantitative PCR (RT-qPCR) (Thermo Scientific, Waltham, MA, United States).

The expression of SST and SSTRs was assessed with Biometria TProfessional Basic Gradient PCR equipment (Biometra GmbH, Göttingen, Germany). Identification of PCR products were performed by agarose (2%) gel electrophoresis.

Relative gene expression ratios were measured by Stratagene Mx3000P QPCR System (Agilent Technologies, Santa Clara, CA, United States). β-*ACTIN* was used as a reference gene based on pilot experiments and literature data (167). Amplification of target genes were carried out using 1x Luminaris HiGreen Low ROX qPCR Master Mix (Thermo Scientific, Waltham, MA, United States). Amplifications were performed under the following conditions: 95°C 10 min, followed by 40 cycles of 95 °C 30 s, 60 °C 30 s, and 72°C 45 s. Dissociation curve analysis were also performed. Primers and product lengths for each gene are listed in the supplementary file of the corresponding publication (153).

3.12. Total RNA isolation and RNA-sequencing

Human heart samples were lysed in 1 ml QIAzol Lysis Reagent (QIAGEN, Hombrechtikon, Switzerland) (153). Total RNA was extracted from 350 μl of the lysates with Direct-Zol RNA Mini Prep System with DNase I treatment according to the manufacturer's protocol (Zymo Research, Irvine, CA, United States). The RNA integrity numbers and RNA concentration were measured by RNA ScreenTape system with 2200 Tapestation (Agilent Technologies, Santa Clara, CA, United States) and RNA HS Assay Kit with Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, United States), respectively.

For Gene Expression Profiling library construction, QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen GmbH, Wien, Austria) was applied following the manufacturer's protocol. The quality and quantity of the library were measured by using High Sensitivity DNA1000 ScreenTape system with 2200 Tapestation (Agilent Technologies, Santa Clara, CA, United States) and dsDNA HS Assay Kit with Qubit 3.0

Fluorometer (Thermo Fisher Scientific, Waltham, MA, United States), respectively. Pooled libraries were diluted to 1.8 pM for 1 × 86 bp single-end sequencing with 75-cycle High Output v2.5 Kit on the NextSeq 500 Sequencing System (Illumina, San Diego, CA, United States) using the manufacturer's protocol. RNA-sequencing datasets of the human samples are deposited in the ArrayExpress database (accession number: E-MTAB-10720). Out of this data set, here, we present the SST and SSTR1-5 genes-related sequencing data from control human cardiac samples.

3.13. Analysis of mRNA-sequencing data

3.13.1. Pig heart samples

mRNA-sequencing data of left ventricle samples of pig myocardial infarction model (168) were analyzed (153). The detailed protocol of the mRNA-sequencing method was described previously in (168). Alignment of the sequencing reads to the Sus scrofa reference genome assembly (Swine Genome Sequencing Consortium Sscrofa10.2/susScr3 UCSC) (169), feature counting for the corresponding reference annotation, and the statistical analysis of differential expression were performed by the TopHat-Cufflinks workflow as described in (170). In this analysis, TopHat version 2.1.1 (171), Bowtie2 version 2.2.3 (172), and Cufflinks version 2.2.1 (170) were used.

3.13.2. Human heart samples

Raw reads determined by RNA-sequencing of the human samples (153) were preprocessed by Cutadapt (version 1.15) (173). During this step adapters, the poly(A) tail and bases below Phred score 30 were trimmed, and reads with a length of less than 19 nt were filtered (174). Quality control was performed using the FastQC (version 0.11.8) and MultiQC (version 1.7) (175) software. HISAT2 (version 2.0.4) (176), featureCounts (version of Subread 2.0.0) (177), and DESeq2 (version 1.10.1) (178) were utilized for alignment, annotation, normalization, and differential expression analysis, respectively. *Homo sapiens* Ensembl GRCh37 reference genome and annotation were applied for the analysis of the human samples (179). Differential expression analysis was performed by the DESeq2 software package (178).

3.14. Gene Ontology (GO) enrichment analysis

GO enrichment analysis (database version released on August 10, 2020) was conducted for each of the possible comparison of the experimental groups (153). In order to obtain GO biological process terms enriched among differentially expressed genes compared to the Sus scrofa reference gene list, online PANTHER Overrepresentation Test [geneontology.org, version released on July 28, 2020 (180) was used. Enrichment analysis was performed by applying Fisher's exact test with false discovery rate adjustment for multiple comparisons (181).

3.15. Statistical analysis

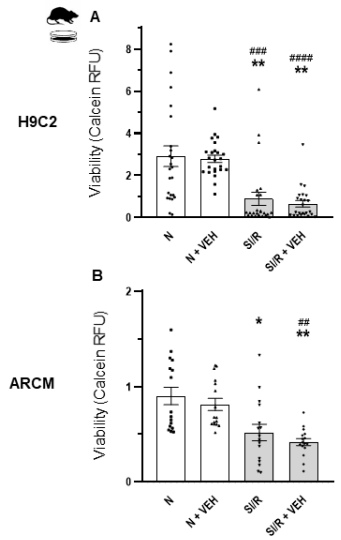
Statistical analyses and Rout outlier analyses were performed and graphs were created using GraphPad Prism version 8 (GraphPad Software, San Diego, CA, United States) (152, 153). Kruskal-Wallis test with Dunn's post hoc test, one-way analysis of variance (ANOVA), two-way ANOVA, unpaired t-test, and Mann–Whitney test were used to find statistically significant differences. Those were considered significant at values of $p < 0.05$. Unless noted otherwise, all data represent the mean ± SEM. In order to avoid the possibility of overlooking significant differences due to small group sizes, ANOVA-like nonparametric bootstrap-based comparison of means with 1,000 times resampling (182) was also performed on relative expression ratios assessed by RT-qPCR.

4. Results

4.1. Investigation of the direct cardiocytoprotective effect of SST against simulated ischemia/reperfusion injury in different cell culture models

4.1.1. SST-treatment increased the viability of H9C2 cells and adult rat cardiomyocytes subjected to simulated ischemia/reperfusion

In order to investigate the potential cardiocytoprotective effect of SST, we performed *in vitro* simulated ischemia/reperfusion experiments in H9C2 cells and adult rat cardiomyocytes, respectively (153). Simulated ischemia/reperfusion significantly decreased both the viability of the H9C2 cells and adult rat cardiomyocytes (Figure 1) compared to normoxic controls.



Comparisons	Summary	p-value
N vs. N + VEH	ns	>0.9999
N vs. SI/R	**	0.0045
N vs. SI/R + VEH	**	0.0013
N + VEH vs. SI/R	###	0.0003
N + VEH vs. SI/R + VEH	####	<0.0001
SI/R vs. SI/R + VEH	ns	>0.9999

Comparisons	Summary	p-value
N vs. N + VEH	ns	>0.9999
N vs. SI/R	*	0.0376
N vs. SI/R + VEH	**	0.0017
N + VEH vs. SI/R	ns	0.0628
N + VEH vs. SI/R + VEH	##	0.0032
SI/R vs. SI/R + VEH	ns	>0.9999

Figure 1. – Effect of simulated ischemia-reperfusion on the cell viability. *Viability of H9C2 (**A**) cells and adult rat cardiomyocytes (ARCM) (**B**) after in vitro simulated ischemia-reperfusion experiment. Data are presented as mean ± SEM. Kruskal-Wallis test, Dunn's posthoc test */#p<0.05 **/##p<0.01, ***/###p<0.001, ****/####p<0.0001. N: Normoxia, VEH: vehicle, SI/R: simulated ischemia/reperfusion, RFU: relative fluorescence unit (153).*

We have found that the simulated ischemia/reperfusion-induced cell death was attenuated by SST treatment at 100 and 300 nM (Figure 2C) showing a concentration-dependent, bell-shaped cardiocytoprotective effect in H9C2 cells. SST did not alter the viability of normoxic cells (Figure 2B), except for the highest concentration of SST (1 μM) that caused a significant cell death in H9C2 cells (153).

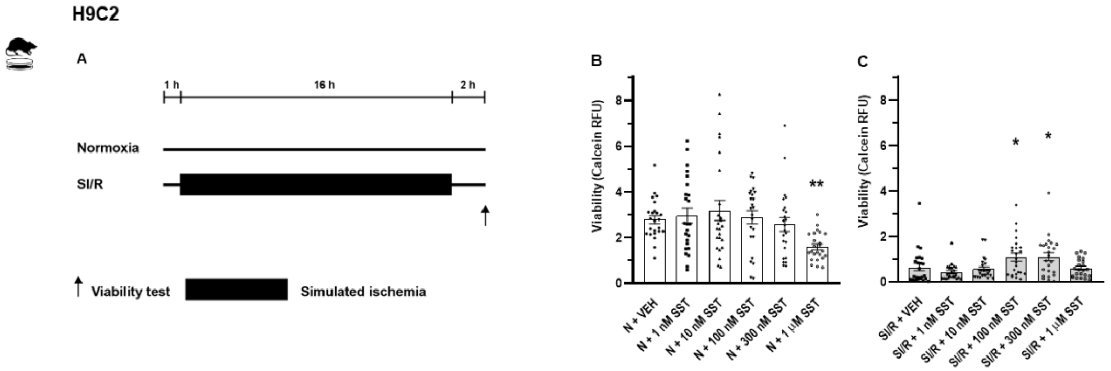


Figure 2. - Effect of SST-treatment on the viability of H9C2 cells. *In vitro simulated ischemia/reperfusion (SI/R) study protocol on H9C2 cell line (**A**). Somatostatin (SST) (1 nM, 10 nM, 100 nM, and 300 nM and 1 μM) or its vehicle (VEH) was added to the cells during the whole experiment. Viability of SST-treated H9C2 cells exposed to normoxia (N) (**B**) or SI/R (**C**). Data are expressed as mean ± SEM. Statistical analysis: Kruskal-Wallis test and Dunn's post hoc test, *p < 0.05; **p < 0.01 vs. VEH from four independent experiments. RFU: relative fluorescence unit (153).*

SST also shows a concentration-dependent, bell-shaped cardiocytoprotective effect against simulated ischemia/reperfusion-induced injury in adult rat cardiomyocytes (Figure 3C) where SST treatment at 1, 10, 100, and 300 nM concentrations increased the viability significantly (153). Although the cell viability shows a similar dose-response pattern in the SST-treated groups in normoxic conditions (Figure 3B), these changes were not statistically significant (153).

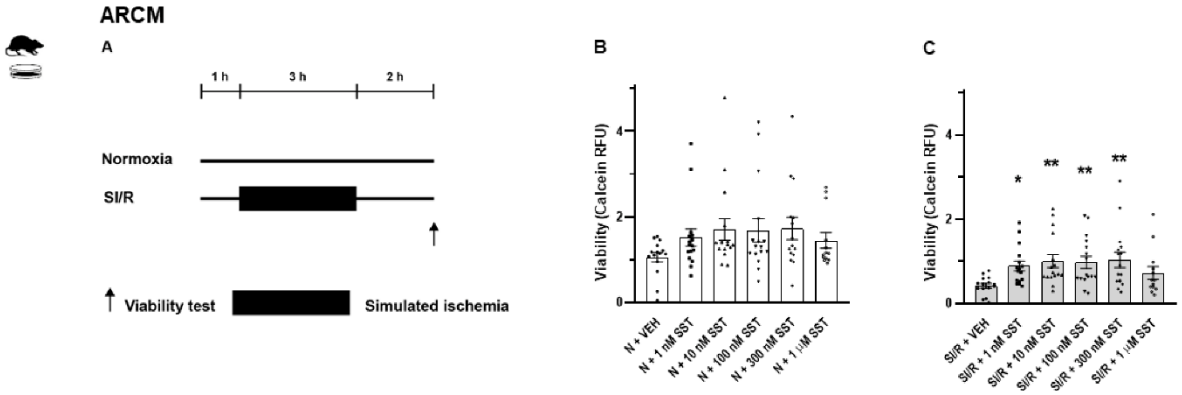


Figure 3. - Effect of SST-treatment on the viability of isolated adult rat cardiomyocytes. *In vitro simulated ischemia/reperfusion (SI/R) study protocol on isolated adult rat cardiomyocytes (ARCM) (**A**). Somatostatin (SST) (1 nM, 10 nM, 100 nM, and 300 nM and 1 µM) or its vehicle (VEH) was added to the cells during the whole experiment. Viability of SST-treated ARCM cells exposed to normoxia (N) (**B**) or SI/R (**C**). Data are expressed as mean ± SEM. Statistical analysis: Kruskal-Wallis test and Dunn's post hoc test, *p < 0.05; **p < 0.01 vs. VEH from four independent experiments. RFU: relative fluorescence unit (153).*

4.2. Investigation of the expression of SST and its receptors in a translationally relevant acute myocardial infarction pig model and healthy human hearts

4.2.1. Ischemic preconditioning increased the expression of SST in the pig plasma

After we have demonstrated that the native SST exerts a direct cardiocytoprotective effect *in vitro*, we have aimed to further support the protective role of SST with data obtained from a translational porcine model of myocardial infarction and cardioprotection, and to investigate the expression of various SST receptors (153). Accordingly, we used tissue and plasma samples collected in our previous porcine myocardial infarction and cardioprotection study with different ischemic conditioning interventions (151). In order to determine SST-like immunoreactivity in pig plasma and left ventricle samples, we performed radioimmunoassay experiments. We detected SST-like immunoreactivity in pig left ventricle samples, but we found no differences between groups (Figure 4C). The plasma SST-like immunoreactivity increased significantly in ischemic preconditioning samples compared to the sham group (Figure 4B) (153).

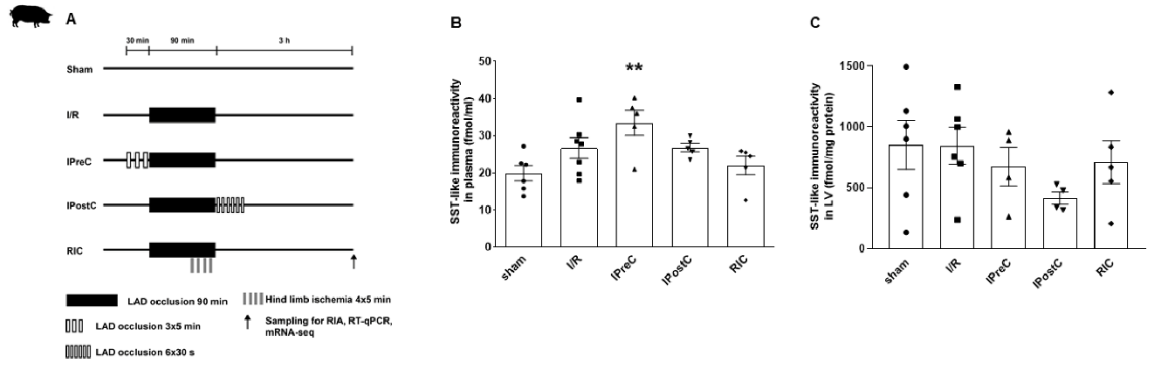


Figure 4. – SST expression in porcine plasma and left ventricle samples. *Closed-chest porcine acute myocardial infarction study protocol (**A**). Somatostatin-like immunoreactivity of plasma (**B**) and left ventricular samples (**C**). Data are expressed as mean ± SEM **p < 0.01 vs. sham (n = 6). Statistical analysis: one-way ANOVA, and Tukey post hoc test, I/R: ischemia-reperfusion (n = 7 in plasma and 6 in LV), IPreC: ischemic preconditioning (n = 5 in plasma and 4 in LV), IPostC: ischemic postconditioning (n = 5 in plasma and 4 in LV), RIC: remote ischemic conditioning (both n = 5), LAD: left anterior descending coronary artery, and LV: left ventricle (153)*

4.2.2. The expression of SSTR1 mRNA increased in porcine left ventricle due to remote ischemic conditioning

Next, we aimed to detect *SST* and its receptor mRNA expression in pig left ventricle, therefore we performed RT-qPCR and bioinformatics analysis of mRNA-sequencing data (153). In addition, we performed a bootstrap-based comparison of means to confirm the RT-qPCR results. We detected the expression of *SSTR1* and *SSTR2* mRNA by PCR, but not *SST* and its other receptors (*SSTR3*, *SSTR4*, and *SSTR5*) (Figure 5A) (153).

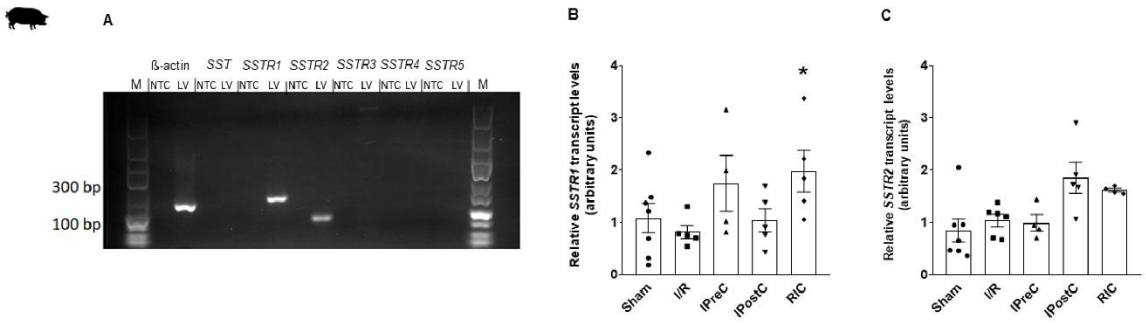


Figure 5. – Expression of SST and its receptors in various porcine left ventricle samples. *Expression of somatostatin and its receptors in healthy porcine left ventricle samples (**A**). The outer lanes contain a DNA ladder (M). No template control (NTC) samples without cDNA did not give any amplification products with the used primers, indicating that contamination was not present. Product sizes: β-actin:*

*133 bp; SST: 117 bp; SSTR1: 139 bp; SSTR2: 82 bp; SSTR3: 110 bp; SSTR4: 112 bp; SSTR5: 98 bp. Relative gene expression ratios of SSTR1 (**B**) and SSTR2 (**C**) in left ventricle samples. Transcript levels were normalized to ß-actin. Data are expressed as mean ± SEM. Statistical analysis: Kruskal-Wallis test and Dunn's post hoc test, *p < 0.05 vs. I/R. SSTR1–5: somatostatin receptors one to five, I/R: ischemia-reperfusion (SSTR1: n = 5, SSTR2: n = 6), IPreC: ischemic preconditioning (n = 4), IPostC: ischemic postconditioning (n = 5), and RIC: remote ischemic conditioning (SSTR1: n = 5, SSTR2: n = 4) and sham-operated group (n = 7) (153).*

The results of the bioinformatics analysis of mRNA-sequencing data confirmed all these results (Table 2) (153). We identified a significantly upregulated relative expression of *SSTR1* in the remote ischemic conditioning group compared to the ischemia/reperfusion group (Figure 5B). There were no significant differences in *SSTR2* mRNA expression between any groups (Figure 5C) (153). The bootstrap analysis also confirmed these results.

Table 2. - Expression of SST and its receptors in porcine left ventricular samples (sham group) measured by mRNA sequencing

Gene	Sample	Presence	FPKM
SST	LV	undetectable	0
SSTR1	LV	detectable	0.3881
SSTR2	LV	detectable	0.3571
SSTR3	LV	undetectable	0
SSTR5	LV	undetectable	0

LV: left ventricle, SST: somatostatin, SSTR1-5: somatostatin receptors one to five, and FPKM: fragments per kilobase of transcript per million mapped reads (153)

4.2.3. SST expression decreased in the interventricular septum samples of patients with ischemic cardiomyopathy

In order to determine SST-like immunoreactivity also in human interventricular septum samples, we performed radioimmunoassay experiments in cardiac samples of both healthy patients and patients with ischemic cardiomyopathy (153). (Since our focus in this study is the cardioprotective effect of SST against ischemia-reperfusion injury cardiac samples of patients with dilated cardiomyopathy were excluded here.) The patient's clinical characteristics are described in Figure 6A. We measured a significant decrease of tissue SST-like immunoreactivity in ICM samples compared to the control group (Figure 6B).

A

Parameter	CON	ICM
Gender (male/female)	4/6	9/1
Age	44.6 (24-44)	64.7 (48-77)
Body mass index (kg/m^2)	26.4 (20.3-33.9)	25.9 (20.0-32.8)
Ejection fraction (%)	-	21 (10-33)
N-terminal pro-BNP (pg/mL)	-	3070 (338-7699)
Medications		
ACE inhibitors	0	8
Beta receptor blockers	1	10
Norepinephrine	10	0
Digoxin	0	2
Amiodarone	0	1
Diuretics	2	10
Mineralocorticoid receptor inhibitor	0	10
Statins	0	10
Antiplatelets	1	3

B

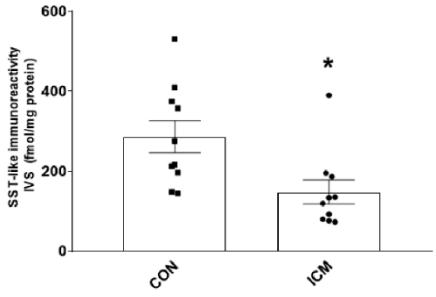


Figure 6. – SST expression in chronic heart failure. *Control and ICM patient's characteristics (**A**). SST-like immunoreactivity of interventricular septum samples (**B**). Data are presented as mean ± SEM. unpaired t-test *p < 0.05 vs. CON (n = 10–10), ICM: ischemic cardiomyopathy, and CON: control (153).*

4.2.4. SSTR1, SSTR2, and SSTR5 mRNA was expressed in human left ventricle

To detect *SST* and its receptor mRNA expression in human left ventricles we performed bioinformatics analysis of the mRNA-sequencing data (153). We detected the expression of the mRNA of *SSTR1, SSTR2,* and *SSTR5* receptors, but not *SST* and its other receptors (*SSTR3* and *SSTR4*) (Table 3).

Table 3. - Expression of SST and its receptors in human left ventricular samples measured by mRNA sequencing.

Gene	Sample	Presence	FPKM
SST	LV	undetectable	0
SSTR1	LV	detectable	3.950
SSTR2	LV	detectable	8.402
SSTR3	LV	undetectable	0
SSTR5	LV	detectable	49.064

LV: left ventricle, SST: somatostatin, SSTR1-5: somatostatin receptors one to five (153).

4.2.5. SSTR1 and SSTR2 mRNA was mainly expressed by vascular endothelial cells of the human left ventricle

In order to determine the SSTR expression more precisely we performed RNAscope *in situ* hybridization assay for the mRNA of *SSTR1 and SSTR2* in human healthy left ventricular samples (Figures 7–10) (153). We found that expression of the mRNA of both *SSTR1* and *SSTR2* are localized primarily in *PECAM-1* mRNA-positive endothelial cells (Figure 7A,B, respectively); however, both *SSTR1* and *SSTR2* mRNA were detected in other cell types, including *TAGLN* mRNA-positive vascular smooth muscle cells (Figure 8) and *RYR2* mRNA-positive cardiomyocytes (Figure 9). There was no detectable signal on the negative control slides (Figure 10) (153).

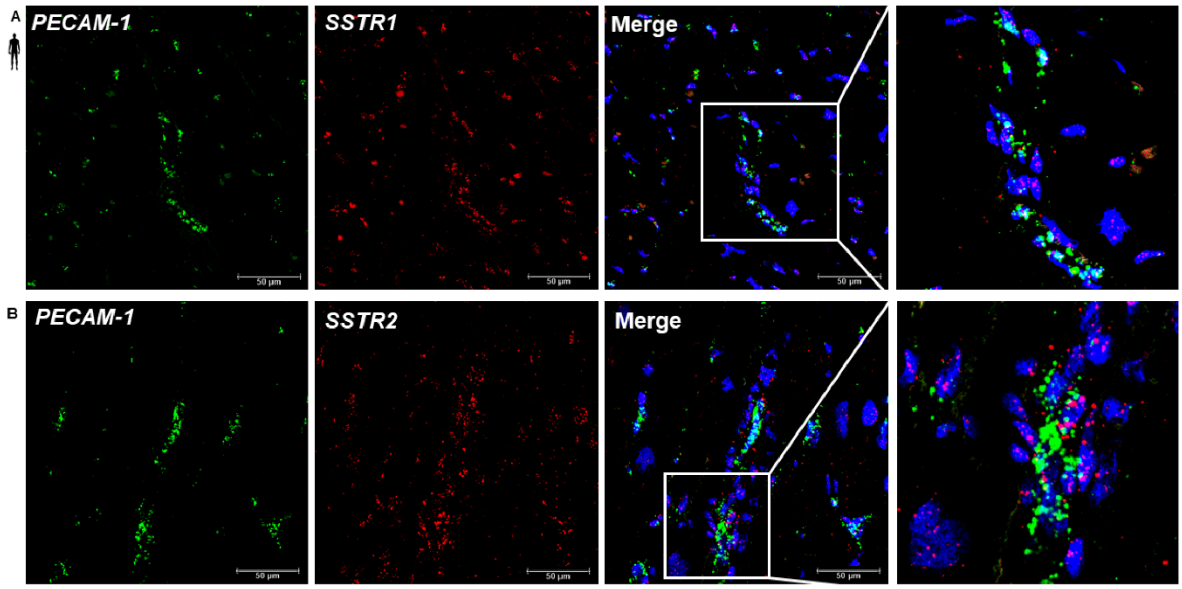


Figure 7. - Representative confocal microscopy images of the SSTR1 and SSTR2 expression in vascular endothelial cells of the healthy human left ventricle. *RNAscope-SSTR1 (**A**) and SSTR2 (**B**) mRNA expression in histological samples of human control left ventricle. Nuclei were stained with DAPI (blue). Fluorescein-labeled tyramide (green) indicates the mRNA of PECAM-1 (endothelial marker) and cyanine 3- (Cy3-) labeled tyramide (red) indicates the mRNA of SSTR1 or SSTR2, respectively. Scale bar = 50 µm (153).*

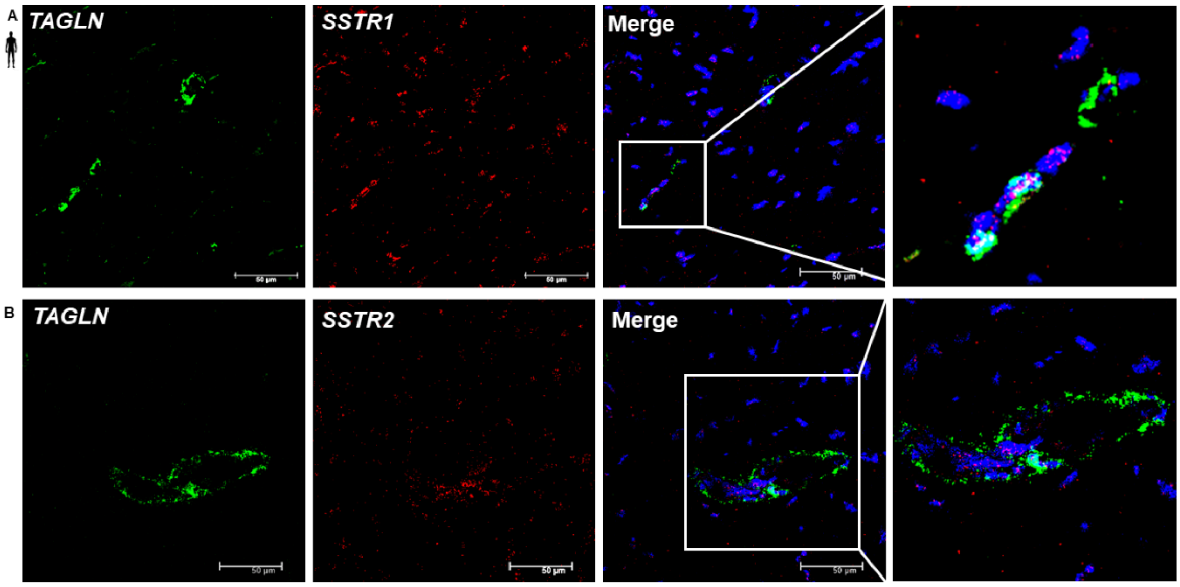


Figure 8. - Representative confocal microscopy images of the SSTR1 and SSTR2 expression in smooth muscle cells of the healthy human left ventricle. *RNAscope-SSTR1 (**A**) and SSTR2 (**B**) mRNA expression in histological samples of human control left ventricle. Nuclei were stained with DAPI (blue). Fluorescein-labeled tyramide (green) indicates the mRNA of TAGLN (smooth muscle marker) and Cy3-labeled tyramide (red) indicates the mRNA of SSTR1 or SSTR2, respectively. Scale bar = 50 µm (153).*

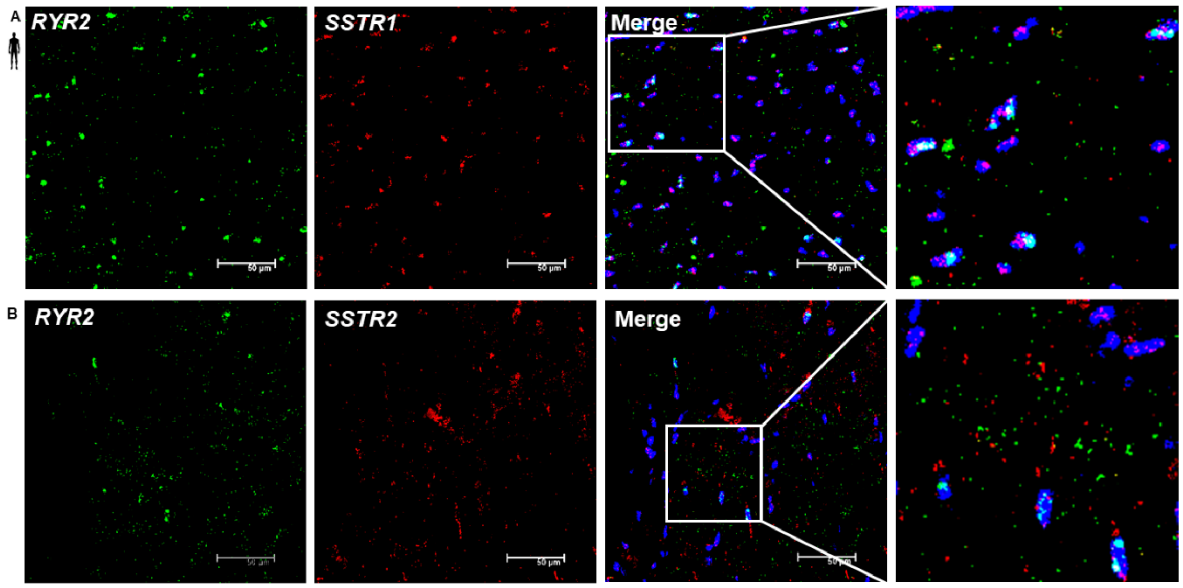


Figure 9. - Representative confocal microscopy images of the SSTR1 and SSTR2 expression in cardiomyocytes of the healthy human left ventricle. *RNA Scope-SSTR1 (**A**) and SSTR2 (**B**) mRNA expression in histological samples of human control left ventricle. Nuclei were stained with DAPI (blue).*

Fluorescein-labeled tyramide (green) indicates the mRNA of RYR2 (myocardial marker) and Cy3-labeled tyramide (red) indicates the mRNA of SSTR1 or SSTR2, respectively. Scale bar = 50 µm (153).

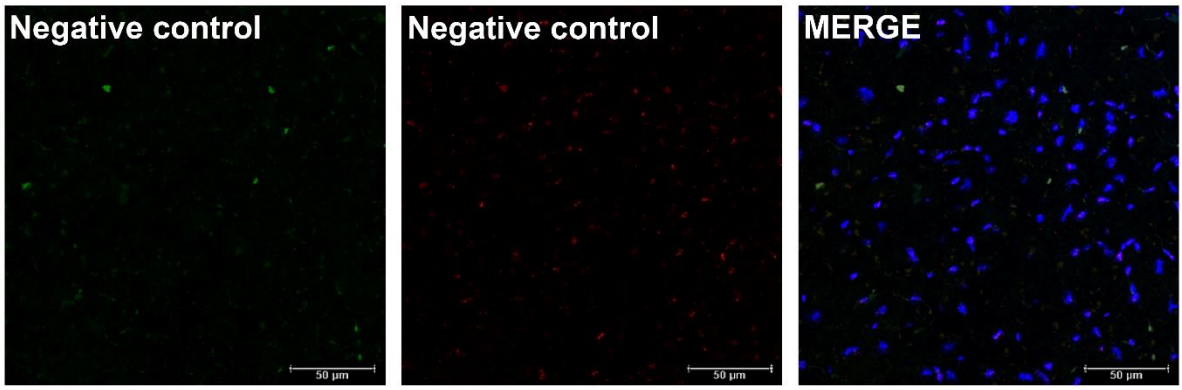


Figure 10. - Representative confocal microscopy images of RNA Scope negative control in histological samples of human control left ventricle. Cells were counterstained with DAPI (blue). Both the fluorescein-labeled tyramide (green) and Cy3-labeled tyramide (red) signals represent the mRNA of Bacillus subtilis dihydrodipicolinate reductase (DapB, negative control) in histological samples of human control left ventricle. Scale bar = 50 µm (153).

4.2.6. GO analysis of mRNA-sequencing data highlighted biological processes altered due to remote ischemic conditioning

To identify the biological processes those could contribute to the cytoprotective effect of SST, SSTR1 and SSTR2 we performed the Gene Ontology (GO) analysis of all differentially expressed mRNAs for each possible comparison of the experimental groups (153). The top thirty results of the comparison between remote ischemic conditioning and ischemia/reperfusion groups—where *SSTR1* expression significantly increased—are presented here. (The full list of these results are available as the Supplementary Table S3 of the published article (153)). Differentially expressed mRNAs were significantly associated with, e.g., cardiac muscle differentiation, skeletal muscle development/regeneration, and response to oxidative stress (Figure 11).

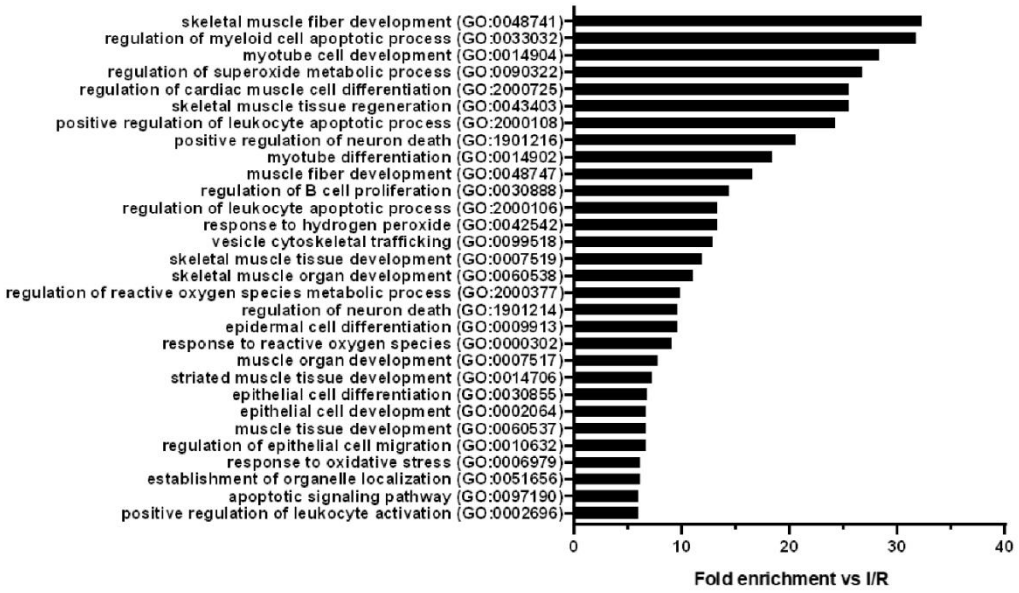


Figure 11. – Biological processes in those the differentially expressed mRNAs enriched in the comparison between remote ischemic conditioning and ischemia/reperfusion groups. *Top thirty Gene Ontology (GO) biological process terms significantly enriched among the mRNAs (n = 143) differentially expressed in the comparison between remote ischemic conditioning (RIC) and ischemia/reperfusion (I/R) groups in the order of decreasing fold enrichment values. GO enrichment analysis highlights the effect of RIC on skeletal muscle fiber development, muscle tissue regeneration, cardiac muscle cell differentiation, superoxide metabolic processes, reactive oxygen species metabolic processes, and response to oxidative stress. Adjusted Fisher's exact p values were <0.05 in case of each shown process after false discovery rate adjustment for multiple comparisons (153).*

4.3. Expression of DPP4 and its neuropeptide substrates in the human heart and cell culture samples

4.3.1. Expression of DPP4 and NPY decreased in human failing heart samples

First, we aimed to determine the expression of DPP4, NPY and SP at the protein level, therefore we performed Western blot, enzyme-linked immunosorbent assay (ELISA), and radioimmunoassay experiments in interventricular septum samples of failing human hearts (ischemic cardiomyopathy (ICM) and dilated cardiomyopathy (DCM)), and also healthy control (CON) hearts (152). Patients' characteristics are presented in Table 4 and 5.

Table 4. – Patient characteristics in Western blot experiments. *This table presents the clinical parameters of the human interventricular tissue samples used in Western blot experiments. Data are expressed as mean and range. CON – control, ICM - ischemic cardiomyopathy, DCM - dilated cardiomyopathy (152).*

Parameter	**CON**	**ICM**	**DCM**
Gender (male/female)	6/2	8/1	8/0
Age (year)	33 (23-52)	58 (39-66)	48 (27-58)
BMI (kg/m^2)	25 (20-32)	27 (20-33)	29 (21-40)
Ejection fraction (%)	-	21 (13-33)	19 (5-33)
N-terminal pro-BNP (pg/ml)	-	3328 (338-8024)	5308 (1001-14750)
Medications			
Norepinephrine	7	0	1
ACE inhibitors	0	7	6
Beta receptor blockers	1	9	8
Mineralocorticoid receptor inhibitors	0	9	2
Furosemide	1	0	0
Mannitol	1	0	0
Digoxin	0	1	1
Mexiletine	0	1	1
Amiodarone	0	3	5
Anticoagulants	1	5	4
Antiplatelets	0	7	0
Dopamine	0	3	3
Dobutamine	0	3	4
Sildenafil	0	3	2
Statins	0	9	2
Allopurinol	0	1	3

Table 5. – Patient characteristics in radioimmunoassay and ELISA experiments. *This table presents the clinical parameters of the human interventricular tissue samples used in radioimmunoassay and ELISA experiments. Data are expressed as mean and range. CON – control, ICM - ischemic cardiomyopathy, DCM - dilated cardiomyopathy (152).*

Parameter	**CON**	**ICM**	**DCM**
Gender (male/female)	4/6	9/1	10/0
Age (year)	38 (17-52)	62 (45-74)	48 (27-60)
BMI (kg/m^2)	26 (20-34)	26 (20-33)	27 (20-39)
Ejection fraction (%)	-	21 (10-33)	17 (5-25)
N-terminal pro-BNP (pg/ml)	-	3071 (338-7699)	8632 (1001 - 35000)
Medications:			
Norepinephrine	10	0	1
ACE inhibitors	0	8	8
Beta receptor blockers	1	10	10
Mineralocorticoid receptor inhibitors	0	10	9
Furosemide	2	0	0
Mannitol	2	0	0

Digoxin	0	2	2
Mexiletine	0	1	1
Amiodarone	0	2	6
Anticoagulants	1	6	6
Antiplatelets	0	7	0
Dopamine	4	2	3
Dobutamine	0	2	4
Sildenafil	0	3	2
Statins	0	10	1
Allopurinol	0	4	3

We identified a significantly decreased expression of DPP4 both in the ICM and DCM samples compared to the healthy hearts (Figure 12A, B). NPY expression significantly decreased in the DCM samples and the ICM samples showed a tendency of decrease compared to the control (Figure 12C). The expression of SP remained on a similar level in each group (Figure 12D) (152).

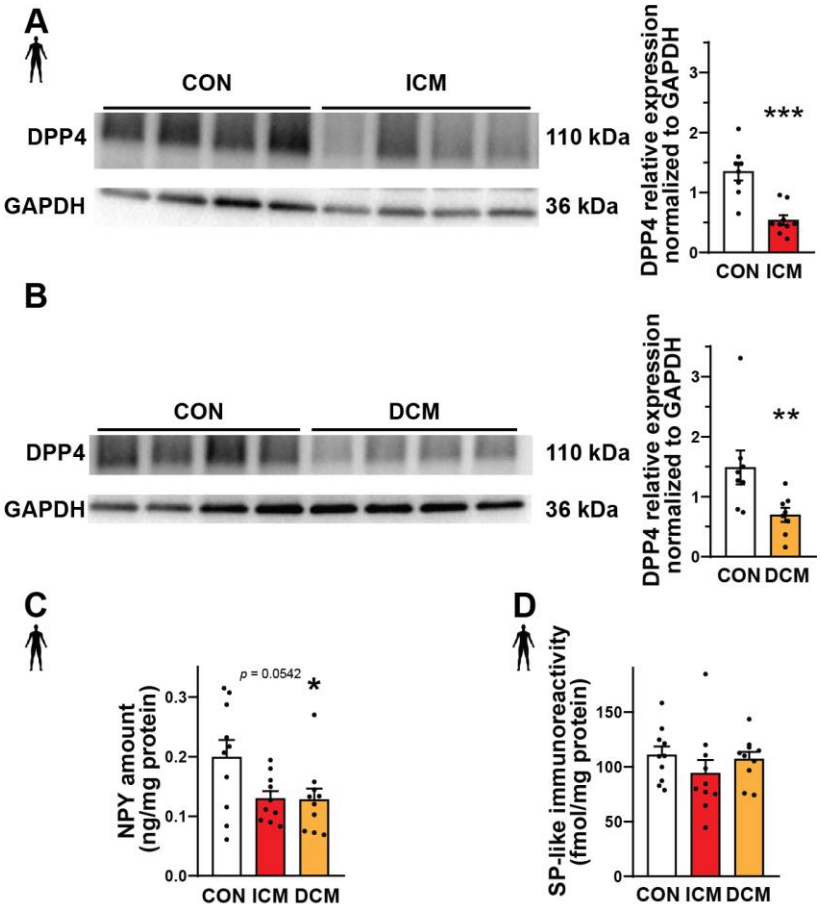


Figure 12. - Expression of DPP4, NPY, and SP in failing human heart samples. *Result of western blot analysis of DPP4 enzyme (**A**, **B**), ELISA (**C**), and radioimmunoassay (**D**). Quantification (**A**–**D**) of the DPP4, NPY, and SP content in interventricular septum samples of healthy patients (CON) or patients with ischemic (ICM) or dilated cardiomyopathy (DCM). Statistical analyses: One-way ANOVA with Tukey's post hoc test, unpaired t-test, and Mann–Whitney test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. CON, group size: $n = 8–10$. Data are expressed as mean ± SEM (152). GAPDH: glyceraldehyde 3-phosphate dehydrogenase (152)*

4.3.2. The mRNA of DPP4 was mainly localized in cardiomyocytes of the human left ventricle

Next, we aimed to determine the cell-type-specific DPP4 expression, therefore RNAscope *in situ* hybridization assay was performed on left ventricular tissue slides of healthy humans (152). We have found that the mRNA of *DPP4* is primarily expressed by ryanodine receptor 2 (*RYR2*) mRNA-positive cardiomyocytes (Figure 13A). We have also detected DPP4 expression to some extent in platelet endothelial cell adhesion

molecule 1 (*PECAM-1*) mRNA-positive endothelial cells (Figure 13B), but not in vimentin (*VIM*) mRNA-positive fibroblasts (Figure 14A) and CD68 mRNA-positive macrophages (Figure 14B). There was no detectable signal on the negative control slides (Figure 15) (152).

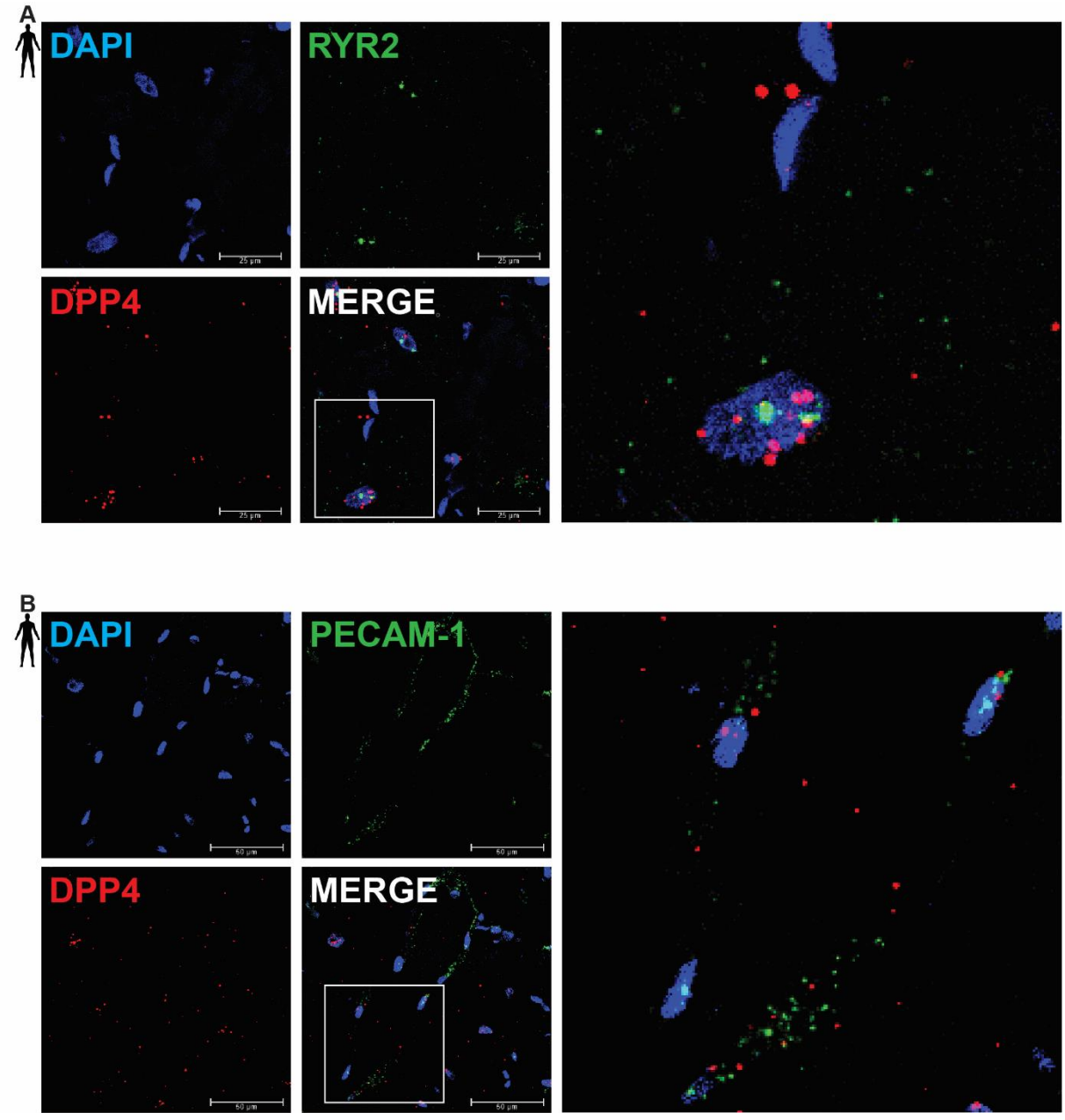


Figure 13. - Representative confocal microscopy images of RNAscope-DPP4 mRNA expression in human control left ventricle. *Nuclei were stained with DAPI (blue). Fluorescein-labeled tyramide (green) signal represents the mRNA of RYR2 (cardiomyocyte marker, (**A**)) or PECAM-1 (endothelium marker, (**B**))*

and Cy3-labeled tyramide (red) signal represents the mRNA of DPP4, respectively. Scale bars = 25/50 μm. (RYR2: ryanodine receptor 2, PECAM-1: platelet endothelial cell adhesion molecule-1) (152)

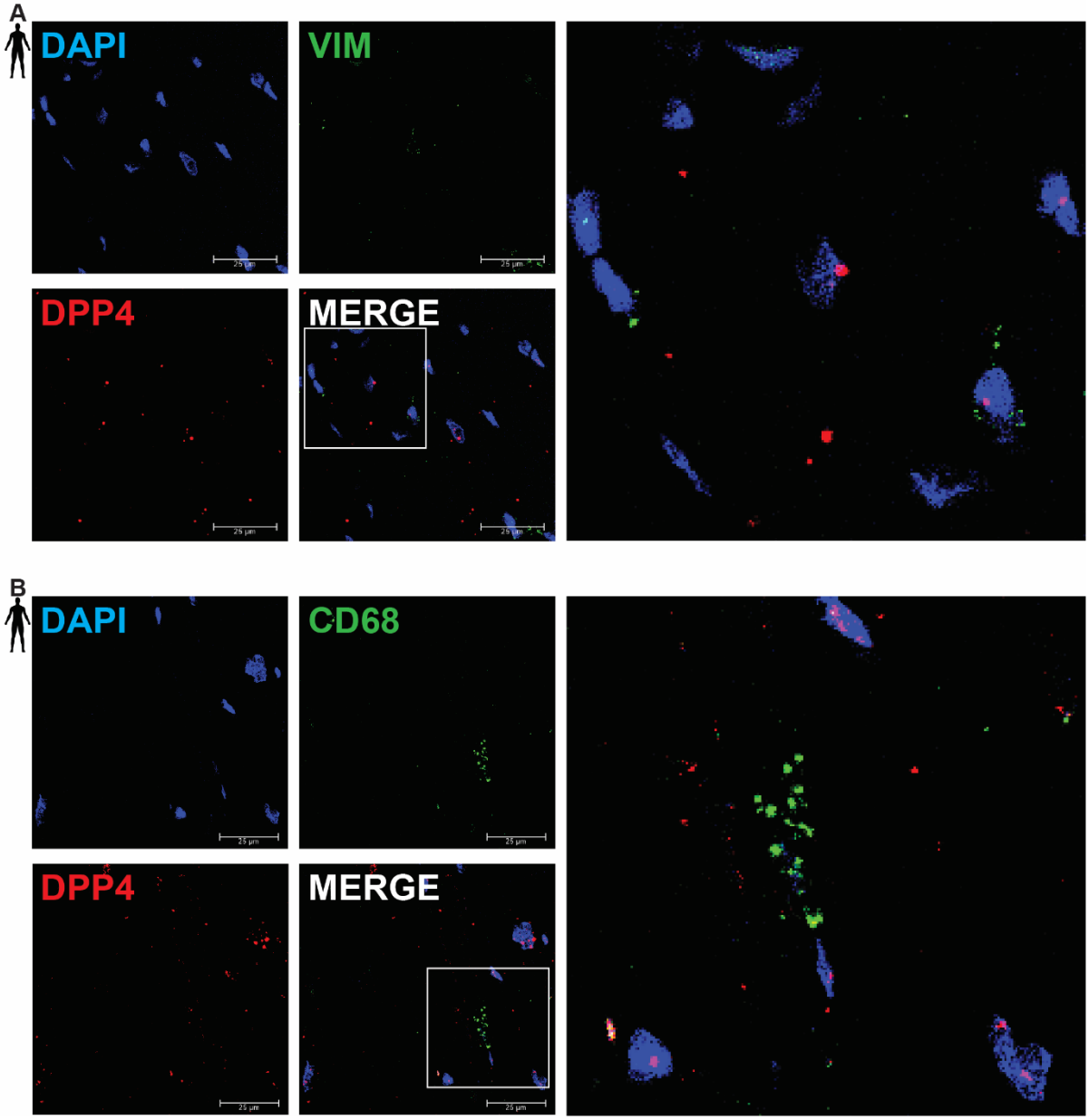


Figure 14. - Representative confocal microscopy images of RNA Scope-DPP4 mRNA expression in human control left ventricle. *Nuclei were stained with DAPI (blue). Fluorescein-labeled tyramide (green) signal represents the mRNA of VIM (fibroblast marker, (**A**)) or CD68 (macrophage marker, (**B**)) and Cy3-labeled tyramide (red) signal represents the mRNA of DPP4, respectively. Scale bar = 25 µm. (VIM: vimentin, CD68: cluster of differentiation 68) (152).*

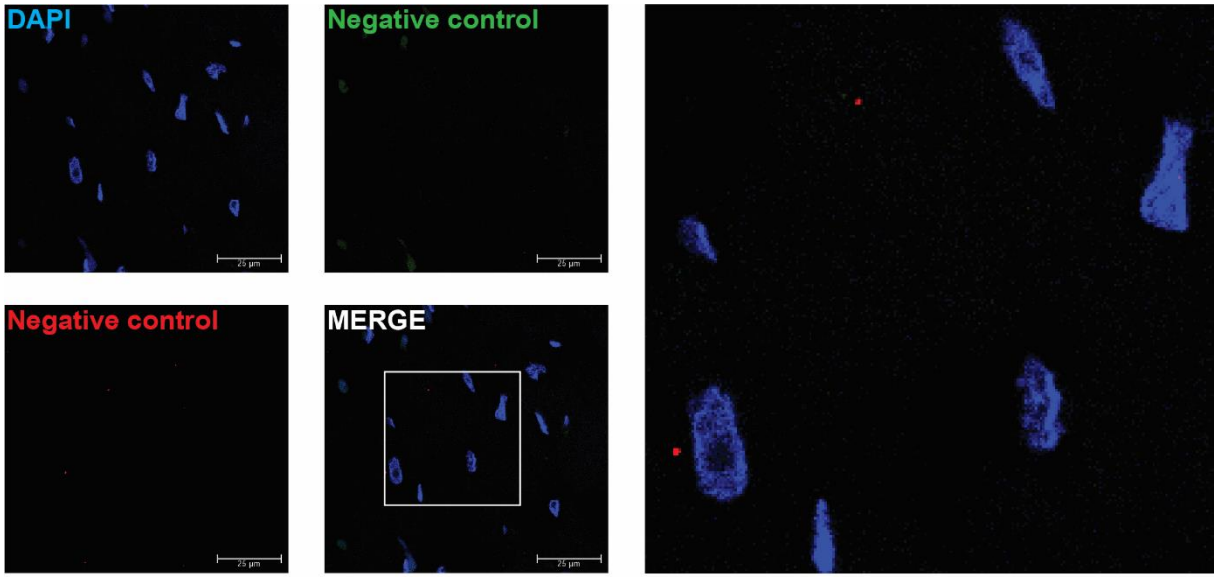


Figure 15. - Representative confocal microscopy images of RNAscope-DapB mRNA expression in human control left ventricle. *Nuclei were stained with DAPI (blue). Both the fluorescein-labeled tyramide (green) and Cy3-labeled tyramide (red) signals represent the mRNA of Bacillus subtilis dihydrodipicolinate reductase (DapB, negative control) in histological samples of human control left ventricle. Scale bar = 25 µm (152).*

These observations indicate that DPP4 is primarily expressed in cardiomyocytes and endothelial cells in the heart tissue (152).

4.4. Cell culture-based experiments for the investigation of the effect of DPP4 inhibition by saxagliptin and the role of its neuropeptide substrates.

4.4.1. Inhibition of DDP4 by saxagliptin and its neuropeptide substrates did not induce changes in the cell viability

After we successfully determined the expression profile of DPP4 and its substrates, next we tested the potential harmful effects of DPP4 inhibition and/or its substrates (SP, NPY) on cardiomyocytes (152). In order to begin the *in vitro* cell culture-based experiments, first an appropriate cell culture model was required. We chose the AC16 cell line since it expresses the DPP4 enzyme according to the results of the Western blot measurements (Figure 16A), also confirming our RNAscope results. In the beginning, we hypothesized

that direct cytotoxicity could be responsible for the increased hospitalization rate caused by saxagliptin, thus we performed calcein-based cell viability tests on AC16 cells treated with saxagliptin or other clinically relevant gliptins. We have found that DPP4 inhibition by any of the tested gliptins (saxagliptin, vildagliptin, linagliptin, alogliptin) alone, at a concentration of 500 nM, does not have a toxic effect on cell viability of AC16 cells (Figure 16B). Next, we shifted our focus to investigating whether decreased DPP4 enzyme activity by saxagliptin exerts cytotoxic effects in the presence of its substrates: NPY and SP (152). Thus, we treated the cells with NPY or SP or in combination with saxagliptin. We have found that neither neuropeptides (NPY or SP) alone nor in combination with saxagliptin decreases the viability of AC16 cells (Figure 16C). These results indicate that both saxagliptin and the neuropeptide substrates of DPP4 do not influence the viability of healthy cells; thus, a different approach and model are required to clarify the potential cardiotoxic effect of saxagliptin (152).

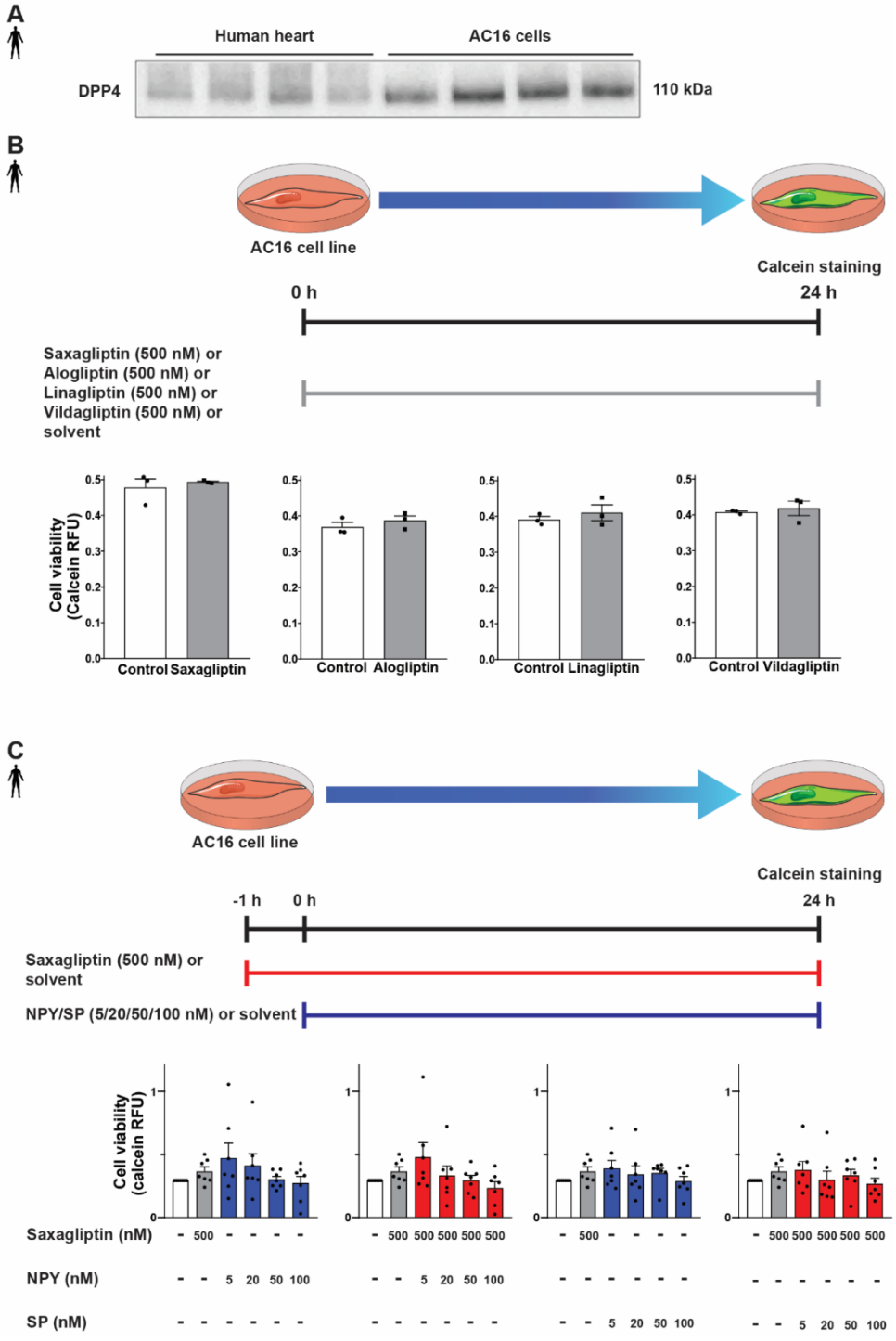


Figure 16. - Effect of DPP4 inhibition and/or neuropeptide substrates on the viability of AC16 cells. *Western blot analysis of DPP4 (**A**) in healthy human left ventricle samples and AC16 cells. Treatment protocol of cell culture experiments with various gliptins on AC16 cells and cell viability (calcein assay) results (**B**). Treatment protocol of cell culture experiments with neuropeptides and their combined administration with saxagliptin and their effect on the viability of AC16 cells (**C**). One-way ANOVA, Tukey's post hoc test, and unpaired t-test. Data are presented as mean ± SEM. Group sizes: (**B**) n = 3 from 1 independent experiment, (**C**) n = 7 from 7 independent experiments. RFU: relative fluorescence unit (152)*

4.4.2. Both saxagliptin and the neuropeptides altered the migration capacity of cardiac fibroblasts

According to the literature data SP and NPY potentially exert their harmful cardiovascular effects through the modulation of fibroblast activities (2, 61, 80, 123). In line with these data and our results that DPP4 is expressed by cardiomyocytes, we continued our experiments with a co-culture model of primary neonatal rat cardiomyocytes and cardiac fibroblasts (152). We performed scratch assay (frequently used *in vitro* model of cell migration and wound healing) experiments (Figure 17A, B) with the same treatment protocol (Figure 17A, C) (neuropeptides and/or saxagliptin) as presented in Figure 16C.

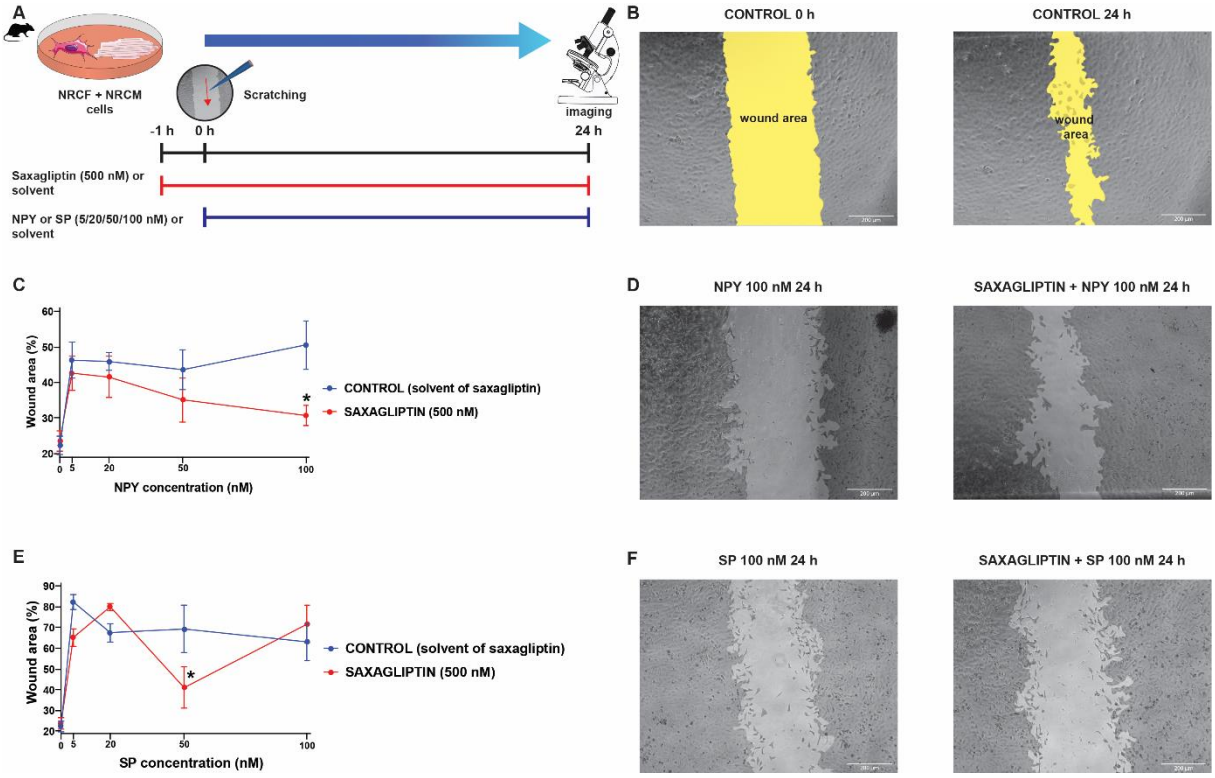


Figure 17. - The cell migration speed in the co-culture of neonatal cardiomyocytes and fibroblasts treated with saxagliptin- and/or neuropeptides. *Treatment protocol (**A**) and the results of the treatment with NPY (**C**) and SP (**E**). Representative bright field microscope images of control (**B**), NPY-treated (**D**), and SP-treated groups (**F**). Statistical analysis: Two-way ANOVA, * $p < 0.05$ vs. CON. Group sizes: n = 8–22 from 3–5 independent experiments. Data are expressed as mean ± SEM in percentage of the wound area compared to each corresponding 0 h (baseline) values (152).*

We have found that administration of either NPY (Figure 17, D) or SP (Figure 17E, F) decreases the migration speed of the cells significantly compared to the control groups

(CONTROL and SAXAGLIPTIN). At the highest concentration of NPY (100 nM), the administration of saxagliptin (500 nM) restored the migration capacity of fibroblasts. In the case of the SP administration, there was a significant but not a meaningful difference caused by saxagliptin (152).

5. Discussion

Neuropeptides and neuropeptide families play a pivotal role both in the physiology and pathology of the cardiovascular system. Some neuropeptides are considered to exert protective or in general beneficial effects, while others e.g. SP and NPY in addition to their potentially protective acute effects often play a detrimental role in various chronic conditions such as adverse cardiac remodeling, hypertrophy, angiogenesis, inflammation, necrosis, and fibrosis depending on the specific cardiac pathology, progression of that disease (60, 74, 83, 122, 123, 183, 184).

An important example for neuropeptides exerting beneficial cardiovascular effects are the SST analogue substances, showing protective effects against ischemic injury in various tissue types (34-38), although the role of the native SST peptide and the SSTR-mediated effects are not yet clarified. Accordingly, in the present work we demonstrated that the neuropeptide SST exerts a cytoprotective effect in rat cardiomyocytes against simulated ischemia/reperfusion injury. In addition, we demonstrated that in samples from a translational pig model of acute myocardial infarction, that plasma SST-like immunoreactivity is increased by the cardioprotective ischemic preconditioning intervention. Previously in this pig model it was found that myocardial necrosis is decreased by ischemic preconditioning, meanwhile myocardial edema is reduced rather by ischemic postconditioning and remote ischemic conditioning. Moreover we have found that microvascular obstruction is decreased by ischemic preconditiong and ischemic postconditioning (151). We found that SST is expressed in the pig left ventricle at the peptide level, but it is not detectable at the mRNA level. *SSTR1* and *SSTR2* mRNAs were expressed in the pig left ventricle, and remote ischemic conditioning upregulated *SSTR1*. We identified that *SSTR1*, *SSTR2*, and *SSTR5* mRNAs were expressed in the healthy human left ventricles, and the expression of *SSTR1* and *SSTR2* mRNAs are mainly localized to vascular endothelial cells. Additionally, expression of the SST peptide decreased in interventricular septum samples of patients with chronic ischemic cardiomyopathy.

We revealed the first time that native SST has a concentration-dependent direct cardiocytoprotective effect against simulated ischemia/reperfusion injury in primary adult rat cardiomyocytes and H9C2 cells. This finding is supported by literature data reporting

that endogenous SST release plays a role in the mechanism of capsaicin-induced protection against ischemia/reperfusion injury in the mouse retina (39) and suggests that SST may exert a general protective effect against ischemia/reperfusion in a various cell/tissue types. The expression of all *Sstrs* was identified in both rat cardiomyocytes and H9C2 cells, although only *Sstr3, Sstr4,* and *Sstr5* were abundant (185, 186). Interestingly, SST, cortistatin-14 (a SST-related peptide with similar receptor mediated effects), and octreotide (synthetic agonist of SSTR2, SSTR3, and SSTR5) decrease infarct size of the brain in a middle cerebral artery occlusion model in rats (187). In addition, octreotide exerts protection against increased intraocular pressure-induced retinal ischemia/reperfusion damage in mice (35), improves the damage caused by ischemia/reperfusion in rat pancreas and rabbit liver (36, 37). Moreover, octreotide decreases infarct size of the myocardium mimicking the effect of ischemic preconditioning in a rat model of coronary occlusion (38). It is notable that incidence of cardiac ischemia was lower in long-acting octreotide treated patients with diabetic retinopathy which was revealed by a recent data analysis of clinical trials (188). Pasireotide (synthetic analog of SST) shows neuroprotective effects *ex vivo* against the chemically induced ischemia/reperfusion injury of the rat retina (34).

After we demonstrated that native SST exerts direct cardiocytoprotection *in vitro* our next objective was to collect additional data supporting the protective role and the translational relevance of SST in a pig model of myocardial infarction and cardioprotection, as well as determine the expression of SST receptors. Accordingly, we used tissue and plasma samples obtained in our previous study of myocardial infarction and cardioprotection in pig with different types of ischemic conditioning interventions (151). In this model we have found that myocardial necrosis is decreased by ischemic preconditioning, meanwhile myocardial edema is reduced rather by ischemic postconditioning and remote ischemic conditioning. Moreover we have found that microvascular obstruction is decreased by ischemic preconditiong and ischemic postconditioning (151). In our present work with these samples, we found an increased plasma SST-like immunoreactivity potentially caused by ischemic preconditioning, when a significant infarct size reduction was observed (151). This finding suggests the potential role of SST in this protective action. Interestingly, in patients with advanced heart failure (left ventricular ejection fraction 18 ± 8%, NYHA classes III-IV), the plasma SST concentration was found to be

around 18 pmol/l (189). This SST level is in a similar range with the one found in pig plasma samples measured in our present study indicating that human and pig plasma SST level might be in the same range. It is also an important finding of our present study that we detected SST-like immunoreactivity in the pig left ventricle, although, without its expression at the mRNA level. Thus, it is suggested that SST in the heart tissue has probably sensory neural origin. TRPV1 receptors of the peptidergic sensory fibers innervating the heart (190) can be activated by various mediators of tissue injury and inflammation (such as protons and prostaglandins) inducing the release of sensory neuropeptides (11) e.g. SST. Our present study reported the first data for the presence of SST in the heart, previous literature data showed that plasma CGRP concentration increased in a rat model of ischemia/reperfusion due to ischemic preconditioning intervention, which also mediates cardioprotection (190-192). In the present study, we also demonstrated that *SSTR1* and *SSTR2* are expressed in the pig left ventricle samples by both RT-qPCR and deep sequencing. We also demonstrated here by RT-qPCR measurements that remote ischemic conditioning caused the upregulation of *SSTR1*. It is crucial to note here that there are different conditioning approaches those exert different types of cardioprotective effects, e.g., infarct size reduction by ischemic preconditioning and vasculoprotective effects (reduction in edema) by ischemic postconditioning and remote ischemic conditioning (151). Accordingly, there are differences in the expression of SST or SSTRs in the experimental groups with different cardioprotective conditioning mechanisms. These results indicate that cardioprotective effects of SST is presumably mediated by SSTR1 and SSTR2 although, other non SSTR-related mechanisms cannot be excluded.

We also revealed biological processes possibly playing a role in the protective mechanism of SST, SSTR1, and SSTR2 performing the GO analysis of the mRNA-sequencing data. The main goal of the GO analysis is the identification of e.g. biological processes affected in the studied condition. According to the aim of our study, we analyzed differentially expressed mRNAs in the comparison between remote ischemic conditioning (RIC) and ischemia/reperfusion (I/R) groups where *SSTR1* shows significantly increased expression. The GO analysis showed that both potentially receptor-linked mechanisms e.g. muscle development, differentiation, and regeneration, and nonreceptor-mediated protective mechanisms of SST are also possible, such as oxidative stress, reactive oxygen

species regulation, and metabolic processes which is also confirmed by the literature data (193, 194). Experimental data of the literature proved that various mechanisms can be related to SSTR1 signaling e.g. the inhibition of Na^+/H exchanger, Rho/Rac, and e-NOS pathways furthermore the activation of protein tyrosine phosphatase and MAPK/ERK pathway (22). Meanwhile Ca^{2+} channel, inward-rectifying K^+ current, e-NOS inhibition, and protein tyrosine phosphatase activation is linked to SSTR2 signaling (22).

In order to further improve the translational relevance of our experimental findings in cells and pig hearts, we decided to perform RNAscope *in situ* hybridization assay for SST receptors in histological section of healthy human heart and demonstrated that *SSTR1* and *SSTR2* is expressed in vascular endothelial cells and cardiomyocytes. Although, due to the diffuse pattern of RNAscope signal, we cannot exclude *SSTR1* and *SSTR2* expression to a lesser extent by other cardiac cell types. We also confirmed that *SSTR1, SSTR2,* and *SSTR5* are expressed in human left ventricle by deep sequencing. In addition, we determined the SST-like immunoreactivity in interventricular septum samples of healthy and ischemic cardiomyopathy patients and provided the first the literature data that SST is expressed at the peptide level in the human heart tissue. Interestingly, SST showed a decreased expression in the heart tissue samples of patients with ischemic cardiomyopathy as compared to the healthy controls. Supporting our present findings, literature data shows that octreotide treatment improves cardiac function in patients with ischemic cardiomyopathy (NYHA class III, ejection fraction < 40%) (195). Our present findings showing that *SSTR1, SSTR2,* and *SSTR5* are expressed at the mRNA level in human heart are supported by others' data presenting the mRNA expression of *SSTR1, SSTR2, SSTR4,* and *SSTR5* in the human atria and ventricle (25). As part of the study, it was identified that cultured human fibroblasts express *SSTR1, SSTR2, SSTR4,* and *SSTR5* receptors, but in case of cardiomyocytes only the expression of *SSTR1* and *SSTR2* were found (25). In contrast to their result the Human Protein Atlas data shows that *SSTR5* is expressed in cardiomyocytes, therefore we can't rule out that *SSTR5* signaling could provide to the potential cardioprotective effects SST. Unfortunately, vascular endothelial cells were not investigated in this study (25). Our results suggest the potential contribution of SSTR1 and SSTR2 to the cardioprotective action of SST; however, other mechanisms cannot be excluded.

In the second part of the present work, we have shown that NPY and SP potentially play a role in the cardiovascular side effects of saxagliptin in HF patients with diabetes, which harmful cardiac adverse effect was originally revealed by the SAVOR-TIMI 53 phase 4 clinical trial (150). Its results suggest the significance of cardiovascular safety testing even in preclinical disease models in order to identify the mechanisms behind this clinically relevant cardiotoxic side effect of saxagliptin. We found that the expression of DPP4 and NPY at the protein level decreased in the myocardium of patients with HF. In addition, we also showed that DPP4 mRNA is expressed primary by cardiomyocytes measured by *in situ* hybridization. Interestingly, we have found that neither DPP4 inhibition alone nor in combination with neuropeptides, NPY or SP exerted any effect on cell viability in AC16 cells, indicating that possibly not direct cytotoxicity is the mechanism behind the clinically observed adverse outcome in those patients (150). Interestingly, both NPY and SP decreased the migration speed of primary neonatal rat cardiomyocytes and cardiac fibroblasts in an *in vitro* co-culture model. Surprisingly, administration of saxagliptin in combination with NPY restored the migration speed of fibroblasts compared to the NPY only treatment.

Dipeptidyl-peptidase-4 enzyme plays a crucial role in the degradation of several endogenous substances such as incretins, neuropeptides, chemokines, etc. DPP4 inhibitors received a solid role in the treatment of type 2 diabetes since they exert an improved regulation of glycemia by the increased levels of glucagon-like peptide-1. Additional *in vivo* experimental results with various gliptins highlighted that DPP4 inhibition possibly plays a role in the improvement of nitric oxide release and the reduction of inflammation in zucker obese rats (196). In addition, DPP4 inhibition reduces the severity of atherosclerotic lesions in diabetic mice (197). Although, the role of DPP4 inhibition in the regulation of cardiomyocyte and fibroblast functions has not been studied so far. Here we demonstrate the first evidence that the expression of DPP4 and NPY at the protein level show a decrease in cardiac tissue of heart failure patients independently from the etiology of the disease. Our results are supported by literature data revealing that cardiac protein level of NPY is reduced by volume-overload-induced heart failure in a rat model, although, it was found that heart failure increased the circulating NPY level (65). Interestingly, in stellate ganglia of patients with heart failure NPY shows a decreased immunoreactivity and a similar mRNA expression as in healthy

patients' samples (198). These results suggest the increased NPY release from the stellate ganglia, further supporting our present results (198). We showed that in the healthy human left ventricle primarily cardiomyocytes and endothelial cells express the mRNA of DPP4. According to literature data DPP4 is known to be widely expressed on the surface of many cell types e.g. leukocytes, epithelial cells, or endothelial cells in several organs/tissues (199-203). In line with the literature data, we confirmed the expression of DPP4 mRNA in endothelial cells of the heart of healthy patients as well. Our data suggest that an important target cell type of saxagliptin in the heart could be cardiomyocytes, therefore those may take part in increasing the non-cleaved substrates' concentration of the DPP4 enzyme. It is also important to mention that the substrates' levels (e.g. SP and NPY) elevated by DPP4 inhibition might exert their effects primarily not only cardiomyoctes but also on different cell types in their vicinity e.g. fibroblasts (2, 61, 80, 123).

There is a major conflict in the literature about the cardiovascular safety of saxagliptin in heart failure patients. Clinical investigations and meta-analyses in general suggest that saxagliptin is potentially harmful or at least controversial regarding to its cardiovascular safety profile (204-207), although certain investigations concluded that saxagliptin is neutral in cardiovascular safety (208-210). In contrast to the clinical data, several preclinical studies show the possible cardioprotective properties of various DPP4 inhibitors in different experimental models (211-213). Therefore, we decided to investigate the direct effects of DPP4 inhibition in the presence of its two neuropeptide substrates on the viability of cardiomyocytes *in vitro*. Our present results revealed that neither DPP4 inhibition alone nor in combination with neuropeptides caused cytotoxic effects on the human cardiomyocyte-derived cell-line: AC16. Several studies investigated the effects of saxagliptin treatment in various models of diabetes and/or acute cardiac damage supporting our results that saxagliptin shows no direct cytotoxicity. Interestingly, saxagliptin treatment was found to exert cytoprotection *in vitro* against glucose and oxygen depletion followed by reoxygenation in primary human brain microvascular endothelial cells (214). Others demonstrated that saxagliptin reduced oxidative stress by modulation of endothelial nitric oxide synthase in a genetics-based model of non-obese type 2 diabetes in rats (215). In addition, saxagliptin is capable to improve isoproterenol treatment-induced cardiac diastolic dysfunction in rats (212) and alleviate cardiac

ischemia-reperfusion injury *ex vivo* in rats with streptozotocin-induced type 2 diabetes (213). The existing controversy between the clinical and preclinical findings is caused by the trend that preclinical, *in vivo* experimental studies in most of the cases focus only on diabetic models and/or acute cardiac damage, but not on chronic conditions such as heart failure, although there are significant differences in the background mechanisms.

It was revealed that sitagliptin reduces cardiac collagen deposition, and activation of pro-fibrotic signaling in rats with HFpEF potentially improving the condition of the animals (216). These data highlighted the potential significance of DPP4 enzyme and its substrates in cardiac fibrosis, cell migration, and remodeling. Accordingly, we aimed to investigate next their potential effect on the migration of cardiac fibroblasts and their potential interaction with adverse cardiac remodeling in a scratch assay model of the cardiomyocytes-cardiac fibroblasts co-culture. We identified that treatment with either NPY or SP significantly decreases the cell migration speed compared to the solvent-treated controls. At the highest (100 nM) concentration of NPY, saxagliptin treatment reverted the fibroblasts' migration speed. In contrast to our results, in different cell types NPY was found to increase the migration of human umbilical endothelial cells (217) and neuroblastoma cells (218), and to promote angiogenesis and vascularization in the ischemic tissue of the rat hind limb (219). Our results revealed that NPY has different effect on rat cardiac fibroblasts, since it reduces their migration speed and possibly preventing cardiac fibrosis. Surprisingly, we found that saxagliptin reverses the effect of NPY on the migration of fibroblast cells; therefore, saxagliptin in the presence of NPY potentially exerts harmful profibrotic effects which effect is problematic in the long term in chronic heart failure. In the case of the SP administration, there was a significant difference caused by the additional administration of saxagliptin, although we consider this result as not a meaningful difference. Probably biological variance of this cell culture-based platform is responsible for the statistical difference. It is also important to mention that DDP4 has several chemokine-type substrates (e.g. RANTES, eotaxins, MPC, SDF-1) have chemoattractant effects increasing the migration of various cell types. Unfortunately, these substrates were not investigated in our study, although their contribution to the cell migration increasing effect of the combined treatment with NPY and saxagliptin is potentially not negligible. Interestingly, both the administration of NPY and SP decreased the migration of fibroblasts, although Y receptors are G_i linked and

NK1 receptors are $G_{q/s}$ linked. A possible explanation for this phenomenon is that Y receptor mediated signaling pathway could work differently in various cell types. Literature data suggest that in isolated rat cardiomyocytes Y_1 activation caused increased phospholipase C activation and Ca^{2+} release (71).

6. Conclusions

We demonstrated the first time that native SST protects cardiomyocytes against ischemia/reperfusion injury. Interestingly, SST peptide is expressed in the heart; although, its mRNA is not detectable, therefore we suspect that it has sensory neural origin. SSTR1 and SSTR2 might be involved in the SST-mediated cardioprotective effects, but other mechanisms such as antioxidant activity cannot be excluded. These results highlight new aspects of SST and SSTR signaling on the pharmacological relevance in cardioprotection. We also showed that in human failing hearts DPP4 expression decreases and the neuropeptide tone changes. Interestingly, neither DPP4 inhibition nor its combined administration with neuropeptides caused cytotoxicity *in vitro*. However, NPY and SP inhibited the cell migration in a co-culture model of primary neonatal rat cardiomyocytes and cardiac fibroblasts. This effect of NPY was reversed by saxagliptin. We revealed that saxagliptin and NPY potentially interferes with cardiac tissue remodeling therefore contributing to the pathophysiology of end-stage chronic heart failure but the contribution of other, chemokine-type DPP4 substrates (e.g. RANTES, eotaxins, MDC, SDF-1) and immune-mediated processes could be significant, although we haven't investigated those is our study. We believe that the DPP4 enzyme could work as a compensatory mechanism against the altered neuropeptide tone caused by the elevated sympathetic activity in heart failure. Inhibition of DPP4 by saxagliptin potentially diminishes this adaptive mechanism, and thereby exacerbates myocardial damage. However, further investigations extended to additional DPP4 substrates are necessary to clarify more deeply their interaction with the long term adverse cardiac remodeling in HF and the exact background mechanisms.

7. Summary

Neuropeptides play an important role the physiology and pathology of the cardiovascular system. Some of them are considered to exert in general rather beneficial or protective effects e.g. SST, while others e.g. SP and NPY also have a detrimental role in various cardiovascular diseases. Despite that administration of synthetic SST analogs exert protective effect against ischemia/reperfusion injury of retina, liver, pancreas, and heart the expression and function of SST in ischemia/reperfusion injury of the heart and its involvement in cardioprotection is not known. In connection to the detrimental effects of neuropeptides the SAVOR-TIMI 53 phase 4 clinical trial revealed that in the saxagliptin -treated patients the rate of hospitalization for heart failure is increased but the mechanism of this side effect is not clarified. We hypothesized that the neuropeptide-type substrates of the DPP4 (SP and NPY) may play a role in the adverse effect of saxagliptin. Accordingly, we aimed to investigate the direct cardiocytoprotective effect of SST against SI/R injury in cardiac cell cultures and determine the expression of SST and its receptors in pig and human hearts. We also aimed to determine the expression of DPP4 and its substrates in the human heart and to set up a relevant cell culture platform to investigate the effect of DPP4 inhibition by saxagliptin and the role of its neuropeptide substrates (SP and NPY). We demonstrated that exogenously administered native SST exerts protective effect against I/R injury of cardiomyocytes. Interestingly, SST peptide is expressed in the heart; although, its mRNA is not detectable. We identified that SSTR1-2 are expressed in the pig heart and the SSTR1 expression increased by various conditioning interventions. Accordingly, SSTR1 and SSTR2 might be involved in the SST-mediated cardioprotective effects. These results highlight perspectives of SST and SSTR signaling on the pharmacological relevance in cardioprotection. We also showed that in human failing hearts the DPP4 and NPY expression decreases. Interestingly, DPP4 inhibition neither alone nor in combination with neuropeptides caused any cytotoxicity *in vitro*. However, NPY and SP inhibited the cell migration of primary neonatal rat cardiac fibroblasts which effect was reversed by saxagliptin in case of NPY administration. We revealed that saxagliptin and NPY potentially interferes with cardiac tissue remodeling therefore playing a role in the pathophysiology of end-stage chronic heart failure but the contribution of other DPP4 substrates could be important as well.

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9. Bibliography of the candidate's publications

9.1. Publications related to the candidate's Ph.D. dissertation

I. **Vörös, I.**, Onódi, Z., Tóth, V. É., Gergely, T. G., Sághy, É., Görbe, A., Kemény, Á., Leszek, P., Helyes, Z., Ferdinandy, P., & Varga, Z. V. (2022). Saxagliptin Cardiotoxicity in Chronic Heart Failure: The Role of DPP4 in the Regulation of Neuropeptide Tone. *Biomedicines*, *10*(7), 1573. https://doi.org/10.3390/biomedicines10071573

IF: 4.7

II. **Vörös, I.***, Sághy, É.*, Pohóczky, K., Makkos, A., Onódi, Z., Brenner, G. B., Baranyai, T., Ágg, B., Váradi, B., Kemény, Á., Leszek, P., Görbe, A., Varga, Z. V., Giricz, Z., Schulz, R., Helyes, Z., & Ferdinandy, P. (2021). Somatostatin and Its Receptors in Myocardial Ischemia/Reperfusion Injury and Cardioprotection. *Frontiers in pharmacology*, *12*, 663655. https://doi.org/10.3389/fphar.2021.663655

IF: 5.988 *equal contribution to this study

ΣIF of dissertation-related publications: 10.688

9.2. Publications independent of the candidate's Ph.D. dissertation

III. Kucsera, D., Tóth, V. E., Gergő, D., **Vörös, I.**, Onódi, Z., Görbe, A., Ferdinandy, P., & Varga, Z. V. (2021). Characterization of the CDAA Diet-Induced Non-alcoholic Steatohepatitis Model: Sex-Specific Differences in Inflammation, Fibrosis, and Cholesterol Metabolism in Middle-Aged Mice. *Frontiers in physiology*, *12*, 609465. https://doi.org/10.3389/fphys.2021.609465

IF: 4.755

IV. Róka, B., Tod, P., Kaucsár, T., Bukosza, É. N., **Vörös, I.**, Varga, Z. V., Petrovich, B., Ágg, B., Ferdinandy, P., Szénási, G., & Hamar, P. (2021). Delayed Contralateral Nephrectomy Halted Post-Ischemic Renal Fibrosis Progression and Inhibited the Ischemia-Induced Fibromir Upregulation in Mice. *Biomedicines*, *9*(7), 815. https://doi.org/10.3390/biomedicines9070815

IF: 4.757

V. Sághy, É., **Vörös, I.**, Ágg, B., Kiss, B., Koncsos, G., Varga, Z. V., Görbe, A., Giricz, Z., Schulz, R., & Ferdinandy, P. (2020). Cardiac miRNA Expression and their mRNA Targets in a Rat Model of Prediabetes. *International journal of molecular sciences*, *21*(6), 2128. https://doi.org/10.3390/ijms21062128

IF: 5.924

VI. Dora, D., **Vörös, I.**, Varga, Z. V., Takacs, P., Teglasi, V., Moldvay, J., & Lohinai, Z. (2023). BRAF RNA is prognostic and widely expressed in lung adenocarcinoma. *Translational lung cancer research*, *12*(1), 27–41. https://doi.org/10.21037/tlcr-22-449

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NYILATKOZAT EREDETISÉGRŐL ÉS SZERZŐI JOGRÓL
a PhD disszertáció elkészítésére vonatkozó szabályok betartásáról

Alulírott **dr. Vörös Imre** jelen nyilatkozat aláírásával kijelentem, hogy a "**Neuropeptides in cardioprotection and cardiovascular diseases**" című PhD értekezésem önálló munkám, a dolgozat készítése során betartottam a szerzői jogról szóló 1999. évi LXXVI tv. vonatkozó rendelkezéseit, a már megjelent vagy közlés alatt álló közlemény(ek)ből felhasznált ábra/szöveg nem sérti a kiadó vagy más jogi vagy természetes személy jogait.

Jelen nyilatkozat aláírásával tudomásul veszem, hogy amennyiben igazolható, hogy a dolgozatban nem saját eredményeimet használtam fel vagy a dolgozattal kapcsolatban szerzői jog megsértése merül fel, a Semmelweis Egyetem megtagadja PhD dolgozatom befogadását, velem szemben fegyelmi eljárást indít, illetve visszavonja a már odaítélt PhD fokozatot.

A dolgozat befogadásának megtagadása és a fegyelmi eljárás indítása nem érinti a szerzői jogsértés miatti egyéb (polgári jogi, szabálysértési jogi, büntetőjogi) jogkövetkezményeket.

Tudomásul veszem, hogy a PhD értekezés nyilvánosan elérhető formában feltöltésre kerül az Országos Doktori Tanács honlapjára.

Budapest, 2023.04.13.

dr. Vörös Imre