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INVESTIGATION OF NOVEL LIPID-DEPENDENT CARDIOVASCULAR PROCESSES

PhD thesis

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1. Abbreviations

2-AG – 2-arachidonoylglycerol

ABC – ATP-binding cassette

ACS – acute coronary syndrome

anandamide – N-arachidonoylethanolamine

Ang II – angiotensin II

AOC – area over the curve

AT_1R – angiotensin receptor type-1

cAMP – cyclic adenosine monophosphate

cGMP – cyclic guanosine monophosphate

CGRP – calcitonin-gene-related peptide

CB_1R – cannabinoid receptor type-1

CB_2R – cannabinoid receptor type-2

CBR – cannabinoid receptor

CF – coronary flow

DAG – diacylglycerol

DAGL – diacylglycerol lipase

THC – delta-9-tetrahydrocannabinol

ED50 – effective dose 50

Edg – endothelial differentiation gene

FAAH – fatty acid amide hydrolase

GPCR – G protein-coupled receptor

HDL – high density lipoprotein

I/R – ischemia-reperfusion

KO – knock-out

LDL – low density lipoprotein

L-NAME – N(gamma)-nitro-L-arginine methyl ester

LVDevP – left ventricular developed pressure

LVDiastP – left ventricular diastolic pressure

NO – nitrogen monoxide

NK cells – natural killer cells

PKC – protein kinase C

PLC – phospholipase C

PTX – pertussis toxin

rimonabant – SR141716A (selective CB_1R inhibitor)

S1P – sphingosine-1-phosphate

$S1P_{1\text{-}5}Rs$ – sphingosine-1-phosphate receptor type-1 to type-5

S1PR – sphingosine-1-phosphate receptor

SphK – sphingosine kinase

SphK1 – sphingosine kinase-1

SphK2 – sphingosine kinase-2

VLDL – very low density lipoprotein

WT – wild-type

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2. Introduction

2.1. Relevance of ischaemic heart disease

In the past decades, life expectancy at birth has remarkably increased alongside shifts in global age structures. While in 1980 global life expectancy was around 61.7 years, this number increased to 73.2 years by 2020. Non-communicable or chronic diseases, including several types of cancers and heart disease, became the leading causes of death globally. Ischaemic heart disease, stroke and diabetes are still considered as leading causes of premature morbidity and mortality in several regions (especially in low and middle income countries), thus putting increased demands on health systems.[1,2]

Atherosclerosis and vascular remodelling are the underlying mechanisms of several cardiovascular conditions, including coronary artery disease, stroke and peripheral artery disease. Among others, obesity, hypertension, diabetes mellitus, smoking and elevated cholesterol levels have been identified as major risk factors. Besides these predisposing factors, numerous other and yet unknown mechanisms might be involved including the diversified cardiovascular effects of lipid mediators.[3,4]

Vascular remodelling refers to alterations in the structure of resistance vessels contributing to elevated systemic vascular resistance, thus impacting both development and complications of hypertension.[5] In the coronary arteries, plaque formation and vascular remodelling may lead to narrowing or occlusion of the lumen and therefore to the reduction of blood flow to the myocardium. Limitation of the blood flow and thus the oxygen supply to the myocardium, causes ischaemia leading to stable or unstable angina pectoris, non-fatal myocardial infarct or sudden cardiac death.[6,4]

In the majority of cases, the occlusion is a consequence of the rupture of a vulnerable atherosclerotic plaque. Upon the rupture, subendothelial tissues and their thrombogenic contents come into direct contact with the bloodstream which leads to platelet activation and aggregation. During platelet activation, vasoactive substances (including lipid mediators) are released. The mural thrombus formation and the emerging vasoconstriction (caused by the released vasoactive substances) lead to the ischemia of the peripherally located areas and this may result in myocyte necrosis.[7,8]

Urgent reestablishment of the blood flow is essential to minimize myocardial injury. On the other hand, besides the adequacy of the treatment, several other factors influence the severity of ischaemia, such as the duration of the occlusion or the vascular and myocardial responses to these pathological events.[9] Release of various lipid mediators in the vessels and/or in the myocardium may be relevant in this context, as they might have an impact on the dynamics and severity of myocardial injury.

2.2. Lipid mediators

Besides their well-known functions as sources of stored energy or components of cell membranes, several groups of lipid molecules function as signalling agents and mediate various physiological and pathophysiological processes. The term "lipid signalling" refers to the lipid-dependent activation or propagation of a signal within a cell.[10] Lipid mediators act on their protein targets (ion channels, transporters, G-protein coupled receptors (GPCRs)) in two well-documented ways. They can act directly, by binding to specific sites of these protein targets thus controlling channel and transporter gating, or they can act non-specifically by modifying the physical environment of channels and transporters, in particular the protein–membrane interface.[11]

According to the classification system developed by The Lipid Maps Consortium, lipids are divided into eight categories, six of which are present in mammals (fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids and prenol lipids). Each group includes members that play dominant roles in intra- and extracellular signalling processes.[10]

Fatty acyls are often found in quantities of pmol/g of tissue making their detection challenging. N-acyl ethanolamines have a long history of physiological and GPCR mediated effects that are directly related to cannabinoid activation, while primary fatty acid amides and N-acyl glycines have demonstrated interactions with serotonin receptors, gap junction proteins and calcium signalling, and a myriad of physiological effects.[12]

Glycerolipids are essential molecules for the regulation of cell functions by hormones, neurotransmitters, growth factors, and inflammatory cytokines. One of their many representatives, diacylglycerol (DAG) functions as a second messenger signalling lipid, and is a product of the hydrolysis of the phospholipid phosphatidylinositol 4,5-bisphosphate by the enzyme phospholipase C (PLC). When synthetized, DAG remains

within the plasma membrane, due to its hydrophobic properties and is a physiological activator of protein kinase C (PKC). The production of DAG in the membrane facilitates translocation of PKC from the cytosol to the plasma membrane.[13]

Glycerophospholipids are the main components of biological membranes. Lysophosphatidic acid (LPA), which is a naturally occurring bioactive lysophospholipid, regulates multiple biological processes including endothelial permeability, angiogenesis, atherogenesis, platelet activation and regulation of vascular tone. LPA activates its specific GPCRs ($LPA_{1-5}R$), which results in the activation of multiple signalling pathways with various downstream physiological and pathological effects.[14,15]

Ceramide, sphingosine and sphingosine-1-phosphate (S1P) are the most important members of the sphingolipid group. Ceramide, in addition to playing a role as a structural component of cellular membranes, may induce vasodilator or vasoconstrictor effects by interacting with several signalling pathways in endothelial and smooth muscle cells.[16]

Structural role of the sterol lipids in cell membranes is well-established. Recent studies uncovered potent biological activities of certain cholesterol metabolic precursors and its oxidized derivatives, oxysterols in the regulation of cellular proliferation and in cancer. They exert their effects on trafficking and signalling of oncogenic epidermal growth factor receptor.[17]

Two representative groups of the most studied and probably the most significant lipid mediators are endocannabinoids and sphingolipids, which are also in the focus of this dissertation.

2.3. The endocannabinoid system

The discovery and investigation of the endocannabinoid system began with the history of *Cannabis Sativa* that has been used for thousands of years to affect human health and for recreational purposes. Its main active ingredient, the delta-9-tetrahydrocannabinol (THC) has been found to be the only one with psychotropic properties and therefore, became the cannabinoid prototype for a long time.[18,19] First investigations about cannabinoids focused on their widespread actions in the brain. They have been found to influence learning and memory in the hippocampus, modulate locomotor activities and reward pathways in the basal ganglia and control appetite in the hypothalamus.[20]

The research of cannabinoid binding sites in mammals led to the discovery of two types of cannabinoid receptors: cannabinoid receptor type-1 (CB_1R), cloned in 1990, and cannabinoid receptor type-2 (CB_2R), cloned in 1993. In humans these two GPCRs share 44% of homologous sequence, however there are major differences in their signalling mechanisms, tissue distribution and sensitivity to certain potent agonists and antagonists.[21]

Although, CB_1Rs exist primarily in central and peripheral neurons and one of their main functions is to inhibit neurotransmitter release, they are located peripherally as well (adipocytes, uterus, myocardium) regulating basic physiological processes such as energy balance and reproduction. CB_2Rs are expressed mainly in immune and haematopoietic cells and they modulate a broad spectrum of immune effects, including the modulation of cytokine release. CB_2Rs are present in the gastrointestinal tract, liver, brain and in the cardiovascular system as well.[22] CB_1R and CB_2R are coupled through inhibitory G proteins ($G\alpha_{i/o}$) to adenylyl cyclase thus negatively modulating cyclic adenosine monophosphate (cAMP) levels, to several types of calcium and potassium channels, and also to mitogen-activated protein kinase.[23]

The presence of CBRs in human tissues led to the discovery of their endogenously produced and metabolized agonists: the endocannabinoids.[24] They are all eicosanoids. Firstly, N-arachidonoylethanolamine (anandamide) was discovered which is a partial agonist of CB_1R and a weak ligand of CB_2R, with a low overall efficiency.[25] Soon after (in 1995) 2-arachidonoylglycerol (2-AG) was isolated from rat brain and canine gut. Contrary to anandamide, 2-AG is produced in much larger amounts and acts as a full agonist on both receptors. Structure-activity relationship studies suggest that 2-AG is the real neutral ligand of these receptors.[26]

Owing to their lipid characteristics they diffuse freely through membranes thus they are not suitable for being stored in lipid vesicles in their active forms. Therefore, endocannabinoids (contrary to many other transmitters) are synthetized "on demand" near their site of action in response to increased intracellular calcium levels in various tissues. 2-AG is produced mainly by diacylglycerol-lipase (DAGL) from DAG, which is produced by PLC from 2-arachidonoyl-phosphatilidinositol, whereas anandamide is biosynthesized from N-arachidonoyl phosphatidylethanolamine via various pathways.[26]

Thereafter, endocannabinoids are efficiently removed through cellular uptake and by specific intracellular enzymes. 2-AG is metabolized mainly by monoacylglycerol-lipase, whereas anandamide is metabolized by fatty acid amide hydrolase (FAAH).[27]

The endocannabinoid system refers not just to the endocannabinoids, but to their synthetizing and metabolizing enzymes, their carrying proteins, the CBRs and their secondary messengers. This system regulates the control of energy balance and metabolism through its central and peripheral effects. In addition, their effects have been discussed in line with obesity, diabetes, inflammation, cancer, epilepsy, affective and neurodegenerative diseases.[19,28,29,30]

Cardiovascular effects of endocannabinoids have also come to the focus of interest in the past decades. Most important cardiovascular effects of endocannabinoids and their receptors are summarized in Table 1. Although, their role has been suggested in various pathophysiological conditions, including their protective role in myocardial and cerebral ischaemia/reperfusion (I/R) injury and preconditioning, their beneficial influence on circulatory shock and hypertension and their controversial impact on the progression of atherosclerosis, this thesis will focus mainly on their physiological short-term effects.[18]

Table 1. Cardiovascular effects of endocannabinoids. (2-AG – 2-arachidonoylglycerol, CBR – cannabinoid receptor, VSMC – vascular smooth muscle cell)[1]

Cardiovascular Effects of Endocannabinoids (Anandamide and/or 2-AG)

Local effect of CB_1R/CB_2R activation	
↓ **Cardiac contractility**	↓ Adhesion of inflammatory cells
↓ **Platelet activation**	↓ Lymphocyte activation
↓ **Monocyte recruitment**	↓ Endothelial cell activation
↓ **Macrophages inflammation**	↓ Release of inflammatory cytokines
↓ **VSMC proliferation and migration**	↓ T cell recruitment/activation
Systemic effect of CB_1R/CB_2R activation	

Protection against ischemic injuries	Mainly CB_2R
Reduction of blood pressure	Mainly CB_1R
Antiarrhythmic effect	Mainly CB_1R
Reduction of shock episodes	CB_1R and CB_2R
Anti-atherogenic activity	Mainly CB_2R

2.4. In vivo cardiovascular effects of endocannabinoids

The cardiovascular effects of (endo)cannabinoids are complex and may involve central and peripheral compounds as well. It has been observed that in humans consumption of *Cannabis Sativa* leads to acute tachycardia without remarkable changes in blood pressure. Intravenously administered THC increased heart rate in healthy volunteers (most likely due to a reflexive response to cannabis induced vasodilation) and enhanced cardiac performance without significant changes in their blood pressure.[31] A single oral dose of the selective CB_1R antagonist SR141716A (rimonabant) abolished marihuana-induced subjective intoxication and tachycardia, confirming the central role of CB_1Rs in mediating the effects of THC.[32] Long-term THC exposition however decreases both heart rate and blood pressure.[33]

Anandamide on the other hand elicits bradycardia and a triphasic blood pressure response in anaesthetised rats.[34] The first phase of the response - a rapid fall in heart rate and blood pressure - seems to be mediated by vagal responses, since this phase is absent after bilateral transection of the vagus nerve.[35] The second, pressor component might be due to peripheral actions and not due to the sympathetic nervous system and is not affected by the inhibition[35], nor the deletion of CB_1R.[36] The most prominent third, depressor phase is contributed mainly to the inhibition of the sympathetic nervous system. Inhibition of the CB_1R by its selective blocker rimonabant reduces the intensity of the depressor phase and bradycardia, indicating that it evolves due to the inhibition of the sympathetic tone mediated by CB_1Rs.[35,34]

2-AG, that has been proposed as the more potent and rather the natural ligand of CBRs, elicits dose-dependent hypotension and moderate tachycardia in anaesthetised mice. In

contrast to anandamide, those effects are unaffected by the CB_1R antagonist rimonabant. An explanation for this discrepancy might be the rapid metabolism of 2-AG. It is degraded rapidly and completely (within 2 minutes) to arachidonic acid when incubated with mouse blood, whereas anandamide stays intact under the same conditions. These results indicate that the observed effects of exogenous 2-AG might be mediated by its metabolite, arachidonic acid through a noncannabinoid mechanism and endogenous, on demand synthetized 2-AG might elicit its effects through CB_1Rs.[37,38]

Besides some contradictions, numerous studies indicate that activation of peripheral CB_1Rs plays the key role in the formation of the prolonged depressor response. HU-210, a potent CB_1R agonist, reduced mean blood pressure similarly as anandamide in anesthetised rats. HU-210, anandamide, and its metabolically stable analogue methanandamide reduced vascular resistance, primarily in the coronaries and the brain. The observed vasodilator effects were prevented in the presence of rimonabant.[39] A series of endocannabinoid analogues with different affinity constants for binding to CB_1R were evaluated for their effects on blood pressure and heart rate. A strong positive correlation has been found between their binding affinity and the intensity of hypotensive and bradycardic responses, further confirming the participation of CB_1R in these effects.[40]

The vasodilator effects of endocannabinoids seem to be mediated by the activation of presynaptic CB_1Rs located on peripheral sympathetic nerve terminals. Their activation inhibits the release of noradrenaline, thus inhibiting sympathetic vasoconstriction and leading to vasodilation. In isolated rat atria and vasa deferentia preloaded with noradrenalin, electrical field stimulation causes norepinephrine release, which is concentration-dependently abolished by anandamide and THC. Their inhibitory effect is completely antagonised by rimonabant.[41]

In spite of their peripheral vasodilator effect, endocannabinoids elicit bradycardia through central cardiovascular regulatory centres. Cannabinoid receptor agonist WIN55212-2, when administered into the cisterna cerebellomedullaris of rabbits, dose-dependently causes bradycardia. Rimonabant attenuates those effects pointing out the involvement of CB_1Rs in central mechanisms as well.[42]

2.5. Direct vasodilator effects of endocannabinoids

Cannabinoids produce vasodilation in various vascular beds. Among others, their vasodilator actions have been described in rat mesenteric and hepatic arteries, guinea pig basilar artery, bovine coronary artery, rabbit aorta, human saphenous vein, thoracic and mesenteric artery. It is suggested that the magnitude of the vasorelaxation and the mechanisms involved are not identical in all species and vascular beds.[43,44] Furthermore, the existence of non-CB_1 and non-CB_2Rs, that might play a key role in cannabinoid-induced vasodilation, have been hypothesized repeatedly in the past years.[18]

In rat hepatic, small mesenteric and guinea pig basilar arteries anandamide caused vasorelaxation, however, other endogenous (2-AG, palmitylethanolamide) and exogenous (THC, WIN55,212-2, HU-210) CBR agonists could not mimic this action. The observed vasodilator response to anandamide might develop through a cannabinoid independent signalling pathway, namely through the activation of vanilloid receptors, located on perivascular sensory nerves and causing release of calcitonin-gene-related peptide (CGRP). In the isolated arteries, CGRP receptor antagonist and capsazepine, a selective vanilloid receptor inhibitor, abolished the anandamide-induced vasorelaxation, however rimonabant had no such effect.[45,36]

Anandamide may produce endothelium-dependent but CBR-independent vasorelaxation as a result of its catabolism to vasodilator eicosanoids such as prostacyclin or epoxyeicosatrienoic acid. In precontracted bovine coronary artery rings anandamide decreases isometric tension, though this effect is absent in endothelium-denuded artery rings. The presence of rimonabant does not affect the vasodilator actions of anandamide, excluding the involvement of CB_1R, however inhibition of its metabolizing enzyme FAAH, the vasodilator actions of anandamide are diminished.[46]

Anandamide and FAAH are also present in the kidney: both in endothelial and mesangial cells. Anandamide, in a concentration of 10^{-6} M, vasodilates juxtamedullary afferent arterioles and this effect can be blocked by the inhibition of NO production, as well as by the blockade of CB_1R. Furthermore, in endothelial cells CB_1R, but not CB_2R mRNA, can be detected and endothelial cells exhibit a specific, high affinity binding of anandamide, suggesting that anandamide stimulates CB_1R-mediated NO release from endothelial cells.[47]

Similar results have been observed in human saphenous vein, internal thoracic artery and right atrium segments. Anandamide stimulates the release of NO in a dose-dependent manner and this process can be antagonised by N(gamma)-nitro-L-arginine methyl ester (L-NAME) and by rimonabant. In the presence of the FAAH inhibitor, anandamide stimulates a higher peak level of NO that remains elevated for a longer period of time, demonstrating the presence of FAAH in human vascular tissues.[48]

In contrast to the studies presented above, methanandamide fails to cause vasodilation in precontracted rat carotid arteries. In fact, methanandamide and HU-210 inhibits forskolin-induced cAMP accumulation and vasodilation in a concentration-dependent manner. This inhibitory effect is prevented by pertussis toxin (PTX) incubation and reduced by a selective CB_1R blocker.[49]

Anandamide is a potent vasorelaxant in the mesentery and this has been described in different species including humans and rodents. In precontracted rat mesenteric arterial beds, anandamide and methanandamide induces a long-lasting and dose-dependent vasodilation, whereas potent CB_1R agonists WIN55,212-2 and HU-210 have only minor dilator effects. 2-AG causes no change in vascular tone, whereas THC and arachidonic acid causes mesenteric vasoconstriction. After endothelial denudation, the dilator response to anandamide is slightly reduced and no longer can be inhibited by rimonabant, indicating that anandamide-induced mesenteric vasodilation is mediated by an endothelium-located rimonabant-sensitive “anandamide receptor” distinct from CB_1R.[50]

In another study the role of an as-yet-unidentified receptor has also been implicated. Abnormal cannabidiol, which is a neurobehaviorally inactive cannabinoid, does not bind to CB_1Rs. Yet, it causes hypotension and vasorelaxation in mice mesenteric arteries. Vasodilator actions can be observed both in WT and in mice lacking CB_1R or both CB_1R and CB_2R. This effect is abolished by endothelial denudation. Interestingly, rimonabant, which is originally a selective CB_1R inhibitor, diminishes the abnormal cannabidiol-induced vasorelaxation, and not just in WT, but also in CBR-KO mice. Furthermore, vasodilation is also blocked in the presence of K^+-channel toxins, that are reported to block the release of endothelium-derived hyperpolarizing factor (EDHF).[36]

In a rat study it has been suggested that anandamide acts via hyperpolarizing mechanisms. Since the identification of NO it has been suspected that there is another endothelial

relaxant factor, referred to as EDHF. Since EDHF-mediated relaxation can be blocked by rimonabant and is accompanied by the accumulation of an arachidonic acid metabolite, which is coeluted on TLC separation with anandamide, it is suggested that EDHF might be a cannabinoid-like substance.[51,52]

In another study it was described that anandamide and methanandamide produce dose-dependent vasodilation in rabbit aortic rings, that is suggested to be partially endothelium dependent and mediated by a rimonabant-sensitive non-CB_1R, that requires $G\alpha_{i/o}$ proteins and NO production. In case of methanandamide, a non-endothelium dependent component was also observed, namely the activation of vanilloid receptor type-1 and subsequent release of CGRP.[53] Similarly to the aforementioned study[50], WIN55,212-2 and other efficacious CB_1R agonists failed to produce this effect.

WIN55,212-2 however, causes a concentration-dependent vasorelaxation in rat aorta. This effect is inhibited by endothelial denudation, inhibition of NO synthesis, blockade of vanilloid receptor type-1, capsaicin desensitization, and a CGRP receptor antagonist. These findings indicate that endothelium and NO-dependent vasorelaxation induced by WIN55212-2 mainly involves vanilloid receptors and CGRP release.[54]

In summary, we can conclude that vasodilator actions of (endo)cannabinoids are complex and several mechanisms have been proposed to explain this effect. Both extra- and intracellular mechanisms can be involved. With regard to the former, stimulation of CB_1R, CB_2R or nonCB_1/nonCB_2 CBRs, vanilloid receptors and subsequent release of the vasodilator neurotransmitter CGRP have been described. With regard to the latter, the main mechanisms implicated include NO release, metabolism to vasoactive arachidonic metabolites or prostanoid analogues, or EDHF release.[43]

2.6. Interplay between endocannabinoid-induced vasorelaxation and Ang II-induced vasoconstriction

Ang II is the main effector molecule of the renin-angiotensin-aldosterone system and plays a key regulatory role in cardiovascular and salt-water homeostasis. It is involved in aldosterone secretion, cell-proliferation, inflammation and atherosclerosis.[55] In the cardiovascular system, its peripheral vasoconstrictor and central pressor effects lead to the elevation of systemic blood pressure.[56] Direct short-term cardiac effects of Ang II

include a decrease in coronary flow (CF) and controversial effects on contractility.[57] Moreover, Ang II produced by the locally activated renin-angiotensin-aldosterone system has adverse effects on the remodelling process of the vascular and cardiac tissues.[56,58]

Ang II activates angiotensin receptor type-1 (AT_1R) and angiotensin receptor type-2 (AT_2R), among which AT_1R mediates its major short-term and long-term actions.[59] AT_1R binding causes AT_1R interaction with heterotrimeric G proteins, including $G\alpha_{q/11}$, $G\alpha_{12/13}$, and $G\alpha_i$.[59] Coupling to $G\alpha_{q/11}$ classically leads to PLC stimulation and activation of downstream inositol-triphosphate and DAG dependent pathways.[59]

It has been previously reported that Ang II via AT_1R induces the release of 2-AG in the vascular tissue, which decreases Ang II-induced vasoconstriction.[60,61] Signalling-induced endocannabinoid release via activation of GPCRs and downstream PLC activation-mediated mechanisms have been detected first in the neural tissue serving as retrograde synaptic inhibition.[62] Endocannabinoid release has also been detected by bioluminescence resonance energy transfer method in isolated cell systems in response to calcium-signal-generating GPCR agonists, such as Ang II via AT_1R, and acetylcholine via muscarinic receptors.[63] GPCR agonist-induced release of 2-AG has also been reported in the vascular tissue previously.[64] The putative mechanism for Ang II-induced 2-AG release is illustrated on Figure 1.

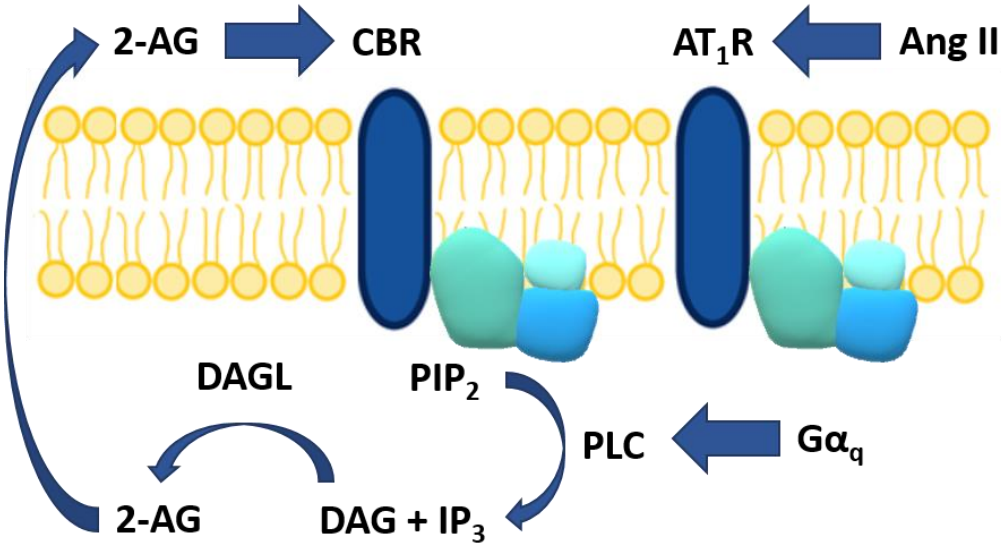

Figure 1. The putative mechanism for Ang II-induced 2-AG release. (Ang II – angiotensin II, AT_1R – angiotensin receptor type-1, PLC - phospholipase C, PIP_2 -

phosphatidylinositol 4,5-bisphosphate, DAG – diacylglycerol, IP_3 - inositol trisphosphate, DAGL - diacylglycerol lipase, 2-AG - 2-arachidonoylglycerol, CBR – cannabinoid receptor)

2.7. Negative inotropic effects of endocannabinoids

Although we own extensive literature about the vascular effects of endocannabinoids, our knowledge about their direct cardiac effects remains insufficient. It is despite the fact that the presence of anandamide and other endocannabinoids, the FAAH enzyme, CB_1R and CB_2R have been determined not only in the vasculature of humans but in cardiomyocytes as well, by distinct methods and by several research groups.[65,66,67,68] Expression of CB_1R in rat cardiac tissue has also been confirmed by immunohistochemistry by our research group. Immunofluorescent visualization of CB_1R revealed its localization on the sarcolemma of cardiomyocytes. Moreover, CB_1R staining was also detectable in the walls of small vessels. CB_1R staining was also present in murine cardiac tissue, but not in the hearts of CB_1R-KO mice.[69]

In Langendorff-perfused isolated rat hearts, anandamide and methanandamide have been reported to cause coronary vasodilation and to decrease left ventricular developed pressure (LVDevP). Selective agonists of CB_1R evoke similar, however selective CB_2R agonists have no such effects. The cardiodepressant and vasodilator effects of anandamide are blocked by rimonabant and other selective CB_1R antagonists, but not by the inhibition of the CB_2R nor the vanilloid receptors, indicating that these effects are attributed mainly to CB_1Rs.[70]

Hypotensive and cardiodepressant activities of endocannabinoids have also been reported in spontaneously hypertensive rats. CB_1R antagonists increased blood pressure and left ventricular contractile performance. Conversely, inhibition of the FAAH, thus inhibiting the degradation of anandamide, reduced blood pressure and cardiac contractility to the levels in normotensive rats and these effects were prevented by CB_1R antagonists, suggesting that CB_1R plays a key role in the hypotensive and cardiodepressant activities of endocannabinoids.[67]

Negative inotropic effects of anandamide, methanandamide and HU-210 have been described in isolated and electrically stimulated human atrial heart muscle preparations. All three substances dose-dependently decrease systolic force and their effects are

prevented by CB_1R, but not by CB_2R antagonists and neither by the inhibition of NO synthesis nor the inhibition of cyclooxygenase. These results suggest that endocannabinoids decrease contractile performance in human atrial muscle via CB_1Rs.[68]

Moreover, endocannabinoids and CB_1R signalling may also have beneficial effects during I/R. CB_1R agonist HU-210 was found to exhibit an infarction-limiting effect during in vitro reperfusion after focal ischemia in isolated hearts.[71]

Taken together, these studies suggest that endocannabinoids and CB_1Rs are present in cardiomyocytes and they play a remarkable role in the modulation of cardiac functions. Endocannabinoids seem to have cardiodepressant, vasodilator and cardioprotective activities in the heart and these are mediated mainly by CB_1Rs. Thus, it is probable that activation of the endocannabinoid system may improve some cardiovascular conditions. It remains for further research to clarify whether the modulation of endocannabinoid system could lead to novel therapeutic approaches in a number of acute or chronic cardiovascular pathologies.[72,73,74]

2.8. Sphingosine-1-phosphate (S1P)

Sphingolipids, one of the six main groups within lipids, are enriched in the lipid rafts of biological membranes and have been detected in all eukaryotic cells to date. The common feature of their structure is the C18 amino alcohol chain and a distinct polar head, by which each member of the groups can be distinguished. Members of the sphingolipid group include sphingosine, ceramide, S1P, ceramide-1-phosphate, and the more complex sphingomyelin and glycophospholipids.[75,76]

Of sphingolipids, S1P is of outstanding importance in mammals, the effect of which was first published in 1990 by Ghosh and his research group, who described a direct and potent calcium-releasing effect of S1P from intracellular calcium stores.[77] Later, its role in the regulation of numerous biological functions was also revealed.

2.9. S1P in the body

In previous studies, S1P has been successfully detected in all types of eukaryotic tissues, both in the intracellular and extracellular spaces. In blood and lymph, it is present in the high nanomolar concentration range, while it is present in lower ranges in other tissues.[78] The concentration of S1P in the blood plasma is relatively high (200-1000 nM), which is

much higher than its concentration in other tissues, thus leading to the formation of an S1P gradient *in vivo*.[79]

In blood plasma only 1-2% of S1P is found in its free form, it is typically bound to other molecules. Most of them bind to lipoproteins, notably to high density lipoprotein (HDL), which can bind up to 60-90% of S1P. Low density lipoprotein (LDL) and very low density lipoprotein (VLDL) are involved in its delivery to a modest extent. Another important transporter molecule of S1P is albumin, which accounts for about 10-20% of S1P binding.[80] The form in which S1P is predominantly present can fundamentally determine its effect *in vivo*. S1P, which specifically binds to HDL's apolipoprotein M, protects endothelial cells from apoptosis, however, the same cannot be claimed about free S1P.[81,82] The dynamics of the S1P effect may also be influenced by the tendency of binding, free S1P tend to degrade faster than the albumin and lipoprotein-bound form.

Plasma S1P is primarily derived from erythrocytes, which are extremely efficient in producing S1P from sphingosine. Because they lack the important S1P-lyase enzyme to degrade S1P, they can release large amounts of S1P. Platelets and endothelial cells are also important sources of S1P.[83,84] Platelets also lack the S1P-lyase enzyme thus large amounts of S1P are stored and released upon platelet activation.[85,86,87] During acute coronary syndrome (ACS), when the rupture of an atherosclerotic plaque leads to platelet activation, the normally minor free S1P fraction is multiplied.[88] This may contribute to pathological changes in coronary circulation and cardiac function, however, only a few and indirect studies of this effect have been performed.

2.10. Metabolism of S1P

S1P is produced primarily intracellularly in the cytoplasm[89] and secreted into the extracellular space via specific ATP-binding cassette (ABC) transporters.[90,91] The major route of S1P formation is by degradation from sphingomyelin - one of the phospholipid components of the plasma membrane. In the first step, the sphingomyelinase enzyme hydrolyses the phosphocholine head group from sphingomyelin to form ceramide.[92] Sphingosine, the precursor of S1P, is produced from ceramide by N-deacetylation by the ceramidase enzyme.[93] Finally, sphingosine is phosphorylated by sphingosine kinases (SphK) to S1P (Figure 2).

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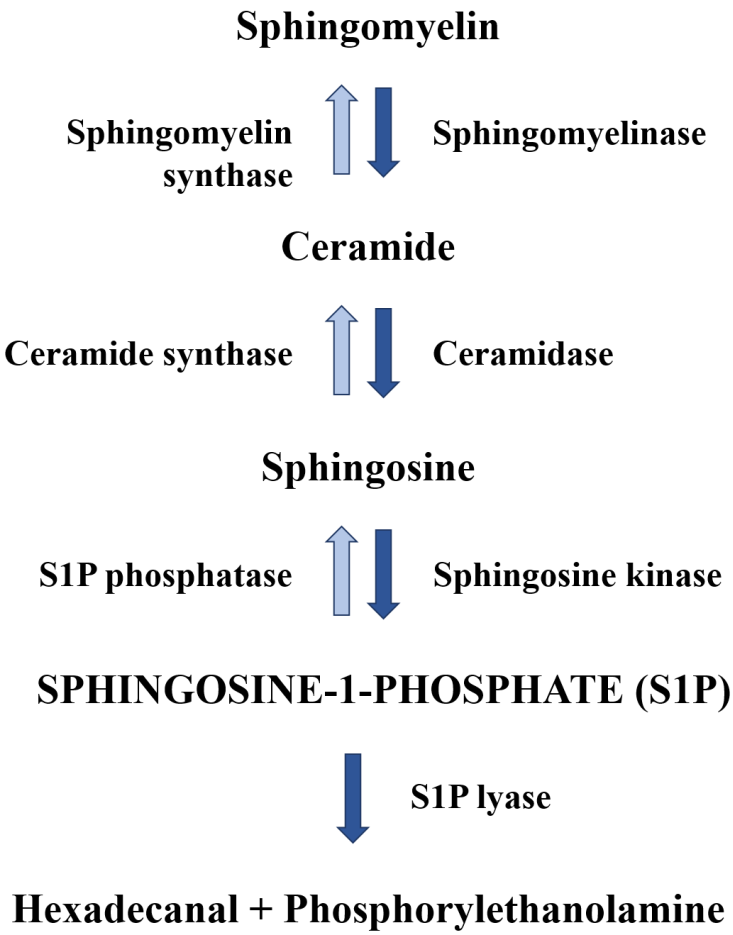

Figure 2. Metabolism of sphingosine-1-phosphate.

SphKs are essential for the synthesis of S1P and thus for the normal development of the cardiovascular and nervous systems.[94] In mammals, two isoforms are known (sphingosine kinase-1 and -2, SphK1, and SphK2) that show high amino acid similarity to each other.[95] They are soluble enzymes and can be found in almost every tissue types, however, the proportion of the isoforms vary. They typically function in the cytoplasm, but can also be secreted into the extracellular space.[96] Their effects are redundant, which is suggested by the observation, that the absence of only one isoform does not cause significant abnormalities, whereas the absence of both enzymes leads to embryonic lethality in mice. In the latter case, defects in angiogenesis (bleeding, oedema) as well as developmental disorders of the nervous system (neural tube defect disorders) have been described in embryos.[94]

Degradation of S1P can be reversible or irreversible. Its reversible dephosphorylation can be catalysed by lipid phosphate phosphatases, of which the best known are S1P-

phosphatase-1 and S1P-phosphatase-2.[97] Its irreversible degradation is catalysed by S1P-lyase to phosphorylethanolamine and hexadecanal (Figure 2).[98]

2.11. S1P receptors

S1P implements its diversified effect through its specific receptors. They are G-protein-coupled, 7-transmembrane receptors, located in the plasma membrane of the target cells and are members of the endothelial differentiation gene (Edg) receptor family.

The numbering of the receptors reflects the order of their discovery. $S1P_1R$ (formerly known as Edg-1) was first recognized by Hla and his research group in 1990.[99] This was followed by the description of $S1P_2R$ (formerly Edg-5) and then $S1P_3R$ (formerly Edg-3) in 1997.[100] $S1P_4R$ (formerly Edg-6) and $S1P_5R$ (formerly Edg-8) were identified in 2000.[101]

Of the S1PRs, $S1P_1R$ is found in the highest amount in the body. It shows high gene expression in the cells of the cardiovascular and nervous systems, spleen, lungs and kidneys.[102] Within the cardiovascular system, $S1P_1R$ is mainly expressed by ventricular, septal, and atrial myocardial cells, as well as by endothelial cells. $S1P_1R$ plays a key role in vascularization. Through $S1P_1Rs$ located in the vascular endothelium, S1P induces cell migration, proliferation, and survival.[103] In addition, it is essential for the formation of cellular connections between endothelial and smooth muscle cells, and thus for the development of vascular integrity, which limits the paracellular transport of fluid and macromolecules.[104] The key role of $S1P_1R$ is supported by the fact that a complete lack of the gene leads to embryonic lethality due to severe bleeding and oedema.[105]

$S1P_1R$ plays a protective role in the progression of atherosclerosis. It mediates anti-inflammatory responses, reduces the production of inflammatory cytokines, and inhibits leukocyte adhesion, thereby inhibiting the growth of atherosclerotic plaques.[106] In addition to the cardiovascular system, $S1P_1R$ is also of great importance in the development of the nervous system. Besides bleeding and oedema, severe nervous system developmental abnormalities have been observed in $S1P_1R$-KO mouse embryos. S1P acts as a chemoattractant, directing the migration of neuronal stem cells through $S1P_1Rs$.[107]

$S1P_2R$ occurs in large amounts in the brain, heart, lungs, and thymus.[108] Within the cardiovascular system, this receptor is expressed in the highest amount in vascular smooth

muscle cells. Together with $S1P_1R$, it plays a redundant role in the normal development of the vascular system. Although, complete deletion of the $S1P_2R$ gene does not lead to embryonic lethality or a clear phenotypic abnormality as opposed to $S1P_1R$, much more severe vascular defects occur in the absence of both genes.[109] This suggests that $S1P_2R$ might has a role in the proper development of the heart.[110] $S1P_2R$ is essential for the normal development of the vestibular and auditory systems. Deafness was observed in $S1P_2R$–KO mice due to vascular abnormality in the inner ear and destruction of hair cells in the Corti organ.[111]

$S1P_3R$ is expressed in large amounts in the brain as well as in the cells of the cardiovascular system: on the surface of vascular endothelial, smooth muscle and myocardial cells.[108] Its role has been described in the modulation of vascular tone and cardioprotection.

According to the current scientific knowledge, there is no evidence that $S1P_4R$ and $S1P_5R$ are expressed in the cardiovascular system significantly and are, therefore, of little interest to this dissertation. $S1P_4R$ is expressed primarily in the lymphatic system: thymus, lymph nodes, spleen, and lungs.[102] It plays an important role in the normal production of platelets. Although $S1P_4R$-KO mice are viable and fertile, they have an increased number of abnormally shaped megakaryocytes, leading to disruption of platelet formation. $S1P_4R$ is hypothesized to have a significant effect on the late stage of megakaryocyte differentiation and platelet production.[112]

$S1P_5R$ is expressed in the brain, lungs, spleen, and skin.[102] High expression of $S1P_5R$ is observed in natural killer cells (NK cells). It promotes NK differentiation and the release of immune cells from bone marrow and lymph nodes in connection with various infections.[113] Its role in the cardiovascular system is not yet known.

S1PRs bind to heterotrimeric G proteins located on the inner surface of the plasma membrane. $S1P_1R$ can bind only to PTX-sensitive $G\alpha_i$ protein, while $S1P_2$ and $S1P_3Rs$ can bind to several heterotrimeric G proteins, including $G\alpha_{i/o}$, $G\alpha_q$, and $G\alpha_{12/13}$. $S1P_4R$ and $S1P_5R$ may be associated with $G\alpha_{i/o}$ and $G\alpha_{12/13}$ proteins.[114] S1PRs and their intracellular signalling pathways are illustrated in Figure 3.

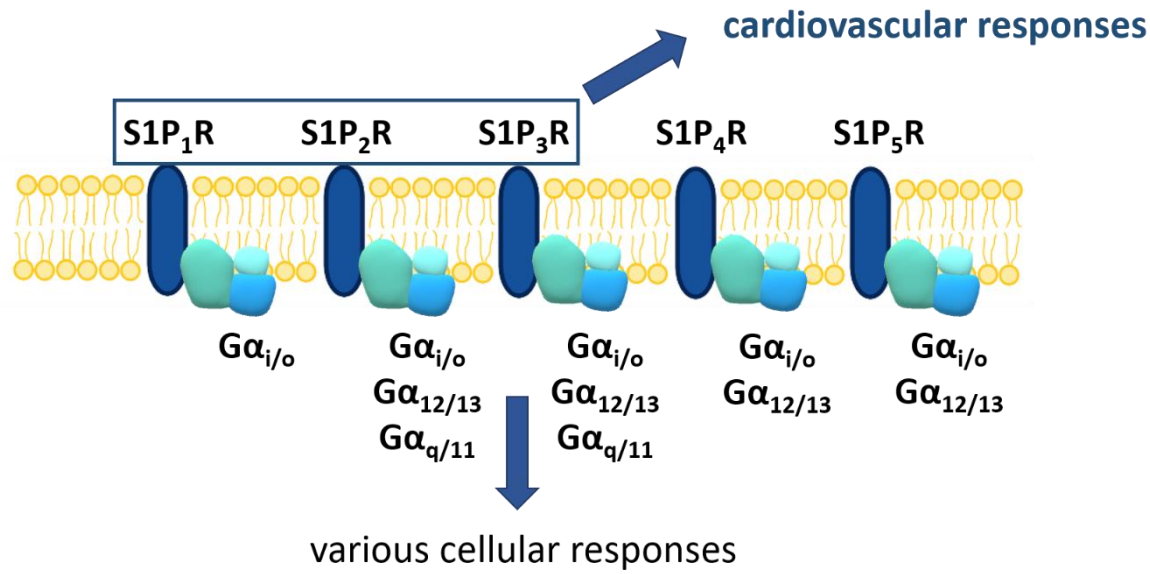

Figure 3. Sphingosine-1-phosphate receptors (S1PRs) and their associated G proteins.[115]

2.12. Cardiovascular effects of S1P

The source of plasma-S1P, which is able to bind to receptors in cardiovascular cells, is not yet fully elucidated. First, Yatomi *et al.* identified platelets as the major source of plasma S1P. The authors explained their finding with the high level of SphK responsible for S1P synthesis in platelets, in conjunction with the complete absence of S1P-lyase, which is responsible for its degradation. As a result, high concentrations of S1P exist in platelets. In addition, stimulation of platelets with thrombin results in substantial S1P release.[116,117]

In contrast, Pappu *et al.* identified erythrocytes as the cells primarily responsible for maintaining plasma S1P concentrations.[118] Moreover, endothelial cells cannot be ruled out as important actors in S1P production, as high activity of SphK in vascular endothelial cells has been reported. Numerous extracellular stimuli, including tumor necrosis factor alpha, are able to activate SphK in endothelial cells and thus contribute to enhanced S1P production.[119] Based on these, it might be concluded that the produced S1P can act in an autocrine/paracrine manner on vascular S1PRs.

Of the S1P receptors, $S1P_1R$, $S1P_2R$, and $S1P_3R$ are commonly found throughout the body, including the cardiovascular system, whereas $S1P_4R$ and $S1P_5$ are found primarily in the lymphatic and nervous systems.[102] $S1P_1R$, $S1P_2R$, and $S1P_3Rs$ are expressed in

vascular endothelium, on the surface of vascular smooth muscle cells, and in cardiomyocytes, but their relative amounts vary cell-specifically. $S1P_1R$ and $S1P_3R$ are expressed in significant amounts in endothelial cells, $S1P_2R$ and $S1P_3R$ in vascular smooth muscle cells, and $S1P_1R$ in cardiomyocytes (Table 2).[120]

Table 2. Sphingosine-1-phosphate receptor (S1PR) expression in the cardiovascular system.[2]

Tissue	Relative S1PR expression
Cardiac myocytes	$S1P_1R >> S1P_3R > S1P_2R$
Vascular endothelial cells	$S1P_1R > S1P_3R >> S1P_2R$
Vascular smooth muscle cells	$S1P_2R > S1P_3R >> S1P_1R$
Cardiac fibroblasts	$S1P_3R >> S1P_1R > S1P_2R$

2.13. The role of S1P in regulating vascular tone

The vascular tone regulating effect of S1P is controversial. It has been studied by several research groups and by distinct methods and both vasoconstrictory and vasodilatorory effects have been attributed to S1P.

Increasing NO production plays one of the major roles in the processes of vascular tone modulation by S1P. In the vascular system, NO is produced primarily in vascular endothelial cells by the endothelial nitric oxide synthase (eNOS). NO produced by eNOS reaches vascular muscle cells through diffusion, where it activates soluble guanylate cyclase, resulting in elevated levels of cyclic guanosine monophosphate (cGMP), which contributes to vascular muscle relaxation.[122] S1PRs in vascular endothelial cells are able to increase NO synthesis through the activation of eNOS and thus producing vasorelaxant effects.

The key role of $S1P_1R$ in mediating vasorelaxant effects has been suggested in early studies. Plasmids encoding eNOS and $S1P_1R$ were introduced into COS-7 cells. The study

found that the addition of S1P greatly increases NO production, however, in cases where a plasmid encoding the $S1P_1R$ gene is not introduced into cells, this effect does not occur.[123] In another study, similar results were obtained in bovine aortic endothelial cell culture, with S1P enhancing NO production in a dose-dependent manner.[124] The most abundant S1PR type is $S1P_1R$ in these cell cultures. "Deletion" of the $S1P_1R$ gene by siRNA technique demonstrates that $S1P_1R$ is essential for NO activation and thus for mediating the vasorelaxant effect of S1P.[125] In the thoracic aortic and mesenteric vascular arteries isolated from rodents, eNOS activation by S1P seems to be mediated by PTX-sensitive GPCR.[126]

Similar to endothelial cells, vascular muscle cells express numerous S1PRs that may be involved in mediating constrictor responses. In contrast to endothelial cells, where $S1P_1R$ is expressed predominantly, $S1P_2$ and $S1P_3$Rs are found in the highest amounts in vascular smooth muscle cells. Solomon *et al.* showed that S1P loses vasoconstrictor activity due to deletion of $S1P_3R$ in cerebral arteries isolated from mice.[127] In contrast, vasoconstrictor effect could not be attributed to $S1P_2R$. A massive vasoconstrictor response was observed in the basilar artery of $S1P_2$-KO mice, to a similar extent as in WT mice.[127] Based on these, the key role of $S1P_3R$ in mediating vasoconstrictor effects was proposed. Murakami *et al.* developed a specific $S1P_3R$ antagonist, TY-52156. WT hearts isolated from rats were examined in Langendorff system. A large reduction in CF was observed in the presence of S1P. This large decrease in CF was also apparent in the presence of $S1P_1R$ and $S1P_2R$ antagonists, however, TY-52156 prevented the flow-reducing effect of S1P.[128] In further studies, the role of $S1P_2R$ in maintaining normal vascular tone was determined. Lorenz *et al.* observed a significant decrease in vascular resistance of the mesenteric artery and renal artery in $S1P_2R$-KO mice.[129] Another research group of our institute, Panta *et al.*, reported the anti-α1-adrenergic vasoconstriction-boosting effect of S1P via the $S1P_2R$ – $G\alpha_{12/13}$ – ROCK pathway.[130]

Thus, S1P is able to mediate vasorelaxant responses in endothelial cells via the eNOS/NO pathway and to mediate vasoconstrictor effects in the vascular smooth muscle. There is no consensus concerning its overall effects, which can vary between specific vascular sections, organs, and species, and can be fundamentally influenced by the experimental set-up, the S1P concentration used, or even prior vasoactive agents used.

2.14. The role of S1P in cardioprotection

S1P and SphK play an important role in the defence against myocardial I/R injury. Numerous studies have addressed the cardioprotective effect of S1P and the vast majority agree that S1P enhances myocardial survival under hypoxic conditions, is involved in the process of ischemic pre- and post-conditioning, and reduces the rate of I/R injury.[131,132,133,134,135,136]

Karliner and colleagues studied the potential cardioprotective effect of S1P in cardiomyocytes. Cardiomyocytes obtained from neonatal rats were exposed to normoxic and hypoxic conditions, and cell viability was measured after 20 h. Under normoxic conditions, cell viability was only slightly reduced, while under hypoxic conditions, it was significantly reduced to 60% of baseline. Incubation of 10 microM S1P prevented damage under hypoxic conditions, and the viability of cells pretreated with hypoxia was similar to that of the normoxic group. Inhibition of PKC and mitochondrial ATP-dependent K^+ channel alone did not, however, co-administration of the two inhibitors abolished the beneficial effect of S1P in hypoxic conditions. The SphK inhibitor dimethylsphingosine significantly impaired cell survival even under normoxic conditions, however, this effect was shown to be preventable by exogenous administration of S1P.[131]

The key enzymes of S1P synthesis, SphK1 and SphK2, play a major role in the release of ischemia-induced S1P from cardiomyocytes and in the process of ischemic pre- and post-conditioning. This is evidenced by experiments showing that hearts from SphK-KO mice had a larger infarct size compared to controls.[137,132,133,138,139]

During ischemic preconditioning, short ischemic periods activate endogenous mechanisms that make the heart more resistant to damage from sudden, persistent ischemia and subsequent reperfusion. Jin *et al.* studied SphK1-KO mouse hearts in the Langendorff system. Ischemic preconditioning consisted of 2-2 minute I/R sections. Isolated hearts were subjected to 50 minutes of global ischemia followed by 40 minutes of reperfusion. In SphK1-KO mice, SphK activity was almost half of that observed in WT, which also resulted in a significant decrease in S1P levels. The experiment showed that the beneficial effect of ischemic preconditioning was completely absent in SphK1-KO mice.[133]

In the Langendorff system, Vessey *et al.* studied the role of SphK in cardioprotection in several configurations. Hearts isolated from rats were subjected to 45 min of ischemia and 45 min of reperfusion after ischemic preconditioning. Their studies showed that SphK activity was greatly reduced due to ischemia and did not recover during reperfusion. In parallel, LVDevP did not recover during reperfusion, and infarct size at the end of reperfusion was significant (47%). In ischemic preconditioned hearts, it was found that the decrease in SphK activity was half of that measured in non-ischemic preconditioned hearts. In parallel, normalization of left ventricular function was observed during the reperfusion period, and much smaller infarct sizes were detected thereafter. Much higher S1P levels were measurable in preconditioned hearts. In the presence of the SphK inhibitor dimethylphosphosine, the cardioprotective effect of ischemic preconditioning was not present.[138]

In another study of the same authors, SphK2-KO mice were studied. In their experimental setup, after 30 min of equilibration of the heart and ischemic preconditioning, they exposed the myocardium to 50 min of global ischemia and then 40 min of reperfusion. In SphK2-KO mouse hearts, SphK activity, which was due to the remaining SphK1, was halved overall. Significant myocardial damage was observed in SphK2-deficient hearts, and postischemic cardiac function parameters were significantly lower than in WT hearts. The beneficial effect of ischemic preconditioning was not detectable in KO animals, and significantly larger infarct sizes were measurable.[139]

Numerous studies have also addressed subtypes of S1PRs involved in mediating the S1P-induced cardioprotective effect. In *in vivo* experiments in mice, Means *et al.* studied the potential role of $S1P_2R$ and $S1P_3R$ in conventional single and double KO animals. The 60-min coronary occlusion used in their experiments was followed by 120 min of reperfusion. I/R injury in $S1P_2R$ and $S1P_3R$ single KO mice was found to be similar to that in WT mice. In contrast, in the hearts of double KO mice deficient in both $S1P_2R$ and $S1P_3R$ genes, infarct size was more than 50% more severe than in control animals.[134]

In a previous study, Yung *et al.* raised the role of RhoA protein and protein kinase D in the process of S1P-induced cardioprotection. To identify S1PRs involved in the effect and their associated G-proteins, each participant was inactivated using siRNA technique. The role of $S1P_3R$ as well as G_{13} protein was confirmed. The role of $S1P_3R$ was also

confirmed in experiments on hearts isolated from $S1P_3R$-KO mice in the Langendorff system. S1P pre-treatment has been found to have a cardioprotective effect, which, however, disappears in $S1P_3R$-KO animals.[135]

In *in vivo* mouse experiments, Theilemier *et al.* showed that co-administration of LDL and S1P significantly reduced infarct size. The importance of the $S1P_3R$ is supported by the observation that mouse hearts treated with exogenous S1P 30 minutes before I/R had a significantly smaller infarct size through $S1P_3R$ and NO-dependent effects than non-treated. The involvement of $S1P_3R$ and NO production in cardioprotection is confirmed by the complete lack of the described protective effect in $S1P_3R$-KO animals and in inhibition of eNOS[136] The importance of S1P-mediated NO synthesis in the above processes may suggest that the effect of S1P in the endothelium in cardioprotection may also be significant.

Although $S1P_2R$ and $S1P_3R$ are primarily implicated in the generation of S1P-mediated cardioprotective effects, a potential role for $S1P_1R$ cannot be ruled out. Goltz *et al.* applied the $S1P_1R$ agonist FTY720 at the onset of reperfusion just right after the ischaemic period in a closed chest model of I/R. Ischaemic postconditioning revealed that FTY720 treated animals had an improved hemodynamic outcome compared to placebo treated animals 21 days after the ischaemic insult.[140] A recent study demonstrated that the specific loss of endothelial $S1P_1R$ exacerbated cardiac remodelling and worsened cardiac dysfunction after myocardial infarct in mouse model.[141] These results indicate the potential role of $S1P_1R$ in cardioprotection.

2.15. Summary of the Introduction

Endocannabinoids and S1P are lipid mediator molecules with diversified effects in several organs, including the cardiovascular system. Both substances are known to act in an autocrine/paracrine manner on their GPCRs ($CB_{1\text{-}2}R$ and $S1P_{1\text{-}5}R$). They regulate vascular tone and cardiac function, the effects of which, although studied by numerous research groups, have often come to contradictory results and conclusions. Both vasoconstrictor and endothelium-dependent vasodilator effects have been published. The cardioprotective effect of S1P has been known for a long time, but this contradicts the vasoconstrictor effects in the coronary system published in the literature, which were also observed by our research group in our own studies. A better understanding of the vascular

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tone regulating, cardiodepressant and cardioprotective effects of endocannabinoids and S1P can contribute to a more accurate understanding of the processes that take place during ACS and thus provide a basis for future research and drug effect studies.

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3. Objectives

Lipid mediators including endocannabinoids and S1P play relevant role in cardiovascular (patho)physiology. Although distinct effects of both compounds have been widely studied by several research groups and different research methods, unanswered questions remain.

Cardiac AT_1R activation has utmost relevance in heart (patho)physiology[56], however, it is yet unknown whether concomitant 2-AG production and CB_1R activation modulate these effects.[142] **The main aim of our "endocannabinoid study" was to reveal the participation of paracrine endocannabinoid mechanisms in Ang II signalling in the heart.** For this purpose, we investigated how inhibition of 2-AG formation and CB_1R activation modify the effects of Ang II administration in isolated Langendorff-perfused rat hearts.

As short-term actions of 2-AG in the heart are complex and controversial[57], we first characterized the responses of the isolated rat heart to these mediators in our experimental setting.[143] The Langendorff system, which is an *ex vivo* method, allows us to examine the heart and only the heart regardless of the autonomic nervous system or other systemic effects.

The cardiac effects of S1P reported in I/R injury are controversial. Activation of S1PRs seems to be cardioprotective, whereas the acute effects of S1P to reduce CF and cardiac contractility are expected to interfere with successful post-ischemic recovery. Moreover, $S1P_2R$ and $S1P_3R$ have been shown to be involved in both mechanisms. In ACS, when S1P is released in large amounts from activated platelets, its favourable and potentially deleterious effects might clash with one another. **In the present study, we aimed to delineate how these opposing S1P actions actually affect postischemic cardiac injury after a non-fatal ischemic insult**.

For this purpose, we conducted experiments in isolated murine hearts mounted on the Langendorff-system. First, we mimicked ACS-related massive S1P release into the blood in order to characterize its coronary effects and consequences on heart function. In these experiments we used albumin as an S1P chaperone and also in subsequent experiments Krebs buffer without added chaperone protein as the vehicle.

Second, using S1PR-KO mouse models, we aimed to identify receptors involved in these cardiac effects. Third, to understand the role of $S1P_3R$ in cardioprotection, we applied a non-fatal I/R protocol in $S1P_3R$-KO hearts.

Finally, the complete sequence of ACS was modelled with an initial exposure of the coronaries to S1P as it occurs during plaque rupture and platelet activation, followed by 20 min of complete ischemia, during which the myocardial S1P-producing machinery can be activated, and concluding with 120 min reperfusion representing successful reopening of the coronary artery in a clinical setting. With this approach, we were able to separate the consequences of intravascular and myocardial S1P-related effects during ACS and also to evaluate their combined effects.

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4. Results

4.1. The endocannabinoid system

All applied procedures regarding our "endocannabinoid study" conform to the guidelines of the Hungarian Law of Animal Protection (28/1998) and were approved by the Government Office of Pest County (Permission number: PEI/001/820-2/2015 and PE/EA/1428-7/2018).

4.1.1. Effects of CB receptor agonists and antagonist on CF and contractile function

To better understand the effects of cannabinoids in isolated Langendorff-perfused rat hearts we examined the effects of CBR endogenous and exogenous agonists and antagonist on CF and contractile function.

The endocannabinoid 2-AG was selected for detailed characterization, since it is produced *in vivo* in larger amounts than other endocannabinoids and is a full agonist on both CB_1R and CB_2R. Furthermore, it has been described that the activation of AT_1R leads to 2-AG release and to the activation of CB_1R. To better understand this interplay between Ang II (a strong vasoconstrictor substance with positive inotropic effects) and 2-AG (to which vasodilator and negative inotropic effects have been attributed) is one of the main goals of this study. Since the metabolism of 2-AG is fast, we also carried out experiments with the more stable CB_1R agonist WIN55,212-2.

After the 30-min equilibration, 2-AG or WIN55,212-2 were infused into isolated hearts for 5 min at a concentration of 10^{-6} M to assess the influence of CBR activation on CF and heart function. 2-AG administration was also repeated in the presence of the CB_1R inhibitor O2050 (10^{-6} M). O2050 infusion was initiated 5 min before the second 2-AG infusion, then continuously coadministered during agonist treatment.

Both CBR agonists enhanced CF (Figure 4A). 2-AG administration had no significant effect on contractile performance; however, WIN55,212-2 produced a marked decline in LVDevP (Figure 4B). 2-AG was also applied in the presence of CB_1R neutral antagonist O2050. The CF-increasing effect of 2-AG was prevented by O2050 (Figure 4A).

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Repeated infusions of 10^{-6} M 2-AG proved to have identical effects in separate experiments (data not shown). We also tested the effects of O2050 infusion alone, and it did not have any significant effect on the cardiac parameters studied (data not shown).

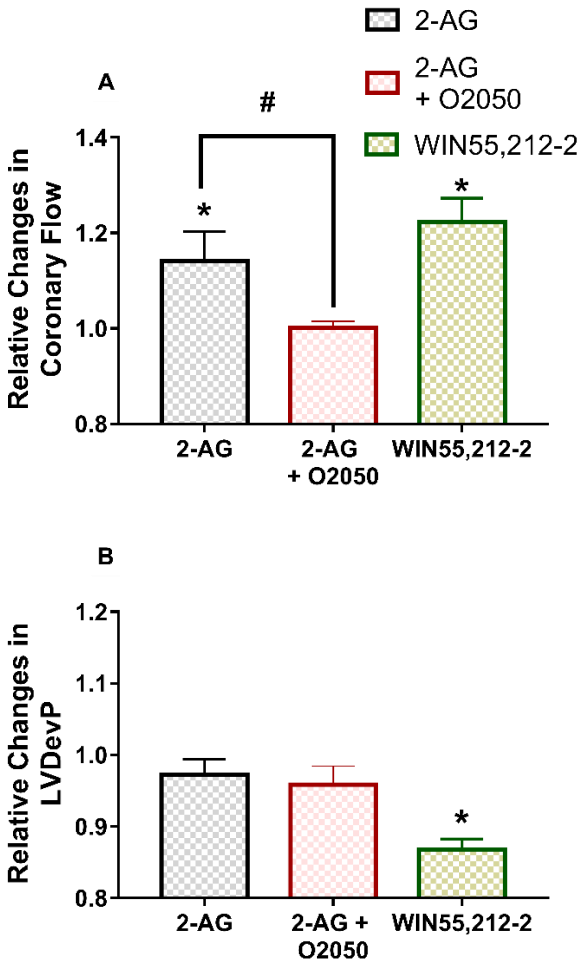

Figure 4. Peak effects of 2-arachidonoylglycerol (2-AG) and synthetic cannabinoid receptor agonist WIN55,212-2 on coronary flow (A) and left ventricular developed pressure (LVDevP) (B) of isolated rat hearts. Data are presented as relative values compared to pre-infusion control data. Mean ± SEM; n = 9 (2-AG and 2-AG + O2050 experiments) and n = 5 (WIN55,212-2 experiments); * $p < 0.05$ vs. pre-infusion value; # $p < 0.05$ vs. 2-AG peak effect, paired t-test.

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4.1.2. The influence of CB_1R antagonist O2050 and DAGL inhibitor Orlistat on Ang II effects

To test how 2-AG-release during AT_1R—$G\alpha_{q/11}$ signalling may modify the primary Ang II responses in the heart via potential transactivation of CB_1R, Ang II (10^{-7} M) was also infused in the presence of CB_1R antagonist O2050 (10^{-6} M) (Figure 5) and DAGL inhibitor Orlistat (10^{-5} M) (Figure 6).

The presence of O2050 in the perfusate substantially altered the effects of Ang II. The deep decline in CF was attenuated by O2050 (Figure 5A). In addition, the temporary decrease in inotropic and lusitropic function, which paralleled the decrease in CF during the first 90 s of the 'Ang II + vehicle' infusion was completely abolished when CB_1R was blocked by O2050 (Figure 5B–D).

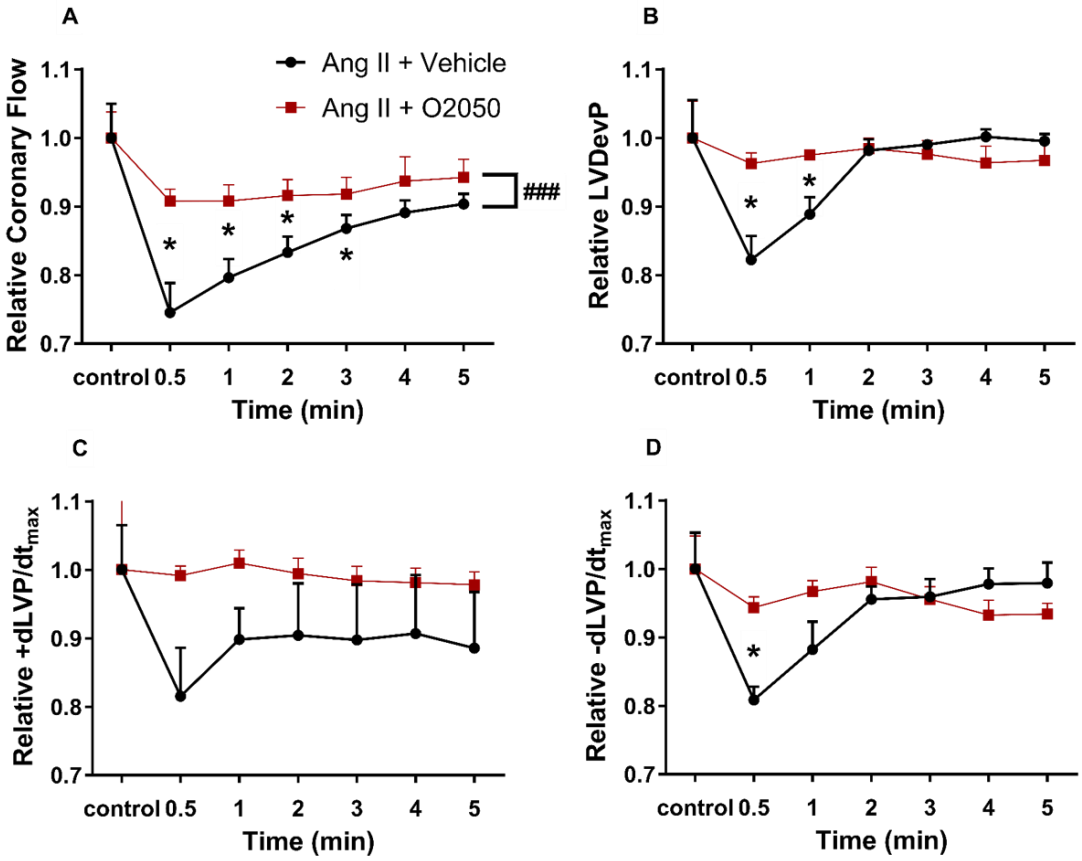


Figure 5. Influence of cannabinoid receptor type-1 inhibitor O2050 on the effects of angiotensin II (Ang II) on coronary flow (A), left ventricular developed pressure (LVDevP) (B), $+dLVP/dt_{max}$ (C) and $-dLVP/dt_{max}$ (D) of isolated rat hearts. The

presented data are expressed as relative values compared to pre-infusion control data. Mean ± SEM; n = 7 (Ang II + vehicle), n = 8 (Ang II + O2050); * $p < 0.05$ vs. control (pre-infusion value); ### $p < 0.001$ vs. vehicle; two-way repeated measurement ANOVA and Dunnett's post-hoc test.

Inhibition of DAGL, the enzyme which is supposed to produce 2-AG from DAG during $G\alpha_{q/11}$-coupled signalling of Ang II, moderated the Ang II-induced peak reduction in CF (Figure 6A) and the significant decrease in LVDevP was not observable (Figure 6B).

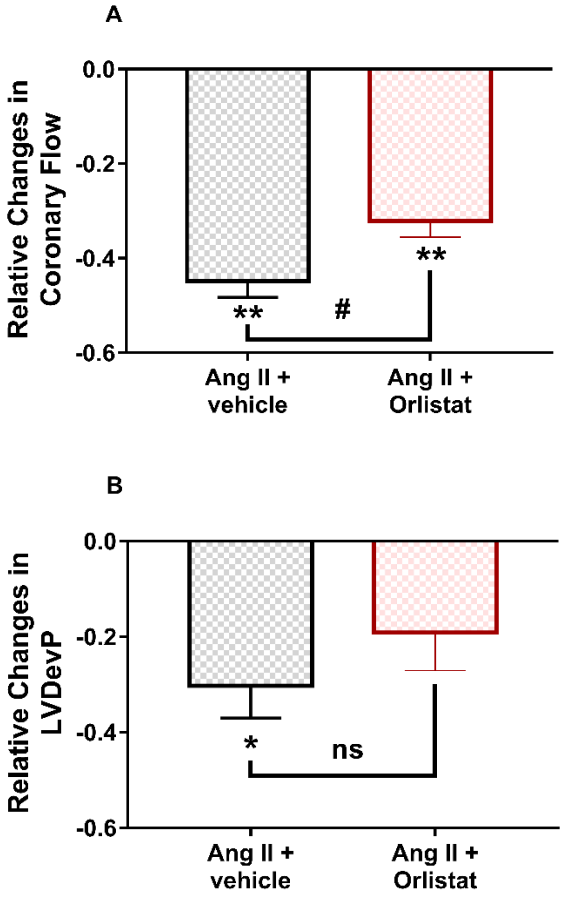


Figure 6. Influence of diacylglycerol lipase inhibitor Orlistat on the peak effects of angiotensin II (Ang II) on coronary flow (A) and left ventricular developed pressure (LVDevP) (B) of isolated rat hearts. The presented data are expressed as relative values compared to pre-infusion control data. Mean ± SEM; n = 4 (Ang II + vehicle), n = 6 (Ang II + Orlistat); * $p < 0.05$ and ** $p < 0.01$ vs. control (pre-infusion value) paired t-test; # $p < 0.05$ vs. Ang II +vehicle; t-test. ns: non-significant

4.2. Sphingosine-1-phosphate

All procedures regarding our "S1P study" were carried out according to the guidelines of the Hungarian Law of Animal Protection (28/1998) and were approved by the Government Office of Pest County (Permission number: PEI/001/820-2/2015).

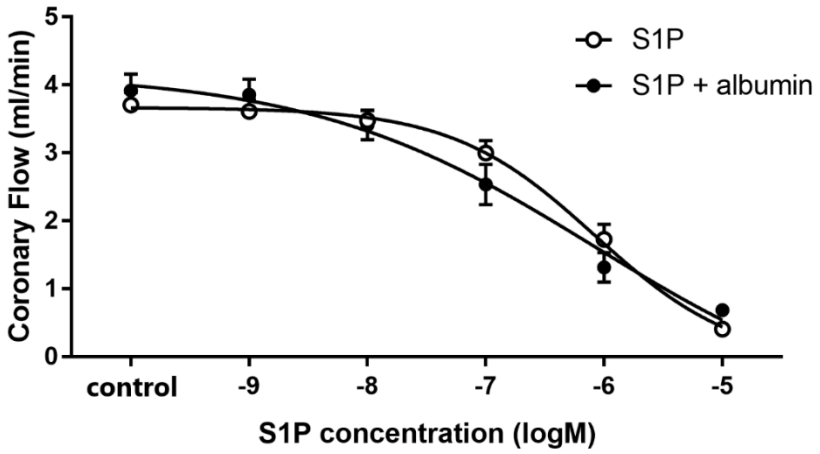
4.2.1. Dose-dependent effects of intravascular S1P on CF administered with or without S1P-chaperon albumin

To characterize the effect of intravascular S1P on CF, we carried out concentration-response experiments in isolated murine hearts either with and without its important chaperone, albumin. Only a small part of S1P is found in its free form under physiological conditions, however, when S1P is released in huge amounts, the ratio of the free form is increased. The dynamics of the S1P effect may be influenced by the tendency of binding, therefore we choose to examine the effect of both free and albumin-bound S1P.

In these experiments S1P was applied in a range of 10^{-9} to 10^{-5} M in cumulative concentrations either without (S1P) or in the presence of its carrier, human serum albumin (S1P + albumin), and its effects on CF were investigated. S1P has been administered for 2 mins in each concentration. Albumin was present in a concentration twice that of S1P.

When administered without a carrier, S1P elicited a concentration-dependent CF reduction in isolated hearts with an ED50 value of 1.17×10^{-6} M. Therefore, the S1P in further experiments was applied in 1 microM - a dose close to its ED50 value.

The coronary effect of S1P was similar in the presence of albumin, however the ED50 value slightly shifted to a smaller concentration range (1.85×10^{-7} M) though it was not



statistically significant ($p = 0.12$, $F = 2.46$) (Figure 7). The maximal reduction in CF was also indistinguishable between groups regardless whether S1P was applied carrier-free or with albumin as vehicle. Based on these, we applied free S1P in our further experiments.

Figure 7. Dose-dependent effects of sphingosine-1-phosphate (S1P) on coronary flow of isolated murine hearts infused alone or in the presence of S1P-carrier albumin. Mean ± SEM; n = 9 (S1P), n = 8 (S1P + albumin). Non-linear regression analysis and comparison of Fits using GraphPad Prism 7.0.

4.2.2. Effects of intravascular S1P exposition on CF and heart function

To investigate the effects of a robust S1P release on CF and cardiac function, 10^{-6} M S1P or its vehicle was administered to the perfusate of isolated WT murine hearts for 5 min. The infusion was followed by a 20-min wash-out period.

Administration of S1P reduced CF by 44 ± 3% (Figure 8A). This remarkable decrease started at the beginning of the S1P infusion and continued progressively during the 5 min. During the 20-min wash-out period, CF did not return to the baseline level and remained at a significantly lower value ($p < 0.0001$).

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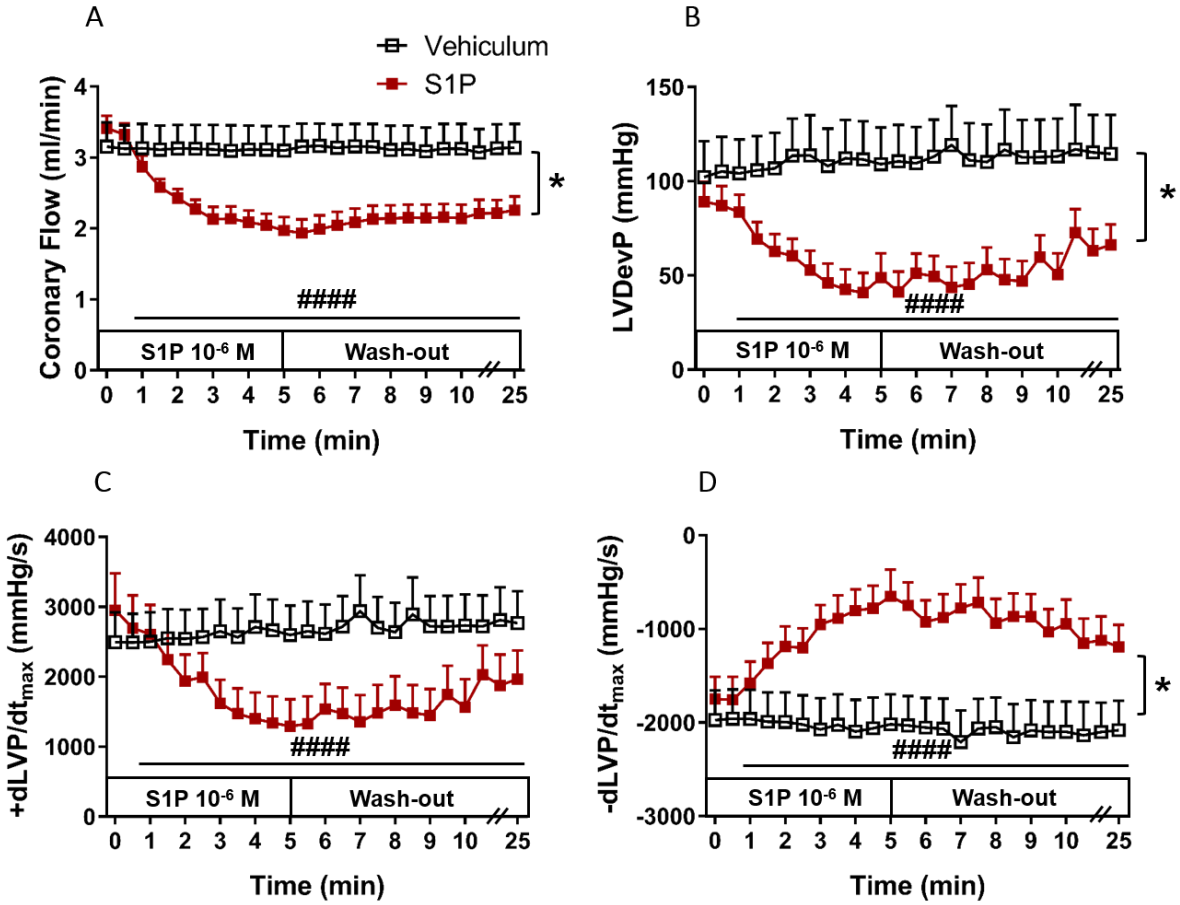

Figure 8. Effects of sphingosine-1-phosphate (S1P) on coronary flow (A), left ventricular developed pressure (LVDevP) (B), +dLVP/dt_{max} (C) and -dLVP/dt_{max} (D) of isolated mouse hearts. Mean ± SEM; n=6 (vehicle), n = 9 (S1P); ####$p < 0.0001$ vs. baseline (pre-infusion value), *$p < 0.05$ vs. vehicle; two-way repeated measurement ANOVA and Dunnett's post hoc test.

CF reduction induced by S1P coexisted with compromised left ventricular contractile performance, which is indicated by a 54 ± 9% drop in LVDevP (Fig. 8B) and by the markedly decreased +dLVP/dt_{max}, and -dLVP/dt_{max} values ($p < 0.0001$) (Figure 8C–D). The vehicle did not affect either CF or other measured heart function parameters (Figure 8A–D).

Earlier studies suggested that S1P might affect coronaries via $S1P_2R$ and $S1P_3R$. Therefore, we aimed to identify which of these receptors mediate(s) the effect of S1P on the CF. For this purpose, we perfused S1P into the isolated hearts of $S1P_2R$-KO (Figure 9) and $S1P_3R$-KO (Figure 10) mice following the experimental protocol described above.

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The CF-reducing effect of S1P developing in S1P$_2$R-KO mice was similar to that of WT littermates (Figure 9A–C). The drop of the LVDevP was also similar in the two groups (Figure 9D–F), with no statistically significant difference.

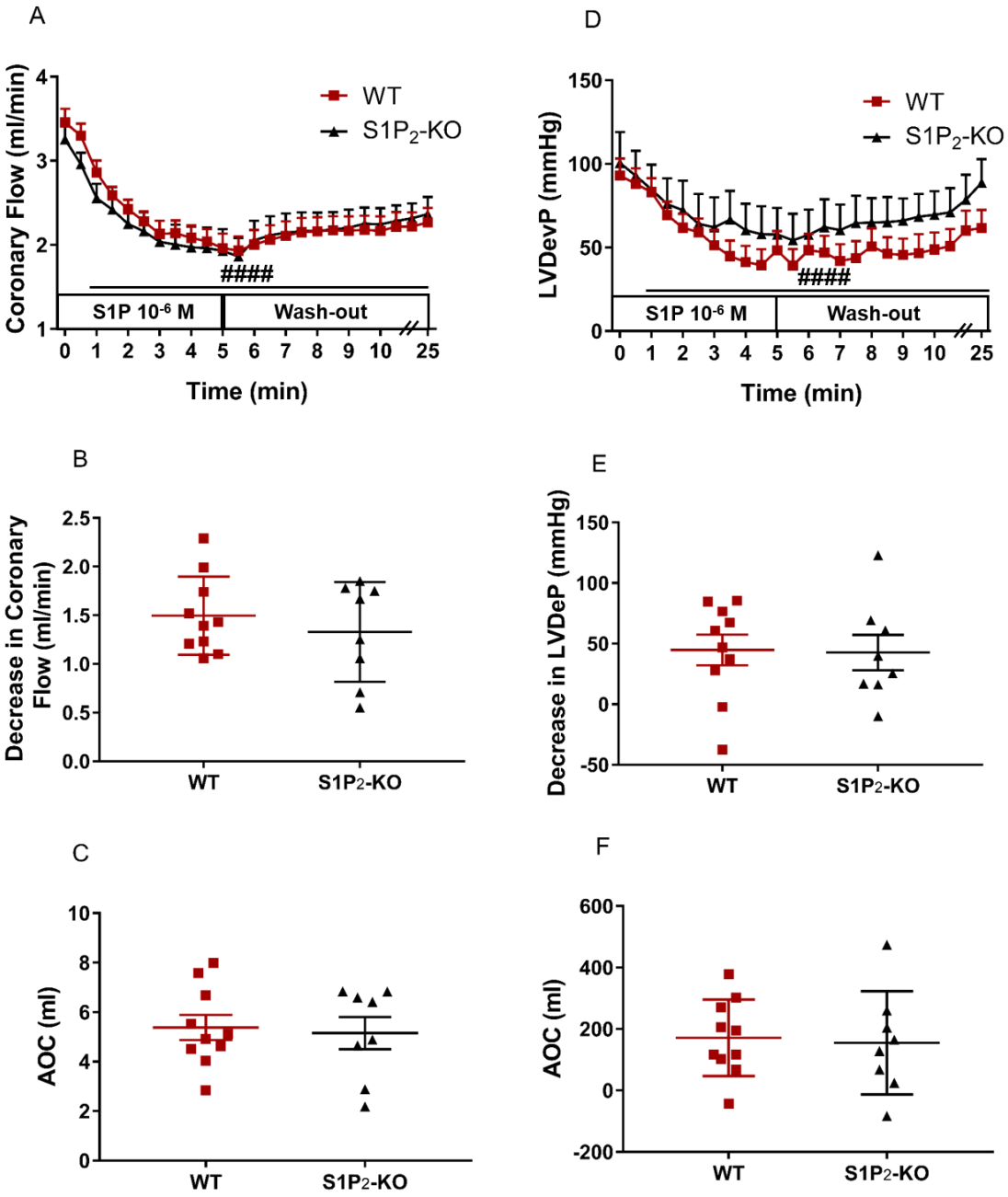

Figure 9. Effects of sphingosine-1-phosphate (S1P) on coronary flow (A–C) and left ventricular developed pressure (LVDevP) (D–F) of hearts isolated from wild-type (WT) and S1P receptor type-2 knock out (S1P$_2$R-KO) mice. Coronary flow and LVDevP were monitored during the entire experiment (panels A and D). Maximal decrease in coronary

flow and LVDevP compared to preinfusion baseline are shown in panels B and E. Values of area over the curve (AOC) during S1P infusion are shown in panels C and F. Mean ± SEM; n=10 (WT), n = 8 ($S1P_2$R-KO); #### $p < 0.0001$ vs. baseline (preinfusion value) in both groups, two-way repeated measurement ANOVA followed by Dunnett's post hoc test.

In $S1P_3$R-KO hearts, the CF-reducing effect of S1P was markedly diminished compared to WT mice (Figure 10A). There was a significant difference in the maximal effects: CF was dropped by 1.95 ± 0.33 mL/min in WT and only by 0.93 ± 0.10 mL/min in $S1P_3$R-KO mice (Fig. 10B). The area over the curve (AOC) used as an index for total perfusion loss during the infusion period showed a similar decrease. During the 5-min S1P infusion, the total perfusion loss was 8.56 ± 1.60 mL in WT vs. 3.70 ± 0.57 mL in $S1P_3$R-KO mice (Figure 10C).

The decrease in left ventricular contractile performance upon S1P infusion was also attenuated in $S1P_3$R-KO mice (Figure 10D): both the maximal drop in LVDevP (Figure 10E) and the area over the LVDevP curve used as a measure of loss of contractile activity (Figure 10F) were significantly reduced compared to WT controls.

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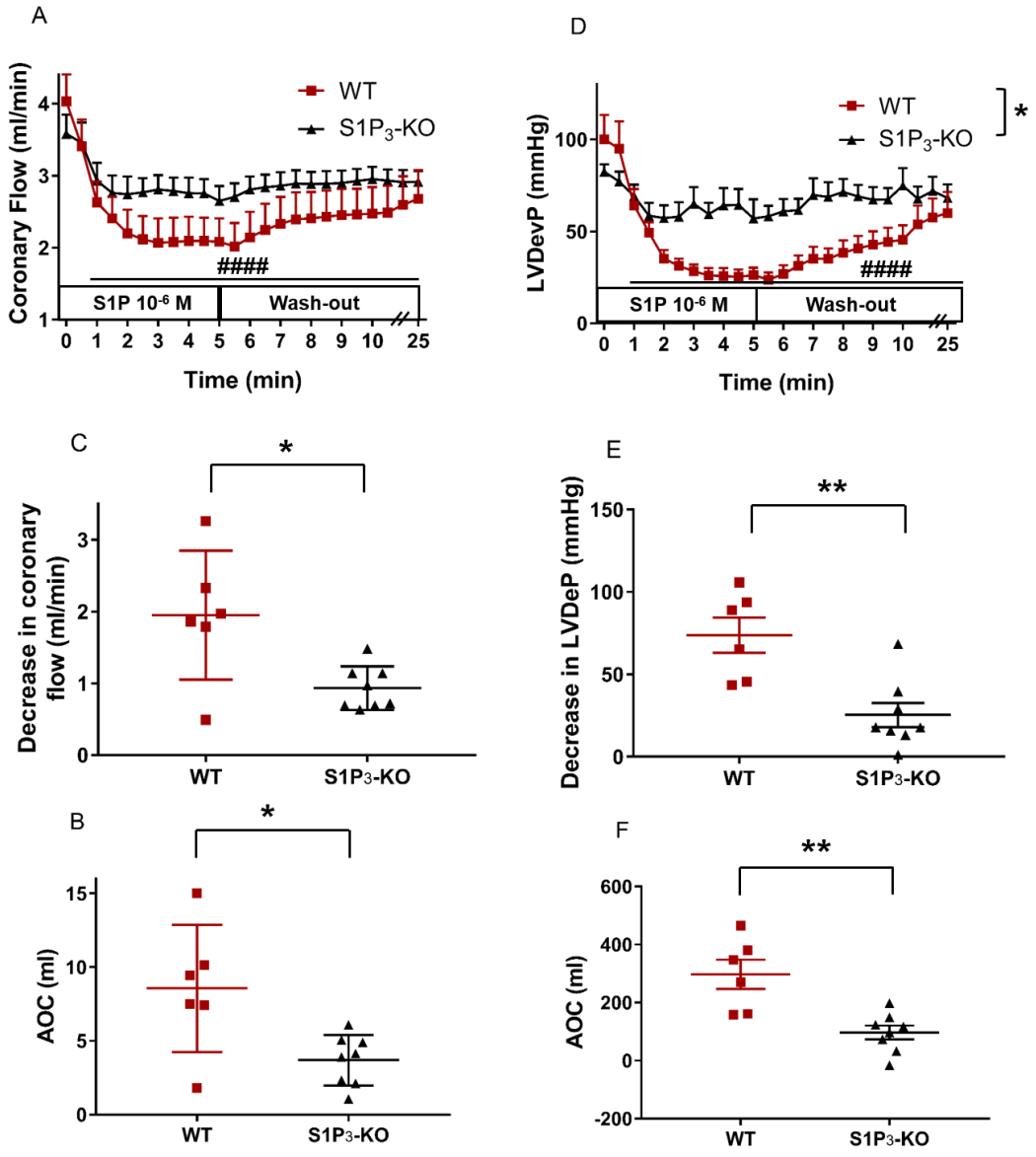

Figure 10. Effects of sphingosine-1-phosphate (S1P) on coronary flow (A–C) and left ventricular developed pressure (LVDevP) (D–F) of hearts isolated from wild-type (WT) and S1P receptor type-3 knock-out ($S1P_3R$-KO) mice. Coronary flow and LVDevP are shown in panels A and D. Maximal decrease in coronary flow and LVDevP compared to preinfusion baseline are shown in panels B and E. Values of AOC during S1P infusion are shown in panels C and F. Mean ± SEM; n=6 (WT), n = 8 ($S1P_3R$-KO); #### $p < 0.0001$ vs. baseline (preinfusion value); * $p < 0.05$, ** $p < 0.01$ vs. WT; two-way repeated measurement ANOVA and Dunnett's post hoc test (A, D) and unpaired t-test (B–F).

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4.2.3. Role of myocardial $S1P_3R$ activation in I/R Injury

To better understand the apparent contradiction between the widely reported cardioprotective and observed cardiosuppressive effects of S1P, we aimed to separate the myocardial and coronary actions of S1P in a model of I/R injury.

The protocol of our I/R experiments was as follows: after 30-min equilibration of the hearts of either WT or $S1P_3R$-KO mice, S1P (10^{-6} M) or its vehicle was perfused for 5 minutes. Then, we applied the 20-min ischemia, which was followed by a 120-min reperfusion period (Suppl. Fig. 1).

First, we investigated the effects of potential $S1P_3R$ activation during I/R in the absence of intravascularly administered S1P. WT and $S1P_3R$-KO hearts were exposed to an I/R protocol, CF and myocardial function were monitored during reperfusion.

During reperfusion CF did not differ significantly between the WT and $S1P_3R$-KO mice (Figure 11A1). In contrast, parameters describing myocardial performance showed marked differences. The lack of $S1P_3R$ resulted in a far worse postischemic functional recovery as evidenced by the drop of the LVDevP (Figure 11B1), decreased $+dLVP/dt_{max}$,, and $-dLVP/dt_{max}$ (Figure 11C1,D1), and elevated left ventricular diastolic pressure (LVDiastP) (Figure 11E1).

These results indicate that $S1P_3R$ plays a beneficial role in preventing ischemia-induced myocardial dysfunction, most probably by activation from S1P generated locally by the tissues of the ischemic heart. However, this myocardial S1P release did not induce $S1P_3R$-mediated coronary vasoconstriction as observed in the previous experiments with intravascular S1P administration.

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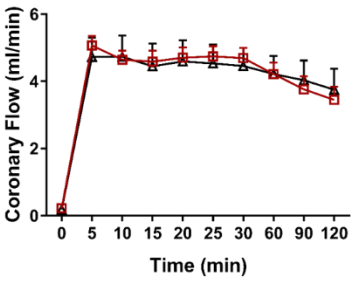
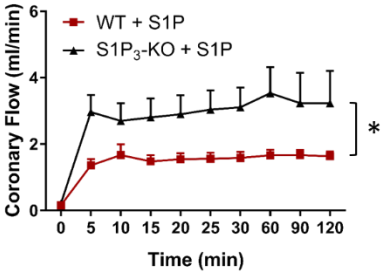
A1



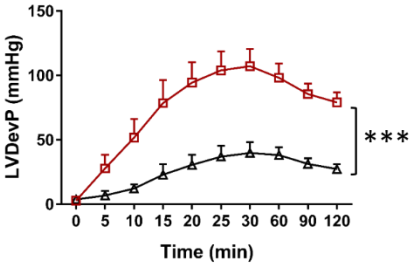
A2



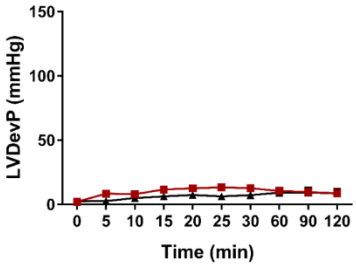
A3

	WT + S1P	S1P$_3$-KO + S1P
WT	ns	ns
S1P$_3$-KO	ns	ns

B1



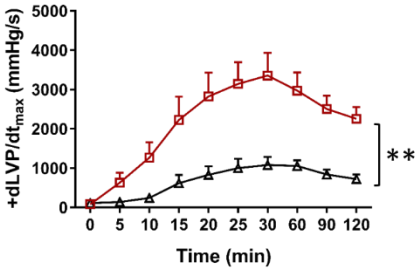
B2



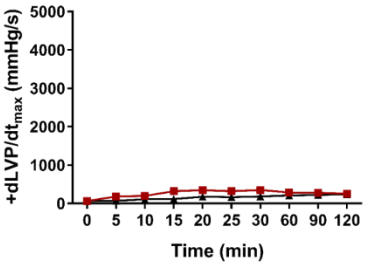
B3

	WT + S1P	S1P$_3$-KO + S1P
WT	***	***
S1P$_3$-KO	*	*

C1



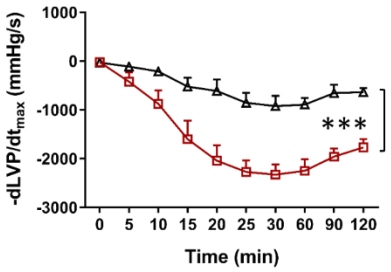
C2



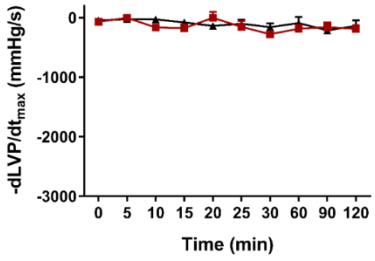
C3

	WT + S1P	S1P$_3$-KO + S1P
WT	****	****
S1P$_3$-KO	ns	ns

D1



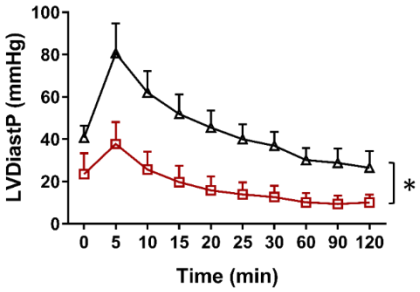
D2



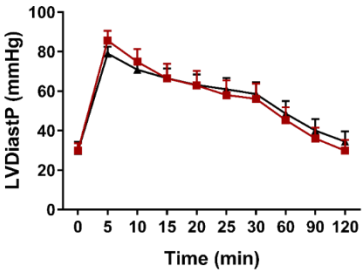
D3

	WT + S1P	S1P$_3$-KO + S1P
WT	****	****
S1P$_3$-KO	*	*

E1



E2



E3

	WT + S1P	S1P$_3$-KO + S1P
WT	ns	*
S1P$_3$-KO	ns	ns

Figure 11. Postischemic coronary flow (A), left ventricular developed pressure (LVDevP) (B), +dLVP/dt_{max} (C), -dLVP/dt_{max} (D) and left ventricular diastolic pressure LVDiastP (E) in isolated wild-type (WT) and sphingosine-1-phosphate receptor type-3 knock-out ($S1P_3$R-KO) mouse hearts without (left panels: A1–E1) or with sphingosine-1-phosphate (S1P) administration (middle panels: A2–E2). The right panels (A3–E3) demonstrate statistical comparison of the parameters captured at the end of the reperfusion period. Mean ± SEM; n=6 (WT), n = 8 ($S1P_3$R-KO), n = 7 (WT + S1P), n = 7 ($S1P_3$R-KO + S1P); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, with two-way repeated measurement ANOVA and Dunnett's post hoc test in the graphs and two-way ANOVA followed by Sidak's post hoc test in the table insets.

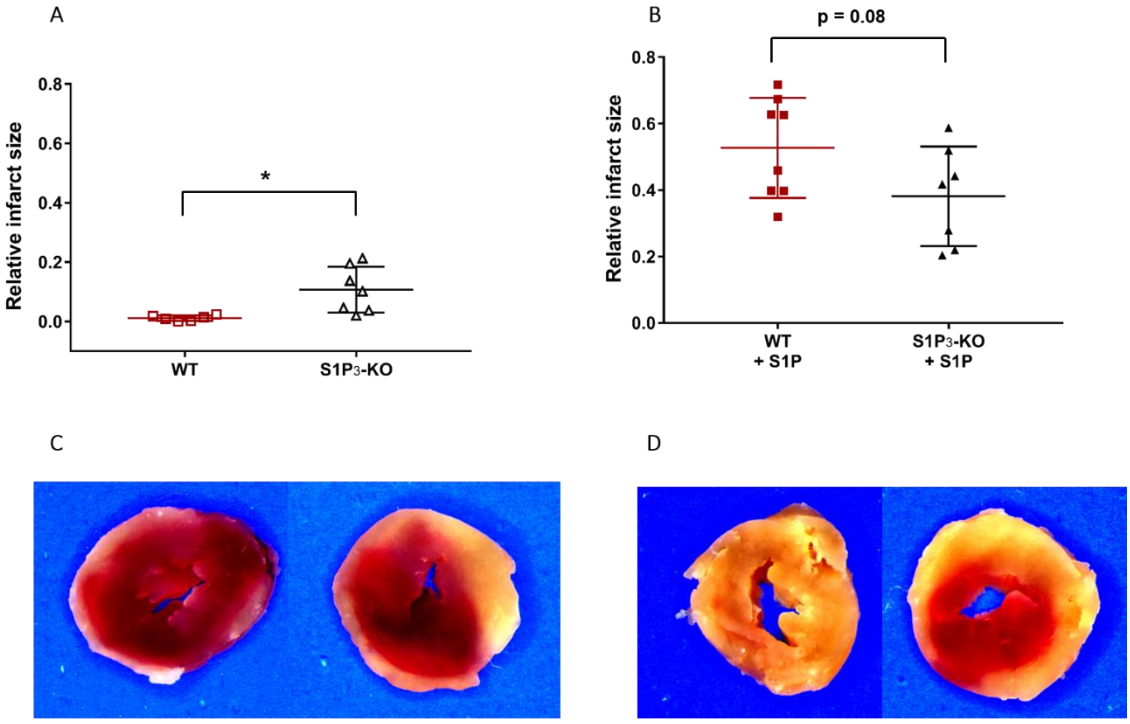
4.2.4. Effects of preischemic intravascular S1P exposure on I/R injury

Next, we investigated the role of intravascular S1P by administering S1P to the perfusion solution before ischaemia at a concentration of 10^{-6} M for 5 min. Under these conditions, CF returned to a significantly higher value during reperfusion in the $S1P_3$R-KO hearts (Figure 11A2) indicating $S1P_3$R-mediated coronary vasoconstriction. Postischemic myocardial function failed to return during the reperfusion without any difference between the two groups (Figure 11B2–E2).

In order to determine the effects of S1P infusion on postischemic CF and cardiac performance, results obtained by the two experimental protocols were compared (see the table panels in Figure 11). S1P-exposed WT hearts showed a marked reduction in post-ischemic functional myocardial recovery as compared to WT or $S1P_3$R-KO hearts without S1P administration (Figure 11 B3–E3). Furthermore, the difference in the functional parameters between WT and $S1P_3$R-KO hearts was not statistically significant (B2–E2 table insets in Figure 11).

Finally, we determined whether alterations in myocardial function were reflected in the irreversible ischemic damage of cardiomyocytes. TTC staining revealed that without S1P administration, the relative infarct size was larger in $S1P_3$R-KO (10.72 ± 2.93%) than in WT (1.12 ± 0.37%) hearts (Fig. 12A & C). In the S1P-exposed groups, the infarcts were substantially larger compared to untreated groups, but they did not differ between $S1P_3$R-KO and WT hearts (Figure 12B,D).

Comparing the size of the infarcted area in S1P-pretreated (Figure 12B,D) to that of the untreated groups (Figure 12A,C), we detected a marked increase in the size of the infarcted myocardium as a result of S1P administration (Figure 12E).



E

	WT + S1P	$S1P_3$-KO + S1P
WT	****	****
$S1P_3$-KO	****	**

Figure 12. Relative infarct size (A, B) and representative sections (C,D) from hearts subjected to ischemia/reperfusion without (A & C) or with (B & D) sphingosine-1-phosphate (S1P) infusion. Mean ± SEM; n=6 (WT), n = 8 ($S1P_3$R-KO), n = 7 (WT + S1P), n = 7 ($S1P_3$R-KO + S1P); * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, with unpaired t-test (A, B) or two-way ANOVA and Sidak's multiple comparison test (E).

4.2.5. Role of myocardial $S1P_2$R activation in I/R Injury

Interestingly, lack of $S1P_2$R did not influence any of these functional parameters nor the infarct size in this I/R model (Figure 13).

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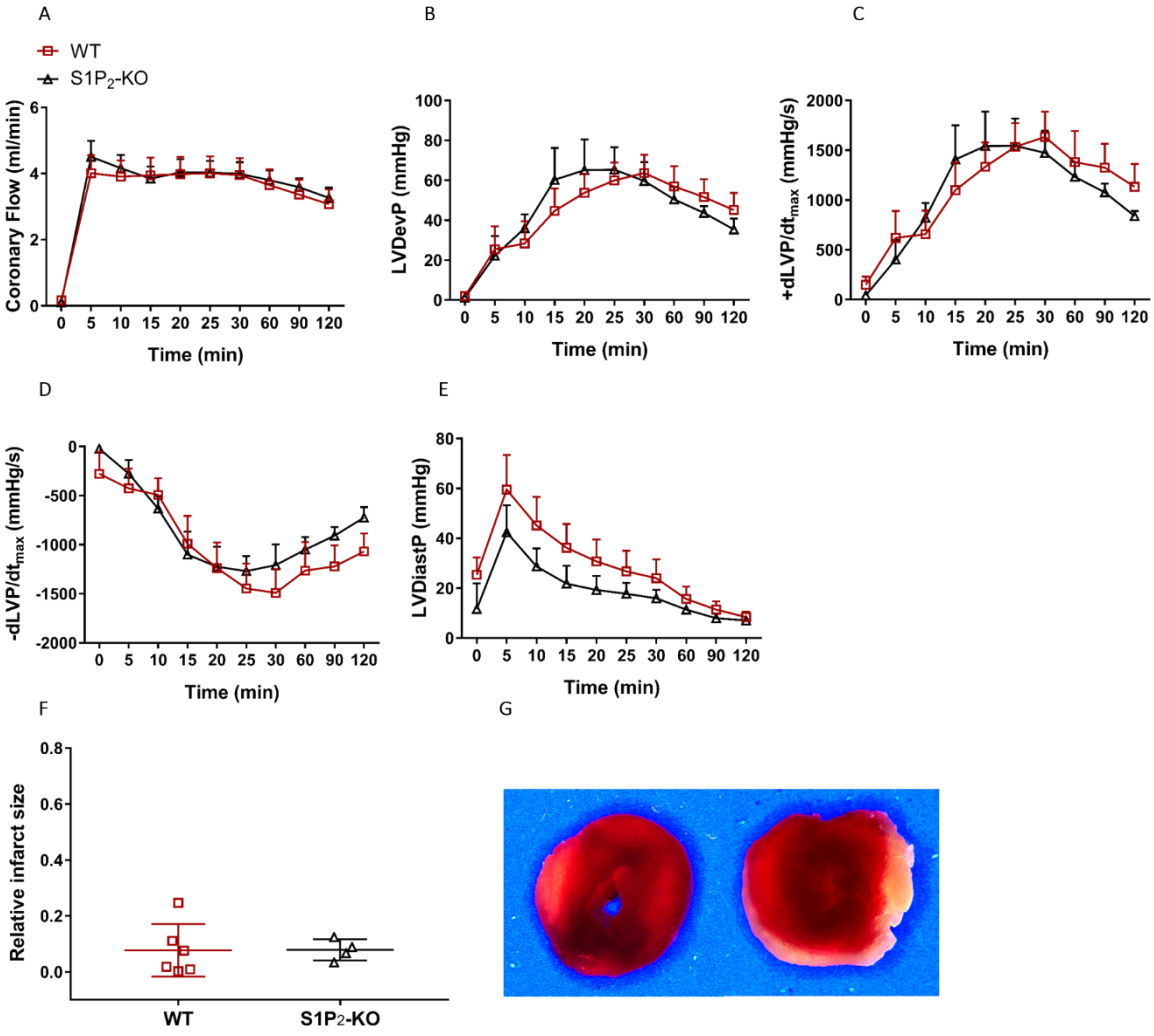

Figure 13. Postischemic coronary flow (A), left ventricular developed pressure (LVDevP) (B), +dLVP/dt_{max} (C), -dLVP/dt_{max} (D) and left ventricular diastolic pressure (LVDiastP) (E) in isolated wild-type (WT) and sphingosine-1-phosphate receptor type-2 ($S1P_2$R-KO) mouse hearts. Vehicle was administered for 5 min to the perfusate before the induction of a 20-min ischemia followed by a 120-min reperfusion period. Relative infarct size (F) and representative sections (G) from hearts subjected to ischemia/reperfusion. Mean ± SEM; n=6 (WT), n = 4 ($S1P_2$R-KO); statistics: two-way repeated measurement ANOVA and Dunnett's post hoc test (A-E) and unpaired t-test (F).

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5. Discussion

5.1. Main findings of the endocannabinoid study

The main finding of our endocannabinoid study is that in an isolated rat heart preparation with intact circulation, the cardiac responses to Ang II are mediated in part by CB_1R activation, most probably through 2-AG release during AT_1—$G\alpha_{q/11}$ signalling. In our experiments, Ang II induced a remarkable decrease in CF, which was associated with a temporary decrease in cardiac function. These effects were effectively blocked by CB_1R and DAGL inhibition. In addition to this observation, direct administration of 2-AG and CB_1R agonist WIN55,212-2 into the perfusate induced a significant increase in CF, indicating that the endocannabinoid system and CB_1R play a relevant role in the regulation of cardiac tissue perfusion.

5.1.1. Effects of endocannabinoids on cardiac function

Endocannabinoids, such as anandamide, 2-AG and 2-arachidonoylglyceryl ether[144], serve as endogenous ligands for CBRs. Cardiovascular effects of cannabinoids are attributed mostly to CB_1R[145,146,147]. The direct and indirect endocannabinoid effects on the heart are diverse. *In vivo*, endocannabinoid-induced bradycardia as well as tachycardia induced by THC have been observed, most probably as the indirect consequences of endocannabinoid effects on autonomic nerves. Moreover, anandamide has been shown to induce a complex triphasic autonomic response in the heart.[62,33,34]

Cannabinoids, and synthetic analogues (e.g. THC, WIN55,212-2) also target vascular tissue cells causing mostly vasodilation and hypotension.[43,51,52,45,46,48,50,53,54] Besides their vascular expression, CB_1Rs have also been detected in the cardiac tissue by several research groups[68,66] and also by us[69]. Cannabinoid actions in the heart are the result of an interplay between coronary and cardiomyocyte effects. Anandamide and 2-AG endocannabinoids have been shown in various experimental settings to cause coronary vasodilation, increase in coronary blood flow and decrease in cardiac contractility.[148,149,71,150]

In Langendorff hearts, anandamide has been reported to cause vasodilation and to decrease LVDevP, effects that are attributed mainly to CB_1R[150]. Similarly, in vasopressin-pretreated Langendorff hearts, the selective CB_1R agonist anandamide concentration-

dependently increased CF[151]. Similar effects were observed with metabolically stable endocannabinoid derivatives, R-methanandamide and noladin ether as well. Moreover, in this study, anandamide and 2-AG were detected in the cardiac tissue[151]. In a previous study performed on isolated coronary vessels, Szekeres *et al.* observed vasodilation of precontracted vessels in response to CB_1R agonist WIN55,212, an effect that was blocked by CB_1R antagonists AM251 and O2050.[152]

In agreement with literature data, we also found that 2-AG and WIN55,212-2 infusion (Figure 4) significantly increased CF. The inhibition of 2-AG-induced CF response by O2050 indicates the functional presence of CB_1R on coronary vessels and suggests that CB_1R has a significant role in the control of coronary perfusion. Notably, we reported CB_1R on rat coronary vessels in a previous study.[152] The negative inotropic response of the heart to cannabinoids reported by other research groups[147] was observed only during WIN55,212-2 administration in our study, and did not develop when 2-AG was applied (Figure 4). This discrepancy can be explained by the fast metabolism of 2-AG, which may not allow a build-up of 2-AG concentration in the close environment of cardiomyocytes which is high enough to elicit the negative inotropy when intracoronary application is used. On the other hand, we need to take into account that the observed 2-AG-induced rise in CF evokes a consequential positive effect on contractility (Gregg phenomenon), which can mask its direct negative inotropic action.[153]

5.1.2. Role of Ang II-Induced Endocannabinoid Release in Cardiac Function

Since *Szekeres et al.* has previously reported that Ang II via AT_1R induces the release of 2-AG in the vascular tissue and CB_1R has also been detected in the cardiac tissue, we postulated that Ang II-induced 2-AG release may also have relevance in the heart. In our study, Ang II remarkably decreased coronary perfusion flow with a moderate negative inotropic and lusitropic effect in Langendorff rat hearts (Figure 5). This is in agreement with the findings of van Esch *et al.*[57,154], but seems to contradict other studies which reported positive inotropy at higher Ang II-concentrations[155] and in isolated cardiomyocytes[156]. However, in our experimental setting, the negative functional effects on the heart are suggested to be an indirect consequence of decreased coronary perfusion.

In our experiments, we found that CB_1R blockade by O2050 (Figure 5) abolished, and inhibition of DAGL by Orlistat attenuated (Figure 6) Ang II-induced CF reduction and cardiac effects. This seems to contradict previous studies on isolated coronary arteries and also on the aorta, which reported augmented vasoconstriction in response to Ang II when CB_1R inhibitor O2050 was applied.[157] However, it is important to note that the Langendorff-perfused heart is a more complex system in which the applied pharmacons act on the cardiac tissue and on the coronary vessels as well, influencing CF and cardiac function in a complex manner. In the intact heart, CF is primarily controlled by the oxygen demand of cardiac work. This regulatory phenomenon often overwrites the direct vascular effects of vasoactive agents and may explain why we observed a CF effect that is opposite to the one expected on the basis of isolated vessel studies. This phenomenon can be most probably explained by the altered cardiac effects of Ang II upon CB_1R inhibition. Specifically, we propose that O2050 blocks the CB_1R-induced negative inotropic effect, which allows the Ang II-induced positive inotropy to develop (which is otherwise masked by flow deprivation). This increases the overall oxygen demand and thus the CF, and overweighs the CF-reducing effect of the loss of direct vasodilator 2-AG action.

Although we did not observe negative inotropy upon 2-AG administration to the coronary perfusate, it was evident when WIN55,212-2 was applied (Figure 4). The lack of 2-AG effect may be caused by its fast metabolism, which may prevent the build-up of a concentration in the cardiac tissue upon intracoronary application, which is sufficiently high to exert this effect. However, we may assume that when 2-AG is released from cardiomyocytes during Ang II signalling, negative inotropy develops as a paracrine/autocrine effect.

In summary, the diminished CF-reducing effect of Ang II during DAGL blockade and CB_1R inhibition can be attributed to the enhanced cardiac oxygen demand, which is evoked by increased cardiac contractility. This overrides Ang II-induced vasoconstriction. Thus, our observation suggests that the acute effects of Ang II on isolated Langendorff hearts are modulated by PLC-signalling-induced endocannabinoid release via CB_1R stimulation. We propose that the short-term cardiac effects of Ang II are exhibited as a delicate balance of the simultaneous cardiac and vascular effects of Ang II and 2-AG (Figure 14).

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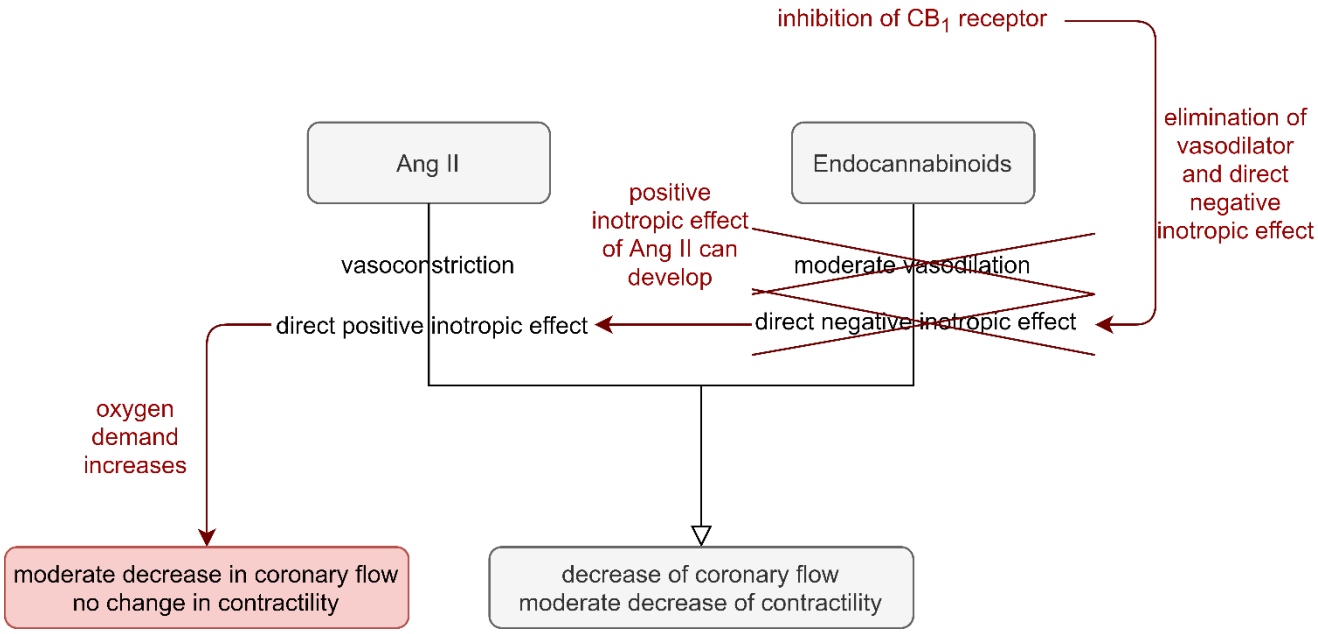

Figure 14. Postulated mechanism of the role of angiotensin II (Ang II)-induced 2-arachidonoylglycerol release in Ang II-induced short-term cardiac effects. Ang II causes vasoconstriction of the coronaries and has positive inotropic effects on cardiomyocytes. On the other hand, 2-arachidonoylglycerol release during Ang II signalling causes moderate vasodilation and has negative inotropic effects. The summation of these opposing effects manifests in diminished coronary flow accompanied by a moderate decrease in contractility in the Langendorff-perfused rat heart (black arrows). Inhibition of cannabinoid receptor type-1 (CB_1R), and 2-arachidonoylglycerol generation eliminates the counteraction of 2-arachidonoylglycerol on the positive inotropic effect of Ang II. The unopposed positive inotropic effect of Ang II enhances cardiac oxygen demand, which counterregulates the vasoconstrictor effect of Ang II. As a result, the flow-reducing effect of Ang II is moderated, and the opposing effects of diminished coronary flow and increased contractility cancel each other out, leaving the contractile function unaltered (red arrows).

5.2. Main findings of the S1P study

In our S1P study, three different experimental protocols were tested in order to understand the complexity of S1P-induced alterations of cardiac function in ACS and also to determine the involvement of S1PR subtypes in mediating these effects.

First, we focused on intravascular S1P release, which occurs at the onset of ACS when plaque rupture initiates platelet activation. We found that S1P caused a concentration-dependent massive reduction of heart perfusion and simultaneous suppression of myocardial contractility. Both effects were attenuated in hearts of $S1P_3$R-KO mice, indicating a major role of $S1P_3$R in signalling.

In the second part of our study, we focused on the effects of S1PR activation within the heart in response to ischemia. Under these conditions, hearts of $S1P_3$R-KO mice exhibited worse postischemic contractile recovery and larger infarct size than WT hearts, indicating that ischemia-induced myocardium-related S1P actions are cardioprotective via activation of $S1P_3$R.

Finally, we proposed to model the complex scenario of ACS, when intravascular and myocardial S1P release may occur simultaneously and influence cardiac function. Under these conditions, WT hearts showed limited coronary perfusion without any sign of postischemic functional recovery. In $S1P_3$R-KO hearts, coronary reflow was better, but this failed to improve cardiac function or to reduce infarct size compared to WT.

These observations indicate that although $S1P_3$R-mediated vasoconstriction contributes to the deleterious no-reflow phenomenon, elimination of this effect in $S1P_3$R-KO hearts does not moderate I/R injury because it also abolishes the benefits of $S1P_3$R-mediated cardioprotection.

5.2.1. Effect of S1P on coronary flow and cardiac function

The major sources of S1P in blood plasma are red blood cells, platelets, and endothelial cells.[158,159] SphK is highly active in platelets and synthetizes S1P from sphingosine taken up from plasma and produces it in the outer leaflet of the platelet plasma membrane.[160] Platelets store S1P abundantly and release it upon activation.[161,87,162] In ACS, when blood clotting is activated by the rupture of an atherosclerotic plaque, substantial amount of S1P might be released to the circulation.[163]

S1P has been reported to have vasoconstrictor and endothelium-dependent vasodilator actions in different vascular beds.[164,165,166] For instance, S1P was shown to have a constrictor effect in isolated porcine pulmonary artery rings[167], in canine, rat, murine and leporine basilar and middle cerebral arteries[168,169], in rat portal veins[170], and in canine

coronaries.[171] Contrary to these S1P increased NO production in cultured HUVEC[172] and in bovine lung microvascular endothelial cells[173], and HDL, a carrier of S1P was shown to cause endothelium-dependent vasodilation in aortic rings of rats and mice mediated via $S1P_3R$ activation.[174]

However, despite its potential pathophysiological relevance, only a few of these studies have investigated the effects of S1P in the coronaries, and none of them have attempted to relate it to myocardial function.[175] In our study, we found that S1P causes dose-dependent reduction in CF of Langendorff-perfused murine hearts (Figure 7). This observation is in agreement with earlier reports that also ascribed vasoconstrictor effects to S1P in the coronaries and other vascular beds.[176,177,178] Murakami *et al.* reported dose-dependent S1P-induced CF reduction in rat hearts in a similar experimental model.[176] When we delivered S1P in the presence of albumin, the coronary effect of S1P remained unchanged (Figure 7). Although, a slight, nonsignificant shift in the ED50 to lower concentration was observed, potentially indicating that albumin may enhance S1P coronary effects by protecting it from degradation by phosphatases in the vessels of isolated hearts.

One of the main aims of our study was to 1) mimic the effect of robust S1P exposure of the coronary arteries that might occur in ACS upon thrombotic platelet activation, and 2) explore its effects on coronary perfusion, and 3) on heart function. For this purpose, we administered S1P to the coronary perfusate of isolated murine hearts at 1 microM, a concentration that might easily occur in a thrombotic coronary artery[158,163,179,180], and was close to the ED50 value, we defined (Figure 7). This produced a remarkable decrease in CF (Figure 8A). The S1P-induced flow deprivation in our study was associated with a significant decline in cardiac performance, which was evidenced by decreased LVDevP, +dLVP/dtmax and -dLVP/dtmax (Figure 8B-D). This might be primarily attributed to CF reduction. However, direct negative inotropic effect of S1P on cardiomyocytes reported by earlier studies might also play a role.[23]

5.2.2. Role of S1PRs in CF reducing and cardiac effects of S1P

The cellular actions of S1P are attributed to the presence of five specific G protein-coupled S1P receptors.[181,143] Among these, $S1P_1R$, $S1P_2R$, and $S1P_3R$ are expressed abundantly in the CV system.[182] Detailed description of S1P signalling in coronaries is

not available in the literature. However, a few studies provide evidence that $S1P_2R$ or $S1P_3R$ might play a role in the regulation of heart function. In a recent study, a dominant role of $S1P_2R$ in S1P-induced enhancement of vasoconstrictor stimuli in the circulation has been reported.[183] Therefore, in the present study we aimed to characterize the role of these two receptors in mediating CF reduction by S1P.

Using an $S1P_3R$-KO mouse model, we showed that the $S1P_3R$ plays a relevant role in mediating S1P-induced CF reduction, because the absence of this receptor diminishes significantly the CF reducing effect of S1P (Figure 10C). This observation confirms the findings of Murakami *et al.*, who proposed the role of $S1P_3R$ in coronary constriction using the $S1P_3R$ antagonist TY-52156 in a similar experimental setting.[176] Levkau *et al.* found that S1P decreases myocardial perfusion *in vivo* and this effect was absent in $S1P_3R$-KO mice.[178]

Other investigators proposed the role of $S1P_2R$ because S1P induced constriction in human coronary smooth muscle cells that was attenuated by the $S1P_2R$ antagonist, JTE-013.[177] However, in our experiments $S1P_2R$-KO mice did not reproduce these pharmacological observations. We acknowledge that this does not necessarily mean that the $S1P_2R$ has no role in regulating coronary vessel tone, because it might be that $S1P_2R$ also activates pathways in the heart which cause coronary dilation, and these and the direct vasoconstrictor effects in smooth muscle cells cancelled out each other in our experiments. However, this putative mechanism requires further investigation. Moreover, $S1P_2R$ activation might also sensitize the smooth muscle to other vasoconstrictor stimuli, as has been shown in the systemic circulation.[183]

5.2.3. Role of S1P and S1PRs in cardioprotection

S1P is frequently implicated in cardioprotection.[184,185,186,187] Indeed, numerous studies have shown that it decreases the infarcted area and apoptotic cell death after I/R injury, and that it plays a role in the mechanism of ischemic pre- and post-conditioning.[188,189,138,190,191,192] However, myocardial function has not yet been evaluated in detail in these previous studies, although the involvement of $S1P_2R$ and $S1P_3R$ has already been suggested.[192,176,177] This protective effect has been inferred from experiments, through the use of fundamentally different methodological approaches. In most of these studies, inhibition of S1P signalling in ischemia was achieved by using

S1PR gene-deficient models or the pharmacological inhibition of SphK1 and SphK2 enzymes, which made I/R injury more severe and/or reduced the benefits of ischemic pre- and post-conditioning. These observations suggest that S1P signalling is stimulated in ischemia most likely by locally generated S1P released from the heart tissue.

The other approach introduced intravascular S1P administration into the coronary blood flow before an ischemic insult. Although this experimental setting can be considered as a relevant model for studying S1P effects in ACS, inasmuch as S1P infusion mimics S1P release during thrombus formation, whereas the flow cessation models thrombotic occlusion, only a few investigators have explored S1P effects this way, and they only assessed tissue damage without monitoring postischemic heart function. Nevertheless, these studies consistently reported a decrease in the infarcted area.[138,192] This is surprising considering that S1P has several short-term effects in the heart by reducing coronary flow (CF) and causing negative inotropy that might be detrimental to postischemic contractile recovery.[193,194,195,196]

Our S1P study was designed to combine these approaches in the context of S1PR signalling. Our choice of focus on $S1P_3R$ signalling was motivated by the results of our experiments shown in Figures 9 and 10 which highlight that the short-term cardiac effects of S1P are mediated in large part by $S1P_3R$. However, S1P2, which is the other receptor proposed to participate in cardioprotection[190], did not have a major role. Moreover, the exposure of $S1P_2R$-KO hearts to our I/R protocol produced similar functional and tissue injury to that observed in control hearts (Figure 13), showing that this receptor has no detectable role in cardioprotection in our experimental setting.

5.2.4. Role of $S1P_3R$ in cardioprotection

First, we aimed to clarify whether intrinsic activation of $S1P_3R$ signalling during ischemia was protective in our experimental setting. Our results showed that, in the absence of $S1P_3R$, murine hearts were more susceptible to a 20-min global ischemia. This was indicated by weaker contractile recovery during the 2-hour reperfusion period (Fig. 9, B1-D1), higher postischemic end-diastolic pressure (Fig. 9, E1) an indicator of more severe myocardial ischemic contracture, and increased infarct size (Fig. 10A & C).

These observations are in agreement with other studies which also implicated the participation of $S1P_3R$ signalling in cardioprotection against I/R injury.[190,191,192] Notably,

the severe functional and morphological injury in $S1P_3R$-KO hearts developed despite a relatively maintained CF, which approached the preischemic value and was not worse than that of WT hearts during the reperfusion period (Fig. 9, A1). The observation that CF during reperfusion was similar in WT and $S1P_3R$-KO hearts indicates that vascular $S1P_3R$ was not exposed to S1P levels sufficient to induce $S1P_3R$-mediated vasoconstriction. This is not surprising if we consider that the perfusion fluid was free of exogenously added S1P.

5.2.5. Role of exogenously administered S1P in cardioprotection

In our study we also investigated the effects of S1P on I/R injury by the other approach described in the literature, where S1P was administered to the coronary circulation before ischemia. After S1P pretreatment, we applied a non-fatal ischemia protocol and followed up the recovery of cardiac function upon reperfusion.

This model allowed for the exploration of S1P actions which may take place in a complex ACS scenario in which simultaneous intravascular and myocardial S1P release may occur. Preischemic S1P infusion can be considered as simulation of platelet-derived S1P release in ACS[161,160,158,163,87,162], whereas the ischemia protocol as S1P release from the myocardium.[189,138]

Furthermore, our experiments investigated S1P effects more broadly than previous studies. In addition to determining infarct size, we also assessed postischemic cardiac function. We found that preischemic S1P exposure exacerbated ischemic injury. After an ischemic insult which is supposed to be non-fatal, the infarcted tissue extended to a large part of the myocardium and restitution of contractile activity was hardly observed in WT hearts. The latter was indicated by extremely low LVDevP, +dLVP/dtmax, and -dLVP/dtmax values (Fig. 9) that failed to approach preischemic levels during reperfusion, although CF partly recovered. Comparing ischemic injury of S1P pretreated and non-treated WT hearts, infarct size was significantly larger (Fig. 10E), whereas LVDevP, +dLVP/dtmax and -dLVP/dtmax (Fig. 9 B3-D3) values were significantly lower at the end of the 2-h reperfusion period.

Interestingly, some researchers observed a decrease in infarct size after preischemic S1P treatment.[138,191,192] This difference might be explained by differences in methodology because the infusion time and concentration of S1P preinfusion were slightly

different.[191,184,138] A possible explanation can be that our infusion protocol, where we applied S1P at 10^{-6} M, might have caused sustained desensitization of cardiomyocytes to S1P. Several studies have shown in different cell types that S1P causes rapid desensitization of S1P receptors which persists for hours.[197,198] It might well be that in our experimental setting the combination of sustained vasoconstriction, which is potentially detrimental to postischemic recovery, and the loss of $S1P_3R$ mediated activation of prosurvival and antiapoptotic protective pathways due to desensitization, results in enhanced I/R injury. Whereas, in experimental settings, where lower doses are used (0.1 micromolar), $S1P_3R$ mediated protection is more active and dominates over effects of more moderate perfusion loss.

Because we observed that the coronary effects of S1P are in part mediated by $S1P_3R$ (Fig. 4), we also explored the effects of preischemic S1P exposure on ischemic damage in $S1P_3R$-KO hearts. Although preischemic CF and the function of $S1P_3R$-KO hearts were better (data not shown, also cf. Fig. 8), their functional recovery was as weak, and infarct size as large, as those of WT hearts.

Interestingly, although CF in $S1P_3R$-KO hearts returned close to the preischemic value, this relatively better perfusion did not provide any benefit for cardiac performance. All these results indicate, that although the absence of $S1P_3R$ might mitigate the detrimental effects of preischemic intravascular S1P exposure by decreasing the CF-reducing effect of S1P and allow for better reflow during reperfusion, the concomitant loss of $S1P_3R$-mediated cardioprotection obliterates this potential benefit. Therefore, the $S1P_3R$ seems to mediate two opposing S1P actions in the heart, as schematically shown in Figure 15.

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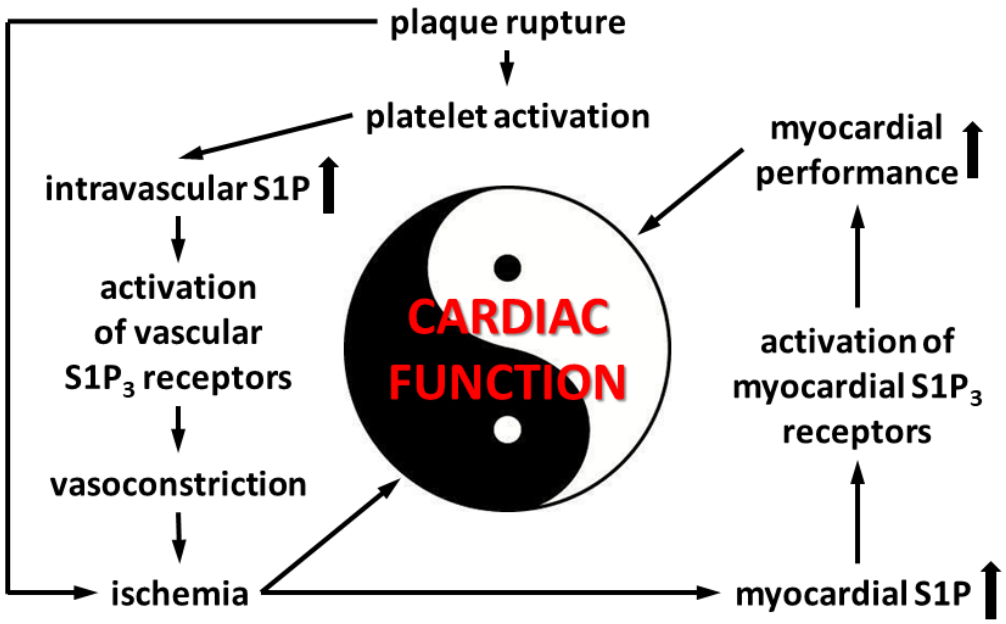

Figure 15. Events in acute coronary syndrome related to sphingosine-1-phosphate receptor type-3 ($S1P_3R$)-mediated alterations of cardiac function.

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6. Conclusions

Lipid mediators are involved in the regulation of numerous physiological and pathophysiological cardiovascular processes. The lipid mediators I studied, endocannabinoids and S1P, also exert their effects through the regulation of numerous cardiovascular processes. Their effects on vascular tone regulation and cardiac function, as well as their role in cardioprotection, are areas of science that have been studied and contradicted by numerous research groups. S1P has been attributed both vasoconstrictive and vasodilator effects and its protective role in I/R injury has been suggested, whereas endocannabinoids have vasodilator and negative inotropic effects in the heart. Their interactions with other substances, especially with substances that also have cardiovascular effects, such as endocannabinoids and Ang II, further nuance the picture of their complex effects.

In the first part of our study, we reported that the short-term effects of Ang II in an isolated heart preparation with intact circulation are modulated by DAGL activation and CB_1R transactivation, indicating a significant role of Ang II-signalling-induced release of the endocannabinoid 2-AG. We propose that the short-term cardiac effects of Ang II are exhibited as the summation of the simultaneous cardiac and vascular effects of Ang II and 2-AG. Whether this holds for long-term effects of Ang II, when the local renin-angiotensin system is upregulated in the heart under pathological conditions, and whether these potential effects are detrimental or beneficial needs further investigation.

In our second study, using isolated perfused murine hearts, we designed experimental models to simulate and explore the actions of S1P release in ACS. Our results suggest that in clinical situations, when thrombotic coronary occlusion causes cardiac ischemia, the released S1P might compromise postischemic recovery due to its unfavourable coronary effects, which might outweigh the presumed cardioprotective effects of S1P produced by the ischemic myocardium. Clearly, further studies are warranted using *in vivo* and *ex vivo* models to obtain a better understanding of the (patho)physiological actions of S1P in ACS.

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7. Summary

Cannabinoids and sphingosine-1-phosphate (S1P) are lipid mediators with diversified effects in the cardiovascular system. The endocannabinoid 2-arachidonoylglycerol (2-AG) has been attributed vasodilator and negative inotropic effects, while angiotensin II (Ang II) mediates vasoconstrictive and positive inotropic effects. Activation of the Ang II receptor type-1 - $G_{q/11}$ signalling pathway leads to 2-AG release and cannabinoid receptor type-1 (CB_1R) activation. We aimed to investigate whether cardiac Ang II effects are modulated by 2-AG-release and to identify the role of CB_1R and potential intracellular pathways in these effects. Administration of Ang II to isolated Langendorff-perfused rat hearts induced a remarkable decrease in coronary flow (CF) and a moderate decrease in cardiac function. Contrarily, administration of 2-AG and CB_1R agonist WIN55,212-2 induced a significant increase in CF. Effects of Ang II were moderated in the presence of CB_1R blocker O2050 and diacylglycerol-lipase inhibitor Orlistat. Our findings indicate that Ang II-induced cardiac effects are modulated by simultaneous CB_1R-activation, most likely due to 2-AG-release during Ang II signalling. The response to 2-AG via cardiac CB_1R may counteract the positive inotropic effect of Ang II, which may decrease metabolic demand and augment Ang II-induced coronary vasoconstriction.

S1P was shown to protect the heart against ischemia/reperfusion (I/R) injury. Other studies highlighted its vasoconstrictor effects. We aimed to separate the beneficial and potentially deleterious cardiac effects of S1P during I/R and identify the signalling pathways involved. Murine hearts were exposed to intravascular S1P administration, I/R protocol, or both. S1P induced a 45% decrease of CF in WT hearts, which was diminished in $S1P_3R$-KO but not in $S1P_2R$-KO hearts, indicating that $S1P_3R$ mediates coronary vasoconstriction. In I/R experiments, $S1P_3R$ deficiency diminished functional recovery and increased infarct size, indicating a cardioprotective effect of $S1P_3R$. Preischemic S1P exposure resulted in a substantial reduction of postischemic CF and cardiac performance and increased the infarcted area. These results indicate a dual role of $S1P_3R$ involving a direct protective action on the myocardium and a cardiosuppressive effect due to coronary vasoconstriction. In acute coronary syndrome when S1P is released abundantly, intravascular and myocardial S1P production may have competing influences on cardiac function via activation of $S1P_3Rs$.

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Kulin, D.; Antali, F.; Kulin, S.; Wafa, D.; Lucz, K.I.; Veres, D.S.; Miklós, Z. Preclinical, Multi-Aspect Assessment of the Reliability of a Photoplethysmography-Based Telemonitoring System to Track Cardiovascular Status. Appl. Sci. 2020, 10, 7977. https://doi.org/10.3390/app10227977

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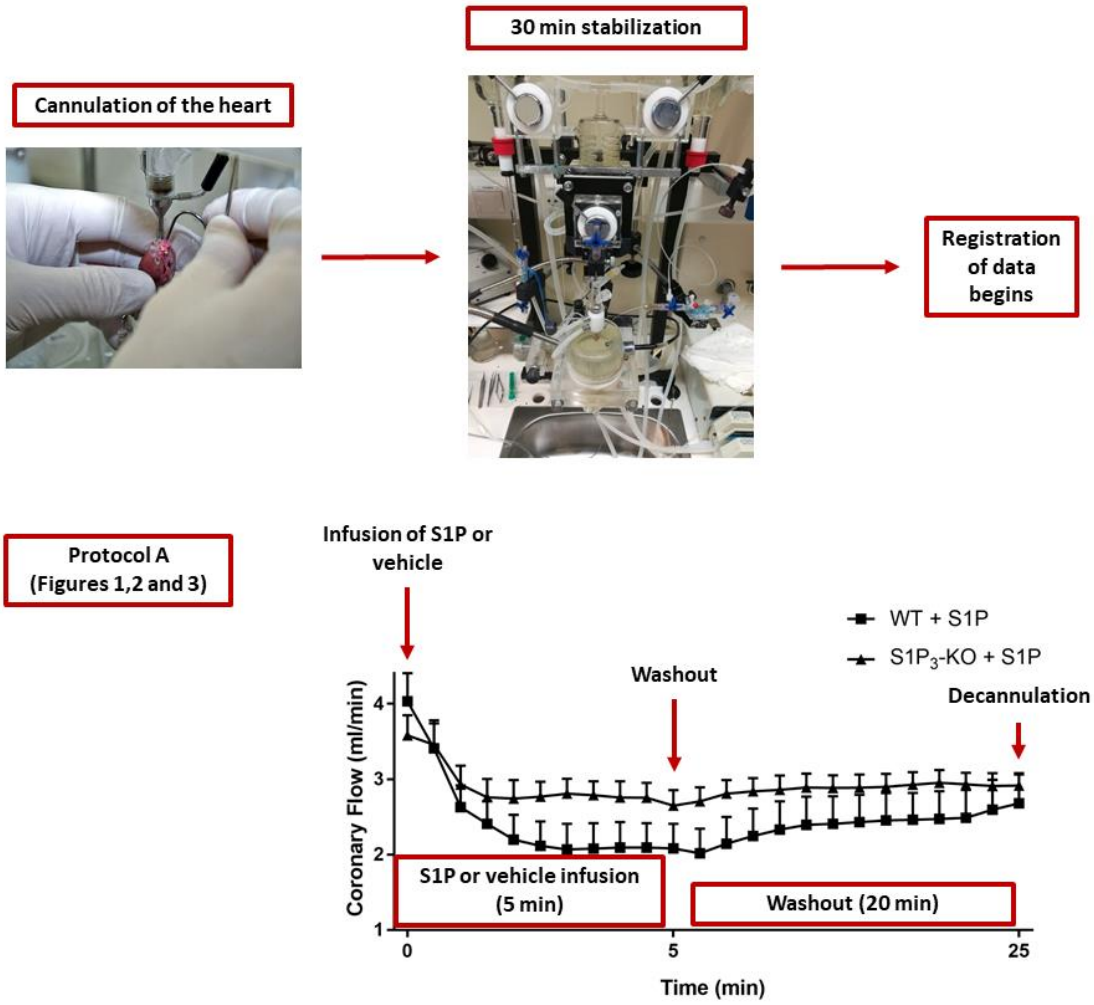
11. Supplementary



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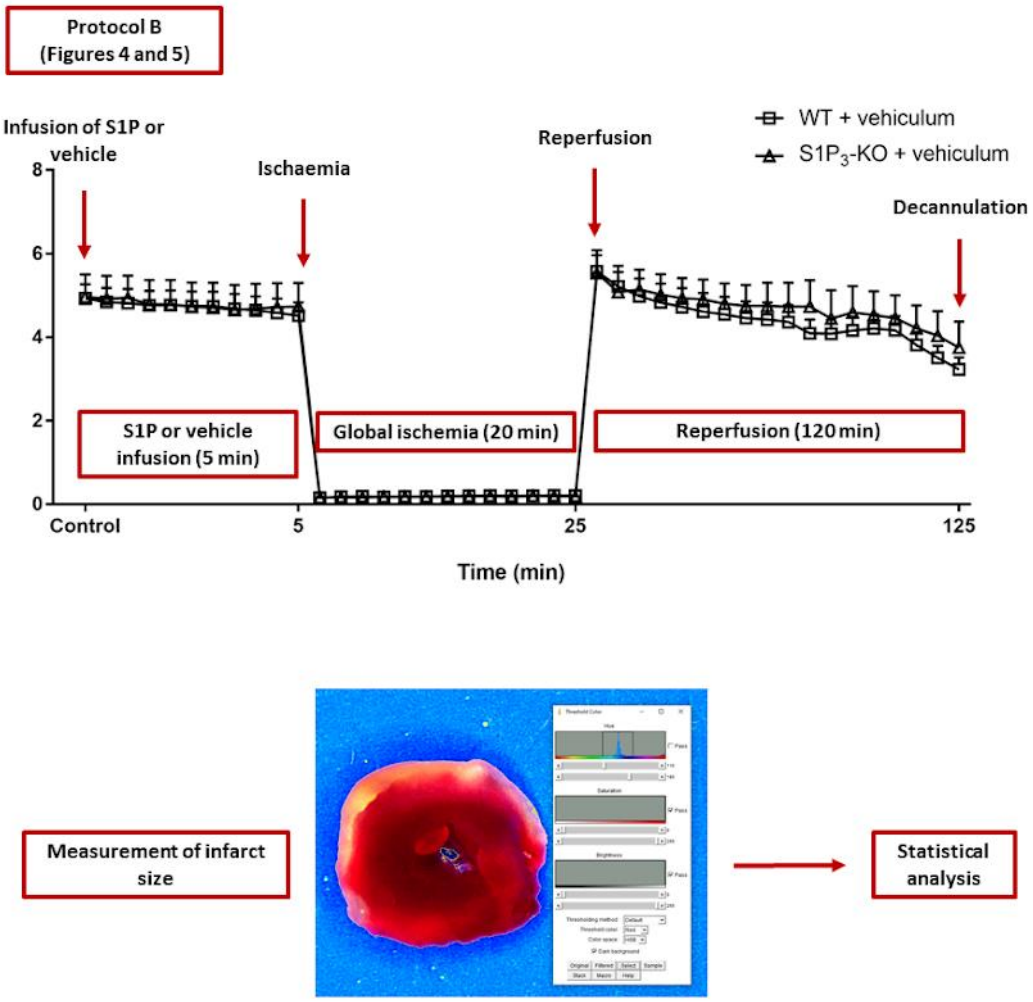

Suppl. Figure 1. Experimental protocols of isolated heart experiments. After cannulation, a 30-min stabilization period was allowed. Subsequently, baseline data were recorded and S1P or vehicle was infused to the perfusion line for 5 min. Afterwards, depending on the experiment, either a 20-min washout period (Protocol A) or a 20-min global ischemia caused by complete cessation of perfusion was applied. At the end of the ischemic period, perfusion was reintroduced and reperfusion was followed up for 2 h (Protocol B).