SEMMELWEIS EGYETEM

DOKTORI ISKOLA

Ph.D. értekezések

3017.

JANOVICZ ANNA

A vérkeringési rendszer normális és kóros működésének mechanizmusai
című program

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Lysophospholipid Signaling in Endothelial Dysfunction

PhD thesis

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Budapest
2024

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List of Abbreviations

ACh	acetylcholine
AC	adenylyl cyclase
ANOVA	analysis of variance
ApoE	apolipoprotein E
ATX	autotaxin
BH_4	tetrahydrobiopterin
CAD	coronary artery disease
cGMP	cyclic guanosine-monophosphate
COX	cyclooxygenase
CRC	concentration-response curve
CXCL	C-X-C motif chemokine ligand
EDG	endothelial differentiation gene
EDHF	endothelium-derived hyperpolarizing factor
ET-1	endothelin-1
eNOS	endothelial nitric oxide synthase
ENPP	ectonucleotid pyrophosphatase/phosphodiesterase
GPCR	G-protein coupled receptor
HBSS	Hanks' Balanced Salt Solution
H_2O_2	hydrogen peroxide
HRP	horseradish peroxidase
KO	knock-out
LCAT	lecithin-cholesterol acyltransferase
LDL	low-density lipoprotein
LPA	lysophosphatidic acid

LPC	lysophosphatidylcholine
LPP	lipid-phosphate phosphatase
NADPH	nicotinamide adenine dinucleotide phosphate hydrogen
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOX	NADPH-oxidase
NUC	nuclease-like
$ONOO^-$	peroxynitrite
OxLDL	oxidized LDL
PC	phosphatidylcholine
Phe	phenylephrine
PDE	phosphodiesterase
PLA2	phospholipase A2
PLC	phospholipase C
qPCR	quantitative real-time polymerase chain reaction
ROS	reactive oxygen species
sGC	soluble guanylate cyclase
SMB	somatomedin B
SNP	sodium nitroprusside
SOD	superoxide dismutase
TRPV	transient receptor potential vanilloid
TXA_2	thromboxane A_2
TP	thromboxane prostanoid
UHPLC-MS	ultra-high performance liquid chromatography-mass spectrometry
WT	wild type

1. Introduction

1.1. Diverse roles of the endothelium

Endothelium, the innermost layer of the vascular wall was once thought of as a simple barrier between the intravascular space and the vessel wall with some tissue-, and organ-specific transport functions (1, 2). However, in the past decades, it has become obvious that this continuous cellular lining that covers the inner surface of the vasculature has a crucial role in maintaining homeostasis. Besides forming a barrier, endothelial cells act as biomechanical transducers transforming blood flow-induced stimuli into biological responses (3, 4). Furthermore, the endothelium is also a key player in the regulation of hemostasis, thrombosis, inflammation, and immune responses (5). The majority of its actions are mediated by membrane proteins as well as by a wide range of autocrine and paracrine mediators released upon various stimuli. The endothelium is the source of multiple vasoactive substances that are involved in the regulation of tissue perfusion and blood pressure (5).

1.1.1. Endothelium and vascular tone regulation

In their seminal work, Furchgott and Zawadzki described that acetylcholine (ACh) -induced vasorelaxation develops only in the presence of intact endothelium, in isolated rabbit arteries (6). They suggested that this effect is mediated by an unknown relaxing factor, produced by endothelial cells (6). In subsequent years, nitric oxide (NO) was identified as this relaxing factor, playing an important role in vascular tone regulation (7). In the vasculature, NO is mainly generated by the endothelial nitric oxide synthase (eNOS) enzyme (5). By conversion of intracellular L-arginine, eNOS produces NO with L-citrulline as a byproduct (8). This locally formed NO then diffuses to smooth muscle cells, activating the soluble guanylate cyclase (sGC) enzyme, which leads to cyclic guanosine monophosphate (cGMP) production and subsequent vasorelaxation (5, 9, 10).

Although NO is a key player in vascular tone regulation, endothelial cyclooxygenase (COX)-1 and 2-derived prostanoids are also important vasoactive mediators (11). Both COX-1 and 2 are expressed in vascular endothelial cells with COX-1 being the more abundant one under physiological conditions (12, 13). While COX-2 is also present in healthy endothelial cells, its expression was found to be upregulated at

sites of inflammation (14). COX enzymes catalyze the conversion of membrane-derived arachidonic acid into prostaglandin H_2, which can be metabolized further by tissue-specific prostaglandin synthases. The most important prostanoid mediators are PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and thromboxane A_2 (TXA_2), each acting on their specific receptors, evoking either vasorelaxant or vasoconstrictor responses (11, 15, 16). PGI_2, a potent vasodilator agent is produced by endothelial cells and has been implicated in flow-mediated vasodilatation (17, 18). However, in inflammatory cardiovascular diseases the effects of prostanoid mediators, including PGI_2, shift from vasorelaxation to vasoconstriction, mostly mediated by thromboxane prostanoid (TP) receptors (19).

It is noteworthy, that NO and COX-derived prostanoids are not the only endothelial mediators that evoke vasodilatation. The so-called endothelium-derived hyperpolarizing factors (EDHF) are also capable of inducing vasorelaxation, mainly by activation of large conductance calcium-activated potassium channels resulting in hyperpolarization of the smooth muscle cells (20). While NO-dependent vasorelaxation is more significant in large arteries, EDHF-mediated actions are dominant in resistance vessels (21, 22).

Besides the above-mentioned mediators, endothelial cells produce endothelin-1 (ET-1), that is known as a vasoconstrictor agent (23, 24). ET-1 elicits its effects via activating ET_A or ET_B receptors (25). ET_A is expressed primarily on vascular smooth muscle cells and its activation leads to vasoconstriction, while ET_B is located predominantly in endothelial cells, mediating mainly vasorelaxant responses (24). Although considered a vasoconstrictor, in a number of species, ET-1 evokes a biphasic response. After an initial reduction of blood pressure, hypertension can be observed, suggesting that ET-1 evokes vasoactive effects on both ET_A and ET_B (12, 26).

1.2. Endothelial dysfunction

As described above, endothelium plays a crucial role in maintaining vascular homeostasis, therefore disturbances in its normal function can lead to severe consequences. The phenomenon, called endothelial dysfunction marks the shift from normally functioning endothelium towards the so-called proinflammatory endothelial phenotype (5). It is characterized by increased adhesion molecule expression, as well as enhanced inflammatory chemokine and cytokine secretion (1, 27). Moreover, the endothelium loses its anti-thrombotic features, resulting in more pronounced thrombotic

events (5). Along with these functional alterations, endothelial cells undergo morphological changes as well, resulting in impaired barrier function and increased permeability (28). The hallmark of endothelial dysfunction is the altered vasodilatory capacity of the vessels, which is generally attributed to the dysregulated metabolism and reduced bioavailability of NO (12, 29). Due to the loss of NO, the fine-tuned balance between endothelium-dependent vasorelaxant and vasoconstrictor responses is disrupted, causing the impaired regulation of vascular tone with subsequent alterations in blood pressure and blood perfusion of tissues and organs (30).

1.2.1. Molecular mechanisms of endothelial dysfunction

The development of endothelial dysfunction is often linked to the functional and structural alterations of eNOS, which is often referred to as the "uncoupling" of the enzyme and results in reduced NO production by endothelial cells (31). Uncoupling of eNOS can be caused by the depletion of either its cofactor, tetrahydrobiopterin (BH_4), or its substrate, L-arginine. Post-translational modifications, most commonly S-glutathionylation can also lead to eNOS uncoupling, by altering the normal protein structure (31). The uncoupled eNOS cannot function properly, hence, instead of producing NO, it shifts to a superoxide-generating phenotype. This not only leads to decreased NO levels but also evokes an oxidative burden, further damaging the cells (31, 32) (Figure 1). Therefore, the uncoupled enzyme itself can be a source of reactive oxygen species (ROS), however, there are other possible sources of oxidative agents as well. Most notably, members of the nicotinamide adenine dinucleotide phosphate hydrogen (NADPH)-oxidase (NOX) family are well-known contributors to ROS production in endothelial dysfunction (33). There are several papers reporting increased expression of these enzymes under pathophysiological conditions that elicit the disruption of redox homeostasis in vascular cells (33-35).

The overproduction of ROS is harmful to the endothelium in many ways. On one hand, superoxide scavenges NO, reducing its ability to induce vasorelaxation and to inhibit the activation of leukocytes and platelets (36, 37). On the other hand, superoxide and other ROS are able to reduce the intracellular levels of BH_4, contributing to the uncoupling of eNOS (38, 39). These changes collectively lead to impaired NO production in endothelial cells, thus shifting the vasoconstrictor-vasorelaxant balance in the vessels.

Although the production of other relaxing factors may partly compensate for this, NO cannot be replaced entirely. Therefore, if the NO deficit lasts long enough, it may result in an abundance of vasoconstrictor stimuli (12). Key players in this phenomenon are the COX-derived vasoconstrictor prostanoids, especially TXA_2, whose production is known to be enhanced in inflammatory diseases (12) and it is also known to induce vasoconstriction via activation of TP receptors (19).

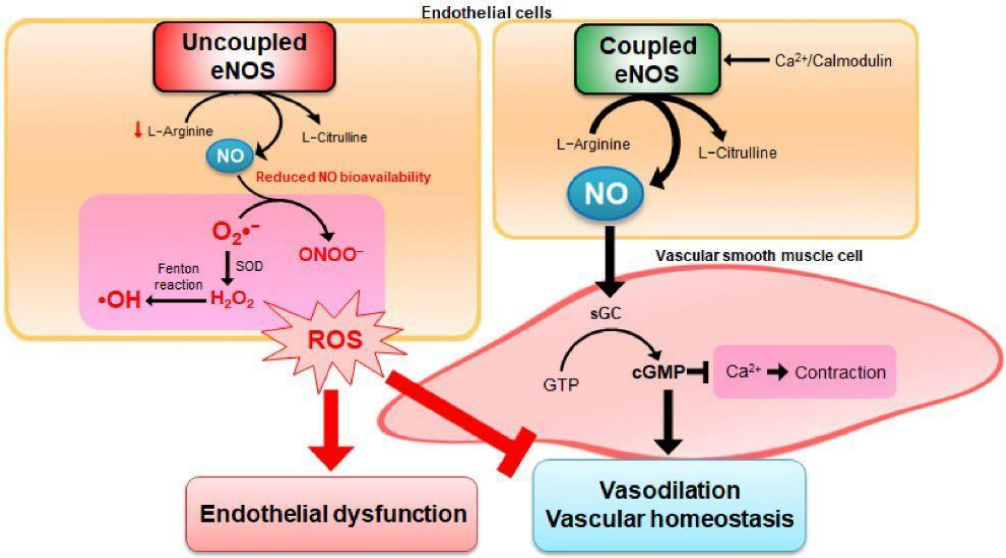

Figure 1. Role of eNOS uncoupling in endothelial dysfunction. In a healthy environment, eNOS converts intracellular L-arginine into NO, that diffuses to smooth muscle cells, where it activates soluble guanylate cyclase (sGC), evoking vasorelaxation. In contrast, when uncoupled, instead of producing NO, eNOS generates superoxide anions ($O2^{\bullet-}$). The locally formed superoxide can interact with NO, leading to the formation of peroxynitrite ($ONOO^-$). Superoxide can be also transformed into hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) enzymes, that can be converted further into hydroxyl radicals (•OH). Overall, the elevated levels of intracellular ROS along with reduced NO bioavailability contribute to the development of endothelial dysfunction. Figure from the publication of Lee et al. (32).

1.2.2. Endothelial dysfunction in cardiovascular diseases

Endothelial dysfunction is often described as an early sign of the development of atherosclerosis (40, 41). In the early stages of the disease, localized accumulation of lipoproteins, mostly low-density lipoprotein (LDL), can be observed in the arterial wall (42, 43). These lipoprotein particles become oxidized on site, resulting in the upregulation of pro-inflammatory mediators that are responsible for endothelial dysfunction and the progression of the disease (1, 30). Before the development of atherosclerotic plaques, loss of endothelial function can be observed in both animal models and humans, characterized

by impaired vasorelaxation (5). For instance, reduced endothelium-dependent vasorelaxation affects patients with coronary artery disease (CAD) (44). In fact, endothelial dysfunction appears to be a good predictor of the outcome of CAD, especially with respect to the occurrence of myocardial infarction (45-47).

Impaired endothelial function can be observed in type 2 diabetes as well (48-50). Chronic hyperglycemia, associated with the disease is responsible for several cardiovascular complications, involving endothelial dysfunction. Besides increased blood glucose levels, diabetes is accompanied by disrupted lipid metabolism, further contributing to the loss of endothelial function. Hyperglycemia and hyperlipidemia, along with pronounced oxidative stress, can trigger inflammatory processes that are harmful to the endothelial cells and lead to decreased vasorelaxant properties of the affected vessels, and in the long term the development of atherosclerotic lesions (51).

Reduced endothelium-dependent vasodilatation can be also observed in animal models of hypertension, as well as in humans affected by the disease (52, 53). In both cases, endothelial dysfunction is associated with reduced NO bioavailability, partly caused by oxidative stress and reduced eNOS function (12).

1.3. Lysophosphatidylcholine in vascular health and disease

Lysophosphatidylcholine (LPC) is a bioactive lipid, that is present in the plasma. Under physiological circumstances, its plasma concentration ranges between 120-150 μM (54), but its level can be elevated in hyperlipidemia (55). Increased plasma levels of LPC has been reported in cardiovascular diseases, thus LPC has long been implicated in the development of vascular inflammation (54).

1.3.1. LPC biosynthesis and metabolism

The majority of plasma LPC is originated from phosphatidylcholine (PC) (56). As a glycerophospholipid, PC is composed of a glycerol backbone with fatty acid chains attached to its sn-1 and sn-2, and a phosphatidylcholine group bound to the sn-3 position (57). The two most important enzymes responsible for the conversion of PC to LPC in the plasma are phospholipase A2 (PLA2) (58) and lecithin-cholesterol acyltransferase (LCAT) (59). Both of these enzymes generate LPC by removing the sn-2 fatty acid group of PC (60, 61). Other LPC-producing enzymes, such as endothelial lipase or hepatic lipase

are sn-1 phospholipases, creating LPC molecules with a fatty acid chain on the sn-2 position (62, 63). Therefore, the name LPC does not refer to a single molecule, but to an entire group of mediators, that can be distinguished based on the position, length, and degree of saturation of their fatty acid group (64).

Different LPC molecules often have distinct cellular effects. In human plasma, the most abundant subtypes are palmitoyl (16:0), oleoyl (18:1), stearoyl (18:0), linoleoyl (18:2) and arachidonoyl (20:4) LPC (65, 66). Most of plasma LPC is bound to and carried by albumin, however, it can be also found in lipoprotein particles, most notably in LDL, and to a lesser extent, as free LPC (64, 67). In the circulation, LPC is metabolized by autotaxin (ATX), an ectoenzyme with lysophospholipase D activity (68), that is expressed in a number of vascular cells, including endothelial cells (69), smooth muscle cells (70), and macrophages (71). ATX cleaves the choline headgroup from the LPC molecule, generating lysophosphatidic acid (LPA), an important lysophospholipid mediator with several cellular functions (72). (Figure 2.)

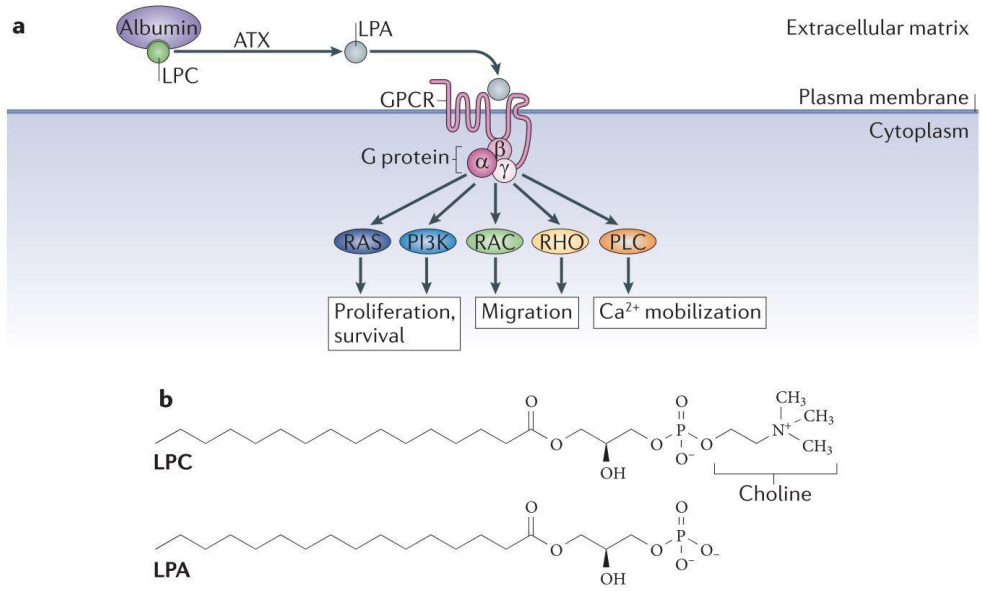

Figure 2. LPC-ATX-LPA signaling pathway. ATX catalyzes the conversion of LPC into LPA, that is, by activating G-protein coupled receptors (GPCR), can induce a number of intracellular signaling pathways. Key players of the LPA-induced signaling are the RAS, phosphoinositide 3-kinase (PI3K), RHO, RAC, and phospholipase C (PLC) mediated pathways (a). Schematic structures of LPC and LPA molecules (b). Figure from the publication of Moolenaar et al. (72).

1.3.2. LPC and endothelial dysfunction

As mentioned earlier, LDL accumulation and oxidation in the vascular wall is a key step in the development of endothelial dysfunction and atherosclerosis. According to Steinbrecher et al., during LDL oxidation a significant amount of its PC content is hydrolyzed by PLA2 enzymes (73), leading to a buildup of LPC in the vascular wall. Furthermore, Yokoyama et al. reported that the vasorelaxant properties of isolated rabbit aortic segments treated with oxidized LDL (OxLDL) were markedly impaired, and they attributed this effect to LPC (74). In the subsequent years, the ability of free LPC to evoke similar disruption in the endothelium-dependent vasorelaxation of isolated vessels was also confirmed (75-77).

There are several theories for the molecular mechanism by which LPC contributes to the impairment of endothelium-dependent vasorelaxation. Many of these point to the possibility that LPC reduces the bioavailability of NO, either by uncoupling eNOS enzymes (75) or by inducing the production of ROS via NOX activation in the vascular cells (78). Furthermore, in more recent publications, it has also been suggested, that the deleterious effects of LPC are associated with neuronal nitric oxide synthase (nNOS) uncoupling as well (76, 77). Although eNOS is considered the main enzyme producing NO in the vasculature, nNOS is expressed in endothelial cells as well and contributes to vasodilator responses. Thus, loss of its function can impair endothelium-dependent vasorelaxation (79, 80). Besides, other research groups claim that the LPC-evoked reduction of vasorelaxation is related to its ability to interfere with vascular COX-mediated pathways. According to the results of Stoll et al., LPC can reduce the ability of endothelial cells to produce the vasorelaxant PGI_2 mediator (81), while others reported that LPC likely induces the overexpression of COX enzymes (82), possibly leading to a vasoconstrictor prostanoid abundance.

Although, it is well known that LPC is involved in the pathogenesis of endothelial dysfunction, the exact molecular mechanism of this effect is obscure. As described earlier, LPC is an amphipathic molecule, having a polar choline headgroup and a nonpolar fatty acid chain attached to its glycerol backbone. Due to its structural characteristics, LPC is a potent detergent, thus it can interact with biological membranes directly, changing their biophysical properties (83). It has been also suggested that by being incorporated into the

lipid bilayer of cell membranes, LPC can interfere with signal transduction pathways leading to altered cellular functions (75, 81).

Besides having direct membrane effects, it has been proposed that LPC has specific G-protein coupled receptors (GPCR) that mediate its effects (54). In the early 2000s, a few publications reported that LPC is capable of activating directly the G2A and GPR4 receptors, evoking cellular responses (84, 85). It is important to note though, that these results could not be reproduced since then, therefore it is highly debated, whether LPC is a bona fide ligand of these receptors. However, we cannot exclude the possibility that LPC acts as a modulator of GPCRs. It has also been suggested that LPC evokes vasoconstriction directly (86), or potentiates the effect of other mediators, such as angiotensin II, resulting in a more pronounced vasoconstriction (87).

When discussing the possible mechanisms of actions of LPC, it is often overlooked that LPC can be converted rapidly to LPA by the ectoenzyme ATX (72). Several papers published in the past years claim that certain atherogenic properties of LPC can be attributed to LPA (64, 88, 89). Therefore, it is reasonable to hypothesize that LPC might evoke some of its effects as converted to LPA by ATX.

1.3.3. Autotaxin

In 1986, Tokumura et al. were the first to report that the bulk of plasma LPA is generated by an enzyme with lysophospholipase D activity (90). It only became obvious in the early 2000s, that this enigmatic enzyme is autotaxin (68). ATX belongs to the ectonucleotid pyrophosphatase/phosphodiesterase (ENPP) family, which consists of seven members (91). While the ENPPs share similarities in terms of their structure and catalytic activity, ATX, also known as ENPP2, is considered unique among these enzymes. First of all, ATX is the only member with lysophospholipase D activity (72). Furthermore, while the other ENPPs have a transmembrane domain, ATX is a secreted enzyme that is anchored to the cell surface through integrin receptors (91). This is especially interesting, as it has been suggested, that besides its catalytic functions, ATX might evoke cellular effects via integrin-signaling as well (72).

ATX is an enzyme with diverse physiological and pathophysiological functions. van Meerten et al. reported in 2006, that the genetic deletion of ATX in mice results in embryonic lethality, due to developmental abnormalities involving the vascular system

(92). Since then, many publications have supported these observations, suggesting that the ATX-LPA axis is a key player in embryonic vascular development (93, 94). Besides, ATX is a well-known tumor motility factor, that is involved in the development of many cancerous diseases such as melanoma (95), breast cancer (96) or non-small cell lung cancer (97). In addition, it has been also suggested that ATX contributes to vascular diseases, such as atherosclerosis (71, 98). ATX present in the circulation is mostly originated from adipose tissue and high endothelial venules (99, 100).

ATX is an enzyme with a rather rigid structure. It has two somatomedin B-like (SMB) domains on the N-terminal, a central catalytically active phosphodiesterase (PDE) domain, and a nuclease-like (NUC) domain on the C-terminal, that maintains structural stability (72). According to the large body of literature data, the SMB domains have multiple roles in regulating ATX functions. It is well-described, that these sequences are responsible for maintaining protein-protein interactions, therefore ATX is most likely to be anchored to the cell surface integrin receptors through these domains (72, 101). It is noteworthy, that the SMB domains are in close interaction with the PDE domain, suggesting that they are involved in regulating the catalytic functions as well (72).

Structural studies examining its domain organization revealed that ATX has a unique tripartite binding site (101). The catalytically active bimetallic site is surrounded by a hydrophilic groove, that is connected to a hydrophobic pocket, and a tunnel, often referred to as hydrophobic channel that ends on the other side of the enzyme (102). ATX is considered a promiscuous enzyme, as beside of LPC, it is able to hydrolyze other lysophospholipids such as lysophosphatidylserine or lysophosphatidylethanolamine (103, 104). Thus, the substrate binding appears to be related to the characteristics of the hydrophobic pocket, as ATX prefers lysophospholipid molecules with shorter and/or unsaturated fatty acid chains (72).

As described previously, ATX has been proven to be involved in the progression of several life-threatening diseases, making it a potential therapeutic target. The proper mapping of the enzyme's structure, hence a more detailed understanding of its functions, has contributed to the development of specific ATX inhibitors. At present, there are five different subgroups of ATX inhibitors, distinguished by their modes of binding (102, 105). Type I inhibitors are known as orthosteric modulators, as they block the active site and the hydrophobic pocket of ATX, similarly to LPC, while Type II compounds bind

only to the hydrophobic pocket. Type III inhibitors occupy the hydrophobic tunnel, and therefore considered as allosteric modulators of the enzyme. The combination of Type II and Type II inhibitors led to the development of Type IV compounds, that have a more complex mode of binding, blocking both the hydrophobic pocket and the tunnel of ATX (106). A prominent member of this group is the GLPG1690 compound, also known by the brand name of Ziritaxestat, which was tested in a clinical trial for the treatment of idiopathic pulmonary fibrosis (107). However, the trial was terminated early, due to unfavorable clinical outcomes (108). In a recent publication by Clark et al., the group of Type V inhibitors was introduced, which includes ATX blockers that bind to the tunnel and the active site as well, however, these substances are not widely used yet (105). The above-mentioned inhibitors are extremely valuable tools for understanding the role of ATX and its product, LPA in different physiological and pathophysiological processes.

1.3.4. LPA

The product of LPC hydrolysis by ATX is the small bioactive lipid molecule, LPA. The plasma concentration of LPA in healthy individuals is in the nanomolar range, with 18:2, 18:1, 18:0, 20:4, and 16:0 LPA species being the most abundant ones (109, 110). In the circulation, newly formed LPA is quickly metabolized by cell-surface lipid-phosphate phosphatases (LPP) (111).

The majority of the actions of LPA are mediated by six G protein-coupled receptors, which are classified into two groups, based on their homology. LPA_{1-3} receptors are members of the endothelial differentiation gene (EDG) family, whereas LPA_{4-6} are known as non-EDG receptors and share similarities with purinergic receptors (112). LPA receptors can couple to at least four types of G proteins, namely $G\alpha_s$, $G\alpha_{q/11}$, $G\alpha_{12/13}$ and $G\alpha_i$ (Figure 3.) (113). Besides activating GPCRs, LPA can also evoke its cellular effects via the intracellular peroxisome proliferator-activated gamma (PPARγ) receptor (114). In addition, LPA has ion channels among its targets (115), including transient receptor potential vanilloid (TRPV) 4 (116) and TRPV1 channels (117).

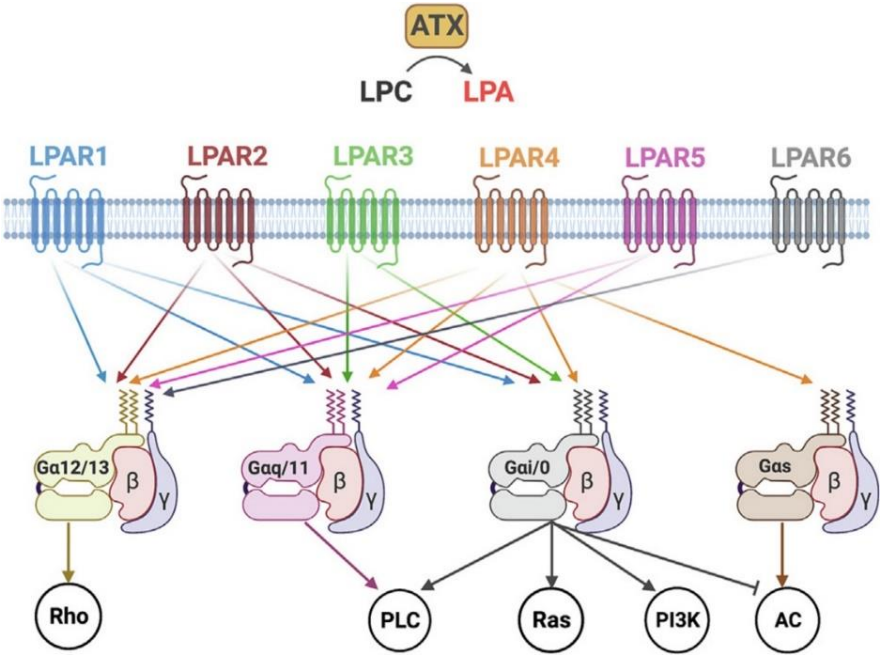

Figure 3. LPA signaling pathways. LPA evokes its effects mostly by activating GPCRs (LPAR1-6). These receptors can couple to four types of G proteins ($G\alpha_s$, $G\alpha_{q/11}$, $G\alpha_{12/13}$ and $G\alpha_i$) activating different intracellular signaling pathways. PLC: phospholipase C; PI3K: phosphoinositide 3-kinase; AC: adenylyl cyclase. Figure from the publication of Dacheux et al. (118).

1.3.4.1. Vasoactive effects of LPA

In the late 1970s, LPA was discovered by Tokumura et al. and was identified as a vasoactive mediator. They found that when administered intravenously, LPA evoked hypertension in guinea pigs and rats (119), and hypotension in cats and rabbits (120), suggesting that LPA is capable of inducing both vasoconstriction and vasorelaxation depending on the species examined.

LPA has been proven to evoke eNOS activation, with consequent NO production in cultured endothelium (121, 122). In accordance with this, our research group found earlier that LPA elicits eNOS-dependent vasorelaxation in isolated mouse aortic segments, and this effect appeared to be mediated by LPA_1 receptor activation (69).

Besides its vasorelaxant properties, LPA is also capable of evoking vasoconstriction in isolated, endothelium-denuded mouse aortic segments via LPA_1 activation. The results suggest, that this effect is mediated by G_i protein, with consequent COX-1 and TP receptor activation (70). In a more recent publication, Kano et al. investigated the mechanism of LPA-evoked hypertension in a mouse model. They found that this effect is mainly mediated by the LPA_4-$G_{12/13}$-Rho-Rho-kinase pathway (123).

In addition to the aforementioned data, it is possible that LPA can evoke vasoactive effects in a GPCR-independent manner. In 2020, Phan et al. described that LPA induces a potent vasoconstriction in isolated skeletal muscle arterioles, that is dependent on TRPV1 channel activation (124).

1.3.4.2. ATX-LPA-LPA receptor axis in vascular inflammation

Apart from being a significant player in vascular homeostasis, the involvement of the ATX-LPA axis has been associated with inflammatory vascular alterations as well. The expression of ATX and LPA receptors was shown to be upregulated in sites of atherosclerotic lesions and vascular injury (71). In addition, the accumulation of unsaturated long-chain LPA species was also observed in atherosclerotic plaques (71). The results of *in vitro* studies suggest that LPA is capable of inducing the expression of C-X-C motif chemokine ligand (CXCL) 1, which is a potent pro-inflammatory chemokine, in endothelial cells (125). Furthermore, LPA can promote smooth muscle proliferation and migration, which could potentially contribute to vascular remodeling (126). The majority of the aforementioned actions of LPA appeared to be mediated by $LPA_{1/3}$ receptors (71, 125). The detrimental effects of LPA on vascular cells include its ability to increase endothelial permeability (127), to induce the expression of adhesion molecules (126, 128), as well as the release of pro-inflammatory cytokines and ROS (126). In view of this, it is not surprising, that genetic deletion of endothelial ENPP2 reduces atherosclerosis in apolipoprotein E (ApoE) -/- mice (98). Furthermore, Yang et al. demonstrated that LPA_4 knock-out (KO) mice develop less severe atherosclerotic lesions (129). Besides the aforementioned alterations, it has also been demonstrated that LPA can induce platelet aggregation as well, confirming its role as a mediator in atherothrombotic events (130, 131).

Although it is well-documented, that ATX and LPA are involved in multiple aspects of vascular inflammation, their possible role in endothelial dysfunction has not been examined yet.

2. Objectives

LPC has long been known as a pro-inflammatory mediator, disrupting the endothelium-dependent vasorelaxation, and contributing to the development of endothelial dysfunction. Despite the fact, that LPC can be metabolized to LPA by the ATX enzyme in the vasculature, the involvement of the ATX-LPA-LPA receptor axis in mediating this process has not been addressed yet. In our experiments, we aimed to investigate:

- the involvement of the ATX-LPA-LPA receptor axis in the development of LPC-induced impairment of endothelium-dependent vasorelaxation,
- the downstream signaling mechanism of LPC-induced endothelial dysfunction, and
- the possible alterations of lysophospholipid metabolism in a mouse model of type-2 diabetes.

3. Methods

3.1. Animals

All procedures were carried out in accordance with guidelines of the Hungarian Law of Animal Protection (28/1998) and were approved by the Government Office of Pest County (PE/EA/924-7/2021). Wild type (WT) mice on C57BL/6 genetic background were obtained from Charles River Laboratories (Isaszeg, Hungary). Mice deficient in *Lpar1* and *Lpar2* were generated and kindly provided by Dr. Jerold Chun (Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA, USA). *Lpar4* KO mice were received from Dr. Satoshi Ishii (Department of Immunology, Graduate School of Medicine, Akita University, Akita, Japan) and the *Lpar5* KO animals were gifts from Lexicon Pharmaceuticals (The Woodlands, TX, USA). COX-1 KO mice were from Dr. Ingvar Bjarnason (Guy's, King's College, and St. Thomas' School of Medical Education, London, United Kingdom) and TP KO mice were kindly provided by Dr. Shuh Narumiya (Kyoto University, Kyoto, Japan). COX-1, TP, and all *Lpar* KO mouse lines had the C57BL/6 genetic background. The BKS db diabetic mouse strain was obtained from Jackson Laboratory (Bar Harbor, ME, USA) and has been maintained in our animal facility by mating repulsion double heterozygotes (*Dock7*^{m} *+/+ Lepr*^{db}). Littermate adult male diabetic (*Lepr*^{db}*/Lepr*^{db}, referred to as *db/db*) and misty (Dock7^{m}/Dock7^{m}, referred to as control) mice were selected for experiments. All mice investigated were male and aged between 90 and 180 days. Animals were housed in a temperature and light-controlled room (12 h light-dark cycle) with free access to food and water. In some experiments, nonfasting blood glucose was measured by a Dcont IDEÁL biosensor-type blood glucose meter (77 Elektronika Kft.; Budapest, Hungary).

3.2. Preparation of thoracic aorta segments

Adult male mice were euthanized in a CO_2-chamber, followed by transcardial perfusion with Krebs solution containing 10 U/mL Heparin as described elsewhere (132). The thoracic aorta was isolated and cleaned of adipose and connective tissues under dissection microscope (M3Z; Wild Heerbrugg AG, Gais, Switzerland). During the preparation, special care was taken to preserve the integrity of the endothelium. The distal region of the thoracic aorta was cut into 3 mm long segments and mounted on two parallel

stainless-steel needles of a myograph chamber filled with 6 ml gassed Krebs solution at 37°C.

3.3. Myography

3.3.1. Examination of vasoconstrictor and vasorelaxant properties of vessels

Vascular tension changes were measured with wire myography as described previously, with a few modifications (69). Before every experiment, the vessels were allowed to rest for 45 min at a passive tension of 15 mN. First, the vessels were exposed to 124 mM KCl containing Krebs solution for 1 min to elicit vasoconstriction. After several washes, when the vessels returned to resting tone, phenylephrine (Phe; 10 μM) and acetylcholine chloride (ACh; 0.1 μM) were added to the chambers to test the smooth muscle and the endothelium function. After repeated washing, the segments were adjusted to 124 mM KCl Krebs solution for 3 min to elicit a reference maximal contraction. After washout, the vessels were pre-contracted using increasing concentrations of Phe (10 nM to 10 μM) followed by increasing concentrations of ACh (1 nM to 10 μM) to evoke NO-dependent vasorelaxation. In some of the experiments, to test the sensitivity of the smooth muscle to NO, sodium nitroprusside (SNP) (0.1 nmol to 10 μmol) was administered after a stable pre-contraction elicited by Phe.

3.3.2. Examination of LPC-induced impairment of vasorelaxation

In those experiments, where we investigated the mechanism of LPC-induced endothelial dysfunction, the above-mentioned experimental protocol was followed by the administration of 124 mM KCl Krebs solution for 3 min, then, the Phe-ACh concentration-response curve (CRC) was repeated to reach the maximal responsiveness of the rings. After washout, the vessels were treated with 10 μM 18:1 LPC for 20 min, followed by the re-administration of the Phe and ACh CRCs. In some experiments, the ATX inhibitor GLPG1690 at 10 μM or 200 U/mL superoxide dismutase (SOD) was applied to the vessels 10 min prior to LPC administration. The superoxide scavenger Tempol (1 mM) was applied right before LPC treatment in some experiments.

3.4. Immunohistochemistry

Thoracic aorta segments, isolated from adult male WT mice, were fixed in 10% formalin for 48 hours, then embedded in paraffin and cut into 2,5 μM slices. Samples were incubated with ATX primer antibody for 16 hours. 3,3'-Diaminobenzidine reagent was used for visualization.

3.5. Quantitative real-time PCR

Whole thoracic aorta was isolated and stored at -80 °C until RNA isolation. Total RNA from the samples was extracted using Tri Reagent. Total RNA was reverse transcribed using RevertAid First Strand cDNA Synthesis kit. Quantitative real-time polymerase chain reaction (qPCR) measurements were performed on CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using SsoAdvanced Universal SYBR Green Supermix. Temperature cycles were as follows: 95 °C for 60 s, 95 °C for 10 s, and 58 °C for 30 s (40 cycles). Specific primer sets were designed by using Primer3Plus and Primer-BLAST software tools and/or ordered from Sigma-Aldrich (133, 134). Primer sequences are listed in Table 1. The beta-2 microglobulin (B2m) gene was considered the housekeeping gene for normalizing gene expression. The delta–delta CT (ΔΔCT) method was used to calculate the gene expressions of B2m, LPA_1, LPA_2, LPA_3, LPA_4, LPA_5, LPA_6 receptors, and ATX (135).

Table 1. Primer sequences used in quantitative PCR analysis**.** The gene identities and forward (F) and reverse (R) primer sequences with the length of the PCR products for qPCR. The specific PCR products were checked by gel electrophoresis for absence of primer-dimers and correct PCR product length. Table adapted from the author's original publication (136). Copyright (2023) by the Society for Experimental Biology and Medicine.

Gene name	**Primer sequence**	**NCBI reference sequence number**	**Size (bp)**
Target genes			
Lpar1 (lysophosphatidic acid receptor 1)	F: GACTCCTACTTAGTCTTCTGG R: CAGACAATAAAGGCACCAAG	NM_010336.2	200
Lpar2 (lysophosphatidic acid receptor 2)	F: CAAGACGGTTGTCATCATTC R: AATATACCACTGCATTGACC	NM_020028.3	167

Lpar3 (lysophosphatidic acid receptor 3)	F: AGGGCTCCCATGAAGCTAAT R: GTTGCACGTTACACTGCTTG	NM_022983.4	124
Lpar4 (lysophosphatidic acid receptor 4)	F: CTGATCGTCTGCCTCCAGAAA R: TTGAGACTGAGGACCAGTAGAG	NM_175271.4	117
Lpar5 (lysophosphatidic acid receptor 5)	F: TCATCTTCCTGCTGTGC R: ATCGCGGTCCTGAATACTGT	NM_001163268.2	98
Lpar6 (lysophosphatidic acid receptor 6)	F: ACTGAAGTAAAGCTGGTTTG R: AACCCATAAAGCTGAAAGTG	NM_175116.4	109
Enpp2 (ectonucleotide pyrophosphatase/ phosphodiesterase 2)	F: CTGTCTTTGATGCTACTTTCC R: TCACAGACCAAAAGAATGTC	NM_001040092.3	129
Reference gene			
B2m (beta-2 microglobulin)	F: CTTTCTGGTGCTTGTCTCACTG R: AGTATGTTCGGCTTCCCATTC	NM_009735.3	105

3.6. Amplex Red Hydrogen Peroxide Assay

LPC-induced ROS release was measured by Amplex Red Assay, a method widely used for the quantification of extracellular H_2O_2 levels (137). Whole descending thoracic aortae were cut longitudinally and allowed to rest in 250 μL Hanks' Balanced Salt Solution (HBSS) for 60 min at 37 °C. To measure the basal H_2O_2 levels, the vessels were incubated with a working solution containing 50 μM Amplex Red reagent and 0,2 U/mL horseradish peroxidase (HRP) in HBSS for 15 min at 37 °C. The supernatant was collected and absorbance was measured at 570 nm. Then, the vessels were incubated with working solution containing 10 μM LPC for 40 min at 37 °C followed by absorbance measurement of supernatant. Absorbance values were normalized to 1 min.

3.7. Measurement of serum phosphorylcholine levels

Serum phosphorylcholine levels of diabetic and control mice were measured in order to examine their lipid profile. Phosphorylcholine is the precursor molecule of phosphatidylcholine (PC) (138, 139), therefore changes in its serum level can indicate an altered lipid metabolism. Blood samples were collected by cardiac puncture. Samples were allowed to clot for 30 min at room temperature, and centrifuged at 2000 × g for 15 min at 4 °C. Serum was snap frozen for phosphorylcholine assay, which was based on the

method described by Hojjati and Jiang (140), using a commercially available kit (Cayman Chemical; Ann Arbor, MI, USA).

3.8. Measurement of plasma LPC levels

3.8.1. Sample preparation procedure

Lipid extracts from mouse plasma were prepared in the following way. Prior to the extraction of plasma, 10 μL lipid internal standard mixture (1,12 μM LPA 17:0, 199 μM LPC 19:0 in methanol) was added to the 100 μL plasma sample. Briefly, after vortex mixing, 2 x 600 μL of ice-cold methanol was added, followed by a vortex mixing, and the mixture was shaken for 10 minutes at room temperature. Upon 10 min of incubation at room temperature, the sample was centrifuged at 15,000 g for 15 min at 22 °C. After centrifugation, 2 x 600 μL of the upper phase was collected and dried under nitrogen at ambient temperature. For analysis, the dried extracts were dissolved in 110 μL of methanol/2-propanol/water (1/1/0.1, v/v/v%).

3.8.2. Targeted ultra-high performance liquid chromatography high-resolution mass spectrometry method

The ultra-high performance liquid chromatography high-resolution mass spectrometry (UHPLC-MS/HRMS) analysis was performed using a Waters Acquity I-Class UPLC™ (Waters, Manchester, UK), equipped with a binary solvent manager, auto-sampler, and column manager. The UHPLC system was coupled to the Thermo Scientific Q Exactive Plus hybrid quadrupole-Orbitrap (Thermo Fisher Scientific, Waltham, MA) mass spectrometer. The UHPLC system was controlled with MassLynx V4.1 SCN 901 (Waters, Milford, MA, USA). The control of HRMS system and HRMS data acquisition were conducted by Xcalibur™ 4.0 software (Thermo Fisher Scientific, Waltham, MA).
The final UHPLC-MS/HRMS method for the analysis of LPC species was as follows: Waters Acquity UPLC HHS T3 (100 x 2.1 mm, 1.8 μm) column with guard column, injection volume 10 μL, and column temperature 50 °C. The mobile phase A was ammonium-formate (0.5 mM) in 60/40/1 methanol/water/formic acid (v/v/v%), and the eluent B was ammonium-formate (0.5 mM) and 1 v% formic acid in methanol. The

gradient program was the following: 0 – 1 min – 10 – 10% B, 15 – 20 min – 100 – 100% B, and 20.1 – 22 min – 10% B. The flow rate was 0.4 mL/min during the analysis.

The high-resolution mass spectrometer was operated in the scheduled negative-ion parallel reaction monitoring mode using a heated electrospray ionization source with the following conditions: capillary temperature 250 °C, S-Lens RF level 50, spray voltage 2.5 kV, sheath gas flow 47.5, spare gas flow 2.25 and auxiliary gas flow 11.25, full scan with a mass range of 100-1000, isolation window 1 Da, and a resolution of 17,500. The automatic gain control setting was defined as 1×10^6 charges and the maximum injection time was set to 30 ms. The 55 eV and 25 eV of optimization energies were used for the fragmentation of LPCs.

3.9. Reagents

Oleoyl-lysophosphatidylcholine (18:1 LPC) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was dissolved in methanol to stock solutions of 10 mM. Required amounts of LPC stock solutions were transferred to glass vials and the vehicle was removed using a stream of nitrogen. LPC was re-dissolved in water containing 0,1% bovine serum albumin before use. SOD was also purchased from Sigma Aldrich and dissolved in water to stock solutions of 20000 U/mL. GLPG1690 was purchased from Cayman Chemicals (Ann Arbor, MI, USA) and DMSO was used as a solvent for preparing a 10 mM stock solution. Amplex™ Red reagent and HRP were purchased from Thermo Fisher Scientific (Waltham, MA, USA) and were diluted in DMSO and aqueous solutions to stock solutions of 10 mM and 0.4 U/mL. Tri Reagent was purchased from Zymo Research (Irvine, CA, USA). RevertAid First Strand cDNA Synthesis kit was purchased from Thermo Scientific (Waltham, MA, USA). SsoAdvanced Universal SYBR Green Supermix was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Tempol was purchased from Sigma Aldrich (St. Louis, MO, USA) and dissolved in water before use. ATX primer antibody was purchased from Abcam (Cambridge, UK) and 3,3'-Diaminobenzidine reagent from Thermo Scientific (Waltham, MA, US).

3.10. Data Analysis

Vascular tension changes were recorded with the MP100 system and analyzed with the AcqKnowledge 3.7.3 software of Biopac System Inc. (Goleta, CA, USA). All data

are presented as mean ± SE, and 'n' indicates the number of vessels tested in myograph experiments or the number of animals tested in the case of body weight, blood glucose, H_2O_2 production, plasma LPC, and serum phosphorylcholine level measurements. In the case of the myography experiments, for each group, vessels were obtained from at least three animals. Three to four aortic segments were isolated per animal. Data analysis was carried out by GraphPad Prism statistical software (version 8.0.1.244; GraphPad Software Inc., La Jolla, CA, USA). Concentration-response curves for ACh and SNP were plotted with responses expressed as percentage of the maximal contraction induced by Phe. When examining the effect of LPC on endothelial function, Two-way ANOVA followed by Tukey's multiple comparisons test was used in order to compare the ACh concentration-response curves. When examining the vascular phenotype of *db/db* mice, the effects of cumulative doses of SNP and ACh were evaluated by dose-response curve fitting for the determination of E_{max} and EC_{50} values. Student's t-test or Mann-Whitney test was used when comparing two variables. $p<0.05$ was considered statistically significant.

4. Results

4.1. Examination of the possible role of ATX in LPC-induced endothelial dysfunction

LPC has been described as a significant pathogenic factor in several inflammatory vascular diseases (see Introduction). More specifically, its involvement in the development of endothelial dysfunction is well-known (141). Therefore, in the first part of the study, we were examining the molecular mechanisms of LPC-evoked endothelial dysfunction.

We observed that in LPC-treated vessels the ACh-induced endothelium-dependent vasorelaxant responses were markedly attenuated (Figure 3A). To investigate the contribution of ATX to this deleterious effect of LPC, vessels were pre-treated with the selective ATX inhibitor GLPG1690. GLPG1690 significantly decreased the LPC-induced endothelial dysfunction (Figure 3A), suggesting the involvement of ATX in the effect of LPC. In order to confirm the presence of ATX on the surface of vascular cells, immunohistochemistry was used. We observed ATX expression in all layers of the aortic tissue (Figure 3B).

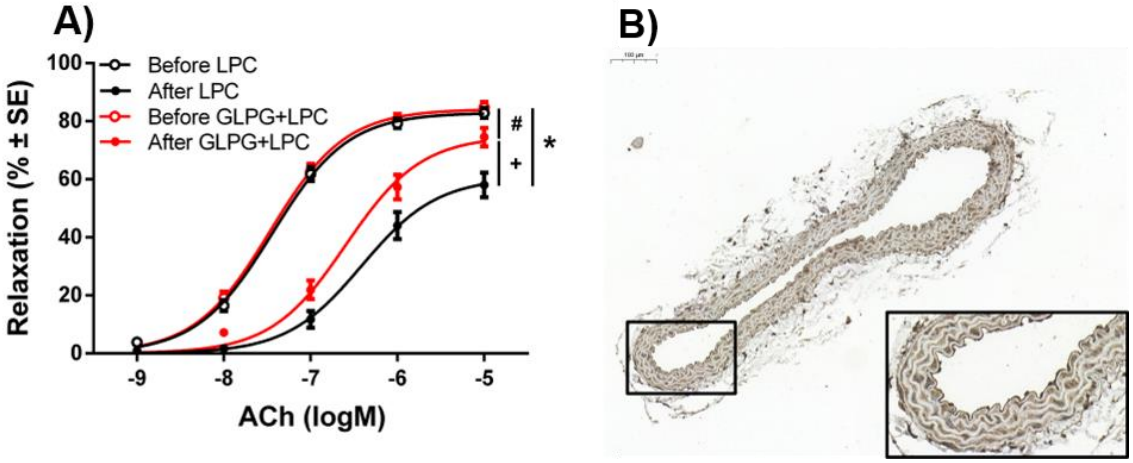

Figure 3. ATX inhibitor GLPG1690 significantly reduced the LPC-evoked attenuation of endothelium-dependent vasorelaxation in WT aortic segments (A). Relaxation values are expressed as mean ± SE percentage of Phe-induced precontraction. n=31-38. Curves were compared using two-way ANOVA, followed by Tukey's multiple comparisons test. #p<0.0001 between before and after GLPG+LPC; +p<0.01 after GLPG+LPC vs. after LPC; *p<0.0001 between before and after LPC. Immunohistochemistry of thoracic aorta segments shows expression of ATX enzyme in all layers of the vascular wall (B). ATX is stained with brown. Figure adapted from the author's original publication (136). Copyright (2023) by the Society for Experimental Biology and Medicine.

4.2. Identification of LPA receptors involved in LPC-induced endothelial dysfunction

Because ATX inhibition attenuated LPC-induced endothelial dysfunction, we hypothesized that it is mediated by LPA and its receptors. Therefore, the effect of LPC was tested on aorta segments isolated from knock-out (KO) mice deficient for type 1, 2, 4, and 5 LPA receptors. In vessels of *Lpar1*, *Lpar2* and *Lpar4* KO mice, the effect of LPC was similar to that observed in wild-type (WT) animals (Figure 4A-C). On the contrary, the impairment of ACh-induced vasorelaxation by LPC was markedly attenuated in *Lpar5* KO mice (Figure 4D). These results indicate that LPC-derived LPA may contribute to the development of endothelial dysfunction through LPA_5 receptor activation.

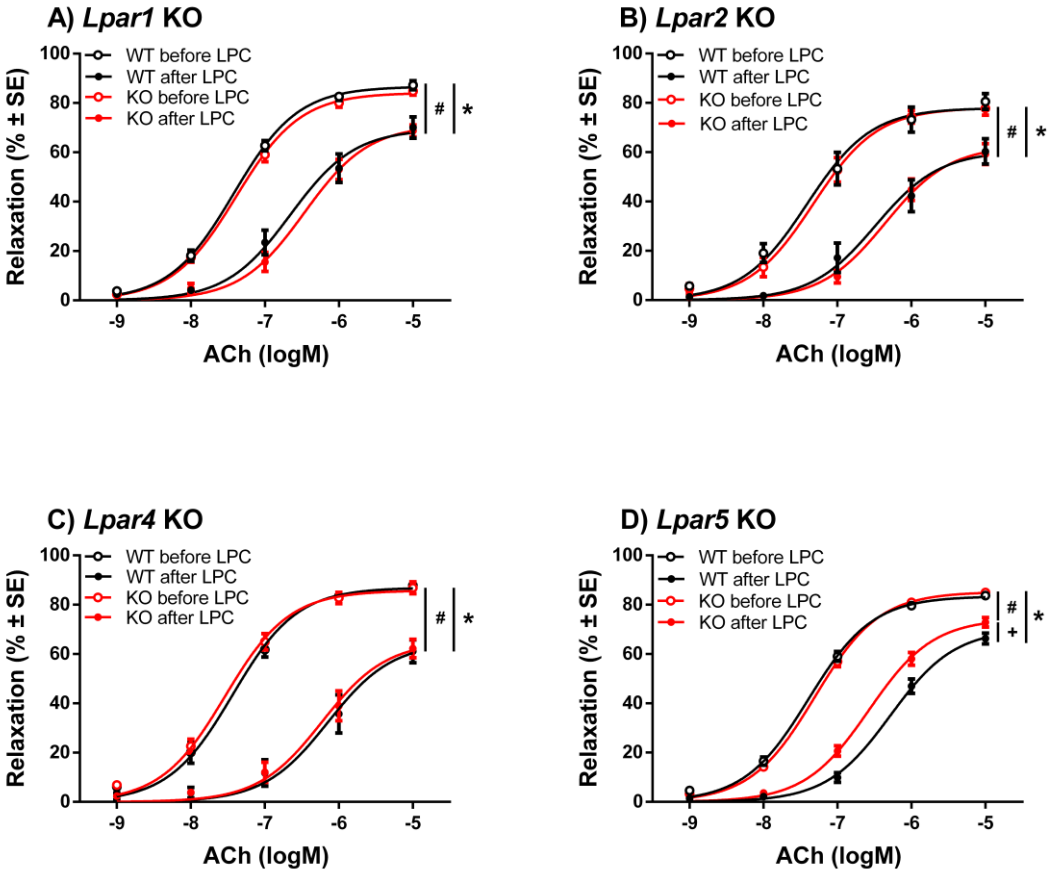

Figure 4. The LPC-induced attenuation of endothelium-dependent vasorelaxation was unaltered in *Lpar1* (A), *Lpar2* (B), and *Lpar4* (C) KO, but it was reduced in *Lpar5* KO (D) vessels. Relaxation values represent mean ± SE percentage of Phe-induced precontraction. A: n=13-15. B: n=9-9 C: n=10-14. D: n=49-60. Curves were compared using two-way ANOVA, followed by Tukey's multiple comparisons test. #p<0.0001 KO before vs. after LPC; *p<0.0001 WT before vs. after LPC"; +p<0.01 KO vs. WT after LPC". Figure adapted from the author's original publication (136). Copyright (2023) by the Society for Experimental Biology and Medicine.

4.3. Expression profile of LPA receptors and ATX in WT and *Lpar5* KO mice

Next, using quantitative real-time PCR, we examined the LPA receptor and ATX expression profile of aortic tissue isolated from WT and *Lpar5* KO mice. We wanted to examine the possibility that LPA_5 deficiency changes the expression of other LPA receptors or ATX, which could be the explanation for preserved endothelial function in *Lpar5* KO vessels. Our data showed that *Lpar5* deletion did not significantly affect the expression of LPA_1, LPA_2, LPA_3, LPA_4, LPA_6 receptors, and ATX as no significant differences in mRNA expression rate were detected relative to WT. In addition, the qPCR analysis confirmed the lack of *Lpar5* in the KO mice (Figure 5).

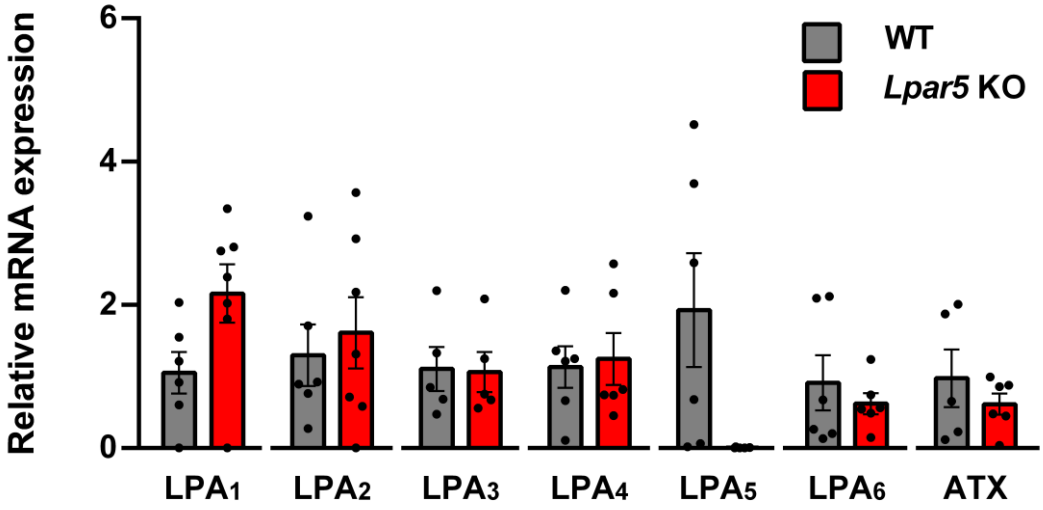

Figure 5. No significant difference was observed in the mRNA expression of LPA_1, LPA_2, LPA_3, LPA_4, LPA_6 receptors, and ATX between WT and *Lpar5* KO aortic segments. *Lpar5* mRNA was not detectable in the KO mice. The mRNA expression of examined genes was normalized to B2m mRNA levels. n=5-8. Mann-Whitney test. Figure adapted from the author's original publication (136). Copyright (2023) by the Society for Experimental Biology and Medicine.

4.4. Examination of the involvement of prostanoid mediators

After confirming the involvement of the ATX-LPA-LPA_5 pathway in LPC-evoked impairment of vasorelaxation, we wanted to clarify, which downstream signaling pathways mediate the LPA-dependent part of the effect. It had been reported before, that LPA can evoke COX-mediated effects, involving the release of vasoconstrictor prostanoid mediators that act on TP receptors (70), thus as a next step, we examined the possible involvement of this pathway. We observed no significant difference in the effect of LPC in COX-1 KO (Figure 6A) and TP KO (Figure 6B) aortic segments compared to WT, suggesting that the COX-1-TP axis is not involved in this process.

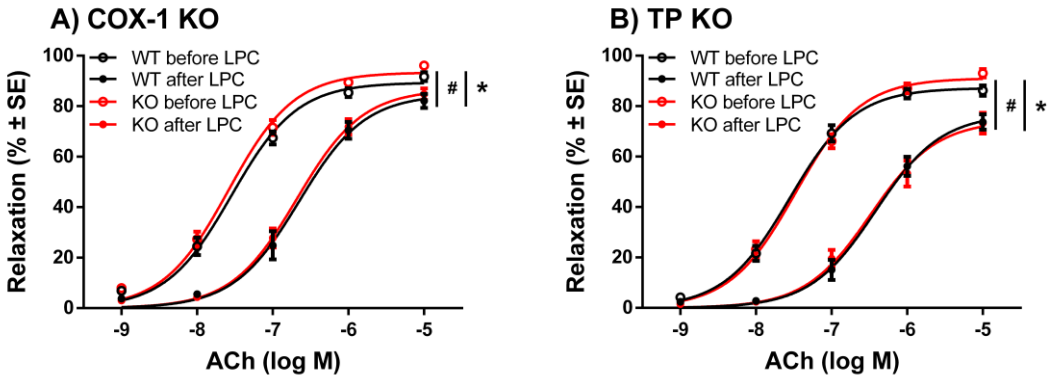

Figure 6. LPC evoked a marked impairment of endothelium-dependent vasorelaxation in COX-1 and TP KO, similar to that of WT vessels. Curves were compared using two-way ANOVA, followed by Tukey's multiple comparisons test. #p<0.0001 KO before vs. after LPC; *p<0.0001 WT before vs. after LPC". A: n=12-18; B: n=13-19.

4.5. Involvement of ROS in LPC-induced endothelial dysfunction

Considering that superoxide is a well-known factor in the development of endothelial dysfunction we tested the effect of superoxide dismutase (SOD) on the deleterious effect of LPC. As shown in Figure 7A, SOD prevented the effect of LPC in WT vessels. Interestingly, this beneficial effect of SOD was absent in *Lpar5* KO vessels (Figure 7B). In addition, Tempol, a membrane-permeable superoxide scavenger, also failed to achieve further improvement in *Lpar5* KO vessels (Figure 7C), suggesting that LPA_5 drives ROS production.

To further confirm the involvement of LPA_5 receptor in ROS generation upon LPC treatment, H_2O_2 production was determined in WT and *Lpar5* KO vessels. LPC induced a marked increase in extracellular H_2O_2 levels in aortic tissue isolated from WT mice, however, its effect was significantly (p<0.05) diminished in *Lpar5* KO vessels (Figure 7D). These data suggest that the LPA_5 activation is involved in LPC-evoked ROS production.

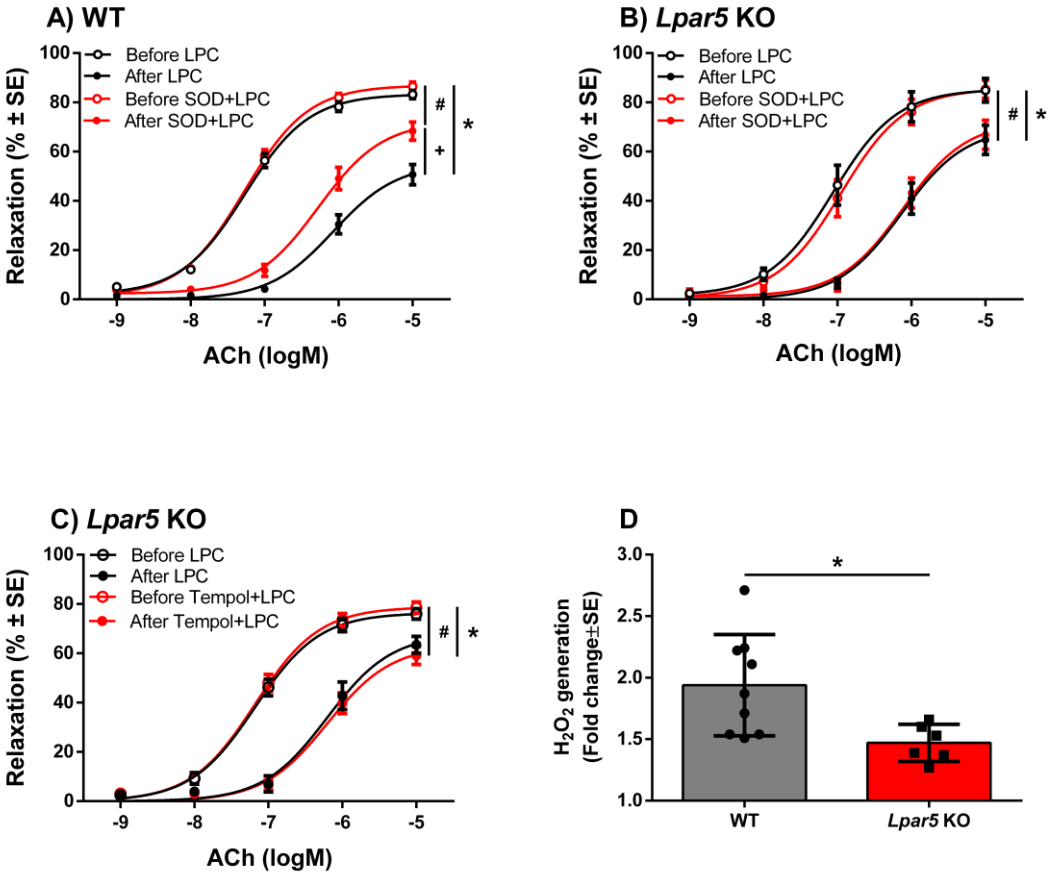

Figure 7. SOD significantly reduced the LPC-evoked attenuation of vasorelaxation in WT (A), but it was ineffective in *Lpar5* KO (B). The membrane-permeable superoxide scavenger Tempol also failed to evoke further improvement in *Lpar5* KO (C) mice. Relaxation values represent mean ± SE percentage of Phe-induced precontraction. Curves were compared using two-way ANOVA, followed by Tukey's multiple comparisons test. A: n=29-30. B: n=9-9. C: n=13-13. A: #p<0.0001 before vs. after SOD+LPC; +p<0.001 after LPC vs. after SOD+LPC; *p<0.0001 before vs. after LPC. B: #p<0.01 before vs. after SOD+LPC; *p<0.001 before vs. after LPC. C: #p<0.0001 before vs. after Tempol+LPC; *p<0.0001 before vs. after LPC. The LPC-evoked H_2O_2 production was significantly reduced in *Lpar5* KO as compared to WT vessels (D). Values are expressed as fold H_2O_2 increase after LPC treatment. WT: n=9, *Lpar5* KO: n=6. *p<0.05 compared to *Lpar5* KO. Mann-Whitney test. Figures adapted from the author's original publication (136). Copyright (2023) by the Society for Experimental Biology and Medicine.

4.6. Manifestation of metabolic and vascular phenotypes of type 2 diabetes in *db/db* mice

In a separate study, we aimed to characterize the general metabolic and vascular phenotypes of the *db/db* mice. These animals reportedly develop obesity with elevated blood glucose levels and hyperinsulinemia (142, 143). Accordingly, both the body weight (Figure 8A) and blood glucose levels increased (Figure 8B) in *db/db* mice as compared to non-diabetic control littermates.

According to literature data, acetylcholine-induced vasorelaxation of the aorta prepared from *db/db* mice is completely eNOS-dependent (144), thus ACh was used to characterize the endothelial function. The vessels of *db/db* animals showed marked endothelial dysfunction, as indicated by the impairment of the concentration-response relationship of ACh-induced vasorelaxation (Figure 8D). In contrast, reactivity of the vascular smooth muscle to NO remained unaltered, as there was no significant difference in the sodium nitroprusside (SNP)-induced vasorelaxation responses between the control and *db/db* mice (Figure 8E).

As type 2 diabetes is associated with altered blood lipid profile, we examined the phosphorylcholine and LPC levels of control and *db/db* animals. Phosphorylcholine is the precursor of phosphatidylcholine (138, 139), which is an important intermediate molecule in the synthesis of several lipid mediators, including LPC (54). Therefore, changes in phosphorylcholine levels can be good indicators of altered lipid metabolism. Serum phosphorylcholine (Figure 8C) levels appeared to be elevated in diabetic animals, as compared to control. Next, we examined the plasma levels of the most abundant LPC species in *db/db* and control mice. All five of the studied LPCs showed a tendency for increased plasma levels in *db/db* mice, with 18:0 and 20:4 reaching the level of statistical significance (Table 2.).

These results indicate that the endothelium-dependent vasoactive responses are disrupted simultaneously with the increase of serum phosphorylcholine and plasma LPC levels in a mouse model of type 2 diabetes.

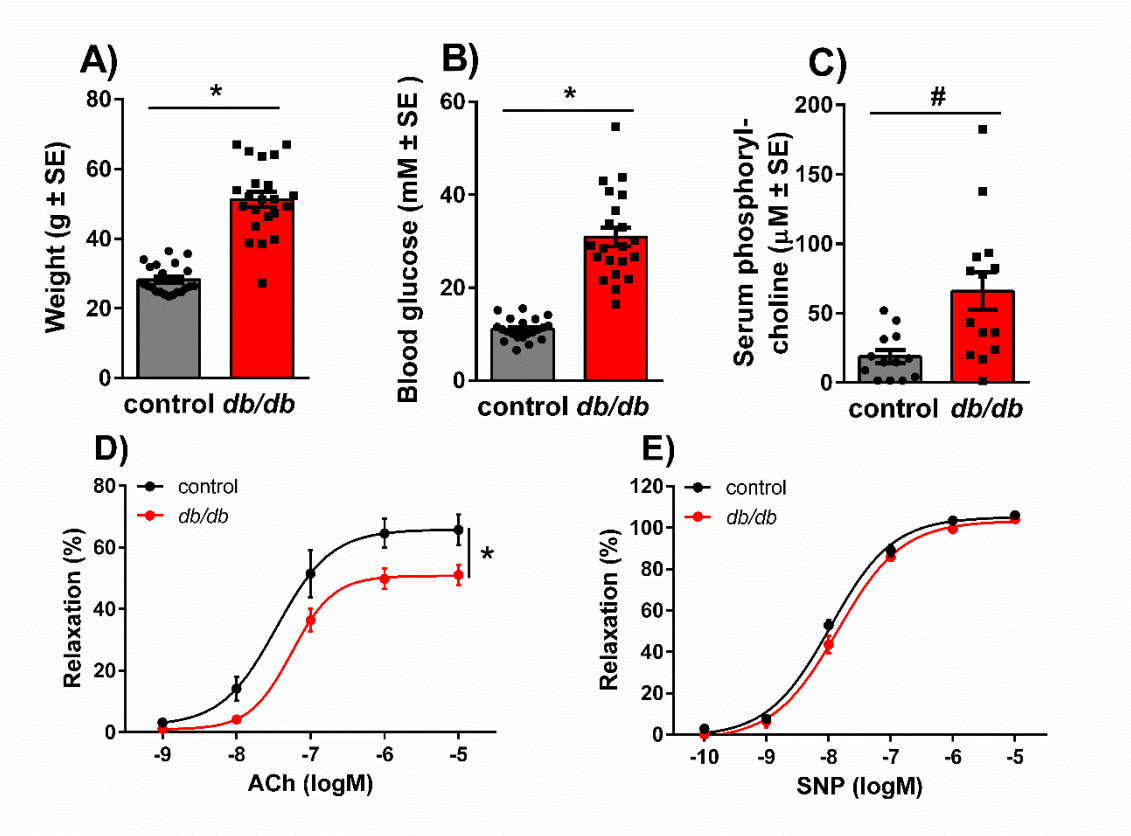

Figure 8. Body weight (A), as well as non-fasting blood glucose (B) and serum phosphorylcholine levels (C), increased in *db/db* mice as compared to controls. n=13–22. *p < 0.001, #p<0.01; Student's unpaired t-test. ACh-induced relaxation diminished (D), while the reactivity of the vascular smooth muscle to sodium nitroprusside (SNP) remained unaltered (E) in vessels of *db/db* mice as compared to controls. Relaxation values are expressed as mean ± SE percentage of Phe-induced precontraction. Dose-response curve fitted to n=12–24. *p<0.001. Figure adapted from the author's original publication (145). Copyright (2023) by Ruisanchez et al. CC BY 4.0.

Table 2. Increased levels (mean±SE) of the most abundant LPC species in the plasma of diabetic animals, with 18:0 and 20:4 being significantly (*p<0.05) higher as compared to controls with Mann-Whitney test, n=4-5.

	Control	*db/db*	*p*-value
16:0 LPC	60.51 ± 6.34 µM	73.45 ± 4.61 µM	0.11
18:0 LPC	43.19 ± 2.69 µM	56.23 ± 4.39 µM	0.03 *
18:1 LPC	16.90 ± 2.08 µM	23.81 ± 4.39 µM	0.19
18:2 LPC	42.69 ± 3.95 µM	44.91 ± 3.79 µM	0.87
20:4 LPC	1.16 ± 0.30 µM	2.48 ± 0.47 µM	0.02 *

5. Discussion

Cardiovascular diseases are leading causes of death worldwide and many of them are associated with inflammatory vascular alterations (146). A characteristic symptom of these conditions is endothelial dysfunction, which often manifests in reduced endothelium-dependent vasorelaxation (12). LPC, a bioactive lipid mediator reportedly participates in the pathogenesis of endothelial dysfunction. It has long been considered as a pro-inflammatory mediator, and its levels in the circulation appeared to correlate with the outcome of atherosclerosis-associated alterations (54). LPC exerts its effects on a number of vascular cells. It has been shown to damage smooth muscle cells by transforming them into a proliferative/secretory phenotype (147). In addition, LPC can activate macrophages and differentiate them into the M1-like, inflammatory phenotype (148). The detrimental effects of LPC on endothelial cells were discussed previously in this Thesis (see Introduction). Along with the aforementioned properties, LPC also upregulates the expression of adhesion molecules (149) contributing to the progression of inflammatory alterations.

As mentioned earlier, in the vasculature, LPC can be converted to LPA, by ATX. LPA has been proposed to participate in several vascular inflammatory processes (150, 151), however, there is a limited amount of data available about its possible involvement in the pathogenesis of endothelial dysfunction. In 2012, Chen et al. studied the effects of LPA on the vasorelaxant functions of porcine coronary arteries (152). After a 24-hour-long incubation with LPA, the vessels showed significantly decreased vasorelaxant features compared to controls, and this effect was attributed to increased ROS production and altered eNOS signaling (152). It is important to highlight though, that in these experiments vessels were treated with LPA for 24 hours, therefore, the effects observed are likely attributable to gene expression changes (152). In contrast, in our experiments, we used a different approach to examine the possible negative effects of LPA on endothelium-dependent vasorelaxation. We addressed whether the locally produced, LPC-derived LPA can have such effects. Our results prove that inhibition of the LPA-producing ATX enzyme is protective against the LPC-induced impairment of vasorelaxation.

As a next step, we aimed to identify the receptor mediating the deleterious effects of the LPC-derived LPA. The majority of the LPA's effects are mediated by six GPCRs, hence we performed our experiments on aortic segments isolated from different LPA receptor KO mice. LPC evoked a marked inhibition of endothelium-dependent vasorelaxation in LPA_1, LPA_2, and LPA_4 KO vessels, while its effect was significantly reduced in LPA_5 KO suggesting that the locally produced LPA activates LPA_5 receptors contributing to endothelial dysfunction. The involvement of this receptor in vascular inflammatory processes has not been addressed thoroughly yet, making our observation especially intriguing.

LPA_5 was identified as a receptor of LPA in 2006, as a result of the deorphanization of GPR92 (153, 154). It belongs to the so-called non-EDG LPA receptors, sharing 35-55% amino acid similarity with the other two members (LPA_4 and LPA_6) of this family (155). LPA_5 can activate $G\alpha_{q/11}$ and $G\alpha_{12/13}$ proteins, but it can induce β-arrestin-dependent signaling as well (118). LPA_5 is expressed in a wide range of mammalian organs including the heart, brain, lungs, and placenta (118). Since its discovery, it has been associated with biological functions such as immune modulation (156) and brain development (157). In the past decade, it has emerged as a key player in several disorders including cancer (118), neuropathic pain (158), and neuroinflammation (159). In the vascular system, LPA_5 mRNA can be found in both endothelial (69) and smooth muscle cells (70). Moreover, LPA_5 is highly abundant in human platelets (118) making it a potential participant in atherothrombotic events. It is well-known that LPA is capable of inducing platelet aggregation (130). In their study published in 2011, Khandoga et al. investigated the signaling mechanisms of LPA-induced activation of human megakaryocytic cells (131). They found that all LPA receptors are expressed in these cells, with LPA_4 and LPA_5 being the most abundant ones. Furthermore, the depletion of LPA_5 inhibited significantly the LPA-induced shape change of these cells suggesting that LPA_5 is the main effector of LPA (131). LPA_5 receptor has also been implicated in the pathogenesis of atherosclerosis, as its expression was observed to be increased in atherosclerotic plaques obtained from human carotid arteries (160). LPA_5 expression correlated with macrophage and endothelial cell markers, hence it has been assumed that along with other LPA receptors, LPA_5 plays a role in endothelial cell activation (160).

This is in line with our hypothesis that LPA_5 is involved as a regulator in vascular inflammation.

In addition, we examined whether the genetic deletion of LPA_5 affects the expression of other LPA receptors and ATX. The results of the qPCR measurement showed no significant difference in the mRNA levels of LPA_1, LPA_2, LPA_3, LPA_4, LPA_6, and ATX between WT and *Lpar5* KO suggesting that neither ATX nor other LPA receptors compensate for the loss of LPA_5.

We can therefore conclude that our results prove the involvement of the ATX-LPA-LPA_5 receptor axis in the pathogenesis of LPC-evoked endothelial dysfunction. Hence, we aimed to investigate further this effect in order to find out which signaling pathways are induced upon LPA_5 receptor activation that contribute to the deleterious consequences. As indicated in the Introduction, a number of publications claim that endothelial dysfunction is characterized by the disruption of the fine-tuned balance of vasoconstrictor-vasorelaxant mediators. One possible mechanism of this phenomenon is the overproduction of COX-derived vasoconstrictor prostanoids in the endothelium leading to an increased tone of the vessels. In a previous publication, our research group described that in isolated, endothelium-denuded aortic segments, LPA evokes a potent vasoconstrictor response (70). The results suggest that this effect is achieved by LPA_1 receptors, leading to COX-1 activation and consequently the production of TXA_2, a mediator that increases the vascular tone via activating TP receptors of smooth muscle cells (70). Based on these observations, we intended to test, whether the COX-1-TP pathway is the one that mediates the effects of LPA in our experiments. We found that LPC evoked a marked impairment of vasorelaxation in both COX-1 and TP KO mice and these effects were similar to that we observed in WT vessels. Therefore, we concluded that LPC-derived LPA induce endothelial dysfunction independently of the activation of the COX-1-TP pathway.

Besides prostanoid upregulation, endothelial dysfunction is also characterized by oxidative stress. Elevated ROS levels reportedly contribute to the reduced NO bioavailability and disrupted endothelial function (see Introduction). The negative effects of LPC on endothelial cells are well-known and mostly attributed to its ability to evoke oxidative stress. A number of studies have reported elevated levels of ROS in cultured endothelial cells after LPC treatment (75, 78). The release of these oxidative agents can

impair normal endothelial function resulting in decreased endothelium-dependent vasorelaxation. In their study, Rao et al. demonstrated (141) that the adverse effect of 18:1 LPC on NO-dependent vasorelaxation can be mitigated almost completely with the superoxide-scavenger Tempol. Our observations align with these results, as we observed a substantial reduction in the 18:1 LPC-induced impairment of vasorelaxation in WT when SOD was administered. It is important to highlight though, that the protective effect of SOD was not complete. The reason for the difference might be that Tempol is a membrane-permeable agent, thus interacting with both intra- and extracellular ROS (161), while SOD has limited membrane permeability and acts primarily extracellularly (162). The involvement of extracellular ROS was further confirmed by the results we obtained with the Amplex Red assay, a method widely used for extracellular H_2O_2 detection (137). Notably, in the supernatant of vessels isolated from WT, a significant amount of H_2O_2 was observed after LPC treatment suggesting that LPC evokes ROS generation in the aortic tissue. In contrast, in *Lpar5* KO vessels we did not see additional improvement with SOD or Tempol in the myograph experiments. Furthermore, we observed reduced ROS release upon LPC treatment in *Lpar5* KO as compared to WT vessels. These findings imply that the activation of LPA_5 receptor by locally produced LPA leads to ROS release contributing to the impairment of NO-dependent vasorelaxation.

Although we confirmed the contribution of the ATX-LPA-LPA_5 pathway to the effect of LPC, the exact intracellular signaling mechanism is yet to be elucidated. It is plausible that LPA_5 activation induces the uncoupling of the eNOS enzyme resulting in enhanced ROS production. Another possibility is that the LPA_5-dependent ROS release is associated with the activation of NOX enzymes, which are major sources of ROS (see Introduction). Vascular cells express four of the known seven isoforms of NOX enzymes, namely NOX1, NOX2, NOX4, and NOX5 (163). Endothelial cells, smooth muscle cells, and macrophages express all four NOX enzymes, while adventitial fibroblasts express NOX2 and 4 (163). The ability of LPA to induce NOX-dependent signaling has been described by others. Lin et al. showed in their *in vitro* studies that LPA evokes ROS release in prostate cancer cells that is dependent on PLC, PKC, and NOX enzymes (164). In addition, in a paper published by Plastria et al., it was described that in microglia (that

are resident macrophages in the brain) LPA acts via LPA_5 receptors to induce PKD enzymes, which results in ROS production (165, 166).

While our results imply that a substantial part of the detrimental effect of LPC requires its conversion to LPA, it is possible that additional signaling pathways independent of LPA also contribute, as we were unable to prevent completely the effect of LPC either with ATX inhibition or with the genetic deletion of LPA_5. Previous results proposed that LPC can exert its effects by directly activating GPCRs like G2A and GPR4. Although there is a possibility that LPC modulates their function, it is still debated whether LPC is a ligand of these receptors, as direct interaction has not been verified (54, 84, 85). Given its amphipathic nature, it is more likely that LPC interacts with the cell membrane, altering its biophysical properties and thereby affecting normal membrane functions (83). In accordance with this hypothesis, it has been suggested that by being incorporated into the endothelial cell membrane, LPC interacts directly with the eNOS enzymes located in caveolae (75, 81). This process could disrupt eNOS leading to reduced NO bioavailability and subsequent endothelial dysfunction.

As mentioned previously, LPC has been linked to the development of a number of inflammatory vascular diseases (54). These pathologies are often associated with altered blood lipid profiles, thus lipid mediators could be good biomarkers of the development and severity of these diseases. Diabetic alterations are often associated with disrupted lipid metabolism (167), therefore, in another study, we aimed to characterize the vascular and metabolic phenotypes of a mouse model of type II diabetes.

It is well known that *db/db* animals have higher body weight and blood sugar levels compared to wild-type littermates (142, 143). Our results support these observations confirming that diabetes was developed. When investigating their vascular features, we found that ACh-dependent vasorelaxation was markedly impaired compared to controls whereas SNP-mediated relaxation remained intact. Since ACh-induced vasorelaxation is completely eNOS-dependent (144), it is a good indicator of endothelial function. Altogether, the results show that the animals developed endothelial dysfunction, but the smooth muscle remained sensitive to exogenous NO.

Serum phosphorylcholine of *db/db* showed elevated levels compared to controls. Phosphorylcholine is a precursor molecule of phosphatidylcholine (PC) (138), an important intermediate in lipid metabolism (168, 169). More specifically, PC serves as a

substrate for PLA2 enzyme in the process of LPC production (see Introduction). Hence, the elevated serum phosphorylcholine can be an indicator of altered lipid metabolism in diabetes and also allows the development of increased LPC levels. In line with this observation, we found that plasma levels of 18:0 and 20:4 LPC were also elevated in diabetic animals further supporting our hypothesis.

Although previous publications examined the levels of different LPC species in diabetes, the results are contradictory. A few have shown increased levels of LPC in diabetic patients, however, there are data available suggesting that LPC is decreased in diabetic alterations (170, 171). A possible explanation of the different findings may be that LPC levels change during the course of the disease, so the results are affected by the stage at which the tests are performed.

Our results raise the question of whether increased plasma LPC and reduced vasorelaxation in diabetic states are related. Further studies are needed to answer this question. Literature data shows, that various inflammatory factors can increase ATX expression, and it may also be upregulated by elevated glucose and insulin levels (71, 172, 173). It has been suggested that obesity increases adipose ATX expression in both animal models and humans (174, 175), that can contribute to elevated ATX levels in the circulation. More specifically, recent data are available showing that ATX is increased in adipose tissue of *db/db* mice (176). Overall, we assume that our results showing increased plasma LPC, accompanied by possibly elevated levels of ATX in the vascular system might contribute to the impaired vasorelaxation observed in *db/db* mice. However, further experiments are needed to prove this hypothesis.

6. Conclusions

In our experiments, we aimed to investigate the molecular mechanisms of LPC-induced endothelial dysfunction and to characterize the vascular and metabolic phenotype of a mouse model of diabetes. Our results indicate that:

- 18:1 LPC induces a marked impairment of ACh-evoked vasorelaxation that can be partially prevented by inhibition of the ATX enzyme implying that LPA, the product of ATX, mediates a significant part of the effect of LPC.

- LPC-derived LPA is likely to evoke its deleterious effects via LPA_5 receptor activation, as the effect of LPC was reduced in *Lpar5* KO mice. LPA_1, LPA_2 and LPA_4 are not involved in this phenomenon.

- The mRNA expression of LPA_1, LPA_2, LPA_3, LPA_4, LPA_6, and ATX do not change in the aortic tissue of *Lpar5* KO mice suggesting the other LPA receptors and ATX do not compensate for the loss of LPA_5.

- COX-1 enzyme and TP receptor are not involved in the LPC-evoked impairment of vasorelaxation, since the effect of LPC remained unaltered in COX-1 and TP receptor KO mice.

- LPC evokes ROS release from the aortic tissue of wild-type mice that is reduced in *Lpar5* KO demonstrating that LPA_5 activation by locally produced LPA results in oxidative stress contributing to endothelial dysfunction.

- Diabetic *db/db* mice present with increased serum phosphorylcholine levels, accompanied by elevated plasma levels of 18:0 and 20:4 LPC indicating an altered blood lysophospholipid profile in type II diabetes.

7. Summary

Lysophosphatidylcholine is a bioactive mediator that has been linked to several inflammatory cardiovascular diseases. Although it has long been known that LPC contributes to the development of endothelial dysfunction by impairing the vasorelaxant properties of the vessels, its exact mechanism of action remains elusive. The purpose of our study was to investigate the possible involvement of the ATX-LPA-LPA receptor pathway in the LPC-evoked impairment of vasorelaxation in the aortic segments and to examine the underlying molecular mechanisms. We found that 18:1 LPC significantly reduces the ACh-induced endothelium-dependent vasorelaxation which could be partially prevented by the inhibition of ATX enzyme. Therefore, we assume that LPC mediates its deleterious effects as converted to LPA, by ATX. In order to further prove our hypothesis, we tested the effect of LPC on the vessels of different LPA receptor deficient mice. LPC evoked endothelial dysfunction remained unaltered in *Lpar1, Lpar2,* and *Lpar4* KO, whereas it was significantly decreased in *Lpar5* KO aortic segments. These observations indicate that the locally produced, LPC-derived LPA activates LPA_5 receptors, thus contributing to the impairment of endothelial function. In the next phase of the study, we intended to examine further the molecular mechanisms involved in this phenomenon. As both LPC and LPA are known to induce prostanoid-dependent signaling, we tested the involvement of this pathway. The effect of LPC developed in both COX-1 and TP receptor KO mice implying that the COX-1-TP axis is unlikely to be involved. In contrast, the effect of LPC decreased significantly in the presence of SOD and LPC evoked a marked increase in H_2O_2 levels in the aortic tissue of WT mice. Interestingly, in *Lpar5* KO we could not achieve further improvement of the endothelium-dependent vasorelaxation with SOD or Tempol and we also found a significantly lower ROS release upon LPC treatment. In a separate study, we examined the serum and plasma lysophospholipid profile in a diabetic mouse model. We found elevated serum phosphorylcholine and plasma LPC levels in the diabetic animals compared to WT. Taken together, our results contribute to a more thorough understanding of the LPC-ATX-LPA axis in vascular functions, possibly providing new pharmacological targets for the treatment of vascular diseases associated with alterations of lysophospholipid metabolism.

8. References

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Ruisanchez É*, **Janovicz A***, Panta RC, Kiss L, Párkányi A, Straky Z, Korda D, Liliom K, Tigyi G, Benyó Z. Enhancement of Sphingomyelinase-Induced Endothelial Nitric Oxide Synthase-Mediated Vasorelaxation in a Murine Model of Type 2 Diabetes. International Journal of Molecular Sciences. 2023 May 6;24(9):8375) **IF: 5.6**

***Contributed equally to this work.*

10. Acknowledgments

I would like to express my gratitude to all those who contributed to the work presented in this thesis. I would like to thank my supervisors, Prof. Zoltán Benyó and Dr. Éva Ruisanchez for all their guidance throughout my PhD studies. I would like to thank my fellow PhD students and other colleagues at the Institute of Translational Medicine for their encouragement and technical support. I would also like to thank my loved ones for their continuous support during my PhD studies.