Original Research *Feature article*

Autotaxin–lysophosphatidic acid receptor 5 axis evokes endothelial dysfunction via reactive oxygen species signaling

Anna Janovicz[1,2], Aliz Majer[1], Mónika Kosztelnik[1,2], Miklós Geiszt[3], Jerold Chun[4], Satoshi Ishii[5], Gábor József Tigyi[1,6], Zoltán Benyó[1,2] and Éva Ruisanchez[1,2]

[1]Institute of Translational Medicine, Semmelweis University, H-1094 Budapest, Hungary; [2]Eötvös Loránd Research Network and Semmelweis University (ELKH-SE) Cerebrovascular and Neurocognitive Disorders Research Group, H-1052 Budapest, Hungary; [3]Department of Physiology, Faculty of Medicine, Semmelweis University, H-1094 Budapest, Hungary; [4]Translational Neuroscience at Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA 92037, USA; [5]Department of Immunology, Graduate School of Medicine, Akita University, Akita 010-8543, Japan; [6]Department of Physiology, University of Tennessee Health Science Center, Memphis, TN 38163, USA
Corresponding author: Éva Ruisanchez. Email: ruisanchez.eva@med.semmelweis-univ.hu

Impact Statement

The autotaxin (ATX)–lysophosphatidic acid (LPA) axis has been proposed to be involved in several cardiovascular diseases; however, its involvement in lysophosphatidylcholine (LPC)-induced endothelial dysfunction has not been studied yet. Here, we demonstrate for the first time that the development of LPC-induced impairment of endothelium-dependent vasorelaxation requires the conversion of LPC to LPA by the ATX enzyme. LPA activates LPA_5, triggering signaling pathways that lead to an elevated production of reactive oxygen species (ROS) and subsequent endothelial dysfunction. The ATX–LPA–LPA_5 receptor pathway might provide new targets to prevent endothelial dysfunction.

Abstract

Lysophosphatidylcholine (LPC) is a bioactive lipid that has been shown to attenuate endothelium-dependent vasorelaxation contributing to endothelial dysfunction; however, the underlying mechanisms are not well understood. In this study, we investigated the molecular mechanisms involved in the development of LPC-evoked impairment of endothelium-dependent vasorelaxation. In aortic rings isolated from wild-type (WT) mice, a 20-min exposure to LPC significantly reduced the acetylcholine chloride (ACh)–induced vasorelaxation indicating the impairment of normal endothelial function. Interestingly, pharmacological inhibition of autotaxin (ATX) by GLPG1690 partially reversed the endothelial dysfunction, suggesting that lysophosphatidic acid (LPA) derived from LPC may be involved in the effect. Therefore, the effect of LPC was also tested in aortic rings isolated from different LPA receptor knock-out (KO) mice. LPC evoked a marked reduction in ACh-dependent vasorelaxation in *Lpar1*, *Lpar2*, and *Lpar4* KO, but its effect was significantly attenuated in *Lpar5* KO vessels. Furthermore, addition of superoxide dismutase reduced the LPC-induced endothelial dysfunction in WT but not in the *Lpar5* KO mice. In addition, LPC increased H_2O_2 release from WT vessels, which was significantly reduced in *Lpar5* KO vessels. Our findings indicate that the ATX–LPA–LPA_5 receptor axis is involved in the development of LPC-induced impairment of endothelium-dependent vasorelaxation via LPA_5 receptor–mediated reactive oxygen species production. Taken together, in this study, we identified a new pathway contributing to the development of LPC-induced endothelial dysfunction.

Keywords: Lysophosphatidylcholine, autotaxin, lysophosphatidic acid, lysophosphatidic acid receptor 5, endothelial dysfunction, reactive oxygen species

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Introduction

Endothelial dysfunction refers to the complex structural and functional alteration of the endothelium that manifests in several cardiovascular diseases including atherosclerosis, diabetes, and hypertension.[1] It is characterized by endothelial cell activation resulting in increased adhesion molecule and cytokine expression and impaired endothelial permeability.[2,3] The hallmark of endothelial dysfunction is the altered endothelium-dependent vasodilation, mostly attributed to the disrupted synthesis and reduced bioavailability of nitric oxide (NO).[1]

Lysophosphatidylcholine (LPC) is a bioactive glycerophospholipid with well-documented toxic effects on the endothelium.[4,5] It is present in the circulation in high micromolar concentrations, mostly bound to carrier proteins such as albumin or lipoproteins.[6] LPC is known as a proinflammatory mediator that is involved in the progression

of several cardiovascular diseases.[7] Moreover, LPC is known to interfere with the NO homeostasis of endothelial cells, which results in an impaired endothelium-dependent vasorelaxation.[8,9]

Although, in the past decades, several papers reported the involvement of LPC in the development of endothelial dysfunction, the mechanism underlying this phenomenon remains unclear. Some suggest that LPC might activate signaling pathways that lead to the increased production of reactive oxygen species (ROS) including superoxide and hydrogen peroxide (H_2O_2). These ROS can damage the endothelial cells directly or react with NO reducing the vasorelaxant features of the endothelium.[8,10,11] It is also possible that LPC disrupts the integrity of the nitric oxide synthase (NOS) enzyme decreasing its activity.[9]

In the vascular system, LPC is metabolized by autotaxin (ATX), an ectoenzyme with lysophospholipase D activity, coded by the ectonucleotide pyrophosphatase/phosphodiesterase 2 (*ENPP2*) gene.[12] ATX is found in the plasma mostly generated by the adipose tissue,[13] but it is also expressed in different vascular cells such as the endothelium,[14] smooth muscle,[15] and macrophages.[16] The product of LPC metabolism by ATX is lysophosphatidic acid (LPA), a bioactive mediator, with multiple vascular functions.[16,17] Most of the effects of LPA are mediated by six G protein-coupled receptors, which are classified into two groups. $LPA_{1–3}$ 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.[18]

The ATX–LPA–LPA receptor axis has been implicated in the pathology of different inflammatory cardiovascular diseases including atherosclerosis. For example, LPA induces the expression of chemokines and adhesion molecules through activating $LPA_{1/3}$.[19] In addition, LPA also plays an important role in neointima formation.[20,21] Despite their documented involvement in progression of vascular dysfunction, the potential role of ATX and LPA in LPC-induced endothelial dysfunction has not yet been reported. In this study, we described the ATX–LPA–LPA_5 axis as a previously unidentified pathway contributing to the LPC-induced impairment of endothelium-dependent vasorelaxation.

Materials and methods

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 a gift from Lexicon Pharmaceuticals (The Woodlands, TX, USA). All transgenic mouse lines had the C57BL/6 genetic background. Animals were housed in a temperature and light controlled room (12 h light–dark cycle) with free access to food and water.

Preparation of vessels

Adult (90–140 days old) male mice were anesthetized in a CO_2-chamber, followed by transcardial perfusion with Krebs solution containing 10 U/mL Heparin as described previously.[22] 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.

Myography

Vascular tension changes were measured with wire myography as described previously, with a few modifications.[14] 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 (PE) and acetylcholine chloride (ACh) 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 precontracted using increasing concentrations of PE (10 nM–10 μM), followed by increasing concentrations of acetylcholine (1 nM–10 μM) to evoke NO-dependent vasorelaxation. After washing, vessels were exposed to 124 mM KCl Krebs solution for 3 min once more, to elicit a reference contraction. Then, the PE–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 or 18:1 LPA for 20 min, followed by the re-administration of the PE and ACh concentrations (Supplementary Figure 1). 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.

Quantification of vascular H_2O_2 release

LPC-induced ROS release was measured by the Amplex Red assay, a method widely used for detection of extracellular H_2O_2 levels.[23] 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.

Table 1. Primer sequences used in quantitative PCR analysis.

Gene name	Primer sequence	NCBI reference sequence number	Size (bp)	Reference
Target genes				
Lpar1 (lysophosphatidic acid receptor 1)	F: GACTCCTACTTAGTCTTCTGG R: CAGACAATAAAGGCACCAAG	NM_010336.2	200	Purchased from Sigma-Aldrich
Lpar2 (lysophosphatidic acid receptor 2)	F: CAAGACGGTTGTCATCATTC R: AATATACCACTGCATTGACC	NM_020028.3	167	Purchased from Sigma-Aldrich
Lpar3 (lysophosphatidic acid receptor 3)	F: AGGGCTCCCATGAAGCTAAT R: GTTGCACGTTACACTGCTTG	NM_022983.4	124	Ye *et al.*[25]
Lpar4 (lysophosphatidic acid receptor 4)	F: CTGATCGTCTGCCTCCAGAAA R: TTGAGACTGAGGACCAGTAGAG	NM_175271.4	117	Ye *et al.*[25]
Lpar5 (lysophosphatidic acid receptor 5)	F: TCATCTTCCTGCTGTGC R: ATCGCGGTCCTGAATACTGT	NM_001163268.2	98	Purchased from Sigma-Aldrich
Lpar6 (lysophosphatidic acid receptor 6)	F: ACTGAAGTAAAGCTGGTTTG R: AACCCATAAAGCTGAAAGTG	NM_175116.4	109	Purchased from Sigma-Aldrich
Enpp2 (ectonucleotide pyrophosphatase/ phosphodiesterase 2)	F: CTGTCTTTGATGCTACTTTCC R: TCACAGACCAAAAGAATGTC	NM_001040092.3	129	Purchased from Sigma-Aldrich
Reference gene				
B2m (beta-2 microglobulin)	F: CTTTCTGGTGCTTGTCTCACTG R: AGTATGTTCGGCTTCCCATTC	NM_009735.3	105	Untergasser *et al.*[24]

NCBI: National Center for Biotechnology Information.
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.

Quantitative real-time polymerase chain reaction analysis

Whole thoracic aorta of WT and *Lpar5* KO mice 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 (Thermo Scientific, Waltham, MA, USA). Quantitative 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 (Bio-Rad Laboratories). 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 using Primer3Plus and Primer-BLAST software tools and/or ordered from Sigma-Aldrich (St. Louis, MO, USA).[24,25] 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.[26] The minimum information for the publication of quantitative real-time PCR experiments (MIQE) guideline was considered during the entire qPCR quantification workflow and the detailed descriptions of methodology can be found in the Supplementary Materials.[27]

Reagents

Oleoyl-lysophosphatidylcholine (18:1 LPC) was purchased from Sigma-Aldrich 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 20,000 U/mL. GLPG1690 was purchased from Cayman Chemicals (Ann Arbor, MI, USA), and dimethyl sulfoxide (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. SsoAdvanced Universal SYBR Green Supermix was purchased from Bio-Rad Laboratories. Tempol was purchased from Sigma-Aldrich and dissolved in water before use.

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 value ± SE, and "*n*" demonstrates the number of vessels tested. 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 were plotted with responses expressed as percentage of the maximal contraction induced by PE. Two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test was used in order to compare the ACh concentration–response curves. Mann–Whitney test was used when comparing two variables. $P < 0.05$ was considered statistically significant.

Results

Inhibition of ATX attenuates LPC-induced endothelial dysfunction

LPC reportedly evokes endothelial dysfunction characterized by reduction in NO-dependent vasorelaxation.[10] In agreement with that, we observed that in LPC-treated WT vessels, the ACh-induced vasorelaxant responses were markedly attenuated (Figure 1). To investigate the contribution of ATX to this deleterious effect of LPC, vessels were pretreated with the selective ATX inhibitor GLPG1690. GLPG1690 significantly ($P < 0.01$) decreased the LPC-induced endothelial dysfunction (Figure 1), suggesting the involvement of ATX in the effect of LPC. ATX expression in the aortic tissue was confirmed with immunostaining (Supplementary Figure 2).

Identification of LPA receptors involved in LPC-induced endothelial dysfunction

Because ATX appeared to be involved in the LPC-induced attenuation of endothelial function, we examined whether its product, LPA also contributes to the effect. Therefore, the effect of LPC was tested on aorta segments isolated from mice KO for different LPA receptors. In the case of *Lpar1*, *Lpar2*, and *Lpar4* KO, the effect of LPC was similar to that observed in WT mice (Figure 2(A) to (C)). On the contrary, the impairment of ACh-induced vasorelaxation by LPC was markedly attenuated in *Lpar5* KO mice (Figure 2(D)). These results indicate that LPC-derived LPA may contribute to the development of endothelial dysfunction through LPA_5 receptor activation. The direct effect of LPA on the endothelial function was also tested. LPA evoked a significant impairment of the ACh-induced vasorelaxation in WT vessels, but its effect was absent in *Lpar5* KO (Supplementary Figure 3).

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

We examined the LPA receptor and ATX expression profile in aortic tissue isolated from WT and *Lpar5* KO mice using quantitative real-time PCR. 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 3).

Involvement of ROS in LPC-induced endothelial dysfunction

In the next phase of the study, we investigated the downstream signaling mechanism involved in the LPA receptor-mediated portion of the LPC-induced endothelial dysfunction. Considering that superoxide is a well-known factor participating in the development of endothelial dysfunction,[1] we tested the effect of SOD on the deleterious effect of LPC. As shown in Figure 4(A), SOD prevented the effect of LPC in WT vessels. Interestingly, this beneficial effect of SOD was absent in *Lpar5* KO vessels (Figure 4(B)). In addition, Tempol, a membrane-permeable superoxide scavenger, also failed to achieve further improvement in *Lpar5* KO vessels

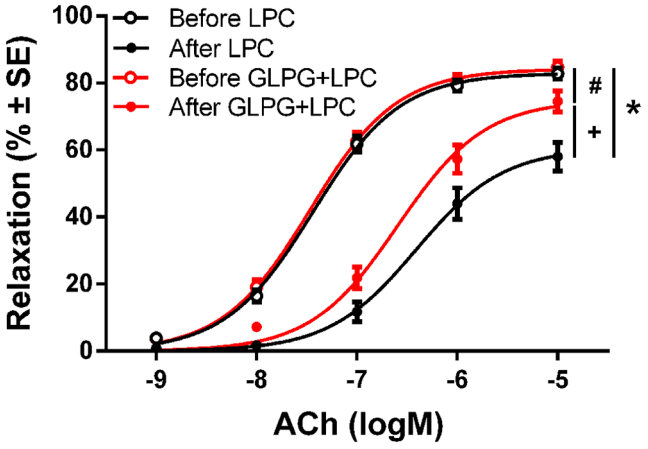

Figure 1. Cumulative concentration–response curves to ACh were performed on WT mouse aortic rings before and after incubation with 18:1 LPC (10 μM, 20 min) in the presence or absence of GLPG1690, a selective ATX inhibitor (10 μM). GLPG1690 significantly reduced the LPC-evoked attenuation of vasorelaxation. Relaxation values are expressed as mean value ± SE percentage of maximal PE-induced contraction. LPC: $n = 31$, GLPG + LPC: $n = 38$. Curves were compared using two-way ANOVA, followed by Tukey's multiple comparisons test. #$P < 0.0001$ vs "Before GLPG + LPC"; +$P < 0.01$ vs "After GLPG + LPC"; *$P < 0.0001$ vs "Before LPC."

(Supplementary Figure 4), 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 assay was performed in the vessels. Baseline H_2O_2 levels of aortic tissue isolated from WT (0.03905 ± 0.003378 μM/min) and *Lpar5* KO (0.03369 ± 0.006017 μM/min) showed no significant difference. 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 5). These data suggest that the LPA_5 activation is involved in LPC-evoked ROS production.

Discussion

In this study, we demonstrated that ATX and LPA_5 receptor contribute to the LPC-induced impairment of endothelium-dependent vasorelaxation. Furthermore, these results suggest that the reduction of NO-dependent vasorelaxation is coupled with elevated ROS production and this effect is mediated, at least in part by LPA_5 activation.

Although ATX and LPA are associated reportedly with inflammatory vascular diseases like atherosclerosis,[20,28] their involvement in the alteration of endothelium-dependent vasorelaxation has not yet been investigated. In contrast, the disruptive effect of LPC on vasorelaxation is well-documented;[8,10,11] however, to the best of our knowledge, the potential involvement of ATX and LPA in this process has not been addressed previously. Here, we demonstrated that the selective inhibition of ATX significantly reduces the LPC-induced impairment of endothelial function, suggesting that LPC achieves this effect partly by conversion to LPA.

The involvement of LPA was further confirmed, as we observed that the deletion of *Lpar5* is protective of the LPC-evoked endothelial dysfunction. Since its discovery in 2006,[29] LPA_5 receptor has been implicated in multiple

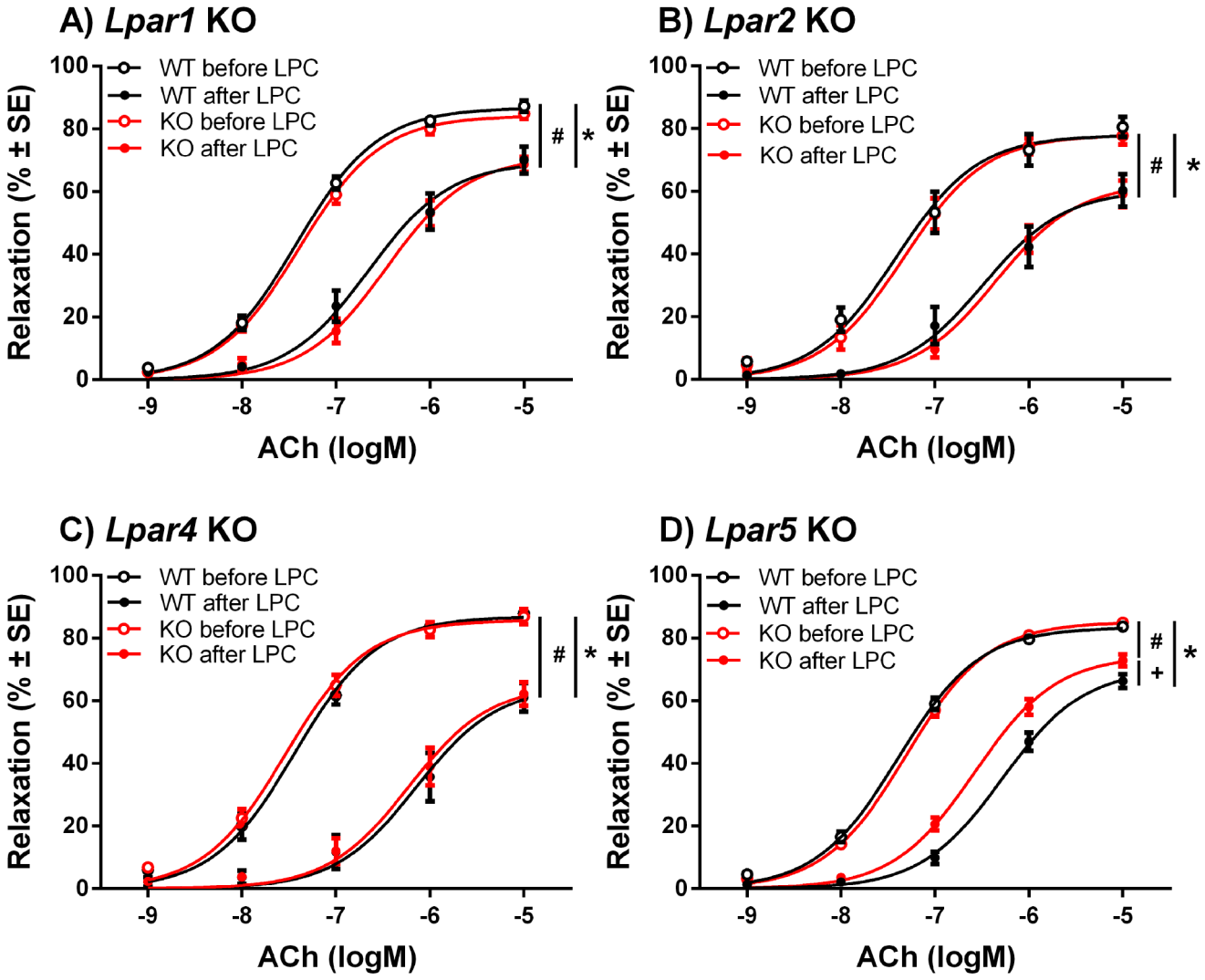

Figure 2. Cumulative concentration–response curves to ACh were performed on aortic rings before and after incubation with 18:1 LPC (10 μM, 20 min). Vessels were isolated from *Lpar1* (A), *Lpar2* (B), *Lpar4* (C), and *Lpar5* (D) KO and WT mice. The LPC-induced attenuation of endothelium-dependent vasorelaxation was unaltered in *Lpar1* (A), *Lpar2* (B), and *Lpar4* (C) KO, but it was reduced in *Lpar5* KO (D). Relaxation values represent mean value ± SE percentage of maximal PE-induced contraction. (A) WT: $n=15$, KO: $n=13$. (B) WT: $n=9$, KO: $n=9$. (C) WT: $n=10$, KO: $n=14$. D: WT: $n=49$, KO: $n=60$. Curves were compared using two-way ANOVA, followed by Tukey's multiple comparisons test.
#$P<0.0001$ vs "KO before LPC"; *$P<0.0001$ vs "WT before LPC"; +$P<0.01$ vs "KO after LPC."

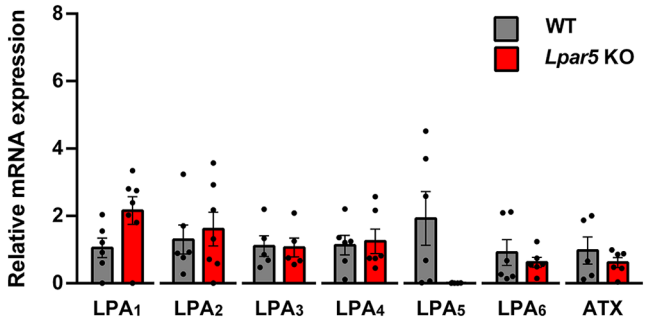

Figure 3. LPA receptor and ATX expression of mouse aortic tissue isolated from WT (gray bars) and *Lpar5* KO (red bars) mice. mRNA expression was determined using quantitative real-time PCR. No statistical difference was observed in LPA_1, LPA_2, LPA_3, LPA_4, LPA_6 receptors, and ATX gene expression between the two groups. *Lpar5* mRNA was not detectable in the KO mice. The changes in mRNA expression of examined genes were normalized to *B2m* mRNA levels. LPA_1: Bl6: $n=6$, *Lpar5* KO: $n=7$. LPA_2: Bl6: $n=6$, *Lpar5* KO: $n=7$. LPA_3: Bl6: $n=5$, *Lpar5* KO: $n=5$. LPA_4: Bl6: $n=8$, *Lpar5* KO: $n=6$. LPA_5: Bl6: $n=6$, *Lpar5* KO: $n=6$. LPA_6: Bl6: $n=6$, *Lpar5* KO: $n=6$. ATX: Bl6: $n=5$, *Lpar5* KO: $n=6$ (Mann–Whitney test).

biological functions such as brain development,[30] immune modulation,[31] and neuropathic pain sensitization.[32] In the vascular system, LPA_5 is expressed in endothelial cells,[14] smooth muscle cells,[33] and platelets.[34] LPA_5 has also been associated with atherosclerosis progression, as its expression was found to be upregulated in atherosclerotic plaques isolated from human carotid arteries.[35] It has been assumed that LPA_5 along with other LPA receptors is involved in endothelial cell activation,[35] which further supports our hypothesis that LPA_5 is a potential regulator of vascular inflammatory processes.

As our results suggested the involvement of the ATX–LPA–LPA_5 axis in the effect of LPC, we examined the direct effects of LPA on the ACh-induced vasorelaxation. 18:1 LPA evoked a small, but significant reduction in endothelium-dependent vasorelaxation in WT, but it was ineffective in *Lpar5* KO, indicating that exogenous LPA can directly impair endothelial function by activating LPA_5 receptors. As the effect of LPA appeared to be less pronounced, compared to

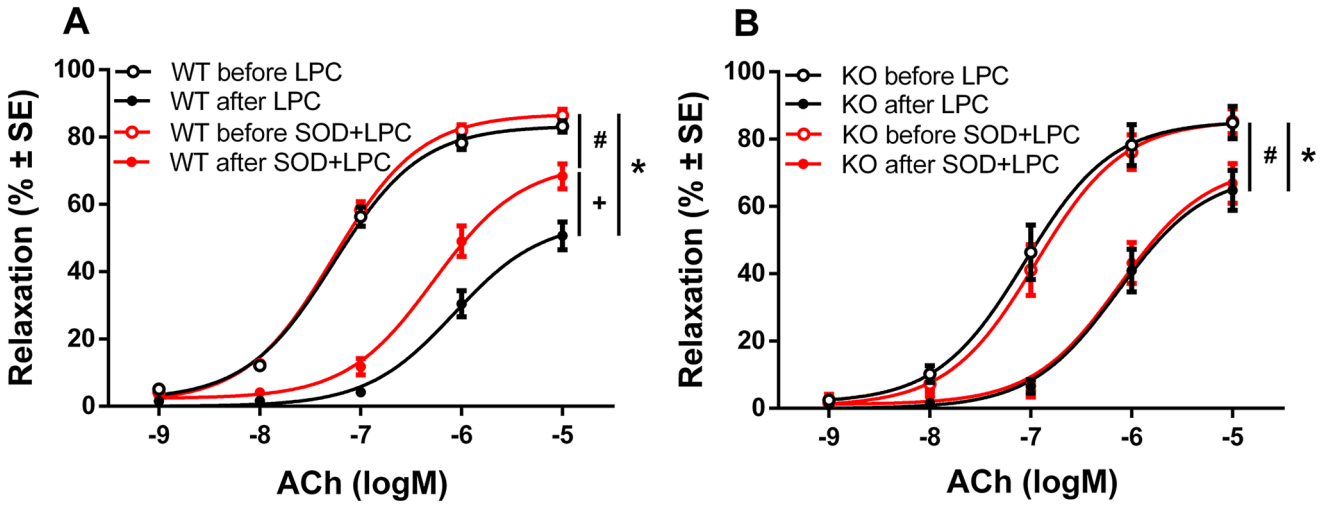

Figure 4. Cumulative concentration–response curves to ACh were performed on aortic rings before and after incubation with 18:1 LPC (10 μM, 20 min) in the presence or absence of SOD (200 U/mL). Vessels were isolated from WT (A) or *Lpar5* KO (B) mice. SOD significantly reduced the LPC-evoked attenuation of vasorelaxation in WT (A), but it was ineffective in *Lpar5* KO (B). Relaxation values represent mean value ± SE percentage of maximal PE-induced contraction. Curves were compared using two-way ANOVA, followed by Tukey's multiple comparisons test. (A): LPC: $n = 29$, SOD + LPC: $n = 30$. (B) LPC: $n = 9$, SOD + LPC: $n = 9$. (A): #$P < 0.0001$ vs "WT before SOD + LPC"; +$P < 0.001$ vs "WT after SOD + LPC"; *$P < 0.0001$ vs "WT before LPC." (B): #$P < 0.01$ vs "KO before SOD + LPC"; *$P < 0.001$ vs "KO before LPC."

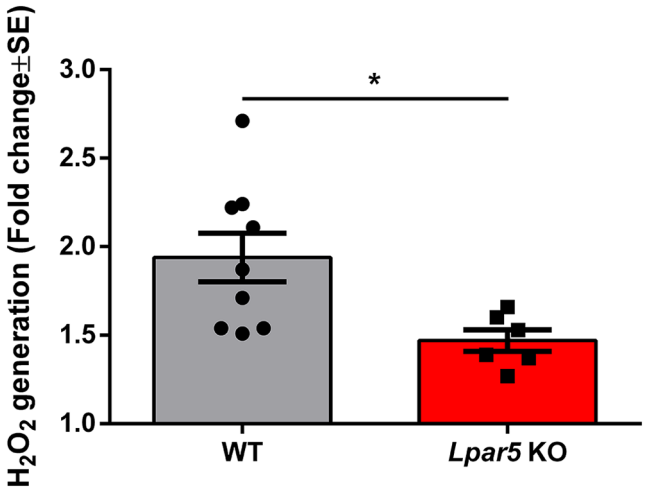

Figure 5. H_2O_2 production of WT (gray bar) and *Lpar5* KO (red bar) mouse aortic rings measured by the Amplex Red assay. Vessels were incubated with working solution containing Amplex Red (50 μM) and HRP (0.2 U/mL) in HBSS at 37°C. Absorbance was measured from supernatant after 15 min. Then, the vessels were incubated with working solution containing 18:1 LPC (10 μM) for 40 min at 37°C, followed by absorbance measurement of supernatant. The LPC-evoked H_2O_2 production was significantly reduced in *Lpar5* KO as compared to WT vessels. Absorbance values were normalized to 1 min. 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).

LPC, we hypothesize that LPA produced *in situ* might be protected by ATX and channeled directly toward the receptors.[36]

We also analyzed the LPA receptor and ATX expression profile of WT and *Lpar5* KO mice. The results showed no significant difference between the two groups in LPA_1, LPA_2, LPA_3, LPA_4, LPA_6, and ATX expression suggesting that the genetic deletion of LPA_5 does not affect the expression of other LPA receptors and ATX.

The deleterious effects of LPC on endothelial cells are well-documented and mostly attributed to the ability of LPC to evoke oxidative stress. Several research groups reported increased ROS production in cultured endothelial cells upon LPC treatment.[8,37] The release of these oxidative agents can contribute to the disruption of the normal endothelial function, leading to decreased endothelium-dependent vasorelaxation.[10] As Rao *et al*.[10] showed earlier, the negative effect of 18:1 LPC on NO-dependent vasorelaxation can be almost entirely abolished by the superoxide-scavenger Tempol. Our results are in agreement with these observations, as SOD enzyme significantly decreased LPC-evoked attenuation of vasorelaxation in WT mice, albeit its protective effect was not complete. One possible explanation for this difference is that while Tempol is a membrane-permeable agent, reacting with both intracellular and extracellular ROS,[38] SOD has poor membrane permeability and acts extracellularly.[39] The involvement of extracellular ROS in this phenomenon was further confirmed by the results we obtained in the Amplex Red assay, a method used for extracellular H_2O_2 detection.[23] In the supernatant of LPC-treated WT vessels, a significant amount of H_2O_2 was detected, indicating that LPC evokes ROS release from vascular cells. Interestingly, in case of *Lpar5* KO vessels, we could not achieve further improvement with SOD or Tempol treatment. In addition, we observed significantly lower ROS release upon LPC stimulation in *Lpar5* KO as compared to WT vessels. These results indicate that LPA_5 receptor activation is involved in the initiation of oxidative stress in mouse aortic tissue.

Whereas our results suggest that a significant part of the deleterious effect of LPC requires its conversion to LPA, it is likely that other, LPA-independent signaling pathways are also involved, as we were unable to prevent the entire LPC effect either with ATX inhibition or the genetic deletion of LPA_5. Previous results suggested that LPC can evoke its effects by directly activating G-protein-coupled receptors (GPCRs) such as G2A and GPR4. Although it is possible that LPC modulates their function, it has been debated whether

LPC is a ligand of these receptors, as direct interaction could not be verified.[7,40,41] Given its amphipathic nature, it is more likely that LPC interacts directly with the cell membrane, changing its biophysical properties leading to an altered membrane function.[42] In line with this hypothesis, it has been speculated that LPC might incorporate into the endothelial cell membrane and interacts with the eNOS enzymes located in caveolae.[8,43] This process may lead to a disrupted eNOS function with decreased NO bioavailability and subsequent endothelial dysfunction.

In conclusion, we have shown that the development of LPC-induced impairment of endothelium-dependent vasorelaxation requires the conversion of LPC to LPA by the ATX enzyme in mouse aortic tissue. This locally formed LPA appears to activate LPA_5 receptor, triggering signaling pathways that lead to an elevated production of ROS and subsequent endothelial dysfunction.

AUTHORS' CONTRIBUTIONS

AJ, AM, MK, MG, GJT, ZB, and ÉR conceived and designed research. JC and SI provided LPA receptor knock-out mice. AJ, AM, MK, and ÉR performed experiments. AJ and AM analyzed data. AJ prepared figures and drafted manuscript. AJ, AM, MK, MG, JC, SI, GJT, ZB, and ÉR edited and revised manuscript. AJ, AM, MK, MG, JC, SI, GJT, ZB, and ÉR approved final version of manuscript.

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DECLARATION OF CONFLICTING INTERESTS

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ORCID IDS

Anna Janovicz https://orcid.org/0000-0003-3383-5576

Gábor József Tigyi https://orcid.org/0000-0001-5371-171X

SUPPLEMENTAL MATERIAL

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