Role of EDG-like lysophospholipid receptors in the regulation of the vascular tone

PhD Thesis

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1. Introduction

The term lysophospholipid includes two lipid families with numerous members: the glycerophospholipids and the sphingolipids. The best-characterized representatives of these two groups are lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) respectively.

The first LPA-related publication is from 1957, when Vogt isolated a mixture of lipid nature, which elicited contractions in the intestine of rabbits. The exact compound and structure remained however undiscovered that time. It was only in 1978, when Akira Tokumura and his group isolated and identified LPA from soybean lecithin, a new lipid mediator that induced hypertension in rats and other species. At the beginning of the 1990s, Tigyi and Miledi identified albumin-bound LPA as an endogenous regulator of cellular functions and proposed that its actions are mediated by plasma membrane receptor(s).

Since then, the number of reports on LPA increased rapidly. However, the molecular target of LPA actions was unclear until 1996, when Chun and colleagues identified the first G protein-coupled receptor (GPCR) of LPA, LPA_1. Since that time, five other LPA receptors have been described and confirmed ($LPA_{2\text{-}6}$). Moreover, an intracellular receptor of the same mediator has also been reported in 2003.

The first studies with S1P date back to the early 1990s as S1P was established a regulator of cell proliferation, growth, and survival. Although the first S1P receptor itself was described already in 1990 by Timothy Hla and his group, it took eight years for $S1P_1$ to be de-orphaned and confirmed as an S1P GPCR in 1998. During the past 20 years, four other S1P receptors were reported ($S1P_{2\text{-}5}$).

Interestingly, three of the six LPA and all S1P GPCRs belong to the endothelial differentiation gene (EDG) family of receptors, sharing several similarities in structure and function as well. Studies conducted with human umbilical vein endothelial cells (HUVECs) showed, that the transcription of these genes increases upon the administration of phorbol-12-myristate-13-acetate, a compound that induces endothelial differentiation, hence the name EDG. Subsequently, it was revealed, that the structure of the EDG encoded protein shows great similarity to the GPCRs.

Following the early reports, the lysophospholipid field developed rapidly. The application of genetically modified animals gave another boost; hence, both LPA and S1P became well-established mediators in several physiological and pathological processes.

Our workgroup has been investigating the vasoactive actions of both mediators since the late 2000s. Interestingly, the literature available on both lipids is contradictory. In case of LPA, we described dual vasoactive effects in isolated murine vessels depending on the presence or absence of the endothelium. On the other hand, S1P, that had a nearly negligible impact on the diameter of vessels applied alone, potentiated the effect of other constrictors.

Our aim was to describe these phenomena and to explore, with the aid of pharmacological and genetic methods, which lysophospholipid receptors and downstream signaling pathways mediate these processes.

2. Aims

Both LPA and S1P play several, physiologically significant roles in the cardiovascular system. As for the regulation of the vascular tone, a large amount of data is available for both mediators. Constrictor and dilator responses have been reported in case of both lysophospholipids. Nonetheless, these results and especially the receptor-dependency of the described effects are unclear and often inconsequent.

Accordingly, in the present study we addressed the following questions:

1. Are there any EDG LPA receptor-mediated vasoactive actions?

2. If yes, are they endothelium-dependent or independent?

3. Which receptors and underlying signaling pathways may be involved?

4. Does the activation of the S1P GPCRs mediate any vasoactive processes?

5. If yes, are they dependent of the endothelium?

6. Which receptors and downstream pathways participate?

3. Materials and Methods

All procedures were carried out according to the guidelines of the Hungarian Law of Animal Protection (28/1998) and were approved by the National Scientific Ethical Committee on Animal Experimentation (PEI/001/2706-13/2014).

3.1. Animals

C57BL/6 and endothelial nitric-oxide synthase (eNOS) knockout (KO) mice were obtained from Charles River Laboratories. C57BL/6 mice are referred to as WT in the text. All transgenic mouse lines were on C57BL/6 genetic background. Mice deficient in LPA_1 or LPA_2 receptors (LPA_1 KO and LPA_2 KO, respectively) were generated as previously described. Cyclooxygenase-1 KO (COX1 KO) mice were from Dr. Ingvar Bjarnason. Thromboxane prostanoid receptor-deficient (TP KO) mice were kindly provided by Dr. Shuh Narumiya. The smooth muscle-specific $G\alpha_{q/11}$ and $G\alpha_{12/13}$ deficient mice ($G\alpha_{q/11}$ KO and $G\alpha_{12/13}$ KO respectively) and their respective controls ($G\alpha_{q/11}$ CTRL and $G\alpha_{12/13}$ CTRL), were generated as described previously. Mice deficient in $S1P_2$- and $S1P_3$ receptors ($S1P_2$ KO, $S1P_3$ KO) and their controls were kindly provided by Dr. Richard L. Proia. In experiments performed with LPA_1 KO, LPA_2 KO or COX1 KO mice, wild-type animals from the same strain served as controls and are referred to as LPA_1 CTRL, LPA_2 CTRL and COX1 CTRL, respectively. Because the TP mice have been maintained in our animal facility with KO x KO mating, WT C57BL/6 mice served as controls (TP CTRL). Pertussis toxin (PTX) was administered intraperitoneally in some of the animals for 5 days prior

to the experiments in a dose of 50 μg/kg body weight in order to inhibit G_i proteins.

3.2. Preparation of Vessels

Adult male animals were perfused transcardially with 10 mL heparinized (10 IU/mL) Krebs solution under deep ether anesthesia as described previously. The aorta was removed and cleaned of fat and connective tissue under a dissection microscope and immersed in a Krebs solution of the following composition (mM): 119 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 2.5 $CaCl_2 \cdot 2\ H_2O$, 1.2 $MgSO_4 \cdot 7\ H_2O$, 20 $NaHCO_3$, 0.03 EDTA, and 10 glucose at room temperature and pH 7.4. Abdominal and thoracic aortae were cut into ~3 mm-long segments and mounted on stainless steel vessel holders (200 μm in diameter) in a myograph. In certain experiments special care was taken to preserve the endothelium of the segments, in all other cases the endothelium was removed intentionally by gently rotating the segments on the holder pins and mechanical ablation with surgical thread. Integrity or absence of the endothelium was confirmed by the presence or lack of acetylcholine-induced vasorelaxation respectively. Thoracic aortae were also cut into segments and subjected, with the endothelium preserved, to thromboxane B_2 ELISA as described below in detail.

3.3. Myography

Chambers of the myographs were filled with 6 mL gassed (95% O_2–5% CO_2) Krebs solution. The vessels were allowed a 30-min resting period, during which the bath solution was warmed up to 37 ^oC and the passive tension was adjusted to 10 mN in case of abdominal and to 15 mN in case of thoracic segments, which was

determined to be optional in a previous study. Subsequently, the tissues were exposed to 124 mM K^+ Krebs solution (made by isoosmolar replacement of Na^+ by K^+) for 1 min, followed by several washes with normal Krebs solution. A contraction evoked by 10 μM phenylephrine (PE) followed by administration of 0.1 μM acetylcholine served as a test of the reactivity of the smooth muscle and the endothelium, respectively. After repeated washing, during which the vascular tension returned to the resting level, the segments were exposed to 124 mM K^+ Krebs solution for 3 min in order to elicit a reference contraction. Subsequently after a 30-min resting period, increasing concentrations of PE (0.1 nM to 10 μM) and acetylcholine (1 nM to 10 μM) were administered to determine the reactivity of the vessel and to verify the integrity or the proper denudation of the endothelium. We proceeded with a 30-min resting period. Thereafter, we followed three distinct protocols, depending on the aim and setup of the given experiment.

3.3.1. Protocol for testing vasoactive effects in precontracted vessels

Thoracic vessels were precontracted to 70–90% of the reference contraction by an appropriate concentration of PE, and after reaching a stabile plateau, the effect of either the LPA_{1-3} agonist VPC31143 in a concentration of 10 μM or that of S1P in 5 μM was determined in vessels of different genetic background. Vasoconstrictions were normalized to the reference contraction induced by 124 mM K^+, whereas vasorelaxations were expressed as percentage of the precontraction produced by PE

3.3.2. Protocol for testing vasoactive effects on resting tone

In this type of protocols, the vessels were exposed to either 10 μM VPC31143 or different concentrations of the LPA_3 agonist T13 or 5 μM S1P at the resting tone. In some experiments, the $LPA_{1/3}$ receptor antagonist Ki16425 or the selective LPA_3 antagonist diacylglycerol pyrophosphate (DGPP) was applied to the bath chambers at a concentration of 10 μM, 30 min prior to the administration of VPC31143. Vasoconstrictions are expressed as percentage of the reference contraction induced by 124 mM K^+.

3.3.3. Protocol for testing the long-term vasoactive effects of S1P

In these experiments, we investigated the potentiating effect of S1P on an α_1 agonist-induced contraction in thoracic vessels. Vasoconstrictions were elicited in every 20 minutes by repeated administration of PE. Mean of the first three contractions served as reference and was considered as 100%. After the third administration of PE, we incubated the vessel with either S1P in a concentration of 5 μM, or its vehicle 0.3 N sodium hydroxide (NaOH). Subsequently, PE was applied every 20 minutes for three hours after the incubation. Vasoconstrictions are expressed as percentage of the mean of the three contractions, evoked before the incubation.

3.4. Quantification of Vascular Thromboxane A_2 Release

Thoracic aortae were cut into 5 segments and allowed a 2-h resting period. In some of the experiments, 3 μg/mL PTX was applied for 2 h in order to inhibit G_i. Thereafter, the vessels were incubated in 200 μL Krebs solution at 37°C for 2 min to obtain a baseline level of TXA_2 release. After the incubation, the supernatant was replaced with 200 μL of Krebs solution containing 10 μM VPC31143 and incubated for 2 min. Supernatants of the resting and the VPC31143-stimulated vessels were snap-frozen and stored at -80°C until the measurement of thromboxane levels. Concentrations of thromboxane B_2 (TXB_2), a non-enzymatically produced stable metabolite of TXA_2, were determined using a TXB_2 EIA kit, purchased from Cayman Chemical Co. TXB_2 production was calculated as pg/min. Vessels with a baseline production of TXB_2 higher than 20 pg/min were considered pre-activated and were excluded from the experiment.

3.5. Expression Analysis of LPA and S1P Receptors in VSM

Endothelium-denuded thoracic and abdominal aortae were isolated, and the adventitia of the vessels was carefully removed under a dissection microscope. Thereafter the vessels were fast-frozen and stored at -80°C until PCR analysis. RNA was isolated from VSM with the RNeasy Micro kit from Qiagen, and RNA concentration and quality were assessed with Nanodrop from Thermo Fischer Scientific. Up to 500 ng total RNA was converted to cDNA using a SuperScript® VILO™ cDNA Synthesis Kit from Invitrogen.

Assessment of mRNA expression was performed by quantitative real-time PCR using cDNA corresponding to 20 ng RNA template. PCR reactions were carried out in triplicate with 300 nmol of each primer in a final volume of 25 μL of 2 x Maxima SYBR Green/ROX qPCR master mix from Thermo Fischer Scientific. Amplification was performed after one initial step of 10 min at 95°C for 40 cycles at 94°C /15 s and 60°C /60 s with a StepOnePlus real-time PCR system from Applied Biosystems. Relative gene expression of each mRNA to GAPDH was determined using the dCt method.

3.6. Reagents

LPA (18:1) and VPC31143 were purchased from Avanti Polar Lipids and dissolved in saline immediately before administration. DGPP was purchased from Avanti Polar Lipids and dissolved in methanol. Ki16425 was purchased from Cayman and dissolved in DMSO to make a 100-fold concentrated stock solution. In these experiments, vehicle treatment served as control. PTX was purchased from List Biological Laboratories, Inc. and dissolved in glycerol. T13 was synthesized as described previously and was dissolved in PBS containing 0.1 % fatty acid free bovine serum albumin. Sphingosine 1-phosphate was purchased from Cayman Chemical Company and dissolved in 0.3 N NaOH before administration. All other drugs and chemicals used in the present study were purchased from Sigma-Aldrich. In myography experiments, all concentrations are expressed as the final concentration in the organ bath.

3.7. Data Analysis

An MP100 system and AcqKnowledge 3.72 software from Biopac System Inc. were used to record and analyze changes in the vascular tone. All data are presented as mean ± SE, and *n* indicates either the number of vessels tested in myography experiments or the number of animals tested in the case of TXB_2 EIA or qPCR. Statistical analysis was performed using the GraphPad Prism software v.6.07 from GraphPad Software Inc. Student's unpaired *t* test was applied when comparing two variables, whereas all other comparisons between the different experimental groups were made by ANOVA followed by either Tukey's or Bonferroni's post hoc test. A *p* value of less than 0.05 was considered statistically significant.

4. Results

4.1. Activation of EDG-like LPA receptors induce endothelium-dependent and -independent changes of the vascular tension

In order to evaluate the potential vascular effects of LPA EDG-like GPCRs, the LPA_{1-3} agonist VPC31143 was administered to WT thoracic aortic segments after PE-induced precontraction. VPC31143 elicited a marked vasorelaxation. The agonist-induced vasorelaxation has an EC_{50} of 15 nM and an E_{max} of 51.9% expressed as percentage of the precontraction.

In order to analyze the mechanism of VPC31143-evoked relaxation the possible role of endothelium-derived relaxing agents was assessed first. To do so, we tested WT vessels, mechanically denuded of endothelium, in which the VPC31143-elicited relaxation was not only abolished but was converted to vasoconstriction indicating a major role of endothelium-derived vasoactive mediators in the vasoactive effect of VPC31143. In order to identify the mediator(s) involved, vessels of either COX1 KO or eNOS KO mice were tested. Whereas the absence of COX1 did not alter the dilator effect of VPC31143, the lack of eNOS, similarly to that of the endothelium, prevented the VPC31143-elicited relaxation and turned it to contraction. Taken together, these results indicate that the vasorelaxant effect of EDG-like LPA receptor activation is mediated by endothelial NO whereas COX1-derived prostanoid mediators are not involved.

In the next phase of our study, we sought an in-depth analysis of the VPC31143-evoked vasoconstriction. In

order to analyze the potential regional differences between the different parts of the aorta the agonist was applied to WT thoracic and abdominal aortic segments (TA and AA, respectively) at resting tension. In vessels with intact endothelium VPC31143 in a dose of 10 μM elicited moderate vasoconstriction compared to the reference contraction induced by 124 mM K^+.
However, removal of the endothelium unmasked the constrictor effect of VPC31143 resulting in an approximately three-fold increase in the AA. The vasoconstriction has an EC_{50} of 4.1 μM and an E_{max} of 87.4% as compared to the reference contraction.

4.2. Identification of the LPA receptor(s) mediating VPC31143-induced vasoconstriction

Since removal of the endothelium augmented the vasoconstrictor effect of VPC31143, our attention turned to LPA GPCRs expressed in the vascular smooth muscle (VSM). First, the gene expression profile of LPA receptors was determined in isolated tunica media of the murine TA and AA. LPA_1, LPA_2, LPA_4, and LPA_6 mRNA were most abundantly detectable, with a slightly higher expression of LPA_4 and LPA_6 in the AA as compared to the TA. LPA_3 transcripts had the lowest abundance, nonetheless they found to be expressed in a higher amount in TA than AA. Taken together, these results identified LPA_1 and LPA_2 as likely candidates for mediating EDG-like LPA receptor related vasoconstriction.
Thereafter, we implemented pharmacological and genetic methodologies to determine the LPA receptor responsible for the vasoconstrictor effect. Whereas the $LPA_{1/3}$ antagonist Ki16425 revoked the 10μM VPC31143-elicited vasoconstriction, the selective LPA_3 antagonist

DGPP failed to influence it as compared to vehicle treatment in the AA of WT mice. Aortic rings isolated from LPA_1 KO mice failed to contract upon VPC31143 application, while the vessels of LPA_2 KO animals showed similar responses to those of WT, indicating that the vasoconstriction is mediated by LPA_1.
To obtain a more detailed evaluation of the possible involvement of LPA_3 in the vasoconstrictor response, the vasoactive effects of T13 were investigated, which had been implied to activate LPA_3 selectively and with high efficiency at 10 nM, however at higher concentrations, it stimulates other LPA GPCRs as well. T13, applied in 10 nM concentration, failed to induce vasoconstriction in AA segments of LPA_1 CTRL animals, however at higher concentrations a dose-dependent contractile response developed. This effect was absent in vessels isolated from LPA_1 KO animals, which is consistent with our hypothesis, that the vasoconstrictor response is solely mediated by LPA_1.

4.3. Identification of the signal transduction pathways of LPA_1-mediated vasoconstriction

LPA signaling has been implicated to interact with the prostanoid system. Previously reported COX1-mediated effects of LPA and the fact that LPA-evoked contractions of the longitudinal smooth muscle layer of guinea-pig ileum were shown to be indomethacin-sensitive, raised our hypothesis, that TXA_2, a potent vasoconstrictor, might have a role in the contractile effect mediated by LPA_1. In order to investigate this possibility, vessels of WT, COX1 KO and TP KO mice were exposed to 10 μM VPC31143. The absence COX1 and TP markedly decreased the agonist-induced contractions, implying that COX1-derived TXA_2 could be the mediator, that

activates TP, thus elicits (or at least contributes to) vasoconstriction.

To verify the presence of the constrictor agent TXA_2, levels of TXB_2, a metabolite of TXA_2 with a longer life span, were measured from the supernatants of vessels exposed to VPC31143 for 2 min. VPC31143 treatment enhanced the TXB_2 production of WT specimens more than two-fold, which was also the situation in case of LPA_2 KO aortae. However, the agonist failed to alter the amount of TXB_2, released from LPA_1 KO vessels.

Aortae of COX1 KO mice showed a diminished basal rate of TXB_2 production, which remained unaltered upon VPC31143 application. Nonetheless, absence of the TP receptors had no such effect on either resting or stimulated release of TXB_2. The above mentioned results are consistent with our hypothesis of LPA_1-mediated COX1 activation and TXA_2 production, leading to TP activation and consequent contraction of VSM.

As LPA_1 is often coupled to $G\alpha_{i/o}$, and $G\alpha_{i/o}$ has been reported to activate PLA_2 and TXA_2 production, we sought evidence of its possible role in LPA_1-mediated vasoconstriction. In consistence with our hypothesis, PTX pretreatment of WT vessels abolished the VPC31143-induced increase in TXB_2 production. Moreover, aortic segments of PTX-pretreated WT animals showed diminished vasoconstriction upon VPC31143 administration.

We also investigated, if the well-established regulators of smooth muscle contraction, $G\alpha_{q/11}$, and $G\alpha_{12/13}$, could play a role in the VPC31143-induced vasoconstriction. The smooth muscle-specific absence of $G\alpha_{q/11}$ as well as that of $G\alpha_{12/13}$ decreased the contraction, evoked by 10 μM VPC31143, however both failed to completely abolish it.

4.4. Vasoactive effects of S1P depend on the presence of other constrictors

In the next part of our experiments, we focused on the vasoactive effects of the other thoroughly investigated lipid mediator, S1P. S1P, administered on the basal tone, had negligible effect in either thoracic or abdominal aortic segments of WT animals. Removal of the endothelium did not influence this minor effect significantly. Nonetheless, after PE-induced precontraction of endothelium-denuded WT vessels S1P elicited a marked vasoconstriction. Although, S1P applied alone failed to significantly influence the basal tone, in presence of an other constrictor, elicited vasoconstriction. Considering the fact, that under physiological conditions, several mediators, (e.g. constrictors, dilators) are present in the circulation and actively regulate the tension of the vessels, the above-mentioned effect could play major roles in cardiovascular regulation in vivo.

In recognition of the imperceptible effect of S1P, applied solely, we investigated if this mediator influences the contractile effect of other constrictors. For this purpose, vasoconstrictions elicited by the α_1 adrenergic receptor agonist PE at 100 nM in WT endothelium-intact TAs were investigated and recorded before and after the administration of S1P.

Incubation with 5 μM S1P for 20 min, increased PE-mediated contractions almost 1.5-fold after 40 min and surprisingly the contractions remained enhanced even 180 min after the incubation. In contrast, 0.3 N NaOH, the vehicle of S1P, had no effect on PE-elicited vasoconstrictions.

4.5. Identification of the S1P receptors, mediating the potentiating effect of S1P

Thereafter, we aimed to identify the S1P receptor responsible for the S1P-mediated augmentation of PE-induced contractions. For this purpose, we first determined the expression profile of S1P GPCRs in VSM of the murine thoracic aorta. In WT specimens, mRNAs of $S1P_1$, $S1P_2$, $S1P_3$, and $S1P_4$ were detectable, with $S1P_1$ as the most abundant S1P GPCR.

In the next phase, we applied a genetic approach, thus we tested vessels prepared from $S1P_2$ KO and $S1P_3$ KO mice two of the GPCRs which were detectable in the isolated tunica media of murine thoracic aorta. In segments of $S1P_3$ KO animals, the S1P-induced potentiation was not different from that observed in WT vessels. Nevertheless, aortae of $S1P_2$ KO mice failed to develop the above mentioned phenomenon after 20 min incubation with S1P, highlighting the role of this receptor in S1P-induced potentiation of PE-mediated contractions.

5. Conclusions

In our experiments we sought to answer the question, how the EDG-like lysophospholipid receptors influence the vascular tone. According to our results, we can conclude that:

- Activation of EDG-like lysophosphatidic acid (LPA) receptors elicits dose-dependent vasorelaxation in the intact murine aorta, which is an endothelium dependent process and independent of prostanoids.

- In the absence of endothelium, the EDG-like LPA agonist VPC31143 evoked dose-dependent vasoconstriction, which was more pronounced in the abdominal than in the thoracic aorta. This effect is mediated by LPA_1, which is relatively highly expressed in murine vascular smooth muscle cells. VPC31143 elicits TXA_2 release in a G_i- and COX1-dependent manner, which, acting on its receptor (TP), causes vasoconstriction. The G proteins $G\alpha_{q/11}$ and $G\alpha_{12/13}$ are also involved in the signal transduction, probably downstream of the TP receptor.

- Exposition to sphingosine 1-phosphate (S1P) potentiates α_1-dependent vasoconstriction in murine aorta, in a long-lasting manner. This impact is mediated by $S1P_2$ receptors, which are expressed in murine vascular smooth muscle.

6. Bibliography of the candidate's publications

6.1. Publication directly related to this thesis

Ruisanchez, E., **Dancs, P.**, Kerek, M., Nemeth, T., Farago, B., Balogh, A., Patil, R., Jennings, B. L., Liliom, K., Malik, K. U., Smrcka, A. V., Tigyi, G., and Benyo, Z.: Lysophosphatidic acid induces vasodilation mediated by LPA1 receptors, phospholipase C, and endothelial nitric oxide synthase. FASEB J. 2014 Feb; 28(2): 880-90 **(IF: 5.043)**

Dancs, P. T., Ruisanchez, E., Balogh, A., Panta, C. R., Miklos, Z., Nusing, R. M., Aoki, J., Chun, J., Offermanns, S., Tigyi, G., and Benyo, Z.: LPA1 receptor-mediated thromboxane A2 release is responsible for lysophosphatidic acid-induced vascular smooth muscle contraction. FASEB J. 2017 Apr; 31(4): 1547-1555 **(IF: 5.595)**

6.2. Publications not related to this thesis

Iring, A., Jin, Y.-J., Albarrán-Juárez, J., Siragusa, M., Wang, S., **Dancs, P. T.**, Nakayama, A., Tonack, S., Chen, M., Künne, C., Sokol, A. M., Günther, S., Martínez, A., Fleming, I., Wettschurek, N., Graumann, J., Weinstein, L. S., and Offermanns, S.: Shear stress-induced endothelial adrenomedullin signaling regulates vascular tone and blood pressure. J Clin Invest. 2019 Jun; 129(7): 2775-2791 **(IF: 12.282)**