

LPA₁ receptor-mediated thromboxane A₂ release is responsible for lysophosphatidic acid-induced vascular smooth muscle contraction

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ABSTRACT: Lysophosphatidic acid (LPA) has been recognized recently as an endothelium-dependent vasodilator, but several lines of evidence indicate that it may also stimulate vascular smooth muscle cells (VSMCs), thereby contributing to vasoregulation and remodeling. In the present study, mRNA expression of all 6 LPA receptor genes was detected in murine aortic VSMCs, with the highest levels of LPA₁, LPA₂, LPA₄, and LPA₆. In endothelium-denuded thoracic aorta (TA) and abdominal aorta (AA) segments, 1-oleoyl-LPA and the LPA₁₋₃ agonist VPC31143 induced dose-dependent vasoconstriction. VPC31143-induced AA contraction was sensitive to pertussis toxin (PTX), the LPA_{1&3} antagonist Ki16425, and genetic deletion of LPA₁ but not that of LPA₂ or inhibition of LPA₃ by diacylglycerol pyrophosphate. Surprisingly, vasoconstriction was also diminished in vessels lacking cyclooxygenase-1 [COX1 knockout (KO)] or the thromboxane prostanoid (TP) receptor (TP KO). VPC31143 increased thromboxane A₂ (TXA₂) release from TA of wild-type, TP KO, and LPA₂ KO mice but not from LPA₁ KO or COX1 KO mice, and PTX blocked this effect. Our findings indicate that LPA causes vasoconstriction in VSMCs, mediated by LPA₁, G_i, and COX1-dependent autocrine/paracrine TXA₂ release and consequent TP activation. We propose that this new-found interaction between the LPA/LPA₁ and TXA₂/TP pathways plays significant roles in vasoregulation, hemostasis, thrombosis, and vascular remodeling.—Dancs, P. T., Ruisanchez, E., Balogh, A., Panta, C. R., Miklós, Z., Nüsing, R. M., Aoki, J., Chun, J., Offermanns, S., Tigyi, G., Benyó, Z. LPA₁ receptor-mediated thromboxane A₂ release is responsible for lysophosphatidic acid-induced vascular smooth muscle contraction. *FASEB J.* 31, 000–000 (2017). www.fasebj.org

KEY WORDS: LPA • vasoconstriction • endothelial dysfunction • platelet activation • TP receptor

Lysophosphatidic acid (LPA) is a multifunctional lipid mediator/second messenger with numerous biological actions in mammalian physiology and pathology. It has diverse roles in embryonic development and in the regulation of the immune, reproductive, nervous, and cardiovascular systems as well as tumor biology and

fibrosis (1–4). LPA exerts most of its actions *via* 6 different subtypes of G protein-coupled receptors (GPCRs). The first GPCR cluster specific to LPA (LPA_{1/2/3}) belongs to the endothelial differentiation gene family (5), which includes the plasma membrane receptors of the other lipid mediator, sphingosine-1-phosphate (6). Subsequently, 3 additional nonendothelial differentiation gene LPA receptors, designated LPA_{4/5/6}, that share similarities with the P2Y (purinergic receptor) gene cluster have been discovered (7). In addition, intracellular LPA activates the peroxisome proliferator-activating receptor γ (PPAR γ) (8). LPA is primarily generated by autotaxin (ATX), an enzyme with lysophospholipase D activity, which cleaves the headgroup of lysophospholipids to generate LPA (9). Subsequent studies revealed possible topological and structural connections between ATX and LPA GPCRs (10, 11).

The first biological effect of LPA described *in vivo* was the regulation of blood pressure (12, 13). Due to these observations, LPA rapidly emerged as a potential

ABBREVIATIONS: AA, abdominal aorta; ATX, autotaxin; COX, cyclooxygenase; CTRL, control; DGPP, diacylglycerol pyrophosphate; GPCR, G protein-coupled receptor; KO, knockout; LPA, lysophosphatidic acid; PPAR γ , peroxisome proliferator-activated receptor γ ; PTX, pertussis toxin; TA, thoracic aorta; TP, thromboxane prostanoid; TXA₂, thromboxane A₂; VSMC, vascular smooth muscle cell; WT, wild-type

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mediator in the cardiovascular system. Early studies conducted by Tokumura *et al.* (13) in rats and guinea pigs revealed a vasoconstrictive and hypertensive response after exposure to different molecular species of LPA. At the same time, the same molecular species elicited a hypotensive response in rabbits and cats. However, the hypotensive effect in cats later proved to be the result of decreased cardiac output induced by pulmonary vasoconstriction produced by platelet aggregation (14). LPA applied to the adventitial surface of pial arteries of newborn piglets in a closed cranial window model caused a dose-dependent vasoconstrictive response (15). Although at that time LPA receptors had not yet been identified, the sensitivity of the pial artery constriction to pertussis toxin indicated the involvement of G_i proteins in mediation of the effect. LPA has also been shown recently to induce endothelium- and shear stress-dependent vasoconstriction when applied to mouse aortic strips (16). In addition, LPA has been implicated in vascular pathologies, such as the development and complications of atherosclerosis (17–20).

We have demonstrated recently that LPA potently elicited vasodilation in isolated aortae mediated by LPA_1 , PLC, and activation of eNOS (21). In the absence of endothelium or eNOS, however, LPA caused vasoconstriction, indicating that it also exerts a direct vasoactive action on vascular smooth muscle cells (VSMCs). The objective of the present study was to characterize the vasoconstrictive effect and the underlying signal transduction mechanisms activated by LPA in murine aortic rings denuded of endothelium. Our results using pharmacological tools and gene knockout (KO) animal models indicate that LPA_1 activation in a cyclooxygenase-1 (COX1)-dependent and pertussis toxin-sensitive manner elicits thromboxane A_2 (TXA₂) production, which in turn causes thromboxane prostanoid (TP) receptor-mediated vasoconstriction.

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).

Animals

C57BL/6 mice were obtained from Charles River Laboratories (Isaszeg, Hungary) and are referred to as wild-type (WT) mice in the text and figures. All transgenic mouse lines were backcrossed to the 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 (22–25). The LPA_1 KO and LPA_2 KO strains have been maintained with constant backcrossing to C57BL/6 since 2008. COX1 KO mice were from Dr. Ingvar Bjarnason (Guy's, King's College, and St. Thomas' School of Medical Education, London, United Kingdom) and were backcrossed to C57BL/6 more than 10 times. TP receptor-deficient (TP KO) mice were kindly provided by Dr. Shuh Narumiya (Kyoto University, Kyoto, Japan) and were backcrossed to C57BL/6 more than 10 times. In experiments performed with

LPA_1 KO, LPA_2 KO, or COX1 KO mice, WT 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 had been maintained in our animal facility with KO $\times$ KO matings, WT C57BL/6 mice served as controls (TP CTRL). Pertussis toxin (PTX) was administered intraperitoneally in some of the animals for 5 d prior to the experiments at a dosage of 50 μ g/kg body weight to inhibit G_i proteins (26, 27).

Preparation of vessels

Adult male animals were perfused transcardially with 10 ml heparinized (10 IU/ml) Krebs solution under deep ether anesthesia as previously described (28). The aorta was removed and cleaned of fat and connective tissue under a dissection microscope (M3Z; Wild Heerbrugg AG, Gais, Switzerland) 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 aortae were cut into ~3-mm-long segments and mounted on stainless steel vessel holders (200 μ m in diameter) in a myograph (610 M multiwire myograph system; Danish Myo Technology A/S, Aarhus, Denmark). The endothelium of the segments was removed intentionally by gently rotating the segments on the holder pins. Absence of the endothelium was confirmed by the lack of acetylcholine-induced vasorelaxation of the denuded vessels. Thoracic aortae were also cut into segments and subjected, with the endothelium preserved, to TXB₂ ELISA as described below in detail.

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 to 37°C and the passive tension was adjusted to 10 mN, which was determined to be optimal in a previous study (28). 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 μ M phenylephrine followed by administration of 0.1 μ M 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 to elicit a reference maximal contraction. After a 30-min washout, increasing concentrations of phenylephrine (0.1 nM to 10 μ M) and acetylcholine (1 nM to 10 μ M) were administered to determine the reactivity of the vessel and to verify the proper denudation of the endothelium. After a 30-min resting period, the vessels at the resting tone were exposed to different concentrations of either LPA, the LPA_{1-3} agonist VPC31143 (29), or the LPA_3 agonist T13 (30). In some experiments, the $LPA_{1\&3}$ receptor antagonist Ki16425 (31) or the selective LPA_3 antagonist diacylglycerol pyrophosphate (DGPP) (32) was applied to the bath chambers at a concentration of 10 μ M, 30 min prior to the administration of VPC31143. Vasoconstriction is expressed as percentage of the reference contraction induced by 124 mM K^+ .

Quantification of vascular TXA₂ release

Thoracic aortae were cut into 5 segments and allowed a 2-h resting period. In some of the experiments, 3 μ g/ml PTX was applied for 2 h to inhibit G_i (33). Thereafter, the vessels were incubated in 200 μ l Krebs solution at 37°C for 2 min to obtain a

baseline level of TXA₂ release. After the incubation, the supernatant was replaced with 200 μ l of Krebs solution containing 10 μ M 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 TXB₂, a nonenzymatically produced stable metabolite of TXA₂, were determined using a TXB₂ enzyme immunoassay kit (501020; Cayman Chemical Co., Ann Arbor, MI, USA). TXB₂ production was calculated as pg/min. Vessels with a baseline production of TXB₂ >20 pg/min were considered preactivated and were excluded from the experiment.

Expression analysis of LPA receptors in the vascular smooth muscle

Endothelium-denuded thoracic and abdominal aortae were isolated, and the adventitia of the vessels was carefully removed under a dissection microscope. The vessels were fast-frozen and stored at -80°C until PCR analysis. RNA was isolated from vascular smooth muscle with an RNeasy Micro kit (74004; Qiagen, Valencia, CA, USA), and RNA concentration and quality were assessed with Nanodrop (Thermo Fischer Scientific, Waltham, MA, USA). Up to 500 ng total RNA was converted to cDNA using a SuperScript VILO cDNA Synthesis Kit (11754-050; Thermo Fisher Scientific).

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 μ l of 2 $\times$ Maxima SYBR Green/ROX quantitative PCR master mix (K0223; Thermo Fischer Scientific). Amplification was performed after an initial step of 10 min at 95°C for 40 cycles at $94^{\circ}\text{C}/15$ s and $60^{\circ}\text{C}/60$ s with a StepOnePlus real-time PCR system (Thermo Fisher Scientific). Relative gene expression of each mRNA to glyceraldehyde 3-phosphate dehydrogenase was determined using the dC_T method. The primer sequences are listed in Table 1.

Reagents

LPA (18:1) and VPC31143 were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and dissolved in saline immediately before administration. DGPP was purchased from Avanti Polar Lipids and dissolved in methanol. Ki16425 was purchased from Cayman Chemical Co. 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 Biologic Laboratories, Inc. (Campbell, CA, USA) and dissolved in glycerol. T13 was synthesized as previously described (30) and was dissolved in PBS containing 0.1% fatty acid free bovine serum albumin. All

other drugs and chemicals used in the present study were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Data analysis

An MP100 system and AcqKnowledge 3.72 software from Biopac System, Inc. (Goleta, CA, USA) were used to record and analyze changes in the vascular tone. All data are presented as means $\pm$ SE, and *n* indicates either the number of vessels tested in myography experiments or the number of animals tested in the case of TXB₂ enzyme immunoassay or quantitative PCR. The number in parentheses after *n* indicates the number of animals the vessel segments were obtained from in the myography experiments. Statistical analysis was performed using GraphPad Prism software (v.6.07; GraphPad Software Inc., La Jolla, CA, USA). Student's unpaired *t* test was applied when comparing 2 variables. All other comparisons between the different experimental groups were made by ANOVA followed by Tukey's or Bonferroni's *post hoc* test. A value of *P* < 0.05 was considered to be statistically significant.

RESULTS

We have previously shown that LPA administration to murine aortic rings with intact endothelium caused relaxation, which disappeared when the endothelium was removed or when eNOS was genetically deleted (21). In our present experiments, LPA elicited a marked vasoconstriction when applied to endothelium-denuded vessels at resting tension (Fig. 1A). In intact vessels, VPC31143 reproduced the vasorelaxant effect of LPA (21). In endothelium-denuded vessels, VPC31143, like LPA, elicited vasoconstriction in segments of the thoracic aorta (TA) and abdominal aorta (AA) (Fig. 1A). Rings exposed to VPC31143 from the AA showed 2-fold higher contraction compared with those from the TA (Fig. 1B). VPC31143 stimulation in the AA evoked higher contraction compared with equimolar LPA (Supplemental Fig. 1), which was similar to that seen in intact vessels for the vasorelaxation response (21). Thus, VPC31143 showed a higher efficacy over the natural ligand LPA in inducing constriction or relaxation, which is consistent with findings obtained in LPA₁-expressing HEK cells (29).

Murine aortic endothelial cells predominantly express LPA₁ and LPA₄ transcripts. Here we determined the gene expression profile in isolated murine TA and AA. LPA₁, LPA₂, LPA₄, and LPA₆ transcripts were abundantly detectable in both types of segments, with a slightly higher

TABLE 1. Primers used for real-time quantitative PCR

Gene	Primer, 5'-3'	
	Forward	Reverse
<i>GAPDH</i>	CTGCACCACCAACTGCTTAG	GGGCCATCCACAGTCTTCT
<i>LPA₁</i>	CACCATGATGAGCCTTCTGA	GCAGCACACATCCAGCAATA
<i>LPA₂</i>	CCAGCCTGCTTGCTTCTCTA	GTGTCCAGCACACCACAAAT
<i>LPA₃</i>	AGGGCTCCCATGAAGCTAAT	TGCACGTTACACTGCTTGC
<i>LPA₄</i>	ACAGTGCCTCCCTGTTTGTC	AAATCAGAGAGGGCCAGGT
<i>LPA₅</i>	TCATCATCTTCCCTGCTGTGC	ATCGCGGTCTCTGAATACTGT
<i>LPA₆</i>	TCGCTCATGAGGACACAGAC	CAAAGCAGCAGTTGGAACA

GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

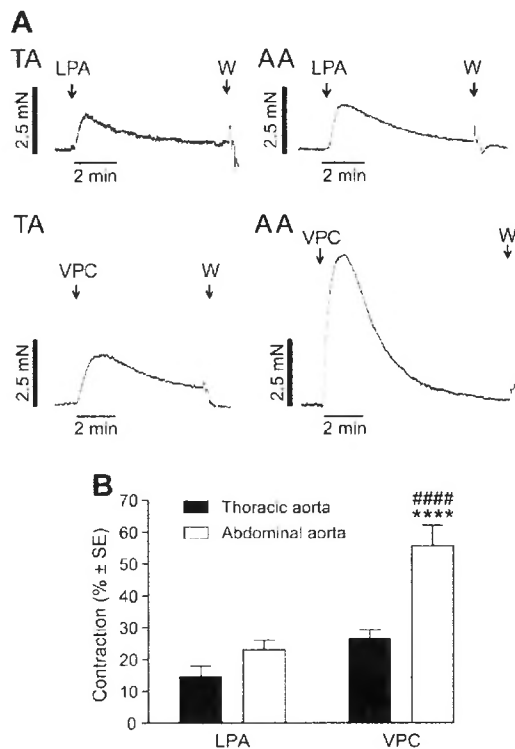


Figure 1. Effects of LPA and VPC31143 on the tone of endothelium-denuded murine TA or AA segments. **A)** Representative recordings of vessels prepared from WT mice. "W" denotes wash-out with Krebs solution. **B)** Abdominal segments showed 2-fold higher contraction compared with thoracic ones when stimulated with VPC31143. VPC31143 stimulation elicited more than twice as forceful contractions in the AA compared with LPA. **** $P < 0.0001$ vs. VPC effect in the thoracic aorta; **** $P < 0.0001$ vs. LPA effect in the abdominal aorta; 2-way ANOVA with Tukey's *post hoc* test ($n = 18$ –32 vessels obtained from 14–32 animals).

abundance of LPA₄ and LPA₆ in the AA specimens (Fig. 2). LPA₃ transcripts were the least abundant; however, they showed higher expression in the TA. To identify the LPA receptor mediating the vasoconstriction response, we applied pharmacological and genetic approaches (Fig. 3). The LPA_{1&3}-selective antagonist Ki16425 abolished contraction evoked by VPC31143 compared with vehicle-treated controls isolated from WT mice (Fig. 3A). Similar to its action in rings isolated from WT mice, VPC31143 elicited vasoconstriction in rings prepared from LPA₂ KO animals. Furthermore, the LPA₃-selective antagonist DGPP failed to alter VPC31143-induced contractions in the AA of WT animals. In contrast, rings prepared from LPA₁ KO mice failed to contract in response to the maximally effective concentration (10 μ M) of VPC31143, indicating that the vasoconstriction is mediated by LPA₁. To evaluate the potential involvement of LPA₃ in mediating vasoconstriction, the vasoactive effects of T13 were determined, which had been shown to induce selective and maximal LPA₃ activation at 10 nM but to also stimulate other LPA receptors at higher concentrations (30). This compound failed to induce contraction of endothelium-denuded AA segments when 10 nM was applied, whereas dose-dependent vasoconstriction developed in response to higher concentrations (Fig. 3B).

However, this vasoconstrictor effect was absent in vessels prepared from LPA₁ KO mice, indicating that LPA₁ is the sole mediator of the vasoactive effect.

Because LPA was reported to induce COX1-mediated effects (34) and LPA-induced contractions of the longitudinal smooth muscle layer of the ileum were shown to be sensitive to indomethacin (35), we hypothesized that TXA₂, a potent activator of VSMC contraction (36), might be involved in LPA₁-mediated vasoconstriction. To test this hypothesis, we isolated aortic rings from WT, COX1 KO, and TP KO mice and exposed them to maximally effective concentrations of VPC31143. Vessels from COX1 KO and TP KO mice showed markedly reduced contractions in response to VPC31143 (Fig. 4A), indicating that COX1-derived TXA₂ could be involved in the downstream activation of TP, which in turn causes contraction.

To gain further insight into this mechanism, we determined the levels of TXB₂ in supernatants collected after exposure of the vessels to VPC31143 for 2 min (Figures 4B and 5). After VPC31143 treatment, a 2-fold increase of TXB₂ production was detected in the supernatant collected from aortae of WT mice, which was absent in the vessels of LPA₁ KO mice but remained unaltered in LPA₂ KO vessels (Fig. 5). Furthermore, in the supernatants of vessels taken from COX1 KO mice, we found a diminished basal rate and a lack of VPC31143-activated elevation of TXB₂ production, whereas deletion of TP receptors failed to alter the resting or stimulated release of TXB₂ (Fig. 4B). These results are in agreement with our hypothesis that LPA₁ elicits vasoconstriction *via* COX1-mediated production of TXA₂, leading to the activation of the TP GPCRs in VSMCs.

LPA- and VPC31143-activated vasorelaxation involves the activation of the canonical PLC–inositol trisphosphate–Ca²⁺ signal transduction pathway in the endothelium (21). Here we hypothesized that the LPA₁-mediated autocrine/paracrine release of TXA₂ and the consequent vasoconstriction may be mediated by G_i, a well-documented regulator of phospholipase A₂ activity and TXA₂ production (37–39). In support of this hypothesis, preincubation of aortic segments with PTX abolished the enhancement of TXB₂ production upon

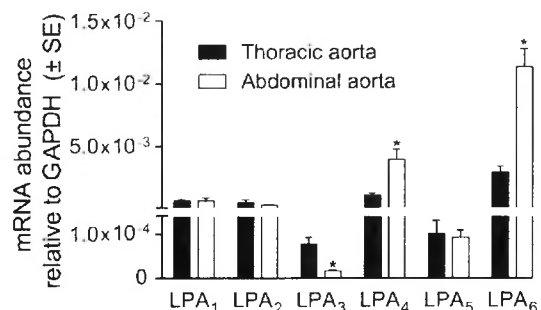


Figure 2. Expression profile of LPA receptors in freshly isolated murine TA and AA VSMCs, determined by quantitative PCR. Murine aortic VSMCs predominantly express LPA₁, LPA₂, LPA₄, and LPA₆. LPA₄ and LPA₆ showed higher abundance in the abdominal, whereas LPA₃, the least abundant subtype, showed higher expression in the thoracic aorta ($n = 3$ –9). * $P < 0.05$ vs. thoracic aorta (unpaired Student's *t* test).

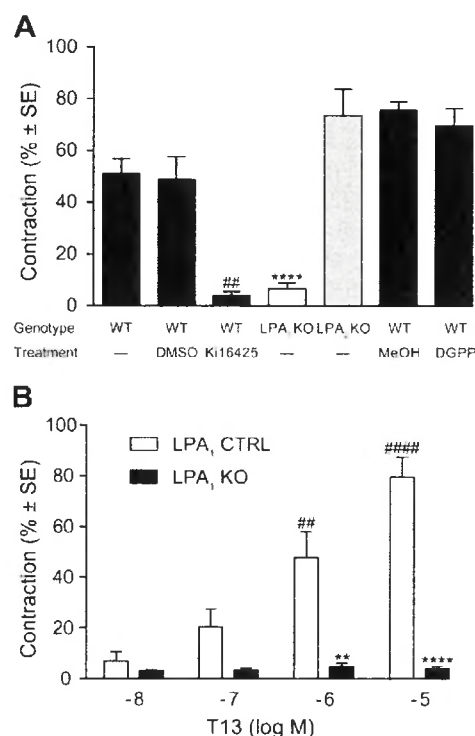


Figure 3. VPC31143-evoked contraction is mediated by LPA₁ receptors. *A*) The LPA_{1&3} antagonist Ki16425 and the lack of LPA₁, but not that of LPA₂ receptors nor the selective LPA₃ antagonist DGPP, abolish VPC31143-elicited vasoconstriction. Vessels of C57BL/6, LPA₁ CTRL, and LPA₂ CTRL mice showed identical responses and are therefore pooled and referred to as WT in the figure. Both Ki16425 and DGPP were applied at 10 μ M for 30 min before the administration of VPC31143. Vehicle-treated control vessels were exposed to 1% DMSO or methanol (MeOH). **** P < 0.0001 *vs.* WT; ## P < 0.01 *vs.* WT with DMSO treatment; 1-way ANOVA with Tukey's *post hoc* test (n = 5–23 vessels obtained from 4–13 animals). *B*) T13 at 10 nM concentration, where it selectively activates LPA₃, failed to elicit vasoconstriction in control vessels. However, the administration of higher concentrations of T13, which are known to stimulate LPA₁, resulted in dose-dependent contraction that was completely missing in LPA₁ KO vessels. ** P < 0.01 *vs.* LPA₁ CTRL, **** P < 0.0001 *vs.* LPA₁ CTRL, ## P < 0.01 *vs.* LPA₁ CTRL 10 nM, and #### P < 0.0001 *vs.* LPA₁ CTRL 10 nM (n = 16–22 vessels obtained from 7–12 animals).

VPC31143-stimulation (Fig. 6A). Furthermore, aortic segments isolated from PTX-pretreated mice showed reduced contractile responses after administration of VPC31143 (Fig. 6B).

DISCUSSION

The diversity of the cardiovascular effects of LPA has long been known, yet no mechanistic explanation has been provided for the marked differences between the experimental findings. The results we present here indicate for the first time that LPA induces vasoconstriction in vessels with damaged endothelium, in contrast to its recently reported vasodilator effect in intact vessels. Interestingly, both the constrictive and dilatory effects are mediated by

LPA₁. The role of LPA₁ is supported by the absence of vasoconstriction in LPA₁ KO mice, the constrictive effect of the LPA₁₋₃ agonist VPC31143 (29), and inhibition of constriction by the LPA_{1&3} antagonist Ki16425 but not by the LPA₃ antagonist DGPP and the lack of LPA₂ receptors. In addition, T13 failed to induce contraction at a concentration that selectively stimulates LPA₃, whereas its contractile effect at higher concentrations was abolished by the absence of LPA₁. Quantitative PCR results obtained from freshly isolated thoracic and abdominal aortic VSMCs showed a rank order of expression of LPA receptor subtype transcripts as $6 > 4 > 1 \geq 2 > 5 > 3$. This rank order of receptor expression also argues in favor of LPA₁ rather than LPA₃ being responsible for the VPC31143 and Ki16425 effects.

In search of the mediator of the vasoconstrictor response, we hypothesized that the constrictor prostanoid TXA₂ could be responsible for this effect. This hypothesis is supported by our finding that LPA increased TXA₂

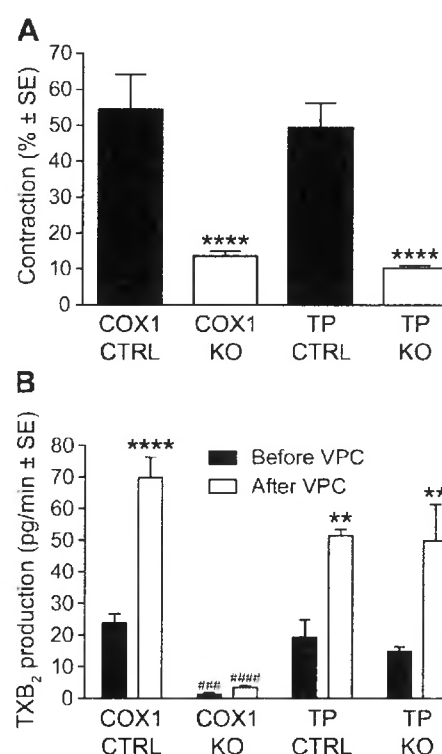


Figure 4. Involvement of constrictor prostanoids in VPC31143-induced contraction. *A*) Reduced vasoconstrictor effects of 10 μ M VPC31143 in AA segments prepared from COX1 KO and TP KO mice as compared with WT animals. **** P < 0.0001 *vs.* the corresponding CTRL; 1-way ANOVA with Tukey's *post hoc* test (n = 8–23 vessels obtained from 5–16 animals). *B*) VPC31143 treatment markedly increased TXA₂ release in COX1 CTRL TA segments, whereas COX1 KO vessels showed diminished basal rate of TXA₂ production that did not increase in response to VPC31143. In contrast, basal and VPC31143-stimulated TXA₂ release from vessels of TP KO mice showed no difference compared with TP CTRL. vessels. ** P < 0.01 *vs.* before VPC, **** P < 0.0001 *vs.* before VPC, ### P < 0.001 *vs.* COX1 CTRL before VPC, and #### P < 0.0001 *vs.* COX1 CTRL after VPC; 2-way ANOVA with Bonferroni's *post hoc* test (n = 3–7).

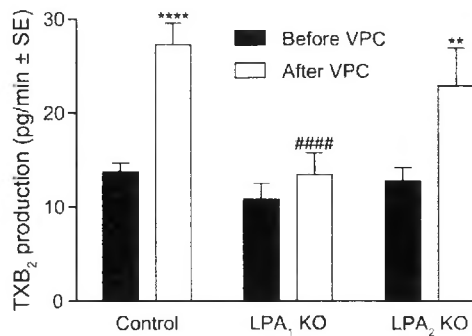


Figure 5. LPA₁ mediates VPC31143-induced vascular TXA₂ release. A lack of LPA₁ but not LPA₂ abolishes the VPC31143-elicited elevation in TXA₂ production of thoracic aortic segments. LPA₁ CTRL and LPA₂ CTRL mice showed identical responses and were therefore pooled and referred to as control. ** $P < 0.01$ vs. before VPC, **** $P < 0.0001$ vs. before VPC, #### $P < 0.0001$ vs. control after VPC; 2-way ANOVA with Bonferroni's *post hoc* test ($n = 6-16$).

production in isolated vessels that required COX1 and LPA₁ but not LPA₂. Moreover, the vasoconstrictor response was diminished in COX1 KO and in TP KO vessels. Our results support a mechanism whereby TXA₂ is generated by LPA₁-mediated COX1 activation. In addition, we found that PTX treatment of the vessels abolished production of TXA₂ and diminished the vasoconstrictor response, indicating that this LPA₁ receptor-mediated effect is coupled to the G_i protein in VSMCs.

LPA has also been reported to induce endothelium-dependent vasoactive effects mediated by TP receptors. Ohata *et al.* (40, 41) demonstrated that under conditions of increased shear stress, LPA stimulates Ca²⁺ influx in murine and bovine aortic endothelial cells *via* mechanosensitive cation channels. LPA in the presence of shear stress increased phenylephrine-induced contraction and reduced acetylcholine-induced relaxation in rat mesenteric arteries in an endothelium-dependent manner. These 2 effects of LPA were sensitive to the COX inhibitor indomethacin and the TP receptor antagonist SQ29548 (16, 42). In addition, LPA induced endothelial shear stress-dependent Ca²⁺ elevation in VSMCs and contraction of the mouse aorta, which could be blocked by inhibitors of COX, TXA₂ synthase, and the TP receptor (16).

In spite of the similar paracrine signaling, the mechanism of this vascular reaction is completely different from that described in the present study because the former develops only in the presence of shear-stress and depends on the presence of intact endothelium, whereas our experiments have been performed in the absence of endothelium and shear-stress. Nevertheless, these 2 lines of findings together indicate that under pathophysiological conditions involving increased shear stress or endothelial damage, LPA stimulates TXA₂ release from the endothelium or VSMCs, leading to vasoconstriction. Reduction in vascular diameter in turn might further increase shear stress and mechanical stimulation or injury of the endothelium, leading to a vicious circle.

Regarding a potential interaction between LPA and prostanoids in the regulation of smooth muscle

contraction, the available literary data are sparse and controversial. In an earlier study, Tokumura *et al.* (35) reported that the constrictor action of LPA in the guinea pig ileum was sensitive to indomethacin, whereas in a subsequent report by the same group COX-inhibition failed to influence LPA-induced contractions of the rat colon (43). Interestingly, in cultured ileal smooth muscle cells, LPA has also been reported to enhance the intracellular Ca²⁺ response to mechanical stimulation, an effect similar to that observed by Ohata *et al.* (44, 45) in endothelial cells, although in these studies the potential involvement of TXA₂ or TP receptors has not been investigated. Moreover, in the uterus LPA was shown to regulate COX2 expression (46). However, in contrast to our findings, this effect is mediated by LPA₂ and results in the release of prostaglandin E₂ and I₂. The present observations provide direct evidence for an LPA-stimulated TXA₂ release from the smooth muscle and a consequent change of the smooth muscle tone.

Our present and previous findings (21) together support the conclusion that LPA, acting on LPA₁ receptors, represents a Janus-faced mediator in the control of vascular tone. To understand the physiological importance of this paradoxical behavior, the mechanism of intravascular LPA production should be considered. LPA production in blood is linked to platelet activation. At the site of vascular

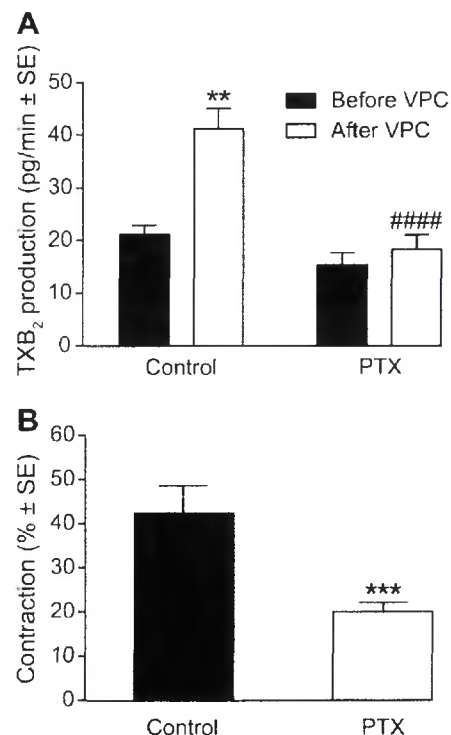


Figure 6. LPA-induced TXA₂ production and constriction is mediated by G_i protein. A) Effect of PTX pretreatment on VPC31143-stimulated TXA₂ production in WT vessels. ** $P < 0.01$ vs. before VPC, #### $P < 0.0001$ vs. control after VPC; 2-way ANOVA with Bonferroni's *post hoc* test ($n = 5-8$). B) PTX treatment inhibited VPC31143-elicited contraction in WT vessels. *** $P < 0.001$ vs. CTRL; unpaired Student's *t* test ($n = 14-19$ vessels obtained from 6-10 animals).

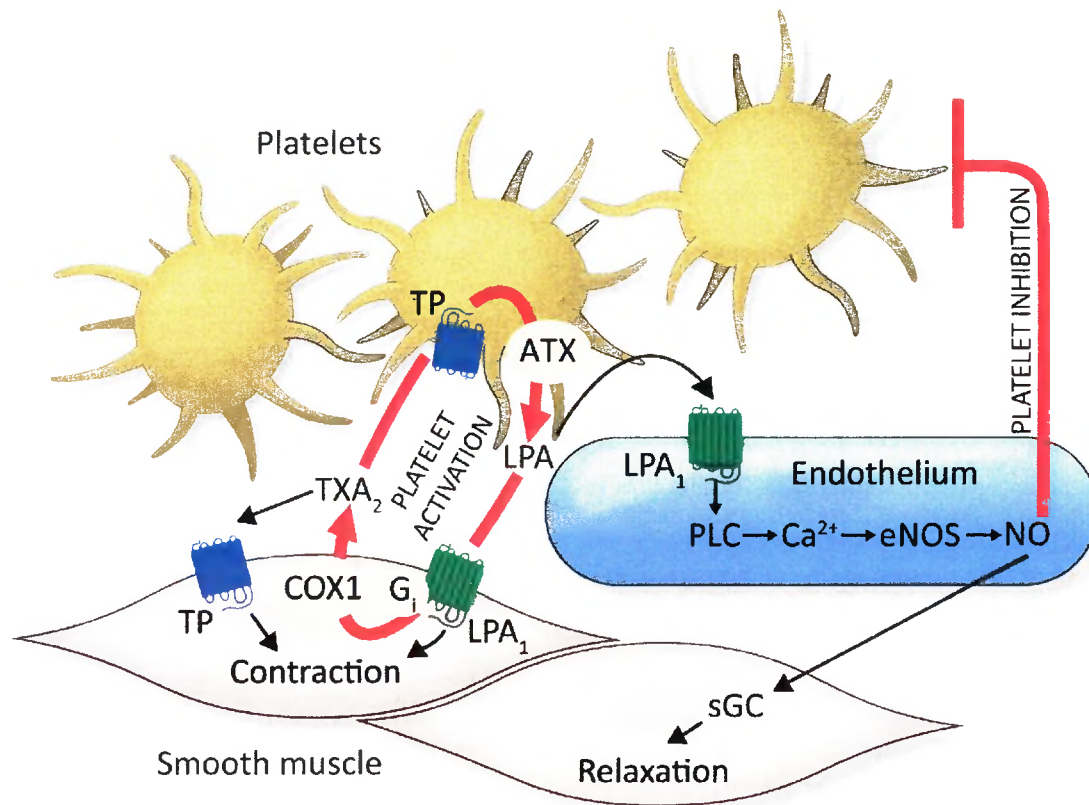


Figure 7. Integrated hypothesis of LPA₁-mediated vasoactive effects in intact vessels *vs.* after endothelial damage. Under physiological conditions LPA stimulates endothelial NO production in an LPA₁/PLC-dependent manner (21), resulting in vasorelaxation and inhibition of platelets. In the absence of the endothelium, however, platelet activation initiates LPA production, which in turn acts on LPA₁ in VSMCs and induces TXA₂ production. TXA₂ elicits contraction *via* the activation of TP in VSMCs but also promotes further platelet activation acting on TP in platelets. TP-mediated activation of platelets results in additional LPA production. This mechanism represents a potential positive feedback loop in which platelet activation promotes contraction and further platelet activation *via* a vicious circle involving LPA/LPA₁ and TXA₂/TP receptors, resulting in a pathophysiological vasoconstriction.

injury, platelets can come into direct contact with VSMCs, and LPA produced by ATX can activate the LPA₁–COX1–TXA₂ axis (Fig. 7). TXA₂, in turn, could lead to vasoconstriction and augmented platelet aggregation, preventing hemorrhage through the damaged vessel wall. A putative positive feedback loop between the release of LPA and TXA₂ production ensures efficient hemostasis, but it may initiate a vicious circle by promoting thrombus formation. Plausibly, when a growing thrombus reaches the intact vascular wall, localized LPA generation can induce NO release *via* the endothelial LPA₁ receptor–PLC–eNOS pathway (Fig. 7) (21). In such a case, endothelial NO, as the negative feedback regulator of the vascular LPA₁ system, can oppose TXA₂-mediated vasoconstriction and inhibit platelet activation.

The opposite effects of LPA on the vascular tone mediated by LPA₁ in VSMCs and the endothelium may not be equally potent in every species. For instance, it was reported that intravenous injection of LPA induces hypertension in rats and guinea pigs, whereas hypotension is induced in cats and rabbits (13). In mice, LPA induces only weak and transient hypertension (47), which may indicate

a balance between vasoconstrictor and vasodilator effects. Interestingly, the pressor effect of LPA is enhanced significantly in spontaneously hypertensive rats as compared with Wistar-Kyoto rats (48). This latter report indicates that the balance between the vasoconstrictor and vasodilator properties of LPA might become disturbed under pathologic conditions. Our present and previous observations indicate that dysfunction or damage of the endothelium can tilt the vasodilator effect of LPA toward vasoconstriction and potentially even to vasospasm. Accumulation of LPA in atherosclerotic plaques has been reported (18–20, 49). Release of LPA from plaque-associated sources coupled with disruption of normal endothelial functions might lead to sustained vasoconstriction. The vasoconstrictor properties of LPA can become dominant after hemorrhage, where it can directly stimulate VSMCs, bypassing the physical and functional barrier provided by the endothelium. Experiments conducted in the late 1990s showed that LPA applied to the subarachnoid space attenuated vasodilator responses and elicited vasospasm (15, 50). Furthermore, in an experimental subarachnoid hemorrhage model 4 d after intrathecal injection of autologous blood, an LPA-like

bioactive mediator was detected in the cerebrospinal fluid (15). These results are consistent with the actions of LPA in posthemorrhagic hydrocephalus, which has been reported to be coupled to LPA₁ (51). The present results might offer a potential mechanism of LPA-induced vasoconstriction/vasospasm after subarachnoid hemorrhage. Platelet-derived LPA in the cerebrospinal fluid comes into direct contact with LPA₁ on the abluminal surface of VSMCs, where it can selectively trigger TXA₂-mediated contraction, the very mechanism we described in the present study. Interestingly, with the same time course (*i.e.*, 3–4 d after subarachnoid hemorrhage), when the LPA-like biologic activity was detected in the cerebrospinal fluid (15), pharmacological inhibition of TXA₂ synthesis attenuated the development of cerebral vasospasm (52, 53). Nevertheless, the role of a potentially vicious circle between LPA and TXA₂ production requires further investigation in models of subarachnoid hemorrhage.

In summary, the present study demonstrates that mouse aortic VSMCs express all known LPA receptor transcripts, with the highest abundance of LPA₁, LPA₂, LPA₄, and LPA₆. The direct effect of LPA on VSMCs is contraction, which is in contrast to its endothelium-dependent vasorelaxant action, even though both are mediated by LPA₁ receptors. The signaling pathways include LPA-induced COX1-mediated autocrine/paracrine TXA₂ release in VSMCs and consequent activation of TP receptors, which is responsible for the development of vasoconstriction. We propose that LPA acts as a Janus-faced mediator in the control of vascular tone and that its overall effect is determined by the functional integrity of the endothelium. The interaction between the LPA/LPA₁ and TXA₂/TP pathways described in the present study might play a significant role not only in vasoregulation but also in hemostasis, thrombosis, and vascular remodeling. **[F]**

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AUTHOR CONTRIBUTIONS

P. T. Dancs, É. Ruisanchez, J. Aoki, G. Tigyi, and Z. Benyó designed research; P. T. Dancs, É. Ruisanchez, A. Balogh, and C. R. Panta performed experiments; P. T. Dancs, Z. Miklós, and É. Ruisanchez analyzed data and prepared figures; P. T. Dancs, G. Tigyi, and Z. Benyó wrote the

paper; R. M. Nüsing, J. Aoki, J. Chun, and S. Offermanns corrected the paper; and J. Chun, R. M. Nüsing, and S. Offermanns contributed transgenic mice to the study.

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LPA₁ receptor –mediated thromboxane A₂ release is responsible for lysophosphatidic acid-induced vascular smooth muscle contraction

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