Published in final edited form as:

Angiogenesis. 2008 ; 11(3): 301–310. doi:10.1007/s10456-008-9113-5.

Lysophosphatidic acid (LPA) and Angiogenesis

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Abstract

Lysophosphatidic acid (LPA) is a simple lipid with many important biological functions such as the regulation of cellular proliferation, cellular migration, differentiation, and suppression of apoptosis. Although a direct angiogenic effect of LPA has not been reported to date, there are indications that LPA promotes angiogenesis. In addition, LPA is a chemo-attractant for cultured endothelial cells and promotes barrier function in such cultures¹. To test the hypothesis that LPA is angiogenic, we used the chicken chorio-allantoic membrane (CAM) assay. Sequence analysis of the cloned, full length chicken LPA receptor cDNAs revealed three receptor types that are orthologous to the mammalian LPA₁, LPA₂ and LPA₃ receptors. We document herein that LPA is angiogenic in the CAM system and further that synthetic LPA receptor agonists and antagonists mimic or block this response, respectively. Our results predict that LPA receptor antagonists are a possible therapeutic route to interdicting angiogenesis.

Keywords

angiogenesis; chorio-allantoic membrane (CAM); lipid mediators; lysophosphatidic acid (LPA); vessels

INTRODUCTION

Lysophosphatidic acid (LPA, 1-acyl-2-hydroxy-*sn*-glycero-3-phosphate) is a simple lipid involved in a number of biologic processes including cellular proliferation, suppression of apoptosis, and modulation of survival and differentiation^{2, 3}. Other important cellular effects induced by LPA are related to cytoskeletal filament reorganization, including regulation of chemotaxis in endothelial cells and smooth muscle contraction^{4, 5}. Many of these actions of LPA are mediated by G protein-coupled receptors (GPCRs) of the Endothelial Differentiation Gene (EDG) family. Three GPCRs of the EDG family have been identified as LPA receptors, LPA₁ (formerly EDG-2), LPA₂ (EDG-4) and LPA₃ (EDG-7). Two additional GPCRs have been suggested to be LPA receptors, GPR23/p2y9/LPA₄⁶ and GPR92/LPA₅⁷, but these are distantly related to the EDG family LPA receptors and have not yet been studied in detail. LPA receptors are developmentally regulated and differ in tissue distribution, but couple similarly to multiple types of G proteins to signal through Ras and Mitogen-Activated Protein Kinase (MAPK)⁸, Rho⁹, phospholipase C (PLC)¹⁰, and several protein tyrosine kinases¹¹. The

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Disclosures: VPC32183 is sold by Avanti Polar Lipids under license from the University of Virginia.

physiological roles of individual LPA receptors are not well understood, but studies with mutant mice have suggested that the LPA₁ and LPA₃ receptors are important for normal development^{12, 13}.

LPA is found in human plasma at a concentration of 0.4 – 0.5 μM ¹⁴ while in serum the concentration range is approximately 10-fold higher¹⁵. It is synthesized through a number of different pathways including de-acylation of phosphatidic acid (PA) by phospholipases A1 and A2 (PLA1 and PLA2), acylation of glycerol 3-phosphate by glycerophosphate acetyltransferase, phosphorylation of monoacylglycerol (MAG) by acylglycerol kinase (AGK), and hydrolysis of plasma lysophosphatidylcholine (LPC) by the lyso-phospholipase D (lyso-PLD), autotaxin¹⁶. Extracellular LPA is degraded by the action of integral membrane lipid phosphate phosphatases (LPPs), which hydrolyze LPA to form monoacylglycerol and inorganic phosphate. LPA can also be metabolized via acylation by LPA acyl transferases (LPAATs) to form PA¹⁷.

Several properties of LPA prompted us to test it as an angiogenic factor. For instance, LPA promotes endothelial cell migration and proliferation *in vitro*^{18, 19}, it enhances matrix metalloproteinase-2 expression in endothelial cells²⁰ and autotaxin (ATX, a plasma lyso-PLD), is angiogenic *in vivo*²¹ and is essential for vascular development in mice²². Although these results suggest that LPA is an angiogenic molecule, there has not been a demonstration that LPA itself induces angiogenesis *in vivo*. To test the hypothesis that LPA is angiogenic, we used the chicken chorio-allantoic membrane (CAM) assay using LPA as well as synthetic compounds that are agonists or antagonists at individual LPA receptors. We report the results of these studies herein.

Angiogenesis – the sprouting of capillaries from existing blood vessels – is required in normal embryogenesis as well as wound healing. However, this process is also critical for solid tumor growth. Tumors attract new blood vessels to receive nutrients and oxygen necessary for expansion. Angiogenesis is a tightly regulated process (by the strict balance of angiostatic/ inhibitors vs. angiogenic/stimulators factors), thus it is important to understand how it is regulated and to identify molecules that are involved in the process. If LPA induces angiogenesis *in vivo*, it would be helpful to determine the function of individual LPA receptors in the regulation of this process so as to direct the development of LPA receptor-directed anti-angiogenic compounds.

METHODS

Materials and Reagents

Lipids (1-oleoyl LPA, S-OMPT, sphingosine 1-phosphate) and the LPA receptor antagonist VPC32183 were purchased from Avanti Polar Lipids, Alabaster, AL. Fatty acid free-bovine serum albumin (FAF-BSA), water-soluble hydrocortisone and Vascular Endothelial Growth Factor (VEGF) were purchased from Sigma-Aldrich, St. Louis, MO. Recombinant spider sphingomyelinase D (SMaseD) and the two catalytically inactive mutants were prepared and purified by Sangderk Lee, Ph.D. in our laboratory.

RNA extraction from chicken embryos

Total RNA was extracted from the chicken embryos using TRI reagent (Sigma-Aldrich, St. Louis, MO). Briefly, tissue from the embryo was cut and immediately transferred to a tube containing RNeasy lysis solution to inhibit degradation of RNA. The tissue was homogenized in TRI reagent and chloroform was added. The mixture was centrifuged ($12,000 \times g$ for 15 minutes at 4°C), the phase containing the RNA was removed, transferred to a new RNase-free tube and isopropanol was added. After centrifugation, the supernatant fluid was removed, the

RNA pellet was washed with 75% ethanol and collected by centrifugation ($7,500 \times g$ for 5 minutes). The RNA pellet was then air-dried and dissolved in DEPC-treated (RNase-free) water (Ambion, Austin, TX).

Cloning of LPA receptors from chicken

The three EDG chicken LPA receptors (LPA_{1, 2, 3}) were cloned using total RNA extracted from a chicken embryo. In the case of cLPA₁, the full translational open reading frame (ORF) was amplified using the following primer sets designed from the nucleotide sequence present in the chicken genome record (sense: 5'- ATG GAT ATC CCC ACT GAT TTG GTG CCA and anti-sense: 5'- TCC ACA GCA ACG ACC ACT CGG TGG TGT AA (for the construct with stop codon (N-terminal tagged)) or anti-sense: 5'- TCC ACA GCA ACG ACC ACT CGG TGG TG (for the construct without the stop codon (C-terminal tagged)). In the case of cLPA₂, we designed the following sense primer (5'- ATG GTA GAG GTG CGG TGT GGA T) from the known nucleotide sequence encoding a fragment (~900 bp) of this receptor. We amplified the full ORF sequence using 3'-rapid amplification of cDNA ends (RACE) using this sense primer and an oligo-dT anti-sense primer using the Generacer kit (Invitrogen, Carlsbad, CA).

Similarly, for cLPA₃, we designed a sense primer from the known nucleotide sequence (5'- ATG AAT GAA TGC TAC TAT GAT AAG CAC AT) and we used 3'-RACE (with the oligo-dT anti-sense primer) to amplify the sequence encoding the entire translational open reading frame (ORF). For all the receptors, the DNA encoding the full translational ORF was sub-cloned into pcDNA3.1/V5-His -TOPO (for C-terminal epitope tag) or pcDNA4/HisMax -TOPO (for N-terminal tag) plasmids for eventual expression in mammalian cells. The fidelity of the plasmid constructs were verified by automated DNA sequencing (UVA Biomedical Research Core Facility).

Chicken Chorio-Allantoic Membrane (CAM) assay for angiogenesis

White Leghorn chicken eggs were purchased from CBT Farms (Chestertown, MD). After shipment, eggs were incubated in a rocking incubator at 37°C for three days (the first day of incubation is considered the first day of embryonic development, or E1). On day E4, a 1 cm² window was cut in the shell of the eggs and the underlying membrane was removed. The window was covered with a plastic cover slip and the eggs were returned to the incubator. At day E8 the CAMs (~8–10 per group) were treated with the appropriate compounds (20 µl at the desired concentrations) or controls. Compounds were delivered by applying to 0.5 cm diameter Whatman GF/C filter paper placed on the surface of the CAM. Eggs were treated daily for three consecutive days (E8, E9 and E10) by adding fresh drug to the same filter paper disc. On day E11, CAMs were harvested by injecting a 37% formaldehyde solution under and around the filter disc, the disc was removed and the number of new vessels (all vessels that intersect the filter paper disc at an angle greater than 45°) was determined using a light microscope. Images of CAM membranes were captured using a dissecting microscope equipped with a digital camera.

Statistical Analysis

One-Way ANOVA or Student *t* test were performed between groups.

RESULTS

Chicken LPA Receptors

Because the CAM system is avian, we first determined the number and type of LPA/EDG receptors encoded in the chicken genome. Repeated queries of the chicken genome with the three human LPA/EDG receptor sequences using TBLASTN or FASTA algorithms revealed

the existence of three orthologous genes, or fragments thereof. As described in the Experimental Methods section, we used this sequence information to clone the corresponding full-length chicken LPA receptor cDNAs. The amino acid sequence identities of the conceptualized human and chicken proteins over their full lengths are 95% (LPA₁), 66% (LPA₂) and 82% (LPA₃) (Figure 1a–c). The LPA₁ sequences are particularly highly conserved, indeed, there is very nearly complete conservation of amino acid sequence except at the amino termini of these proteins. We note in passing that this extraordinary degree of conservation among LPA₁ receptor sequences extends to other non-mammalian vertebrates (*e.g.* the fish *Tetraodon*, the amphibian *Xenopus*) (data not shown). Such a high degree of similarity virtually assures that our LPA₁ receptor-directed antagonist compounds (see below) are active at the orthologous chicken receptor. To determine which of the three LPA receptor genes are expressed in the chicken embryos, we analyzed RNA extracted from day E7 chicken embryos by RT/PCR using oligonucleotide primers specific for each of the three chicken receptors. We detected a signal for each of the three receptors using this method (data not shown).

LPA induces angiogenesis quantitatively similar to vascular endothelial growth factor (VEGF) and sphingosine 1-phosphate (S1P)

The CAM assay is a well-established system for gauging the angiogenic activity of small molecules, and thus is a logical first choice for assessing LPA in this regard. We began by treating eggs daily for three consecutive days with 1 μ M 1-oleoyl (18:1) LPA using sterile de-ionized water and 50 ng vascular endothelial growth factor-A (VEGF-A) as negative and positive controls, respectively. With this protocol, LPA evoked a robust angiogenic response quantitatively equivalent to that of VEGF (Figure 2A). Interestingly, LPA-induced vessels were larger than those induced by VEGF (Figure 2B), suggesting that LPA-induced vessels are more mature than VEGF-induced vessels.

We also tested another known angiogenic molecule, sphingosine 1-phosphate (S1P)²³, which is a phospholipid that is structurally similar to LPA. As presented in Figures 2C and 2D, 1 μ M of S1P induced an angiogenic response similar to the same concentration of LPA, confirming that S1P, like LPA, is an angiogenic molecule *in vivo*.

LPA-induced angiogenesis is blocked by an LPA_{1,3} antagonist

Although there is not yet a complete set of LPA receptor selective antagonists, several antagonists active at both the LPA₁ and LPA₃ receptors have been developed. After determining that LPA induces angiogenesis *in vivo*, we determined if the response was blocked by an antagonist for LPA₁ and LPA₃ receptors, VPC32183²⁴. This compound does not have agonist activity at the LPA₁, LPA₂ or LPA₃ receptors, rather it is antagonist for LPA₁ and LPA₃ receptors²⁴. We treated the CAMs for three consecutive days with 1 μ M LPA or 1 μ M LPA with 10 μ M VPC32183. In these experiments, VPC32183 (Figures 3A and 3B) blocked the angiogenic response obtained with 1 μ M 18:1 LPA. At this antagonist concentration (10 μ M), the compound alone does not have any significant effect in the number of vessels, as compared to the vehicle control (Figures 3A and 3B). Further, VPC32183 (at 10 μ M) had no effect in VEGF-induced angiogenesis (Figure 3C). Together, these results suggest that the angiogenic response obtained with LPA was due to the activation of LPA₁ and/or LPA₃.

An LPA₃-selective agonist induces angiogenesis

There is a paucity of LPA receptor selective compounds, particularly agonists; the few that have been discovered are directed to the LPA₃ receptor. One of these is 1-oleoyl-2-*O*-methylglycerophosphothionate (OMPT). *S*-OMPT is a selective agonist for the LPA₃ receptor if used at a relatively low concentration (*i.e.* 100nM)²⁵. We used *S*-OMPT to study the role of LPA₃ receptor in angiogenesis in the CAM system, applying compound daily to eggs for three days. Due to the low solubility of this compound in pure water, we used water containing 3%

fatty acid free BSA as a vehicle. As documented in Figures 4A and 4B, S-OMPT was angiogenic at this concentration. When we collided the LPA₃ receptor selective agonist, S-OMPT, with the antagonist VPC32183, the angiogenic effect of this LPA₃-selective agonist was not observed (Figure 4C).

Arachnid lysoPLD is an angiogenic factor *in vivo* and its action is blocked by VPC32183

An arachnid sphingomyelinase D ("SMaseD") that is a component of the venom of *Loxosceles reclusa* spiders was demonstrated recently by us²⁶ and others²⁷ to also catalyze the formation of LPA from lysophosphatidylcholine (LPC). Indeed, this enzyme activity is far more robust than that of the mammalian lysoPLD, autotaxin (ATX), and it lacks the broad-spectrum nucleotide phosphodiesterase and pyrophosphatase activities of autotaxin. Since the spider enzyme generates LPA, we wanted to determine if it is able to induce angiogenesis *in vivo*, similar to autotaxin (known to be angiogenic in mice²¹). We tested recombinant SMaseD produced in our laboratory and compared its effects with two catalytically inactive mutant SMaseD proteins (H37N, H73N)²⁶. As documented in Figure 5A, the brown recluse spider enzyme evokes an angiogenic response, and the magnitude of its effect is comparable to the response induced by LPA. In contrast, the two catalytically inactive mutants did not mimic the angiogenic response observed with the wild type enzyme (Figure 5B). Importantly, the angiogenesis that results from the treatment with the wild type spider enzyme is blocked fully by the LPA_{1,3} receptor antagonist, VPC32183 at 10 μ M (Figure 5C). Combined, these results further suggest that LPA is an angiogenic factor *in vivo* and that its actions are due to the activation of the LPA₁ receptor, the LPA₃ receptor, or both.

LPA-induced angiogenesis is not due to a non-specific inflammatory response

LPA has been shown to be involved in some inflammatory responses after activation of its receptors²⁸. To discount the possibility that the angiogenesis obtained with LPA in the CAM membranes is a result of a non-specific inflammatory response, we treated them with hydrocortisone, an anti-inflammatory corticosteroid. CAMs were treated for three days with LPA, hydrocortisone alone or LPA together with 30 ng hydrocortisone and then the number of new vessels was determined²⁹. We found that hydrocortisone is unable to block LPA-induced angiogenesis (Figures 6A and 6B), suggesting that the response we observed with LPA is not due to a non-specific inflammatory response. Importantly, hydrocortisone alone has no effect in the normal angiogenesis/development of the CAMs.

DISCUSSION

Angiogenesis is the sprouting and growth of new capillaries from pre-existing blood vessels. It is a tightly regulated process that controls a number of normal biological functions such as normal development and reproduction, but also regulates pathological conditions such as tumor growth and cancer metastasis³⁰. Angiogenesis has been studied for many years and the discovery of new angiogenic (stimulants) and angiostatic (inhibitors) factors makes it an active area of research. Two essential processes that are involved in the growth of new blood vessels are proliferation and migration of endothelial cells to start lining the new vessels. Lysophosphatidic acid (LPA) has been shown to induce both of these processes *in vitro* using endothelial cell cultures. Because of these results obtained in the last few years, we were interested in studying LPA and its role in angiogenesis *in vivo*. Using the chicken chorio-allantoic membrane (CAM) assay, we document that LPA is a direct angiogenic factor *in vivo*. Importantly, this response is completely blocked by an LPA₁ and LPA₃ receptor antagonist, VPC32183, suggesting that the response obtained with LPA is due to the activation of either of these receptors or both working in a synergistic manner. To study further the role of individual LPA receptors in LPA-induced angiogenesis, we tested an LPA₃ receptor-selective agonist, S-OMPT. At a concentration where this compound does not activate other

LPA receptor subtypes, S-OMPT induces a strong angiogenic response in the CAMs. Moreover, its effect is completely blocked by the LPA₁/LPA₃ antagonist VPC32183. This suggests that LPA₃ could be inducing the angiogenic response, but we cannot discard the possibility that LPA₁ is also involved in the chicken assay. A better tool kit of LPA receptor-selective compounds is required to test such hypotheses, but such molecules are lacking at present.

In 2001 a group of researchers showed that autotaxin (ATX; one of the LPA-producing enzymes) is angiogenic *in vivo*²¹ while, another group showed that this enzyme is essential for normal vascular development²². To complement these results, we tested a similar LPA-producing enzyme present in brown recluse spider (*Loxosceles reclusa*) venom. This enzyme also has lyso-PLD activity and robustly generates LPA from LPC. As we documented herein, the spider enzyme is able to induce angiogenesis as well. Interestingly, the angiogenic response obtained from the enzyme is dependent on its lyso-PLD activity, since two different catalytically-inactive mutants are not able to produce the same response. Moreover, the response of the wild type construct appears to be dependent on the activation of LPA receptors, because it is completely abolished by the antagonist VPC32183. *In toto*, our results suggest that LPA is an angiogenic factor *in vivo* and that the response is via the activation of LPA₁, LPA₃ or both.

An interesting detail that we noticed in the course of our studies is that LPA-induced vessels appear larger and more robust than VEGF-induced vessels. It is known that VEGF induces vasculogenesis and angiogenesis and also that it produces immature/leaky vessels that need to continue a maturation process induced by other factors (arteriogenic factors)^{31, 32}. Perhaps LPA is an angiogenic as well as arteriogenic (or vessel maturation) factor *in vivo*. However, this hypothesis needs to be studied in detail with other arteriogenesis-specific experiments. It is clear now that LPA induces angiogenesis *in vivo* in the CAM assay. However, more experiments using other angiogenic models in rodents are needed to determine the biological effects of blocking LPA receptors and LPA-induced angiogenesis. Also, there is a need for new and more selective compounds to target (positively or negatively) individual LPA receptors. Another option is to use LPA₁ and/or LPA₃ null mice to study further the role of each of these receptors in angiogenesis and angiogenesis-dependent diseases such as tumor metastasis. If we are able to determine the role that each of these receptors play in angiogenesis, then it would be helpful to have a receptor-selective antagonist that blocks the unwanted response and that could be used in the future to treat angiogenesis-related diseases.

Acknowledgements

We want to thank Sangderk Lee, Ph.D. (Lynch laboratory) for providing us with helpful advice for cloning the chicken LPA receptors.

Funding sources: This work was supported by research grants from the National Institutes of Health (R01 GM052722 (KRL) and F31 HL079881 (CRL)).

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hLPA1	MAAISTSIP.	.VISQPQF..	.TAMNEPQCF	YNESIAFFYN	RSGKHLATEW
cLPA1	MDIPTDLVPS	SMMSQPEVIE	STAMSEPQCY	YNETIAFFYN	RSGKYLATEW
Consensus	M-----P-	---SQP----	-TAM-EPQC-	YNE-IAFFYN	RSGK-LATEW
	51				100
hLPA1	NTVSKLVMGL	GITVCIFIML	ANLLVMVAIY	VNRRFHFPIY	YLMANLAAAD
cLPA1	NTVSKLVMGL	GITVCIFIML	ANLLVMVAIY	VNRRFHFPIY	YLMANLAAAD
Consensus	NTVSKLVMGL	GITVCIFIML	ANLLVMVAIY	VNRRFHFPIY	YLMANLAAAD
	101				150
hLPA1	FFAGLAYFYL	MFNTGPNTRR	LTVSTWLLRQ	GLIDTSLTAS	VANLLAIAIE
cLPA1	FFAGLAYFYL	MFNTGPNTRR	LTVSTWLLRQ	GLIDTSLTAS	VANLLAIAIE
Consensus	FFAGLAYFYL	MFNTGPNTRR	LTVSTWLLRQ	GLIDTSLTAS	VANLLAIAIE
	151				200
hLPA1	RHITVFRMQL	HTRMSNRRVV	VVIVVIWTMA	IVMGAIPSVG	WNCICDIENC
cLPA1	RHITVFRMQL	HTRMSNRRVV	VVIVVIWTMA	IVMGAIPSVG	WNCICDITHC
Consensus	RHITVFRMQL	HTRMSNRRVV	VVIVVIWTMA	IVMGAIPSVG	WNCICDI--C
	201				250
hLPA1	SNMAPLYSDS	YLVFWAIFNL	VTFVVMVVLV	AHIFGYVRQR	TMRMSRHSSG
cLPA1	SNMAPLYSDS	YLVFWAIFNL	VTFVVMVVLV	AHIFGYVRQR	TMRMSRHSSG
Consensus	SNMAPLYSDS	YLVFWAIFNL	VTFVVMVVLV	AHIFGYVRQR	TMRMSRHSSG
	251				300
hLPA1	PRRNRDTMMS	LLKTVVIVLG	AFIICWTPGL	VLLLLDVCCP	QCDVLAYEKF
cLPA1	PRRNRDTMMS	LLKTVVIVLG	AFIICWTPGL	VLLLLDVCCP	QCNVLAYEKF
Consensus	PRRNRDTMMS	LLKTVVIVLG	AFIICWTPGL	VLLLLDVCCP	QC-VLAYEKF
	301				350
hLPA1	FLLLAEFNSA	MNPPIISYRD	KEMSATFRQI	LCCQRSENPT	GPTEGSDRSA
cLPA1	FLLLAEFNSA	MNPPIISYRD	KEMSATFKQI	LCCQRSESTN	GPTEGSDRSA
Consensus	FLLLAEFNSA	MNPPIISYRD	KEMSATF-QI	LCCQRSE---	GPTEGSDRSA
	351		369		
hLPA1	SSLNHTILAG	VHSNDHSVV			
cLPA1	SSLNHTILAG	VHSNDHSVV			
Consensus	SSLNHTILAG	VHSNDHSVV			

B

	1				50
hLPA2	~~~~~	~~~~MVIMGQ	CYYNETIGFF	YNNSGKELSS	HWRPKDVVVV
cLPA2	MVEVRCGCRG	CVCRPAAMKH	CFYNESVGFF	YNNSRKELTT	YWRTKDILVV
Consensus	-----	-----M--	C-YNE--GFF	YNN--KEL--	-WR-KD--VV
	51				100
hLPA2	ALGLTVSVLV	LLTNLLVIAA	IASNRRFHQP	IYYLLGNLAA	ADLFAGVAYL
cLPA2	ALGLTVSLIV	LLTNLLVIAA	IIINRRFHYP	IYYLLGNLAA	ADLFAGIAYM
Consensus	ALGLTVS--V	LLTNLLVIAA	I--NRRFH-P	IYYLLGNLAA	ADLFAG-AY-
	101				150
hLPA2	FLMFHTGPRT	ARLSLEGWFL	RQGLLDTSLT	ASVATLLAIA	VERHRVMAV
cLPA2	FLMFHTGPRT	AELSLKTWFI	RQSLLDTSLT	ASVVNLLAIA	VERHQTVLTM
Consensus	FLMFHTGPRT	A-LSL--WF-	RQ--LLDTSLT	ASV--LLAIA	VERH--V---
	151				200
hLPA2	QLHSRLPRGR	VVMLIVGVWV	AALGLGLLPA	HSWHCLCALD	RCSRMAPLLS
cLPA2	QLHSKMSNQR	VMILIFCIWA	TALLLGLIPS	YGWHCLCALE	ECSSMAPLYS
Consensus	QLHS-----R	V--LI---W-	-AL-LGL-P-	--WHCLCAL-	-CS-MAPL-S
	201				250
hLPA2	RSYLAVWALS	SLLVFLLMVA	VYTRIFFYVR	RRVQRMAEHV	SCHPRYRETT
cLPA2	RSYLTFWAIS	NLLVFLLMAV	VYAHIFIYVK	RKMGRMSAHT	SFHPRYRETV
Consensus	RSYL--WA-S	-LLVFLLM--	VY--IF-YV-	R---RM--H-	S-HPRYRET-
	251				300
hLPA2	LSLVKTVVII	LGAFVVCWTP	GQVVLLEDGL	GCESC NVLAV	EKYFLLLAEA
cLPA2	VGLVKTVTII	LGAFXIFWTP	RQGVXL DGL	GCKSC NVLAG	EKYVLLLA EI
Consensus	--LVKTV-II	LGAF---WTP	-Q-VL-LDGL	GC-SCNVA-	EKY-LLLAE-
	301				350
hLPA2	NSLVNAAVYS	CRDAEMRRTF	RRLCCACLR	QSTRESVHYT	SSA.QGGAST
cLPA2	NSLINAIVYS	YRDNEMRSXF	RRILCTICYR	NPKYT	PTAVKPN GAD
Consensus	NSL-NA-VYS	-RD-EMR--F	RR-LC--C-R	-----YT	--A-----
	351				366
hLPA2	RIMLPENGHP	LMDSTL			
cLPA2	RGSLSLNGHF	TVDSSI			
Consensus	R--L--NGH-	--DS-			

C

	1				50
cLPA3	MNECYDYDKHM	DDFYNKTNTH	TADEWTGPPL	IGVLCFGTFF	CLFIFISNSL
hLPA3	MNECHYDKHM	DDFYNRSNTD	TVDDWTGTL	VIVLCVGTFF	CLFIFFSLSL
Consensus	MNEC-YDKHM	DDFYN--NT-	T-D-WTG--L	--VLC-GTFF	CLFIF-SNSL
	51				100
cLPA3	VIAAVVKNKR	FHFPPYYLLA	NLAAADFFAG	IAYVFLMFHT	GPVSKTLTVN
hLPA3	VIAAVIKNRK	FHFPPYYLLA	NLAAADFFAG	IAYVFLMFNT	GPVSKTLTVN
Consensus	VIAAV-KN--	FHFPPYYLLA	NLAAADFFAG	IAYVFLMF-T	GPVSKTLTVN
	101				150
cLPA3	RWFLRQGLLD	TSLTASLVNL	LVI AVERHMS	IMRMKIHSNL	TKKRVTFLLI
hLPA3	RWFLRQGLLD	SSLTASLTNL	LVI AVERHMS	IMRMVHSNL	TKKRVTLILL
Consensus	RWFLRQGLLD	-SLTASL-NL	LVI AVERHMS	IMRM--HSNL	TKKRV-TLI-
	151				200
cLPA3	SIWAIAIFMG	AVPTLGWNCL	CDISACSSLA	PIYSRSYLVF	WSVLNLVVF
hLPA3	LVWAIAIFMG	AVPTLGWNCL	CNISACSSLA	PIYSRSYLVF	WTVSNLMAFL
Consensus	--WAIAIFMG	AVPTLGWNCL	C-ISACSSLA	PIYSRSYLVF	W-V-NL--F-
	201				250
cLPA3	IMVVVYIRIY	MYVQRKTNL	SSHTSGSISR	RRTPVKLMKT	VMTLLGAFVV
hLPA3	IMVVVYLRIY	VYVQRKTNL	SPHTSGSISR	RRTPMKLMKT	VMTVLGAFVV
Consensus	IMVVVY-RIY	-YV-RKTNL	S-HTSGSISR	RRT-P-KLMKT	VMT-LGAFVV
	251				300
cLPA3	CWTPGLVVLL	LDGLNCTNCG	IQNVKRWFL	LALLNSVMNP	VIYSYKDDM
hLPA3	CWTPGLVVLL	LDGLNCRQCG	VQHVKRWFL	LALLNSVNP	IIYSYKDDM
Consensus	CWTPGLVVLL	LDGLNCG--	-Q-VKRWFL	LALLNSV-NP	-IYSYKDD-M
	301				350
cLPA3	WGTMKRMLCC	SSDDRNQERR	SSRIPSTVLG	RSTDITGQYI	EDSIIQGTIC
hLPA3	YGTMKMKICC	FSQE.NPERR	PSRIPSTVLS	RS.DTGSQYI	EDSISQGAVC
Consensus	-GTMK-M-CC	-S---N-ERR	-SRIPSTVL-	RS-DT--QYI	EDSI-QG--C
	351	361			
cLPA3	GKGDLEKGN	S			
hLPA3	NKSTS~~~~~	~			
Consensus	-K-----	-			

Figure 1. LPA₁, LPA₂, and LPA₃ receptors are expressed in chicken embryos

Peptide sequence alignments of chicken lysophosphatidic acid receptors: cLPA₁ (1A), cLPA₂ (1B), and cLPA₃ (1C) with the respective human LPA receptors (database accession numbers for human peptide sequences used are: LPA₁: NM_001401, LPA₂: NM_004720, LPA₃: NM_012152). The “Pretty” sequence comparison program was used to align the peptide sequences and calculate a consensus. These newly identified chicken LPA receptor sequences have been deposited in GenBank. Their accession numbers are: cLPA₁ (EU339317), cLPA₂ (EU339318) and cLPA₃ (EU339319).

B

vehicle-treated CAM

LPA-treated CAM

VEGF-treated CAM

C

D

Figure 2. LPA induces angiogenesis *in vivo* that is quantitatively similar to VEGF and S1P

To determine the effects of LPA in angiogenesis, we treated the chorio-allantoic membranes (CAMs) with 1 μ M 18:1 LPA, sterile water as a negative control or 50 ng vascular endothelial growth factor-A (VEGF-A) as a positive control. We also treated CAMs with S1P, another angiogenic factor that is structurally related to LPA. A piece of GF/C filter paper (0.5 cm diameter) was used as the drug carrier. After three consecutive days of treatment, we counted the number of vessels that intersected the filter paper disc at an angle greater than 45°. These vessels are the new vessels that represent the angiogenic response. **2A** LPA induces angiogenesis, compared to the control. Interestingly, LPA-induced angiogenesis is comparable with VEGF-induced angiogenesis in terms of the number of new vessels induced. **2C** LPA-

induced angiogenesis is also comparable with S1P-induced angiogenesis. One-Way analysis of variance (ANOVA) test was performed between groups. *** $P < 0.0001$. ** $P < 0.001$. Error bars represent standard deviation (SD). Images were captured using a dissecting microscope (Makroscope) connected to a digital camera, at a magnification of 6 ×. In Figures 2B and 2D we show one membranes representative of each group. Scale bar: 0.125 cm.

A

B

C

Figure 3. LPA-induced angiogenesis is blocked by an LPA_{1,3} receptor antagonist

To analyze the role of LPA receptors in LPA-induced angiogenesis we used the LPA₁ and LPA₃ receptor antagonist, VPC32183. Using the same approach aforementioned, we show in **3A and 3B** that LPA-induced angiogenesis is blocked completely by this compound at 10 μ M. Importantly, VPC32183 alone (at 10 μ M) had no discernable effect in angiogenesis and does not block VEGF-induced angiogenesis (Figure 3C). One-Way ANOVA test was performed between groups. ** P<0.001. Error bars represent standard deviation (SD). In figure 3B (scale bar: 0.125 cm), we present the image of a single membrane representative of each group.

Figure 4. S-OMPT, an LPA₃-selective agonist, is also an inducer of angiogenesis *in vivo* and its effect is antagonized by VPC32183

S-1-oleoyl-2-O-methylglycerophosphothionate (S-OMPT), at 100nM, is able to induce a strong angiogenic response, similar to the LPA response at this concentration (4A–4C). CAMs treated with S-OMPT (100nM) together with VPC32183 (10μM) document that the response is completely blocked by inclusion of this antagonist (4C). For statistical analysis, Student *t* test was performed between groups. ** P<0.001. Error bars represent standard deviation (SD). Figure 4B is the image of a single membrane that is representative of each group. Scale bar: 0.125 cm.

A

B

Figure 5. Arachnid PLD is an angiogenic factor *in vivo* and its action is blocked by VPC32183 CAMs were treated with recombinant sphingomyelinase D (SMaseD) and the number of vessels that intersected the disc was counted. This enzyme is able to induce an angiogenic response (**5A**) similar to the LPA response. Two catalytically inactive forms of this enzyme (H37N and H73N) were unable to induce angiogenesis (**5B**), suggesting that the response obtained is dependent on the catalytic activity (lyso-PLD) of the arachnid enzyme. Moreover, SMaseD-induced angiogenesis is blocked by the LPA receptor antagonist VPC32183 at 10 μ M (**5C**), suggesting that the positive response obtained is dependent on the activation of LPA_{1,3} receptors. One-Way ANOVA test was performed between groups. ** P< 0.001. Error bars represent standard deviation (SD).

Figure 6. LPA-induced angiogenesis is not due to a non-specific inflammatory response

To test the possibility of LPA producing a non-specific inflammatory response, we treated the CAMs with hydrocortisone (30ng). As shown in Figures 6A and 6B, hydrocortisone did not block the LPA-induced angiogenesis in the CAMs, suggesting that the LPA response is not due to a non-specific inflammatory response. The One-Way ANOVA test was performed between groups. ** $P < 0.001$. Error bars represent standard deviation (SD). Figure 6B shows one membrane representative of each group. The vehicle and LPA images in this figure are replicated from Figure 2B because it was one large experiment wherein we used the same controls (LPA and vehicle) to analyze two results. Scale bar: 0.125 cm.