Primary human endothelial cells secrete agents that reduce responsiveness to lysophosphatidic acid (LPA)

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Synopsis
The plasma level of LPA (lysophosphatidic acid) (200–600 nM) is well within the range that promotes proliferation and migration of vascular ECs (endothelial cells), yet vessels are quiescent and stable. In this report, we considered one explanation for this paradox: that ECs secrete agents that attenuate responsiveness to LPA. Indeed, we observed that CM (conditioned medium) from confluent, quiescent cultures of primary HUVECs (human umbilical vein ECs) contained an agent that inhibited LPA-mediated signalling events and cellular responses. The putative inhibitor, which we tentatively call ILMR (inhibitor of LPA-mediated responsiveness) seemed to act on cells (instead of at the level of LPA) by suppressing the ability of LPA receptor 1 to signal. The amount and/or activity of ILMR was regulated by growth factors; exposing HUVECs to VEGF-A (vascular endothelial growth factor A), but not bFGF (basic fibroblast growth factor), reduced the amount and/or activity of ILMR in CM. We conclude that in addition to promoting angiogenesis directly, VEGF-A can also act indirectly by modulating the bioactivity of angiomodulators such as LPA.

Key words: conditioned medium, endothelial cell, intracellular signalling, lysophosphatidic acid, vascular endothelial growth factor A.


INTRODUCTION

LPA (lysophosphatidic acid) stimulates a variety of cellular activities (cytoskeleton remodelling, ion transport and cell survival) by engaging one or more of its six GPCRs (G-protein-coupled receptors) [1–4]. For instance, LPA promotes migration of ECs (endothelial cells), and mice that lack autotaxin, an enzyme that produces LPA, display vascular defects and embryonic lethality [5,6]. Furthermore, LPA promotes regression of unstable vascular beds [7]. These observations indicate that LPA is one of the agents that contribute to angiogenic homeostasis.

ECs lining vessels of healthy individuals are quiescent despite their continual exposure to an activating concentration of LPA [1]. This phenomenon supports the idea that the quantity/availability of LPA and expression of LPA receptors is only a subset of mechanisms that govern responsiveness to LPA. Indeed, circulating proteins such as albumin and gelsolin bind LPA and regulate its activity [8–10]. Cells express LPPs (lipid phosphate phosphatases), which are transmembrane enzymes that dephosphorylate and thereby inactivate LPA [11]. Furthermore, intracellular signalling can desensitize LPA receptors, and thereby reduce LPA responsiveness [12,13]. These findings indicate that the bioactivity of LPA can be regulated at multiple levels, and one or more of these mechanisms are likely explanations for how vascular ECs remain quiescent when exposed to circulating LPA.

In the present study, we developed a cell-based assay to gauge the bioactivity of LPA and used it to discover that primary human ECs secreted agents that attenuated the responsiveness of cells to LPA. This agent appeared to suppress LPA-mediated signalling of the LPA1 (LPA receptor 1). Finally, the amount/activity of this agent was reduced by VEGF-A (vascular endothelial growth factor A), but not bFGF (basic fibroblast growth factor).

We conclude that primary human ECs have the potential to...
self-regulate their responsiveness to LPA, and that this phenomenon is under the control of VEGF-A.

**EXPERIMENTAL**

**Cell culture**
The rat hepatoma cell line (RH7777) containing an empty vector (V) or LPA1 (L1) were kindly provided by Dr Xianjun Fang (Department of Biochemistry, Virginia Commonwealth University, Richmond, VA, U.S.A.) [14]. They were maintained in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% fetal bovine serum, 80 units/ml penicillin and streptomycin C. HUVECs (human umbilical vein ECs) were purchased from Lonza and plated on gelatin (0.2%)-coated dishes. HDMVECs (human dermal microvessel ECs) were kindly provided by Dr Rong Shao (Department of Veterinary and Animal Science, University of Massachusetts, Amherst, MA, U.S.A.). All EC lines were maintained in M199 media supplemented with 20% bovine calf serum, 80 units/ml penicillin and streptomycin C, 12 μg/ml bovine brain extract and 100 μg/ml heparin at 37° C in 5% CO2.

**Antibodies and reagents**
Anti-LPA1 (NBPI-03363) was purchased from Novus Biology. Anti-gelsolin (sc-6405) and anti-c-Met (sc-10) were purchased from Santa Cruz Biotechnology. Anti-phospho-ERK (extracellular-signal-regulated kinase) (#9101) and U0126 (39903) was purchased from Cell Signaling. Gelsolin (G1538) and cycloheximide were purchased from Sigma. Ki16425 and SU6656 were purchased from Cayman Chemicals and Calbiochem respectively. LPA (L7260; Sigma) was dissolved in PBS containing 0.5% fatty-acid-free BSA (#3117405001; Roche) to a final stock concentration of 3.5 mM. VEGF-A and bFGF were kindly provided by NCBI.

**LPA bioassay**
L1 and V cells were plated at a density of 2×10⁴ cells/well into a 48-well plate coated with polylysine and incubated overnight in culture media. Cells were subsequently serum starved for 48 h, during which time the medium was changed every day. After starvation, cells were treated with LPA in either serum-free DMEM, or CM (conditioned medium) for 1 h at 37° C in the 5% CO2 incubator. In pre-treatment experiments, cells were pre-treated with either serum-free DMEM or CM for 1 h, washed with serum-free DMEM once, and then treated with LPA in DMEM for an additional 1 h. CM was generated by growing HUVEC to confluence, incubating for 16–18 h in serum-free DMEM with or without 10 ng/ml VEGF-A or 10 ng/ml bFGF. The resulting CM was centrifuged and the supernatant was used in experiments as described below.

To monitor phosphorylation of paxillin, cells were stimulated as described below, rinsed once with ice-cold PBS and lysed for 30 min at room temperature (21° C) with rocking in SDS sample buffer [2 mM EDTA, 2% SDS, 50 mM Tris/HCl (pH 6.8), 10% glycerol, 5% 2-mercaptoethanol and 0.01% Bromophenol Blue]. Western blot analysis was performed as described previously [15] with the following minor modifications. Samples were boiled for 7 min and resolved by SDS/PAGE (8% gel). Membranes were incubated overnight with a 1:5000 dilution of anti-phospho-paxillin (p-paxillin) Western blot analysis. The signal intensity was quantified and normalized to the loading control (RasGAP). The LPA1-specific response was calculated by subtracting the normalized phospho-paxillin response in V cells from L1 cells. The LPA1-specific response was plotted as a function of LPA concentration. The linear range of this assay was 0–200 nM.

![Figure 1 LPA induced the phosphorylation of paxillin in a dose-dependent manner](image)

**Radioenzymatic assay**
Radioenzymatic assay was performed as described previously [13,17]. In this assay, LPA was converted into [14C]PA (phosphatidic acid) in the presence of recombinant LPAAT (LPA acyltransferase) and [14C]oleoyl-CoA. LPAAT was prepared from...
Figure 2  **HUVECs secreted agent(s) that suppressed the bioactivity of LPA**

(A) The cells described in Figure 1 were either left resting (−), or stimulated for 1 h with 50 nM LPA (+) in the presence of CM or DMEM (mock CM). Cells were harvested and subjected to Western blot analysis as described in Figure 1. The lower panel is a histogram of the means ± S.E.M. from three independent experiments. *P < 0.05. CM was generated by incubating serum-free DMEM with confluent, quiescent HUVECs for 16–18 h. (B) 50 nM LPA was incubated with either DMEM or CM for 1 h and then the amount of LPA was quantified using the radioenzymatic assay described in the Experimental section. The top panel is a representative autoradiogram. The normalized intensity was calculated by subtracting the signal of control samples (−) from the signal of LPA-containing samples (+). The lower panel is a histogram of means ± S.E.M. from three independent experiments. n.s.: not significant. These data indicate that CM attenuated the response to LPA without degrading it.

**RESULTS**

A bioassay for LPA

Since the activity of LPA can be regulated by a variety of factors [10,13,19] knowing the concentration of LPA is not sufficient to accurately predict its bioactivity. Consequently, we sought to develop an LPA bioassay. To this end, we used a rat hepatoma cell line (RH7777), which is unresponsive to LPA [20], and was modified to overexpress LPA receptor 1 (L1) or the corresponding empty vector (V) [14] (Figure 1A). Phosphorylation of paxillin, one of the signalling events induced by LPA [21], was markedly better in L1 versus V cells at a number of doses of LPA (Figure 1B). Further characterization of LPA-triggered phosphorylation of paxillin indicated that the response reached a plateau by 1 h and was linear up to 200 nM LPA (Figure 1C and results not shown). Although we have not ruled out the possibility that RH7777 cells express additional LPA receptors, they did not make a large contribution to phosphorylation of paxillin (Figure 1B). We conclude that monitoring phosphorylation of paxillin in this pair of cell lines is one approach to assess the bioactivity of LPA.
ECs produced an ILMR (inhibitor of LPA-mediated responsiveness)

LPA is present in plasma and mediates EC migration and proliferation [1,2,22], yet the endothelium is stable and quiescent in most vessels of adult animals. While there are many explanations for this apparent paradox, we considered the hypothesis that vascular ECs in adults are stable and quiescent because they produce a soluble agent that suppresses their ability to respond to LPA. To test this hypothesis we determined if CM from HUVECs reduced LPA-mediated phosphorylation of paxillin. CM was generated by incubating serum-free DMEM with confluent, quiescent HUVECs for 18 h. LPA was consistently less potent in the presence of CM than in the presence of mock CM (DMEM) (Figure 2A). This reduction in activity was not because the CM degraded the LPA; the amount of LPA was not decreased by incubation with CM, rather it tended to increase; however, the difference did not reach statistical significance (Figure 2B). We conclude that HUVECs produced a soluble inhibitor of LPA-mediated responsiveness, which we tentatively name ILMR.

CM appeared to influence the LPA responsiveness of cells

Possible mechanisms by which ILMR reduced the bioactivity of LPA involved limiting the ability of LPA to activate its receptor, or reducing the ability of cells to respond to LPA. Others reported that, at a sufficiently high concentration, the LPA-binding protein gelsolin inhibits LPA. While CM contained gelsolin, it was 30-fold below the reported inhibitory concentration [10] (Figure 3A, left-hand panel). Furthermore, purified gelsolin, at the concentration present in CM, had no effect on LPA’s bioactivity (Figure 3A, right-hand panel). We conclude that even though gelsolin was present in CM, its concentration/effect was insufficient to account for the CM-mediated attenuation of LPA activity.

To investigate whether CM was acting on cells instead of LPA, we altered the experimental protocol to both maximize the exposure of cells to CM and minimize the exposure of LPA to CM. Instead of treating both cells and LPA with CM, we pre-incubated cells with CM for 1 h, removed it, rinsed the cells and then stimulated with LPA. Substantially minimizing exposure of LPA to CM did not prevent the CM from blunting the response to LPA (Figure 3B). These observations support the idea that CM acted on cells instead of on LPA. This concept is consistent with our finding that diabetes reduced the ability of retinal neovessels to regress in response to LPA, and that this phenomenon could be recapitulated by high glucose treatment of primary retinal ECs [13]. Thus, there is precedent for the idea that the responsiveness of cells to LPA is a variable, and subject to environmental conditions.

In the following series of experiments, we investigated the mechanism by which CM reduced the responsiveness of cells to LPA. We considered whether CM reduced expression of LPA1, and found that this did not appear to be the case. There was no statistically significant decline in the level of LPA1 upon exposure to CM (compare the unstimulated lanes in the ‘DMEM’ and ‘CM’ groups of Figure 4A).

Next, we tested whether CM altered LPA-induced signalling events. While LPA promotes internalization of LPA1 [23], and internalized GPCRs can be either recycled to the cell surface, or degraded [24], the fate of internalized LPA1 has not been investigated. We observed that, in LPA-treated cells, the level of LPA1 in the membrane fraction decreased (Figure 4A), which suggests that LPA-induced activation of LPA1 promotes its degradation. Importantly, this event was substantially reduced in CM-treated cells (Figure 4A), suggesting that CM blunted LPA-induced signalling at a very early step. If this were true, then phosphorylation of paxillin would not be the only signalling pathway that was compromised. Indeed, LPA-induced phosphorylation of ERK was also reduced by CM (Figure 4B). As expected from the work of other investigators [2,25], we found that LPA-dependent activation of ERK involved signalling intermediates that were not required for phosphorylation of paxillin (Figure 4C). The findings that CM suppressed a spectrum of LPA-dependent signalling events suggest that ILMR interfered at an early step in the LPA-triggered signalling cascade.
A potential explanation for how CM broadly reduced the response to LPA is by promoting degradation of LPA. While Figure 2(B) ruled out the possibility that CM degraded LPA, CM may increase the expression and/or activity of LPPs. This did not appear to be the case since the level of all three LPPs remained unchanged within the 1 h timeframe of a typical experiment (results not shown). Furthermore, while there was less LPA at the end of the assay, and this decline was dependent on the presence of cells (i.e. there was no decrease in the concentration of LPA in the absence of cells), the magnitude of the decline was comparable in both DMEM and CM pre-treated cells (Figure 4D). These results do not support the idea the CM attenuated LPA-dependent responsiveness by accelerating the cell-mediated degradation of LPA (presumably by LPPs). The data in Figures 3 and 4 indicate that HUVECs produce and secrete ILMR, and that the mechanism of action seems to be at the level of the cells, perhaps by compromising LPA-triggered activation of LPA1.

**VEGF-A reduced amount and/or activity of ILMR produced by HUVECs**

Since LPA is associated with angiogenesis [26], one may expect that angiogenic ECs would cease producing ILMR. To test this idea we investigated whether VEGF-A influenced the amount or activity of ILMR produced by HUVECs. As shown in Figure 5(A), CM produced from VEGF-A-stimulated cells failed to suppress the bioactivity of LPA. This was not a universal feature of pro-angiogenic agents; stimulating HUVECs with bFGF did not influence production/activity of ILMR. Since VEGF-A was present in the CM from VEGF-A-stimulated cells, it was...
E. Y. Park and A. Kazlauskas

Figure 5 VEGF, but not bFGF, reduced the amount of LPA inhibitory activity in CM

(A) The standard protocol to generate CM was modified such that instead of collecting medium from unstimulated HUVECs, it was harvested from cells that were exposed to 10 ng/ml of either VEGF-A or bFGF during the 16–18 h interval to generate CM. The resulting panel of CM, which included DMEM (mock CM) CM (collected from unstimulated cells) VCM (collected from VEGF-A-stimulated cells) and FCM (collected from bFGF-stimulated cells), was supplemented with nothing (−) or 100 nM LPA (+) and added to either V or L1 cells. After 1 h cells were harvested, analysed and the resulting data normalized and quantified as described in Figure 1. The top panel is a representative blot of L1 cells, while the bottom panel shows results from three independent experiments; means ± S.E.M. *P < 0.05. The data indicate that VEGF-A reduced the amount and/or activity of ILMR produced by HUVECs. Controls and experimental samples were run on a single gel and always analysed in parallel. The lines were added to the Western blotting to indicate where lanes were removed in order to juxtapose the lanes shown in the Figure. (B) V and L1 cells were left resting or stimulated with either LPA (100 nM), VEGF-A (10 ng/ml), bFGF (10 ng/ml), LPA + VEGF-A, or LPA + bFGF for 1 h. Cells were harvested, analysed and the resulting data normalized and quantified as described in Figure 1. The top panel is a representative blot of L1 cells, while the bottom panel shows results from three independent experiments; means ± S.E.M. These data reveal that VEGF-A and bFGF failed to promote phosphorylation of paxillin in RH7777 cells, and did not alter the ability of LPA to trigger this response. n.s., not significant.

Figure 6 ILMR suppressed LPA-induced migration of HDMVECs

(A) HDMVECs were plated on to the filter insert of a Boyden chamber and placed on top of wells containing one of the four types of CM described in Figure 5 that was (black bars) or was not (white bars) supplemented with 500 nM LPA. Pilot experiments indicated that this dose of LPA optimally induced migration (results not shown). The duration of the assay was 4 h. Means ± S.E.M. of three independent experiments are presented in the histogram. *P < 0.05; n.s.: not significant. The results indicate that the impact of the panel of CM on LPA-induced migration of ECs was comparable with its effect on LPA-induced phosphorylation of paxillin in RH7777 cells (Figure 4A). (B) HDMVECs were subjected to a Boyden-chamber-based migration assay in which the bottom wells contained DMEM alone (0), 100 nM LPA, 10 ng/ml VEGF-A or bFGF. The assay was ended after 4 h and the data were processed as in (A). (C) Similar to the experiment shown in (B), except that the time course was extended to 16–18 h. The results confirm that VEGF-A and bFGF promoted migration of ECs, and demonstrated that the time course of this response was slower than when driven with LPA. *P < 0.05 and **P < 0.01.

possible that VEGF-A promoted phosphorylation of paxillin, which had an impact upon the interpretation of the effect of ILMR. However, this did not seem to be the case; VEGF-A failed to stimulate phosphorylation of paxillin, and did not influence the...
magnitude of this response in LPA-treated cells (Figure 5B). We conclude that VEGF-A reduced the amount and/or activity of ILMR produced by HUVECs.

Finally, we assessed whether ILMR attenuated LPA-mediated migration of ECs. To this end, we monitored LPA-induced migration of HDMVECs (Figure 6A). CM produced from unstimulated or bFGF-stimulated HUVECs attenuated this response, whereas CM from VEGF-stimulated cells was ineffective (Figure 6A). In this set of experiments, we avoided the contribution of VEGF and bFGF (which were present in the CM collected from VEGF-A and bFGF-stimulated cells) by monitoring migration before these growth factors had a detectable influence. While 4 h was sufficient to observe LPA-induced migration (Figure 6B), 16–18 h was required for HDMVECs to respond to VEGF-A and bFGF. We conclude that ILMR had an impact upon not only LPA-triggered signalling events in a model cell line (RH7777), but also attenuated LPA-stimulated cellular responses in at least one type of EC.

DISCUSSION

In the present paper, we report that VEGF-A suppressed the amount and/or activity of ILMR, a secreted agent that attenuated responsiveness of cells to LPA. ILMR appeared to act at the level of cells, by blocking LPA-dependent signalling events and cellular responses such as migration.

A shortcoming of our newly developed LPA bioassay is that it is limited to LPA1. This raises the obvious question of whether the effect of ILMR extends to the other five LPA receptors. The fact that ECs express multiple LPA receptors [27], and the observation that ILMR blunted LPA-mediated migration of HDMVECs (Figure 6), suggests that LPA1 is not the only receptor subject to the influence of ILMR.

A question that arises from our observations is the mechanism of action of ILMR. Since ILMR attenuated multiple, early LPA-dependent signalling events without influencing the amount of LPA, we speculate that ILMR desensitizes LPA1. Supporting this idea is the observation that GPCR kinase 2, a kinase that phosphorylates and desensitizes GPCRs [28], also desensitizes LPA1 [29]. Furthermore, activation of protein kinase C family members is associated with internalization of LPA1 independently of agonist occupation [12]. Additional studies are required to elucidate the mechanism of action by which ILMR attenuates LPA-dependent events and to determine how many of the LPA receptor family members are subject to this mode of regulation.

An additional burning question is the identity of ILMR. Such information is likely to advance our understanding of the indirect action of VEGF-A and reveal additional regulators of LPA-mediated events. In light of the breadth of action of LPA, identification of ILMR, an agent that blunts responsiveness to LPA, is likely to guide development of therapeutic strategies to combat cancer and other angiogenesis-dependent pathologies.

The canonical view of how VEGF-A promotes angiogenesis is by engaging its receptors that trigger signalling events leading to cellular responses required for the angiogenic programme. Our finding that VEGF-A reduced antagonism of LPA-mediated responses suggests that VEGF-A may also promote angiogenesis by increasing responsiveness of ECs to additional pro-angiogenic agents such as LPA.

AUTHOR CONTRIBUTION

Andrius Kazlauskas and Eun Young Park designed the experiments, interpreted the data and wrote the paper. Eun Young Park performed all of the experiments.

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