Lysophosphatidic Acid and Receptor-Mediated Activation of Endothelial Nitric-Oxide Synthase†

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ABSTRACT: Both lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) are platelet-derived phospholipids that elicit diverse biological responses. In endothelial cells, S1P stimulates the EDG-1 receptor-mediated activation of the endothelial isoform of nitric oxide synthase (eNOS), but the role of LPA in eNOS regulation is less well understood. We now report that LPA treatment of bovine aortic endothelial cells (BAEC) activates eNOS enzyme activity in a pathway that involves phosphorylation of eNOS on serine 1179 by protein kinase Akt. In contrast to the cellular responses elicited by S1P in COS-7 cells, LPA can stimulate the activation of eNOS and Akt independently of EDG-1 receptor transfection. LPA-stimulated enzyme activation was significantly attenuated in an eNOS mutant lacking the site that is phosphorylated by kinase Akt (eNOS S1179A). In BAEC, activation of eNOS by LPA is completely blocked by pertussis toxin, by the intracellular calcium chelator BAPTA (1,2-bis(aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid), and by the phosphoinositide 3-kinase (PI3−K) inhibitor wortmannin, but is unaffected by U0126, an inhibitor of mitogen-activated protein (MAP) kinase pathways. Analysis of the LPA dose response for eNOS activation reveals an EC_{50} of ∼40 nM, a concentration well below the potency of LPA at the EDG-1 receptor. Taken together, these results indicate that LPA potently activates eNOS in BAEC in a pathway distinct from the EDG-1 receptor, but mediated by a similar receptor-mediated pathway dependent on pertussis toxin-sensitive G proteins and involving activation of the PI3-K/Akt pathway. These studies have identified a role for the phospholipid LPA in eNOS activation, and point out the complementary role of distinct platelet-derived lipids in endothelial signaling pathways.

Lysophosphatidic acid (LPA)[1] is a bioactive phospholipid released into the extracellular environment by blood platelets that have been activated by platelet agonists such as thrombin (*1*−*4*). In vascular tissues, LPA binds to members of a family of G protein-coupled EDG receptors, and elicits diverse responses such as platelet aggregation (*5*, *6*) and endothelial cell migration (*7*, *8*). The regulatory mechanisms involved in receptor-mediated LPA release are not yet fully elucidated. Although LPA is present in serum at a concentration in the low-micromolar range (*9*−*11*), a significant fraction of serum LPA appears to be protein-bound, in association with serum albumin (*9*, *12*) and gelsolin (*13*) among other proteins. LPA is one of several bioactive platelet-derived phospholipids and sphingolipids that have been discovered to have important roles in intercellular signaling (for review, see refs *2*, *14*, and *15*).

Two of the best-characterized signaling phospholipids, LPA, and sphingosine 1-phospate (S1P), represent structurally distinct molecules that are synthesized in separate enzymatic pathways. The formation of LPA, a glycerophospholipid, appears to involve the action of phospholipase A2 (*16*, *17*) and phospholipase D (*18*, *19*) on preexisting phospholipids. By contrast, S1P is a sphingolipid that is formed by the action of sphingosine kinase on sphingosine (*20*). Both S1P and LPA serve as ligands for different members of the G protein-coupled EDG receptor family. Eight distinct subtypes of EDG receptors have been identified in mammalian cells, four of which (EDG-1, -2, -3, and -5) appear to be expressed in bovine aortic endothelial cells (*7*, *21*). EDG-1, -3, and -5 are selectively activated by S1P, whereas EDG-2 preferentially binds to LPA. Responses mediated by the EDG-2 receptor are blocked by pertussis toxin and involve the mobilization of intracellular calcium (*7*). Recent work from our group (*22*) has established that one important response to S1P-mediated activation of the EDG-1 receptor is the stimulation of the beta isoform of the phosphoinositide 3-kinase (PI3-Kβ) in vascular endothelial cells. Activation of PI3-Kβ by S1P leads to the activation of the endothelial isoform of nitric-oxide synthase (eNOS) (*22*), a key signaling enzyme. The effects of S1P on PI3-K and eNOS pathways have been explored in recent reports

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[1] Abbreviations: LPA, lysophosphatidic acid; S1P, sphingosine 1-phosphate; MAP kinase, mitogen-activated protein kinase; eNOS, endothelial isoform of nitric-oxide synthase; PI3-K, phosphoinositide 3-kinase; FBS, fetal bovine serum; BAPTA, 1,2-bis(aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; BAEC, bovine aortic endothelial cells; FLAG/EDG-1, EDG-1 receptor epitope-tagged with FLAG peptide; PAGE, polyacrylamide gel electrophoresis.

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(*22*, *23*), but the roles of LPA in eNOS regulation by PI3-K are less well characterized.

eNOS is a key determinant of vascular tone and platelet function, and may also play an important role in the regulation of angiogenesis (see review in refs *24* and *25*). eNOS is a calcium-calmodulin dependent enzyme, and undergoes activation in response to diverse calcium-mobilizing agonists in endothelial cells. Several protein kinases are known to phosphorylate eNOS, of which the protein kinase Akt, which is regulated by PI3-K, has been extensively characterized (*26*−*28*). Phosphorylation of eNOS at Ser^{1179} by protein kinase Akt activates eNOS, but different receptor pathways modulate distinct kinases in the process of eNOS activation. For example, S1P-mediated stimulation of eNOS involves the G protein-mediated activation of PI3-Kβ, whereas vascular endothelial growth factor (VEGF) activates eNOS via the G protein-independent stimulation of PI3-Kα as well as PI3-Kβ (*22*). By contrast, the agonist bradykinin activates pertussis toxin-insensitive G proteins and appears to activate eNOS independently of the PI3-K pathway (*29*). The role of LPA in eNOS activation is not well understood, but LPA has been previously shown to activate the PI3-K pathway in other cell types including COS-7 cells (*3*, *30*−*33*). We now report that LPA treatment of bovine aortic endothelial cells (BAEC) activates eNOS via a receptor-mediated pathway that appears to be distinct from the eNOS activation elicited by S1P.

EXPERIMENTAL PROCEDURES

Materials. Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT); all other cell culture reagents, media, and LipofectAMINE were from Life Technologies Inc. (Frederick, MD). S1P and LPA were from Biomol (Plymouth Meeting, PA). U0126, wortmannin, BAPTA, and pertussis toxin were from Calbiochem (San Diego, CA). Anti-phospho-eNOS antibody (phospho-Ser^{1177} in the human eNOS sequence, corresponding to Ser^{1179} in bovine eNOS), anti-phospho-Akt antibody (Ser^{473}) and anti-phospho-ERK1/2 antibody (Thr^{202}/Tyr^{204}) were from Cell Signaling Technologies (Beverly, MA). Anti-eNOS monoclonal antibody was from Transduction Laboratories (Lexington, KY). Super Signal substrate for chemiluminescence detection and secondary antibodies conjugated with horseradish peroxidase were from Pierce (Rockford, IL). L-[^{3}H] Arginine was from Amersham Pharmacia Biotech (Piscataway, NJ). Protein determinations were made with the BioRad protein assay kit. FuGENE 6 was from Roche (Indianapolis, IN). All other reagents including anti-FLAG monoclonal antibody were from Sigma.

Plasmids. Plasmids used in these studies have been described previously including wild-type bovine eNOS (*34*) and Akt phosphorylation site-deficient (S1179A) mutant eNOS cDNA (*35*). cDNA encoding full-length human EDG-1 receptor epitope-tagged with FLAG peptide (FLAG/EDG-1) was provided by Timothy Hla, University of Connecticut (*36*), and was subcloned into pcDNA3 (Invitrogen) and characterized as described (*37*).

Cell Culture, Transfection and Drug Treatment. Bovine aortic endothelial cells (BAEC) were obtained from Cell Systems (Kirkland, WA) and maintained in culture in Dulbecco's Modified Eagle's medium supplemented with FBS (10% in V/V) as described (*38*). Cells were plated onto gelatin-coated culture dishes and studied prior to cell confluence between passage 5 and 9. COS-7 cells were maintained in culture as described (*39*) and transfected with 1 μg of wild-type or S1179A mutant eNOS cDNA using LipofectAMINE following the manufacturer's protocol. For cotransfection experiments, cDNA encoding FLAG/EDG-1 (0.47 μg) plus wild-type eNOS (0.3 μg) or an identical amount of vector DNA was transfected using 3 μL of FuGENE 6 following the protocol provided by the manufacturer. Prior to analysis, cells were incubated overnight with culture medium without FBS to exclude the effects of S1P and LPA contained in FBS (*7*, *29*). LPA was dissolved in water followed by short-term sonication. U0126 was dissolved in dimethyl sulfoxide. Other drugs were prepared as described previously (*29*). For drug treatment, see ref *29*.

Immunoblot Analysis and NOS Activities Assay. Cell lysates were prepared using a cell lysis buffer containing Nonidet P-40, and the degree of protein expression and phosphorylation were assessed as we previously described in detail (*35*). eNOS activity was quantified as the formation of L-[^{3}H] citrulline from L-[^{3}H] arginine as described previously (*29*, *40*). Briefly, reactions were initiated by adding L-[^{3}H] arginine (10 μCi /mL, diluted with unlabeled L-arginine to give a final concentration of 10 μM) plus various drug treatments as described below; each treatment was performed in triplicate cultures, which were analyzed in duplicate. NOS activity, measured as L-[^{3}H] citrulline formation, was expressed as fmol of L-[^{3}H] citrulline produced (mg of cellular protein)$^{-1}$ min^{-1}.

Other Methods. All experiments were performed at least three times. Mean values for individual experiment were expressed as mean ± SE. EC_{50} values (half-maximal effective concentrations) were determined by interpolation between the maximal and minimal concentrations yielding (respectively) basal and maximal responses; for EC_{50} determinations from immunoblots, the signals were quantitated by densitometry. Statistical differences were analyzed by unpaired t-test. A p value less than 0.05 was considered statistically significant.

RESULTS

We first studied the effect of LPA treatment on eNOS activity in bovine aortic endothelial cells (BAEC). Cultured BAEC were labeled with L-[^{3}H]-arginine and treated with increasing concentrations of LPA for 10 min. eNOS activity was quantitated by measuring the formation of L-[^{3}H] citrulline, the coproduct of NOS enzyme reaction, as described in the experimental procedures section. As shown in Figure 1, LPA dose-dependently increases eNOS activity in BAEC, activating the enzyme by more than 2-fold. The EC_{50} for LPA-induced activation of eNOS is 40 ± 20 nM (n = 4).

We next studied the effects of LPA on eNOS Ser^{1179} phosphorylation, as well as Akt and ERK phosphorylation in BAEC. We exploited antibodies that specifically recognize phosphorylated (activated) forms of eNOS (Ser^{1179}), kinase Akt, and the MAP kinases ERK1/2. As shown in Figure 2, after the addition of LPA (1 μM) to BAEC, eNOS Ser^{1179} and kinase Akt phosphorylation increased reaching a maximum ∼2-fold increase within 1∼2 min, and persisting for

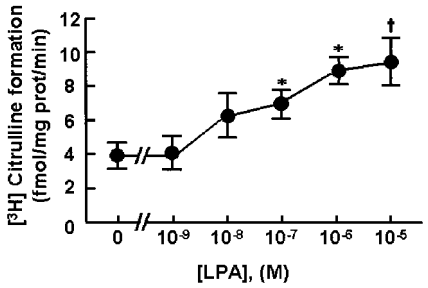

FIGURE 1: eNOS activation by LPA in BAEC. Shown are the results of eNOS activity assays analyzed in BAEC treated with varying concentrations of LPA for 10 min. eNOS activity was quantitated in intact cells by measuring the formation of L-[^{3}H] citrulline from L-[^{3}H] arginine, as described in the text. Each data point represents the mean ± SE derived from four independent cell preparations, each performed in triplicate. ∗: $P < 0.05$, †: $P < 0.01$.

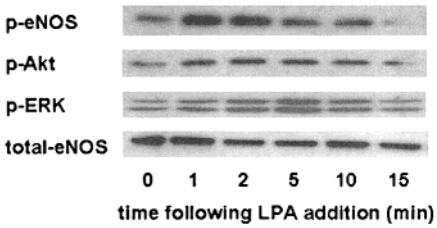

FIGURE 2: Time course of LPA-induced phosphorylation of Akt, ERK1/2, and eNOS Ser1179 in BAEC. Shown are the results of immunoblots prepared from BAEC treated with LPA (1 μM) for varying times. Cell lysates were resolved by SDS−PAGE and analyzed in immunoblots probed separately with specific antibodies directed against Ser^{1179}-phospho-eNOS, phospho-Akt, phospho-ERK1/2, and total eNOS, as indicated. The experiment shown is representative of six independent experiments that yielded similar results.

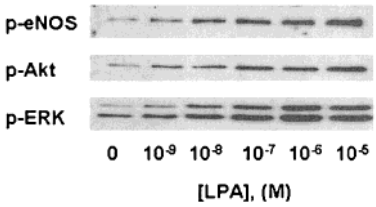

FIGURE 3: Dose response for LPA-induced phosphorylation of Akt, ERK1/2, and eNOS Ser^{1179} in BAEC. BAEC were treated with LPA for 5 min at indicated concentrations, and cell lysates were resolved by SDS−PAGE and analyzed in immunoblots probed with antibodies directed against phospho-Akt, phospho-ERK, and Ser^{1179}-phospho-eNOS, as indicated. The experiment shown is representative of four experiments that gave equivalent results.

at least 10 min. Kinase ERK1/2 also underwent robust and rapid phosphorylation following the addition of LPA (Figure 2). These same immunoblots were probed with an antibody against total eNOS, and documented equivalent protein loading under the different experimental treatments. Figure 3 shows a dose response to LPA for Akt and ERK1/2 phosphorylation and Ser^{1179}-phospho-eNOS formation in BAEC. Treatment of BAEC with LPA (5 min) induced the dose-dependent formation of Ser ^{1179}-phospho-eNOS, as well as the phosphorylation of Akt and ERK. These responses to LPA became apparent at 1 nM and had an EC_{50} of 43 ± 19 nM LPA ($n = 6$), similar to the EC_{50} value determined for LPA-induced eNOS enzyme activation (Figure 1).

We next characterized the effects of LPA on eNOS phosphorylation in COS-7 cells transiently transfected with either wild-type eNOS or an eNOS mutant in which the

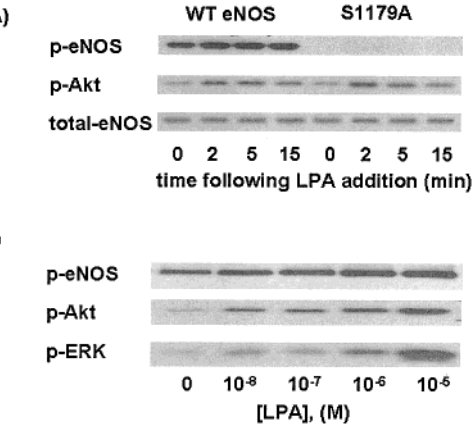

FIGURE 4: Effects of LPA on phosphorylation of Akt, ERK, and eNOS Ser^{1179} in COS-7 cells transfected with wild-type or S1179A mutant eNOS. Shown are the results of immunoblot analyses of COS-7 cells transfected with cDNA encoding wild-type eNOS or S1179A mutant eNOS, as indicated. Cell lysates were resolved by SDS−PAGE, and immunoblots were probed with antibodies against phospho-Akt, phospho-ERK, or Ser^{1179}-phospho-eNOS, as shown. This figure is representative of four independent experiments that gave equivalent results. In panel A, the cells were treated with 1 μM LPA for indicated times. In panel B, the cells were treated for 5 min with increasing concentrations of LPA, as indicated.

putative Akt phosphorylation site (Ser^{1179}) was replaced by alanine (S1179A). Analyses of immunoblots probed with an antibody specific for Ser^{1179}-phospho-eNOS showed that LPA induced the rapid phosphorylation of wild-type eNOS, while there was no phospho-eNOS signal detected in COS-7 cells transfected with the S1179A mutant eNOS (Figure 4A). LPA treatment also induced the phosphorylation (activation) of kinase Akt in COS-7 cells, whether transfected with the wild-type eNOS or the S1179A mutant eNOS. Figure 4B shows the results of a LPA dose response experiment performed in COS-7 cells transfected with wild-type eNOS. This experiment reveals that LPA promotes the phosphorylation (activation) of kinase Akt as well as eNOS with an EC_{50} of 46 ± 24 nM ($n = 4$), similar to the EC_{50} for LPA-induced eNOS activation and kinase phosphorylation documented in BAEC (Figures 1 and 3).

The response of COS-7 cells to LPA suggested that there may be a LPA-responsive EDG receptor endogenously expressed in COS cells. This was in contrast to our previous observations studying responses to the sphingolipid S1P, in which we found that S1P-mediated Akt phosphorylation and eNOS activation in COS-7 cells were both completely dependent upon transfection of cDNA encoding the EDG-1 receptor (*29*, *37*). We therefore characterized the responses to LPA and S1P in COS-7 cells transfected with eNOS cDNA, with or without cotransfected EDG-1 cDNA. As shown in Figure 5, COS-7 cells treated with LPA show robust phosphorylation of kinase Akt and eNOS in either the presence or absence of cotransfected EDG-1. By contrast, the same phosphorylation responses to S1P are completely dependent upon the cotransfection of the EDG-1 receptor.

Having established that LPA-induced eNOS and Akt phosphorylation in COS-7 cells were both independent of the EDG-1 receptor, we characterized the LPA response in greater detail. COS-7 cells were transfected with either wild-type or S1179A mutant eNOS, treated with increasing concentrations of LPA and assayed for eNOS activity as described above. In comparison to the activation of wild-

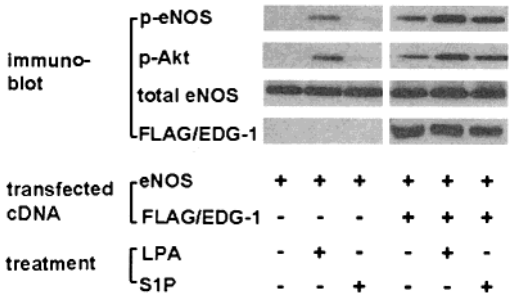

FIGURE 5: Differential effects of EDG-1 transfection on Akt and eNOS Ser^{1179} phosphorylation induced by LPA and S1P in COS-7 cells. COS-7 cells were cotransfected with cDNA encoding eNOS, with or without FLAG/EDG-1; the total amount of DNA was normalized by the addition of empty vector DNA for each transfection. The cells were serum starved overnight 36 h after transfection and then treated for 5 min with either LPA (1 μM), S1P (100 nM), or vehicle, as indicated. Cell lysates (20 μg/lane) were resolved by SDS−PAGE and analyzed in immunoblots probed with antibodies directed against phospho-Akt and Ser^{1179}-phospho-eNOS, as indicated; immunoblots probed with antibody against total eNOS showed equivalent levels of eNOS expression. The experiment shown is representative of four independent experiments.

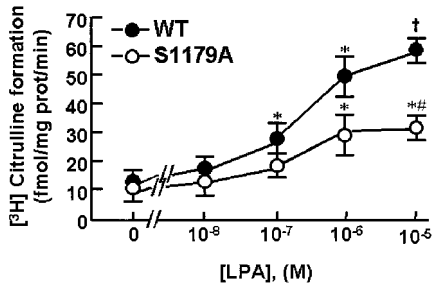

FIGURE 6: LPA treatment and enzyme activation of wild-type and S1179A mutant eNOS. Shown are the results of eNOS activity assays performed in COS-7 cells transfected with cDNA encoding wild-type eNOS or S1179A mutant eNOS. Cells were incubated with L-[^{3}H] arginine and treated with increasing concentration of LPA; eNOS activity was assayed as the formation of L-[^{3}H] citrulline, as described in detail in the text. Equivalent levels of eNOS protein expression were confirmed by immunoblot analysis (Figure 4A). Results shown are representative of three experiments, each performed in duplicate. $*$ indicates $p < 0.05$ versus vehicle treatment. † indicates $p < 0.01$ versus vehicle treatment. # indicates $p < 0.05$ versus wild-type eNOS transfected cells.

type eNOS, LPA-induced activation of the S1179A mutant eNOS was significantly attenuated (Figure 6).

We next explored the more proximal signaling pathways of LPA-mediated eNOS Ser^{1179} phosphorylation in BAEC (Figure 7). Pretreatment of BAEC with wortmannin, an inhibitor of PI3-K, blocked the LPA-induced phosphorylation of kinase Akt and also blocked formation of Ser^{1179}-phospho-eNOS, but did not affect the phosphorylation of ERK induced by LPA. In contrast, pretreatment of BAEC with U0126, an inhibitor of MAP kinase kinase, completely blocked LPA-induced ERK phosphorylation, but had no effect on either eNOS or Akt phosphorylation induced by LPA. Treatment of BAEC with BAPTA, an intracellular calcium chelator, completely blocked LPA-mediated formation of Ser^{1179}-phospho-eNOS as well as blocking the phosphorylation of Akt. As we have previously observed (*29*), treatment of endothelial cells with BAPTA results in a marked increase in ERK1/2 phosphorylation (Figure 7). In exploring the G-protein pathways involved in the response to LPA, we found that BAEC treated with pertussis toxin showed

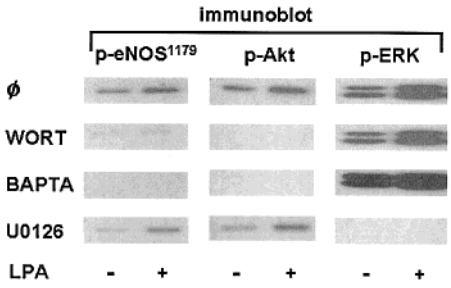

FIGURE 7: Pharmacological characterization of LPA-mediated phosphorylation of Akt, ERK1/2, and eNOS. BAEC cells were pretreated with BAPTA (20 μM), wortmannin (WORT, 500 nM), U0126 (10 μM), or vehicle (ϕ) as indicated for 30 min prior to LPA treatment (1 μM for 5 min). Cell lysates were resolved by SDS−PAGE and analyzed in immunoblots probed separately, as indicated, using specific antibodies directed against Ser^{1179}-phospho-eNOS, phospho-Akt, and phospho-ERK1/2. The results shown are representative of three independent experiments that yielded similar results.

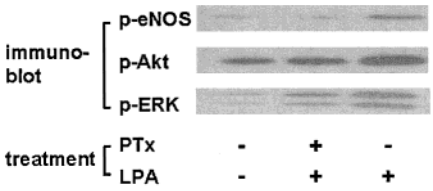

FIGURE 8: Effects of pertussis toxin on LPA-mediated phosphorylation of kinase Akt, ERK1/2, and eNOS. BAEC cells were incubated overnight with pertussis toxin (PTx, 50 ng/mL) or vehicle, then treated with LPA (1 μM for 5 min). Cell lysates were resolved by SDS−PAGE and analyzed in immunoblots probed separately, as indicated, with specific antibodies directed against Ser^{1179}-phospho-eNOS, phospho-Akt, or phospho-ERK1/2. The results shown are representative of three independent experiments that gave equivalent results.

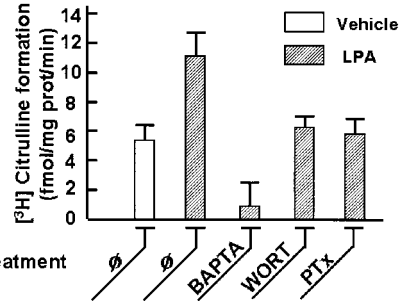

FIGURE 9: Effects of pharmacological treatments on eNOS activation by LPA. Shown are the results of eNOS activity assays analyzed in BAEC subjected to various treatments as indicated: pertussis toxin (PTx, 50 ng/mL overnight), BAPTA (20 μM for 30 min), wortmannin (WORT, 500 nM for 30 min), or vehicle (ϕ). The treated cells were exposed to LPA (1 μM) or vehicle (ϕ) for 5 min, and eNOS activity was assayed as described in the text. Each data point represents the mean ± SE from four independent cell preparations, each analyzed in triplicate.

significantly attenuated LPA-induced phosphorylation of eNOS as well as Akt and ERK1/2 (Figure 8).

We next characterized the effects of different pharmacological interventions on LPA-induced eNOS enzyme activity (Figure 9). In BAEC, LPA-mediated eNOS activation was abolished by the calcium chelator BAPTA as well as by pertussis toxin treatment. The MAP kinase kinase inhibitor U0126 had no effect on eNOS activation induced by LPA (eNOS activity observed with LPA plus U0126 was 115 ± 13% of the activity seen with LPA treatment alone; $n = 4$,

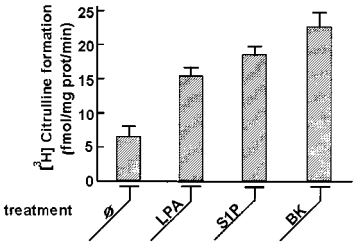

FIGURE 10: Relative levels of eNOS activation induced by LPA, S1P, and bradykinin in BAEC. Shown are the results of eNOS activity assays performed in BAEC treated with LPA (1 μM), S1P (100 nM), bradykinin (BK, 1 μM), or vehicle (ϕ) as indicated in the figure. Each data point represents the mean ± SE from three independent cell preparations, each performed in triplicate.

$p = 0.36$). By contrast, the PI3-K inhibitor wortmannin, which completely abrogated eNOS Ser1179 phosphorylation (Figure 7), also blocked eNOS activation induced by LPA in BAEC; wortmannin had no effect on LPA-induced ERK phosphorylation (Figure 7). The magnitude of eNOS activation induced by LPA was slightly less than that induced by the eNOS agonists S1P or bradykinin (Figure 10), and there was no additivity of the eNOS response when these individual agonists were added at their maximally effective concentrations (data not shown).

DISCUSSION

These studies have shown that LPA induces eNOS activation in bovine aortic endothelial cells, a rapid and robust response mediated by pertussis toxin-sensitive G proteins and associated with the LPA-promoted activation of kinase Akt and phosphorylation of eNOS on Ser^{1179}. In BAEC, the EC_{50} for LPA-induced eNOS activation was found to be ~40 nM (Figure 1), and a similar EC_{50} was found for LPA-induced phosphorylation of kinase Akt and of MAP kinases ERK1/2, as well as the formation of Ser^{1179}-phospho-eNOS (Figure 3). The serum concentration of LPA is probably in the low micromolar range (*9*−*11*), yet it is known that a considerable fraction of LPA is protein-bound both in serum (*9*, *12*) and in the extracellular matrix (*13*). The concentration of free LPA is thus considerably lower, and is likely to be determined both by the dynamics of its protein binding, and may also be influenced by the local release of LPA from activated platelets, the principal source of LPA in the blood (*9*). It is plausible that alterations in LPA binding to proteins, as well as platelet activation, may serve to alter the local concentrations of LPA at the vascular wall.

We were surprised at first to observe that LPA elicits significant eNOS responses in COS-7 cells in the absence of any transfected EDG receptors (Figures 4 and 5). We had previously reported (*37*) that the receptor-mediated effects of S1P on COS-7 cells were completely dependent upon transfection of the EDG-1 receptor, an observation confirmed in the present studies (Figure 5). By contrast, we found that LPA was able to promote the robust activation of transfected eNOS, as well as the phosphorylation of endogenous kinases Akt and ERK1/2, without the requirement for EDG-1 receptor transfection (Figures 4 and 5). Even taken alone, the dose response to LPA in BAEC and COS-7 indicates that involvement of the EDG-1 receptor is implausible: although LPA can serve as an agonist at EDG-1 receptors, it does so only at a high (micromolar) LPA concentrations (*41*). We observed an EC_{50} for LPA-induced kinase activation and eNOS phosphorylation of ~40 nM; this EC_{50} for LPA is characteristic of the potency of LPA at its cognate EDG receptors (*42*−*45*), but is orders of magnitude below the potency of LPA at the EDG subtypes that are typically responsive to S1P (e.g. EDG-1, *41*). By contrast, the EDG-2 receptor is known to couple LPA to intracellular responses mediated by pertussis toxin-sensitive G proteins (*42*). Detection of EDG-2 receptor protein in has been problematic because there are currently no commercially available EDG subtype-specific antibodies capable of detecting the low levels of EDG-2 protein expression that likely are characteristic of this G protein-coupled receptor. However, several prior studies have detected EDG-2 mRNA in both BAEC and COS-7 cells (*7*, *46*). Moreover, EDG-2 is involved in LPA induced G protein-kinase Akt-mediated cell survival (*30*), and the heterologous expression of EDG-2 cDNA is required for LPA-induced ERK2 activation (*47*) in HEK293 cells, which otherwise do not express EDG-2 receptor transcripts (*48*). All these prior data taken together, it seems most plausible that eNOS responses to LPA in BAEC are modulated by EDG-2 receptors, which are known to be expressed in this cell type. However, since subtype-selective EDG receptor antagonists are not available, it is difficult to perform more definitive pharmacological characterizations, but at the least, our data argue strongly against a role for the EDG-1 receptor in LPA-mediated eNOS responses in endothelial cells. Despite their both belonging to the broad category of signaling phospholipids, the essential structural differences between S1P (sphingosine-based) and LPA (glycerol-based), undoubtedly underlie the receptor subtype selectivity of these two ligands. Another level of complexity derives from the heterogeneity of signaling pathways elicited by EDG receptor isoforms, which themselves are differentially expressed in different endothelial cell types (*7*, *49*, *50*).

LPA treatment of BAEC exhibits a similar time course (Figure 2) and dose−response (Figure 3) for activation (phosphorylation) of the kinase Akt as well as eNOS (Figures 2 and 3). It must be noted that our results are at variance with a recent report wherein LPA was found to activate eNOS without inducing the formation of Ser^{1179} phospho-eNOS in NIH-3T3 cells that were transfected with eNOS. These differences may reflect the fact that we have focused on characterizing the role of LPA in eNOS regulation in native endothelial cells, whereas the prior study (*51*) largely confined its analyses to LPA responses studied in heterologous overexpression systems (transfected NIH-3T3 cells). These differences may identify an important caveat in extrapolating to native cells the characteristics of signaling systems analyzed in transfected cells. Indeed, in our studies of eNOS activation in transfected COS-7 cells, we found that LPA was still able to promote some activation (albeit to a lesser extent) of the S1179A mutant eNOS. By contrast, in BAEC, wortmannin completely blocked any LPA-induced eNOS activation. Thus, in COS-7 cells, but not in endothelial cells, there appears to be a small but significant component of LPA-induced eNOS activation that occurs independently of the PI3-K/Akt pathway. In COS-7 cells, it is possible that other LPA-mediated cellular responses, perhaps involving calcium mobilization or the activation of distinct protein kinase pathways, may be sufficient to support eNOS activation independent of the PI3-K/Akt pathway.

The MAP kinase pathway inhibitor U0126 has no substantive effect on kinase Akt phosphorylation (Figure 7) or eNOS activation, under conditions in which this inhibitor completely blocks LPA-mediated ERK1/2 phosphorylation. These data argue against a major role for the MAP kinase pathway in eNOS regulation induced by LPA treatment in these endothelial cells, although LPA-mediated MAP kinase activation may play other important roles, such as endothelial cell proliferation and migration (*7*). The calcium chelator BAPTA completely blocks LPA-induced Akt phosphorylation and eNOS activation, but apparently leads to an increased phosphorylation of ERK1/2 (Figure 7), as we have previously reported in experiments exploring the S1P/EDG-1 response (*29*). These observations argue for a role of calcium signaling in potentiating the PI3-K/Akt response, while inhibiting the MAP kinase pathway. Again, these data are at variance with the same recent report cited above (*51*) in which it was found that BAPTA blocked neither the insulin-mediated kinase Akt phosphorylation nor the formation of Ser^{1179}-phospho-eNOS. Perhaps part of the explanation for this discrepancy may reflect the differential role of calcium-dependent signaling in different receptor pathways.

Our studies indicate that the signaling phospholipid LPA should be added to the growing list of eNOS agonists. The magnitude of LPA-induced eNOS activation in endothelial cells, while significant, is slightly less than that induced by the classical agonist bradykinin or by the sphingolipid S1P. What is perhaps most striking about these new results is the fact that another platelet-derived phospholipid appears to play a role in receptor-mediated eNOS regulation. These findings raise the possibility that intercellular signaling between platelets and endothelial cells may importantly modulate NO-dependent signaling pathways in the vascular wall.

REFERENCES

1. Goetzl, E. J., and An, S. (1998) *FASEB J. 12*, 1589−1598.
2. Moolenaar, W. H. (1995) *J. Biol. Chem. 270*, 12949−12952.
3. Kranenburg, O., and Moolenaar, W. H. (2001) *Oncogene 20*, 1540−1546.
4. Goetzl, E. J. (2001) *Prostaglandins 64*, 11−20.
5. Gueguen, G., Gaige, B., Grevy, J. M., Rogalle, P., Bellan, J., Wilson, M., Klaebe, A., Pont, F., Simon, M. F., and Chap, H. (1999) *Biochemistry 38*, 8440−8450.
6. Siess, W., Zangl, K. J., Essler, M., Bauer, M., Brandl, R., Corrinth, C., Bittman, R., Tigyi, G., and Aepfelbacher, M. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 6931−6936.
7. Lee, H., Goetzl, E. J., and An, S. (2000) *Am. J. Physiol. 278*, C612−C618.
8. Panetti, T. S., Nowlen, J., and Mosher, D. F. (2000) *Arterioscler. Thromb. Vasc. Biol. 20*, 1013−1019.
9. Eichholtz, T., Jalink, K., Fahrenfort, I., and Moolenaar, W. H. (1993) *Biochem. J. 291*, 677−680.
10. Baker, D. L., Desiderio, D. M., Miller, D. D., Tolley, B., and Tigyi, G. J. (2001) *Anal. Biochem. 292*, 287−295.
11. Saulnier-Blache, J. S., Girard, A., Simon, M. F., Lafontan, M., and Valet, P. (2000) *J. Lipid. Res. 41*, 1947−1951.
12. Tigyi, G., and Miledi, R. (1992) *J. Biol. Chem. 267*, 21360−21367.
13. Goetzl, E. J., Lee, H., Azuma, T., Stossel, T. P., Turck, C. W., and Karliner, J. S. (2000) *J. Biol. Chem. 275*, 14573−14578.
14. Moolenaar, W. H. (2000) *Ann. N. Y. Acad. Sci. 905*, 1−10.
15. Tigyi, G. (2001) *Prostaglandins Other Lipid Mediators 64*, 47−62.
16. Fourcade, O., Le Balle, F., Fauvel, J., Simon, M. F., and Chap, H. (1998) *Adv. Enz. Regul. 38*, 99−107.
17. Gerrard, J. M., and Robinson, P. (1989) *Biochim. Biophys. Acta 1001*, 282−285.
18. van Dijk, M. C., Postma, F., Hilkmann, H., Jalink, K., van Blitterswijk, W. J., and Moolenaar, W. H. (1998) *Curr. Biol. 8*, 386-392.
19. Eder, A. M., Sasagawa, T., Mao, M., Aoki, J., and Mills, G. B. (2000) *Clin. Cancer Res. 6*, 2482−2491.
20. Olivera, A., and Spiegel, S. (2001) *Prostaglandins 64*, 123−134.
21. Wang, F., Van Brocklyn, J. R., Hobson, J. P., Movafagh, S., Zukowska-Grojec, Z., Milstien, S., and Spiegel, S. (1999) *J. Biol. Chem. 274*, 35343−35350.
22. Igarashi, J., and Michel, T. (2001) *J. Biol. Chem. 276*, 36281−36288.
23. Morales-Ruiz, M., Lee, M. J., Zoellner, S., Gratton, J. P., Scotland, R., Shiojima, I., Walsh, K., Hla, T., and Sessa, W. C. (2001) *J. Biol. Chem. 276*, 19672−19677.
24. Loscalzo, J., and Welch, G. (1995) *Prog. Cardiovasc. Dis. 38*, 87−104.
25. Folkman, J., and D'Amore, P. A. (1996) *Cell 87*, 1153−1155.
26. Gallis, B., Corthals, G. L., Goodlett, D. R., Ueba, H., Kim, F., Presnell, S. R., Figeys, D., Harrison, D. G., Berk, B. C., Aebersold, R., and Corson, M. A. (1999) *J. Biol. Chem. 274*, 30101−30108.
27. Fulton, D., Gratton, J. P., McCabe, T. J., Fontana, J., Fujio, Y., Walsh, K., Franke, T. F., Papapetropoulos, A., and Sessa, W. C. (1999) *Nature 399*, 597−601.
28. Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R., and Zeiher, A. M. (1999) *Nature 399*, 601−605.
29. Igarashi, J., Bernier, S. G., and Michel, T. (2001) *J. Biol. Chem. 276*, 12420−12426.
30. Weiner, J. A., and Chun, J. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 5233−5238.
31. Roche, S., Downward, J., Raynal, P., and Courtneidge, S. A. (1998) *Mol. Cell Biol. 18*, 7119−7129.
32. Murga, C., Fukuhara, S., and Gutkind, J. S. (2000) *J. Biol. Chem. 275*, 12069−12073.
33. Hawes, B. E., Luttrell, L. M., van Biesen, T., and Lefkowitz, R. J. (1996) *J. Biol. Chem. 271*, 12133−12136.
34. Robinson, L. J., and Michel, T. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 11776−11780.
35. Bernier, S. G., Haldar, S., and Michel, T. (2000) *J. Biol. Chem. 275*, 30707−30715.
36. Lee, M. J., Evans, M., and Hla, T. (1996) *J. Biol. Chem. 271*, 11272−11279.
37. Igarashi, J., and Michel, T. (2000) *J. Biol. Chem. 275*, 32363−32370.
38. Michel, T., Li, G. K., and Busconi, L. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6252−6256.
39. Lamas, S., Marsden, P. A., Li, G. K., Tempst, P., and Michel, T. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6348−6352.
40. Chen, Z., Yuhanna, I. S., Galcheva-Gargova, Z., Karas, R. H., Mendelsohn, M., and Shaul, P. W. (1999) *J. Clin. Invest. 103*, 401−406.
41. Lee, M. J., Thangada, S., Liu, C. H., Thompson, B. D., and Hla, T. (1998) *J. Biol. Chem. 273*, 22105−22112.
42. An, S., Bleu, T., Hallmark, O. G., and Goetzl, E. J. (1998) *J. Biol. Chem. 273*, 7906−7910.
43. Jalink, K., van Corven, E. J., and Moolenaar, W. H. (1990) *J. Biol. Chem. 265*, 12232−12239.
44. Jalink, K., Hengeveld, T., Mulder, S., Postma, F. R., Simon, M. F., Chap, H., van der Marel, G. A., van Boom, J. H., van Blitterswijk, W. J., and Moolenaar, W. H. (1995) *Biochem. J. 307*, 609−616.
45. Fukushima, N., Kimura, Y., and Chun, J. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 6151−6156.
46. Furui, T., LaPushin, R., Mao, M., Khan, H., Watt, S. R., Watt, M. A., Lu, Y., Fang, X., Tsutsui, S., Siddik, Z. H., Bast, R. C., and Mills, G. B. (1999) *Clin. Cancer Res. 5*, 4308−18.
47. Cunnick, J. M., Dorsey, J. F., Munoz-Antonia, T., Mei, L., and Wu, J. (2000) *J. Biol. Chem. 275*, 13842−13848.

48. Fischer, D. J., Liliom, K., Guo, Z., Nusser, N., Virag, T., Murakami-Murofushi, K., Kobayashi, S., Erickson, J. R., Sun, G., Miller, D. D., and Tigyi, G. (1998) *Mol. Pharm. 54*, 979−988.
49. Tigyi, G., Fischer, D. J., Baker, D., Wang, D. A., Yue, J., Nusser, N., Virag, T., Zsiros, V., Liliom, K., Miller, D., and Parrill, A. (2000) *Ann. N. Y. Acad. Sciences 905*, 34−53.
50. Rizza, C., Leitinger, N., Yue, J., Fischer, D. J., Wang, D. A., Shih, P. T., Lee, H., Tigyi, G., and Berliner, J. A. (1999) *Lab. Invest. 79*, 1227−1235.
51. Montagnani, M., Chen, H., Barr, V. A., and Quon, M. J. (2001) *J. Biol. Chem. 276*, 30392−30398.

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