Lysophosphatidic acid induces endothelial cell death by modulating the redox environment

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Submitted 30 August 2006; accepted in final form 13 November 2006

Lysophosphatidic acid (LPA), a key intermediate in glycerolipid synthesis, is particularly abundant in the brain (16); its concentration increases further during injury (64) associated with oxidative stress and ensuing activation of phospholipases (7, 50), which catalyze LPA formation (52). LPA exerts its effects by activation of four G protein-coupled receptors, LPA1, LPA2, LPA3, and the distantly related and newly discovered LPA4 (reviewed in Ref. 31), which are coupled to G proteins Gα1, Gα11,14 (LPA1, LPA2, and LPA3), and G12,13 (LPA1 and LPA2). LPA1, LPA2, and LPA3 are expressed by the majority of cells in the brain (reviewed in Ref. 69); however, LPA1 is the most ubiquitous and conducts crucial functions (14, 15). For a variety of cells, LPA is a known proliferative and survival factor (69) contributed by activation of the multicomponent serum-response factor (25). In this context, during brain development LPA significantly influences neuronal morphology, proliferation, and migration, as well as myelination (reviewed in Ref. 69). However, potent inflammatory functions also have been attributed to LPA, including initiation of calcium mobilization (1, 20), activation of phospholipases (20) and NF-κB (53), and induction of expression of cyclooxygenase (COX)-2, inducible nitric oxide synthase (iNOS) (20, 24), adhesion molecules (53, 56), and cytokines (53); these effects of LPA could have impact on cell survival (22, 63).

Compared with other cells of the microvasculature, endothelial cells are more susceptible to oxidative stress and inflammation (5, 8, 39, 54). Our group has previously shown that LPA triggers inflammatory pathways in cerebrovascular endothelial cells (20). However, the potential cytoidal action of LPA on brain microvascular cells has yet to be described. We hypothesized that LPA modulates the cell redox environment in a receptor-dependent manner to elicit endothelial cell death and corresponding neuromicrovascular rarefaction. Lysophosphatidic acid (LPA), a key intermediate in glycerolipid synthesis, is particularly abundant in the brain (16); its concentration increases further during injury (64) associated with oxidative stress and ensuing activation of phospholipases (7, 50), which catalyze LPA formation (52). LPA exerts its effects by activation of four G protein-coupled receptors, LPA1, LPA2, LPA3, and the distantly related and newly discovered LPA4 (reviewed in Ref. 31), which are coupled to G proteins Gα1, Gα11,14 (LPA1, LPA2, and LPA3), and G12,13 (LPA1 and LPA2). LPA1, LPA2, and LPA3 are expressed by the majority of cells in the brain (reviewed in Ref. 69); however, LPA1 is the most ubiquitous and conducts crucial functions (14, 15). For a variety of cells, LPA is a known proliferative and survival factor (69) contributed by activation of the multicomponent serum-response factor (25). In this context, during brain development LPA significantly influences neuronal morphology, proliferation, and migration, as well as myelination (reviewed in Ref. 69). However, potent inflammatory functions also have been attributed to LPA, including initiation of calcium mobilization (1, 20), activation of phospholipases (20) and NF-κB (53), and induction of expression of cyclooxygenase (COX)-2, inducible nitric oxide synthase (iNOS) (20, 24), adhesion molecules (53, 56), and cytokines (53); these effects of LPA could have impact on cell survival (22, 63).

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MATERIALS AND METHODS

Animals. Yorkshire newborn pigs (Fermes Menard, L’Ange-Gardien, Quebec, Canada) and Sprague-Dawley rat pups (Charles River, St. Constant, Quebec, Canada) were used according to a protocol of the Hôpital Ste-Justine Animal Care Committee.

Endothelial and astroglial cell culture. Endothelial and astroglial cells were isolated from newborn pig brain, purified, and cultured as

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previously described (29). Adult porcine brain microvascular endothelial cells and human umbilical vein endothelial cells (HUVEC) were obtained from Cell Systems andCambrex.

**Cell viability assay.** Cell viability was assessed as previously described (5, 35) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma). Cells were treated with stearyl-LPA (s-LPA), palmitoyl-LPA (p-LPA), oleyl-LPA (o-LPA), oleyl-lysophosphatidylcholine, oleyl-lysophosphatidylethanolamine, dioleoylphosphatidic acid, cyclic phosphatidic acid (1 μmol/l; all from Avanti Polar Lipids), the thromboxane A₂ mimetic U-46619 (1 μmol/l; Cayman Chemical), 15-F₂t-isoprostane (15-F₂t-IsoP; 1/ H9262 mol/l; Cayman Chemical), or H₂O₂ (100 μmol/l; Fisher) for 24 h. In other experiments, cells were pretreated with established concentrations of the following inhibitors: PTX (25 ng/ml overnight; Calbiochem), the allosteric LPA₁ antagonist THG1603 (1–100 μmol/l; Calbiochem), the xanthine oxidase inhibitor apocynin (50 μmol/l; Sigma), the iNOS inhibitor 1400W (1 μmol/l; Calbiochem), the COX inhibitor ibuprofen (1 μmol/l; Sigma), the cytochrome P-450 inhibitor ketoconazole (30 μmol/l; ICN Biochemicals), the phospholipase A₂ inhibitor dicitydine-5-phosphocholine (20 μmol/l; Calbiochem), the p38 MAPK inhibitor SB-203580 (5 μmol/l; Calbiochem), and the JNK inhibitor SP600125 (1 μmol/l; Biomol). To avoid interference of MTT assay by NAC, the culture medium was removed and cells were rinsed with fresh medium before incubation with MTT (10). Cell viability was expressed as a percentage of optical density relative to control.

The nature of cell death (necrosis), defined as oncocytic (associated with plasma membrane leakage) or apoptotic [characterized by DNA fragmentation leading to terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) positivity] (47) was determined using the biochemical live-dead assay (Molecular Probes) and TUNEL (TACS TdT fluorescein kit; R&D Systems) according to the instructions of the manufacturer. The live-dead assay recognizes two parameters of cell viability: intracellular esterase activity and plasma membrane integrity, which are measured, respectively, by the active incorporation of calcein-AM in living cells and the passive incorporation of ethidium homodimer-1 (EthD-1) in oncotic cells (Molecular Probes).

**Phase-contrast and electron microscopy.** Phase-contrast micrographs of porcine cerebromicrovascular endothelial cells (pCMVEC) treated for 24 h with vehicle (control), s-LPA (10 μmol/l), or H₂O₂

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**Fig. 1.** Effects of lysophosphatidic acid (LPA) on survival of newborn porcine cerebral microvascular endothelial cells (pCMVEC; A–E), adult pCMVEC, human umbilical vein endothelial cells (HUVEC), and astroglia (Astro) (all D). A: dose-response (EC₅₀ ≈ 0.2 μmol/l); B: response to stearyl-LPA (s-LPA; 1 μmol/l) at different times of exposure. C: response of pCMVEC to various lipids related or not to s-LPA (1 μmol/l for all lipids); effects of H₂O₂ (100 μmol/l) were also determined for comparison. Veh, vehicle; c-PA, cyclic phosphatidic acid (PA); o-PA, dipalmitoyl-PA; o-LPA, oleyl-LPA; p-PA, palmitoyl-LPA; o-LPC, oleyl-lysophosphatidylcholine; o-LPE, oleyl-lysophosphatidylethanolamine; iP₃, 15-F₂t-isoprostane. D: cell specificity to s-LPA (1 μmol/l)-induced cytotoxicity at 24 h; all cells were unaffected by vehicle and are represented by a single bar regardless of cell type. E: s-LPA-induced endothelial cell death in the presence or absence of the LPA₁ allosteric antagonist THG1603 (IC₅₀ ≈ 60 μmol/l) or the Gₛₐ, inhibitor pertussis toxin (PTX; 25 ng/ml). Cell survival was quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Values are means ± SE of 3–5 experiments, each performed in triplicate. *P < 0.01 compared with control and/or vehicle.
(100 μmol/l) were taken before fixation and processing for electron microscopy, according to standard techniques (20).

Western blot analysis of activated phosphorylated p38 MAPK, phosphorylated Jnk, and cleaved spectrin and caspase-3. pCMVEC were treated with s-LPA (10 μmol/l) for different durations in the presence or absence of THG1603 (100 μmol/l), suramin (100 μmol/l), NAC (2 mM), and the NADPH oxidase inhibitors apocynin (500 μmol/l) and diphenyleneiodonium (DPI; 10 μmol/l; Biomol). Total cell lysates were prepared using conventional RIPA buffer, and protein content was determined using the Bradford method. Cell samples were solubilized in Laemmli buffer, resolved by SDS-PAGE (9%), transferred to polyvinylidene difluoride (PVDF) membranes, and blotted with the following antibodies: anti-JNK (polyclonal, 1:1,000; Cell Signaling), anti-phosphorylated-JNK (polyclonal, 1:1,000; Cell Signaling), anti-p38 MAPK (monoclonal, 1:250; Chemicon), anti-phosphorylated p38 MAPK (monoclonal, 1:1,000; Chemicon), anti-α-spectrin (monoclonal, 1:1,000; Chemicon), and anti-caspase-3 (polyclonal, 1:200; Chemicon).

Fig. 2. Effects of s-LPA on calcein-AM and ethidium homodimer-1 (EthD-1) incorporation (live-dead assay) and DNA fragmentation (TUNEL) in pCMVECs. A: cells were exposed for 24 h to vehicle control (Ctl), s-LPA (1 and 10 μmol/l), or a hypotonic shock (Osm) that consists of culture medium diluted with an equal volume of distilled sterile H2O, as a positive control for oncosis (43), and the live-dead assay was performed. Calcein-AM (green) and EthD-1 (red) are respective markers of live and oncosis cells. Note the intense green staining of cells treated with vehicle (top image) and the decreased number of cells, diminished intensity of green staining, and increased incorporation of EthD-1 (red, arrows) in s-LPA-treated cells (bottom image). B: DNA fragmentation assessed by TUNEL (green, arrows). Cells were treated for 24 h with vehicle (Ctl) or s-LPA (1 μmol/l), processed for TUNEL, and counterstained using propidium iodide (red). Actinomycin D (0.5 μmol/l) treatment served as a positive control for DNA fragmentation. Values are means ± SE of 3 preparations. *P < 0.05 compared with control.
Western blot analysis of iNOS. pCMVEC were treated for 5 h with vehicle (control) or s-LPA (10 μmol/l) in the presence or absence of NAC, SB-203580, and SP-600125. Crude cytosolic fractions were prepared as described previously (20). With standard procedures, immobilized proteins were blotted with antibodies against iNOS (monoclonal, 1:200; Transduction Laboratory), and protein expression was compared with that of control (vehicle-treated cells) after normalization to β-actin (monoclonal, 1:40,000; Novus Biological) using ImagePro Plus 4.1 (Media Cybernetic, Silver Spring, MD).

Measurement of reduced glutathione. Reduced glutathione (GSH) was measured in pCMVEC treated with vehicle (control) or s-LPA (10 μmol/l) for 24 h in the presence or absence of THG1603 and NAC. Cells were harvested in 2% metaphosphoric acid (in HPLC H2O grade) for subsequent measurement of levels (6).

Detection of protein nitration, protein S-nitrosylation, and measurement of 15-F2t-IsoP and hydroperoxides. Tyrosine nitration of proteins, a marker for nitrosative stress, was determined by slot blot of pCMVEC stimulated for 5 and 24 h with vehicle, s-LPA (10 μmol/l), or the peroxynitrite generator SIN-1 (0.1 mmol/l; Cayman Chemical), as previously described (6). pCMVEC also were separately harvested for the measurement of 15-F2t-IsoP (40) and hydroperoxides (9).

S-nitrosylation of proteins was determined on pCMVEC treated with s-LPA (10 μmol/l) using the S-nitrosylated protein detection assay kit (Cayman Chemical); this assay is based on the "biotin-switch" method (32). IL-1β (10 ng/ml) was used as a positive control. Endogenous biotinylated proteins were detected on vehicle-treated cell lysates. Proteins were loaded on PVDF membrane by slot blot, and biotinylated nitrosocysteine residues were detected using streptavidin-horseradish peroxidase (as supplied).

Brain explants and quantification of vascular density. Brain explants of rat pups (postnatal days 6 to 10) were cultured in vitro based on a modification of a retinal explant protocol (12, 55). Brains were sectioned in ice-cold culture medium using a vibratome. Sections obtained (~200 μm thick) were delicately placed in six-well dishes on top of a free-floating membrane (Nucleopore polycarbonate Track Etch, pore size 0.03 μm; Whatman, Brentford, UK). Brain explants were cultured for 4 days in endothelial basal medium (Clonetics), without FBS and supplemented with growth factors (EBM bullet kit; Clonetics), containing vehicle (control), s-LPA (1 μmol/l), or s-LPA with THG1603 (100 μmol/l), NAC (2 mmol/l), SP600125 (1 μmol/l), and SB-203580 (5 μmol/l) or t-NNA (1 mmol/l). Ex vivo monitoring of vascular degeneration was visualized by live-staining the endothelium using FITC-conjugated lectin (Griffonia simplicifolia; Sigma), and vascular density was quantified using a software program (ImagePro Plus 4.1). Control explants did not show signs of vascular degeneration for up to 7 days of culture.

Determination of vascular density in retinas of eyes injected with LPA. In addition to brain, microvascular toxicity of LPA was assessed on another neural tissue, the retina, to which administration of compounds in vivo is less invasive and more easily performed than in the brain. Newborn rat pups were anesthetized with isoflurane and injected intravitreally using glass capillaries (~60 gauge) on postnatal days 1 and 3 with 3–5 μl of vehicle (saline), s-LPA (~1–10 μmol/l) alone, or s-LPA with THG1603 (~100 μmol/l), NAC (~2 mmol/l), SP600125 (~1 μmol/l), and SB-203580 (~5 μmol/l), or t-NNA (~1 mmol/l) [final intracoacral concentrations, based on estimated eye volume (57)]. Pups were killed at postnatal day 6, and eyes were enucleated and fixed in 4% formaldehyde. Retinas were isolated and

Table 1. Intracellular levels of GSH in pCMVEC

<table>
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<tr>
<th>Treatment</th>
<th>GSH Levels (at 24 h), mmol/mg protein</th>
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<tr>
<td>Control (vehicle)</td>
<td>363 ± 24</td>
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<tr>
<td>s-LPA (10 μmol/l)</td>
<td>247 ± 38*</td>
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<tr>
<td>s-LPA (10 μmol/l) + THG1603 (100 μmol/l)</td>
<td>385 ± 46</td>
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<tr>
<td>s-LPA (10 μmol/l) + NAC (2 mmol/l)</td>
<td>330 ± 15</td>
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Values are means ± SE; n = 3. GSH, reduced glutathione; pCMVEC, porcine cerebrovascular endothelial cells; s-LPA, stearoyl lysophosphatidic acid; THG1603, allosteric LPA1 agonist; NAC, N-acetyl-cysteine.

*P < 0.05 compared with all other values.
their microvasculature stained using TRITC-conjugated lectin. Quantification of vascular density was performed on retinal flat mounts (57).

Statistical analysis. Data were analyzed using one-way ANOVA followed by post hoc Dunnett’s test for comparison among means. Values are presented as means ± SE. Statistical significance was set at P < 0.05.

RESULTS

Effects of LPA on endothelial cell survival. s-LPA caused a concentration-dependent death of pCMVEC (EC50 ≈ 0.2 μmol/l) (Fig. 1A). Cell survival was markedly and comparably diminished 5 and 24 h after exposure to s-LPA (Fig. 1B). Moreover, a 10-min duration of exposure to s-LPA (1 μmol/l) was sufficient to trigger pCMVEC death detected 24 h later (14.2 ± 1.6% cell death, P < 0.01). All LPA species tested (s-, α-, and p-LPA) were equivalently cytotoxic; the naturally occurring cyclic PA, which exhibits partial LPA antagonistic activity (49), was ineffective, as expected (Fig. 1C). As well, closely related phospholipids and lysophospholipids did not affect endothelial cell survival (Fig. 1C). s-LPA was as effective as if not superior to other previously described cytotoxic lipid mediators, namely, thromboxane A2 (5, 8, 55), platelet-activating factor (4), the isoprostane 15-F2t-isoP (8), and H2O2 (Fig. 1C). Endothelial cells from different vascular beds, species, and age were also sensitive to s-LPA; astrocytes were resistant (Fig. 1D).

s-LPA induced endothelial cell death was prevented by the LPA1 allosteric antagonist THG1603 (IC50 ≈ 60 μmol/l) (Fig. 1E) as well as by the nonselective G protein blocker suramin (11, 65) (cell survival after exposure to s-LPA alone for 24 h was 60.2 ± 2.1% and was augmented to 82.3 ± 3.6% in suramin-pretreated s-LPA-exposed cells, P < 0.01); PTX did not affect the cytotoxicity of s-LPA (Fig. 1E).

Fig. 4. A: survival of pCMVEC treated with s-LPA (1 μmol/l) in the absence (vehicle) or presence of allopurinol (Allo; xanthine oxidase inhibitor and free radical scavenger, 1 mmol/l), N-acetyl-cysteine (NAC; glutathione precursor, 2 mmol/l), U-74389G (lipid peroxidation inhibitor, 1 μmol/l), catalase (500 units), 1400W [inducible nitric oxide synthase (iNOS) inhibitor, 1 μmol/l], N-α-nitro-arginine (l-NAME, t-NA; nonselective NOS inhibitor, 1 mmol/l), ibuprofen (Ibu; cyclooxygenase inhibitor, 1 μmol/l), MK886 (5-lipoxygenase inhibitor, 2 μmol/l), dicytidine-5-phosphocholine (Dicyt; phospholipases A2 inhibitor, 20 μmol/l), and ketoconazole (Keto; cytochrome P-450 inhibitor, 30 μmol/l). Cell survival was quantified by MTT assay. B: protein S-nitrosylation of pCMVEC treated with vehicle, s-LPA (1 μmol/l) in the absence or presence of THG1603 (THG; 100 μmol/l), or IL-1β (10 ng/ml, positive control) for 5 h. Baseline refers to endogenous biotinylated proteins. C: Western blot of iNOS [130 kDa, normalized to β-actin (48 kDa)] from pCMVEC treated or untreated with s-LPA (1 μmol/l) for 5 h in the absence or presence of NAC (2 mmol/l) or combined p38 and JNK inhibitors [SB-203580 (SB), 5 μmol/l; SP-60125 (SP), 1 μmol/l]. D: Western blot of the phosphorylated and nonphosphorylated forms of JNK (arrows, 46 and 54 kDa) and p38 MAPK (38 kDa) on protein extracts of pCMVEC treated with s-LPA (1 μmol/l) in the absence or presence of the LPA1 allosteric antagonist THG1603 (100 μmol/l), NAC (2 mmol/l), or the NADPH oxidase inhibitors diphenylenediamonium (DPI; 10 μmol/l) and apocynin (Apo; 500 μmol/l). E: combined effects of p38 and JNK inhibitors (SB and SP) on pCMVEC survival in response to s-LPA (1 μmol/l). Values are means ± SE of 3–5 experiments, each performed in triplicate. *P < 0.05 compared with vehicle or control. †P < 0.05 compared with s-LPA-treated cells.
Nature of s-LPA-induced endothelial cell death. The biochemical live-dead assay (Molecular Probes) corroborated the MTT data obtained as cell density diminished in presence of s-LPA (Fig. 2A). Furthermore, in pCMVECs still attached after s-LPA treatment, incorporation of calcein-AM (a marker for live cells) decreased, whereas staining with EthD-1 (a marker for oncotic cells) increased (Fig. 2A). Also, endothelial cells treated with s-LPA did not show signs of classic apoptosis such as cell rounding, nuclear condensation, and apoptotic bodies (Fig. 3, B, B', and B'') and did not exhibit TUNEL positivity (Fig. 2B) or cleaved fragments of spectrin and caspase-3 (data not shown). s-LPA-treated cells resembled H$_2$O$_2$-treated cells as indicated by the overall morphology and ultrastructural features (Fig. 3, B, B', B'', C, C', and C''). Numerous vacuoles were present in s-LPA- and H$_2$O$_2$-treated cells but not in control cells (Fig. 3, A', A'', B', B'', C', C'', and insets); these
phospholipase A2, and cytochrome E). Interestingly, NAC and the NADPH oxidase inhibitors p38 and JNK inhibitors blocked LPA-induced cell death (Fig. D). These observations are consistent with the notion that oxidative stress is involved in LPA-induced cell death.

Effects of inhibitors of major oxidative and nitrosative stress-generating systems on s-LPA-induced cell death. s-LPA treatment significantly decreased endothelial intracellular GSH levels (Table 1). In an attempt to identify the source and nature of ROS involved, we screened numerous antioxidants on endothelial cell death. The majority of antioxidants were ineffective at preventing s-LPA-induced cell death, with the exception of NAC (Fig. 4A); NAC normalized GSH levels (Table 1). The specific iNOS inhibitor 1400W and the nonspecific ROS inhibitor t-NNa also significantly attenuated LPA-induced cell death (Fig. 4A). A major source of oxidant, NADPH oxidase, seemed to partake in LPA-induced cell death; its inhibitor apocynin (50 μmol/l, corresponding to IC50) diminished LPA-triggered cytotoxicity [cell survival: 59.5 ± 2.6% with s-LPA (1 μmol/l) vs. 72.0 ± 1.7% with apocynin pretreatment, P < 0.01]. Inhibitors of COX, 5-lipoxygenase, phospholipase A2, and cytchrome P450 were ineffective (Fig. 4A).

Indexes of oxidant stress, notably isoprostanes (5.9 ± 1.9 and 8.0 ± 2.3 pg of 15-F2t-Isop/mg protein at 24 h in control and s-LPA-treated cells, respectively), hydroperoxides (not detected in control and s-LPA-treated cells at 24 h), and protein tyrosine nitration (nonsignificant 1.3-fold increase in nitrotyrosine in s-LPA-treated cells compared with control cells at 24 h) were minimally affected by LPA. On the other hand, protein nitrosylation was markedly (12-fold) augmented (Fig. 4B); this effect was inhibited by NAC (not shown). Concomitantly, s-LPA induced within 5 h a robust increase in iNOS (Fig. 4C) and an associated increase in nitrite production [control, 10.1 ± 0.7 nmol nitrite/mg protein−1·30 min−1; s-LPA (1 μmol/l), 25.4 ± 4.3 P < 0.05]. Inhibitors of the p38/JNK pathways (SB-203580 and SP-600125) significantly diminished LPA-evoked iNOS expression (Fig. 4C). These pathways were rapidly activated (phosphorylated) in response to s-LPA (Fig. 4D). Correspondingly, the combined effects of p38 and JNK inhibitors blocked LPA-induced cell death (Fig. 4E). Interestingly, NAC and the NADPH oxidase inhibitors DPI and apocynin attenuated JNK phosphorylation and, in turn, iNOS expression in response to LPA (Fig. 4, C and D).

Neuromicrovascular degeneration by s-LPA. Endothelial cytotoxicity of LPA was corroborated ex vivo and in vivo on rat pup brain explants and retinas, respectively. Brain and retinas exposed to s-LPA exhibited diminished microvasculature; these effects were prevented by THG1603, NAC, t-NNa, and combined SP-600125 and SB-203580 (Fig. 5).

DISCUSSION

The present study reveals that physiologically encountered concentrations of LPA (EC50 ~ 0.2 μmol/l) (64, 68) promote endothelial cell death, resulting in cerebral and retinal microvascular rarefaction. This effect associated with protein nitrosylation depends on the modulation of the cell redox state toward a less reducing environment and interrelated induction of iNOS and is depicted in a model presented in Fig. 6. The findings disclose novel properties of LPA, specifically regarding endothelial cytotoxicity, by involving nitrosylation, which also has yet to be described for LPA.

Although the adverse effects of LPA on cell viability appear to be an exception to its commonly known proliferative/antiapoptotic properties seen in various cell types (70), the current observation of cytotoxic effects of LPA is compatible with emerging findings, highlighting its Janus faces in inflammatory states. For instance, toxic effects of LPA have been reported in T cells and neurons, and the latter have been proposed to play a role in brain injury (26, 27, 41, 60). As demonstrated in the current study, LPA also may partake in brain injury by causing endothelial cell demise and microvascular degeneration.

Several lines of evidence support an active participation of the G protein-coupled receptor LPA1 in LPA-mediated cytotoxic responses in endothelial cells. 1) LPA1 is abundant in pCEMVEC (20, 21), and HUVECs, which only express LPA1 (44), were susceptible to s-LPA (Fig. 1D). 2) LPA1-mediated responses occur independently of LPA’s degree of fatty acid saturation (3, 20), consistent with cytotoxic results obtained using various LPAs (Fig. 1C). 3) Effects of LPA are G protein dependent given that they were inhibited by suramin. 4) The specific LPA1 peptide antagonist THG1603 (21) prevented s-LPA-induced cell death in vitro (Fig. 1E) as well as ex vivo and in vivo (Fig. 5).

The s-LPA-induced cell death did not seem to proceed via classic apoptosis as revealed by an absence of TUNEL, cleaved caspase-3 and spectrin, chromatin condensation, and cellular blebbing (Figs. 2 and 3). The cell death process appeared more related to oncosis, since cell swelling and increased incorporation of EthD-1 were observed (Figs. 2 and 3). Nevertheless, numerous intermediate pathways of cell death have been described (37, 46, 59) spanning a spectrum between apoptosis and oncosis. Of relevance are the vacuolized organelles (e.g., endoplasmic reticulum) observed in electron micrographs (Fig. 3); these are indicative of ongoing autophagy (38, 42), a process also referred to as type II programmed cell death, which is activated in response to oxidant stress and found to occur in multiple brain injuries, including cerebral ischemia (17, 71).

LPA has been suggested to signal through generation of ROS (13, 30, 34, 58); these in turn are key effectors in cell death (26, 60). A number of observations point to an LPA-induced oxidant stress in causing endothelial cytotoxicity. LPA

![Fig. 6. Model depicting the mechanism by which s-LPA exerts its cytotoxic actions on neuromicrovascular endothelium. s-LPA via its LPA1 receptor activates NADPH oxidase (28) as well as the p38 MAPK and JNK pathways, which are themselves modulated by the redox environment. Activation of these enzymes causes an increase in iNOS expression and NO formation, as well as a decrease in GSH, which also may be partly attributed to its nitrosylation. These redox changes favor protein nitrosylation and, in turn, endothelial cell death and microvascular rarefaction. EC, endothelial cell; P, phosphorylation/activation of the kinases.](http://ajpregu.physiology.org/)
reduced GSH levels (Table 1) and caused an increase in iNOS (Fig. 4), nitrites, and nitrosylation (Fig. 4), yet this oxidant stress was not overwhelming because other indexes of oxidation (hydroperoxides, isoprostanes, and protein nitration) were not augmented. The cell death was prevented by NOS blockers and by renormalization of glutathione with its precursor NAC (Figs. 4 and 5). Also, protection against LPA-induced cytotoxicity was observed with inhibitors of the major superoxide generator NADPH oxidase, which reestablish a redox balance (2, 62), inferring a significant role for this enzyme in LPA-induced redox changes (Table 1). This also suggests a coupling between LPA1 and NADPH oxidase, which has been reported to involve the small GTPases Rac1 and Rac2 (28) through a G$_{i/o}$-independent pathway (61), as we have observed (Fig. 1).

In the inflammatory environment of the endothelium, s-LPA also may contribute to toxic mediators present during HI injury, such as cytokines (53, 56), the endothelial cytotoxicity conveyed by LPA broadens its purposed pathological role in cerebral ischemia by causing microvascular degeneration. By modulating the redox environment of the endothelium, s-LPA also may contribute to the establishment of an environment favorable for other cytotoxic mediators present during HI injury, such as cytokines (33, 66).

**ACKNOWLEDGMENTS**

We thank Hendrika Fernandez for technical assistance.

**GRANTS**

This study was supported in part by grants from the Canadian Institute of Health Research (CIHR) and the Heart and Stroke Foundation of Québec. S. Chemtob is recipient of a Canada Research Chair. S. Brault is a recipient of studentships from the CIHR. F. Gobeil is a recipient of a scholarship from the Fonds de la Recherche en Santé du Québec.

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