Activation of Lysophosphatidic Acid Receptor Type 1 Contributes to Pathophysiology of Spinal Cord Injury

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Lyosphosphatidic acid (LPA) is an extracellular lipid mediator involved in many physiological functions that signals through six known G-protein-coupled receptors (LPA1–LPA6). A wide range of LPA effects have been identified in the CNS, including neural progenitor cell physiology, astrocyte and microglia activation, neuronal cell death, axonal retraction, and development of neuropathic pain. However, little is known about the involvement of LPA in CNS pathologies. Herein, we demonstrate for the first time that LPA signaling via LPA1 contributes to secondary damage after spinal cord injury. LPA levels increase in the contused spinal cord parenchyma during the first 14 d. To model this potential contribution of LPA in the spinal cord, we injected LPA into the normal spinal cord, revealing that LPA induces microglia/macrophage activation and demyelination. Use of a selective LPA1 antagonist or mice lacking LPA1 linked receptor-mediated signaling to demyelination, which was in part mediated by microglia. Finally, we demonstrate that selective blockade of LPA1 after spinal cord injury results in reduced demyelination and improvement in locomotor recovery. Overall, these results support LPA–LPA1 signaling as a novel pathway that contributes to secondary damage after spinal cord contusion in mice and suggest that LPA1 antagonism might be useful for the treatment of acute spinal cord injury.

Key words: demyelination; lysophosphatidic acid; microglia; neuroprotection; oligodendrocytes; spinal cord injury

Introduction

Spinal cord injury (SCI) causes functional deficits produced by the loss of axons and neurons and contributes to the death of oligodendrocytes and the limited ability of the CNS to regenerate axons (Rowland et al., 2008; David et al., 2012a). The pathophysiology of SCI involves two stages of tissue degeneration known as primary and secondary injury. Primary injury results from the direct mechanical trauma to the spinal cord, which is followed by secondary injury, consisting of a wave of tissue degeneration that occurs over a period of several weeks, and is associated with inflammation and other mechanisms triggered by injury (David et al., 2012a,b; Popovich, 2014). Secondary injury is thought to contribute importantly to the functional deficits seen after SCI and may be preventable. Repairing the damaged spinal cord is still a clinical challenge. Neuroprotective strategies aimed at preventing damage arising from secondary injury processes provide some hope for tissue sparing and improved functional outcome. However, there is currently no effective clinical treatment for SCI. Hence, there is a need to identify factors that are involved in triggering secondary damage in SCI and to develop drug interventions to limit damage and improve functional deficits.

Significance Statement

This study reveals that LPA signaling via LPA receptor type 1 activation causes demyelination and functional deficits after spinal cord injury.
Lyso-phosphatic acid (LPA; 1-acyl-sn-glycerol 3-phosphate) is a bioactive lipid that has a wide variety of biological activities, including cellular proliferation, prevention of apoptosis, cell migration, cytokine and chemokine secretion, platelet aggregation, smooth muscle contraction, and neurite retraction (Bot et al., 2010; Choi et al., 2010; Choi and Chun, 2013; Yung et al., 2014). LPA exerts this wide variety of effects by binding to specific G-protein-coupled receptors, such as LPA receptors (LPARs) LPA₁–LPA₁₀ which belong to the endothelial differentiation gene family, and the genetically more distant LPA₁₂–LPA₁₄, which belong to the P2Y purinergic receptor family (Choi et al., 2010; Choi and Chun, 2013; Kihara et al., 2014). These receptors differ in their ability to alter downstream signaling pathways, including intracellular Rho levels, mobilize Ca²⁺, contribute to phosphoinositol turnover, and to activate intracellular kinases, such as MAPK and ERK (Choi et al., 2010; Choi and Chun, 2013).

In vivo studies demonstrate that LPA is involved in the etiology of fetal hydrocephalus (Yung et al., 2011), fetal hypoxic brain damage (Herr et al., 2011), and the development of neuropathic pain after sciatic nerve injury and cerebral ischemia (Inoue et al., 2004; Frisca et al., 2012; Lin et al., 2012). Because of the wide variety of LPAR subtypes, it is likely that LPA may exert helpful or harmful effects in the CNS depending on the receptors it signals through. Thus, there is a need to know which LPARs contribute to neurodegeneration and/or neuroprotection. Here we show that LPA levels increase in the spinal cord parenchyma after contusion injury. We provide evidence suggesting that LPA leads to demyelination via activation of microglia LPA₁. Moreover, we demonstrate that selective blockade of LPA₁ after SCI reduces functional deficits and demyelination, altogether revealing important contributions of LPA–LPA₁ signaling in secondary damage after SCI.

Materials and Methods

Surgical procedure

All surgical procedures were approved by the Autonomous University of Barcelona Animal Care Committee and followed the guidelines of the European Commission on Animal Care. Adult (8–10 weeks old) female C57BL/6 mice (Charles River), LPA₁-deficient mice (maLPA₁-null; Estivill-Torrués et al., 2008), or wild-type littermates (C57BL/6; 129X1/Sv background) were anesthetized with ketamine (90 mg/kg, i.m.) and xylazine (10 mg/kg, i.m.). After performing a laminectomy at the 11th thoracic vertebrae, the exposed spinal cord was either injected intraspinally or contused. Intraspinal injections were performed using a glass needle (30 µm internal diameter; Eppendorf) coupled to a 10 ml Hamilton syringe (catalog #701; Hamilton). One microliter of saline, saline containing 5 nmol of LPA (18:1 LPA; Sigma-Aldrich), or 5 nmol of LPA plus the selective LPA₁ antagonist AM095 sodium ([4′,5′-methyl-4-((R)-1-phenyl-ethoxycarbonylaminoo)-isoxazol-5-yl]-biphenyl-4-yl)-acetate 0.22 nmol; kindly provided by Bristol-Myers Squibb; Swaney et al., 2011) was injected into the dorsal column of the uninjured spinal cord. Injections were made at a perfusion speed of 2 µl/min controlled by an automatic injector (KDS 310 Plus; KD Scientific), and the tip of the needle was withdrawn to remove all LPA from the medium, or saline was administered orally at a dose of 30 mg/kg. Treatment was initiated 1 h after SCI and then given every 12 h for 1 week.

Functional assessment

Locomotor recovery was evaluated at 1, 3, 5, 7, 10, 14, 21, and 28 d post-injury (dpi) in an open-field test using the nine-point Basso Mouse Scale (BMS; Basso et al., 2006), which was developed specifically for locomotor testing after contusion injuries in mice. The BMS analysis of hindlimb movements and coordination was performed by two independent assessors who were blinded to the experimental groups and the consensus score taken. In addition, at the end of the follow-up (28 dpi), the highest locomotion speed of the mice was evaluated on a belt of a motorized treadmill. Briefly, each mouse was allowed to explore the treadmill compartment, with the motor speed set to zero, for 5 min. Then speed was gradually increased from 0 up to 35 cm/s, and the maximum speed at which each mouse was to perform for at least 5 s was recorded.

Motor-evoked potentials

At day 28, electrophysiological tests were used to evaluate spared motor central pathways after SCI. Motor-evoked potentials (MEPs) were recorded from the gastrocnemius muscle (GM) with microneedle electrodes, in response to transcranial electrical stimulation of the motor cortex by single rectangular pulses of 0.1 ms duration. Pulses were delivered through needle electrodes inserted subcutaneously, with the cathode over the skull overlying the sensorimotor cortex and the anode at the nose (García-Álasis et al., 2003). Compound muscle action potential from GM muscle was recorded for internal control of normal peripheral conduction. In this case, the sciatic nerve was stimulated percutaneously by means of single pulses of 0.02 ms duration (Grass S88; Grass Instruments) delivered through a pair of needle electrodes placed at the scatic notch. All potentials were amplified and displayed on a digital oscilloscope (Tektronix 4505).

Cell cultures

Mouse oligodendrocyte cultures. Primary oligodendrocyte progenitor cells (OPCs) were isolated from mouse cerebral cortex at postnatal day 2 (P2) to P4 as described previously (O’Meara et al., 2011). Briefly, after removal of the meninges, cortical tissue was minced using a P1800 pipette tip and dissociated by incubating in a solution of 1.54 mg/ml papain (Worthington Biochemical), 400 µg/ml t-cysteine (Sigma), and 1 mg/ml DNP (Roche) in MEM (Gibco) for 20 min at 37°C. Mixed glial culture media [MGCM; 10% inactivated and filtered fetal bovine serum (FBS; Sigma), 0.33% penicillin–streptomycin (P/S; Sigma), and 1% Glutamax 100X (Gibco) in DMEM (Gibco)] was added to stop papain and DNase I activity. Cells were plated into T25 tissue culture flasks coated with 10 µg/ml poly-β-lysine for 1 h at 37°C and cultured at 37°C in an humidified incubator with 5% CO₂ supplementation. Three hours after plating, the floating cells were discarded by replacing the medium. Two thirds of the MGCM was replaced every 3 d with new MGCM supplemented with 5 µg/ml insulin (Seralab). Nine days later, OPCs were harvested by shaking at 37°C at 220 rpm overnight. The collected cells were plated into 10 µg/ml poly-β-lysine-coated coverslips with DMEM (Gibco) supplemented with 1% Glutamax 100X, 2% B27 (Gibco), 0.5% FBS, 50 µg/ml recombinant mouse ciliary neurotrophic factor (BioTrend), and 1% OL supplement (10 µl/ml N-2 supplement 100X (Gibco), 10 mg/ml bovine serum albumin (Sigma), and 40 µg/ml 3,3′,5-triiodo-l-thyronine (Sigma). OPC maturation in oligodendrocytes was achieved after 7 d in vitro.

Mouse microglia cultures. Primary microglial cells were isolated from P4 mouse cerebral cortex as described previously (Saura et al., 2003). Tissue dissociation and cell isolation was performed as described in OPC culture. Mixed glial cultures were prepared into T25 tissue culture flasks without any coating. Cells were seeded at a density of 300,000 cells/ml in DMEM/F-12 (Gibco) with 10% FBS and 5% P/S and cultured at 37°C in a humidified incubator with 5% CO₂ supplementation. Medium was replaced every 4–5 d, and confluence was achieved after 10–12 d. At this point, mixed cells were incubated with 0.25% trypsin–EDTA (Gibco) diluted 1:4 in DMEM/F-12 for 30 min at 37°C. This trypsinization resulted in the detachment of the upper layer of cells in one piece, and the remaining adherent microglial cells were cultured for 24 h before stimulation. Microglial cells were stimulated with LPA (1 µM) or LPA plus AM095 (12.5 µM) or 0.025% DMSO (vehicle for AM095) as control for 24 h. Afterward, cells were washed to remove the LPA from the medium, and microglia were then incubated with oligodendrocyte conditioned media for 24 h and then used for cytotoxicity assays in oligodendrocyte cell cultures.
Table 1. Real-time PCR primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
</tr>
</thead>
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<tr>
<td>LPA1</td>
<td>TCTGTCGGCTGATGAAAGTCTC</td>
<td>TTGTGGGAGGAGTATGTAAGA</td>
</tr>
<tr>
<td>LPA2</td>
<td>CTCAGTGCTGACGATGCTATAT</td>
<td>GAGACGGGAGGAAAGG</td>
</tr>
<tr>
<td>LPA3</td>
<td>GGGACATTCCCTGTCTTATTA</td>
<td>GAAGGAGGAACCTGTGGTTG</td>
</tr>
<tr>
<td>LPA4</td>
<td>GATGAAGGGCTCTTATGAAGCGA</td>
<td>GTTTGATGACCACTCCGTGATA</td>
</tr>
<tr>
<td>LPA5</td>
<td>CCACTGATCATTGGACACTGATGG</td>
<td>CAGCATATCTTCCTCCACCT</td>
</tr>
<tr>
<td>LPA6</td>
<td>ACCGTGTTCGGACCAATG</td>
<td>AAGGCTGTATGATGTTG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TCAAGAAGACCTCAGCCCTCA</td>
<td>ACCGTTGTCTGAGCCGATTC</td>
</tr>
</tbody>
</table>

Assessment of oligodendrocyte cell death

Oligodendrocytes were stimulated with LPA (0.01, 0.1, or 1 μM), LPA plus AM095 (12.5 μM), and DMSO (0.025%) as control, or with conditioned media of microglia harvested from C57BL/6 mice, ml-LPA1 null mice, or wild-type littermates, stimulated with DMSO, LPA (1 μM), or LPA (1 μM) plus AM095 (12.5 μM) for 24 h. Afterward, coverslips were fixed in 4% paraformaldehyde for 20 min, washed in PBS, and incubated overnight with anti-rat or anti-rabbit Alexa Fluor 594-conjugated antibody (1:500; Invitrogen) and DAPI (Sigma), and the total number of oligodendrocytes (MBP+ cells) or oligodendrocyte precursor cells (A2B5 and NG2) were counted under fluorescence microscope (Olympus BX51). Four independent replicates were done for each of the experiments.

Histology

At 4 d after intraspinal injection and 28 d after spinal cord contusion, mice were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). A 10 mm length of spinal cord containing the injection or the contusion site was harvested, postfixed with 4% paraformaldehyde in 0.1 M PB for 1 h, and cryoprotected with 30% sucrose in 0.1 pb PB at 4°C for a minimum of 48 h. The samples were fast frozen at −80°C in cryoembedding compound (Tissue-Tek OCT; Sakura) and cut on a cryostat (Leica). Ten series of 10-μm-thick transversal sections were picked up on glass slides, so adjacent sections on the same slide were 100 μm apart. For demyelination and myelin staining analyses, sections were stained with Luxol fast blue (LFB; Sigma) or fluoromyelin (Invitrogen). After graded dehydration, sections were placed in a 1 mg/ml LFB solution in 95% ethanol and 0.05% acetic acid overnight at 37°C. Sections were then washed in 95% ethanol and distilled water before being placed in a solution of 0.5 mg/ml Li2CO3 in distilled water for 1 min at RT. After washes in distilled water, sections were dehydrated and mounted in DPX mounting media (Sigma). For immunofluorescence staining, sections were dehydrated in PBS and blocked with 5% FBS in PBS with Tween 20 (PBST) for 1 h at RT. The sections were then incubated overnight at 4°C with primary antibodies against Neut for (neurons; 1:200; Millipore), neurofilament-200 (NF-200; for axons; 1:1000; Millipore), GFAP (for astrocytes; 1:500; Invitrogen), ionized calcium-binding adapter molecule 1 (Iba1; for macrophage/microglia; 1:400; Abcam), and MBP (for myelin; 1:100; Abcam). After several washes in PBST, sections were incubated for 1 h at RT with the appropriate Alexa Fluor 594 or 488-conjugated secondary antibodies (1:200; Invitrogen) and then coverslipped in Mowiol mounting media containing DAPI (1 μg/ml; Sigma). Tissue sections were viewed with a Philips CM 10 electron microscope. Then, sections were cut at 90 nm, stained with lead citrate, and viewed with a Philips CM 10 electron microscope.

Flow cytometry

Immune cells from the injured spinal cord were analyzed by flow cytometry at 7 dpi, as described previously (Klopstein et al., 2012). Briefly, spinal cords were cut in little pieces and passed through a cell strainer of 70 μm ( Falcon; BD Bioscience Discovery Labware), and the cell suspension was centrifuged twice at 300 × g for 10 min at 4°C. Samples were divided, and cells alone and isotype–matched control samples were generated to control for nonspecific binding of antibodies and for autofluorescence. Isotype control antibodies were purchased from eBioscience and included phycoerythrin–cyantee-7 (PE–Cy7)-labeled rat IgG2b, adenosomatous polyposis coli (APC)-labeled rat IgG2b, peridinin chlorophyll-a protein cyanine 5.5 (PerCP–Cy5.5)-conjugated rat IgG2b, and fluorescein isothiocyanate-conjugated rat IgG2b and IgG2M. The following antibodies were also purchased from eBioscience: CD45–PerCP–Cy5.5, CD11b–PE–Cy7, and F4/80–APC. After 30 min of incubation with combinations of antibodies at 4°C, the samples were washed and fixed in 1% paraformaldehyde. Microglial cells were identified as CD45–PE–Cy5.5 and CD11b–PE–Cy7 cells, whereas myeloid cells were identified as CD45–PerCP and CD11b–PE–Cy7 cells (Stirling and Yong, 2008). Macrophages were identified from myeloid cells based on F4/80 expression (CD45–PerCP, CD11b–PE–Cy7, F4/80–APC). At least 3000 CD45–PE–Cy5.5 cells were analyzed per spinal cord sample using FlowJo software on a FACSCanto flow cytometer (BD Biosciences). Four contused mice treated with saline or AM095 were used in this experiment.

Isolation of microglia from CNS tissue

Briefly, spinal cord and brain from adult C57BL/6 mice (8–10 weeks old) were removed and digested enzymatically with 0.2% collagenase B (Roche Diagnostics) and 0.2% trypsin–EDTA at 37°C for 30 min and then passed through a cell strainer of 40 μm (Falcon; BD Bioscience Discovery Labware). Cell suspension was centrifuged twice at 300 × g for 10 min at 4°C, and microglial cells were first isolated by magnetic sorting using a CD11b antibody (Miltenyi Biotec) and then stained with PerCP–Cy5.5-conjugated CD45 and PE–Cy7-conjugated CD11b antibodies for additional purification on a cell sorter (FACSARIA III; BD Bioscience). Microglia cells were assessed on a flow cytometer (FACSCalibur; BD Biosciences), and only populations presenting >90% purity were used for gene expression analysis.

RNA isolation, reverse transcription, and real-time PCR

Mice were perfused with sterile saline, and a 5-mm-length of uninjured spinal cord was removed. Tissue was homogenized with QIAzol lysis reagent (Qiagen) and RNA extracted using the RNAeasy Lipid Tissue kit (Qiagen), according to the protocol of the manufacturer. RNA was treated with DNaseI (Qiagen) to eliminate genomic DNA contamination. mRNA from cultured and in vivo sorted microglia was extracted using the RNAeasy Micro kit following the guidelines of the manufacturer. One and 0.5 μg of RNA obtained from tissue or microglia, respectively, was primed with random hexamers (Promega) and reverse transcribed using the Omniscript RT kit (Qiagen). RNase inhibitor (Roche) was added (1 U/μl final concentration) to avoid RNA degradation. Primer sequences are specified in Table 1. Glyceraldehyde–3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. The amount of cDNA was calculated based on the tissue, whereas neuronal survival was assessed by counting the number of NeuN+ cells in the ventral horns. Moreover, axonal and demyelination in the dorsal column was assessed by counting the NF+ structures as well as fibers double stained for NF and MBP at the lesion epicenter.
Table 2. Quantification of the different LPA species in SCI

<table>
<thead>
<tr>
<th>LPA species</th>
<th>16:0</th>
<th>18:1</th>
<th>18:0</th>
<th>20:4</th>
<th>20:1</th>
<th>22:6</th>
<th>22:4</th>
<th>22:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>0</td>
<td>33.5±7.1</td>
<td>34.6±3.2</td>
<td>0</td>
<td>7.1±1.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6 h</td>
<td>7.7±0.3*</td>
<td>166.4±9.0*</td>
<td>79.7±4.4*</td>
<td>10.5±0.9*</td>
<td>77.8±5.8*</td>
<td>55.0±4.5*</td>
<td>15.1±0.7*</td>
<td>0.4±0.1</td>
</tr>
<tr>
<td>1 d</td>
<td>7.4±0.9*</td>
<td>167.8±18.6*</td>
<td>80.5±8.3*</td>
<td>13.1±1.8*</td>
<td>94.0±8.4*</td>
<td>60.3±6.1*</td>
<td>14.9±1.9*</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>3 d</td>
<td>13.4±1.0*</td>
<td>290.5±21.2*</td>
<td>143.3±10.5*</td>
<td>20.2±1.1*</td>
<td>144.0±12.9*</td>
<td>85.0±6.2*</td>
<td>25.5±1.7*</td>
<td>0.7±0.0</td>
</tr>
<tr>
<td>7 d</td>
<td>12.9±0.8*</td>
<td>255.4±7.1*</td>
<td>125.4±4.1*</td>
<td>21.9±0.7*</td>
<td>119.8±6.1*</td>
<td>73.4±2.3*</td>
<td>22.3±0.5*</td>
<td>0.7±0.0</td>
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<tr>
<td>14 d</td>
<td>0</td>
<td>307.7±35.7*</td>
<td>125.1±8.2*</td>
<td>25.7±2.0*</td>
<td>52.1±8.2*</td>
<td>82.0±7.6*</td>
<td>13.6±2.3*</td>
<td>0</td>
</tr>
</tbody>
</table>

*p < 0.01 versus intact spinal cord.

Results

LPA levels are increased in the injured mouse spinal cord

We first assessed whether LPA levels increased in the spinal cord after contusion injury. Using mass spectrometry, we detected the presence of three LPA molecular variants (18:1, 18:0, and 20:1) in the uninjured spinal cord (Table 2). After contusion injury, these three LPA species were elevated significantly at all time points analyzed (one-way ANOVA; post hoc Bonferroni’s test, p < 0.01; n = 4 per time point). Moreover, additional LPA variants (16:0, 20:4, 22:6, and 22:4) were also detected in the contused spinal cord (Table 2). Quantification of the different LPA species revealed that the total level of LPA increased very rapidly in the injured spinal cord (Fig. 1), being approximately fivefold higher at 6 h, rising to ~10-fold at day 3, and the remaining approximately eightfold increased from 7 to 14 dpi compared with intact spinal cord (one-way ANOVA; post hoc Bonferroni’s test, p < 0.01; n = 4 per time point). These results demonstrate that LPA levels increase in the spinal cord after contusion injury.

Exogenous LPA exposure leads to demyelination

To assess the potential contribution of increased levels of LPA in the spinal cord parenchyma, we injected 1 μl of saline containing 5 nmol of LPA into the dorsal column of uninjured spinal cord or saline alone as a control group. The LPA variant used in these experiments was the 18:1 oleoyl LPA, because our mass spectrometry results revealed that this LPA species was the most abundant in the contused spinal cord. Microglia/macrophage activation in the spinal cord parenchyma was assessed by measuring the presence of reactive Iba1+ cells, 4 d after injection. Microglia/macrophage activation was restricted to the injection area in saline-injected mice, probably because of the mechanical injury caused by the needle insertion (Fig. 2A–C). By comparison, spinal cord sections from mice injected with LPA showed a marked activation of microglia/macrophages 4 d after injection (Fig. 2D).

The presence of these inflammatory cells was not limited to the injection site but were also distributed within the whole dorsal columns (Fig. 2D). In addition, the immunoreactivity for Iba1+...
cells was also increased in rostral and caudal areas from the injection site in LPA-injected compared with saline-injected mice (Fig. 2; two-way RM-ANOVA; post hoc Bonferroni’s test, \( p < 0.05; n = 4 \) per group). Mild activation of astrocytes was also observed but were only restricted to areas close to the LPA injection (data not shown), indicating that LPA activates both microglia and astrocytes but has a greater preference for microglia.

We then assessed whether activation of microglia/macrophages triggered by LPA led to demyelination. At 4 dpi, LFB staining revealed the presence of a small demyelinating lesion in the dorsal column of saline-injected spinal cords (Fig. 3), which was restricted to the area of the needle insertion (Fig. 3B). We also observed a demyelinating lesion in the spinal cords injected with LPA (Fig. 3D). The area of this lesion was significantly increased in LPA-injected compared with PBS-injected mice at the injection epicenter and in adjacent areas (Fig. 3A–E; two-way RM-ANOVA; post hoc Bonferroni’s test, \( p < 0.05; n = 4 \) per group). To assess whether demyelination induced by LPA was caused by axonal or myelin damage, we double stained spinal cord sections to assess whether demyelination induced by LPA was caused by axonal or myelin damage, we double stained spinal cord sections to assess whether demyelination induced by LPA was caused by axonal or myelin damage, we double stained spinal cord sections.

Figure 2. Intraspinal injection of LPA leads to microglia/macrophage activation. A, Quantification of microglia/macrophage immunoreactivity at 4 d after intraspinal injection of saline or LPA. B–E, Representative images of spinal cords stained against Iba1 at the epicenter (B, D) and 1800 \( \mu \text{m} \) rostral to the injection site (C, E) of saline-injected (B, C) and LPA-injected (D, E) mice (\( n = 4 \) per group). \( * p = 0.006 \). Error bars indicate SEM. Scale bar, 250 \( \mu \text{m} \).

Exogenous LPA exposure mediates demyelination via LPA1

Because LPA1 is expressed in mature oligodendrocytes (Weiner et al., 1998), we assessed whether LPA-induced demyelination was attributable to a toxic effect of LPA on oligodendrocytes. Cell culture work indicated that LPA treatment led to a mild reduction in the viability of oligodendrocytes (~20%) at concentrations of 1 \( \mu \text{M} \) (Fig. 6A; \( n = 4 \) per group). However, LPA did not have an effect on oligodendrocyte precursor cell survival (Fig. 6B). Administration of AM095 failed to rescue cultured oligodendrocytes from cell death, suggesting that the LPA1 signaling does not mediate a direct cytotoxic effect in cultured oligodendrocytes (Fig. 6C; \( n = 4 \) per group), albeit with the caveat that these cells only approximate what occurs \emph{in vivo}. Because LPA also triggers microglia activation when injected into the spinal cord and activated microglial cells were associated with areas of demyelination (Fig. 6D), we also assessed whether microglial LPA1 was responsible for the LPA-induced demyelination. In agreement with previous work (Moller et al., 2001; Tham et al., 2003) real-time PCR analysis identified LPA1 gene expression in cultured microglia (Fig. 6F; \( n = 4 \)). Moreover, microglial cells \emph{in vivo} have constitutive expression of LPA1, showing similar levels of LPA1 relative to cultured microglia (Fig. 6E,F; \( n = 4 \)). Interestingly, we observed that conditioned medium of primary microglial cells stimulated with LPA led to a marked reduction (~85%) in oligodendrocyte survival (Fig. 6G,I–F; one-way ANOVA; post hoc Bonferroni’s test, \( p < 0.001; n = 4 \) per group), suggesting that microglia become cytotoxic after LPA stimulation.

Figure 6. Quantification of microglia/macrophage immunoreactivity at 4 d after intraspinal injection of saline or LPA.

A–C, Representative images of spinal cords stained against Iba1 at the epicenter (B, D) and 1800 \( \mu \text{m} \) rostral to the injection site (C, E) of saline-injected (B, C) and LPA-injected (D, E) mice (\( n = 4 \) per group). \( * p = 0.006 \). Error bars indicate SEM. Scale bar, 250 \( \mu \text{m} \).

Histological analysis revealed that 4 d after LPA administration, the demyelinating lesion was reduced substantially when LPA1 was blocked (Fig. 5A–C). Quantification of the demyelinated areas revealed that mice treated with AM095 showed reduced myelin loss at the injection site and in the adjacent sections corresponding to 600 \( \mu \text{m} \) rostral and caudal to the epicenter (two-way ANOVA; post hoc Bonferroni’s test, \( p < 0.05; n = 4 \) per group). Spinal cord tissue sections from LPA1 null mice (maLPA1 variant) also displayed smaller demyelinating lesions (Fig. 5D–F) after intraspinal injection of LPA, further implicating LPA1 signaling in LPA-induced demyelination.

Because LPA1 is expressed in mature oligodendrocytes (Weiner et al., 1998), we assessed whether LPA-induced demyelination was attributable to a toxic effect of LPA on oligodendrocytes. Cell culture work indicated that LPA treatment led to a mild reduction in the viability of oligodendrocytes (~20%) at concentrations of 1 \( \mu \text{M} \) (Fig. 6A; \( n = 4 \) per group). However, LPA did not have an effect on oligodendrocyte precursor cell survival (Fig. 6B). Administration of AM095 failed to rescue cultured oligodendrocytes from cell death, suggesting that the LPA1 signaling does not mediate a direct cytotoxic effect in cultured oligodendrocytes (Fig. 6C; \( n = 4 \) per group), albeit with the caveat that these cells only approximate what occurs \emph{in vivo}. Because LPA also triggers microglia activation when injected into the spinal cord and activated microglial cells were associated with areas of demyelination (Fig. 6D), we also assessed whether microglial LPA1 was responsible for the LPA-induced demyelination. In agreement with previous work (Moller et al., 2001; Tham et al., 2003) real-time PCR analysis identified LPA1 gene expression in cultured microglia (Fig. 6F; \( n = 4 \)). Moreover, microglial cells \emph{in vivo} have constitutive expression of LPA1, showing similar levels of LPA1 relative to cultured microglia (Fig. 6E,F; \( n = 4 \)). Interestingly, we observed that conditioned medium of primary microglial cells stimulated with LPA led to a marked reduction (~85%) in oligodendrocyte survival (Fig. 6G,I–F; one-way ANOVA; post hoc Bonferroni’s test, \( p < 0.001; n = 4 \) per group), suggesting that microglia become cytotoxic after LPA stimulation.

Oligodendrocyte cell death was reduced approximately...
three times when microglia LPA1 was blocked with AM095 (Fig. 6G, I–F; one-way ANOVA; post hoc Bonferroni’s test, p < 0.05; n = 4 per group). Similar results were obtained from conditioned medium of LPA-stimulated microglia cells isolated from maLPA1 mice (Fig. 6H; two-way ANOVA; post hoc Bonferroni’s test, p < 0.05; n = 4 per group). These data indicate that the activation of LPA1 in microglial cells is responsible, in part, for the cytotoxic effects mediated by LPA-stimulated microglia. Overall, our results suggest that demyelination triggered by intraspinal injection of LPA is mediated, in part, by the activation of microglial LPA1, with unclear participation of oligodendrocyte LPA signaling in vivo.

**LPA1 contributes to SCI pathophysiology**

LPA–LPA1 signaling was assessed for its role in secondary damage and functional impairment after SCI in mice. Because maLPA1 null mice showed locomotor deficits (data not shown), probably attributable to its developmental brain defects (Santin et al., 2009), we used AM095 to assess the role of LPA1 in SCI. Oral administration of AM095 significantly reduced locomotor impairments based on the BMS assessment (Fig. 7A; p < 0.001, two-way RM-ANOVA; n = 8 per group). At the end of the follow up (28 dpi), the BMS score was improved by 1.5 points after AM095 treatment (Fig. 7A). Mice treated with saline showed occasional plantar stepping and no coordination, whereas AM095 showed frequent/consistent plantar stepping and most of them (87.5%) had coordination. In addition, the BMS subscore, which
assess fine aspects of locomotion, was markedly improved after LPA1 blockade (Fig. 7B; \( p < 0.001 \), two-way RM-ANOVA; \( n = 8 \) per group), whereas mice treated with AM095 were able to run at significantly higher speeds on a treadmill (Fig. 7C; \( p = 0.011 \), Mantel–Cox test; \( n = 8 \) per group).

Electrophysiological tests designed to assess the preservation of descending pathways of the spinal cord based on MEP analysis were used on 28 dpi mice treated with AM095: treated animals showed significantly greater MEP amplitudes for the GM compared with controls (\( p = 0.018 \), \( t \) test; \( n = 8 \) per group), suggesting an increase in spared functional descending tracts in the spinal cord after the lesion (Fig. 7D,E). Mice administered AM095 were further assessed for amelioration of secondary tissue damage. Histological sections of the spinal cord stained with fluoromyelin revealed that animals treated with the LPA1 antagonist showed less demyelination at the injury epicenter and in adjacent regions (Fig. 7F–H; \( p < 0.001 \), two-way ANOVA; \( n = 8 \) per group). We also assessed whether the greater myelin sparing observed after AM095 treatment was attributable to reduced axonal loss or reduced demyelination. Spinal cord tissue section at the injury epicenter, the most damaged area of the spinal cord, revealed that AM095 did not enhance the number of NF fibers but increased the percentage of axons labeled with MBP (Fig. 7I–K; \( p = 0.034 \), \( t \) test; \( n = 8 \) per group). However, AM095 treatment led to only minor improvement in neuronal sparing, seen only at 400 \( \mu \)m caudal to the injury epicenter (Fig. 7L–N). These results suggest that the increased levels of LPA in the injured spinal cord contributes to demyelination and loss of function by signaling via LPA1.

We finally assessed whether AM095 attenuated the inflammatory response after SCI. FACS analysis of injured spinal cord harvested at 7 dpi, when microglial cell and macrophages peak and demyelination increases, revealed that AM095 did not reduce microglia counts (Fig. 8). Similarly, AM095 did not attenuate the infiltration of macrophages into the injured spinal cord (Fig. 8). Overall, our data suggest that AM095 confers protection against demyelination by reducing the cytotoxic actions of microglia LPA1 signaling but not its density.

**Discussion**

The results of the present study provide clear evidence that the LPA–LPA1 pathway contributes to secondary damage after SCI. LPA levels increase in the spinal cord after contusion injury, contributing to activation of microglia/macrophages and demyelination that are, in part, mediated by LPA1, signaling. Moreover, our *in vitro* experiments suggest that detrimental actions of LPA1 involve signaling in microglial cells. Finally, we demonstrate that selective inhibition of LPA1 after SCI confers protection from demyelination and enhances locomotor skills. Overall, this work demonstrates for the first time that the LPA–LPA1 pathway contributes to secondary damage and is detrimental to functional recovery after SCI.

Lysophospholipids, which include LPA and sphingosine 1-phosphate (SIP), have emerged as key modulators of inflammation, including in nervous system disorders (Chun and Brinkmann, 2011; David et al., 2012c; Choi and Chun, 2013). The contribution of lysophospholipids to CNS pathology includes experiments identifying lysoselcitin (also known as lysophosphatidylcholine) as a potent demyelinating and inflammation-inducing agent in the CNS (Ousman and David, 2000, 2001). SIP has been demonstrated to play a key role in multiple sclerosis (Brinkmann et al., 2010) through the actions of fingolimod (FTY720; Chun and Brinkmann, 2011), a nonselective S1P receptor modulator, that was the first worldwide oral treatment for relapsing forms of multiple sclerosis (Brinkmann et al., 2010).

LPA is a lipid signaling molecule capable of evoking multiple physiological responses in a wide variety of cells (Choi et al., 2010;
LPA is present in low concentrations in mammalian cells and tissues, but its concentrations in the blood can range from 0.1 μM in plasma to >10 μM in serum. LPA is synthesized in vivo from membrane phospholipids by multiple pathways (Aoki et al., 2002; Choi et al., 2010; Choi and Chun, 2013). Here, we observed that LPA levels rise rapidly in the spinal cord parenchyma after injury. This is likely attributable to a combination of its de novo production in the contused spinal cord, including through phospholipase A2 enzymes that are upregulated in SCI (Titsworth et al., 2009; López-Vales et al., 2011; David et al., 2012c). However, plasma leakage and blood vessel disruption, as well as serum production during the clotting process, likely contribute to increases in LPA levels in SCI, as has been modeled for post-hemorrhagic hydrocephalus wherein LPA

**Figure 6.** Microglia LPA₁ mediates oligodendrocyte cell death. A, Effects of several concentrations of LPA on oligodendrocyte survival. B, Effects of LPA (1 μM) on OPC survival. C, Quantification of oligodendrocyte cell death after stimulation with LPA or LPA plus AM095. D, Representative spinal cord sections from mice that received intraspinal injection of LPA showing that the demyelinating lesion is associated with areas enriched in microglia. E, Representative FACS analysis of microglia immunopanned with the CD11b antibody from adult CNS before and after being cell sorted. F, Quantification of LPA₁ expression in cultured and cell-sorted microglial cells. Note the microglia show similar levels of LPA₁ in both conditions. G, Assessment of conditioned medium of unstimulated (control) microglia or conditioned medium of microglia stimulated with LPA or LPA plus AM095 on oligodendrocyte survival. H, Assessment of conditioned medium of unstimulated (DMSO; control) or LPA-stimulated microglial cells harvested from wild-type or LPA₁ null mice. I, K, Representative images of oligodendrocytes (MBP⁺) from cultures treated with conditioned medium from untreated microglia (DMSO; control) or microglia treated with LPA or LPA plus AM095 stimulated microglia. Note that gene deletion of pharmacological blockade of microglia LPA₁ significantly reduces oligodendrocyte cell death. Scale bar, 100 μm. *p < 0.05 versus control; #p < 0.05 versus LPA or LPA wild type). Error bars indicate SEM (n = 4 per group).
Figure 7. LPA1 blockade enhances functional outcomes and reduces demyelination after SCI. A, B, Animals treated with AM095 show significant improvement in locomotor performance compared with saline-treated mice using the nine-point BMS (A) and the 11-point BMS subscore (B), as well as faster locomotion on a treadmill (C). D, E, Mice treated with AM095 show greater preservation of MEPs. D, Quantification of MEP recordings from GM at 28 d after SCI. E, Representative MEP recordings from mice treated with saline or AM095. F, Quantification of myelin sparing at various distances rostral and caudal to the injury epicenter reveals significant reduction in tissue loss in mice treated with AM095 at the epicenter of the injury and in adjacent sections. G, H, Representative micrographs showing myelin sparing at the injury epicenter in section stained against fluoromyelin from mice treated with saline (G) or AM095 (H; n = 8 per group). Scale bar, 500 μm. *p < 0.05. I, Quantification of axon and myelin sparing in the dorsal column at the epicenter of the injury. Treatment with AM095 resulted in greater myelin but not axonal preservation. J, K, Representative micrographs of the dorsal columns stained against NF and MBP from mice treated with saline (J) and AM095 (K). L, Quantification of ventral (Figure legend continues.)
is an active factor in hemorrhagic fluids (Yung et al., 2011). Indeed, the presence of the 20:4 LPA in the injured spinal cord, which is a major form of LPA in plasma (Scherer et al., 2009) but that was not found in the intact cord, supports hemorrhagic blood fractions as a source of LPA in SCI.

Exogenous LPA can initiate a large range of neural sequelae within the nervous system that includes effects on most cell types and activities in disease models. Cell types include developing neural cells (Dubin et al., 1999, 2010; Kingsbury et al., 2003), astrocytes (Shano et al., 2008; Spohr et al., 2008), microglia (Möller et al., 2001; Ma et al., 2013), oligodendrocytes and Schwann cells (Weiner et al., 1998, 2001), and neurons (Fukushima et al., 2002; Trimbach et al., 2009; Lin et al., 2012). Disease models include pain (Inoue et al., 2004; Lin et al., 2012; Ma et al., 2013), hypoxia and ischemia (Herr et al., 2011; Halder et al., 2013), demyelination (Inoue et al., 2004, 2008a,b; Nagai et al., 2010), and behavioral disorders (Contos et al., 2000, 2002; Santin et al., 2009; Castillo-Ortega et al., 2012; Pedraza et al., 2014). These physiological responses linked to LPA activation are triggered by a variety of downstream pathways, including Ca2+ mobilization, adenylyl cyclase inhibition, activation of Rho, mitogen-activated protein kinase, phospholipase C, and Akt (Yung et al., 2014, 2015; Sheng et al., 2015). The findings here identify LPA as a new trigger of secondary damage in SCI. The mechanism involves LPA1, which is the most highly expressed LPAR in the spinal cord, suggesting that the detrimental actions of LPA are likely to be mediated by binding to LPA1. The maLPA1 knock-out mice and mice treated with AM095, a selective LPA1 inhibitor (Swaney et al., 2011), showed reduced myelin loss when LPA is injected into the intact spinal cord, revealing that LPA1 signaling is involved in the LPA-demyelinating lesion, consistent with previous studies within the peripheral nervous system (Inoue et al., 2004, 2008a,b; Xie et al., 2008; Nagai et al., 2010; Halder et al., 2013). SCI effects appear to involve microglia/macrophages. Although we also noticed mild astrogliaosis in the spinal cord after LPA injection, the effects of the LPA were more pronounced in microglia/macrophages, suggesting that they are more susceptible to activation by LPA. Importantly, we also observed that intraspinal injection of LPA led to the development of a demyelinating lesion in the spinal cord. This is of crucial importance because myelin loss contributes to functional impairments after SCI and in other neural conditions, such as multiple sclerosis.

LPA1 is found on oligodendrocytes (Weiner et al., 1998), and its expression appears shortly before maturation/myelination (Garcia-Diaz et al., 2014). Although LPA1 signaling plays a key role in the regulation of oligodendrocyte differentiation and myelination in the CNS (Garcia-Diaz et al., 2014), little is known about the effects of LPA on mature oligodendrocytes. Our data indicate that LPA exerts mild toxicity in oligodendrocytes at doses ≥1 μM but not in oligodendrocyte precursor cells. The mechanism underlying LPA toxicity is unknown, but this effect is not mediated via LPA1 because AM095 failed to rescue cell death, at least in cell culture. Hence, LPA-mediated demyelination is likely to be mediated by the activation of LPA1 in other cell types rather than to direct activation of LPA1 in oligodendrocytes, consistent with our data showing that intraspinal injection of LPA leads to activation of microglia/macrophages that express LPA1, in vivo and in vitro, as we and other authors have demonstrated (Tham et al., 2003). Conditioned medium of microglia stimulated with LPA mediates marked oligodendrocyte cell death, indicating that microglial become cytotoxic during LPA stimulation of LPA1 signaling. Interestingly, selective blockade or gene deletion of LPA1 in LPA-stimulated microglial cells appears to increase oligodendrocyte cell survival. This result suggests that microglia LPA1 is involved in the development of the demyelinating injury triggered by LPA injection into the spinal cord. However, LPA1 inhibition did not completely suppress the harmful effects of LPA-stimulated microglia, suggesting that other microglial LPARs may contribute to oligodendrocyte cell death. Similarly, myelin loss was not completely abrogated in the maLPA1 null mice or in mice administered AM095, indicating that other LPARs or other non-LPA-mediated mechanisms also contribute to demyelination. However, LPA1 blockade does not reduce microglia or macrophage accumulation in SCI, but it likely attenuates the harmful effects linked to LPA1 activation. The soluble factors released by microglial cells during LPA1 activation that mediate oligodendrocyte cell death are unidentified yet, but it can be attributed to cytokine and free radical production because LPA regulates the production of these factor in several conditions (Cummings et al., 2004; Fang et al., 2004; Sevastou et al., 2013).

Activation of Rho, which can be induced by LPA, is one of the most important intracellular pathways that inhibit axonal regen-

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(Figure legend continued.) horn neuron survival at various distances rostral and caudal to the injury epicenter reveals significantly greater neuronal survival in mice treated with AM095. **M**, **N**, Representative micrographs showing sparing of ventral horn neurons in mice administered with saline (**M**) and AM095 (**N**) in sections stained against NeuN at 600 μm rostral to the injury epicenter. Error bars indicate SEM. n = 8 per group.

**Figure 8.** AM095 does not attenuate inflammatory response. **A**, **B**, Representative density plots of FACS analysis showing microglia and myeloid cells in the injured spinal cord of saline-treated (**A**) or AM095-treated (**B**) mice at 7 dpi. Myeloid cell were further discriminated in macrophages based on F4/80 expression. **C**, Graph showing microglia and macrophage counts in the injured spinal cord after LPA1 blockade. Note that treatment with AM095 did not reduce the numbers of microglia and macrophages. Error bars indicate SEM. n = 8 per group.
eration. Previous works revealed that LPA triggers axonal collapse and retraction (Tigyi et al., 1996; Birgbauer and Chun, 2006), in part by activating Rho (Fincher et al., 2014). However, the inhibitory effects of LPA on axonal growth are not triggered by LPA1, at least in vitro (Birgbauer and Chun, 2010).

Reports that a “B3” antibody that can bind to LPA and other lysophospholipids and mediates protection after spinal cord hemisection and traumatic brain injury (Goldshmidt et al., 2012; Crack et al., 2014) are consistent with increased LPA in CNS injuries. However, mechanistic validation, including demonstration that B3 specifically lowers pathogenic LPA availability to account for its effects, remain to be established. Nevertheless, possible therapies targeting LPA, its biosynthetic enzymes, and/or its receptors may represent viable strategies for improving clinical outcomes for SCI in the future. In particular, our results suggest that LPA1 could be a novel therapeutic target for the treatment of acute SCI, as well as other CNS conditions in which demyelination contributes to the pathology.

References


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