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Research article

Lysophosphatidic acid propagates post-injury Schwann cell dedifferentiation through LPA_1 signaling

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ABSTRACT

Lysophosphatidic acid (LPA) is a pleiotropic signaling lipid that acts as ligand for at least six specific G-protein coupled receptors. Schwann cells (SC) are known to mainly express the LPA_1 receptor subtype. An emerging body of evidence has linked LPA with injury-induced peripheral nerve demyelination as well as neuropathic pain. However, the molecular mechanisms underlying its demyelinating effect have not been conclusively elucidated.

We aimed to decipher the demyelinating effect *in vitro* as well as *in vivo* by studying markers of SC differentiation and dedifferentiation: Myelinated dorsal root ganglia (DRG) cultures were treated either with LPA, LPA plus AM095 (LPA_1 antagonist) or vehicle. Myelin content was subsequently investigated by Sudan Black staining and immunocytochemistry. *In vivo*, we performed sciatic nerve crush in C57BL/6 mice treated with AM095 at 10 mg/kg.

In DRG cultures, LPA caused a significant reduction of myelin as demonstrated by both Sudan Black staining and immunocytochemical analysis of myelin basic protein. Demyelination was paralleled by an upregulation of TNF-alpha as well as downregulation of Sox10, a marker for SC differentiation. LPA mediated effects were largely blocked by the addition of the LPA_1 receptor antagonist AM095. In the *in vivo* model, AM095 treatment prior to crush injury increased Sox10 expression in SCs in the distal nerve stump while reducing the number of cells expressing the SC dedifferentiation marker Sox2. Additionally, TNF-alpha immunofluorescence was reduced in CD11b-positive cells. These data indicate that LPA may be a critical factor that shifts SCs towards a post-injury phenotype and contributes to the onset of Wallerian degeneration.

1. Introduction

Sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA) represent the two major bioactive members of the lysophospholipid family. Both lipids have been ascribed significant physiological and pathophysiological roles in the developing and adult nervous, vascular and immune system by acting as ligands for specific G-protein coupled receptors [4]. While the clinical efficacy of S1P receptor modulation has been established for multiple sclerosis [9], LPA receptors represent an emerging therapeutic target for a variety of inflammatory diseases, with phase II clinical trials being completed for systemic sclerosis and idiopathic pulmonary fibrosis [15].

In the peripheral nerve, myelinating Schwann cells are known to be responsive to LPA mainly via expression of the LPA receptor 1 (LPA_1) [3,6]. Activation of LPA_1 has been associated with neuropathic pain after partial sciatic nerve injury and a single intrathecal injection of LPA was found to be sufficient to trigger demyelination in dorsal roots [12]. In this context, LPA treatment was shown to result in the downregulation of myelin basic protein (MBP) and myelin protein zero (P_0) [12]. Additionally, upregulation of LPA_1 has been recognized to occur in the distal nerve stump following injury [6]. However, significant gaps in our understanding of the mechanism by which LPA facilitates demyelination in the peripheral nervous system have remained.

Interestingly, it was previously reported that LPA induces proliferation in Schwann cells isolated from axotomized sciatic nerves [17]. The induction of proliferation in otherwise quiescent adult Schwann cells is a major hallmark of Wallerian degeneration [1]. In response to injury, Schwann cells rapidly (within 24 h) undergo dedifferentiation and start to proliferate. Furthermore, Schwann cells are involved in the initial cytokine/chemokine response by secreting cues

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such as tumor necrosis factor alpha (TNF-alpha) and monocyte chemoattractant protein 1 (MCP-1) [1]. Given that LPA has been demonstrated to induce cytokine expression in a variety of cell types [21], we hypothesized that LPA may not only promote Schwann cell proliferation, but may actually contribute to Schwann cell dedifferentiation and the emergence of a post-injury phenotype that is typically associated with peripheral nerve injury.

2. Materials and methods

2.1. Preparation of murine dorsal root ganglia (DRG) cultures

Undissociated DRG cultures were prepared essentially as previously described [14]. DRGs were harvested from E 15.5 C57BL/6 embryos (Janvier Labs, Le Genest-Saint-Isle, France) by opening the cutis and subcutis along the spine and removing the spinal cord not completely enclosed by the vertebral column. DRGs were collected in Leibovitz's L-15 medium (Thermo Fisher Scientific/Gibco, Waltham, MA, USA) stored on wet ice, centrifuged at 800 rpm for 5 min and carefully resuspended in Neurobasal medium (NBM). NBM consisted of Dulbecco's modified eagle's medium (DMEM, low glucose; Lonza, Basel Switzerland) containing 10% horse serum (Invitrogen, Carlsbad, CA, USA), 50 U/ml penicillin/streptomycin (Sigma-Aldrich, Munich, Germany), 10 ng/ml nerve growth factor beta (Sigma-Aldrich), 4 g/l D-glucose (Sigma-Aldrich) and 2 mM L-glutamine (Gibco). DRGs were plated on collagen-coated 24-well plates. Following incubation in NBM for seven days, cultures were incubated with mouse myelination medium (MMM). MMM consisted of minimal essential medium (MEM; Invitrogen) containing 10% horse serum, 4 g/l D-glucose, 50 U/ml penicillin/streptomycin, 2 mM L-glutamine, 5 ng/ml nerve growth factor beta, 0.02 mg/ml bovine pituitary extract (Merck Millipore, Billerica, MA, USA), 0.005% ascorbic acid (Sigma-Aldrich), 500 nM forskolin (Sigma-Aldrich) and N2 supplement (Invitrogen). MMM was renewed every 3-4 days. Cultures were kept at 37 °C and 10 % CO_2 atmosphere.

2.2. Treatment of cultures with LPA and AM095

LPA (1-oleoyl-2-hydroxy-sn-glycero-3-phosphate) was obtained from Avanti Polar Lipids (Alabaster, AL, USA) and the LPA_1 antagonist AM095 from ApexBio (Boston, MA, USA). LPA was dissolved in PBS containing 0.1% bovine serum albumin (BSA, fatty acid free; Sigma Aldrich). AM095 was dissolved in DMSO (Sigma-Aldrich). After 10 days incubation in MMM, cultures were treated with forskolin-omitted MMM containing either vehicle (0.01% BSA and 0.1% DMSO), 10 µM LPA + 0.1% DMSO or 10 µM LPA + 10 µM AM095 for 24 h.

2.3. Sciatic nerve crush and administration of AM095

Male, age-matched (3–4 months) C57BL/6 mice (Janvier Labs) were anesthetized for surgery via intraperitoneal injection of a mixture of xylazine (Rompun; Bayer, Leverkusen, Germany) (10 mg/kg) and ketamine (Actavis, Munich, Germany) (100 mg/kg) and placed on a heating plate (37 °C) to maintain constant body temperature. All instruments were sterilized. The fur of the lower back was removed with an electric razor, and the skin was disinfected using 70% ethanol. A small incision (1 cm) was made in the skin above the right hindlimb between the mm. gluteus maximus and biceps femoris. Opening the facial plane between both muscles revealed the sciatic nerve which was carefully lifted using bent forceps and crushed right before its distal branches using a lockable non-serrated clamp at maximum intensity for 30 s. The nerve was replaced under the muscle, and the incision was closed using non-absorbable suture material.

AM095 was administered at a concentration of 10 mg/kg [7] via intraperitoneal injection immediately before surgery. Controls received an equal volume of vehicle.

2.4. Tissue preparation and immunohistochemistry

Mice were sacrificed 24 h post-crush via cervical dislocation. Preparation of sciatic nerves and immunohistochemical procedures were performed essentially as described previously [2]. Images were taken using a Zeiss Axioplan 2 microscope.

2.5. Antibodies

The following antibodies were used and diluted in Antibody Diluent (Dako) as indicated: mouse anti-MBP monoclonal SMI-94 (Biolegend, San Diego, CA, USA) 1:500; rabbit anti-neurofilament L polyclonal (Merck-Millipore) 1:800; rat anti-TNF-alpha monoclonal MP6-XT22 (Acris, Herford, Germany) 1:100; rabbit anti-Sox2 polyclonal (abcam, Cambridge, UK) 1:1000; rabbit anti-Sox10 polyclonal (abcam) 1:1000; rat anti-mouse CD11b clone 5C6 (Serotec) 1:200; Alexa Fluor 488/594 goat anti-rabbit IgG (Life Technologies, Carlsbad, CA, USA) 1:200; Alexa Fluor 594 goat anti-rat IgG (Life Technologies) 1:200; biotinylated goat anti-rabbit IgG (Vector Laboratories) 1:200; DyLight 594 Streptavidin (Vector Laboratories) 1:200.

2.6. Immunocytochemistry

Cultures were fixed with 4% PFA for 20 min, washed and incubated with blocking solution (PBS containing 10% normal goat serum and 0.1% Triton X-100) for 1 h at room temperature (RT). Cultures were incubated with primary antibodies for 1 h at RT. Following two washing steps in PBST (PBS containing 0.1% Triton X-100), secondary antibodies were applied and incubated for 1 h at RT. Following three washing steps in PBST, PBS was applied to each well and fluorescence images were immediately taken in randomly selected fields by a blinded investigator using a Nikon Eclipse TE200 microscope.

2.7. Sudan black staining

Cultures were stained with Sudan Black dye as described previously [14] to assess the extent of myelination. Myelin quantification was performed by counting the total number of internodes and correlating them to the total number of neurons within the individual culture wells. Images were taken on a Zeiss LSM510 microscope.

2.8. Image and data analysis

Image analysis was performed using ImageJ (NIH, Bethesda, MA, USA). Data analysis and compilation of graphs was performed using Microsoft Excel 2010 and GraphPad Prism 5. Statistical analysis was done by Student's *t*-test, for multiple comparisons one-way ANOVA followed by Newman-Keuls post hoc test was performed. Statistical significance is indicated by asterisks with $P \leq 0.05$*, $P \leq 0.01$**, and $P \leq 0.001$***.

3. Results

3.1. LPA causes demyelination of DRG cultures in an LPA_1 dependent manner

To study the effect of LPA on demyelination *in vitro*, myelinated DRG cultures were treated with LPA, a combination of LPA and the LPA_1 receptor antagonist AM095 or vehicle for 24 h and subsequently stained with Sudan Black (Fig. 1A). LPA caused a reduction of myelin as determined by measuring the number of internodes per neurons (Fig. 1A, B). A significant reduction of myelin in response to LPA treatment was not observed when applied in combination with AM095, suggesting that the demyelinating effect of LPA is dependent on the LPA_1 receptor.

To substantiate these findings, we assessed myelination via