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The phospholipase D pathway mediates the inflammatory response of the retinal pigment epithelium

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ABSTRACT

The retinal pigment epithelium (RPE) plays an important immunological role in the retina and it is involved in many ocular inflammatory diseases that may end in loss of vision and blindness. In this work the role of phospholipase D (PLD) classical isoforms, PLD1 and PLD2, in the inflammatory response of human RPE cells (ARPE-19) was studied.

ARPE-19 cells exposed to lipopolysaccharide (LPS, 10 μg/ml) displayed increased levels of NO production and diminished mitochondrial function after 48 h of incubation. Furthermore, 24 h LPS treatment strongly induced cyclooxygenase-2 (COX-2) expression and activation of extracellular signal-regulated kinase (ERK1/2). EGFP-PLDs showed the typical subcellular localization, perinuclear for PLD1 and plasma membrane for PLD2. LPS increased PLD activity by 90% with respect to the control. The presence of PLD1 inhibitor (EVJ 0.15 μM) or PLD2 inhibitor (APV 0.5 μM) reduced LPS-induced COX-2 induction but only PLD2 inhibition reduced ERK1/2 activation. Mitochondrial function was restored after inhibition of PLD2 and ERK1/2. These findings evidence the participation of PLD2 as a promoter of RPE inflammatory response through ERK1/2 and COX-2 regulation. Our results demonstrate for the first time distinctive roles of PLD isoforms in pathological conditions in RPE.

1. Introduction

The retinal pigment epithelium (RPE) is located between vessels of the choriocapillaris and the light-sensitive retina layer, and closely interacts with photoreceptors (PR) in the process of vision. RPE cells are essential for the maintenance of a healthy retina, and any impairment of their functionality may have deleterious effects on vital activities such as phagocytosis of PR rod outer segments or maintenance of the blood retinal barrier (Lamb et al., 2007; Strauss, 2005). Analyses of hereditary types of retinal degeneration disclose a strong dependence of PR on RPE cells. In line with this, mutations in genes which are expressed in RPE are responsible for primary PR degeneration (Sparrow et al., 2010). Increasing knowledge on the multiple functions performed by the RPE has contributed to the understanding of many diseases leading to blindness, such as Stargardt's disease (STGD), retinitis pigmentosa (RP) and age-related macular degeneration (AMD). RPE cells also mediate the immune response in the eye and can (Perez et al., 2013; Ishida et al., 2003) secrete immune modulatory factors, such as interleukin-8 (IL-8), complement factor H (CFH) or monocyte chemotactic protein-1 (MCP1) (Strauss, 2005).

In spite of the close homology of RPE with immune system cells, phospholipase D (PLD) expression and their role in this specialized epithelium remain unknown. PLD isoforms have been shown to be involved in many functions of the blood immune response. PLD catalyzes phosphatidylcholine (PC) hydrolysis to generate the lipid second messenger, phosphatidic acid (PA), and choline. PA generated by PLD can be further hydrolyzed by lipid phosphate phosphatases (LPPs) in order to generate diacylglycerol (DAG), another lipid second messenger (Peng and Frohman, 2012). The two most studied isoforms, PLD1 and PLD2, and their product, PA, have been involved in a variety of signaling and membrane

Abbreviations: AA, arachidonic acid; AMD, age-related macular degeneration; COX, cyclooxygenase; DAG, diacylglycerol; DR, diabetic retinopathy; EGFP, enhanced green fluorescent protein; ERK, extracellular signal-regulated kinase; EtOH, ethanol; HRP, horseradish peroxidase; iNOS, inducible nitric oxide synthase; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; LPPs, lipid phosphate phosphatases; MAPKs, mitogen-activated protein kinases; MEK, MAPK kinase; NO, nitric oxide; OA, oleic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PEth, phosphatidylethanol; PGs, prostaglandins; PIP_2-PLC, phosphatidylinositol bisphosphate-phospholipase C; PLD, phospholipase D; PR, photoreceptor; PVDF, polyvinylidene fluoride; RPE, retinal pigment epithelium; RP, retinitis pigmentosa; STGD, Stargardt's disease; TTBS, Tween-tris buffer solution.

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trafficking events in different immunity cells (Jenkins and Frohman, 2005; McDermott et al., 2004; Zeng et al., 2009; Peng and Frohman, 2012).

RPE inflammatory processes are known to be associated with the above-mentioned pathologies. Prostaglandins (PGs) are the most important lipid mediators involved in the generation of the inflammatory response. They are generated from arachidonic acid (AA) by cyclooxygenase 2 (COX-2) action and their biosynthesis is significantly increased in inflamed tissue, thus contributing to the development of acute inflammation. COX-2 regulation involves gene transcriptional and post-transcriptional events, such as COX-2 mRNA stability and translational efficiency, specific microRNAs and RNA-binding proteins, and COX-2 alternative polyadenylation (Dixon et al., 2013). However, the intracellular signaling pathways involved in the regulation of COX-2 activity remain obscure.

Several lines of evidence described a functional role for PLD in COX-2 regulation during cell activation (He et al., 2008; Ahn et al., 2007; Kim et al., 2004). However, the contribution of PLD to COX-2 expression, posttranslational regulation and PGs production during inflammatory processes in the RPE has not yet been elucidated. Based on all these evidences, we tested the hypothesis of PLD participation in RPE inflammatory process. Specifically, we explored the crosstalk between PLD-generated signaling and COX-2 regulation in human retinal epithelium cells (ARPE-19). To this end, ARPE-19 cells were challenged with increasing lipopolysaccharide (LPS) concentrations (as an inflammatory model) and the activity, expression, subcellular localization of PLD1 and PLD2 isoforms and their involvement in COX-2 regulation and PGs production were analyzed.

2. Materials and methods

2.1. Reagents

Triton X-100, dimethyl sulfoxide (DMSO), U0126, U73122, Celecoxib, LPS from *Pseudomona aeruginosa* (L7018) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). VU0359595 or EVJ, VU0285655-1 or APV, oleic acid (OA) and arachidonic acid (AA) were from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Radiolabeled oleic acid [9,10-^{3}H(*N*)] ([^{3}H]-OA) (15–60 Ci/mmol) and arachidonic acid [5,6,8,9,11,12,14,15-^{3}H(*N*)] ([^{3}H]-AA) (60–100 Ci/mmol) were purchased from New England Nuclear-Dupont, Boston, MA, USA. Preblended dry fluor 2a70 (98% PPO and 2% bis-MSB) was obtained from Research Products International Corp. (Mount Prospect, IL, USA). All other chemicals were of the highest purity available.

2.2. Antibodies

Rabbit polyclonal antibodies anti-ERK1/2 (#9102), anti-phospho-ERK1/2 (#9101), and anti-PLD1 (#3832) were from Cell Signaling (Beverly, MA, USA). Rabbit polyclonal antibodies anti-TLR4 (H-80) (sc-10741) and anti-CD14 (M-305) (sc-9150), goat polyclonal anti-PLD2 antibody (sc-48269), polyclonal horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG and polyclonal HRP-conjugated goat anti-mouse IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Mouse monoclonal anti-α Tubulin (DM1-A) (CP06) was from EMD/Biosciences-Calbiochem (San Diego, CA, USA). Rabbit polyclonal antibody anti-COX-2 (160116) and mouse monoclonal antibody anti-COX-1 (160110) were from Cayman Chemical (Ann Arbor, MI, USA). Mouse monoclonal antibody anti-EGFP (MMS-108P) was from Covance Inc. (Santa Cruz, CA, USA).

2.3. Retinal-pigmented epithelium cell culture and treatments

Human retinal-pigmented epithelium cells (ARPE-19) from the American Type Culture Collection (ATCC, Manassas, VA) were generously donated by Dr. E. Politi and Dr. N. Rotstein (INIBIBB, Bahía Blanca, Argentina). ARPE-19 cells, a spontaneously arising human RPE cell line, were routinely passaged by dissociation in 0.25% trypsin and 5 mM EDTA in Hanks' balanced salt solution without calcium and magnesium (HBSS), followed by replating at a split ratio 1:3. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Natocor, Argentina), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B at 37 °C under 5% CO_2. Confluent ARPE-19 cell cultures were serum-starved for 2 h prior to stimulation and treated for 2, 24, 48 or 72 h with different doses (5, 10 or 100 μg/ml) of *P. aeruginosa* LPS in serum-free DMEM. In control conditions the same volume LPS was replaced for sterile ultra pure water. Inhibition of specific signaling pathways was achieved by incubation of ARPE-19 cells with selective inhibitors for 1 h at 37 °C prior to cell stimulation with LPS. DMSO (vehicle of the inhibitors) was added to all conditions (including control conditions) to achieve a final concentration of 0.025%.

2.4. MTT reduction assay

MTT, a water-soluble tetrazolium salt, is reduced by metabolically viable cells to a colored, water-insoluble formazan salt. This reduction depends on the activity of mitochondrial dehydrogenases. After LPS treatment (for 24, 48 or 72 h), MTT (5 mg/ml, prepared in phosphate buffer saline) was added to the cell culture medium at a final concentration of 0.5 mg/ml. The culture dishes (35 mm) were incubated for 1 h at 37 °C in a 5% CO_2 atmosphere, cells were washed twice with phosphate buffer saline (PBS) and lysed with 600 μl of a buffer containing 10% Triton X-100 and 0.1 N HCl in isopropanol. The extent of MTT reduction was measured spectrophotometrically at 570 nm using a V-630 spectrophotometer (JASCO, Analytical Instruments). Results are expressed as arbitrary units with respect to the control condition.

2.5. LDH assay

Lactate dehydrogenase (LDH) leakage was evaluated as a parameter of plasma membrane integrity using kit LDH-P UV AA (Wiener laboratory, Rosario, Argentina). After LPS treatment (for 24, 48 and 72 h) LDH activity was measured spectrophotometrically in 50 μl of the culture medium following the manufacturer's instructions. Briefly, the rate of conversion of reduced nicotinamide adenine dinucleotide (NADH) to oxidized nicotinamide adenine dinucleotide (NAD^+) was followed at 340 nm using a V-630 spectrophotometer (JASCO Analytical Instruments).

2.6. Determination of NO production

Nitrites (NO_2^-) were measured in the incubation media as a stable and non-volatile breakdown product of the nitric oxide (NO) released by ARPE-19 cells, employing the spectrometric Griess reaction as previously described (Cutini et al., 2012).

2.7. COX-2 activity assay

To determine COX-2 activity, the generation of PGs F and E (PGF_2 and PGE_2) was measured. Confluent 60 mm dishes (2 per condition) were incubated at 37 °C with [^{3}H]-AA. The labeled fatty acid was mixed with unlabeled AA (final concentration of 1.5 μM and 0.25 μCi/dish) in the presence of lipid-free bovine serum albumin (BSA) (4 mol AA/mol BSA) in serum-free DMEM. Cells were