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Epigenetic regulation of the formyl peptide receptor 2 gene

Felice Simiele [a,c], Antonio Recchiuti [a,c], Sara Patruno [a,c], Roberto Plebani [a,c], Anna Maria Pierdomenico [b,c], Marilina Codagnone [a,c], Mario Romano [a,c,*]

[a] Department of Medical, Oral, and Biotechnological Science, "G. D'Annunzio" University of Chieti-Pescara, Via Luigi Polacchi 11/13, 66100 Chieti, Italy
[b] Department of Medicine and Aging Science, "G. D'Annunzio" University of Chieti-Pescara, Via Luigi Polacchi 11/13, 66100 Chieti, Italy
[c] Center of Aging Sciences and Translational Medicine (CeSI-MeT), "G. D'Annunzio" University of Chieti-Pescara, Via Luigi Polacchi 11/13, 66100 Chieti, Italy

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ABSTRACT

Lipoxin (LX) A_4, a main stop signal of inflammation, exerts potent bioactions by activating a specific G protein-coupled receptor, termed formyl peptide receptor 2 and recently renamed ALX/FPR2. Knowledge of the regulatory mechanisms that drive ALX/FPR2 gene expression is key for the development of innovative anti-inflammatory pharmacology. Here, we examined chromatin patterns of the ALX/FPR2 gene. We report that in MDA-MB231 breast cancer cells, the ALX/FPR2 gene undergoes epigenetic silencing characterized by low acetylation at lysine 27 and trimethylation at lysine 4, associated with high methylation at lysine 27 of histone 3. This pattern, which is consistent with transcriptionally inaccessible chromatin leading to low ALX/FPR2 mRNA and protein expression, is reversed in polymorphonuclear leukocytes that express high ALX/FPR2 levels. Activation of p300 histone acetyltransferase and inhibition of DNA methyltransferase restored chromatin accessibility and significantly increased ALX/FPR2 mRNA transcription and protein levels in MDA-MB231 cells, as well as in pulmonary artery endothelial cells. In both cells types, changes in the histone acetylation/methylation status enhanced ALX/FPR2 signaling in response to LXA_4. Collectively, these results uncover unappreciated epigenetic regulation of ALX/FPR2 expression that can be exploited for innovative approaches to inflammatory disorders.

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1. Introduction

Resolution of inflammation, an active process that prevents tissue damage and re-establish homeostasis, is governed by specific mediators [1]. The arachidonic acid (AA)-derived lipoxins (LX), were the first chemical autacoids recognized to have dual anti-inflammatory and pro-resolution activities [2–4]. LXA_4 (5,6,15S-trihydroxy-7,9,11,13-trans-11-cis-eicosatetraenoic acid) is biosynthesized primarily during cell–cell interactions that occur in vivo by trans-cellular routes involving 5-Lipoxygenase (LO) and 15-LO or 5- and 12-LO [4]. In addition, 15-epi-LXA (5,6,15R-trihydroxy-7,9,11,13-trans-11-cis-eicosatetraenoic acid), formed in the presence of aspirin via acetylation of endothelial cyclooxygenase-2 (COX-2) [5], proved to mediate anti-inflammatory actions of low-dose aspirin in humans [6]. LXA_4 and 15-epi-LXA_4 inhibit polymorphonuclear leukocyte (PMN) infiltration in inflamed tissues, promote inflammation resolution in vivo by stimulating phagocytosis of apoptotic cells and microbes, and have protective actions in the cardiovascular district by stimulating the production of prostacyclin and nitric oxide, upregulating heme oxygenase-1, and reducing oxidative stress in endothelial cells [4].

LXA_4 and 15-epi-LXA_4 bind and activate a specific G protein-coupled receptor (GPCR), initially identified in human leukocytes and termed formyl peptide receptor like-1 (FPRL-1) or FPR2 [7], recently renamed ALX/FPR2, because of its high affinity for LXA_4 [8]. This receptor is also recognized by resolvin (Rv)D1 [9,10] and by the glucocorticoid-induced protein annexin-1 and its N-terminal peptides [11], two additional main components of the resolution cascade. Thus, ALX/FPR2 represents the first identified GPCR able to mediate anti-inflammatory and pro-resolving actions of both lipid and peptide endogenous mediators.

Human ALX/FPR2 is expressed in several cell types, including leukocytes, endothelial and epithelial cells [4]. Although a number of pro-inflammatory peptides can activate this receptor in vitro [11], its main function in vivo appears to be anti-inflammatory and pro-resolutive, as documented by studies of targeted overexpression and genetic deletion of both human and murine orthologs of this GPCR [12,13]. Along these lines, Morris et al. reported that 15-epi-LXA_4 biosynthesis and ALX/FPR2 expression determine the magnitude and duration of inflammatory reaction in humans [14], while other studies demonstrated decreased biosynthesis of LXA_4 and expression of ALX/FPR2 in patients with chronic disease, like asthma [12,13] in which LXA_4 is pro-resolutive and organ protective [15]. These results unveil the importance of the LX-ALX/FPR2 axis in orchestrating anti-inflammatory and pro-resolution pathways in humans, underscoring the need for knowledge of regulatory mechanisms of ALX/FPR2 expression as proper

* Corresponding author at: CeSI-MeT, Via Luigi Polacchi 11/13, 66013 Chieti, Italy.
E-mail address: mromano@unich.it (M. Romano).

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background for the development of innovative anti-inflammatory pharmacology. Along these lines, we recently identified a ALX/FPR2 gene promoter region and a heritable genetic variant associated with reduced receptor expression [16]. More recently, we demonstrated that ALX/FPR2 expression and pro-resolution actions is regulated by microRNA 181b [17].

Here, we carried out a detailed analysis of epigenetic mechanisms in control of ALX/FPR2 expression. We report that the pattern of methylated and acetylated histones along the ALX/FPR2 gene is very different between cells with high or low ALX/FPR2 expression and that by unlocking the repressive profile it is possible to considerably upregulate receptor expression and response to agonist.

2. Materials and methods

2.1. Cells

Polymorphonuclear cells (PMN) isolated as in [9] were maintained at the density of 2×10^5/mL in Roswell Park Memorial Institute medium (RPMI 1640, PAA, Pasching, Austria) with 10% (vol/vol) fetal bovine serum (FBS, Invitrogen, San Giuliano Milanese, Italy). Human breast cancer MDA-MB231 cells were grown in high glucose Dulbecco's Modified Eagle Medium (DMEM, PAA) with 10% (vol/vol) FBS.

2.2. Chromatin immunoprecipitation

Cells were fixed (10 min, r.t.) to cross-link DNA-protein complexes, nuclei were extracted following cell lysis and dounce homogenization and then precipitated (5.000 rpm, 10 min, 4 °C). Pellets were suspended, sheared and sonicated (10 cycles of 20 s) in ice in appropriate buffer with protease inhibitors. Nuclear extracts were mixed with protein G beads (2 h, 4 °C) followed by specific anti K27me3, K4me3, K27 ac antibodies, or non-binding IgG as negative control. After incubation (16 h, 4 °C) on a rotating wheel, protein G beads were added to each of the antibody/chromatin incubations for 1.5 h at 4 °C. Immunoprecipitated DNA was eluted from the washed Protein G beads; cross-links were disrupted by heating at 65 °C overnight, and DNA was purified after proteinase K digestion (2 h, 42 °C) using silica gel columns (ChiP-IT kit, Active Motifs, Carlsbad, CA). Purified DNA was then used as a template for real-time PCR analyses of enrichment in select histones (Post Translational Modification = PTM) in several regions of the ALX/FPR2 gene (minus 7 kb, plus 200 kb, TSS, and TES) using the primers listed in Table 1.

2.3. PAEC isolation

Segments of pulmonary arteries were chirurgical removed and exposed to antibiotics (250 µg/ml of linezolid, 50 U/ml of colistin, 25 µg/ml of cotrimoxazole and 25 µg/ml of amphotericin B) to prevent bacterial contamination. After eliminating the excess of fat and connective tissue, the artery segments were washed 3 times with PBS containing 1% penicillin/streptomycin (P/S) and 1% amphotericin B to remove blood cells. Specimens were then incubated 20 min at 37 °C within tubes containing PBS without calcium and magnesium supplemented with 2 mg/ml collagenase type II. Arteries were then transferred to a Petri dish containing culture medium (DMEM-M199 medium (50% vol/vol), 10% FBS, 1% L-glutamine, 1% P/S, 1% ECGF, 1% heparin), and cells were collected by gentle agitation. Cells were harvested by centrifugation (330 ×g, 7 min) and seeded in fibronectin-coated plates (1 mg/cm^2) (Sigma-Aldrich, St. Louis, MO, USA). The endothelial phenotype was assessed by flow cytometric evaluation of von Willebrand Factor and CD31 expression.

Table 1
Primers used for chromatin immunoprecipitation and evaluation of ALX/FPR2 mRNA expression.

Primer name	Sequence (5′ → 3)
Forward TTS	TCTCTCACAGAAGGCTAGAG
Reverse TTS	CAGAAGACACCTTGGGTATG
Forward TES	CTTCCGAGAGAGACTGATCC
Reverse TES	CAGGAGGTGAAGCAGAATTG
Forward plus 200 kb	AAATCCTGAGTTTCTGTTAGGG
Reverse plus 200 kb	TGTGTGCTTCTTGGCTTC
Forward minus 7 kb	AAAGCAGTGGACTGAGGTGG
Reverse minus 7 kb	GCTTGTCCGGAGCTAGTGAA
Forward Murphy	TGGAAACCAACTTCTCCACTC
Reverse Murphy	TGGAAACCAACTTCTCCACTC
Forward Gapdh	CCACCCATGGCAAATTCCATGGCA
Reverse Gapdh	TCTAGACGGCAGCTCAGGTCCACC
Forward ALX/FPR2	GGCCAAGACTTCCGAGAGAG
Reverse ALX/FPR2	CCGTGTCATTAGTTGGGGCT

2.4. Quantitative real-time PCR for detection ALX/FPR2 mRNA

Total RNA was extracted using a total RNA extraction kit (Norgen, Thorold, ON, Canada) and reverse transcribed using M-MLV Reverse Transcriptase (Sigma-Aldrich, Milan). ABI PRISM 7900 HT real-time PCR thermal cycler was used to carry out quantitative PCR. Expression of ALX/FPR2 mRNA was quantified using Forward ALX/FPR2 and Reverse ALX/FPR2 primers (Table 1) and Syber green. Ct values were normalized using GAPDH (primers listed in Table 1) as housekeeping gene in the comparative ΔΔCT method [18].

2.5. Flow cytometry

MDA-MB231 cells (5×10^5) were fixed with 3% paraformaldehyde (10 min, 4 °C), permeabilized (10 min, r.t.) with BD Permeabilizing Solution 2 (Becton Dickinson, Franklin Lakes, NJ, USA) and incubated (30 min at 4 °C) with an *anti*-ALX/FPR2 (Genovac GmbH, Freiburg, Germany) antibody in Dulbecco's Phosphate Buffered Saline (DPBS) plus 0.5% bovine serum albumine (BSA) and sodium azide (0.05%). Fluorochrome-conjugated anti-mouse secondary antibody (Sigma-Aldrich) was used in the same buffer (30 min at 4 °C) to label primary antibody. Analyses were carried out on a FACSCalibur flow cytometer (BD) and results were analyzed using the CELLQuest software (BD).

2.6. Cell proliferation

MDA-MB231 (1.0×10^6) were seeded in 100 mm tissue culture dishes and treated for 48 h with cell permeable CTPB (Axxora, LCC Farmingdale, NY, USA) (180 µM), followed by LXA_4 (100 nM) with or without WRW4 (10 µM). Viable cells were identified by Trypan blue staining and counted after 48 h.

2.7. Impedance measurements

We used the xCELLigence real-time cell analyzer (RTCA) by ACEA. This system calculates the impedance of cell monolayers, which is influenced by proliferation, adhesion and spreading. PAEC (8×10^3) were seeded in each well of a *E*-Plate, previously coated with 1,5% gelatin. The E-Plates were incubated at 37 °C, 5% CO_2 and monitored every min for 10 min. Replicates were carried out for each experimental condition [19]. LXA_4 (100 nM) and WRW4 (10 µM) were added 48 h after acetylating and/or demethylating agents.

2.8. Statistical analysis

Results are reported as mean ± S.D. Statistical analysis was performed using Student's *t*-test with *P* values <0.05 considered to be significant.

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