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Original research article

Specialized pro-resolvin mediators induce cell growth and improve wound repair in intestinal epithelial Caco-2 cell cultures

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ABSTRACT

Specialized pro-resolvin mediators (SPMs) are a superfamily of bioactive molecules synthesized from polyunsaturated fatty acids (arachidonic, eicosapentaenoic and docosahexaenoic acids) that include resolvins, protectins and maresins. These metabolites are important to control the resolution phase of inflammation and the epithelial repair, which is essential in restoring the mucosal barriers. Unfortunately, the effects of SPMs on intestinal epithelial cell growth remain poorly understood. Caco-2 cell were used as intestinal epithelial cell model. Cell growth/DNA synthesis, cell signalling pathways, western blot and wound repair assay were performed. Our data demonstrated that SPMs such as lipoxin LxA_4, resolvin (Rv) E1, RvD1, protectin D 1 and maresin 1 were able to enhance intestinal epithelial Caco-2 cell growth and DNA synthesis. Furthermore, our results provide evidence that these effects of RvE1 and RvD1 were associated with a pertussis toxin-sensitive G protein-coupled receptor, and that leukotriene B_4 receptor 2 could be involved, at least in part, in these effects of RvE1/RvD1. Moreover, these mitogenic effects induced by SPMs were dependent on the ERK 1/2 and p38 MAPK pathways as well as phospholipase C and protein kinase C activation. Thus, these mitogenic effects of RvE1/RvD1 on intestinal epithelial cells could be involved in this signalling circuit involved in wounded epithelium and the catabasis process.

Abbreviations

AA	arachidonic acid
BLT1/BLT2	leukotriene B_4 receptors
ChemR23	chemerin receptor 1
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
FBS	fetal bovine serum
FPR2/ALX	N-Formyl peptide receptor 2/LxA_4 receptor
GPR	G-coupled protein receptor
LxA_4	lipoxin A_4
Rv	resolvin
SPMs	specialized pro-resolvin mediators

1. Introduction

The intestinal epithelium is a dynamic structure that is continuously renewed through cell proliferation, cell differentiation and apoptosis processes. Furthermore, it is an important interface with the external environment. Thus, the homeostatic maintenance of the epithelium architecture and epithelial barrier function are necessary to the digestive health and host well-being. Epithelial barrier disruption induces a spatiotemporal recruitment of immune cells with a consequent biosynthesis/release of a complex group of mediators that interact with the intestinal mucosa to orchestrate inflammation resolution and intestinal epithelium repair. The perturbation of the delicate balance of inflammation, resolution and repair processes is a key element involved in the chronicity of the intestinal inflammation [1].

Dietary linoleic acid can be conversed to arachidonic acid (AA) by mammal cells. This polyunsaturated fatty acid (PUFA) is esterified in the phospholipid membranes and can be precursor of numerous bioactive eicosanoids biosynthesised by intestinal epithelial cells [2]. These lipid mediators are key elements involved in the regulation of intestinal epithelial structure/function [3,4]. In addition to classical inflammatory mediators such as prostaglandins or leukotrienes, epithelial mucosa can release specialized pro-resolvin mediators (SPM) such as maresin-1 [5],

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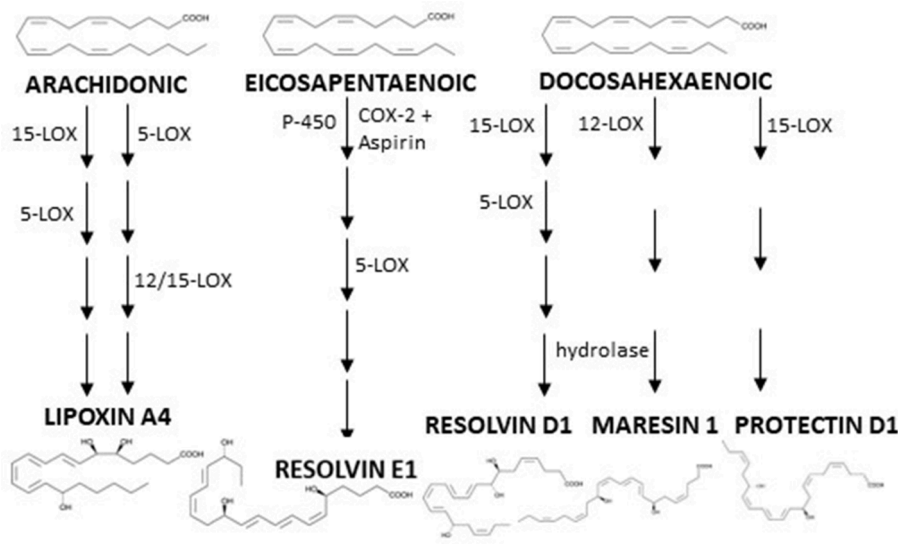

Fig. 1. Main specialized pro-resolvin mediators (SPM) synthetised from eicosapentaenoic or docosahexaenoic acids. COX, cyclooxygenase; LOX, lipoxygenase; P-450, cytochrome P-450.

which have been demonstrated to play a main role to restore intestine homeostasis. Le Faouder et al. [5] also reported the SPM synthesis by infiltrated immune cells during intestinal inflammatory processes. Interestingly, Quiros and Nusrat [1] recently reported that SPM are important to direct the resolution phase of inflammation and the epithelial repair, which is essential in restoring the intestinal mucosal barrier.

SPMs constitute a superfamily of bioactive metabolites of PUFAs that include lipoxin A_4 (LxA_4) from AA, eicosapentaenoic acid (EPA)-derived resolvins (Rvs) (the E-series Rvs) including RvE1, and docosahexaenoic acid (DHA)-derived Rvs (the D-series Rvs) such as RvD1, protectin D1, maresins 1 and 2 and maresin conjugates [6] (Fig. 1). These lipid mediators are produced in response to physiological stress as well as tissue injury through the modulation of immune and nonimmune cells in dextran sodium sulphate-induced experimental intestinal inflammation [7]. Interestingly, Quiros et al. [8] have recently demonstrated that RvE1 induces intestinal epithelial wound healing in murine colonic mucosa. Nevertheless, the effects of SPMs on intestinal epithelial cell growth remain poorly understood. Considering these antecedent, the goal of this study was analysed the role of representative SPMs on intestinal epithelial cell growth and wound closure, and to explore the mechanism involved in these events.

2. Materials and methods

2.1. Materials

LxA_4, RvD1, RvE1, maresin 1, protectin D1, U-73,122, U-75,302 and PBP10 were purchased from Cayman Chemical (Ann Arbor, MI, USA). Pertussis toxin (PTX), PD98059, Go6983, KT5720, SB203580, α-NETA, ethidium bromide and acridine orange were from Sigma-Aldrich (St. Louis, MO, USA). LY 255,283 and PSB-CB5 were supplied by Tocris Biosc. (Bristol, UK). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), trypsin, penicillin, streptomycin, and cell culture supplementary products were supplied by GIBCO (Paisley, UK).

2.2. Cell culture and cell growth assay

Caco-2 cells derived from a moderately well-differentiated primary colon adenocarcinoma were provided by American Type Culture Collection (HTB-37, Manassas, VA, USA). The cells (passages 127–136) were routinely grown in 75 or 150 cm^2 plastic flasks at a density of 10^4 cells/cm^2 and cultured in DMEM supplemented with 4.5 g/l D-glucose, 1% (v/v) nonessential amino acids, 2 mM L-glutamine, 10% (v/v) heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a modified atmosphere of 5% CO_2 in air. The growth medium was replaced twice per week and the day before the experiment.

Cell growth/DNA synthesis assays were performed in pre-confluent cultures (≤ 80% confluency) and consequently, in non-differentiated Caco-2 cells. To perform the cell growth assay, cells were harvested using trypsin/EDTA and passed to 12 mm plastic clusters at a density of 10^4 cells/cm^2. After 4 days in culture, pre-confluent cell cultures (3–4 × 10^4 cells/cm^2) were replaced with FBS-free culture medium before to be incubated with treatments for a period of 48 h. Finally, cells were washed, trypsinized and counted microscopically using ethidium bromide/acridine orange staining to measure viable cells [9].

2.3. Analysis of DNA synthesis

DNA synthesis was assessed by a colorimetric immunoassay based on the measurement of BrdU incorporation during DNA synthesis (Cell proliferation ELISA BrdU Kit, from Roche, Basel, Switzerland). Caco-2 cells were cultured at 1000–1500 cell/well in 96 well plates for 96 h in DMEM supplemented with 10% FBS. Then, cells were incubated for 48 h in the presence of compounds. Finally, DNA synthesis were measured following the manufacturer instructions. Absorbance was measured at 450 nm in a plate reader (TECAN, Sunrise, Grödig, Austria).

2.4. Measurement of the cell signalling pathways

Caco-2 cells were seeded in 60 mm plastic clusters (10^4 cells/cm^2) and, after 4 days, the cultures were incubated with the treatments (5 or 15 min). To measure the kinase activation, cells were lysed using a denaturing cell lysis buffer containing 6 M urea and protease (leupeptin 2 μg/ml, pepstatin 10 μM, aprotinin 3 μg/ml) and phosphatase (NaF 5 mM, $Na_4P_2O_7$ 2 mM, Na_3VO_4 1 mM) inhibitors (Sigma-Aldrich). Proteins in cell samples were measured using Bio-Rad protocol with Coomassie brilliant blue G-250 (Bio-Rad, Hercules, CA, USA) as a dye reagent. 80–100 μg of proteins were then added to kinase ELISA plate and the assay was performed following the manufacturer's recommendations (Symansis, Auckland, New Zealand). Optical density was measured at 450 nm. Thus, we studied the effect of SPMs on the phosphorylation of AKT1 (pS473), AKT2 (pS474), ERK1/2 (pT202/Y204; pT185/Y187), GSK3β (pS9), p38α (pT180/Y182) and CREB (pS133).

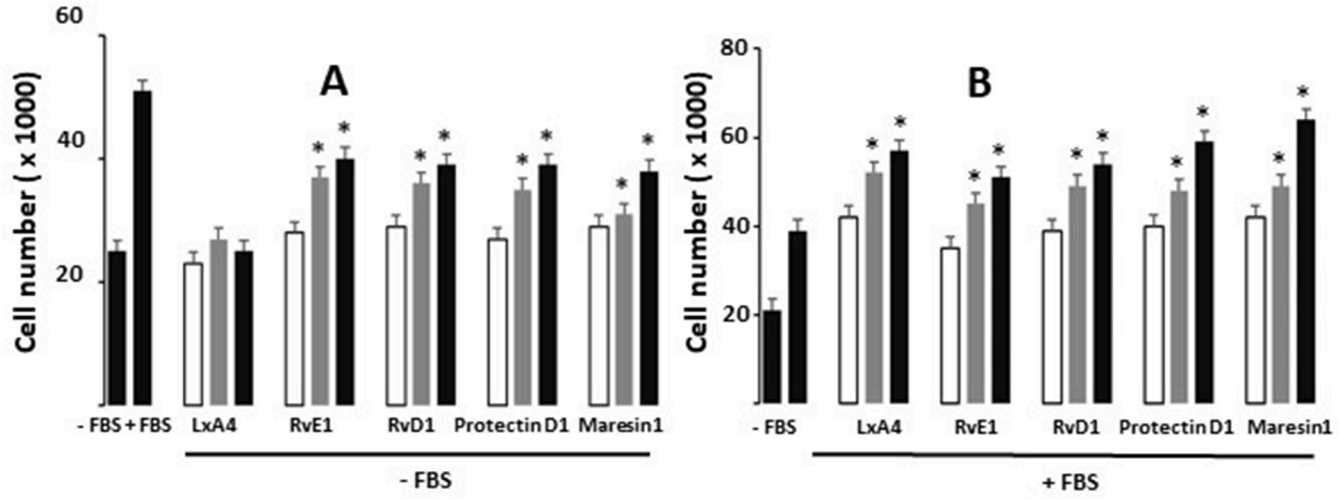

Fig. 2. Effect of SPMs on Caco-2 cell growth. Pre-confluent Caco-2 cell cultures were incubated for 48 h with LxA4, RvE1, RvD1, protectin D 1 and maresin 1 (0.001, 0.01 and 0.1 μM; white, grey, and black bars, respectively) in the absence of growth factors (FBS) (A) and presence of 10% FBS (B). Results are expressed as means ± SEM of 3 determinations performed in triplicate. * $P \leq 0.05$ vs. control Caco-2 cell cultures.

2.5. Western blot

Caco-2 cells were seeded in 60 mm plastic clusters (104 cells/cm^2) and, after 4 days, the cultures were incubated with the treatments 15 min. Finally, cells were washed twice with ice-cold PBS, scrapped of into PBS, and pelleted. Protein content was measured as above mentioned. The pellets were then sonicated in lysis buffer containing 2 m M sodium EDTA, 20 μg/mL aprotinin, 20 μg/mL leupeptin, 20 μg/mL phenylmethylsulphonyl fluoride, 200 μg/mL diethyldithiocarbamic acid, 50 mM Tris–HCl, 150 mM NaCl, 0.5% Igepal CA-630 and 1 mM dithiothreitol (Sigma-Aldrich). 30 μg of protein was mixed with buffer containing 0.5 M Tris–HCl, 10% glycerol, 10% SDS, 2% mercaptoethanol and 0.5% bluebromopherol (Sigma-Aldrich) and heated at 100°C for 5 min. Samples were separated by 12% SDS-PAGE gel and blotted for 2 h at voltage of 100 V and constant amperage of 400 mA onto a nitrocellulose membrane (Trans-Blot, 0.45 μm pore size, Bio-Rad) using a Miniprotean II system (Bio-Rad). A pre-stained SDS-PAGE protein standard (Bio-Rad) was used as molecular weight marker to check transfer efficiency. Membranes were blocked with 5% non-fat milk power in Tris-buffered saline (TBS) 0.1% Tween-20 (TBS-T20) for 1 h. Monoclonal antibodies against dephosphorylated/phosphorylated extracellular signal-regulated kinase 1/2 (ERK 1/2) and p38 MAPK were applied in a 1:2000 dilution overnight. The blot was washed several times with PBS-0.1% Tween 20 and incubated with a horseradish peroxidase-conjugated goat anti-mouse antibody in a 1:2000 dilution for 1 h. The above antibodies were obtained from Sigma Aldrich. Antibody binding was visualized by an enhanced chemical luminescence technique using Supersignal West Femto Maximun Sensitivity Substrate (ThermoFisher Scientific) and KodakX-OMAT film (Rochester, NY, USA).

Table 1
DNA synthesis induced by specialized pro-resolvin mediators (SPMs) in Caco-2 cell cultures.

FBS	DNA synthesis (Optical density, 450 nm)	+ FBS
Control	0.32 ± 0.02	0.63 ± 0.05
Lipoxin A4 (0.01 μM)	0.36 ± 0.04	1.06 ± 0.06*
12 HEPE (0.01 μM)	0.53 ± 0.05	0.94 ± 0.07*
Resolvin E1 (0.01 μM)	0.62 ± 0.03*	0.95 ± 0.06*
Resolvin D1 (0.01 μM)	0.58 ± 0.02*	0.93 ± 0.04*
Maresin 1 (0.01 μM)	0.69 ± 0.04*	1.03 ± 0.04*
Protectin D1 (0.01 μM)	0.76 ± 0.03*	1.12 ± 0.07*

Non-differentiated Caco-2 cells were incubated with SPMs in presence or absence of FBS for 48 h and DNA synthesis were measured. Results are shown as means ± 2–3 determinations performed in triplicate. * $P \leq 0.05$ vs control (Caco-2 cells cultures in the absence of SPMs).

2.6. Wound repair assay

Caco-2 cells were seeded on 13 mm round Thermanox tissue culture coverslips (Miles Laboratories, Naperville, IL), which were placed into 12-well plates (Costar, Cambridge, MD), containing DMEM with 10% FBS. Then, 48 h before wounding, cultures were transferred to medium supplemented with FBS at 2.5% instead of 10% to decrease the potential influence of serum-derived factors on wound repair responses while maintaining the health and stability of the cultures. Thereafter, two 2-mm-wide wounds were made radially in a cell monolayer using a sterile Teflon rake. The wound area affected 50 mm^2 of cell surface (approximately 20% of the total surface). Finally, wounded monolayers on coverslip were incubated in DMEM containing the SPMs at 37°C in a humidified chamber supplemented with 95% air/5% CO_2 and the wound closure of the cell cultures was determined.

Measurement of the wound area Images of the wounds were collected at specific times with a Nikon Diaphot 300 inverted microscope equipped with an integrating charge-coupled device camera and a digital image processor. The wounded area was measured using a computer-assisted image analysis system (Soltaire, Seescan, VIDS III, Analytical Measuring Systems). Images were obtained at the initial time of wounding and after wounding. Images of the wounds in each well were obtained at three points by well. The measurements were averaged, and the results are presented as a simple data point for that experiment and expressed as% recovery using the formula:% recovery = (1 − wound area at t_x/wound area at t_o) x 100%, where t_x is 12 h post-injury and t_o is time of injury [10].

2.7. Statistics

Results are expressed as mean ± SEM. All data were compared by one-way ANOVA and Student's *t*-test using SPSS software (SPSS Inc., Chicago, IL). $P < 0.05$ was considered to denote significance.

3. Results

3.1. SPMs induced Caco-2 cell growth and DNA synthesis

Caco-2 cell cultures is a good cellular model of intestinal epithelium.

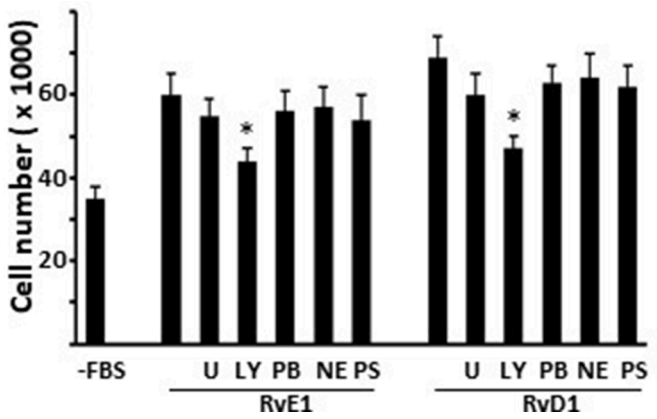

Fig. 3. Effects of receptorial antagonists on Caco-2 cell growth induced by SPMs. Pre-confluent Caco-2 cell cultures were incubated for 48 h with RvE1 or RvD1 (0.1 μM) in presence of U75302 (U, 1 μM), LY255283 (LY, 10 μM), PBP 10 (PB, 1 μM), α-NETA (NE, 10 μM) or PSB-CB5 (PS, 1 μM). These experiments were performed in the absence of FBS. Results are expressed as means ± SEM of 3 determinations performed in triplicate. * $P \leq 0.05$ vs. Caco-2 cell cultures incubated with resolvins.

Table 2
Effects of specialized pro-resolvin mediators (SPMs) on cell signalling pathways involved in Caco-2 cell growth.

Pathway	Control	RvE1	RvD1
AKT1	0.16 ± 0.02	0.11 ± 0.02	0.14 ± 0.03
AKT2	0.17 ± 0.02	0.12 ± 0.03	0.14 ± 0.03
P38α	0.19 ± 0.04	0.42 ± 0.14*	0.47 ± 0.12*
GSKβ	0.18 ± 0.04	0.22 ± 0.09	0.26 ± 0.08
CREB	0.21 ± 0.05	0.33 ± 0.10	0.38 ± 0.09
ERK1/2	0.17 ± 0.03	0.42 ± 0.11*	0.41 ± 0.08*

Caco-2 cells were incubated with SPMs (100 nM) for 5 or 15 min, cells were then collected and phosphorylated AKT1, AKT2, p38α, GSKβ, CREB, and ERK 1/2 were measured as described in the Material and Methods section. Findings are expressed as optical density (450 nm). Data are expressed as means ± SEM of two to four experiments performed in triplicate. * $P < 0.05$ vs. Control (Caco-2 cell cultures in the absence of resolvins).

Furthermore, AA cascade is well characterized in this intestinal epithelial cell. Considering all together, Caco-2 cells were cultured in the presence of representative SPMs (0.001–0.1 μM) to study their mitogenic effects. RvE1, RvD1, protectin D 1 and maresin 1 induced a significant Caco-2 cell growth in absence of growth factors (FBS). These effects were concentration-dependent and reached a plateau at 0.1 μM (Fig. 2A). In contrast, no appreciable proliferative response was seen in presence of LxA_4 in this experimental condition. However, LxA_4 was able to induce Caco-2 cell proliferation in presence of 10% FBS (Fig. 2B), as well as other SPMs studied. Our results also show that RvE1 and RvD1, protectin D 1 and maresin 1 induced a significant Caco-2 DNA synthesis in absence and presence of FBS. In contrast, LxA_4 induced DNA synthesis exclusively in presence of FBS (Table 1). Thus, these results demonstrate that SPMs showed mitogenic action on intestinal epithelial cells besides in absence of growth factors (FBS). RvE1 was chosen as representative EPA derived SPM and RvD1 as DHA derived SPM to advance in the knowledge of SPM effects on intestinal epithelial cell growth and wound closure.

3.2. BLT2 receptor could be involved in Caco-2 cell growth induced by RvE1 and RvD1

Diverse receptors have been postulated to be involved in the SPMs biological effects such as leukotriene B_4 receptors (BLT1/BLT2), N-formyl peptide receptor 2/LxA_4 receptor (FPR2/ALX), chemerin receptor 1 (ChemR23), and several G-coupled protein receptors (GPR) such as GPR18, GPR32 and GPR37. Our findings show that Caco-2 cell growth induced by RvE1 and RvD1 was inhibited by LY255283, a BLT2 antagonists [11], whereas BLT1 antagonists (U75302), FPR2/ALX antagonist (PBP10), ChemR23 antagonist (α-NETA) or GPR18 antagonist (PSB-CB5) were not effective to modulate RvE1/RvD1 effects on Caco-2 proliferation (Fig. 3).

Table 3
Cell signalling pathway elements involved in Caco-2 cell growth induced by resolvins.

	Viable cell number (10^3/cm^2)	DNA synthesis (0.D. 450 nm)
Control	36.2 ± 2.1	0.31 ± 0.02
RvE1	59.1 ± 2.6*	0.64 ± 0.03*
RvD1	63.4 ± 3.7*	0.73 ± 0.03*
RvE1 + PTX	44.8 ± 3.1$^{\neq}$	0.43 ± 0.03$^{\neq}$
RvE1 + SB203580	46.7 ± 2.6$^{\neq}$	0.48 ± 0.02$^{\neq}$
RvE1 + PD 98,059	65.2 ± 2.9	0.68 ± 0.03
RvE1 + KT5720	66.7 ± 3.1	0.67 ± 0.04
RvE1 + Go9683	48.1 ± 2.3$^{\neq}$	0.50 ± 0.03$^{\neq}$
RvE1 + U73122	41.9 ± 3.1$^{\neq}$	0.51 ± 0.02$^{\neq}$
RvD1 + PTX	41.5 ± 2.8$^{\neq}$	0.43 ± 0.03$^{\neq}$
RvD1 + SB203580	46.7 ± 2.6$^{\neq}$	0.48 ± 0.02$^{\neq}$
RvD1 + PD 98,059	65.2 ± 2.9	0.68 ± 0.03
RvD1 + KT5720	66.7 ± 3.1	0.67 ± 0.04
RvD1 + Go9683	48.1 ± 2.3$^{\neq}$	0.50 ± 0.03$^{\neq}$
RvD1 + U73122	49.2 ± 2.5$^{\neq}$	0.52 ± 0.05$^{\neq}$

Non-differentiated Caco-2 cells were incubated with RvE1 or RvD1 (100 nM) in presence of toxin pertussis (PTX, 100 ng/ml), SB203580 (15 μM), PD 98,059 (1 μM), KT5720 (10 μM), Go9683 (10 μM) or U73122 (10 μM) for 48 h and cell growth and DNA synthesis were measured. Results are shown as means ± 2–3 determinations performed in triplicate. * $P \leq 0.05$ vs control Caco-2 cells.
$^{\neq}$ $P \leq 0.05$ vs RvD1 or RvE1.

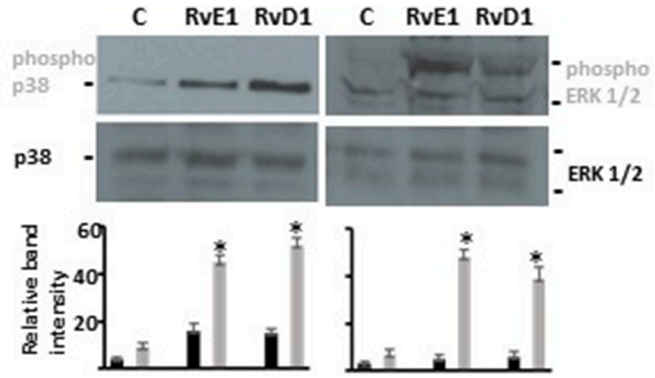

Fig. 4. Western blot of phosphorylated ERK 1/2 and p38MAPK in cultured Caco-2 cells in presence of resolvins. Cells were incubated with RvE1 or RvD1 (0.1 μM) for 15 min and phosphorylated and non-phosphorylated ERK 1/2 or p38 were determined using specific antibodies. Relative band intensity of non-phosphorylated (black bands) and phosphorylated (grey bands) proteins were normalized and are expressed as means of relative units ± SEM. of three experiments. *$P < 0.05$ compared with non-phosphorylated protein.

3.3. G protein sensible to PTX, ERK 1/2 and p38MAPK are involved in the control of Caco-2 cell growth induced by RvE1/RvD1

Once the mitogenic effect of SPMs on Caco-2 cell proliferation was established, we examined the cell signalling pathway through which these lipid mediators act. Table 2 shows that RvE1 and RvD1 activate p38α and ERK 1/2 pathways whereas these SPMs were not able to induce AKT1/AKT2, GSKβ or CREB pathways.

To further elucidate the significance of G proteins in the mitogenic actions of SPMs, we examined the effects of PTX, a compound that

Table 4
Resolvins stimulate wound repair in Caco-2 cell cultures.

	Wound recovery (%)	
	- FBS	+ FBS
Wounded cultures (WC)	3.04 ± 0.02	36.21 ± 1.51
WC + LxA4	4.26 ± 0.05	59.74 ± 2.32*
WC + RvE1	23.73 ± 1.67*	49.56 ± 3.07*
WC + RvD1	25.18 ± 2.34*	51.78 ± 2.19*
WC + Protectin 1	31.25 ± 1.76*	53.87 ± 2.25*
WC + Maresin 1	30.42 ± 2.54*	55.28 ± 1.89*
WC + RvE1 + PTX	8.76 ± 1.2$^{\neq}$	
WC + RvE1 + SB203580	12.32 ± 2.4$^{\neq}$	
WC + RvE1 + PD 98,059	15.14 ± 1.7$^{\neq}$	
WC + RvE1 + Go9683	13.11 ± 1.6$^{\neq}$	
WC + RvE1 + U73122	14.23 ± 2.5$^{\neq}$	
WC + RvE1 + LY255283	17.11 ± 1.3$^{\neq}$	
WC + RvD1 + PTX	10.43 ± 1.8$^{\neq}$	
WC + RvD1 + SB203580	12.97 ± 1.4$^{\neq}$	
WC + RvD1 + PD 98,059	16.14 ± 2.1$^{\neq}$	
WC + RvD1 + Go9683	13.05 ± 1.1$^{\neq}$	
WC + RvD1 + U73122	15.03 ± 1.6$^{\neq}$	
WC + RvD1 + LY255283	18.25 ± 1.2$^{\neq}$	

Wounded cell cultures (WC) were incubated with LxA4, RvE1, RvD1, protectin 1 or maresin 1 (100 nM) in absence or presence of FCS (10%) and toxin pertussis (PTX, 100 ng/ml), SB203580 (15 μM), PD 98,059 (1 μM), Go9683 (10 μM), U73122 (10 μM) or LY255283 (10 μM) for 12 h. The percentage of wound recovery was then determined. Measurements were performed in triplicate and expressed as the mean ± SEM of two experiments. *$P < 0.05$, significantly different from wound cultures. $^{\neq}P < 0.05$, significant different from wound cultures cells incubated with RvE1 or RvD1.

inhibits ADP-ribosylate specific G-proteins [12] and has been used as a probe for G-protein involvement in cell signalling. Pre-treatment of Caco-2 cultures with PTX blocked the mitogenic effects induced by RvE1 and RvD1 (Table 3). Similar effects were observed when was used PD 98, 059, a specific ERK 1/2 inhibitor [13] or SB 203,580, a highly selective p38 MAPK inhibitor [14] (Table 3). Thus, these findings suggest that receptor(s) associated to G protein sensible to PTX as well as ERK 1/2 and p38MAPK pathways could be also involved in the control of Caco-2 cell growth induced by these SPMs. To confirm this hypothesis, we performed the corresponding Western blot analysis. These experiments show that both Rvs increased phosphorylated ERK 1/2 and p38 MAPK levels (Fig 4), findings that support the role of these cell signalling pathways in the mitogenic effects of both Rvs. Finally, our findings using a phospholipase C inhibitor (U73122) and protein kinase C inhibitor (Go9683) treatments also suggested an implication of both cell signalling elements in the mitogenic action of RvE1 and RvD1 (Table 3).

3.4. RvE1/RvD1 induce wound closure in Caco-2 cell cultures

Based on previous reports that several elements and metabolites of AA cascade contributes to cell growth and wound closure [15–23], and that wound closure form part of the resolution of inflammation. We proposed that SPMs could regulate wound healing in intestinal epithelial cultures. The findings of Table 4 confirms this hypothesis. Thus, RvE1, RvD1, protectin D 1 and maresin 1 induced the intestinal epithelial culture wound healing in absence of FBS and increase the wound repair process, whereas LxA_4 stimulated the wound repair exclusively in presence of FBS. Furthermore, we demonstrated that wound repair process induced by RvE1 and RvD1 were reverted by PTX, SB203580, PD 98,059, Go9683, and U73122 treatments as well as a BLT2 antagonist.

4. Discussion and conclusions

In the last decade, several studies have focused on the regulation and resolution of inflammation by SPMs during tissue homeostasis [1,6]. Healing of the intestinal inflamed mucosa is a key element to the remission of the inflammatory bowel disease. This process is initiated by intestinal epithelial cell migration residing near to the wound area and by the subsequent intestinal epithelial cell proliferation induced by growth factors, cytokines, and hormones. Interestingly, Gobbetti et al. [24] and Lee and co-workers [7] reported the biosynthesis of endogenous SPMs during intestinal inflammation and suggested a role of these lipid mediators on intestinal wound healing. Interestingly, resolvin administration induced mucosal wound repair in several models of damaged intestine [8,25], although, unfortunately, the involved mechanisms are poorly understood.

Our data demonstrated that LxA_4, RvE1, RvD1, protectin D 1 and maresin 1 were able to enhance intestinal epithelial Caco-2 cell growth induced by FBS. Our observations further demonstrate that these SPMs, except for LxA_4, have mitogenic action in absence of growth factors. Furthermore, these mitogenic effects can be related to the capacity of these SPMs to induce a wound repair process in intestinal epithelial cultures. The effect of SPMs on wound repair has been previously reported in several tissues. Thus, RvD1 induces periodontal ligament repair [26], skeletal wound repair and osteoblastic bone formation [27], corneal epithelial repair [28], skin epithelial repair [29], RvE1 stimulates dentin regeneration [30], and LxA_4 repairs fibroblast wound cultures [31]. In agreement with these antecedents, these findings are the first time that was demonstrated a mitogenic effect of SPMs on intestinal epithelial cell.

The restoration of the intestinal epithelium is a critical step of intestine mucosa repair, and our study clearly demonstrated that SPMs could stimulate intestinal epithelium repair promoting wound closure by inducing proliferation of intestinal epithelial cells as we previously reported by dietary fatty acid precursors [32] and eicosanoids [21,22, 33]. These findings are agreeing with the recent findings of Quiros et al. [8] that reported a wound repair induced by RvE1 on IEC cultures and in vivo wounding of murine colonic mucosa. Interestingly, our results also demonstrated the effects of DHA-derived SPMs such as RvD1, protectin 1 and maresin 1 on the intestinal epithelial Caco-2 cell culture wound closure by the first time.

Understanding the mechanism by which SPMs contribute to cell growth may be a critical issue in cell growth/wound repair, cancer, and lipid homeostasis. SPMs are reported to mediate their biological effects via several GPRs such as BLT1 and BLT2, ChemR2, FPR2/ALX, and the orphan receptors GPR18, GPR32 and GPR37 [34]. Interestingly, BLT1 [35] and BLT2 [23], ChemR23 [36], FPR2/ALX [37], GPR18 [38] and GPR37 [39] have been expressed in intestine and/or intestinal cell cultures. Interestingly, our results provide evidence that the effects of RvE1 and RvD1 on Caco-2 cell growth /DNA synthesis are associated with PTX-sensitive GPRs, presents probably in plasma membrane. Furthermore, our findings suggest that BLT2 is involved, at least in part, in the reported effects of RvE1/RvD1. BLT2 is a receptor to several eicosanoids such as LTs, hydroxyeicosatetraenoic and hydroxyoctadecadienoic acids [11,40,41]. Moreover, BLT2 had been involved in intestinal epithelial cell growth as well as the mitogenic effects of these eicosanoids [21,23,33]. Considering these antecedent and our findings, it is plausible considering that BLT2 is involved in the mitogenic effects of SPMs in intestinal epithelial cultures. It is important consider that our results did not exclude the additional role of other receptors in the above-mentioned effects of RvE1/RvD1.

Several other potential pathways by which these metabolites may stimulate cellular growth have been also explored. In this regard, our results showed that RvE1 and RvD1 induced ERK 1/2 and p38 MAPK pathways, and that these actions were involved in the control of the Caco-2 growth induced by both Rvs. MAPK family is an essential part of the signal transduction machinery in signal transmissions from cell surface receptors and environmental stimulation. This family includes three major MAPK subfamilies: ERK, p38 and JNK [42]. These have been proposed to serve as signal elements in cell growth. These events are of relevance, since our study provides additional evidence that the enhancement of intestinal epithelial cell growth induced by both Rvs are

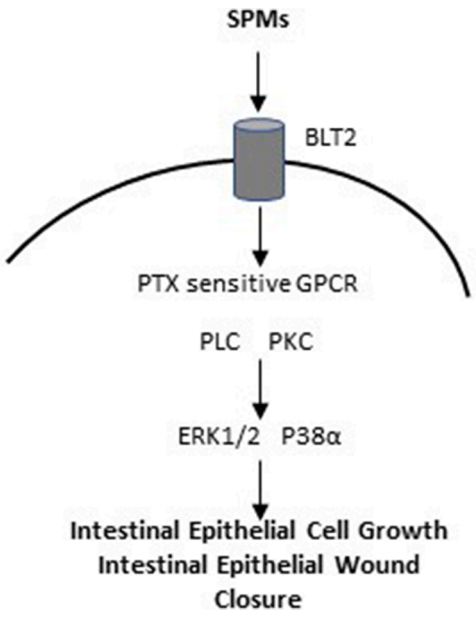

Fig. 5. Main elements involved in the SPMs effects on intestinal epithelial cell growth. The mitogenic effects of RvE1/RvD1 on intestinal epithelial cells are dependent on the ERK 1/2 and p38 MAPK pathways and propose that this effect is mediated through a PTX-sensitive G protein-coupled receptor (GPR), probably leukotriene B4 receptor 2 (BLT2) at least in part, and phospholipase C (PLC) and protein kinase C (PKC) activation.

sensitive to ERK 1/2 and p38 MAPK pathway inhibitors, treatments that also blocked Caco-2 cell culture wound healing induced by both Rvs.

We conclude that the mitogenic effects of RvE1/RvD1 on intestinal epithelial cells are dependent on the ERK 1/2 and p38 MAPK pathways and propose that this effect is mediated through a PTX-sensitive GPR, probably BLT2 at least in part, and phospholipase C and protein kinase C activation (Fig. 5). Suggesting a link for the SPMs to this signalling circuit involved in wounded epithelium and the catabasis process.

Author's contributions

CES and JJM designed experiments and CES, MP and AV performed these experiments. CES, MP, AV and JJM carried out the data analysis and CES and JJM wrote the manuscript. All authors read and approved the manuscript.

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Availability of data and materials

All data needed to evaluate the conclusions of the paper are presented in the manuscript. Additional data related to this study are requested from the authors.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest.

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