Papel de la cascada del ácido araquidónico en el control de la proliferación de las células epiteliales intestinales humanas

Marisol Cabral Salvadores

2 OBJETIVOS

La inflamación contribuye al microambiente tumoral de manera que los eicosanoides sintetizados por diversas células pueden participar en el inicio de la tumorogénesis y promover la progresión de la enfermedad. Aunque la relación de algunos eicosanoides proinflamatorios con el CRC está ampliamente estudiada (Wang y DuBois, 2008; Dixon *et al.*, 2013), es necesario tener una visión holística del papel de los eicosanoides, estudiar la interacción con sus receptores y la posterior activación de vías de señalización que pueden estar implicadas en el desarrollo y progresión del CRC (Ferrer y Moreno, 2010).

Considerando estos antecedentes, el objetivo general de este trabajo es estudiar el papel de la cascada del AA en el control de la proliferación de células epiteliales intestinales humanas.

Para alcanzar este objetivo se proponen los siguientes objetivos específicos:

- Analizar los eicosanoides sintetizados por células epiteliales intestinales no diferenciadas.
- Estudiar el efecto de diferentes metabolitos representativos de todas las vías de la cascada del AA sobre el crecimiento y la inducción de la apoptosis.
- Estudiar los receptores y las vías de transducción de señales a través de los cuales los eicosanoides regulan el crecimiento celular.
- Analizar el efecto del EPA y de sus metabolitos derivados más representativos sobre la proliferación de las células intestinales epiteliales.

Como modelo experimental se ha utilizado la línea celular Caco-2. Estas células se caracterizan porque en preconfluencia presentan un crecimiento celular propio de un adenocarcinoma mientras que a partir de la confluencia se diferencian para dar lugar a una monocapa de células que presentan características morfológicas, bioquímicas y funcionales similares a las de los enterocitos del intestino delgado (Martín-Venegas *et al.*, 2006).

3 RESULTADOS

Artículo 1

.

Marisol Cabral, Raquel Martín-Venegas, Juan J. Moreno.

Role of arachidonic acid metabolites on the control of non-differentiated intestinal epithelial cell growth

Int J Biochemi Cell Biol 45 (2013) 1620-1628

Índice de impacto (JCR, 2013): 4,240
Categoria y posición; quartil: Biochemistry and Molecular biology: 75/291; Q2
Cell biology: 69/185; Q2

Los resultados de esta publicación se han presentado en los siguientes congresos:

- Papel de la vía de las ciclooxigenasas y de la 5-lipoxigenasas en el control de la proliferación de las células Caco-2.
  Cabral M, Martin-Venegas R, Moreno JJ.
  Jornada Científica COLOMICS: Avances en cáncer de colon. Madrid (España) 14 Abril 2008.

- Eicosanoids derived from lipoxygenase pathway are involved in intestinal epithelial cell growth.
  Cabral M, Martin-Venegas R, Moreno JJ.
  Third European Workshop on Lipid Mediators. Paris (Francia) 3-4 Junio 2010.

- PGE_2 interaction with EP_1 and EP_4 receptors are involved in intestinal epithelial cell growth.
  Cabral M, Martin-Venegas R, Moreno JJ.
  Third European Workshop on Lipid Mediators. Paris (Francia) 3-4 Junio 2010.

- Hydroxyeicosatetranoics acids are involved in intestinal epithelial cancer cell growth.
  Moreno JJ, Cabral M, Martin-Venegas R.
  22nd Biennial Congress of the Eureopean Association for Cancer Research Barcelona (España), 10 Julio 2012.
  Resumen publicado en la revista European Journal of Cancer, 48:83 (2012)

- Cell signaling pathways involved in Caco-2 cell growth induced by eicosanoids.
  Cabral M, Martin-Venegas R, Moreno JJ.
  Fourth European Workshop on Lipids Mediators. Paris (Francia), 27-28 Septiembre 2012.

Resumen artículo 1:

Objetivo Estudiar el papel de las vías de la cascada del AA en la proliferación de células epiteliales intestinales no diferenciadas procedentes de un adenocarcinoma.

Material y Métodos Este estudio se ha realizado utilizando células Caco-2 no diferenciadas. Las células se incubaron con inhibidores de las vías COX y LOX, así como agonistas o antagonistas receptoriales de los principales eicosanoides de estas vías y se determino: los eicosanoides presentes en el sobrenadante por HLPC-MS/MS; el crecimiento celular por contaje microscópico usando bromuro de etidio/naranja de acridina y la síntesis de ADN por incorporación de [^{3}H]thimidina; el análisis del ciclo celular por citometría de flujo y la degradación del ADN cromosómico con el método TUNEL; la síntesis de PGE_2 y AMPc mediante un inmunoensayo enzimático (EIA); y finalmente la fosforilación de Akt1, Akt2, ERK1/2, GSK3, p38, CREB y la desfosforilación de β-catenina por ELISA.

Resultados El crecimiento de las células Caco-2 y la síntesis de ADN disminuyó significativamente en presencia de inhibidores de la COX-1, COX-2, 5- LOX y 12-LOX. Además hemos demostrado que el efecto mitogénico inducido por PGE_2 es debido a su interacción con EP_1 y EP_4, el inducido por LTB_4 es debido a su unión con BLT_1 y BLT_2 y el inducido por 12-HETE a su interacción con BLT_2. Los eicosanoides sintetizados por las células Caco-2 en ausencia de factores de crecimiento fueron PGE_2, LTB_4, 5-HETE, 12-HETE y 15-HETE. No observamos formación de LTD_4, 20-HETE, 11,12-EET, 14,15-EET, 11,12-DHETE o 14,15-DHETE. Los eicosanoides sintetizados por las células Caco-2 incrementan el crecimiento celular y la síntesis de ADN. Respecto a las vías de señalización, la PGE_2 incrementó la fosforilación de ERK1/2, p38, CREB y GSK. La fosforilación de CREB inducida por PGE_2 depende de ERK y AMPc-PKA. En cambio, el LTB_4 y el 12-HETE solo incrementaron la fosforilación de ERK y p38 respectivamente. Finalmente, en este estudio se demostró que tanto el LTB_4 como 12-HETE, metabolitos representativos de la vía 5-LOX y 12/15 LOX, activan la síntesis de PGE_2 y que el efecto proliferativo de ambos eicosanoides disminuyó significativamente con la utilización de inhibidores de las COXs y antagonistas de EP_1 y EP_4.

Conclusión PGE_2, LTB_4, 5-HETE, 12-HETE y 15-HETE están involucrados en la regulación del crecimiento celular y la síntesis de ADN en las células Caco-2 a través de la interacción con receptores específicos y la posterior activación de vías de señalización. Además, los efectos proliferativos de LTB_4 y 12-HETE son, al menos en parte, debidos a la síntesis de PGE_2.

The International Journal of Biochemistry & Cell Biology 45 (2013) 1620–1628

Contents lists available at SciVerse ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Role of arachidonic acid metabolites on the control of non-differentiated intestinal epithelial cell growth

Marisol Cabral, Raquel Martín-Venegas, Juan J. Moreno*

Departament de Fisiologia, Facultat de Farmàcia, Universitat de Barcelona, Avda Joan XXIII s/n, 08028 Barcelona, Spain

ARTICLE INFO

Article history:
Received 12 December 2012
Received in revised form 26 April 2013
Accepted 9 May 2013
Available online xxx

Keywords:
Prostaglandin
Leukotriene
Hydroxyeicosatetraenoic acid
Colorectal cancer
Cell signaling

ABSTRACT

Increasingly evidence indicates that enzymes, receptors and metabolites of the arachidonic acid (AA) cascade play a role in intestinal epithelial cell proliferation and colorectal tumorigenesis. However, the information available does not provide a complete picture and contains a number of discrepancies. For this reason it might be appropriate a thorough study into the impacts of the AA cascade on intestinal epithelial cell growth. Our data show that non-differentiated Caco-2 cells cultured with 10% fetal bovine serum (FBS) synthesize appreciable amounts of prostaglandin E_2 (PGE_2), leukotriene B_4 (LTB_4) and 5-, 12 and 15-hydroxyeicosatetraenoic acid (HETE) but not LTD_4, 20-HETE and epoxyeicosatrienoic acids. We also found that inhibitors of PGE_2, LTB_4 and 5-, 12-, 15-HETE synthesis as well as receptor antagonists of PGE_2 and LTB_4 blocked Caco-2 cell growth and DNA synthesis induced by 10% FBS without cytotoxic or apoptotic activity. Interestingly, PGE_2, LTB_4 and 5-, 12- and 15-HETE at concentrations reached in 10% FBS Caco-2 cultures (1–10 nM) were able to induce Caco-2 cell growth and DNA synthesis. This was due to the interaction of PGE_2 with EP_1 and EP_4 receptors and LTB_4 and HETEs with BLT_1 and BLT_2 receptors. Moreover, we provide evidence that PGE_2 stimulates several cell signaling pathways such as ERK, P38α, CREB and GSKβ/β-catenin involved in the regulation of Caco-2 growth. Finally, we provide evidence that the mitogenic effects of LTB_4 and HETEs can be dependent, at least in part, on PGE_2 synthesis.

1. Introduction

Arachidonic acid (AA) are found esterified in the phospholipids of mammalian cell membranes. When AA is released by phospholipase A_2 (PLA_2) activation in response to physiological stimuli it is oxidized by oxygenases. The cyclooxygenases (COX), COX-1 and COX-2, produce prostaglandins (PG) and thromboxanes. AA is also metabolized by 5-lipoxygenase (5-LOX) yielding 5-hydroxyeicosatetraenoic acid (5-HETE) and leukotriene A_4 (LTA_4), the precursor of LTB_4 and cysteinyl leukotrines such as LTC_4, LTD_4 and LTE_4. 12- and 15-LOX produces 12-HETE, 15-HETE and lipoxins. Finally, cytochrome P-450 (CYP450) metabolizes AA by one or more of the following reactions: bis-allylic oxidation to generate 5-, 8-, 9-, 11-, 12-, and 15-HETEs; ω/ω-1 hydroxylation to give 16-, 17-, 18-, 19- and 20-HETEs; and olefin epoxidation to produce 5,6-, 8,9-, 11,12- and 14,15-epoxyeicosatrienoic acids (EETs), which can be catalyzed by epoxide hydrolases to produce dihydroxyeicosatetraenoic acids (DHETEs) (Natarajan and Reddy, 2003). Thus, AA is the precursor of many biologically active products, named eicosanoids, which are typically involved directly in the development of inflammation. However, these bioactive lipids are also pleiotropic modifiers of biological response and modulate diverse physiological responses including cell growth, differentiation and pathological conditions involving altered cellular proliferation (Moreno, 2009; Ferrer and Moreno, 2010).

A well-established connection exists between inflammatory bowel disease and colon cancer (Ekbom et al., 1999), which is one of the leading causes of death in western countries. The development of colon cancer is a typical multistep process involving several mutations in key oncogenes such as ki-Ras, APC and p53. Besides these mutations, as much as 80% of colon carcinomas show an enhanced COX-2 expression compared with normal intestinal mucosa. Consequently there is excessive production of PGs (Eberhart et al., 1994). Accumulated evidence from epidemiological and laboratory studies indicates that chronic ingestion of non-steroidal anti-inflammatory drugs decreases the incidence of colon adenomas (Giardiello et al., 1993) and reduces the risk of colorectal cancer (Thun et al., 1991). Grosch et al. (2001) reported COX-2-independent induction of cell cycle arrest and apoptosis in these colon cancer cells by selective COX-2 inhibitors. However, it is not clear how these effects are mediated predominantly through COX-dependent or through COX-independent mechanisms, or both.

Abbreviations: AA, arachidonic acid; COX, cyclooxygenase; CYP450, cytochrome P-450; DHETE, dihydroxyeicosatetraenoic acid; EET, epoxyeicosatrienoic acid; HETE, hydroxyeicosatetraenoic acid; LOX, lipoxygenase; LT, leukotriene; NDGA, nordihydroguaiaretic acid; PG, prostaglandin; PLA_2, phospholipase A_2.

* Corresponding author. Tel.: +34 93 4024505; fax: +34 93 4035901.
E-mail address: jjmoreno@ub.edu (J.J. Moreno).

1357-2725/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biocel.2013.05.009

Regarding LOX pathway, Shureiqi and Lippman (2001) reported that 5-LOX and 12-LOX, through LTs and HETEs production, are procarcinogenic. On the other hand, Shureiqi et al. (1999, 2005) reported that 15-LOX-1 is expressed at lower levels in human colorectal carcinoma tissues, with a decrease of hydroxyoctadecadienoic acids synthesis. With regard to CYP450s, several isoforms have been described in epithelial colon cells. Yamane et al. (1998) reported that ω-hydroxylation activity appears to be associated with the presence of CYP450s in Caco-2 cells. However, there are no studies available on AA-CYP450 metabolites produced by intestinal epithelial cells and the role of these mediators on cell growth.

Considering the fragmentation of the knowledge in this area, it might be appropriate a thorough study of AA cascade relevance on intestinal epithelial cell growth. Therefore, here we aim to study the role of the three AA cascade pathways on intestinal epithelial cell proliferation.

2. Materials and methods

2.1. Materials

LTD_4, PGE_2, PGE_2-d_4, LTB_4, LTB_4-d_4, 14,15-DHETE, 11,12-DHETE, 5-HETE, 5-HETE-d_8, 12-HETE, 12-HETE-d_8, 12-HETE, 15-HETE, 15-HETE-d_8, 20-HETE, 20-HETE-d_6, 5,6-EET and 5,6-EET-d_{11}, 8,9-EET, 11,12-EET, 14,15-EET, 14,15-EET-d_{11}, U 75302, MK 571 and zileuton were purchased from Cayman Chemical (Ann Arbor, MI). Baicalein, butaprost, carbacyclin, ketoprofen, nordihydroguaiaretic acid (NDGA), LY 171883, MK 886, NS 398, SC 560 and SC 19220, KT 5720, PD 98059, SB 203580, ethidium bromide and acridine orange were purchased from Sigma Chemical (St. Louis, MO). LY 255283 was from Tocris Biosc. (Bristol, UK). [Methyl-^{3}H]thymidine (20 Ci/mmol) was from American Radiolabeled Chemicals Inc. (St. Louis, MO). ONO-329 was kindly provided by Ono Pharmaceutical Co. Ltd. (Osaka, Japan) and AH 23838 by Glaxo-Wellcome (Stevenage, UK).

2.2. Cell culture and cell growth assay

Caco-2 cells were derived from a moderately well-differentiated primary colon adenocarcinoma and were provided by American Type Culture Collection (HTB-37, Manassas, VA, USA). The cells 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.

Table 1
Inhibitors and agonists/antagonists used in this study.

	Abbreviation	Effect
Ketoprofen	Kp	COX-1/COX-2 inhibitor
SC 560	SC5	COX-1 inhibitor
NS 398	NS	COX-2 inhibitor
Zileuton	Z	5-LOX inhibitor
Baicalein	B	12-LOX inhibitor
MK 886	MK8	5-LOX inhibitor
Nordihydroguaiaretic acid	NDGA	non-specific LOX inhibitor
SC 19220	SC1	EP_1 antagonist
AH 23848	AH	EP_4 antagonist
U 75302	U	BLT1 antagonist
LY 255283	LY2	BLT2 antagonist
MK 571	MK5	$CysLt_1R$ antagonist
LY 171883	LY1	$CysLt_1R$ antagonist
Butaprost		EP_2/EP_4 agonist
Carbacyclin		EP_1 agonist
ONO-AE-329		EP_4 agonist

All the experiments were performed in pre-confluent cultures and consequently, in non-differentiated cells. Caco-2 cell differentiation started when they reached the confluence and was completed after 2 weeks post-confluence, a process which was previously described (Martin-Venegas et al., 2006).

To perform the cell growth assay, cells were harvested with 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 cells ($3–4 \times 10^4$ cells/cm^2) were incubated with treatments (Table 1) for a period of 48 h. Finally, cells were washed, trypsinized, and counted with a microscope using ethidium bromide/acridine orange staining to measure viable cells.

2.3. Analysis of DNA synthesis

DNA synthesis was measured by a [^{3}H]thymidine incorporation assay. Caco-2 cell were cultured on 24-well plates in DMEM with 10% FBS at a density of 10^4 cells/cm^2. After 4 days in culture, cells were incubated for 48 h with the treatments, and [^{3}H]thymidine (0.1 μCi/well). Media containing [^{3}H]thymidine were aspirate, cells were washed and overlaid with 1% Triton X-100 and then scraped off the wells. Finally, radioactivity present in the cell fraction was measured by scintillation counting, using a Packard Tri-Carb 1500 counter.

2.4. Fluorescence-activated cell sorting analysis/flow cytometry cell cycle analysis

Caco-2 cells were seeded in 60 mm dishes (10^4 cells/cm^2). After 4 days in culture, cells were incubated for 48 h with the

Table 2
Eicosanoid levels in Caco-2 supernatant cultures measured by HPLC-MS/MS.

	Without FBS	10% FBS				
			Kp	Z	B	NDGA
Eicosanoids						
PGE_2	0.42 ± 0.15	1.98 ± 0.52*	0.51 ± 0.21#	1.85 ± 0.43	1.75 ± 0.38	1.48 ± 0.28#
LTB_4	0.37 ± 0.26	2.01 ± 0.13*	1.92 ± 0.17	1.21 ± 0.03#	1.13 ± 0.08	1.54 ± 0.16#
5-HETE	2.61 ± 0.22	7.76 ± 1.12*	7.81 ± 2.33	4.90 ± 0.21#	8.25 ± 0.92	6.12 ± 0.21#
12-HETE	1.34 ± 0.91	3.67 ± 0.21*	3.79 ± 0.28	4.54 ± 0.69	2.71 ± 0.23#	2.43 ± 0.12#
15-HETE	3.41 ± 0.52	7.98 ± 1.42*	7.17 ± 1.75	6.42 ± 1.82	5.80 ± 0.71#	4.70 ± 0.23#

Non-differentiated Caco-2 cells (10^4 cells/cm^2) were cultured with or without 10% FBS in presence or absence of ketoprofen (Kp, 5 μM), zileuton (Z, 5 μM), baicalein (B, 25 μM) or NDGA (10 μM) for 24 h. Finally, eicosanoids present in culture supernatants were extracted and measured as we described in Section 2. Eicosanoid concentrations are presented as ng/ml. LTD_4, 20-HETE, 11,12-EET, 14,15-EET, 11,12-DHETE and 14,15-DHETE were not detected in our experimental conditions. Results are shown as means ± SEM of 2–3 experiments performed in duplicate.

* $P < 0.05$ between Caco-2 cells cultured with and without 10% FBS.

\# $P < 0.05$ vs. Caco-2 cells cultured with 10% FBS.

treatments. Thereafter, they were trypsinized, fixed with 70% ethanol, and stored at 4 °C for at least 2 days. Low molecular weight DNA was then extracted from the cells, which were stained for 1 h at room temperature with a 20 μg/ml propidium iodide solution in PBS containing 0.1% Triton X-100 and 0.2 mg/ml DNase-free RNase A. Cells were analyzed on an Epics XL flow cytometer (Coulter Corp., Hialeah, FL). DNA was analyzed (ploid analysis) on single fluorescence histograms using Multicycle software (Phoenix Flow Systems, San Diego, CA).

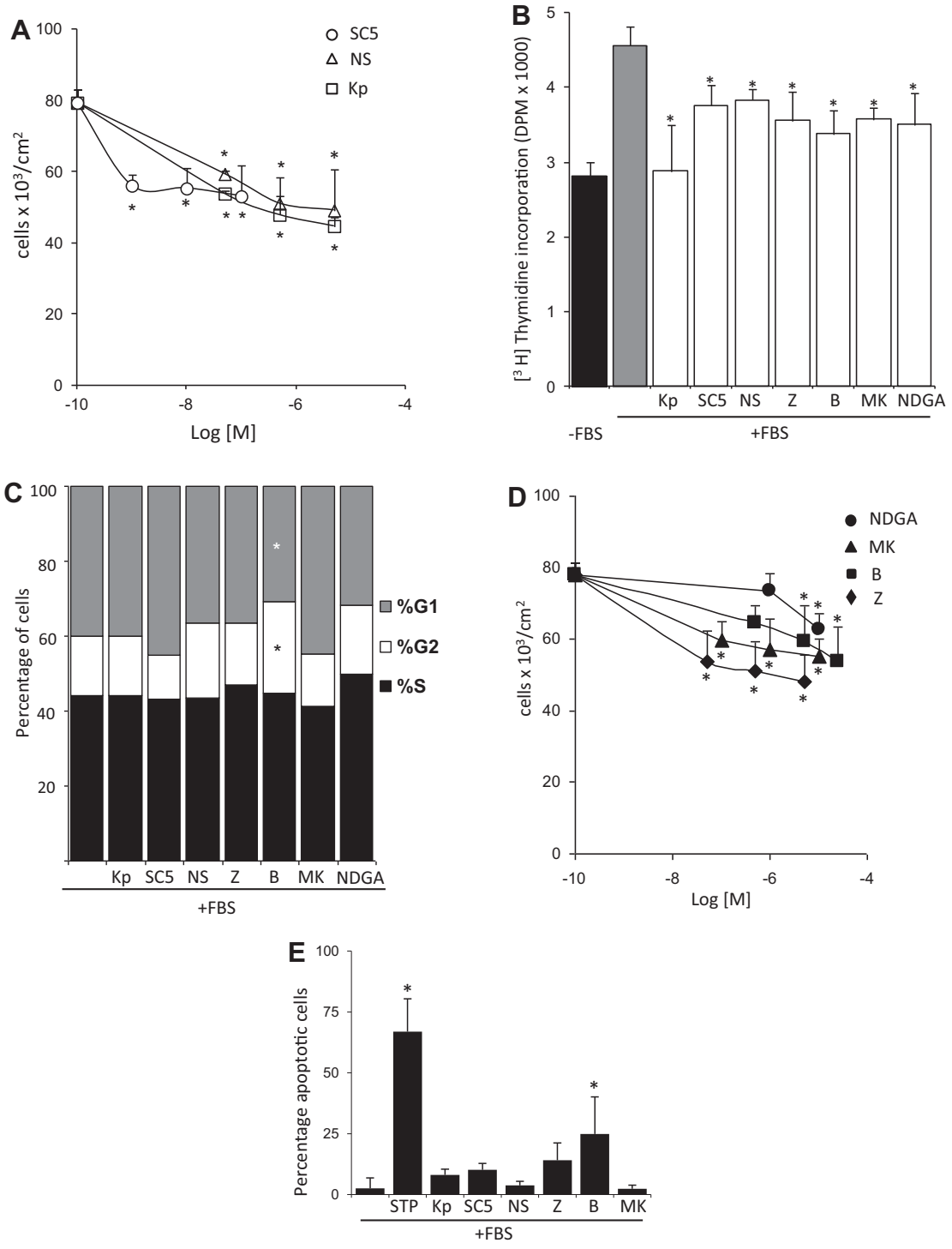


Fig. 1. Effect of COX or LOX inhibitors on cell growth, DNA synthesis, cell cycle and apoptosis in Caco-2 cultures. Cells were incubated for 48 h in the absence or presence of ketoprofen (Kp, open square, 0.05–5 μM), SC 560 (SC5, open circle, 0.001–0.1 μM), NS 398 (NS, open triangle, 0.05–5 μM), baicalein (B, closed square, 0.5–25 μM), NDGA (closed circle, 1–10 μM), MK 886 (MK, closed triangle, 1–10 μM) and zileuton (Z, closed rhomb, 0.05–5 μM) in 10% FBS and cells were then counted (A, D). [³H]thymidine incorporation (B), changes in cell cycle (C) and apoptosis (E) were measured in cells incubated for 48 h in the absence or presence of with Kp (5 μM), SC5 (0.1 μM), NS (5 μM), B (25 μM), NDGA (10 μM), MK (10 μM) and Z (5 μM) in 10% FBS. Staurosporine (STP, 1 μM) was used as a positive control in the apoptosis assay. Results are expressed as means ± SEM of 3–5 determinations performed in triplicate. * $P < 0.05$ vs. 10% FBS Caco-2 cell cultures without treatment.

2.5. Measurement of DNA fragmentation

Degradation of chromosomal DNA was evaluated with the TUNEL method. Subconfluent cell cultures (10^4 cells/cm^2) seeded in 60 mm dishes were incubated in media containing 10% FBS with treatments for 48 h. Cells present in the medium and attached trypsinized cells were then collected, fixed with 4% paraformaldehyde, and permeabilized with 70% ethanol. Thereafter, 3′-OH DNA ends generated by DNA fragmentation were labeled with fluorescein-dUTP, mediated by terminal deoxynucleotidyl transferase, and then analyzed using an Epics XL flow cytometer (Coulter Corporation, Hialeah, FL).

2.6. Liquid chromatography/tandem mass spectrometry measurements of eicosanoids

Cells were seeded in 12 mm plastic clusters (10^4 cells/cm^2) and, after 4 days, the cultures were incubated with the treatments (48 h).

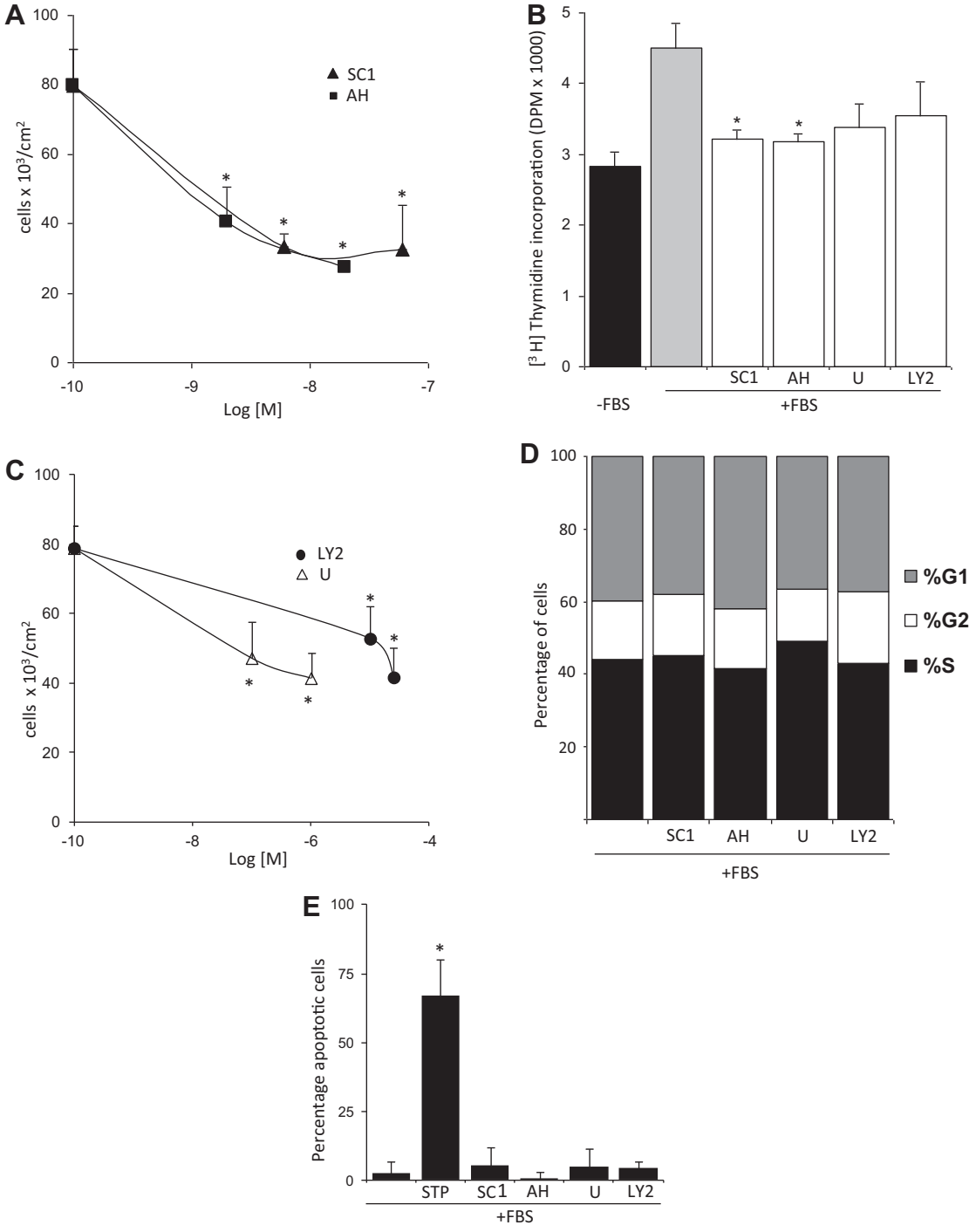


Fig. 2. Effect of PGE_2 and LTB_4 antagonists on cell growth, DNA synthesis, cell cycle and apoptosis in Caco-2 cultures. Cells were incubated for 48 h in the absence or presence of SC 19220 (SC1, closed triangle, 6–60 nM), AH 23848 (AH, closed rhomb, 2–20 nM), U 75302 (U, open triangle, 0.1–1 μM) and LY 255283 (LY2, closed circle, 10–25 μM) in 10% FBS and cells were then counted (A, C). [^{3}H]thymidine incorporation (B), changes in cell cycle (D) and apoptosis (E) were measured in cells incubated for 48 h in the absence or presence of SC1 (60 nM), AH (20 nM), U (1 μM) and LY2 (25 μM) in 10% FBS. Staurosporine (STP, 1 μM) was used as a positive control in apoptosis assay. Results are expressed as means ± SEM of 3–5 determinations performed in triplicate. *$P < 0.05$ vs. 10% FBS Caco-2 cell cultures without treatment.

Then, eicosanoids in the cell culture medium were extracted using a solid phase method. To simultaneously separate 13 eicosanoids and deuterated internal standards we used a liquid chromatograph Perkin Elmer series 200 (Norwalk, CT) equipped with a quarternary pump and thermostated autosampler. To obtain the MS/MS data we used a triple quadrupole mass spectrometer API3000 (ABSciex, Concord, Ontario, Canada) equipped with a TurboIonspray source operating in negative ion mode as described previously (Martin-Venegas et al., 2011).

2.7. PGE_2 and cAMP analysis by EIA

Cells maintained in 12 mm plastic clusters at a density of 10^4 cells/cm^2. After 4 days in culture, Caco-2 cells were incubated for 60 min at 37 °C with LTB_4 (0.1–1 μM) and 12-(S)-HETE (1–10 μM). Finally supernatants were harvested and PGE_2 determined a by competitive EIA kit (Cayman, Ann Arbor, MI) following the manufacturer's instructions.

Subconfluent cells maintained in flasks were incubated for 30 min at 37 °C with eicosanoids. After they were incubated for 20 min at room temperature with 2.5 ml of 0.1 N HCl and then harvested and homogenized. The homogenate was then centrifuged (1000 × *g*, 10 min) and cAMP was measured in supernatants using a competitive EIA kit (Cayman, Ann Arbor, MI) following the acetylation procedure (sensitivity 0.1 pM) of the manufacturer's.

Proteins in cell samples were measured using Bio-Rad protocol with Coomassie brilliant blue G-250 as a dye reagent.

2.8. Measurement of the cell signaling pathways activated by eicosanoids

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 with total cellular lysates, Caco-2 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. The resulting solutions containing 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). Finally, optical density was measured at 450 nm. Thus, we studied the effect of eicosanoids on the phosphorylation of AKT1 (pS473), AKT2 (pS474), ERK1/2 (pT202/Y204;pT185/Y187), GSK3β (pS9), p38α (pT180/Y182), CREB (pS133) and on the dephosphorylation of β-catenin (DP S33/S37/S41).

2.9. 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. Non-differentiated Caco-2 cells synthesize AA metabolites by COX and LOX pathways

We observed that non-differentiated Caco-2 cells, cultured in the absence of FBS, synthesized considerable amounts of PGE_2, LTB_4, 5-HETE, 12-HETE and 15-HETE but not LTD_4, 20-HETE, 11,12-EET, 14,15-EET, 11,12-DHETE or 14,15-DHETE (Table 2). The results also showed that 10% FBS, which did not contain appreciable amount of these eicosanoids (data not shown), significantly increased the synthesis of all of them. Moreover, we found that ketoprofen only reduced PGE_2, zileuton impaired LTB_4 and 5-HETE, and that baicalein reduced 12-HETE and 15-HETE levels. Finally, NDGA decreased these eicosanoids in the Caco-2 culture media (Table 2).

3.2. Modulation of COX and LOX pathways affect Caco-2 cell growth and DNA synthesis

We observed that Caco-2 growth induced by 10% FBS was reduced not only by ketoprofen, a COX inhibitor (Sanchez and Moreno, 1999), but also by both SC 560 and NS 398, specific COX-1 (Smith et al., 1998) and COX-2 (Barnett et al., 1994) inhibitor, respectively (Fig. 1A). Fig. 1B shows how ketoprofen blocked the uptake of [^{3}H]thymidine in Caco-2 cells cultured in 10% FBS whereas specific COX-1 and COX-2 inhibitors partially reduce DNA synthesis. We did not find that COX inhibitors induced an arrest or an appreciable change in the cell cycle (Fig. 1C). Similarly, to determine the role of the LOX pathway in Caco-2 growth induced by 10% FBS, cells were exposed to specific LOX inhibitors. Our results show that Caco-2 proliferation was inhibited not only by NDGA, a non-specific LOX inhibitor (Tang et al., 1996), but also by zileuton, a 5-LOX inhibitor (Carter et al., 1991), MK 886, a 5-LOX-activating protein inhibitor (Dixon et al., 1990), and baicalein, a 12-LOX inhibitor (Sekiya and Okuda, 1982) (Fig. 1D). Moreover, these LOX inhibitors were able to inhibit DNA synthesis (Fig. 1B) but did not change the cell cycle, except in the case of baicalein (Fig. 1C). The above-mentioned treatments did not cause cell detachment nor a decrease in cell viability at the concentrations tested, as confirmed by microscopic observation (data not shown). Moreover, significant apoptotic activity was only observed under the baicalein treatment (Fig. 1E).

Recently, we reported the immunolocalization of PGE_2 receptors such as EP_1 and EP_4 in differentiated Caco-2 cells (Rodríguez-Lagunas et al., 2010). Our results show that SC 19220, an EP_1 antagonist (Funk et al., 1993) and AH 23838, an EP_4 antagonist (Nishigaki et al., 1995), inhibited Caco-2 proliferation (Fig. 2A) and DNA synthesis (Fig. 2B) induced by 10% FBS. Similar results have been obtained with LTB_4 antagonists such as U 75302 and LY 255283 (Yokomizo et al., 2000) (Fig. 2B and C) but there were no significant effects on cell cycle (Fig. 2D) or apoptosis (Fig. 2E).

3.3. Several eicosanoids have proliferative effect on non-differentiated Caco-2 cells

The above findings suggest a mitogenic effect of PGE_2 and LTB_4 in Caco-2 cell growth induced by FBS. As shown in Fig. 3A and B, not only PGE_2 and LTB_4 but also 5-, 12-S- and 15-HETE significantly increased Caco-2 growth and [^{3}H]thymidine incorporation, respectively, at 1–100 nM, at the range of concentrations reached in 10% FBS Caco-2 cell supernatant, whereas 12-R-HETE did not have these effects. Fig. 4A shows that Caco-2 growth induced by PGE_2 was totally inhibited by an EP_1 antagonist (SC 19220) or an EP_4 antagonist (AH 23838). Moreover, Caco-2 cell proliferation was induced by carbacyclin and butaprost, EP_1 and EP_2/EP_4 agonists, respectively (Sugimoto and Narumiya, 2007) and by ONO-AE-329, which is an EP_4 agonist (Yokomizo et al., 2001) (Fig. 4A). Furthermore, Caco-2 cell growth induced by carbacyclin or butaprost was reverted by their specific antagonist. Caco-2 growth induced by LTB_4 was also inhibited by LTB_4 antagonists (U 75302, LY 255283) (Fig. 4B/C).

Specific HETE receptors have not yet been identified. However, it has been described that HETEs can bind to the BLT_2 described as the low affinity receptor for LTB_4 (Yokomizo et al., 2001). Moreover, we observed that the effects of 12-HETE on Caco-2 proliferation and DNA synthesis were reversed by BLT_2 but not by BLT_1 antagonists (Fig. 4B/C). Furthermore, $CysLT_1R$ antagonists (MK 571 and LY 171883) did not modify Caco-2 cell growth induced by 12-HETE, whereas the proliferative effects of LTB_4 and 12-HETE were completely reverted by a COX inhibitor as well as by an EP_1 antagonist (Fig. 4B/C).

3.4. LTB_4 and 12-HETE induce PGE_2 synthesis but not cAMP

LTB_4 (100 nM) and 12-HETE (100 nM) were able to induce a significant PGE_2 synthesis in Caco-2 cell cultures (control, 40.6 ± 4.0 pg/ml; LTB_4, 82.6 ± 16.2 pg/ml; 12-HETE, 66.7 ± 8.1 pg/ml; $P < 0.05$ respect to the control condition). Moreover, PGE_2 (10 nM) increases intracellular cAMP in Caco-2 cells from 23.45 ± 1.58 nM (control condition) to 64.22 ± 2.15 nM ($P < 0.05$), whereas LTB_4 (up to 1 μM) and 12-HETE (up to 10 μM) have no effect in this short period of time tested.

3.5. Eicosanoids activate cell signaling pathways involve in cell growth

Finally, we studied the capacity of these eicosanoids to phosphorylate pivotal elements in the cell signaling pathways implicated in the regulation of cell growth. Maximal phosphorylation was observed after 5 min incubation for ERK, AKT and p38, and after 15 min for GSKβ and CREB, as well as for β-catenin dephosphorylation. Our results show that PGE_2 (1 nM) was able to increase the phosphorylation of ERK1/2, p38α, CREB and GSKβ (Fig. 5A). Meanwhile, LTB_4 (10 nM) and 12-HETE (100 nM) only increase the phosphorylation of ERK or p38α, respectively, in a lesser extent than PGE_2. Moreover, our results also show that ERK and AMPc-PKA inhibitors (PD 98059 and KT 5720, respectively) significantly reduced CREB phosphorylation induced by PGE_2 (Fig. 5B).

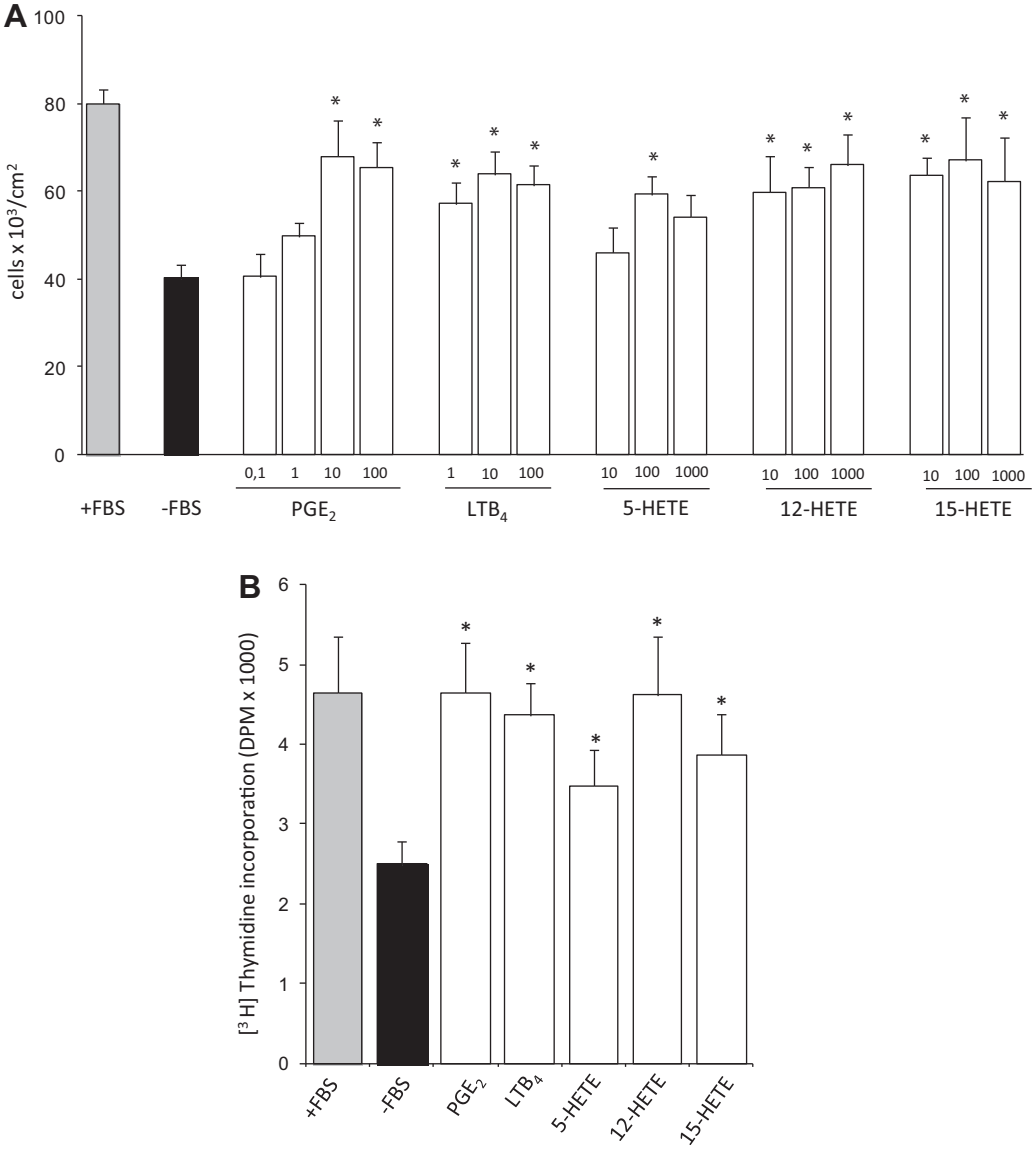


Fig. 3. Effect of eicosanoids on Caco-2 cell growth. PGE_2 (1 nM), LTB_4 (10 nM), 5-HETE (100 nM), 12-HETE (100 nM) and 15-HETE (100 nM) were incubated for 48 h in the absence of growth factors (FBS) and Caco-2 cells were counted (A) and [^{3}H]thymidine uptake measured (B). Results are expressed as means ± SEM of 2–3 experiments performed in triplicate. * $P < 0.05$ vs. Caco-2 cell cultured in absence of FBS.

4. Discussion

Previous findings demonstrating that AA released by calcium-independent PLA_2 participates in the signaling pathways involved in the control of intestinal epithelial proliferation (Sanchez and Moreno, 2002) also show that its subsequent metabolism by COX-2 could be involved in the control of intestinal epithelial cell growth. Several authors also reported that Caco-2 cells were able to synthesize LTB_4 (Wachtershauser et al., 2000) and cysteinyl LTs (Paruchuri et al., 2006) while there are contradictory reports regarding the production of 15-HETE (Kamitani et al., 1998; Wachtershauser et al., 2000), and there is no information about HETEs/EETs/DHETEs produced via the CYP450 pathway. Our data show for the first time an overall picture of the eicosanoids synthesized by non-differentiated intestinal epithelial cells cultured with 10% FBS, confirm the production of PGE_2, LTB_4 and 15-HETE together with 5-HETE and 12-HETE by Caco-2 cells. An important consideration is that Paruchuri et al. (2006) reported that Caco-2 cells release cysteinyl LTs reached 5 pM concentration, which is notably lower than the limits of detection (0.3 nM) for LTD_4 in our experimental conditions (Martin-Venegas et al., 2011).

Our findings also confirm that the COX pathway metabolites are involved in the control of Caco-2 growth, by both COX isoforms expressed in Caco-2 cells (Grosch et al., 2001; Martin-Venegas et al., 2006). Non-specific LOX inhibitors also blocked Caco-2 growth induced by 10% FBS to a similar extent as COX inhibitors. Microscopic and cytometric studies clearly showed that these effects were not a consequence of an impairment of cell viability and/or an apoptotic effect of treatments.

PGE_2, LTB_4 and HETEs synthesized by LOXs (5-, 12-, 15-HETE) at concentrations reached in 10% FBS Caco-2 supernatants (5–20 nM) were able to induce cell proliferation and DNA synthesis. Moreover, we demonstrate that the PGE_2 proliferative effect was a consequence of the interaction with EP_1 and EP_4 receptors using specific receptor. Similarly, we propose that BLT_1 and BLT_2 receptors expressed in intestinal epithelial cells (Ihara et al., 2007; Iizuka et al., 2010) are involved in Caco-2 cell growth induced by LTB_4.

Understanding the mechanism by which HETEs are involved in cell growth may be a critical issue in cell growth/cancer and

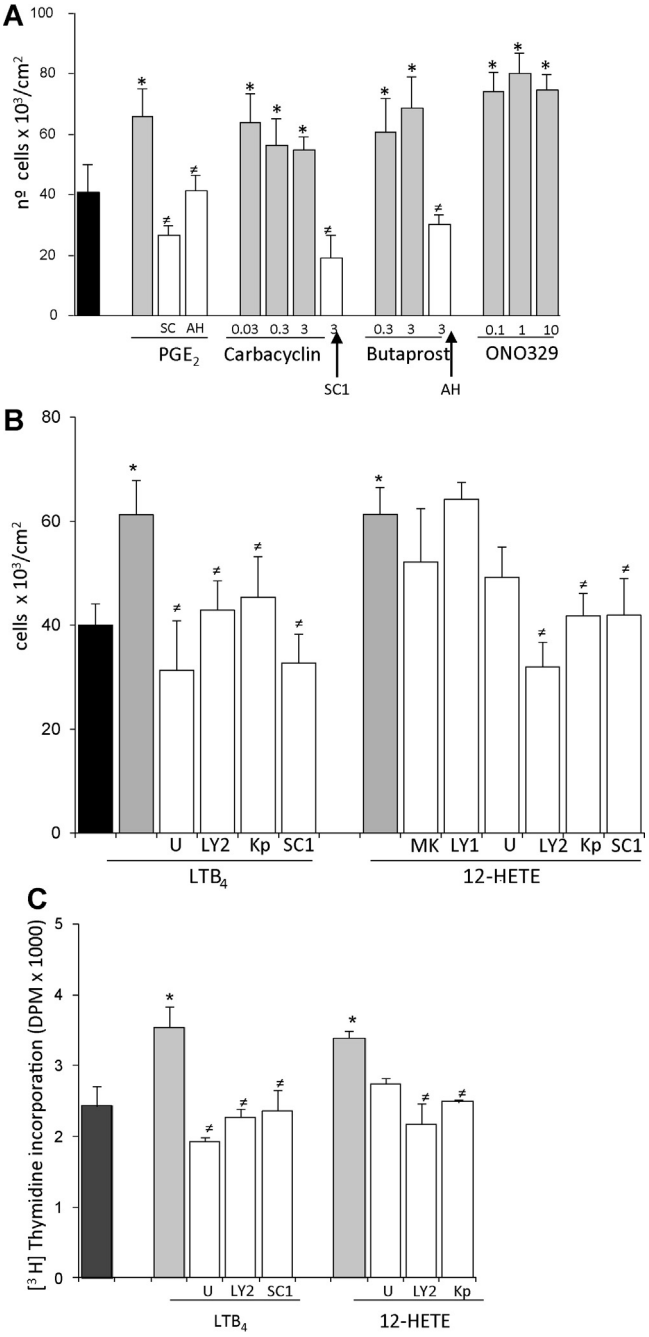


Fig. 4. Effect of agonist and antagonist of PGE_2 and LTB_4 receptors on Caco-2 cell growth. Caco-2 cells were incubated for 48 h with PGE_2 (10 nM) or PGE_2 (10 nM) plus SC 19220 (SC, 60 nM) or AH 23848 (AH, 20 nM) with carbacyclin, carbacyclin plus SC 19220 (SC, 60 nM), butaprost, butaprost plus AH 23848 (AH, 20 nM) or ONO 329. Cells were then counted (A). Caco-2 cells were incubated for 48 h with LTB_4 (10 nM) or 12-HETE (100 nM) in the absence or presence of U 75302 (U, 5 μM), LY 255283 (LY2, 25 μM), ketoprofen (Kp, 5 μM), SC 19220 (SC, 60 nM), MK 571 (MK, 25 μM) or LY 171883 (LY1, 25 μM) and cells were counted (B). The uptake of [^{3}H]thymidine uptake was also measured (C). Data are expressed in means ± SEM of 3–4 experiments performed in triplicate. $^*P < 0.05$ vs. Caco-2 cell cultures in the absence of FBS, $^{\#}P < 0.05$ vs. cells incubated without antagonist/agonist.

lipid homeostasis that has not yet been fully elucidated. Recently, Melstrom et al. (2008) observed that 5-LOX is up-regulated in adenomatous colon polyps and cancer. However, Shureiqi et al. (2010) reported no significant differences with regard to LTB_4, 12- and 15-HETE levels between normal, polyp, and cancer mucosa. Although the translation of an in vitro Caco-2 cell data into in vivo situation necessitates further investigation using colorectal cancer models, this study proposes that HETEs produced by LOXs together COX metabolites might be important elements involved in the control of intestinal epithelial cell growth. Findings in agreement with Cianchi et al. (2006) who reported that 5-LOX inhibitors could augment the antitumor activity of COX inhibitor; and with Mohammed et al. (2011) who observed that dual 5-LOX/COX inhibitor dramatically suppresses colonic tumor formation.

No cellular receptors for HETEs have yet been identified. However, it has been reported that HETEs bind to the BLT_2 receptor (Yokomizo et al., 2001). Taking into account that both LTB_4 receptors are expressed in Caco-2 cells (Ihara et al., 2007; Iizuka et al., 2010) our findings suggest that BLT_2 receptor is involved in the 12-HETE biological effect on Caco-2 cell growth, whereas BLT_1 receptor has no effect. These results are in agreement with recent data demonstrating the role of BLT_2 in the biological effects of 12-HETE (Kim et al., 2009).

Although there is evidence supporting the importance of PGE_2 in cell growth and colon carcinogenesis, there are few reports delineating the signaling pathways involved. Recently, Pham et al. (2008) demonstrated that CREB phosphorylation via angiotensin II was significantly impaired in intestinal epithelial IEC-18 cells when the expression of prostaglandin dehydrogenase increased and, consequently, the synthesis of PGE_2 was dramatically impaired. Regulated activation of CREB has a significant impact on cellular growth, proliferation and survival (Siu and Jin, 2007). Thus, phosphorylation of CREB at its critical Ser-133 residue by ERK has been reported to increase its DNA binding activity (Shaywitz and Greenberg, 1999). Here, we demonstrate that PGE_2 activation of ERK leads to the phosphorylation of CREB at Ser-133 as reported by Cherukuri et al. (2007) using HCA-7 colon cancer cells. In addition, we demonstrate the role of p38α and especially AMPc-PKA in this event. This role of p38α in CREB phosphorylation was recently reported by Pham et al. (2008) using angiotensin II stimulated rat intestinal epithelial cells. The role of AMPc-PKA has been described as a consequence of the increase in AMPc induced by PGE_2 in Caco-2 cell cultures (Rodríguez-Lagunas et al., 2010). However, in disagreement with Tessner et al. (2004), we did not observe a role for AKT1/2 in cell signaling induced by PGE_2. Similarly, we observed, for the first time, that LTB_4 and 12-HETE induced the activation of ERK and p38α in intestinal epithelial cells. However, both eicosanoids did not active CREB, event that may be consequence that these eicosanoids were unable to increase intracellular AMPc levels after a short period of incubation. At any rate, we could not discard that PGE_2 synthesis induced by these two eicosanoids might then modify AMPc and consequently CREB. The Wnt signaling cascade is activated during the regulation of epithelial proliferation and in the majority of colorectal cancers. In resting cells, β-catenin forms a complex with APC, axin and GSK-3β. Failure to phosphorylate β-catenin results in its translocation to the nucleus where it activates Tcf/Lef. Using a human colon cancer cell line with a mutant APC, Castellone et al. (2005) reported that PGE_2 activated components of the canonical Wnt signaling cascade and the Tcf/Lef transcription factors. In the present study, using Caco-2 cells with an APC mutation in the region responsible for APC binding and consequently an important basal dephosphorylation of β-catenin, we observed that PGE_2 and LTB_4 could not induce a significant additional dephosphorylation of β-catenin.

In conclusion, PGE_2, LTB_4, 5-HETE, 12-HETE and 15-HETE are involved in the regulation of Caco-2 cell growth induced by 10% FBS through the interaction of specific eicosanoid receptors and the subsequent cell signaling pathways. In addition, we can suggest that the proliferative effects of LTB_4 and 12-HETE are, at least in part, due to PGE_2 synthesis, as we propose in Fig. 6. This thorough in vitro study would contribute to establish new clinical strategies regarding the relevance of the data presented herein.

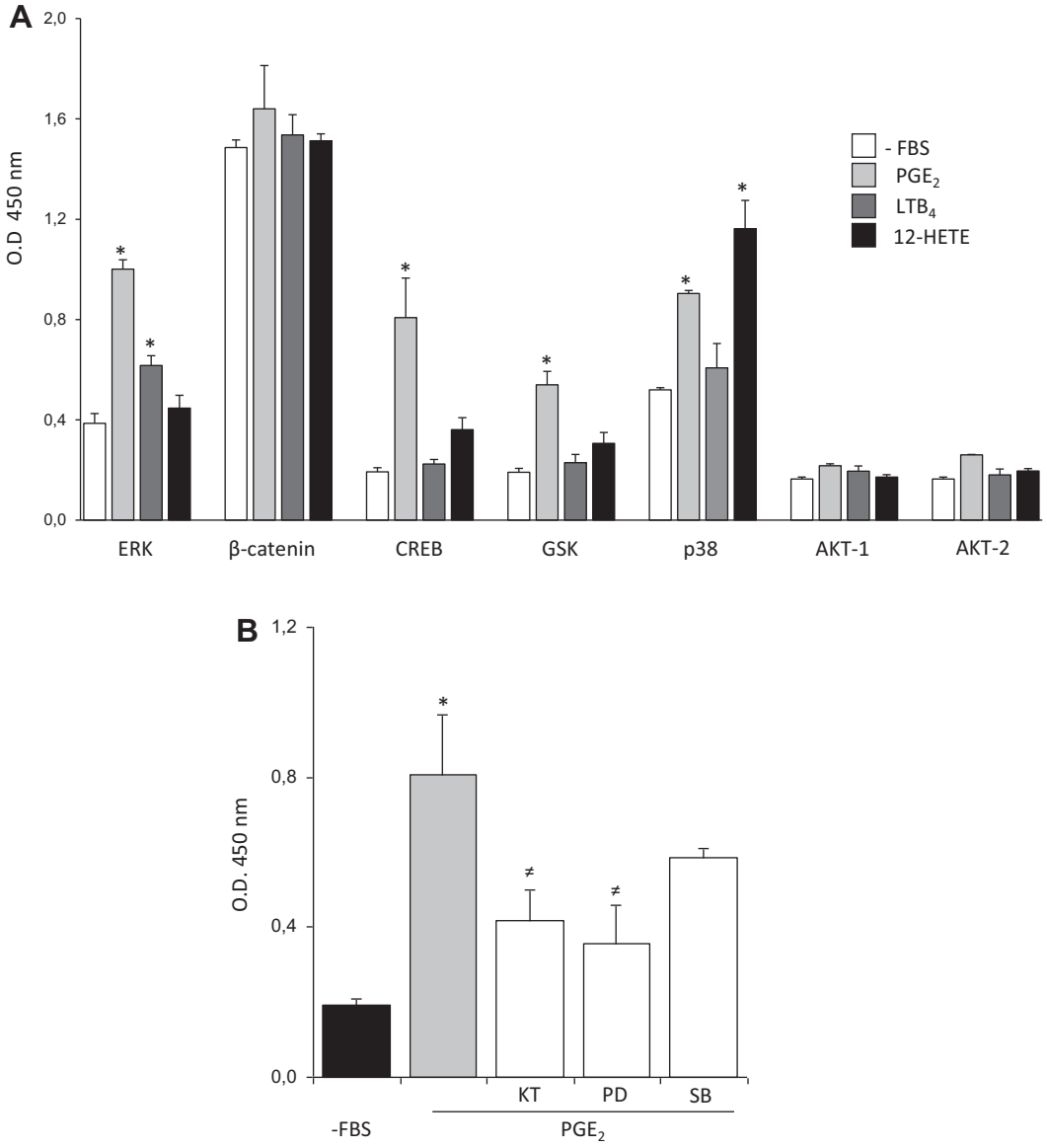


Fig. 5. Effect of PGE_2, LTB_4 and 12-HETE on cell signaling. Caco-2 cells were incubated with PGE_2 (1 nM), LTB_4 (10 nM) and 12-HETE (100 nM) for 5 or 15 min, cells were collected and finally phosphorylated AKT1, AKT2, ERK1/2, p38α, CREB and GSKβ, and dephosphorylated β-catenin were measured as we described in Section 2 (A). CREB phosphorylation induced by PGE_2 was also assayed in presence of PD 98059 (PD, 1 μM), KT 5720 (KT, 10 μM) or SB 203580 (SB, 1 μM). These inhibitors were pre-incubated 30 min and finally cultures were incubated with PGE_2 (1 nM) (B). Data are expressed in means ± SEM of 2–4 experiments performed in triplicate. $^{*}P < 0.05$ vs. Caco-2 cell cultures in the absence of FBS, $^{\#}P < 0.05$ vs. cells incubated with PGE_2.

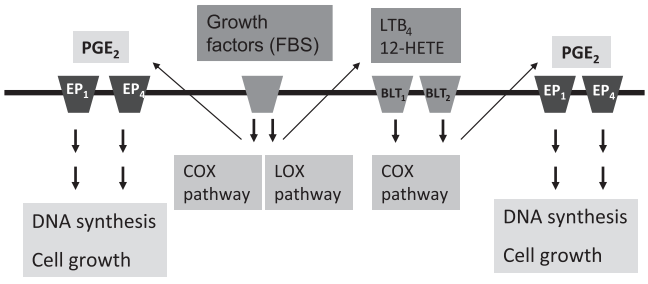


Fig. 6. Role of PGE_2 in the control of Caco-2 cell growth. PGE_2 presents a direct effect on Caco-2 cell growth through its interaction with EP_1 and EP_4 receptors and the activation of several cell signaling pathways. Moreover, PGE_2 can have an indirect effect through its synthesis induced after LTB_4 and HETEs (12-HETE) interaction with BLT_1 and BLT_2 receptors.

Acknowledgements

This work has been supported by Ministerio de Ciencia e Innovación (BFU2007-61727/BFI) and by Generalitat de Catalunya (2009SGR0438). HPLC-MS/MS and cytometry analyses were performed at the Serveis Científic-Tècnics, University of Barcelona. We thank Olga Jáuregui and Jaume Comas for their assistance with liquid chromatography mass spectrometry and cytometry techniques, respectively.

References

Barnett J, Chow J, Ives D, Chiou M, MacKenzie R, Osen E, et al. Purification, characterization and selective inhibition of human prostaglandin G/H synthase 1 and 2 expressed in the baculovirus system. Biochimica et Biophysica Acta 1994;1209:130–9.

Carter GW, Young PR, Albert DH, Bouska J, Dyer R, Bell RL. 5-Lipoxygenase inhibitory activity of zileuton. Journal of Pharmacology and Experimental Therapeutics 1991;256:929–37.

Castellone MD, Teramoto H, Williams BO, Druey KM, Gutkind JS. Prostaglandin E_2 promotes colon cancer cell growth through a Gα-axin-β-catenin signalling axis. Science 2005;310:1504–10.

Cherukuri DP, Chen XBO, Goulet AC, Young RN, Han Y, Heimark RL, et al. The EP_4 receptor antagonist, L-161,982, blocks prostaglandin E_2-induced signal transduction and cell proliferation in HCA-7 colon cancer cells. Experimental Cell Research 2007;313:2969–79.

Cianchi F, Cortesini C, Magnelli L, Fanti E, Papucci L, Schiavone N, et al. Inhibition of 5-lipoxygenase by MK886 augments the antitumor activity of celecoxib in human colon cancer cells. Molecular Cancer Therapeutics 2006;5:2716–26.

Dixon RAF, Diehl RE, Opas E, Rands E, Vickers PJ, Evans JF, et al. Requirement of a 5-lipoxygenase-activanting protein for leukotriene synthesis. Nature 1990;343:282–4.

Ekbom A, Helmick C, Zack M, Adami HO. Ulcerative colitis and colorectal cancer. A population-based study. New England Journal of Medicine 1999;323:1228–33.

Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adeno carcinomas. Gastroenterology 1994;107:1183–8.

Ferrer R, Moreno JJ. Role of eicosanoids on intestinal epithelial homeostasis. Biochemical Pharmacology 2010;80:431–8.

Funk CD, Furci L, Fitzgerald GA, Grygorczk R, Rochette C, Bayne MA, et al. Cloning and expression of a cDNA for the human prostaglandin E receptor EP1 subtype. Journal of Biological Chemistry 1993;268:26767–72.

Giardiello FM, Hamilton SR, Krush AJ, Piantadosi S, Hylind LM, Celano P, et al. Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. New England Journal of Medicine 1993;328:1313–6.

Grosch S, Tegeder I, Niederberger E, Brautigam L, Geisslinger G. COX-2 independent induction of cell cycle arrest and apoptosis in colon cancer cells by the selective COX-2 inhibitor celecoxib. FASEB Journal 2001;15:2742–4.

Ihara A, Wada K, Yoneda M, Fijisawa N, Takahashi H, Nakajima A. Blockade of leukotriene B_4 signaling pathway induces apoptosis and suppresses cell proliferation in colon cancer. Journal of Pharmacological Sciences 2007;103:24–32.

Iizuka Y, Okuno T, Saeki K, Uozaki H, Okada S, Misaka T, et al. Protective role of the leukotriene B_4 receptor BLT_2 in murine inflammatory colitis. FASEB Journal 2010;24:4678–90.

Kamitani H, Geller M, Eling T. Expression of 15-lipoxygenase by human colorectal carcinoma Caco-2 cells during apoptosis and cell differentiation. Journal of Biological Chemistry 1998;73:21569–77.

Kim GY, Lee JW, Cho SH, Seo JM, Kim JH. Role of the low-affinity leukotrienen B_4 receptor BLT_2 in VEGF-induced angiogenesis. Arteriosclerosis, Thrombosis, and Vascular Biology 2009;29:915–20.

Martin-Venegas R, Roig-Perez S, Ferrer R, Moreno JJ. Arachidonic acid cascade and epithelial barrier function during Caco-2 cell differentiation. Journal of Lipid Research 2006;46:1416–23.

Martin-Venegas R, Casillas R, Jáuregui O, Moreno JJ. Rapid simultaneous analysis of cyclooxygenase, lipoxygenase and cytochrome P-450 metabolites of arachidonic and linoleic acids using high performance liquid chromatography/mass spectrometry in tandem mode. Journal of Pharmaceutical and Biomedical Analysis 2011;56:976–82.

Melstrom LG, Bentrem DJ, Salabat MR, Kennedy TJ, Ding XZ, Strouch M, et al. Overexpression of 5-lipoxygenase in colon polyps and cancer and the effect of 5-LOX inhibitors in vitro and in a murine model. Clinical Cancer Research 2008;14:6525–30.

Mohammed A, Janakiram NB, Li Q, Choi CI, Zhang Y, Steele VE, et al. Chemoprevention of colon and small intestinal tumorigenesis in APC (Min/+) mice by licofelone, a novel dual 5-LOX/COX inhibitor: potential implications for human colon cancer prevention. Cancer Prevention Research 2011;4:2015–26.

Moreno JJ. New aspects of the role of hydroxyeicosatetraenoic acids in cell growth and cancer development. Biochemical Pharmacology 2009;77:1–10.

Natarajan R, Reddy MA. HETEs/EETs in renal glomerular and epithelial cell functions. Current Opinion in Pharmacology 2003;3:198–203.

Nishigaki N, Negishi M, Honda A, Sugimoto Y, Namba T, Narumiya S, et al. Identification of prostaglandin E receptor "EP_2" cloned from mastocytoma cells as EP_4 subtype. FEBS Letters 1995;364:339–41.

Paruchuri S, Mezhybovska M, Juhas M, Sjölander A. Endogenous production of leukotrienen D4 mediates autocrine survival and proliferation via CysLT1 receptor signaling in intestinal epithelial cells. Oncogene 2006;25:6660–5.

Pham H, Eibl G, Vincenti R, Chong B, Tai HH, Slice LW. 15-hydroxyprostaglandin dehydrogenase suppresses K-Rasv12-dependent tumor formation in Nu/Nu mice. Molecular Carcinogenesis 2008;47:466–77.

Rodríguez-Lagunas MJ, Martín-Venegas R, Moreno JJ, Ferrer R. PGE_2 promotes Ca^{2+}-mediated epithelial barrier disruption through EP_1 and EP_4 receptors in Caco-2 cell monolayers. American Journal of Physiology: Cell Physiology 2010;299:324–34.

Sanchez T, Moreno JJ. Ketoprofen S(+) enantiomer inhibits prostaglandin production and cell growth in 3T6 fibroblast cultures. European Journal of Pharmacology 1999;370:63–7.

Sanchez T, Moreno JJ. Calcium-independent phospholipase A_2 through arachidonic acid mobilization is involved in Caco-2 cell growth. Journal of Cellular Physiology 2002;193:293–8.

Sekiya K, Okuda H. Selective inhibition of platelet lipoxygenase by baicalein. Biochemical and Biophysical Research Communications 1982;105:1090–5.

Shaywitz AJ, Greenberg ME. CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. Annual Review of Biochemistry 1999;68:821–61.

Shureiqi I, Wojno KJ, Poore JA, Reddy RG, Moussalli MJ, Spindler SA, et al. Decreased 13-S-hydroxyoctadecadenoic acid levels and 15-lipoxygenase-1 expression in human colon cancers. Carcinogenesis 1999;20:1985–95.

Shureiqi I, Lippman SM. Lipoxygenase modulation to reverse carcinogenesis. Cancer Research 2001;61:6307–12.

Shureiqi I, Wu Y, Chen D, Yang XL, Guan B, Morris JS, et al. The critical role of 15-lipoxygenase-1 in colorectal epithelial cell terminal differentiation and tumorigenesis. Cancer Research 2005;65:11486–92.

Shureiqi I, Chen D, Day RS, Zuo X, Hochman FL, Ross WA, et al. Profiling lipoxygenase metabolism in specific steps of colorectal tumorigenesis. Cancer Prevention Research 2010;3:829–38.

Siu YT, Jin DY. CREB a real culprit in oncogenesis. FEBS Journal 2007;274:3224–32.

Smith CJ, Zhang Y, Koboldt CM, Muhammad J, Zweifel BS, Shaffer A, et al. Pharmacological analysis of cyclooxygenase-1 in inflammation. Proceedings of the National Academy of Sciences of the United States of America 1998;95:13313–8.

Sugimoto Y, Narumiya S. Prostaglandin E receptors. Journal of Biological Chemistry 2007;282:11613–7.

Tang DG, Chen YQ, Honn KV. Arachidonate lipoxygenases as essential regulators of cell survival and apoptosis. Proceedings of the National Academy of Sciences of the United States of America 1996;93:5241–6.

Tessner TG, Muhale F, Riehl TE, Anant S, Stenson WF. Prostaglandin E_2 reduces radiation-induced epithelial apoptosis through a mechanism involving AKT activation and bax translocation. Journal of Clinical Investigation 2004;114:1676–85.

Thun MJ, Namboodiri MM Jr, Health CW. Aspirin use and reduced risk of fatal colon cancer. New England Journal of Medicine 1991;325:1593–6.

Wachtershauser A, Steinhilber D, Loitsch SM, Stein J. Expression of 5-lipoxygenase by human colorectal carcinoma Caco-2 cells during butyrate-induced cell differentiation. Biochemical and Biophysical Research Communications 2000;268:778–83.

Yamane M, Shimizu S, Abe A, Yamane S. Docosahexaenoic/arachidonic acid ω-hydroxylation system and differentiation in the human colonic adenocarcinoma cell line, Caco-2. Cancer Letters 1998;122:51–9.

Yokomizo T, Kato K, Terawaki K, Izumi T, Shimizu T. A second leukotriene B_4 receptor, BLT_2: a new therapeutic target in inflammation and immunological diseases. Journal of Experimental Medicine 2000;192:421–31.

Yokomizo T, Kato K, Hagiya H, Izumi T, Shimizu T. Hydroxyeicosanoids bind to and activate the low affinity leukotriene B_4 receptor, BLT_2. Journal of Biological Chemistry 2001;276:12454–9.

Artículo 2

Marisol Cabral, Raquel Martín-Venegas, Juan J. Moreno.

Differential cell growth/apoptosis behavior of 13-hydroxyoctadecadienoic acid enantiomers in a colorectal cancer cell line

Am J Physiol Gastrointest Liver Physiol (2014) 307: 664-671

Índice de impacto (JCR,2014): 3,798
Categoria y posición; quartil: Physiology: 17/83; Q1
Gastroenterology and Hepatology: 21/76; Q2

Los resultados de esta publicación se han presentado en el siguiente congreso:

- 13-HODE derived from lipoxygenase pathway is involved in intestinal epithelial cell growth.
  Moreno JJ, Cabral M, Martin-Venegas R.
  10th World Congress on Inflammation. Paris (Francia). 25-29, Junio 2011.
  Resumen publicado en la revista Inflammation Research, 60:192 (2011).

Resumen artículo 2:

Objetivo Investigar el papel de los dos enantiómeros del 13-HODE sobre el crecimiento de las células epiteliales intestinales no diferenciadas procedentes de un adenocarcinoma.

Material y Métodos Este estudio se ha realizado utilizando células Caco-2 no diferenciadas. Las células se incubaron con 13-HODE en ausencia y presencia de inhibidores de la COX, así como con antagonistas receptoriales de eicosanoides y se determinó: el 13-HODE presente en el sobrenadante por HLPC-MS/MS; el crecimiento celular por contaje microscópico usando bromuro de etidio/naranja de acridina y la síntesis de ADN por incorporación de [^{3}H]thimidina; el análisis del ciclo celular por citometría de flujo y la degradación del ADN cromosómico con el método TUNEL; la síntesis de PGE_2 mediante un inmunoensayo enzimático (EIA); y finalmente la fosforilación de Akt1, Akt2, ERK1/2, GSK3, p38, CREB y la desfosforilación de β-catenina por ELISA. La unión entre los HODEs y el receptor PPARγ se determinó por fluorescencia de polarización.

Resultados El 13-S-HODE no tiene efecto sobre el crecimiento celular ni sobre la síntesis de ADN. En cambio, en presencia de FBS, presenta un efecto anti-proliferativo e incrementa el porcentaje de células apoptóticas. El 9-S-HODE presenta un comportamiento similar. El efecto antiproliferativo y la apoptosis producida por el 13-S-HODE se reducen drásticamente cuando se incuban junto con un antagonista de PPARγ y además observamos que el 13-S-HODE se une al PPARγ de forma similar a la rosiglitazone, conocido ligando del PPARγ. En cambio, el 13-R-HODE presenta los efectos contrarios, ya que incrementa el crecimiento celular y la síntesis de ADN y, además, disminuye el efecto apoptótico del 13-S-HODE. El efecto proliferativo del 13-R-HODE es revertido utilizando antagonistas de los receptores BLT_1 y BLT_2. Estos resultados indican que el 13-R-HODE puede interacciona con los receptores del LTB_4. Por otra parte, el 13-R-HODE incrementa la fosforilación de ERK y CREB, y además esta fosforilación de CREB se reduce con un inhibidor de ERK. Nuestros resultados muestran también que el efecto proliferativo del 13-R-HODE es revertido por antagonistas receptoriales de la PGE_2 e inhibidores de las COXs. Así mismo observamos que 13-R-HODE induce la síntesis de PGE_2.

Conclusión 13-S-HODE y 13-R-HODE tienen efectos contrarios sobre el crecimiento de células epiteliales intestinales. Así, el 13-S-HODE presenta un efecto antiproliferativo a través de su unión con PPARγ, mientras que el 13-R-HODE tiene un efecto mitogénico al interactuar con los BLTs y activar la vía COX dando lugar a la síntesis de PGE_2 que ejerce el efecto proliferativo.

Am J Physiol Gastrointest Liver Physiol 307: G664–G671, 2014.
First published July 17, 2014; doi:10.1152/ajpgi.00064.2014.

Differential cell growth/apoptosis behavior of 13-hydroxyoctadecadienoic acid enantiomers in a colorectal cancer cell line

Marisol Cabral, Raquel Martín-Venegas, and Juan José Moreno

Departament de Fisiologia, Facultat de Farmàcia, Universitat de Barcelona, Barcelona, Spain

Submitted 12 February 2014; accepted in final form 11 July 2014

Cabral M, Martín-Venegas R, Moreno JJ. Differential cell growth/apoptosis behavior of 13-hydroxyoctadecadienoic acid enantiomers in a colorectal cancer cell line. *Am J Physiol Gastrointest Liver Physiol* 307: G664–G671, 2014. First published July 17, 2014; doi:10.1152/ajpgi.00064.2014.—Cyclooxygenases (COXs) and lipoxygenases (LOXs) are important enzymes that metabolize arachidonic and linoleic acids. Various metabolites generated by the arachidonic acid cascade regulate cell proliferation, apoptosis, differentiation, and senescence. Hydroxyoctadecadienoic acids (HODEs) are synthesized from linoleic acid, giving two enantiomeric forms for each metabolite. The aim was to investigate the effect of 13-HODE enantiomers on nondifferentiated Caco-2 cell growth/apoptosis. Our results indicate that 13(S)-HODE decreases cell growth and DNA synthesis of nondifferentiated Caco-2 cells cultured with 10% fetal bovine serum (FBS). Moreover, 13(S)-HODE showed an apoptotic effect that was reduced in the presence of a specific peroxisome proliferator-activated receptor-γ (PPARγ) antagonist. In addition, we observed that 13(S)-HODE but not 13(R)-HODE is a ligand to PPARγ, confirming the implication of this nuclear receptor in 13(S)-HODE actions. In contrast, 13(R)-HODE increases cell growth and DNA synthesis in the absence of FBS. 13(R)-HODE interaction with BLT receptors activates ERK and CREB signaling pathways, as well as PGE_2 synthesis. These results suggest that the proliferative effect of 13(R)-HODE could be due, at least in part, to COX pathway activation. Thus both enantiomers use different receptors and have contrary effects. We also found these differential effects of 9-HODE enantiomers on cell growth/apoptosis. Therefore, the balance between (R)-HODEs and (S)-HODEs in the intestinal epithelium could be important to its cell growth/apoptosis homeostasis.

cyclooxygenase; lipoxygenase; cell growth; apoptosis; PPARγ

THE ARACHIDONIC ACID (AA) cascade is responsible for the generation of a wide variety of bioactive metabolites involved in many different pathological states, including inflammation and cancer (1, 18). Thus these metabolites regulate cell proliferation, apoptosis, differentiation, and senescence. Indeed, the functional relationship between polyunsaturated fatty acid metabolism, inflammation, and carcinogenesis has been examined in numerous molecular studies, revealing possible new targets for chemoprevention and/or treatment of a number of cancers (9).

Cyclooxygenases (COXs) and lipoxygenases (LOXs) are two important enzymatic groups that metabolize polyunsaturated fatty acids. COX has two isoforms (COX-1 and COX-2) and produces prostaglandins (PGs) and thromboxanes. LOXs constitute a family of dioxygenases that insert molecular oxygen into free and/or esterified polyunsaturated fatty acids with regional specificity and are designated 5-, 8-, 12- and 15-LOX, accordingly (12, 13). Thus these enzymes metabolize AA to the biologically active metabolites hydroperoxyeicosatetraenoic acids, which on reduction form corresponding hydroeicosatetranoic acids (HETEs), while the metabolism of linoleic acid preferentially results in the formation of hydroxyoctadecadienoic acids (HODEs). Therefore, HETEs are the main AA metabolites formed by mammalian LOXs, and 9- and 13-HODE are the principal reaction products of linoleic acid oxygenation by 12-LOX and 15-LOX, respectively. 15-LOX can be subclassified, according to the specificity of tissue distribution and enzymatic characteristics, into 15-LOX-1 and 15-LOX-2. While 15-LOX-2 metabolizes AA but not linoleic acid, 15-LOX-1 converts linoleic acid and AA to their metabolites, 13-HODE and 15-HETE, respectively, and is expressed preferentially in reticulocytes, eosinophils, macrophages, tracheobronchial epithelial cells, skin, and colon (21, 29). Interestingly, 15-LOX-1 prefers linoleic acid over AA as substrate and produces 13-HODE in excess when both fatty acids are available. Moreover, we must consider that there are two stereoisomers of 13-HODE, 13-sinister-HODE [13(S)-HODE] and 13-rectus-HODE [13(R)-HODE]. While 13(S)-HODE is only produced in the metabolism of linoleic acid by 15-LOX-1 (38, 44), 13(R)-HODE can be generated by several LOXs and COXs, as well as by autooxidation processes (3, 4, 5, 36, 43).

HETEs and HODES are known to modulate inflammation and may be important factors in carcinogenesis (32, 38, 44). In this sense, 15-LOX metabolites have been shown to play both pro- and antitumorigenic actions, whereas COX metabolites are described to be tumorigenic (42). Thus, while the expression of 15-LOX-1 inhibited intestinal epithelial cell proliferation (12, 13), the expression of COXs is mitogenic (15). Although several studies have implicated the pathways that synthesize 13-HODE in colorectal cancer (46), the results obtained appear to be contradictory. Indeed, although 13(S)-HODE is described as having an antiproliferative effect in colorectal cancer (35), a recent contradictory reference relates the racemic mixture of 13-HODE with a proliferative effect in intestinal epithelial HT-29 culture cells (14), suggesting the hypothesis that 15-LOX-1 metabolites [13(S)-HODE and 15-HETE] as well as both 13-HODE enantiomers may have contrary effects on intestinal epithelial growth. However, few studies have examined the biological actions of 13(R)-HODE specifically, and no studies relate this metabolite to cell proliferation. The aim of the present study was to evaluate the role of 13(R)-HODE and 13(S)-HODE in intestinal epithelial cell growth and to give a new perspective within the dynamically evolving research into the role of the two enantiomers in carcinogenesis.

Address for reprint requests and other correspondence: J. J. Moreno, Departament de Fisiologia, Facultat de Farmàcia, Universitat de Barcelona, Avda Joan XXIII s/n, 08028 Barcelona, Spain (e-mail: jjmoreno@ub.edu).

MATERIALS AND METHODS

Materials. DMEM, trypsin, penicillin, and streptomycin were supplied by GIBCO (Paisley, UK). Fetal bovine serum (FBS), sterile phosphate-buffered saline (PBS), 13(R)-HODE, 13(S)-HODE, 9(R)-HODE, 9(S)-HODE, U-75302, ketoprofen, SC 19220, PD98059, LY171883, bovine serum albumin (BSA), ribonuclease A from bovine pancreas, ethidium bromide, acridine orange, and propidium iodide solution were purchased from Sigma Chemical (St. Louis, MO). LY 255283 was from Tocris Bioscience (Bristol, UK). GW 9662 was purchased from Cayman Chemical (Ann Arbor, MI). AH 23838 was kindly provided by Glaxo-Wellcome (Stevenage, UK). [^{3}H]thymidine (20 Ci/mmol) was from American Radiolabeled Chemicals (St. Louis, MO). Tissue culture supplies were obtained from Costar (Cambridge, MA). Biogreen 3 was supplied by Scharlau Chemie (Barcelona, Spain).

Cell culture and cell growth assay. Caco-2 cells, derived from a colon adenocarcinoma, were provided by the American Type Culture Collection (HTB-37, Manassas, VA). The cells were routinely grown in plastic flasks at a density of 10^4 cells/cm^2 and cultured in DMEM supplemented with 4.5 g/l D-glucose, 1% (vol/vol) nonessential amino acids, 2 mM L-glutamine, 10% (vol/vol) heat-inactivated FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37°C in a modified atmosphere of 5% CO_2 in air, as previously described (27). The growth medium was replaced twice per week and the day before the experiment.

To perform the cell growth assay, cells were harvested with trypsin-EDTA and passed to 12-mm plastic clusters at a density of 10^4 cells/cm^2. After 4 days in culture, cells were incubated with treatments for a period of 48 h. Finally, cells were washed, trypsinized, and counted with a microscope with the use of ethidium bromide-acridine orange staining to assess the number of viable cells (37).

Analysis of DNA synthesis. DNA synthesis was measured by a [^{3}H]thymidine incorporation assay. Caco-2 cells were cultured on 24-well plates in DMEM with 10% FBS at a density of 10^4 cells/cm^2. After 4 days in culture, cells were incubated for 48 h with the treatments and [^{3}H]thymidine (0.1 μCi/well). Then media containing [^{3}H]thymidine were aspirated and cells were washed, overlaid with 1% Triton X-100, and then scraped off the wells. Finally, radioactivity present in the cell fraction was measured by scintillation counting with a Packard Tri-Carb 1500 counter (Downers Grove, IL).

Fluorescence-activated cell sorting analysis-flow cytometry cell cycle analysis. Caco-2 cells were seeded in 60-mm dishes (10^4 cells/cm^2). After 4 days in culture, cells were incubated for 48 h with the treatments. The culture was then trypsinized, fixed with 70% ethanol, and stored at 4°C for at least 2 days. Low-molecular-weight DNA was then extracted from the cells, which were stained for 1 h at room temperature with a 20 μg/ml propidium iodide solution in PBS containing 0.1% Triton X-100 and 0.2 mg/ml DNase-free RNase A. Cells were analyzed on an Epics XL flow cytometer (Coulter, Hialeah, FL). DNA was analyzed on single-fluorescence histograms by Multicycle software (Phoenix Flow Systems, San Diego, CA).

Measurement of apoptosis. Degradation of chromosomal DNA as a final result of apoptosis was evaluated with the TUNEL method. Subconfluent cell cultures (10^4 cells/cm^2) seeded in 60-mm dishes were incubated in medium containing 10% FBS with treatments for 48 h. Cells present in the medium and attached trypsinized cells were then collected, fixed with 4% paraformaldehyde, and permeabilized with 70% ethanol. Thereafter, 3′-OH DNA ends generated by DNA fragmentation were labeled with fluorescein-dUTP mediated by terminal deoxynucleotidyl transferase with the MEBSTAIN Apoptosis Kit (MBL, Woburn, MA) and then analyzed by an Epics XL flow cytometer (Coulter).

Prostaglandin E_2 analysis by enzyme immunoassay. PGE_2 was determined by a competitive enzyme immunoassay kit (Cayman) according to the manufacturer's instructions. Briefly, cells were maintained in 12-mm plastic clusters at a density of 10^4 cells/cm^2. After 4 days in culture, Caco-2 cells were incubated for 60 min at 37°C with 13(R)-HODE (0.1–10 μM) and BLT antagonists or COX inhibitors. Finally, supernatants were harvested and PGE_2 was determined.

Protein assay. Proteins in cell samples were measured with the Bio-Rad protocol with Coomassie brilliant blue G-250 as a dye reagent. The protein amount in each sample was calculated by a standard curve of BSA.

PPARγ ligand assay. HODE binding to peroxisome proliferator-activated receptor-γ (PPARγ) was studied with a fluorescence polarization-based single-step PPARγ ligand screening assay (Cayman). The assay was adapted to be performed in a microcuvette with a luminescence spectrometer (AMINCO-Bowman Series 2, Spectronic Unicam, Leeds, UK).

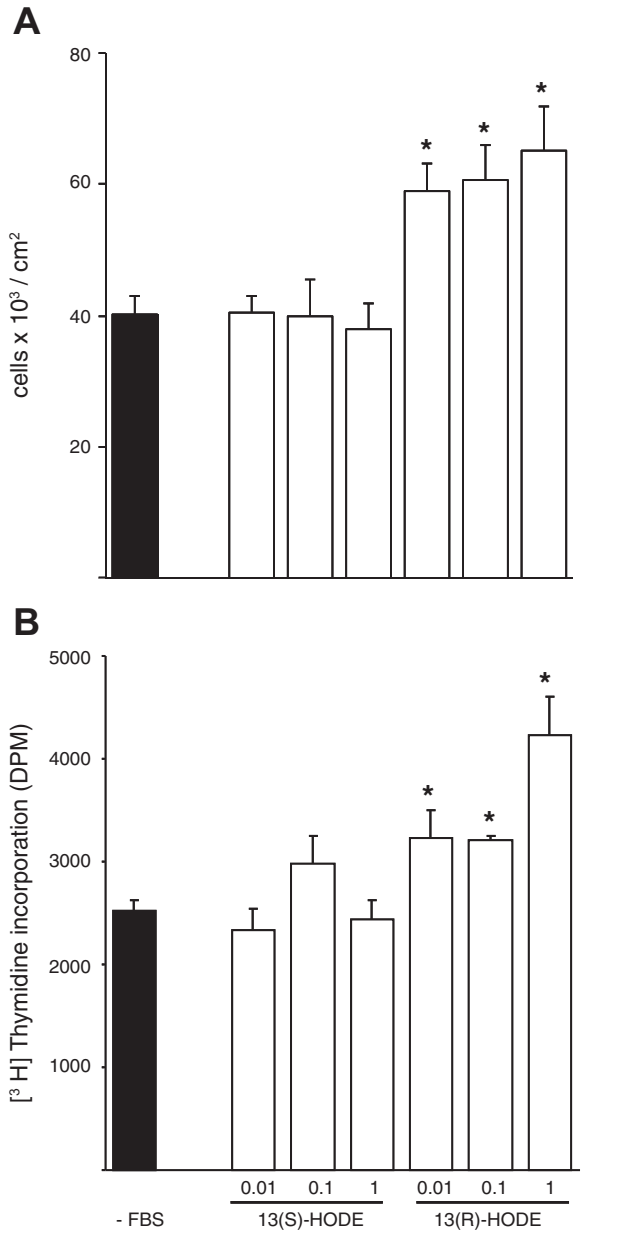


Fig. 1. Effect of 13(S)-hydroxyoctadecadienoic acid (HODE) and 13(R)-HODE on Caco-2 cell growth. Cells were incubated for 48 h with 13(S)-HODE and 13(R)-HODE (0.01–1 μM) in the absence of growth factors [fetal bovine serum (FBS); filled bar]. Caco-2 cells were counted (*A*), and [^{3}H]thymidine uptake was measured (*B*). Results are expressed as means ± SE of 5 determinations performed in triplicate. *$P < 0.05$ vs. Caco-2 cell cultured in the absence of FBS.

Measurement of cell signaling pathways activated by eicosanoids. 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 activity with total cell lysates, Caco-2 cells were lysed with a denaturing cell lysis buffer containing 6 M urea, protease (2 μg/ml leupeptin, 10 μM pepstatin, 3 μg/ml aprotinin), and phosphatase (5 mM NaF, 2 mM $Na_4P_2O_7$, 1 mM Na_3VO_4) inhibitors. The resulting solutions containing 80–100 μg of proteins were then added to a kinase ELISA plate, and the assay was performed according to the manufacturer's recommendations (Symansis, Auckland, New Zealand). Finally, optical density was measured at 450 nm with a TECAN absorbance reader (Tecan Austria, Salzburg, Austria). This methodology provides a qualitatively better alternative to Western blotting for simultaneous assay for activation of multiple kinases. We studied the effect of eicosanoids on the phosphorylation of AKT1 (pS473), AKT2 (pS474), ERK1/2 (pT202/Y204; pT185/Y187), GSK3β (pS9), p38α (pT180/Y182), and CREB (pS133) and on the dephosphorylation of β-catenin (DP S33/S37/S41). The phosphorylation of AKT, ERK, and p38 was measured after 5-min incubation with 13(R)-HODE, whereas the phosphorylation of CREB and GSK and the dephosphorylation of β-catenin were assayed after 15 min.

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

RESULTS

The results showed that 13(S)-HODE (0.01–1 μM) had no effect either on cell growth or on DNA synthesis in the absence of growth factors. In contrast, 13(R)-HODE (0.01–1 μM) significantly increased Caco-2 growth and [^{3}H]thymidine incorporation compared with cells incubated in the absence of FBS (Fig. 1). In light of these results, a concentration of 1 μM was chosen to further investigate the effects of HODE enantiomers. Interestingly, similar effects were found with 9-HODE enantiomers: 9(S)-HODE has no effect, while 9(R)-HODE had a proliferative effect without growth factors (Table 1).

Caco-2 cell growth and DNA synthesis induced by FBS were reverted by 13(S)-HODE, showing the marked antiproliferative effect of this metabolite (Fig. 2), while 13(R)-HODE did not show this effect. Moreover, both in the presence and in the absence of FBS, 13(S)-HODE significantly increased the percentage of apoptotic cells (Fig. 3) in Caco-2 cells, as did 9(S)-HODE (Table 1). Thus the two HODE enantiomers have opposite effects on cell growth and apoptosis. It has been described that 13(S)-HODE binds to the nuclear PPARγ (7, 34). Thus the incubation of 13(S)-HODE in the presence of a specific antagonist of PPARγ (GW 9662) (2) significantly reverted not only the antiproliferative effect (determined by cell growth and DNA synthesis) but also the apoptosis induced by this metabolite (Figs. 2 and 3). In contrast, the proliferative effect of 13(R)-HODE was not inhibited by GW 9662 and, interestingly, was partially reverted by 13(S)-HODE (Fig. 2). On the other hand, the apoptotic effect of 13(S)-HODE (Fig. 3) decreased in the presence of 13(R)-HODE, thus indicating that one HODE enantiomer reduced the effect of the other one.

Leukotriene B_4 (LTB_4) binds to BLT_1 and BLT_2 receptors. BLT_2 is a low-affinity receptor that was also recognized by HETEs (22, 26). Given the similar structure of LTB_4, HETEs, and HODEs, we tested the role of BLTs in 13(R)-HODE acting on Caco-2 cell growth. We found that the presence of BLT_1 or BLT_2 antagonists (U-75302 or LY 255283) (50) reverted the effect of 13(R)-HODE on Caco-2 proliferation and DNA synthesis (Fig. 4). Recently, we reported that the proliferative effect of HETEs is due to COX pathway activation and PGE_2 synthesis (8). In this sense, the proliferative effect of 13(R)-HODE was completely reverted by a COX inhibitor such as ketoprofen (40), as well as by an EP_4 antagonist (AH 23838) (33) (Fig. 4). The presence of an EP_1 antagonist (SC 19220) (16) also reduced both cell growth and DNA synthesis induced by 13(R)-HODE, although this effect was not statistically significant. In contrast, the antagonism of cysteinyl leukotriene receptor (LY171883) did not modify cell growth induced by 13(R)-HODE. Moreover, 13(R)-HODE (0.1–10 μM) induced PGE_2 synthesis in Caco-2 cell cultures (Fig. 5). This synthesis was abolished by ketoprofen and, interestingly, was reduced by BLT_1 and BLT_2 antagonists.

Fatty acids and fatty acid metabolites are ligands to PPARγ. Here we observed that 13(S)-HODE bound to PPARγ in a form similar to rosiglitazone, whereas 13(R)-HODE did not (Fig. 6).

To investigate which cell signaling pathways were involved in the proliferative effect of 13(R)-HODE, we determined the phosphorylation of AKT1, AKT2, ERK1/2, p38α, CREB, and GSKβ, as well as the dephosphorylation of β-catenin, in the absence of FBS and after a short incubation period (5 and 15 min) with the metabolite studied. Our results showed that 13(R)-HODE increased the phosphorylation of ERK1/2 and CREB. Moreover, CREB phosphorylation induced by 13(R)-HODE is inhibited by PD98059, an ERK inhibitor (Fig. 7).

DISCUSSION

The aim of this study was to investigate the differential role of 13-HODE enantiomers on nondifferentiated Caco-2 cell growth. Our results indicate that 13(S)-HODE decreases cell growth and DNA synthesis, in addition to having an apoptotic effect, thus confirming the antiproliferative role of this metabolite in colonic cells. In this sense, Kamitani et al. (24) and Yoshinaga et al. (51) indicated that the induction of differen-

Table 1. *Effect of 9(S)- and 9(R)-HODE (1 μM) on cell growth, DNA synthesis, and apoptosis*

	−FBS			+FBS	
		9(S)-HODE	9(R)-HODE		9(S)-HODE
Cells × 10^3/cm^2	40.0 ± 2.1	35.3 ± 2.9	56.1 ± 7.4*	80.0 ± 6.1	48.2 ± 2.8†
[^{3}H]thymidine incorporation, dpm	2,525 ± 108	2,031 ± 76	3,600 ± 195*	7,153 ± 402	3,337 ± 203†
% Apoptotic cells	n.d.	n.d.	n.d.	2.6 ± 0.9	9.0 ± 0.7†

Cells were incubated for 48 h in the absence or presence of growth factors [fetal bovine serum (FBS)] and/or 9-hydroxyoctadecadienoic acid (9-HODE) enantiomers (1 μM). Cells were then counted, and [^{3}H]thymidine uptake and apoptosis were measured in Caco-2 cells. Results are expressed as means ± SE of 5 determinations performed in triplicate. *$P < 0.05$ vs. Caco-2 cells cultured in absence of FBS; †$P < 0.05$ vs. Caco-2 cells cultured in presence of FBS. n.d., Not determined.

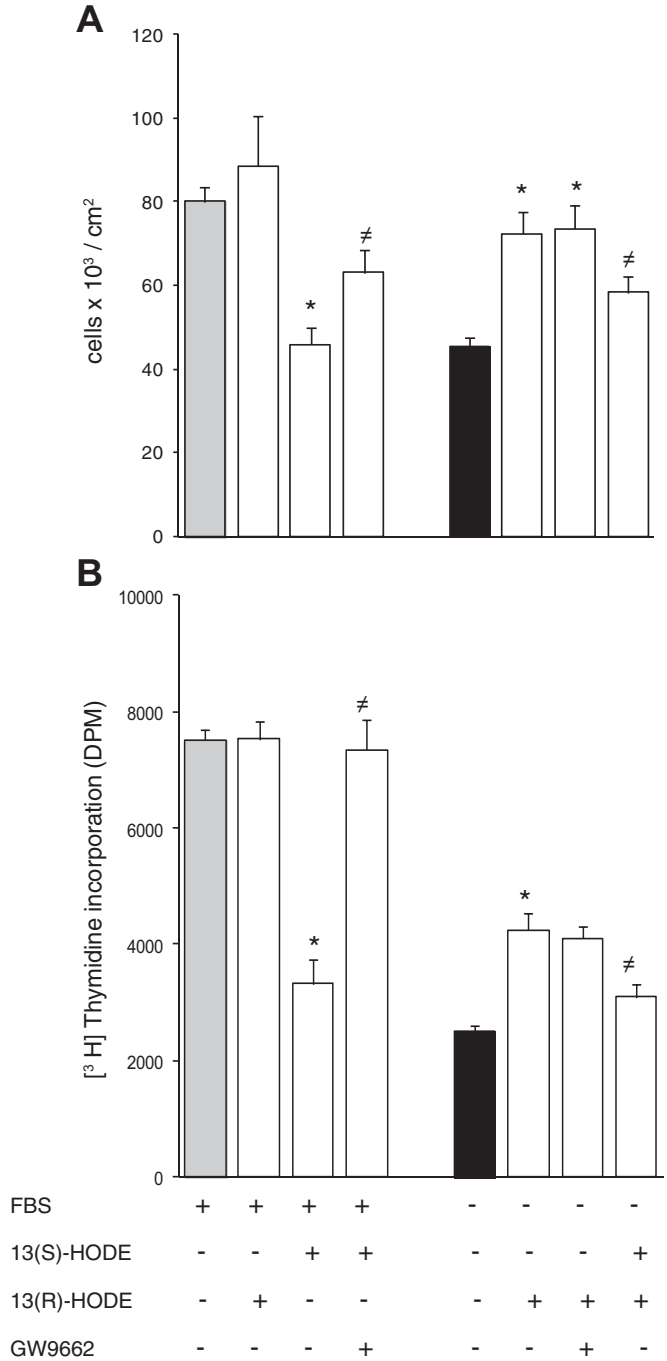


Fig. 2. Role of peroxisome proliferator-activated receptor-γ (PPARγ) in cell growth and DNA synthesis induced by 13(S)-HODE and 13(R)-HODE in Caco-2 cultures. Cells were incubated for 48 h with 13(S)-HODE (1 μM) + PPARγ antagonist GW 9662 (20 μM) in the presence of growth factors (FBS) or with 13(R)-HODE (1 μM) + GW 9662 (20 μM) in the absence of FBS. Cells were also incubated with 13(R)-HODE (1 μM) + 13(S)-HODE (1 μM) in the presence and absence of growth factors (FBS). Cells were then counted (*A*), and [³H] thymidine incorporation was measured (*B*). Results are expressed as means ± SE of 3–5 determinations performed in triplicate. $^{*}P < 0.05$ vs. 10% FBS or vs. absence of FBS in Caco-2 cell cultures; $^{\neq}P < 0.05$ vs. 13(S)-HODE or 13(R)-HODE.

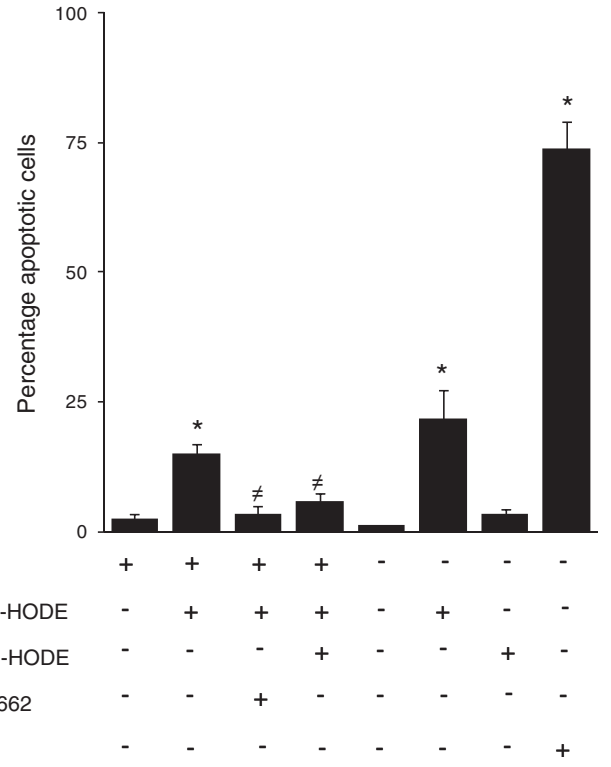


Fig. 3. Role of PPARγ in apoptosis induced by 13(S)-HODE in Caco-2 cultures. Cells were incubated for 48 h with 13(S)-HODE (1 μM) in the absence of growth factors (FBS) and in the presence of FBS + 13(R)-HODE (1 μM) or PPARγ antagonist GW 9662 (20 μM). Cells were also incubated with 13(R)-HODE (1 μM) in the absence of FBS. Staurosporine (STP, 1 μM) was used as positive control. Finally, apoptosis was measured, and the results are expressed as means ± SE of 3–5 determinations performed in triplicate. $^{*}P < 0.05$ vs. 10% FBS or vs. absence of FBS in Caco-2 cell cultures; $^{\neq}P < 0.05$ vs. 13(S)-HODE.

tiation in Caco-2 cells is associated with linoleic conversion to 13(S)-HODE. Since 1999, Shureiqi and collaborators have been working with different colonic cells and with patients and have concluded that 13(S)-HODE inhibits cell proliferation (23, 44, 52).

PPARγ is one of the three PPARs that make up a subfamily of the nuclear hormone receptor superfamily. 13(S)-HODE has been described as an agonist for PPARγ in colorectal cell lines (10, 42). The activation of PPARγ, a transcription factor that is expressed in epithelial cells and has been shown to inhibit cell proliferation, induces differentiation and promotes cell cycle arrest and apoptosis in colon cancer cell lines (13, 19, 30, 49). Accordingly, our results indicate that 13(S)-HODE binds to PPARγ and that its antiproliferative effect is reverted in the presence of a PPARγ inhibitor, which reinforces the data that the binding of 13(S)-HODE to PPARγ possesses anti-tumor-promoting properties.

Although there are few studies of 13(R)-HODE biological effects, Shibata et al. (43) indicate that 13(R)-HODE is a PPARγ agonist on the atherosclerotic plaques of human carotid arteries, thus suggesting an antiproliferative role for this metabolite. In contrast, the results given here regarding Caco-2 cell growth and DNA synthesis after incubation with 13(R)-HODE suggest that this metabolite has a significant proliferative effect in the absence of any other growth factors and show,

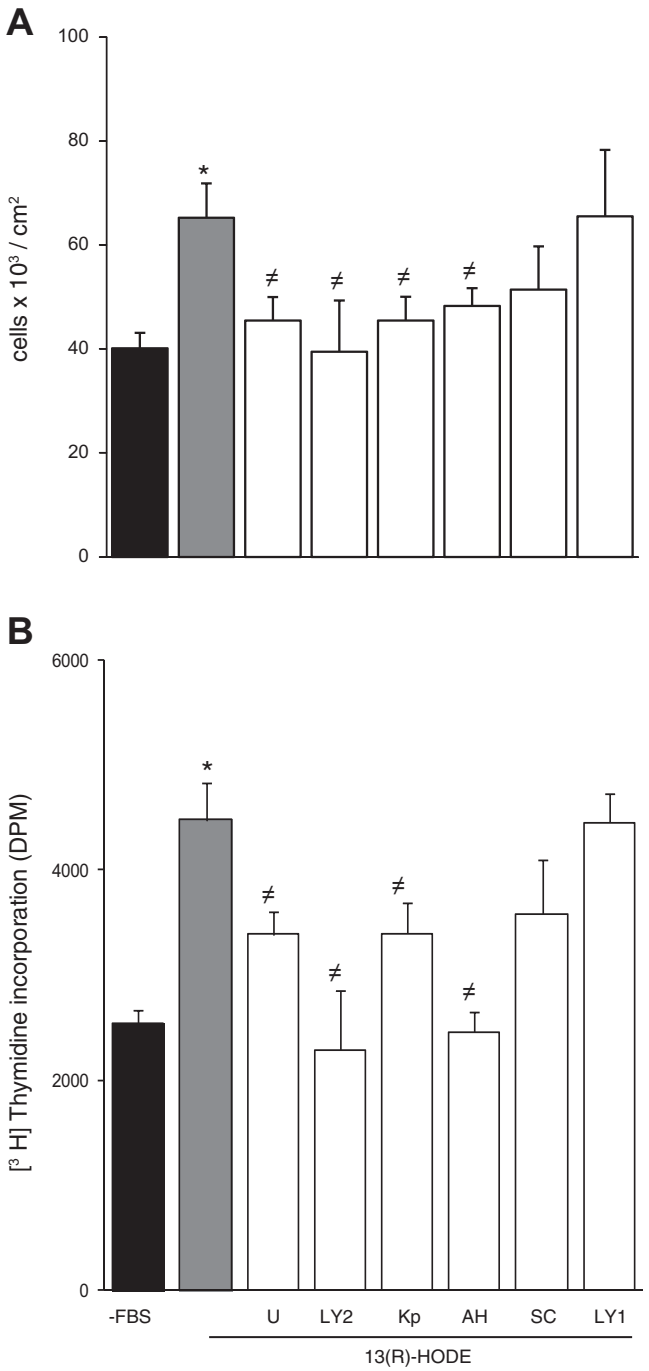


Fig. 4. Effect of BLT antagonists, EP antagonists, and COX inhibitor on cell growth and DNA synthesis induced by 13(R)-HODE. Caco-2 cells were incubated for 48 h with 13(R)-HODE (1 μM) + U-75302 (U, 5 μM), LY 255283 (LY2, 25 μM), ketoprofen (Kp, 5 μM), AH 23838 (AH, 20 nM), SC 19220 (SC, 60 nM) and LY171883 (LY1, 25 μM) in the absence of growth factors (FBS). Cells were then counted (*A*), and [^{3}H]thymidine uptake was measured (*B*). Results are expressed as means ± SE of 5 determinations performed in triplicate. $^*P < 0.05$ vs. Caco-2 cell cultures in the absence of growth factors; $^{\neq}P < 0.05$ vs. cells incubated with 13(R)-HODE.

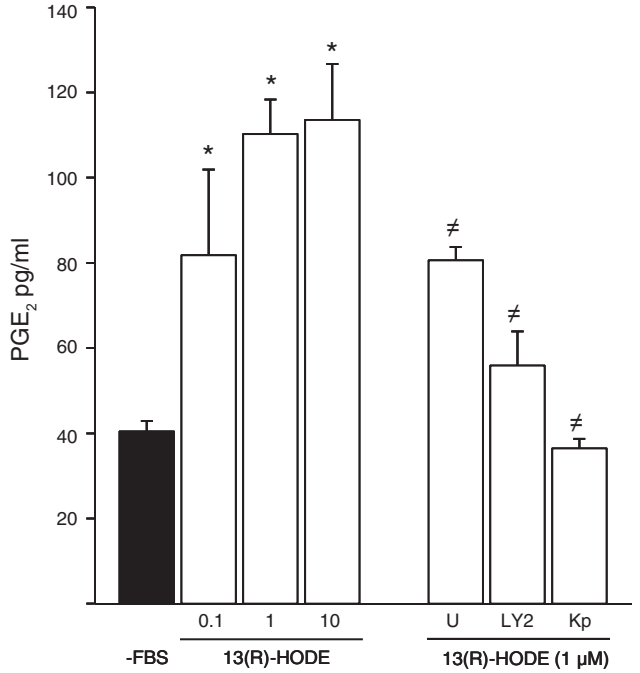


Fig. 5. Effect of 13(R)-HODE on PGE_2 synthesis. Caco-2 cells were incubated with 13(R)-HODE (0.1–10 μM) for 60 min, and PGE_2 synthesis was determined. PGE_2 synthesis induced by 13(R)-HODE (1 μM) was studied in presence of U-75302 (U, 5 μM), LY 255283 (LY2, 25 μM), or ketoprofen (Kp, 5 μM). Results are expressed as means ± SE of 5 determinations performed in triplicate. $^*P < 0.05$ vs. Caco-2 cell cultures in the absence of growth factors (FBS); $^{\neq}P < 0.05$ vs. cells incubated with 13(R)-HODE (1 μM).

accordingly, that 13(R)-HODE effects are PPARγ independent. Moreover, the proliferative effect of 13(R)-HODE is confirmed by the activation of certain signaling pathways after a short incubation of this metabolite. In particular, 13(R)-HODE activates the ERK and CREB pathways. The Ras/MAPK/ERK signaling pathway is known to be important for growth in many cell types (11). Activation of ERK occurs through phosphorylation of threonine and tyrosine residues. There are several recent studies that indicate the importance of the Ras/ERK pathway in colorectal cancer; Ras mutations are

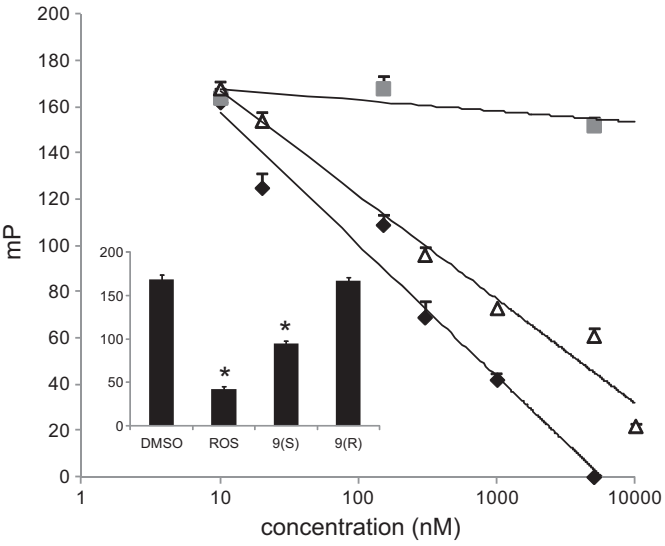


Fig. 6. PPARγ ligand assay for HODEs. The binding of 13(S)-HODE (△) and 13(R)-HODE (□) as well as 9(S)-HODE and 9(R)-HODE [9(S) and 9(R), respectively] to PPARγ was studied with the fluorescence polarization-based assay described in MATERIALS AND METHODS. Results are expressed as means ± SE of 2 determinations performed in triplicate. ROS, rosiglitazone (◆). $^*P <$ 0.05 vs. DMSO.

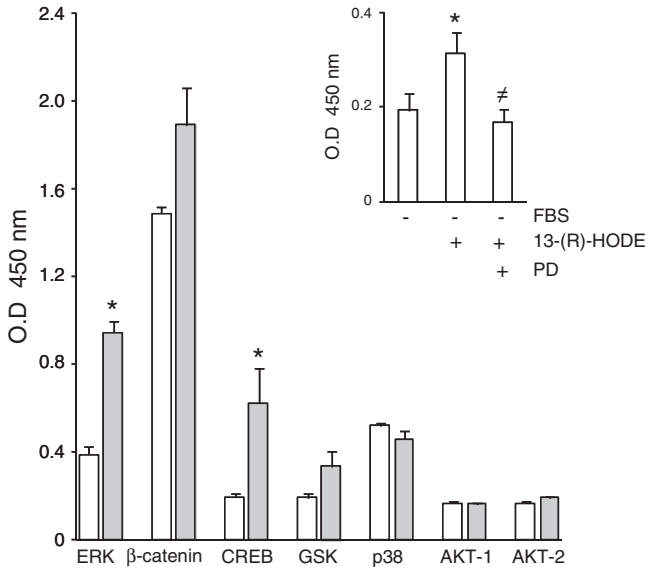


Fig. 7. Cell signaling activation by 13(R)-HODE on Caco-2 cells. Cells were incubated with 13(R)-HODE (1 μM) for 5 or 15 min and collected, and phosphorylated AKT1, AKT2, ERK1/2, p38α, CREB, GSKβ, and dephosphorylated β-catenin were measured. PD98059 (PD, 10 μM) was used to inhibit ERK1/2. Results are expressed as means ± SE of 4 determinations performed in triplicate. $*P < 0.05$ vs. Caco-2 cell cultures in the absence of growth factors (FBS). $\neq P < 0.05$ vs. cell cultures in presence of 13(R)-HODE.

found in 45% of colon carcinomas (47), and inhibition of the Ras/ERK pathway also promotes ABCB1 protein degradation to diminish cellular multidrug resistance in human colorectal cancer cell lines (25). On the other hand, the activation of CREB also has a significant impact on cell growth, proliferation, and survival (39). Thus CREB phosphorylation at its critical Ser-133 residue by ERK has been reported to increase its DNA binding activity (31). In this report, we demonstrate that 13(R)-HODE activation of ERK leads to CREB phosphorylation at Ser-133. In line with this, Sauer et al. (41) indicated that 13-HODE activates the ERK pathway in colorectal tumors, although they did not refer to 13(S)-HODE or 13(R)-HODE. Therefore, our results suggest that the stimulation of cell proliferation by 13(R)-HODE may be mediated, at least in part, by the activation of the ERK and CREB pathways.

Kim et al. (26) reported that HETEs could bind to BLT_1 and BLT_2, LTB_4 receptors. Given the similar structure pattern of LTB_4, HETEs, and HODEs, we also investigated the role of BLTs in Caco-2 growth induced by 13(R)-HODE. The results obtained indicate that BLT_1 and BLT_2 are involved in the proliferative effect induced by 13(R)-HODE. In addition, 13(R)-HODE activates the synthesis of PGE_2, and this effect is reversed by COX inhibitors and, interestingly, by BLT_1 and BLT_2 antagonists. We observed that the proliferative effect of 13(R)-HODE is also inhibited by a COX inhibitor and by EP_1 and EP_4 antagonists. PGE_2, known to be high in malignant tissue, is the predominant prostaglandin found in tumors in both animal and human models of colorectal cancer (10, 48). Moreover, we demonstrated that PGE_2 synthesized by COX pathway is involved in the control of Caco-2 cell growth (27). A recent study in our laboratory (8) concluded that PGE_2 induces Caco-2 cell growth and that PGE_2 production by LTB_4 and 12-HETE is also involved in the proliferation of undifferentiated Caco-2 cells. Taken together, our results suggest that the proliferative effect of 13(R)-HODE could be due, at least in part, to COX pathway activation and, subsequently, to PGE_2 synthesis.

Fewer studies on the role of 9(S)-HODE in cell proliferation were found. Hattori et al. (20) concluded that this metabolite inhibits keratinocyte proliferation. In line with this, our results also suggest an antiproliferative effect of 9(S)-HODE in nondifferentiated Caco-2 cells, whereas 9(R)-HODE presents the opposite action.

There is no information about the HODE enantiomer synthesis by cell cultures or HODE enantiomer tissue concentrations, but we observed that 13-HODE reaches a concentration around 10 nM (2.96 ± 0.22 ng/ml with FBS and 0.96 ± 0.02 without FBS) measured by LC-MS/MS technology (28) in Caco-2 cell culture. Shureiqi et al. (46) reported 13-HODE levels around 40 ng/μg protein in colorectal mucosa, which can be equivalent to ~0.1–1 μM, the HODE concentrations used in our experimental conditions. A future chiral separation/quantification of HODE enantiomers will be necessary to study HODE enantiomer synthesis by intestinal epithelial cells and normal and colorectal cancer tissues.

In conclusion, we provide new evidence that 13-HODE enantiomers have contrary effects on intestinal epithelial growth (Fig. 8). Our results show that the proliferative effect of 13(R)-HODE is PGE_2 synthesis dependent. These findings add an additional feature to the cross talk between the COX and LOX pathways, not only because of the activation by themselves of different cell signaling pathways, but also because of COX pathway activation. In fact, many studies show that in colorectal cancer the expression of 15-LOX-1 [which synthesizes 13 (S)-HODE] decreases while the expression of COX-2 [which can produce 13 (R)-HODE] increases (20, 28, 45, 48). Thus variations in the activity of these pathways could change the balance between the two enantiomers. In colorectal cancer, this balance may be favorable to 13(R)-HODE, which could be activated PGE_2 synthesis, thus causing proliferation. Meanwhile, in differentiated epithelium the balance is pro-13(S)-

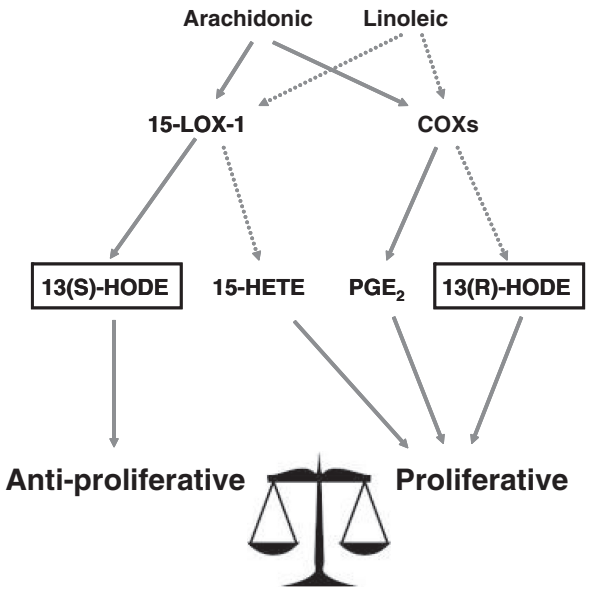


Fig. 8. Schematic illustration of the effect of 13-HODE enantiomers on cell proliferation. Linoleic acid metabolism by 15-lipoxygenase-1 (15-LOX-1) or cyclooxygenase (COX) induces 13(S)- and 13(R)-HODE synthesis, respectively, thus leading to differential behavior in intestinal epithelial cell growth.

HODE, by reducing cell growth and activating apoptotic processes. Moreover, in this study, the effect of one enantiomer could be reverted by the other, thus reinforcing the importance of the balance between these different pathways in cell growth homeostasis in the intestinal epithelium. Further investigations should be conducted to analyze the presence of both enantiomers along the crypt-villus axis as well as in colorectal cancer pathogenesis.

ACKNOWLEDGMENTS

The authors thank Jaume Comas from the Centres Científics i Tecnològics of the Universitat de Barcelona for valuable help with apoptosis analyses and Dra. M. A. Busquests for valuable help with fluorescence polarized assay. The Language Services of the Universitat de Barcelona are also gratefully acknowledged.

GRANTS

This research was supported by the Spanish Ministry of Science and Innovation (BFU2007-61727/BFI) and by the Generalitat (Autonomous Government) of Catalonia (2009SGR0438).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

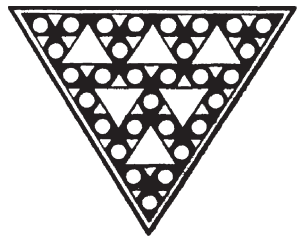
AUTHOR CONTRIBUTIONS

Author contributions: M.C. performed experiments; M.C., R.M.-V., and J.J.M. analyzed data; M.C., R.M.-V., and J.J.M. interpreted results of experiments; M.C. prepared figures; M.C., R.M.-V., and J.J.M. edited and revised manuscript; M.C., R.M.-V., and J.J.M. approved final version of manuscript; R.M.-V. and J.J.M. conception and design of research; R.M.-V. and J.J.M. drafted manuscript.

REFERENCES

1. **Aggarwal BB, Shishodia S, Sandur SK, Pandey MK, Sethi G.** Inflammation and cancer: how hot is the link? *Biochem Pharmacol* 72: 1605–1621, 2006.
2. **Allred CD, Kilgore MW.** Selective activation of PPARgamma in breast, colon, and lung cancer cell lines. *Mol Cell Endocrinol* 235: 21–29, 2005.
3. **Baer AN, Costello PB, Green FA.** Stereospecificity of the hydroxyeicosatetraenoic and hydroxyoctadecadienoic acids produced by cultured bovine endothelial cells. *Biochim Biophys Acta* 1085: 45–52, 1991.
4. **Baer AN, Costello PB, Green FA.** Stereospecificity of the products of the fatty acid oxygenases derived from psoriatic scales. *J Lipid Res* 32: 341–347, 1991.
5. **Bayer M, Mosandl A, Thaçi D.** Improved enantioselective analysis of polyunsaturated hydroxy fatty acids in psoriatic skin scales using high-performance liquid chromatography. *J Chromatogr B Analyt Technol Biomed Life Sci* 819: 323–328, 2005.
6. **Brash AR.** Lipoxygenases: occurrence, functions, catalysis, and acquisition. *J Biol Chem* 274: 23679–23682, 1999.
7. **Bull AW, Steffensen KR, Leers J, Rafter JJ.** Activation of PPAR gamma in colon tumor cell lines by oxidized metabolites of linoleic acid, endogenous ligands for PPAR gamma. *Carcinogenesis* 24: 1717–1722, 2003.
8. **Cabral M, Martín-Venegas R, Moreno JJ.** Role of arachidonic acid metabolites on the control of non-differentiated intestinal epithelial cell growth. *Int J Biochem Cell Biol* 45: 1620–1628, 2013.
9. **Cathcart MC, Lysaght J, Pidgeon GP.** Eicosanoid signalling pathways in the development and progression of colorectal cancer: novel approaches for prevention/intervention. *Cancer Metastasis Rev* 30: 363–385, 2011.
10. **Chell S, Kaidi A, Williams AC, Paraskeva C.** Mediators of PGE_2 synthesis and signalling downstream of COX-2 represent potential targets for the prevention/treatment of colorectal cancer. *Biochim Biophys Acta* 1766: 104–119, 2006.
11. **Cherukuri DP, Chen XB, Goulet AC, Young RN, Han Y, Heimark RL, Regan JW, Meuillet E, Nelson MA.** The EP4 receptor antagonist, L-161,982, blocks prostaglandin E_2-induced signal transduction and cell proliferation in HCA-7 colon cancer cells. *Exp Cell Res* 313: 2969–2979, 2007.
12. **Çimen I, Tunçay S, Banerjee S.** 15-Lipoxygenase-1 expression suppresses the invasive properties of colorectal carcinoma cell lines HCT-116 and HT-29. *Cancer Sci* 100: 2283–2291, 2009.
13. **Çimen I, Astarci E, Banerjee S.** 15-Lipoxygenase-1 exerts its tumor suppressive role by inhibiting nuclear factor-kappaB via activation of PPARgamma. *J Cell Biochem* 112: 2490–2501, 2011.
14. **Dauchy RT, Dauchy EM, Mao L, Belancio VP, Hill SM, Blask DE.** A new apparatus and surgical technique for the dual perfusion of human tumor xenografts in situ in nude rats. *Comp Med* 62: 99–108, 2012.
15. **Ferrer R, Moreno JJ.** Role of eicosanoids on intestinal epithelial homeostasis. *Biochem Pharmacol* 80: 431–438, 2010.
16. **Funk CD, Furci L, Fitzgerald GA, Grygorczk R, Rochette C, Bayne MA, Abramovitz M, Adam M, Metters KM.** Cloning and expression of a cDNA for the human prostaglandin E receptor EP1 subtype. *J Biol Chem* 268: 26767–26772, 1993.
17. **Funk CD.** Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* 29: 1871–1875, 2001.
18. **Greene ER, Huang S, Serhan CN, Panigrahy D.** Regulation of inflammation in cancer by eicosanoids. *Prostaglandins Other Lipid Mediat* 96: 27–36, 2011.
19. **Gupta RA, Brockman JA, Sarraf P, Willson TM, DuBois RN.** Target genes of peroxisome proliferator-activated receptor gamma in colorectal cancer cells. *J Biol Chem* 276: 29681–29687, 2001.
20. **Hattori T, Obinata H, Ogawa A, Kishi M, Tatei K, Ishikawa O, Izumi T.** G2A plays proinflammatory roles in human keratinocytes under oxidative stress as a receptor for 9-hydroxyoctadecadienoic acid. *J Invest Dermatol* 128: 1123–1133, 2008.
21. **Hsi LC, Wilson LC, Eling TE.** Opposing effects of 15-lipoxygenase-1 and -2 metabolites on MAPK signaling in prostate. Alteration in peroxisome proliferator-activated receptor gamma. *J Biol Chem* 277: 40549–40556, 2002.
22. **Iizuka Y, Okuno T, Saeki K, Uozaki H, Okada S, Misaka T, Sato T, Toh H, Fukayama M, Takeda N, Kita Y, Shimizu T, Nakamura M, Yokomizo T.** Protective role of the leukotriene B4 receptor BLT2 in murine inflammatory colitis. *FASEB J* 24: 4678–4690, 2010.
23. **Il Lee S, Zuo X, Shureiqi I.** 15-Lipoxygenase-1 as a tumor suppressor gene in colon cancer: is the verdict in? *Cancer Metastasis Rev* 30: 481–491, 2011.
24. **Kamitani H, Geller M, Eling T.** Expression of 15-lipoxygenase by human colorectal carcinoma Caco-2 cells during apoptosis and cell differentiation. *J Biol Chem* 273: 21569–21577, 1998.
25. **Katayama K, Yoshioka S, Tsukahara S, Mitsuhashi J, Sugimoto Y.** Inhibition of the mitogen-activated protein kinase pathway results in the down-regulation of P-glycoprotein. *Mol Cancer Ther* 6: 2092–2102, 2007.
26. **Kim HJ, Kim DK, Kim H, Koh JY, Kim KM, Noh MS, Lee S, Kim S, Park SH, Kim JJ, Kim SY, Lee CH.** Involvement of the BLT2 receptor in the itch-associated scratching induced by 12-(S)-lipoxygenase products in ICR mice. *Br J Pharmacol* 154: 1073–1078, 2008.
27. **Martín-Venegas R, Roig-Pérez S, Ferrer R, Moreno JJ.** Arachidonic acid cascade and epithelial barrier function during Caco-2 cell differentiation. *J Lipid Res* 47: 1416–1423, 2006.
28. **Martín-Venegas R, Casillas R, Jauregui O, Moreno JJ.** Rapid simultaneous analysis of cyclooxygenase, lipoxygenase and cytochrome P-450 metabolites of arachidonic and linoleic acids using high performance liquid chromatography/mass spectrometry in tandem mode. *J Pharm Biomed Anal* 56: 976–982, 2011.
29. **McDonnell M, Davis W Jr, Li H, Funk CD.** Characterization of the murine epidermal 12/15-lipoxygenase. *Prostaglandins Other Lipid Mediat* 63: 93–107, 2001.
30. **Michalik L, Desvergne B, Wahli W.** Peroxisome-proliferator-activated receptors and cancers: complex stories. *Nat Rev Cancer* 4: 61–70, 2004.
31. **Morel AP, Lièvre M, Thomas C, Hinkal G, Ansieau S, Puisieux A.** Generation of breast cancer stem cells through epithelial-mesenchymal transition. *PLoS One* 3: e2888, 2008.
32. **Moreno JJ.** New aspects of the role of hydroxyeicosatetraenoic acids in cell growth and cancer development. *Biochem Pharmacol* 77: 1–10, 2009.
33. **Nishigaki N, Negishi M, Honda A, Sugimoto Y, Namba T, Narumiya S, Ichikawa A.** Identification of prostaglandin E receptor "EP2" cloned from mastocytoma cells as EP4 subtype. *FEBS Lett* 364: 339–341, 1995.
34. **Nixon JB, Kamitani H, Baek SJ, Eling TE.** Evaluation of eicosanoids and NSAIDs as PPARgamma ligands in colorectal carcinoma cells. *Prostaglandins Leukot Essent Fatty Acids* 68: 323–330, 2003.

35. **Nixon JB, Kim KS, Lamb PW, Bottone FG, Eling TE.** 15-Lipoxygenase-1 has anti-tumorigenic effects in colorectal cancer. *Prostaglandins Leukot Essent Fatty Acids* 70: 7–15, 2004.
36. **Oliw EH, Brodowsky ID, Hörnsten L, Hamberg M.** Bis-allylic hydroxylation of polyunsaturated fatty acids by hepatic monooxygenases and its relation to the enzymatic and nonenzymatic formation of conjugated hydroxyl fatty acids. *Arch Biochem Biophys* 300: 434–439, 1993.
37. **Parks DR, Bryan VM, Oi VT, Herzenberg LA.** Antigen-specific identification cloning of hybridomas with a fluorescence-activated cell sorter. *Proc Natl Acad Sci USA* 76: 1962–1969, 1979.
38. **Pidgeon GP, Lysaght J, Krishnamoorthy S, Reynolds JV, O'Byrne K, Nie D, Honn KV.** Lipoxygenase metabolism: roles in tumor progression and survival. *Cancer Metastasis Rev* 26: 503–527, 2007.
39. **Sabnis AJ, Cheung LS, Dail M, Kang HC, Santaguida M, Hermiston ML, Passegué E, Shannon K, Braun BS.** Oncogenic Kras initiates leukemia in hematopoietic stem cells. *PLoS Biol* 7: e59, 2009.
40. **Sanchez T, Moreno JJ.** Ketoprofen S(+) enantiomer inhibits prostaglandin production and cell growth in 3T6 fibroblast cultures. *Eur J Pharmacol* 370: 63–67, 1999.
41. **Sauer LA, Dauchy RT, Blask DE, Armstrong BJ, Scalici S.** 13-Hydroxyoctadecadienoic acid is the mitogenic signal for linoleic acid-dependent growth in rat hepatoma 7288CTC in vivo. *Cancer Res* 59: 4688–4692, 1999.
42. **Schneider C, Pozzi A.** Cyclooxygenases and lipoxygenases in cancer. *Cancer Metastasis Rev* 30: 277–294, 2011.
43. **Shibata N, Toi S, Shibata T, Uchida K, Itabe H, Sawada T, Kawamata T, Okada Y, Uchiyama S, Kobayashi M.** Immunohistochemical detection of 13(R)hydroxyoctadecadienoic acid in atherosclerotic plaques of human carotid arteries using a novel specific antibody. *Acta Histochem Cytochem* 42: 197–203, 2009.
44. **Shureiqi I, Lippman SM.** Lipoxygenase modulation to reverse carcinogenesis. *Cancer Res* 61: 6307–6312, 2001.
45. **Shureiqi I, Wu Y, Chen D, Yang XL, Guan B, Morris JS, Yang P, Newman RA, Broaddus R, Hamilton SR, Lynch P, Levin B, Fischer SM, Lippman SM.** The critical role of 15-lipoxygenase-1 in colorectal epithelial cell terminal differentiation and tumorigenesis. *Cancer Res* 65: 11486–11492, 2005.
46. **Shureiqi I, Chen D, Day RS, Zuo X, Hochman FL, Ross WA, Cole RA, Moy O, Morris JS, Xiao L, Newman RA, Yang P, Lippman SM.** Profiling lipoxygenase metabolism in specific steps of colorectal tumorigenesis. *Cancer Prev Res* 3: 829–838, 2010.
47. **Tabu K, Kimura T, Sasai K, Wang L, Bizen N, Nishihara H, Taga T, Tanaka S.** Analysis of an alternative human CD133 promoter reveals the implication of Ras/ERK pathway in tumor stem-like hallmarks. *Mol Cancer* 19: 9–39, 2010.
48. **Wang D, Dubois RN.** Prostaglandins and cancer. *Gut* 55: 115–122, 2004.
49. **Yang WL, Frucht H.** Activation of the PPAR pathway induces apoptosis and COX-2 inhibition in HT-29 human colon cancer cells. *Carcinogenesis* 22: 1379–1383, 2001.
50. **Yokomizo T, Kato K, Terawaki K, Izumi T, Shimizu T.** A second leukotriene B4 receptor, BLT2: a new therapeutic target in inflammation and immunological diseases. *J Exp Med* 192: 421–431, 2000.
51. **Yoshinaga M, Murao H, Kitamura Y, Koga K, Tsuruta S, Igarashi H, Nakamura K, Takayanagi R.** The 15-lipoxygenase-1 expression may enhance the sensitivity to non-steroidal anti-inflammatory drug-induced apoptosis in colorectal cancers from patients who are treated with the compounds. *J Gastroenterol Hepatol* 22: 2324–2329, 2007.
52. **Zuo X, Shureiqi I.** Eicosanoid profiling in colon cancer: emergence of a pattern. *Prostaglandins Other Lipid Mediat* 104–105: 139–143, 2013.



Artículo 3

Marisol Cabral, Raquel Martín-Venegas, Juan J. Moreno.

Leukoytriene D_4-induced Caco-2 cell proliferation is mediated by prostaglandin E_2 synthesis

Physiological Reports (2015)

Revista *oppen access*, de reciente creación por la *American Physiological Society*, por lo que no dispone todavía de índice de impacto

Los resultados de esta publicación se han presentado en el siguiente congreso:

- LTD4 is involved in the control of non-diferentiated intestinal epithelial cell growth.
  Martin-Venegas R, Cabral M, Moreno JJ.
  6th International Immunonutrition Workshop. Palma de Mallorca (España). 15-17 Octubre 2012.
  Esta comunicación recibió el premio "Francisco Roses" como mejor poster del congreso.
  Resumen publicado en la revista Proceedings of the Nutrition Society, 72:52 (2012).

Resumen artículo 3:

Objetivo Estudiar el papel del LTD_4 sobre el crecimiento celular en las células epiteliales intestinales no diferenciadas procedentes de un adenocarcinoma.

Material y Métodos. Este estudio se ha realizado utilizando células Caco-2 no diferenciadas. Las células se incubaron con LTD_4 en ausencia y presencia de inhibidores de la vía COX y antagonistas receptoriales de eicosanoides. Se determino: el crecimiento celular por contaje microscópico usando bromuro de etidio/naranja de acridina y la síntesis de ADN por incorporación de [^{3}H]thimidina; la síntesis de PGE_2 mediante un inmunoensayo enzimático (EIA); la liberación de AA fue determinada por la cantidad [^{3}H] AA radiactivo liberado; la expresión de la COX-2 se detectó por Western blot y la fosforilación de Akt1, Akt2, ERK1/2, GSK3, p38, CREB y la desfosforilación de β-catenina por ELISA.

Resultados. El LTD_4 induce el crecimiento celular y la síntesis de ADN en las células Caco-2. Este efecto disminuye significativamente en presencia de antagonistas específicos del Cis-LT_1R, así como de los receptores de la PGE_2, EP_1 y EP_4 y de inhibidores de las COXs. El LTD_4 induce la liberación de [^{3}H] AA, que fue reducida por un inhibidor inespecífico de las PLA_2 y por un inhibidor específico de las PLC. Además la liberación de [^{3}H] AA inducida por LTD_4 fue bloqueada por antagonistas del Cis-LT_1R y por un inhibidor de la PKC. Por otra parte, el LTD_4 induce la expresión de la COX-2 y la síntesis de PGE_2 y ambas son revertidas por antagonistas del Cis-LT_1R. Respecto a las vías de señalización, el LTD_4 activa la fosforilación de ERK, CREB y la desfosforilación de β-catenina. La fosforilación de ERK fue revertida por un inhibidor de las COXs, y por antagonistas de los receptores EP_1, EP_4 y Cis-LT_1R. La fosforilación de CREB también fue bloqueada por un inhibidor de las COXs.

Conclusión. El efecto proliferativo del LTD_4 es debido a su interacción con Cis-LT_1R que activa la COX-2 y por tanto originará un incremento de la síntesis de PGE_2, todo lo cual de lugar a la activación de diversas vías de señalización mitogénicas.

Physiological Reports

Open Access

Physiological Reports ISSN 2051-817X

ORIGINAL RESEARCH

Leukotriene D_4-induced Caco-2 cell proliferation is mediated by prostaglandin E_2 synthesis

Marisol Cabral, Raquel Martín-Venegas & Juan J. Moreno

Departament de Fisiologia, Facultat de Farmàcia, Universitat de Barcelona, Barcelona, Spain

Keywords
5-lipoxygenase, arachidonic acid cascade, cell cycle, cell growth, colon cancer.

Correspondence
Juan José Moreno, Departament de Fisiologia, Facultat de Farmàcia, Universitat de Barcelona, Avda. Joan XXIII s/n, 08028 Barcelona, Spain.
Tel: +34 93 402 4505
Fax: +34 93 403 5901
E-mail: jjmoreno@ub.edu

Funding Information
This research was supported by Spanish Ministry of Science and Innovation (BFU2007-61727/BFI) and by the *Generalitat* (Autonomous Government) of Catalonia (2009SGR0438). Universitat de Barcelona has supported open access publication charge.

Received: 11 March 2015; Revised: 5 May 2015; Accepted: 6 May 2015

doi: 10.14814/phy2.12417

Physiol Rep, 3 (7), 2015, e12417, doi: 10.14814/phy2.12417

Abstract

Leukotriene D_4 (LTD_4) is a pro-inflammatory mediator formed from arachidonic acid through the action of 5-lipoxygenase (5-LOX). Its biological effects are mediated by at least two G-coupled plasmatic cysteinyl LT receptors ($CysLT_{1\text{-}2}R$). It has been reported an upregulation of the 5-LOX pathway in tumor tissue unlike in normal colon mucosa. Colon tumors generally have an increased expression of $CysLT_1R$ and colon cancer patients with high expression levels of $CysLT_1R$ have poor prognosis. We previously observed that the cyclooxygenase pathway is involved in the control of intestinal epithelial cancer cell growth through PGE_2 production. The aim of this study was therefore to assess the effect of LTD_4 binding with $CysLT_1R$ on Caco-2 cell growth. We note a number of key findings from this research. We observed that at a concentration similar to that found under inflammatory conditions, LTD_4 was able to induce Caco-2 cell proliferation and DNA synthesis. Moreover, with the use of a specific receptor antagonist this study has demonstrated that the effect of LTD_4 is a result of its interaction with $CystLT_1R$. We also note the possible participation of the PLC-IP_3-Ca^{2+}/DAG-PKC signaling pathways in cytosolic PLA_2 and [^{3}H]AA release induced by LTD_4-$CystLT_1R$ interaction. Finally, we found that the resulting activation of the AA cascade and the production of PGE_2 eicosanoid could be related to the activation of cell signaling pathways such as ERK and CREB. These findings will help facilitate our understanding of how inflammatory mediators can affect the survival and dissemination of intestinal carcinoma cells.

Introduction

Leukotriene D_4 (LTD_4) is a powerful pro-inflammatory mediator, which is formed from arachidonic acid through the action of 5-lipoxygenase (5-LOX) (Samuelsson 1979). LTD_4 mediates its effects through specific cell surface receptors that belong to the G protein-coupled receptor family, cysteinyl leukotriene receptors (CysLTR). Two such receptors have previously been cloned: $CysLT_1R$ (Lynch et al. 1999) and $CysLT_2R$ (Heise et al. 2000). $CysLT_1R$ has the highest affinity of the two receptors (Heise et al. 2000). As such it has a higher affinity for LTD_4 than $CysLT_2R$ (Lynch et al. 1999).

LTD_4 is associated with the pathogenesis of several inflammatory disorders such as inflammatory bowel disease (IBD) (Stenton 1990). Not only is there a well-established connection between IBD and increased frequency of neoplastic transformation (Smalley and Dubois 1997), but a more general link between chronic inflammation and an increased risk of developing cancer has been suggested in previous studies (Coussens and Werb 2002). A cause-and-effect link has been established between chronic inflammation and colon cancer, which occurs via the activation and over-expression of the enzymes 5-LOX and cyclooxygenase-2 (COX-2). These enzymes are responsible for regulating the production of

LTs and prostaglandins (PGs), respectively (Coussens and Werb 2002; Qiao and Li 2014). Unlike non-transformed human epithelial cells, $CysLT_2R$ is downregulated in the colon cancer cell lines (Magnusson et al. 2007). In contrast, it has been demonstrated that $CysLT_1R$ is upregulated in colon cancer tissue and that the binding of LTD_4 to this receptor facilitates the survival of the cells in this tissue and negatively correlates with patient survival (Öhd et al. 2000, 2003). In accordance with this trend, Magnusson et al. (2007, 2010) recently observed that colon cancer patients with high expression levels of $CysLT_1R$ exhibited a poor prognosis. Moreover, as noted by Yudina et al. (2008), LTD_4 upregulates 5-LOX, COX-2, and $CysLT_1R$ levels in intestinal epithelial cells providing a mechanism for maintaining inflammation and tumor progression.

In our study, we observed that through $CysLT_1R$ binding, LTD_4 increases the release of arachidonic acid (AA) and the synthesis of PGE_2. In addition, we found that this prostaglandin is responsible for the proliferative effects induced by LTD_4 on intestinal epithelial Caco-2 cells.

Materials and Methods

Materials

LTD_4, PGE_2 and murine COX-2 were purchased from Cayman Chemical (Ann Arbor, MI). Non-essential amino acids, FBS, BSA, Fura-2 acetoxymethylester (Fura-2 AM), U73122, dantrolene, Gö 6983, ketoprofen, LY 171883, MK 571, NS 398, SC560 and SC19220, PD98059, ethidium bromide and acridine orange were purchased from Sigma Chemical (St. Louis, MO). LY 255283 was purchased from Tocris Biosc. (Bristol, UK). Arachidonyl trifluoromethylketone ($AACOCF_3$) and bromoenol lactone (BEL) were acquired from Alexis Corp. (San Diego, CA). [Methyl-^{3}H]thymidine (20 Ci/mmol) and [5,6,8,9,11,12,-14,15-^{3}H] arachidonic acid ([^{3}H]AA) (60-100 Ci/mmol) were from American Radiolabeled Chemicals Inc. (St. Louis, MO), and AH 23838 was kindly provided by Glaxo-Wellcome (Stevenage, UK).

Cell culture and cell growth assay

Caco-2 cells, derived from a colon adenocarcinoma, were provided by the American Type Culture Collection (HTB-37, Manassas, VA). The cells were routinely grown in 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 mmol/L L-glutamine, 10% (v/v) heat-inactivated FBS, 100 U/mL penicillin and 0.1 mg/mL streptomycin at 37°C in a modified atmosphere of 5% CO_2 in air, as previously described (Martín-Venegas et al. 2006). The growth medium was replaced twice per week and the day before the experiment. All the experiments were performed in pre-confluent cultures and consequently, in nondifferentiated cells. Caco-2 cell differentiation began when they reached the confluence and finished after 2 weeks postconfluence, following a previously described process (Martín-Venegas et al. 2006).

To perform the cell growth assay, cells were harvested with trypsin/EDTA and passed to 12 mm plastic clusters at a density of 10^4 cells/cm^2. After 4 days in culture, cells were incubated with treatments for a period of 48 h. Then, cell density was around 40 and 80 $\times$ 10^3 cells/cm^2 in absence or presence of FBS, respectively. Consequently all experiments were performed before reaching cell confluence. Cells were then washed, trypsinized, and counted with a microscope using ethidium bromide/acridine orange staining to view the number of viable cells (Parks et al. 1979).

Analysis of DNA synthesis

DNA synthesis was measured using a [^{3}H]thymidine incorporation assay. Caco-2 cell cultures were kept on 24-well plates in DMEM with 10% FBS at a density of 10^4 cells/cm^2. After 4 days in culture, the cells were incubated for 48 h with the treatments; [^{3}H]thymidine (0.1 μCi/well) was added for the last 24 h. The media containing [^{3}H]thymidine were then aspirated and cells were washed, overlaid with 1% Triton X-100 and scraped off the wells (Cabral et al. 2013). Finally, radioactivity present in the cell fraction was measured by scintillation counting, using a Packard Tri-Carb 1500 counter (Downers Grove, IL).

Prostaglandin E_2 (PGE_2) analysis by enzyme immunoassay

PGE_2 determination was performed using a competitive EIA kit (Cayman, Ann Arbor, MI) following the manufacturer's instructions. Briefly, following a previously described process cells were maintained in 12 mm plastic clusters at a density of 10^4 cells/well (Cabral et al. 2013). After 4 days in culture, Caco-2 cells were incubated for 60 min at 37°C with LTD_4 (10 nmol/L) in the absence or presence of $CysLT_1R$ antagonists or a COX inhibitor. Finally supernatants were harvested and PGE_2 was determined.

Incorporation and release of [^{3}H]AA

Cells were harvested with trypsin/EDTA and passed to 24-well plates at a density of 10^4 cells/cm^2. After 4 days, cells were FBS starvated during 24 h and then the medium was replaced by 0.5 mL DMEM containing 0.1% fatty acid free

BSA and 0.1 μCi [^{3}H]AA (1 nmol/L) for a period of 6 h. Cells were then washed three times with 0.5% BSA-containing medium to remove any unincorporated [^{3}H]AA. After the study period (2 h), the medium was removed to determine the amount of [^{3}H] radioactivity release. The amount of [^{3}H]AA released into the medium was expressed as a percentage of cell-incorporated [^{3}H]AA, which was determined in solubilized cells, as previously described (Martín-Venegas et al. 2006).

Western blot analysis

Cells were seeded in 60 mm plastic clusters (10^4 cells/cm^2) and after 4 days the cultures were washed twice with ice-cold PBS, scraped off into PBS containing 2 mmol/L sodium EDTA and pelleted. These pellets were sonicated in PBS containing 4 mmol/L sodium EDTA, 500 μg/mL aprotinin, 500 μg/mL leupeptin, 500 μg/mL PMSF, and 400 μg/mL diethyldithiocarbamic acid, then resuspended in a lysis buffer containing 200 mmol/L Tris-HCl, 200 mmol/L NaCl, 2% Igepal CA-630 and 200 μmol/L DTT. Finally, an immunoblot analysis for COX-2 was performed, as previously described (Martín-Venegas et al. 2006). For β-actin immunoblotting, the monoclonal actin antibody (1:500) was used (Santa Cruz, Dallas, TX).

Measurement of the cell signaling activated by eicosanoids

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) as previously described (Cabral et al. 2013). To measure the kinase activity with total cell lysates, Caco-2 cells were lysed using a denaturing cell lysis buffer containing 6 mol/L urea and protease (leupeptin 2 μg/mL, pepstatin 10 μmol/L, aprotinin 3 μg/mL) and phosphatase (NaF 5 mmol/L, $Na_4P_2O_7$ 2 mmol/L, Na_3VO_4 1 mmol/L) inhibitors. The resulting solutions containing 80–100 μg of proteins were then added to kinase ELISA plate and the assay was performed, following the manufacturer's instructions (Symansis, Auckland, New Zealand). Optical density was then measured at 450 nm using a TECAN absorbance reader (Tecan Austria Gmbh, Salzburg, Austria). This simultaneous assay for the activation of multiple kinases provides a qualitatively better alternative to western blotting. We studied the effect of eicosanoids on the phosphorylation of Akt1 (pS473), Akt2 (pS474), ERK1/2 (pT202/Y204; pT185/Y187), GSK3β (pS9), p38α (pT180/Y182) and CREB (pS133) on the dephosphorylation of β-catenin (DP S33/S37/S41). The phosphorylation of Akt, ERK and p38 was measured after 5 min incubation with LTD$_4$, whereas the phosphorylation of CREB, GSK and the dephosphorylation of β-catenin was assayed after 15 min.

Statistical analysis

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.

Results

Figure 1 shows that LTD$_4$ (1–100 nmol/L) increases the number of viable cells in Caco-2 cell cultures in comparison with the results obtained in the absence of any growth factor. We note that the effect induced by LTD$_4$ (10 nmol/L) was reduced in the presence of CysLT$_1$R antagonists (MK 571 and LY 171883), a COX inhibitor (ketoprofen), a specific COX-2 inhibitor (NS 398), and EP$_1$ or EP$_4$ antagonists (SC 19220 and AH 23848, respectively) (Fig. 1), whereas the effect of specific COX-1 inhibitor (SC 560) did not reach significance. The mentioned treatments did not cause cell detachment nor a decrease in cell viability at the concentrations tested, as confirmed by microscopic observation (data not shown).

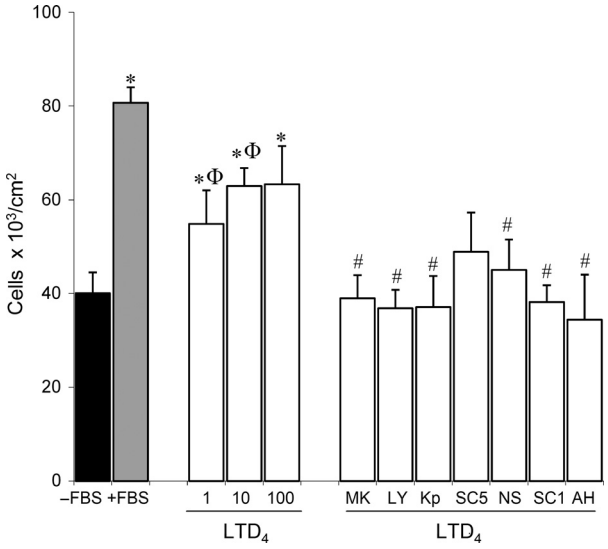


Figure. 1. The effect of Leukotriene D$_4$ (LTD$_4$) on Caco-2 cell growth. Cells were incubated for 48 h in the presence of LD$_4$ (1–100 nmol/L) and in the absence of growth factors (FBS); cells were then counted. Cell growth in control conditions (in the absence or presence of FBS) was included. LTD$_4$ (10 nmol/L) was incubated in the presence of MK 571 (MK5, 25 μmol/L), LY171883 (LY1, 25 μmol/L), ketoprofen (Kp, 5 μmol/L), SC 560 (SC5, 60 nmol/L), NS 398 (NS, 5 μmol/L), SC 19220 (SC1, 60 nmol/L) or AH 23838 (AH, 20 nmol/L). Results are expressed as mean ± SEM of 4-5 determinations performed in triplicate. *$P < 0.05$ versus Caco-2 cells cultured without FBS; $^{\Phi}P < 0.05$ versus Caco-2 cells cultured with FBS; $^{\#}P < 0.05$ versus Caco-2 cells cultured with LTD$_4$ (10 nmol/L).

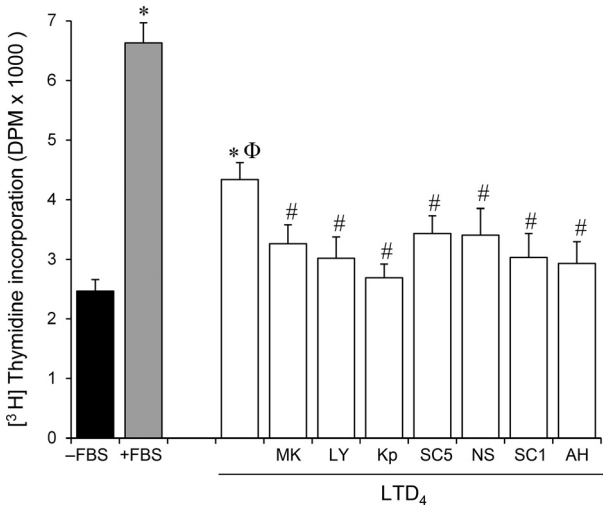


Figure 2. The effect of Leukotriene D_4 (LTD_4) on DNA synthesis. Caco-2 cells were incubated for 48 h in the presence of LTD_4 (10 nmol/L) and in the absence of growth factors (FBS); [^{3}H] thymidine incorporation into cells was then measured. DNA synthesis in control conditions (in the absence or presence of FBS) was included. LTD_4 (10 nmol/L) was incubated in the presence of MK 571 (MK5, 25 μmol/L), LY171883 (LY1, 25 μmol/L), ketoprofen (Kp, 5 μmol/L), SC 560 (SC5, 60 nmol/L), NS 398 (NS, 5 μmol/L), SC 19220 (SC1, 60 nmol/L) or AH 23838 (AH, 20 nmol/L). Results are expressed as mean ± SEM of 4–5 determinations performed in triplicate. $^*P < 0.05$ versus Caco-2 cells cultured without FBS; $^{\Phi}P < 0.05$ versus Caco-2 cells cultured with FBS; $^{\#}P < 0.05$ versus Caco-2 cells cultured with LTD_4.

The mitogenic effect observed with LTD_4 was confirmed using [^{3}H]thymidine incorporation (Fig. 2). We found that LTD_4 (10 nmol/L) induced DNA synthesis, whereas the above mentioned $CysLT_1R$ antagonists (i.e., MK 571 and LY 171883), the COX inhibitors, and the EP antagonists significantly inhibited the incorporation of [^{3}H]thymidine into Caco-2 cells induced by LTD_4.

LTD_4 (10 nmol/L) was also able to induce a significant release of [^{3}H]AA by Caco-2 cells, which was blocked in the presence of $CystLT_1R$ antagonists (MK 571 and LY 171883) (Table 1). Moreover, the release of [^{3}H]AA induced by LTD_4 was blocked by a number of inhibitors: a PLC inhibitor (U 73122); an inhibitor that prevents the release of calcium from the endoplasmic reticulum (dantrolene), and by a PKC inhibitor (Gö 6983). We observed that the release of [^{3}H]AA induced by LTD_4 was also inhibited by a nonspecific phospholipase A_2 (PLA_2) inhibitor ($AACOCF_3$) but not by a specific calcium-independent PLA_2 inhibitor, for example, BEL. In addition, our study showed that LTD_4 (10 nmol/L) increases the expression of COX-2, and that this effect was reverted by a $CysLT_1R$ antagonist (Fig. 3A). In addition, we found

Table 1. [^{3}H]AA release induced by leukotriene D_4 (LTD_4).

	[^{3}H]AA (%)
Control	4.1 ± 0.3
LTD_4	23.6 ± 1.8*
LTD_4 + $AACOCF_3$ (10 μmol/L)	8.2 ± 0.7*,#
LTD_4 + BEL (10 μmol/L)	21.3 ± 1.7*
LTD_4 + MK 571 (25 μmol/L)	7.2 ± 1.1#
LTD_4 + LY 171883 (25 μmol/L)	7.9 ± 1.5#
LTD_4 + U 73122 (0.1 μmol/L)	11.5 ± 2.1*,#
LTD_4 + Dantrolene (50 μmol/L)	12.6 ± 1.8*,#
LTD_4 + Gö 6983 (1 μmol/L)	13.4 ± 1.3*,#

[^{3}H]AA was determined in non-differentiated Caco-2 cell cultures, as described in the Materials and Methods section. Cells were incubated in the presence of LTD_4 (10 nmol/L) or LTD_4 plus treatments and [^{3}H]AA was determined 30 min after LTD_4 incubation, respectively. Values are means ± SEM of three experiments performed in triplicate, $^*P < 0.05$ versus control; $^{\#}P < 0.05$ versus LTD_4.

that LTD_4 induced the synthesis of PGE_2 and that this action was reverted by $CystLT_1R$ antagonists (MK 571 and LY 171883) and ketoprofen (Fig. 3B).

Finally, we studied the capacity of LTD_4 to phosphorylate pivotal elements in the cell signaling pathways implicated in the regulation of cell growth. For ERK, phosphorylation was highest after 5 min incubation and for CREB after 15 min. Dephosphorylation of β-catenin also increased after 15 min incubation (Fig. 4A). In addition, our results show that ERK phosphorylation induced by LTD_4 was reverted by a $CysLT_1R$ antagonist as well as by a COX inhibitor (Fig. 4B) and that CREB phosphorylation induced by LTD_4 was also blocked by ketoprofen (Fig. 4B).

Discussion

Colorectal cancer is the third most common cancer in the Western world and almost half of patients die of metastatic disease. This highlights the importance of research into the molecular mechanisms involved and their role in prognosis. Previous findings in our laboratory have demonstrated that the release of AA by PLA_2s participates in the signaling pathways involved in the control of intestinal epithelial cell proliferation (Sanchez and Moreno 2002a), and that its subsequent metabolism by COX-2 could be involved in the control of Caco-2 cell growth. Research has shown that in tumor tissue, COX and 5-LOX pathways are upregulated, which is not seen in normal colon mucosa (Cianchi et al. 2006). Moreover, as Cianchi et al. (2006) reported, 5-LOX inhibition increases the antitumor activity of COX inhibitors in human colon cancer cells. These findings support the hypothesis that

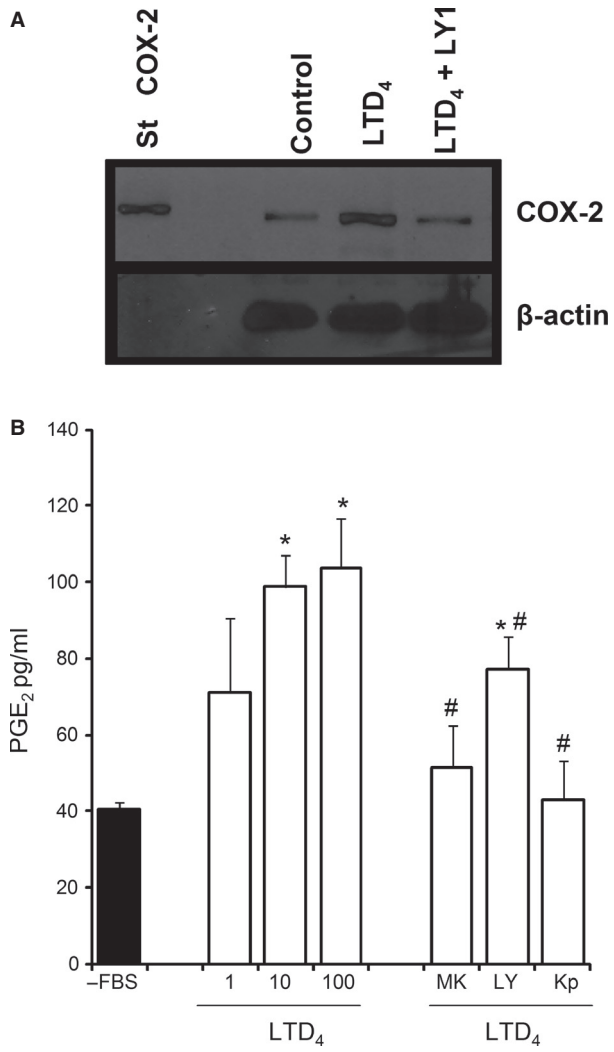


Figure 3. The effect of Leukotriene D_4 (LTD_4) on COX-2 expression and PGE_2 synthesis. (A) Cells were incubated in the absence of FBS (Control) and in the presence of LTD_4 (10 nmol/L) or LTD_4 plus LY171883 (LY1, 25 μmol/L) for 30 min and COX-2 expression was determined using a specific antibody. Murine COX-2 (20 ng) was used as standard (St COX-2). Western blot was used in three experiments. (B) Caco-2 cells were incubated with LTD_4 (1–100 nmol/L) for 15 min and PGE_2 synthesis was determined. PGE_2 synthesis induced by LTD_4 (10 nmol/L) was studied in the presence of MK 571 (MK5, 25 μmol/L), LY171883 (LY1, 25 μmol/L) or ketoprofen (Kp, 5 μmol/L). Results are expressed in mean ± SEM of 5 determinations performed in triplicate. $^*P < 0.05$ versus Caco-2 cell cultures in the absence of growth factors (FBS); $^{\#}P < 0.05$ versus cells incubated with LTD_4 (10 nmol/L).

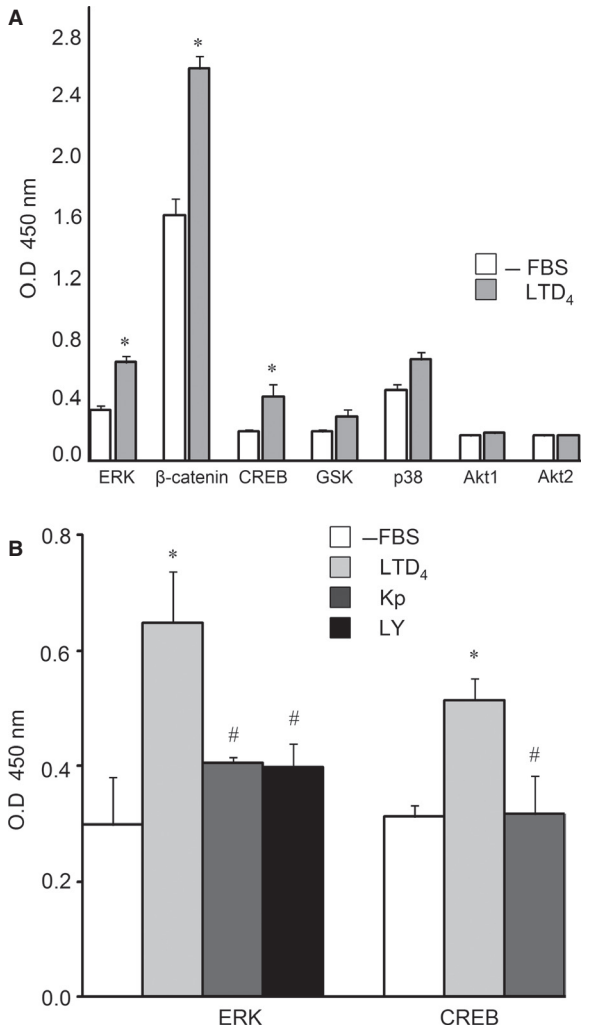


Figure 4. The effect of Leukotriene D_4 (LTD_4) on cell signaling. (A) Caco-2 cells were incubated with LTD_4 (10 nmol/L) for 5 or 15 min and then cells were collected. Phosphorylated Akt1, Akt2, ERK1/2, p38α, CREB, and GSKβ and dephosphorylated β-catenin were then measured, as described in the Material and Methods section. (B) The effect of LTD_4 (10 nmol/L) on ERK1/2 and CREB pathways. Cells were incubated with LTD_4 (10 nmol/L) for 5 min in the presence of LY171883 (LY1, 25 μmol/L) or ketoprofen (Kp, 5 μmol/L). Values are mean ± SEM of triplicate determinations, the experiment was repeated twice. $^*P < 0.05$ versus Caco-2 cells cultures in the absence of LTD_4 (10 nmol/L); $^{\#}P < 0.05$ versus cells incubated with LTD_4 (10 nmol/L).

the key elements of the AA cascade are involved in the regulation of intestinal epithelial structure/function (Ferrer and Moreno 2010).

Recently, we reported that pre-confluent Caco-2 cells were able to synthesize LTB_4 and 5-, and 12- and 15-HETE eicosanoids, which were found to be involved in the regulation of Caco-2 cell growth (Cabral et al. 2013). Dreyling et al. (1986) reported that human gastrointestinal tissues could synthesize cysteinyl leukotrienes, however, we were unable to detect LTD_4 in Caco-2 cell culture supernatants (Cabral et al. 2013). Paruchuri et al. (2006) reported that cysteinyl LTs released from Caco-2

cells reached a concentration of 5 pmol/L, which is notably lower than the limits of detection (0.3 mmol/L) for this eicosanoid in our experimental conditions (Martín-Venegas et al. 2011).

It is important to consider that the tumor microenvironment has often been associated with infiltrating leukocytes in the tumor tissue and the surrounding stroma (Negus et al. 1997). Consequently, the activation of macrophages and mast cells in inflammatory processes and cancer might induce an additional release of cysteinyl LTs, such as LTD_4, in the intestinal mucosa. Thus, our findings demonstrate that at a concentration range of 1–100 nmol/L, which is a level likely reached under tumorigenic conditions, LTD_4 can induce Caco-2 cell proliferation and DNA synthesis. Moreover, our results have confirmed, using specific receptorial antagonists, that this effect is a consequence of the interaction with Cys-tLT_1R.

This effect was also reported by Magnusson et al. (2007), who demonstrated that unlike $CysLT_2R$, $CysLT_1R$ is involved in intestinal epithelial proliferation. Moreover, it has been described that this receptor is upregulated in colon cancer and correlates with a poorer prognosis (Öhd et al. 2003; Magnusson et al. 2010). Similarly, we recently reported that the LTD_4-$CystLT_1R$ interaction increases intracellular Ca^{2+} concentrations in Caco-2 cells, indicating that PLC activation as well as stores of extracellular Ca^{2+} and intracellular Ca^{2+} are involved in this event (Rodríguez-Lagunas et al. 2013). Our results indicate that the PLC-IP_3-Ca^{2+}/DAG-PKC signaling pathways and cytosolic PLA_2 participate in the release of $[^3H]$AA induced by the LTD_4-$CystLT_1R$ interaction. Consequently this also demonstrates their participation in the activation of the AA cascade and eicosanoid production. These findings are consistent with Parhamifar et al. (2005) who reported that cytosolic PLA_2 was activated and translocated to the nucleus upon LTD_4 stimulation via a Ca^{2+}-dependent mechanism that involves the activation of PKC in Caco-2 cells.

Furthermore, we observed that the interaction of LTD_4 with the $CystL_1T$ receptor stimulated COX-2 expression, which is consistent with previous research, carried out by Yudina et al. (2008) using different intestinal epithelial cells. We can therefore surmise that PGE_2 synthesis was induced by LTD_4, which is consistent with the results obtained by Öhd et al. (2000) and Massoumi et al. (2003). Given that Caco-2 cell proliferation induced by LTD_4 was reverted by nonspecific and specific COX inhibitors as well as EP_1 and EP_4 antagonists, we propose that this action is completely dependent of PGE_2 synthesized by both COX as well as by PGE_2 interaction with EP_1 and EP_4 that stimulated cell signaling pathways that are crucial in the progression of the cell cycle as previously described in our group (Cabral et al. 2013; Sanchez and Moreno 2002b). Thus, $[^3H]$AA release, metabolism by both COXs, and the interaction of PGE_2 with EP_1 and EP_4 receptors are not only important events of acute and chronic inflammation, but are also essential regulators of the proliferation of transformed intestinal epithelial cancer cells induced by LTD_4.

We recently demonstrated the signaling pathways involved in Caco-2 cell growth induced by PGE_2 to be ERK, CREB, GSK, and p38, which is consistent with Pham et al. (2008) and Cherukuri et al. (2007). Our results also indicate that LTD_4 also induces ERK and CREB phosphorylation and that both signaling pathways were completely blocked when COX was inhibited. This therefore indicates that the activation of both ERK and CREB could be attributed to PGE_2 synthesis induced by LTD_4. Furthermore, while Caco-2 cells have a high basal β-catenin dephosphorylation level, we found that LTD_4 induces an additional dephosphorylation, which is consistent with research by Öhd et al. (2000). A previous study on Caco-2 cells in our laboratory revealed that this signaling pathway was not activated by a mitogenic factor such as PGE_2. Thus, since the mitogenic effect of LTD_4 is completely PGE_2-dependent, it is unlikely that the activation of the β-catenin pathway by LTD_4 is directly related to cell proliferation, and so as reported by Salim et al. (2014), the dephosphorylation of β-catenin induced by LTD_4 could be linked to the migration of colon cancer cells.

In conclusion, the effects of LTD_4 appear to occur through the increased expression and activation of COX-2, the production of PGE_2, and the interaction of PGE_2 with its cell-surface receptors. These findings will help improve our understanding of how inflammatory mediators can affect the survival and dissemination of intestinal carcinoma cells.

Acknowledgments

The authors thank Jaume Comas from the *Centres Científics i Tecnològics* of the *Universitat de Barcelona* for his valuable help with apoptosis analyses. The Language Services of the *Universitat de Barcelona* are also gratefully acknowledged.

Conflict of Interest

No conflicts of interest, financial or otherwise, are declared by the authors.

References

Cabral, M., R. Martín-Venegas, and J. J. Moreno. 2013. Role of arachidonic acid metabolites on the control of non-

differentiated intestinal epithelial cell growth. Int. J. Biochem. Cell Biol. 45:1620–1628.

Cherukuri, D. P., X. B. O. Chen, A. C. Goulet, R. N. Young, Y. Han, R. L. Heimark, et al. 2007. The EP_4 receptor antagonist, L-161,982, blocks prostaglandin E_2-induced signal transduction and cell proliferation in HCA-7 colon cancer cells. Exp. Cell Res. 313:2969–2979.

Cianchi, F., C. Cortesini, L. Magnelli, E. Fanti, L. Papucci, N. Schiavone, et al. 2006. Inhibition of 5-lipoxygenase by MK886 augments the antitumor activity of celecoxib in human colon cancer cells. Mol. Cancer Ther. 5:2716–2726.

Coussens, L. M., and Z. Werb. 2002. Inflammation and cancer. Nature 420:860–867.

Dreyling, K. W., U. Hoppe, B. A. Peskar, K. Morgenroth, W. Kozuschek, and B. M. Peskar. 1986. Leukotrienes synthesis by human gastrointestinal tissues. Biochim. Biophys. Acta 878:184–193.

Ferrer, R., and J. J. Moreno. 2010. Role of eicosanoids on intestinal epithelial homeostasis. Biochem. Pharmacol. 80:431–438.

Heise, C. E., B. F. O'Dowd, D. J. Figueroa, N. Sawyer, T. Nguyen, D. S. Im, et al. 2000. Characterization of the human cysteinyl leukotriene 2 receptor. J. Biol. Chem. 275:30531–30536.

Lynch, K. R., G. P. O'Neill, Q. Liu, D. S. Im, N. Sawyer, and K. M. Metters. 1999. Characterization of the human cycteinyl leukotriene Cys LT1 receptor. Nature 399:789–793.

Magnusson, C., R. Ehrnstrom, J. Olsen, and A. Sjolander. 2007. An increased expression of cysteinyl leukotriene 2 receptor in colorectal adenocarcinoma correlates with high differentiation. Cancer Res. 67:9190–9198.

Magnusson, C., M. Mezhybovska, E. Lörinc, E. Fernebro, M. Nilbert, and A. Sjölander. 2010. Low expresión of CysLT1R and high expresión of CysLT2R mediate good prognosis in colorectal cancer. Eur. J. Cancer 46:826–835.

Martín-Venegas, R., S. Roig-Pérez, R. Ferrer, and J. J. Moreno. 2006. Arachidonic acid cascade and epithelial barrier function during Caco-2 cell differentiation. J. Lipid Res. 47:1416–1423.

Martín-Venegas, R., R. Casillas, O. Jauregui, and J. J. Moreno. 2011. Rapid simultaneous analysis of cyclooxygenase, lipoxygenase and cytochrome P-450 metabolites of arachidonic and linoleic acids using high performance liquid chromatography/mass spectrometry in tandem mode. J. Pharm. Biomed. Anal. 56:976–982.

Massoumi, R., C. K. Nielsen, D. Azemovic, and A. Sjölander. 2003. Leukotriene D_4-induced adhesion of Caco-2 cells is mediated by prostaglandin E_2 and upregulation of alpha2beta1-integrin. Exp. Cell Res. 289:342–351.

Negus, R. P., G. W. Stamp, J. Hadley, and F. R. Balkwill. 1997. Quantitative assessment of the leukocyte infiltrate in ovarian cancer and its relationship to the expression of C-C chemokines. Am. J. Pathol. 150:1723–1734.

Öhd, J. F., K. Wikström, and A. Sjölander. 2000. Leukotrienes induce cell-survival signaling in intestinal epithelial cells. Gastroenterology 119:1007–1018.

Öhd, J. F., C. K. Nielsen, J. Campbell, G. Landberg, H. Löfberg, and A. Sjölander. 2003. Expression of the leukotriene D_4 receptor CysLT1, COX-2, and other cell survival factors in colorectal adenocarcinomas. Gastroenterology 124:57–70.

Parhamifar, L., B. Jeppsson, and A. Sjölander. 2005. Activation of $cPLA_2$ is required for leukotriene D4-induced proliferation in colon cancer cells. Carcinogenesis 26:1988–1998.

Parks, D. R., V. M. Bryan, V. T. Oi, and L. A. Herzenberg. 1979. Antigen-specific identification cloning of hybridomas with a fluorescence-activated cell sorter. Proc. Natl Acad. Sci. USA 76:1962–1969.

Paruchuri, S., M. Mezhybovska, M. Juhas, and A. Sjölander. 2006. Endogenous production of leukotrienen D4 mediates autocrine survival and proliferation via CysLT1 receptor signaling in intestinal epithelial cells. Oncogene 25:6660–6665.

Pham, H., G. Eibl, R. Vincenti, B. Chong, H. H. Tai, and L. W. Slice. 2008. 15-hydroxyprostaglandindehydrogenase suppresses K-Rasv12-dependent tumor formation in Nu/Numice. Mol. Carcinog. 47:466–477.

Qiao, L., and X. Li. 2014. Role of chronic inflammation in cancers of the gastrointestinal system and the liver: where we are now. Cancer Lett. 345:150–152.

Rodríguez-Lagunas, M. J., C. E. Storniolo, R. Ferrer, and J. J. Moreno. 2013. 5-Hydroxyeicosatetraenoic acid and leukotriene D_4 increase intestinal epithelial paracellular permeability. Int. J. Biochem. Cell Biol. 45:1318–1326.

Salim, T., J. Sand-Dejmek, and A. Sjölander. 2014. The inflammatory mediator leukotriene D_4 induces subcellular β-catenin translocation and migration of colon cancer cells. Exp. Cell Res. 321:255–266.

Samuelsson, B. 1979. Prostaglandins, thromboxanes and leukotrienes: formation and biological roles. Harvey Lect. 75:1–40.

Sanchez, T., and J. J. Moreno. 2002a. Calcium-independent phospholipase A2 through arachidonic acid mobilization is involved in Caco-2 cell growth. J. Cell. Physiol. 193:293–298.

Sanchez, T., and J. J. Moreno. 2002b. Role of EP_1 and EP_4 PGE_2 subtype receptors in serum-induced 3T6 fibrobalst cycle progression and proliferation. Am. J. Physiol. Cell Physiol. 282:C280–C288.

Smalley, W. E., and R. Dubois. 1997. Colorectal cancer and nonsteroidal anti-inflammatory drugs. Adv. Pharmacol. 39:1–20.

Stenton, W. F. 1990. Role of eicosanoids as mediators of inflammation in inflammatory bowel disease. Scand. J. Gastroenterol. 172:13–18.

Yudina, Y., L. Parhamifar, A. M. L. Bengtsson, M. Juhas, and A. Sjölander. 2008. Regulation of the eicosanoid pathway by tumour necrosis factor alpha and leukotriene D_4 in intestinal epithelial cells. Prostaglandins Leukot. Essent. Fatty Acids 79:223–231.

Artículo 4

Marisol Cabral, Carolina Storniolo, Raquel Martín-Venegas, Juan J. Moreno.

Eicosapentaenoic acid and eicosapentaenoic acid-derived eicosanoids are involved in human colorectal carcinoma cell line proliferation

Pendiente de aceptación a J Nutr Biochem

Índice de impacto (JCR, 2014): 3,794
Categoria y posición; quartil: Nutrition and Dietetics: 14/77; Q1
Biochemistry and Molecular biology: 85/290; Q2

Los resultados de esta publicación se han presentado en una comunicación a un congreso:

- Effect of n-3 and n-6 eicosanoids on intestinal Caco-2 cell growth.
  Cabral M, Martin-Venegas R, Moreno JJ. 3rd International Immunonutrition Workshop. Platja d'Aro (España), 21-24 octubre 2009.
  Resumen publicado Proceedings of the Nutrition Society, 69:E309 (2010)

Resumen artículo 4:

Objetivo. Investigar el efecto del EPA y de eicosanoides derivados del EPA sobre el crecimiento de las células epiteliales intestinales no diferenciadas procedentes de un adenocarcinoma.

Material y Métodos. . Este estudio se ha realizado utilizando células Caco-2 no diferenciadas. Las células se incubaron con EPA y sus derivados, tanto en presencia como ausencia de inhibidores de las vías COX y LOX, así como agonistas o antagonistas receptoriales de eicosanoides y se determino: el crecimiento celular por contaje microscópico usando bromuro de etidio/naranja de acridina y la síntesis de ADN por incorporación de BrdU, y la fosforilación de Akt1, Akt2, ERK1/2, GSK3, p38, CREB y la desfosforilación de β-catenina por ELISA.

Resultados. Aunque a concentraciones altas (100µM) el EPA presenta un efecto apoptótico, a 10µM induce el crecimiento celular y la síntesis de ADN en las células Caco-2. Este efecto mitogénico se reduce significativamente con la utilización de inhibidores de las COXs, de la 5-LOX, 12-LOX y antagonistas de los receptores EP_4 y BLT_2. Los eicosanoides derivados del EPA como la PGE_3 y el 12-HEPE inducen la proliferación celular. El efecto inducido por la PGE_3 disminuye significativamente cuando se utilizan antagonistas específicos de EP_1 y EP_4, pero no cuando se utiliza un antagonista del EP_3. En el caso del 12-HEPE el crecimiento celular es inhibido por antagonistas de los receptores BLT_1 y BLT_2 y por un inhibidor de las COXs. La PGE_3 activa la fosforilación de ERK, CREB, GSKβ y p38. Según estos resultados, los efectos de la PGE_3 y el 12-HEPE fueron similares a los observados con los metabolitos correspondientes del AA. En cambio el LTB_5 no induce el crecimiento celular a diferencia del LTB_4, que tiene un marcado efecto mitogénico.

Conclusión. El EPA a concentraciones bajas tiene un efecto proliferativo y a concentraciones altas un efecto apoptótico. La PGE_3 y el 12-HEPE inducen la proliferación celular de forma similar a la PGE_2 y al 12-HETE y estos metabolitos pueden estar implicados en el efecto proliferativo del EPA. En cambio el LTB_5 no induce el crecimiento celular. El efecto mitogenico del 12-HEPE se produce al menos en parte por la activación de la vía de las COXs.

Eicosapentaenoic acid and eicosapentaenoic acid-derived eicosanoids are involved in human colorectal carcinoma cell line proliferation

Marisol Cabral, Carolina E. Storniolo, Raquel Martín-Venegas, Juan José Moreno*

Faculty of Pharmacy, University of Barcelona, Barcelona, Spain

Institute of Nutrition (INSA), University of Barcelona, Barcelona, Spain

* Corresponding author: JJ Moreno, Department of Physiology, Faculty of Pharmacy, University of Barcelona, Avda. Joan XXIII s/n 08028 Barcelona, Spain. Telephone: (+34) 934024505, Fax: (+34) 934035901, E-mail jjmoreno@ub.edu

Running title: EPA and cell proliferation

Grants: BFU2007-61727/BFI (Spanish Ministry of Science and Innovation) and 2009SGR0438 and 2014SGR0773 (Autonomous Government of Catalonia).

Keywords: eicosanoids; EPA; prostaglandin E_3; leukotriene B_5; 12-hydroxyeicosapentaenoic acid; Caco-2 cell growth

Abstract

Numerous studies have suggested that n-3 fatty acids, such as eicosapentaenoic acid (EPA), have a considerable protective effect against colorectal cancer. It is unclear how this phenomenon is triggered and what mechanisms are implicated, but it could be due, at least partly, to the synthesis of EPA-derived eicosanoids. Although EPA can induce apoptosis, we show that low EPA concentrations (5-15 µM) induced Caco-2 cell proliferation, and that this effect was reverted by cyclooxygenase and lipoxygenase inhibitors, as well as by prostaglandin (PG) and leukotriene (LT) receptor antagonists. This suggests that EPA-derived eicosanoids are involved in these effects. We observed that PGE_3 (1-10 nM) and 12-S-hydroxyeicosapentaenoic acid (12-S-HEPE) (100-1000 nM) induced Caco-2 cell growth through interaction with EP_1/EP_4 PGE_2 receptors and BLT_1/BLT_2 LTB_4 receptors, respectively. Moreover, the mitogenic action of 12-S-HEPE was dependent on prostaglandin synthesis. However, LTB_5 did not have a mitogenic effect induced by LTB_4. Finally, we reported that the mitogenic effect of PGE_3 could be related with the activation of several cell signalling pathways, such as ERK 1/2, CREB, GSKβ and p38. In conclusion, EPA and EPA metabolites such as PGE_3 and 12-S-HEPE have a mitogenic effect on intestinal epithelial cells.

Keywords: eicosanoids, EPA, prostaglandin E_3, leukotriene B_5, 12-hydroxyeicosapentaenoic acid, Caco-2 cell growth

1. Introduction

Colorectal cancer (CRC) is the second leading cause of cancer-related death in developed countries, and dietary factors can account for the considerable differences in incidence observed around the world [1]. CRC can develop sporadically or when there is a background of well-characterised familial syndromes. Importantly, patients with inflammatory bowel diseases (IBD) have an increased risk of developing colitis-associated CRC. Thus, it is now well-established that inflammation plays a key role in colon carcinogenesis in both a sporadic and IBD setting [2]. Eicosanoids produced by arachidonic acid (AA) play a pivotal role in inflammation and cancer. AA is the substrate for the production of eicosanoids such as 2-series prostaglandins (PGs), 4-series leukotrienes (LTs) or hydroxyeicosatetraenoic acids (HETEs) that may facilitate colon cancer progression by stimulating cell proliferation and survival, tumour cell invasiveness, and angiogenesis [3,4]. Thus, overexpression of cyclooxygenase-2 (COX-2) has been reported in 90% of colon tumours and premalignant colorectal adenomas [5]. More recently, Melstrom et al. [6] observed that 5-lipoxygenase (5-LOX) is also upregulated in adenomatous colon polyps and cancer. However, Shureiqi et al. [7] reported no significant differences in eicosanoids levels between normal, polyp, and cancer mucosa. Thus, this aspect is controversial and needs additional studies for clarification.

Mammals are unable to synthesize n-3 and n-6 PUFAs, and must obtain them from their diet. Both n-3 and n-6 PUFAs are important constituents of cell membranes and may affect many membrane properties, such as permeability, gene expression, activity of specific proteins, or signal transduction [8, 9]. Some

of these changes could ultimately drive the antineoplastic effect of n-3 PUFAs. Thus, several epidemiologic and case-control studies have suggested a decrease in CRC risk among individuals who consume diets high in n-3 PUFA [10-13]. A genetic model that produces endogenous *de novo* n-3 PUFAs showed reduced colonic tumours [14]. Furthermore, multiple reports using a variety of rodent models of early-stage colorectal carcinogenesis have demonstrated the efficacy of eicosapentaenoic acid (EPA) [15,16], and a recent study has shown that this PUFA is effective as a chemopreventive agent in familial adenomatous polyposis [17]. However, effects of n-3 PUFAs in patients and experimental models of IBD have yielded conflicting data [18,19], and prospective studies have revealed little evidence of protection against colon cancer risk [20]. Thus, the effects of dietary n-3 PUFAs on cancer prevention in colitis-associated CRC are far from completely elucidated.

It is hypothesised that the beneficial effects of n-3 PUFA on colorectal tumours may partly be related to the inhibition of AA-derived eicosanoid synthesis and the release of EPA-derived eicosanoids, such as 3-series PGs, 5-series LTs and hydroxyeicosapentaenoic acids (HEPE). This study was conducted to investigate the role of EPA and EPA-derived eicosanoids on intestinal epithelial cell proliferation. Human colon adenocarcinoma Caco-2 cells were used because they produce a wide eicosanoid spectrum [21] and may represent a stage of colorectal carcinogenesis.

2. Materials and methods

2.1. Materials

EPA, PGE_2, PGE_3, PGD_2, LTB_4, LTB_5, 12-S-HETE, 12-S-HEPE, ketoprofen, baicalein, MK571, MK886 and U75302 were purchased from Cayman Chemical (Ann Arbor, MI). SC19220, ethidium bromide and acridine orange were purchased from Sigma Chemical (St. Louis, MO). LY255283 was from Tocris Biosc. (Bristol, UK). ONO-AE3-240 was kindly provided by Ono Pharmaceutical Co. Ltd. (Osaka, Japan) and AH23838 by Glaxo-Wellcome (Stevenage, UK). Stock solutions of EPA, eicosanoids and treatments were made in dimethylsulfoxide (DMSO) and were stored away from light exposure at -20ºC. Working dilutions were made directly in cell culture medium. Control conditions contained a DMSO concentration similar to those present in EPA/eicosanoid conditions and never higher than 0.1%.

2.2. Cell culture and cell growth assay

Caco-2 cells were derived from a moderately well-differentiated primary colon adenocarcinoma and were provided by American Type Culture Collection (HTB-37, Manassas, VA, USA). The cells were routinely grown in plastic flasks at a density of 10^4 cells/cm^2 and cultured in DMEM containing 2 mM L-glutamine supplemented with 4.5 g/l D-glucose, 1% (v/v) non-essential amino acids, 2 mM L-glutamine, 10% (v/v) heat-inactivated FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37ºC in a modified atmosphere of 5% CO_2 in air.

All the experiments were performed in pre-confluent cultures and, consequently, in non-differentiated cells [22]. To perform the cell growth assay,

cells were harvested with 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 cells (3-4 x 10^4 cells/cm^2) were incubated with treatments for a period of 48 h without FBS. Finally, cells were washed, trypsinised, and counted with a microscope using ethidium bromide/acridine orange staining to measure viable cells.

2.3. Analysis of DNA synthesis

DNA synthesis was assayed using a colorimetric immunoassay based on the measurement of BrdU incorporation during DNA synthesis (Cell proliferation ELISA, BrdU Kit, from Roche). Caco-2 cells were cultured at 1000-1500 cell/well in 96 well plates for 4 days in DMEM medium supplemented with 10% FBS. Then, cells were washed and incubated for 48 h in DMEM without FBS but with the test compounds. Thereafter, cell cultures were treated following the manufacturer's instructions. Final absorbance was measured at 450 nM in a plate reader.

2.4. Measurement of apoptosis

Degradation of chromosomal DNA as a final result of apoptosis was evaluated with the TUNEL method. Sub-confluent cell cultures (10^4 cells/cm^2) were seeded in 60 mm dishes for 4 days and were then incubated in media containing the treatments for 48 h. Cells present in the medium and attached trypsinised cells were then collected, fixed with 4% paraformaldehyde and permeabilised with 70% ethanol. Thereafter, 3'-OH DNA ends generated by DNA fragmentation were labelled with fluorescein-dUTP mediated by terminal deoxynucleotidyl transferase using a MEBSTAIN Apoptosis Kit (MBL, Woburn,

MA) and then analysed by an Epics XL flow cytometer (Coulter Corp., Hialeah, FL).

2.5. Measurement of cell signalling pathways

Cells were seeded in 60 mm plastic clusters (10^4 cells/cm^2). After 4 days, the cultures were incubated with the treatments (5 or 15 min). To measure the kinase activation with total cellular lysates, Caco-2 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. The resulting solutions containing 80-100 µg of proteins were then added to a kinase ELISA plate and the assay was performed following the manufacturer's recommendations (Symansis, Auckland, New Zealand). Finally, optical density was measured at 450 nm. Thus, we studied the effect of eicosanoids on the phosphorylation of AKT1 (pS473), AKT2 (pS474), ERK1/2 (pT202/Y204;pT185/Y187), GSK3β (pS9), p38α (pT180/Y182) and CREB (pS133).

2.6. PPARγ ligand assay

EPA binding to peroxisome proliferator-activated receptor γ (PPARγ) was studied with a fluorescence polarization-based single step PPARγ ligand screening assay (Cayman chemical Co., Ann Arbor, MI) as we previously described [23]. In this assay, a ligand of PPARγ was conjugated to fluorescein and is used as the displacement probe measuring as mP units.

2.7. Statistics

The results are expressed as mean ± SEM. All data were compared by one-way ANOVA and the Student's t-test, using SPSS software (SPSS Inc., Chicago, IL). Significance was taken as $p < 0.05$

3. Results

3.1. EPA has a proliferative effect that is dependent on its metabolism by cyclooxygenase and lipoxygenase pathways

EPA induced DNA synthesis and Caco-2 cell growth between 5-15 µM. Interestingly, this effect disappeared when EPA concentrations were increased (Table 1). Indeed, Caco-2 cells incubated with EPA 100 µM for 48 h produced apoptosis in 82.4 ± 3.1 % of cells (data not shown). Thus, at high concentrations (≥ 50 µM), EPA reduces cell number as a consequence of the apoptotic effect of this fatty acid. Moreover, this EPA-induced mitogenic effect was reverted by ketroprofen (COX inhibitor), MK886 (5-LOX inhibitor), baicalein (12-LOX inhibitor), AH23838 (EP_4 antagonist), and LY255283 (BLT_2 antagonist). In contrast, SC19220 (EP_1 antagonist) and U75302 (BLT_1 antagonist) decreased the mitogenic effect of EPA, but this decrease was not significant. Interestingly, the mitogenic effect of EPA was inhibited by a cysteinyl leukotriene antagonist (MK571).

3.2. PGE_3 has a proliferative effect on Caco-2 cells

Previously we observed that PGE_2 (0.1-10 nM) and 12-HETE (10-1000 nM) were mitogenic on Caco-2 cells, concentrations that could be reached in these cell cultures [21]. We did not measure PGE_3 and 12-HEPE but the replacement of AA by EPA in membrane phospholipids can result in a decrease of AA derived eicosanoids and the increase of EPA derived eicosanoids [24] that can reach concentrations similar to those used in this study. Here, we observed that PGE_3 increased Caco-2 growth and DNA synthesis in a concentration-dependent manner up to 10 nM (Fig. 1), with similar values to those observed with PGE_2 (Fig. 2). Fig. 1 also shows that Caco-2 growth and DNA synthesis induced by PGE_3 was totally inhibited by an EP_1 antagonist (SC19220) and by an EP_4 antagonist (AH23838). However, an EP_3 antagonist (ONO-AE3-240, 2nM) did not have any effect on Caco-2 cell growth. Therefore, the results indicate that PGE_3 acts through EP_1 and EP_4 receptors, but not through the EP_3 receptor.

3.3. PGD_2 does not have a proliferative effect on Caco-2 cells

Our results show that PGD_2 (1-100 nM) did not modify cell growth and DNA synthesis in Caco-2, while PGE_2 (10 nM) had a strong proliferative effect (Fig. 2).

3.4. 12-S-HEPE has a proliferative effect on Caco-2 cells

Our results show that 12-S-HEPE (100-1000 nM) induced significant cell growth and DNA synthesis in Caco-2 cell cultures in the absence of growth factors, in a similar way to 12-S-HETE (100 nM). The mitogenic action of 12-S-HEPE was blocked by a COX inhibitor and by BLT_1 and BLT_2 antagonists (Fig. 3).

3.5. LTB_5 does not have a proliferative effect on Caco-2 cells

Previously we observed that LTB_4 has a mitogenic effect on Caco-2 cells [21]. However, our findings show that LTB_5 derived from EPA did not induce proliferation in the range of 1-100 nM (Fig. 4).

3.6. PGE_3 induces mitogenic cell signalling pathways

Finally, we studied the capacity of PGE_3 to phosphorylate pivotal elements in the cell signalling pathways implicated in the regulation of cell growth. Maximal phosphorylation was observed after 5 min incubation for ERK, AKT and p38, and after 15 min for GSKβ and CREB. Our findings show that PGE_3 presents a similar pattern to PGE_2. Thus, PGE_3 (10 nM) could increase the phosphorylation of ERK 1/2, CREB, GSKβ and p38 (Fig. 5).

4. Discussion

There is a great debate about the role of n-3 and n-6 PUFAs in colorectal carcinogenesis. However, few studies have been conducted on the potency of PUFAs and/or PUFA metabolites in modulating human colon cancer cell line growth. Dommels et al. [25] reported that AA and EPA (10-100 μM) induced Caco-2 cell growth inhibition and cytotoxicity through peroxidation products generated during lipid peroxidation and COX activity. However, our findings indicate that although EPA, at 25-100 μM, decreases cell numbers and induces apoptosis in Caco-2 cells at the highest concentration, this PUFA has a mitogenic effect at 10 μM that is COX- and LOX-pathway dependent. This suggests that some EPA metabolites synthesised by both pathways could induce Caco-2 cell growth. Recently, EPA at low concentrations has been proposed as FFA4/GPR120 ligand [26], receptor abundantly expressed in the intestine [27]. These events could be involved in the mitogenic action observed in our experimental conditions, as also observed on chondrocyte growth [28]. However, EPA at 100 μM binds to PPARγ (Control: 113.7 ± 3.5 mP; Rosiglitazone 10 μM: 43.2 ± 2.1 MP; EPA: 68.1 ± 1.9) and these results can explain, at least in part, the decrease of Caco-2 cell growth and the apoptotic effects of this n-3 fatty acid at high concentrations.

Virtanen and co-workers [29] studied the association between fish, n-3 PUFA consumption, and the risk of total major chronic disease. In this large, prospective cohort study, no significant associations were seen between EPA consumption and cancer incidence. Higurashi et al. [30] proposed the first randomised controlled trial to explore the effect of EPA against CRC, but unfortunately the findings have not yet been published. However, Cockbain et

al. [13] recently observed that EPA did not change the CRC proliferation index, but reduced vascularity in patients undergoing liver resection surgery for colorectal cancer liver metastases.

Experimental studies have shown that diets rich in fish oil significantly reduce the amount of AA present in membrane phospholipids [31] and consequently the synthesis of AA metabolites such as PGE_2 [32], but increase the release of EPA metabolites. Even though the theory of formation of the 3-series PGs by EPA has been studied for decades, we still do not fully understand the role of PGE_3 in cancer cells. The literature has little information on this specific aspect [33], but Yang et al. [34] show that PGE_3 has anti-tumorigenic activity on human lung cancer cells. However, our data show, for the first time, that PGE_3 increased cell growth and DNA synthesis in non-differentiated intestinal epithelial cells cultured without FBS, and that this effect was similar to the proliferative action of PGE_2. These data are, apparently, in disagreement with Fan et al. [35] who reported that PGE_3 diminished the ability to support colonic stem cell expansion, compared to PGE_2 effects. However, we must consider that these authors used a non-physiological PGE_3 concentration (10 µM).

PGE_3 shares the same EP receptor system as PGE_2, with similar binding affinities and potencies [36]. Thus, we demonstrated that the PGE_3 proliferative effect was a consequence of interaction with the PGE_2 receptor EP_1 and EP_4. Our results are in agreement with the affinities of these prostanoids to their EP receptors, and with a recent report showing similar effects of PGE_2 and PGE_3 on the disruption of the intestinal epithelial barrier function [37].

Although there is evidence supporting the importance of PGs in cell growth and colon carcinogenesis, there are few reports delineating the signalling

pathways involved. Recently, we reported that the activation of ERK 1/2, CREB, GSKβ and p38 pathways by PGE_2 are involved in its mitogenic effects on intestinal epithelial Caco-2 cells [21]. Now, we observed that the same cell signalling pathways are also involved in the mitogenic action of PGE_3 on Caco-2 cell cultures.

Whereas PGE_2 has been linked with the promotion of proliferation cells, the role of PGD_2 in colon cancer has not been clarified. PGD_2 is also a metabolite of AA formed by the prostaglandin D_2 synthetase. The disruption of the PGD synthase gene in ApcMin/+ mice accelerates intestinal tumour growth, while ApcMin/+ mice with transgenic human hematopoietic PGD synthase exhibit fewer intestinal adenomas than controls [38], which suggests that PGD_2 serves as a tumour suppressor in colorectal cancer. In our experimental conditions, PGD_2 did not have any effect on Caco-2 cell growth.

12-LOX produces 12-HETE from AA and 12-HEPE from EPA. 12-S-HETE has a proliferative effect and our data show, for the first time, that 12-S-HEPE also has similar cell growth action on intestinal epithelial cell cultures. Numerous studies have suggested that there is a pro-tumorigenic impact of 12-S-HETE on CRC, related to both the proliferation of colonic mucosa cells and the formation of colorectal carcinoma [39, 40]. By contrast, the effect and mechanism of 12-S-HEPE has hardly been studied. No cellular receptors for HETEs have been identified to date. However, 12-S-HETE has been found to bind to the orphan receptor GPR31 [41]. Moreover, it has been reported that the binding of 12-HETE to the BLT_2 receptor may be involved in its mitogenic action [21]. Talking into account the similar structure of 12-HETE and 12-HEPE, we hypothesised that both eicosanoids may have a similar mitogenic mechanism.

Here, we demonstrate that Caco-2 cell growth induced by 12-S-HEPE can be reverted by BLT_1 and BLT_2 antagonists and a COX inhibitor, which suggests, for the first time, that this 12-S-HEPE mitogenic action is at least partly due to PGs synthesis after 12-S-HEPE interaction with both BLT receptors; a mechanism previously described for LTB_4 and 12-HETE [21] as well as 13-R-HODE [23].

LTB_4 synthesis has been associated with inflammatory diseases, promotion of carcinogenesis, tumour progression, and apoptosis resistance [6,42,43]. The major metabolite of 5-LOX from EPA is LTB_5. Ikehata et al. [44] reported that EPA administered to patients with Crohn's disease can increase the generation of LTB_5 and increase the LTB_5-LTB_4 ratio, which produces an improvement in these patients. EPA has already been shown to have an anti-inflammatory effect in psoriasis [45] and ulcerative colitis [46] that could be due to the formation of LTB_5 from EPA. In our study, LTB_5 had no proliferative effect, while LTB_4 significantly induced Caco-2 cell growth. These findings are in agreement with Bortuzzo et al. [39] who found a lower affinity of LTB_5 to the receptor of LTB_4, using HT-29 and HCT-15 human colon carcinoma cells. Moreover, LTB_5 showed 10% of the chemotactic potency of LTB_4 [47, 48], and did not induce polymorphonuclear transmigration across endothelial cell cultures [49], in agreement with the different action of LTB_4 and LTB_5 on Caco-2 cell proliferation. Furthermore, since both the treatment with 5-LOX inhibitor and cysteinyl LT receptor antagonist reduced the mitogenic effect of EPA, our results indirectly suggest that 5-serie cysteinyl LTs could also be involved in this event. There are little literature about the affinity of EPA derived LTs and cysteinyl LT receptors but Wallace and McKnight [50] reported that LTC5 or LTD5 have biological activity although several times less potent than LTs

derived from AA. Caco-2 cell line expresses a lot of AA enzyme cascade and consequently produces a great number of eicosanoids as we previously reported [21, 23], being a suitable cellular model to study the role of EPA and EPA eicosanoids synthetized by COX and LOX pathways on intestinal epithelial cell growth. However, we believe that future research should analyse the role of EPA and EPA eicosanoids on non transformed intestinal epithelial cells.

In conclusion, although LTB_5 did not have the mitogenic effect of its AA-derived partner, several EPA metabolites such as PGE_3 and 12-S-HEPE have considerable mitogenic effects on intestinal epithelial Caco-2 cells, and may be involved in cell proliferation induced by EPA.

Acknowledgments

This research was supported by the Spanish Ministry of Science and Innovation (BFU2007-61727/BFI) and by the Autonomous Government of Catalonia (2009SGR0438 and 2014SGR0773).

References

[1] Parkin DM. International variation. Oncogene 2004; 23:6329-6340.

[2] Ullman TA, Itzkowitz SH. Intestinal inflammation and cancer. Gastroenterol 2011; 140:1807-1816.

[3] Moreno JJ. Arachidonic acid cascade enzyme inhibition and cancer. Current Enzyme Inhibition 2005; 1:131-145.

[4] Moreno JJ. New aspects of the role of hydroxyeicosatetraenoic acids in cell growth and cancer development. Biochem Pharmacol 2009; 77:1-10.

[5] Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, Dubois RN. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterol 1994; 107:1183-1188

[6] Melstrom LG, Bentrem DJ, Salabat MR, Kennedy TJ, Ding XZ, Strouch M, et al. Overexpression of 5-lipoxygenase in colon polyps and cancer and the effect of 5-LOX inhibitors in vitro and in a murine model. Clin Cancer Res 2011; 14:6525-6530.

[7] Shureiqi I, Chen D, Day RS, Zuo X, Hochman FL, Ross WA. Profiling lipoxygenase metabolism in specific steps of colorectal tumorigenesis. Cancer Prevention Res 2010; 3:829-838

[8] Siddiqui RA, Shaikh SR, Sech LA, Yount HR, Stillwell W, Zaloga GP. Omega 3-fatty acids: health benefits and cellular mechanisms of action. Mini Rev Med Chem 2004; 4:859-871.

[9] Grosch S, Maier TJ, Schiffmann S, Geisslinger G. Cyclooxygenase-2 (COX-2)-independent anticarcinogenic effects of selective COX-2 inhibitors. J Natl Cancer Inst 2006; 98:736-747.

[10] Tavani A, Pelucchi C, Parpinel M, Negri E, Franceschi S, Levi F, La Vecchia C. n-3 polyunsaturated fatty acid intake and cancer risk in Italy and Switzerland. Int J Cancer 2003 ; 105:113-116.

[11] Courtney ED, Matthews S, Finlayson C, Di Pierro D, Belluzzi A, Roda E, et al. Eicosapentaenoic acid reduces crypt cell proliferation and increases apoptosis in normal colonic mucosa in subjects with a history of colorectal adenomas. Int J Colorectal Dis 2007; 22:765-776.

[12] Hall MN, Chavarro JE, Lee IM, Willett WC, Ma J. A 22-year prospective study of fish, n-3 fatty acid intake, and colorectal cancer risk in men. Cancer Epidemiol Biomarkers Prev 2008; 17:1136-1143.

[13] Cockbain AJ, Volpato M, Race AD, Munarini A, Belluzzi A, Loadman PM, et al. Anticolorectal cancer activity of the omega-3 polyunsaturated fatty acid eicosapentaenoic acid. Gut 2014; 63:1760-1768.

[14] Nowak J, Weylandt KH, Habbel P, Wang J, Dignass A, Glickman JN, Kang JX. Colitis-associated colon tumorigenesis is suppressed in transgenic mice rich in endogenous n-3 fatty acids. Carcinogenesis 2007; 28:1991-1995.

[15] Cockbain AJ, Toogood GJ, Hull MA. Omega-3 polyunsaturated fatty acids for the treatment and prevention of colorectal cancer. Gut 2012; 61:135-149.

[16] Piazzi G, D'Argenio G, Prossomariti A, Lembo V, Mazzone G, Candela M, et al. Eicosapentaenoic acid free fatty acid prevents and suppresses colonic neoplasia in colitis-associtated colorectal cancer acting on Notch signaling and gut microbiota. Int J Cancer 2014; 135:2004-2013.

[17] West NJ, Clark SK, Phillips RK, Hutchinson JM, Leicester RJ, Belluzzi A, Hull MA. Eicosapentaenoic acid reduces rectal polyp number and size in familial adenomatous polyposis. Gut 2010; 59:918-925.

[18] Belluzzi A, Boschi S, Brignola C, Munarini A, Cariani G, Miglio F. Polyunsaturated fatty acids and inflammatory bowel disease. Am J Clin Nutr 2000; 71:339S-342S.

[19] Matsunaga H, Hokari R, Kurihara C, Okada Y, Takebayashi K, Okudaira K, et al. Omega-3 fatty acids exacerbate DSS-induced colitis through decreased adiponectin in colonic subepithelial myofibroblasts. Inflammatory Bowel Dis 2008; 14:348-357.

[20] MacLean CH, Newberry SJ, Mojica WA, Khanna P, Issa AM, Suttorp MJ, et al. Effects of omega-3 fatty acids on cancer risk: a systematic review. JAMA 2006; 295:403-415.

[21] Cabral M, Martín-Venegas R, Moreno JJ. Role of arachidonic acid metabolites on the control of non-differentiated intestinal epithelial cell growth. Int J Biochem Cell Biol 2013; 45:1620-1628.

[22] Martín-Venegas R, Roig-Pérez S, Ferrer R, Moreno JJ. Arachidonic acid cascade and epithelial barrier function during Caco-2 cell differentiation. J Lipid Res 2006; 46:1416-1423.

[23] Cabral M, Martín-Venegas R, Moreno JJ. Differential cell growth/apoptosis behavior of 13-hydroxyoctadecadienoic acid enantiomers in a colorectal cancer cell line. Am J Physiol Gastroenterol Liver Physiol 2014; 307:G664-G671.

[24] Nieves D, Moreno JJ. Effect of arachidonic acid and eicosapentaenoic acid metabolism on RAW 264.7 macrophage proliferation. J Cell Physiol 2006;208:428-434.

[25] Dommels YEM, Haring MMG, Keestra NGM, Alink GM, van Bladeren PJ, van Ommen B. The role of cyclooxygenase in n-6 and n-3 polyunsaturated fatty acid mediated effects on cell proliferation, PGE_2 synthesis and cytotoxicity in human colorectal carcinoma cell lines. Carcinogenesis 2003; 24:385-392

[26] Ulven T, Christiansen E. Dietary fatty acids and their potential for controlling metabolic diseases through activation of FFA4/GPR120. Annu Rev Nutr 2015;35:239-263.

[27] Hirasawa A, Tsumaya K, Awaji T, Katsuma S, Adachi T, Yamada M et al. Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. Nat Med 2005;11:90-94.

[28] Koren N, Simsa-Maziel S, Shahar R, Schwartz B, Monsonego-Ornan E. Exposure to omega-3 fatty acids at early age accelerate bone growth and improve bone quality. J Nutr Biochem 2014;25:623-633.

[29] Virtanen JK, Mozaffarian D, Chiuve SE, Rimm EB. Fish consumption and risk of major chronic disease in men. A. J Clin Nutr 2008; 88:1618-1625.

[30] Higurashi T, Hosono K, Endo H, Takahashi H, Lida H, Uchiyama T, et al. Eicosapentaenoic acid (EPA) efficacy for colorectal aberrant crypt foci (ACF): a double-blind randomized controlled trial. BMC Cancer 2012; 12:413.

[31] Mitjavila MT, Rodríguez MC, Saiz MP, Lloret S, Moreno JJ. Effect of degree of unsaturation in dietary fatty acids on arachidonic acid mobilization by peritoneal macrophages. Lipids 1996; 31:661-666.

[32] Moreno JJ, Carbonell T, Sánchez T, Miret S, Mitjavila MT. Olive oil decreases both oxidative stress and the production of arachidonic acid metabolites by the prostaglandin G/H synthase pathway in rat macrophages. J Nutr 2001; 131:2145-2149.

[33] Yang P, Jiang Y, Fischer SM. Prostaglandin E_3 metabolism and cancer. Cancer Lett 2014; 348:1-11.

[34] Yang P, Chan D, Felix E, Cartwright C, Menter DG, Madden T, et al. Formation and antiproliferative effect of prostaglandin E_3 from eicosapentaenoic acid in human lung cancer cells. J Lipid Res 2004; 45:1030-1039.

[35] Fan YY, Davidson LAD, Callaway ES, Goldsby JS, Chapkin RS. Differential effects of 2- and 3-series E-prostaglandins on in vitro expansion of Lgr5+ colonic stem cells. Carcinogenesis 2014; 35:606-612

[36] Wada M, DeLong CJ, Hong YH, Rieke CJ, Song I, Sidhu RS, et al. Enzymes and receptors of prostaglandin pathways with arachidonic acid-derived versus eicosapentaenoic acid-derived substrates and products. J Biol Chem 2007; 282:22254-22266.

[37] Rodríguez-Lagunas MJ, Ferrer R, Moreno JJ. Effect of eicosapentaenoic acid-derived prostaglandin E_3 on intestinal epithelial barrier function. Prostaglandins Leukotrienes Essential Fatty Acids 2013; 88:339-345.

[38] Park JM, Kanaoka Y, Eguchi N, Antake K, Gruic S, Materi AM, et al. Haematopoietic prostaglandin D synthase suppresses intestinal adenoma in ApcMin/+ mice. Cancer Res 2007;67:881-889.

[39] Bortuzzo C, Hanif R, Kashfi K, Staiano-Coico L, Shiff SJ, Rigas B. The effect of leukotrienes B and selected HETEs on the proliferation of colon cancer cells. Biochim Biophys Acta 1996; 1300:240-246.

[40] Klampfl T, Bogner E, Bednar W, Mager L, Massudom D, Kalny I, et al. Up-regulation of 12(S)-lipoxygenase induces a migratory phenotype in colorectal cancer cells. Exp cell Res 2012; 318:768-778.

[41] Guo Y, Zhang W, Giroux C, Cai Y, Ekambaram P, Dily AK, et al. Identification of the orphan G protein-coupled receptor GPR31 as a receptor for 12-(S)-hydroxyeicosatetraenoic acid. J Biol Chem 2011; 286:33832-33840

[42] Bachi AL, Kim FJ, Nonogaki S, Carneiro CR, Lopes JD, Jasiulionis MG, Correa MA. Leukotriene B_4 creates a favorable microenvironment for murine melanoma growth. Mol Cancer Res 2009;7:1417-1424.

[43] Ihara A, Wada K, Yoneda M, Fujisawa N, Takahashi H, Nakajima A. Blockade of leukotriene B_4 signaling pathway induces apoptosis and suppresses cell proliferation in colon cancer. J Pharmacol Sci 2007; 103:24-32.

[44] Ikehata A, Hiwatashi N, Kinouchi Y, Yamazaki H, Ito K, Kayaba Y, Toyota T. Effect of intravenously infused eicosapentaenoic acid on the leukotriene generation in patients with active Crohn disease. Am J Clin Nutr 1992; 56:938-942.

[45] Salomon P, Kornbluth WF, Janowitz HD. Treatment of ulcerative colitis with fish oil n-3 fatty acid: an open trial. J Clin Gastroenterol 1990; 12:157-161.

[46] Bittiner SB, Tucker WF, Cartwright I, Bleehen SS. A double blind, randomised, placebo-controlled trial of fish oil in psoriasis. Lancet 1988; 1:378-380.

[47] Heidel JR, Taylor SM, Laegreid WW, Silflow RM, Liggitt HD, Leid RW. In vivo chemotaxis of bovine neutrophils induced by 5-lipoxygenase metabolites of arachidonic acid and eicosapentaenoic acid. Am J Pathol 1989; 134:671-676.

[48] Smith WL. Cyclooxygenase, peroxide tone and the allure of fish oil. Curr Opin Cell Biol 2005; 17:174-182.

[49] Moreno JJ. Differential effects of arachidonic and eicosapentaenoic acid-derived eicosanoids on polymorphonuclear transmigration across endothelial cell cultures. J Pharmacol Exp Ther 2009; 331:1111-1117.

[50] Wallace JL, McKnight GW. Comparison of the damage-promoting effects of leukotrienes derived from eicosapentaenoic acid and arachidonic acid on the rat stomach. J Exp Med 1990;171:1827-1832.

Figure legends

Fig. 1. Effect of PGE_3 on Caco-2 cell growth. Caco-2 cells were incubated for 48 h with PGE_3 (0.1-10 nM) or PGE_3 (10 nM) plus SC19220 (SC, 60 nM) or AH 23848 (AH, 20 nM) or ONO-AE3-240 (ONO, 2 nM). Cells were then counted (A) and DNA synthesis was measured (B). Data are expressed as means ± SEM of 3-4 experiments performed in triplicate. * $P < 0.05$ *vs.* Caco-2 cell cultures in the absence of FBS, ≠ $P < 0.05$ *vs.* cells incubated with 10 nM PGE_3.

Fig. 2. Effect of PGD_2 on Caco-2 cell growth. Caco-2 cells were incubated for 48 h with PGD_2 (1-100 nM) or PGE_2 (10 nM). Cells were then counted (A) and DNA synthesis was also measured (B). Data are expressed as means ± SEM of 3-4 experiments performed in triplicate. * $P < 0.05$ *vs.* Caco-2 cell cultures in the absence of FBS.

Fig. 3. Effect of 12-S-HEPE on Caco-2 cell growth. Caco-2 cells were incubated for 48 h with 12-S-HEPE (10-1000 nM) or with 12-S-HEPE (100 nM) plus U 75302 (U, 5 µM) or LY 255283 (LY, 25 µM) or ketoprofen (Kp, 5 µM). Caco-2 cells were also incubated with 12-S-HETE (100 nM). Cells were then counted (A) and DNA synthesis was measured (B). Data are expressed as means ± SEM of 3-4 experiments performed in triplicate. * $P < 0.05$ *vs.* Caco-2 cell cultures in the absence of FBS, ≠ $P < 0.05$ *vs.* cells incubated with 12-S-HEPE (100 nM).

Fig. 4. Effect of LTB_5 on Caco-2 cell growth. Caco-2 cells were incubated for 48 h with LTB_5 (1-100 nM) or LTB_4 (10 nM). Cells were then counted (A) and the uptake of [^{3}H]thymidine incorporation was also measured (B). Data are expressed as means ± SEM of 3-4 experiments performed in triplicate. * $P < 0.05$ *vs.* Caco-2 cell cultures in the absence of FBS.

Fig. 5. Effect of PGE_3 on cell signalling. Caco-2 cells were incubated with PGE_2 or PGE_3 (10 nM) for 5 or 15 min, cells were then collected and finally phosphorylated. ERK 1/2, CREB, GSKβ, p38α, Akt1 and Akt2 were measured as described in the Material and methods section. Data are expressed as means ± SEM of 2-4 experiments performed in triplicate. * $P < 0.05$ *vs.* Caco-2 cell cultures in the absence of FBS.

Table 1. Effect of EPA on Caco-2 cell growth and DNA synthesis

	Cells (x 10^3/cm^2)	DNA synthesis (OD 450 nm)
Control	30.58 ± 1.04	0.29 ± 0.02
Control + MK886 10 µM	28.95 ± 1.76	0.27 ± 0.03
Control + LY255283 25 µM	28.12 ± 2.02	0.26 ± 0.05
EPA 1 µM	35.71 ± 2.27	ND
EPA 5 µM	46.62 ± 2.08*	ND
EPA 10 µM	49.26 ± 2.56*	0.69 ± 0.04*
EPA 15 µM	39.63 ± 1.78*	ND
EPA 25 µM	28.67 ± 1.72	ND
EPA 50 µM	24.56 ± 3.42*	ND
EPA 100 µM	25.90 ± 2.20*	ND
EPA 10 µM + Kp 5 µM	32.24 ± 2.53≠	0.41 ± 0.03≠
EPA 10 µM + MK886 10 µM	29.60 ± 1.24≠	0.23 ± 0.01≠
EPA 10 µM + baicalein 25 µM	21.76 ± 1.36≠	0.27 ± 0.02≠
EPA 10 µM + SC19220 60 nM	39.81 ± 7.48	0.52 ± 0.03
EPA 10 µM + AH23838 20 nM	31.83 ± 2.56≠	0.55 ± 0.03
EPA 10 µM + U75302 5 µM	34.61 ± 4.33	0.57 ± 0.02
EPA 10 µM + LY255283 25 µM	27.45 ± 3.10≠	0.37 ± 0.02≠
EPA 10 µM + MK571 10 µM	32.61 ± 1.68≠	0.43 ± 0.02≠

Caco-2 cells were incubated for 48 h with the treatments. Then, cells were counted and DNA synthesis was measured. Data are expressed as means ± SEM of 2-5 experiments performed in triplicate. ND, non-determined. * $P < 0.05$ *vs.* control group (cells incubated in the absence of FBS), ≠ $P < 0.05$ *vs.* cells incubated with EPA 10 µM.

Figure 1

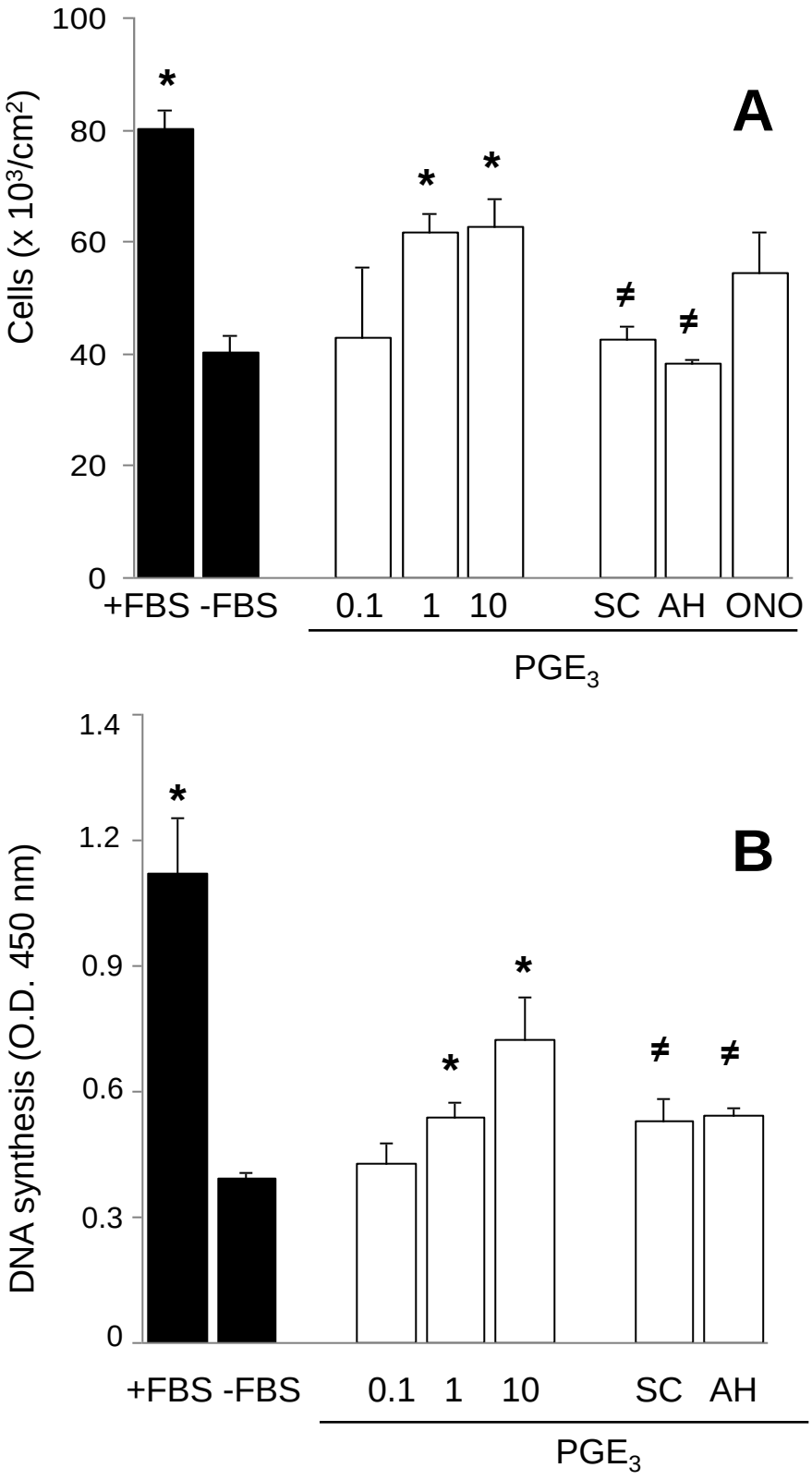

A
Cells (x 10³/cm²)
100
80
60
40
20
0
*
*
*
≠
≠
+FBS
-FBS
0.1
1
10
SC
AH
ONO
PGE3
B
DNA synthesis (O.D. 450 nm)
1.4
1.2
0.9
0.6
0.3
0
*
*
*
≠
≠
+FBS
-FBS
0.1
1
10
SC
AH
PGE3

Figure 2

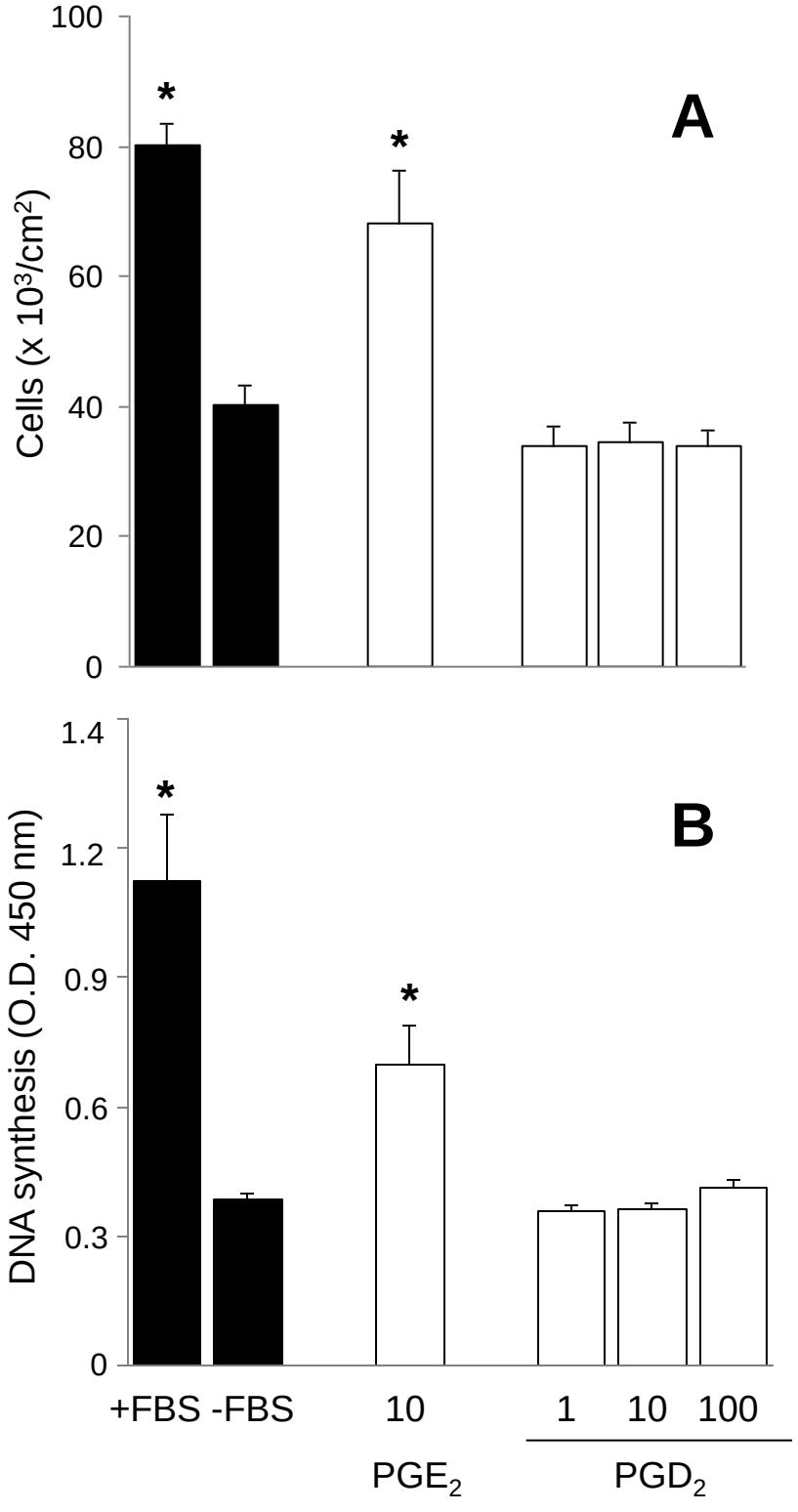

A
B
Cells (x 10³/cm²)
DNA synthesis (O.D. 450 nm)
100
80
60
40
20
0
1.4
1.2
0.9
0.6
0.3
0
*
+FBS
-FBS
10
1
10
100
PGE2
PGD2

Figure 3

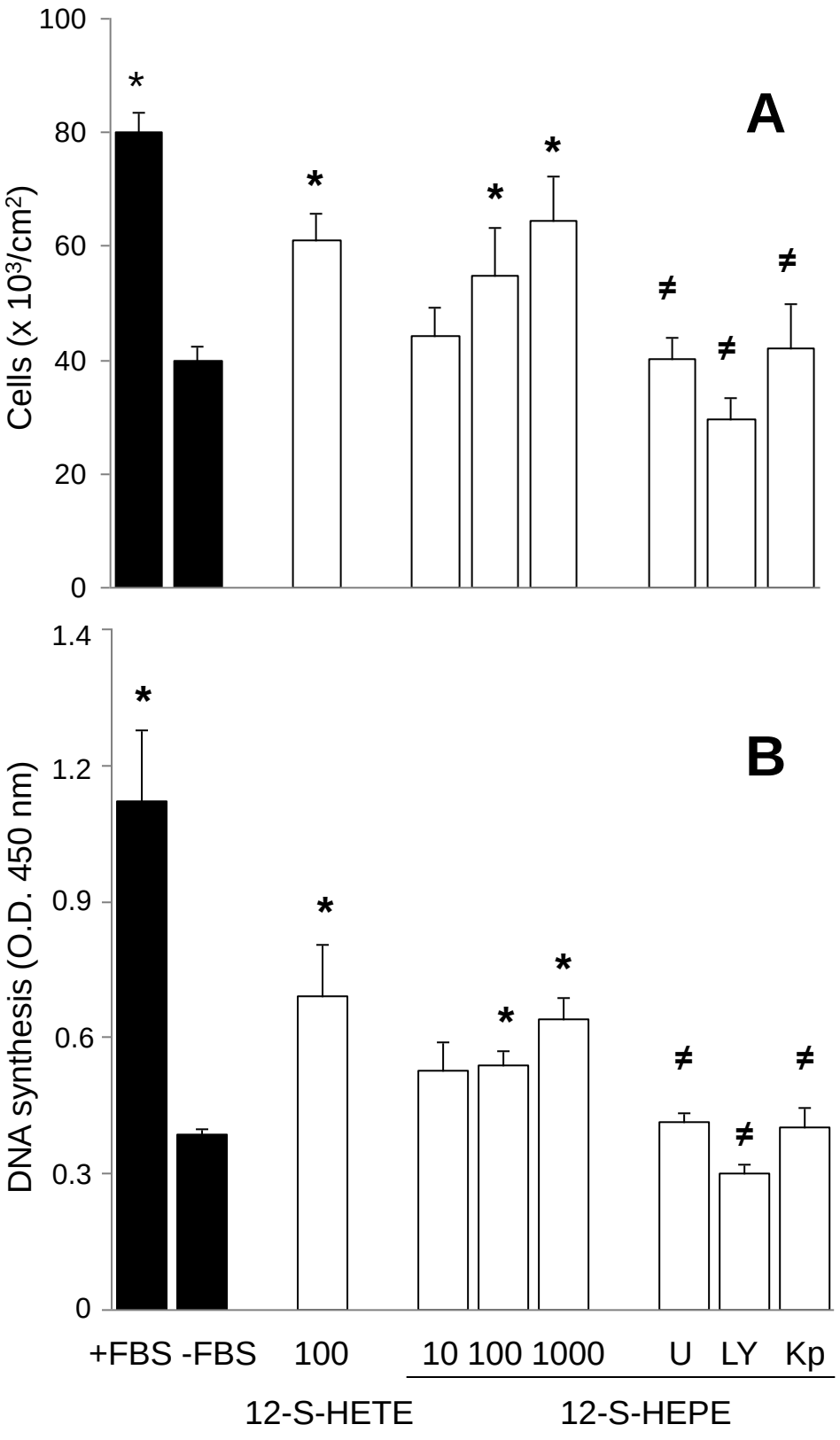

A
B
Cells (x 10³/cm²)
DNA synthesis (O.D. 450 nm)
100
80
60
40
20
0
1.4
1.2
0.9
0.6
0.3
0
*
≠
+FBS
-FBS
100
10
100
1000
U
LY
Kp
12-S-HETE
12-S-HEPE

Figure 4

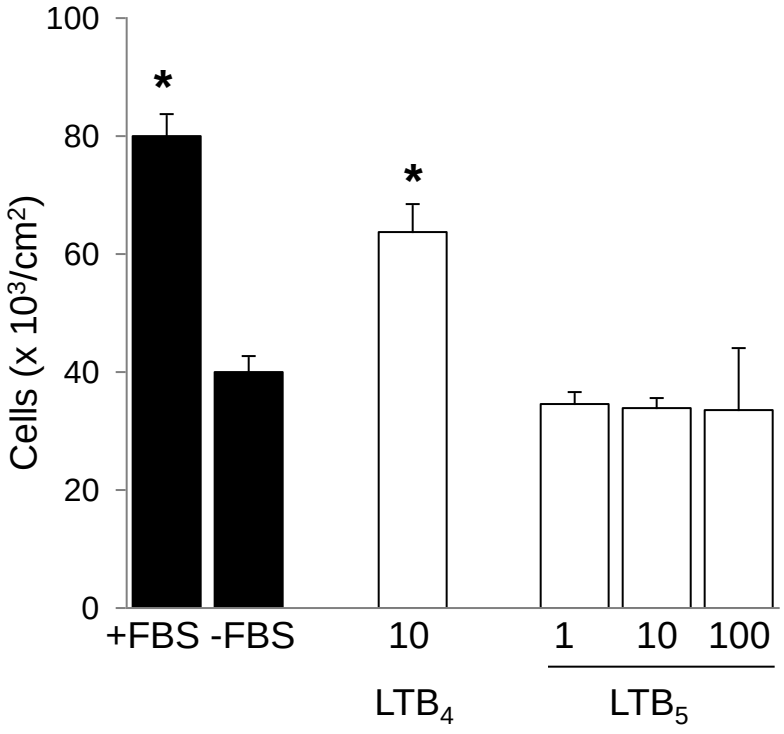
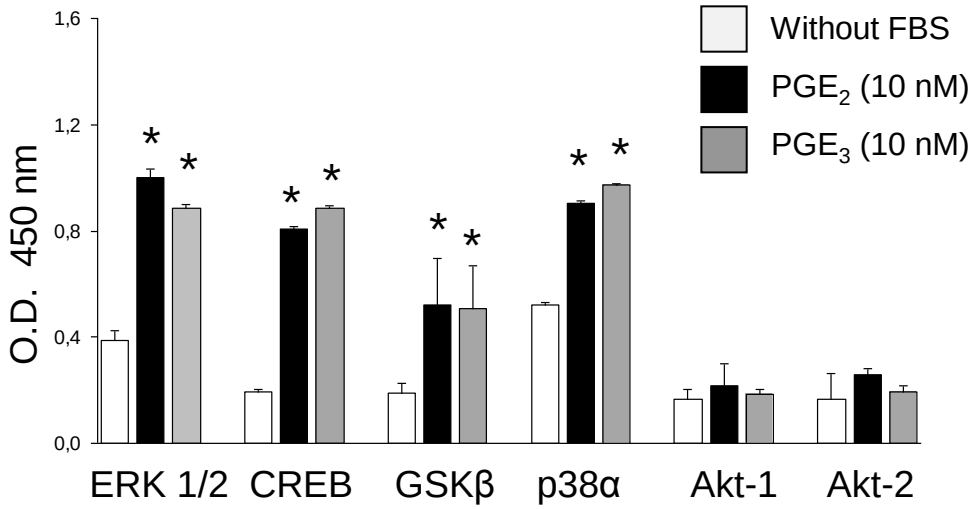


Figure 5


1,6
1,2
0,8
0,4
0,0
O.D. 450 nm
Without FBS
PGE2 (10 nM)
PGE3 (10 nM)
*
ERK 1/2
CREB
GSKβ
p38α
Akt-1
Akt-2