available at www.sciencedirect.com

ScienceDirect

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

Role of 5-lipoxygenase pathway in the regulation of RAW 264.7 macrophage proliferation

Diana Nieves, Juan José Moreno *

Department of Physiology, Faculty of Pharmacy, University of Barcelona, Avda. Joan XXIII s/n, E-08028 Barcelona, Spain

ARTICLE INFO

Article history:
Received 31 March 2006
Accepted 24 July 2006

Keywords:
Leukotriene
Leukotriene receptor
Cell growth
Cell cycle
Apoptosis

Abbreviations:
AA, arachidonic acid
LOX, lipoxygenase
NDGA, nordihydroguaiaretic acid
LT, leukotriene
MAPK, mitogen-activated protein kinase
PI3K, phosphatydilinositol 3-kinase
PG, prostaglandin
COX, cyclooxygenase
HETE, hydroxyeicosatetraenoic acid
CysLT, cysteinyl leukotriene
CysLTR, cysteinyl leukotriene receptor
BLT, LTB_4 receptor
EGF, epidermal growth factor
DMEM, Dulbecco's modified Eagle's medium
FBS, foetal bovine serum
FACS, fluorescent-activated cell sorting

ABSTRACT

Arachidonic acid (AA) metabolites control cell proliferation, among other physiologic functions. RAW 264.7 macrophages can metabolise AA through the cyclooxygenase and lipoxygenase (LOX) pathways. We aimed to study the role of AA-metabolites derived from 5-LOX in the control of RAW 264.7 macrophage growth. Our results show that zileuton, a specific 5-LOX inhibitor, and nordihydroguaiaretic acid (NDGA), a non-specific LOX inhibitor, inhibit cell proliferation and [^{3}H]-thymidine incorporation in a concentration-dependent fashion. Growth inhibition induced by NDGA can be explained by an apoptotic process, while zileuton does not seem to induce apoptosis. Moreover, these treatments delay the cell cycle, as analysed by flow cytometry. On the other hand, the leukotriene (LT) B_4 receptor antagonist U-75302, the LTD_4 receptor antagonists LY-171883 and MK-571, and the cysteinyl-LT receptor antagonist REV-5901 also inhibit cell proliferation and [^{3}H]-thymidine incorporation in a concentration-dependent manner, and delay the RAW 264.7 cell cycle. However, these antagonists did not induce annexin V staining, caspase activation or DNA fragmentation. Furthermore, we demonstrated that exogenous addition of LTB_4 or LTD_4 revert the cell growth inhibition induced by zileuton or the leukotriene receptor antagonists mentioned above. Finally, we observed that LTB_4 and LTD_4, in the absence of growth factors, have pro-proliferative effects on macrophages, and we obtained preliminary evidences that this effect could be through mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways. In conclusion, our results show that the interaction between LTB_4 and LTD_4 with its respective receptor is involved in the control of RAW 264.7 macrophage growth.

* *Corresponding author*. Tel.: +34 93 4024505; fax: +34 93 4035901.
E-mail address: jjmoreno@ub.edu (J.J. Moreno).
0006-2952/$ – see front matter
doi:10.1016/j.bcp.2006.07.021

1. Introduction

Arachidonic acid (AA) is usually esterified in the glycerophospholipids of membranes. Upon stimulation, it is released by selective lipases, and free intracellular AA can be oxidised by the enzymes of the AA cascade: the cyclooxygenases (COXs), which produce prostaglandins (PGs) and thromboxanes; the lipoxygenases (LOXs), which form leukotrienes (LTs), hydroxyeicosatetraenoic acids (HETEs) and lipoxins, and cytochrome P-450 monooxygenases. 5-, 12- and 15-LOX are considered the main LOXs catalyzing the biosynthesis of biologically active compounds. Thus, AA is metabolised by 5-LOX yielding 5-HETE and LTA_4, the precursor of LTB_4 and cysteinyl leukotrienes (CysLTs) such as LTC_4, LTD_4 and LTE_4. Finally, 12- and 15-LOX produce HETEs and lipoxins [1].

In macrophages, AA is mainly metabolised through COXs and LOXs enzymes, which have been described in this cell type [2]. 5-, 12- and 15-LOXs are expressed in murine macrophages [3–5]. Here, we focused on 5-LOX, the key enzyme of leukotriene biosynthesis, and its role in macrophage proliferation.

The biological effects of LTs depend on its interaction with specific receptors. Four leukotriene receptors that belong to the G protein-coupled seven transmembrane domain receptor family have been characterised: two receptors for LTB_4 (BLTs) and two receptors for $LTC_4/D_4/E_4$ (CysLTRs). BLT1 is a high-affinity receptor specific for LTB_4, whereas BLT2 is a low-affinity receptor that also binds other eicosanoids [6]. BLT2 is expressed ubiquitously, in contrast to BLT1, which is expressed predominantly in leukocytes. Pharmacological studies have determined that CysLTs activate at least two receptors, referred to as CysLTR1 and CysLTR2. In macrophages, the main receptor expressed is the former, which preferentially responds to LTD_4 [7].

Cell growth is controlled by mitogens like growth factors. The interaction of growth factors with their cell surface receptors leads to multiple signalling events including the activation of several phospholipases, which in turn can lead to the release of AA and the production of AA metabolites. Thus, epidermal growth factor (EGF) induces LOX metabolism in A431 cells, HeLa cells and fibroblasts [8], and LTB_4 synthesis in guinea pig gastric chief cells [9]. LOX metabolites are also involved in basic fibroblast growth factor-induced endothelial cell proliferation [10]. In addition, LTs and HETEs have recently been described as survival factors. Thus, there is evidence that 12-LOX regulates the serum-supported survival of W256 carcinosarcoma cells of monocytoid origin [11]. Moreover, Ghosh and Myers [12] observed that only 5-LOX inhibitors produce apoptosis in human prostate cancer cells, while 12-LOX, COX and cytochrome P-450 inhibitors do not induce programmed cell death. Thus, a number of studies have suggested that 5-LOX is involved in cell proliferation, although its effect on cell growth is cell-type specific. Thus, the 5-LOX pathway appears to facilitate the growth of several cell types such as pulmonary artery endothelial cells [13] and mesangial cells [14]. In contrast, it suppresses the growth of other cells, e.g. glioma cells [15] and murine Leidig cells [16].

Little is known about the role of the 5-LOX pathway in the proliferation of macrophages. Recently, Titos et al. [17] suggested that 5-LOX pathway metabolites participate in the regulation of Kupffer cell growth and survival in a model of liver fibrosis. The aim of this study was to explore the effects of 5-LOX pathway metabolites on serum stimulated RAW 264.7 macrophage proliferation. Our results suggest that LTB_4 and LTD_4 interaction with its specific receptors, are involved in RAW 264.7 cell cycle progression and serum-induced macrophage proliferation. Moreover, we show that the inhibition of macrophage proliferation by 5-LOX pathway inhibition was not consequence of a pro-apoptotic effect. Furthermore, we show that LTB_4 and LTD_4 have mitogenic effects in absence of growth factors, suggesting the involvement of mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways in these events.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS), penicillin, streptomycin and versene-EDTA were purchased from BioWhittaker Europe (Verviers, Belgium). Nordihydroguaiaretic acid (NDGA), zileuton, LTB_4, LTD_4, PD098059, wortmannin, propidium iodide, Triton X-100, ribonuclease A from bovine pancreas, acridine orange, ethidium bromide and Bay u9773 were provided by Sigma Chemical Co. (St. Louis, MO, USA). Leukotriene antagonist receptors U-75302, LY-171883, MK-571 and REV-5901 were supplied by Cayman Chemical Co. (Ann Arbor, MI, USA). [Methyl-^{3}H]thymidine (20 Ci/mmol) was from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA). All chemicals were of the highest quality available commercially.

2.2. Cell culture

Murine RAW 264.7 macrophages (ATCC TIB-71) were cultured in DMEM supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were seeded in 24-well plates (tissue-culture cluster 24; Costar Cambridge, MA, USA) or in tissue-culture 100 mm-dishes (Costar), and allowed to adhere at 37 °C in 5% CO_2/95% air for 24 h. Finally, cells were re-seeded by scraping off with versene-EDTA.

2.3. Cell growth assay

The effect of the treatments was assessed on macrophages plates at 25×10^3 cells/well in 24-well plates, cultured for 24 h in DMEM supplemented with 10% FBS. After 24 h of serum starvation, cells were incubated for 48 h in 10% FBS medium in the presence of various compounds. Finally, the medium was aliquoted to determine floating cells and attached cells were scraped off with versene-EDTA. Cells were counted under a microscope using ethidium bromide/acridine orange staining to assess viability.

2.4. Analysis of DNA synthesis

DNA synthesis was measured by a [^{3}H]-thymidine incorporation assay, which involved culturing macrophages in

24-well plates in DMEM with 10% FBS at a density of 25×10^3 cells/well. After 24 h, serum starvation cells were incubated for 48 h with the treatments and [^{3}H]-thymidine (0.25 μCi/well) was added for the last 24 h. [^{3}H]-thymidine-containing media were aspirated, cells were overlaid with 1% Triton X-100 and then scraped off the dishes. Finally, the radioactivity present in the cell fraction was measured by scintillation counting, using a Packard Tri-Carb 1500 counter.

2.5. *Fluorescent-activated cell sorting (FACS) analysis/flow cytometry cell cycle analysis*

Macrophages were seeded in 100 mm-dishes and 24 h later, they were serum starved. After 24 h without FBS, the percentage of cells in G_0/G_1 was about 80%. Cells were then cultured in 10% FBS–DMEM containing the treatments. Thereafter, they were scraped, fixed with 70% ethanol and stored at 4 °C for at least 2 h. Next, low-molecular weight DNA was extracted from 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 analysed on an Epics XL flow cytometer (Coulter Corporation, Hialeah, FL). DNA was analysed (Ploidy analysis) on single fluorescence histograms by Multicycle software (Phoenix Flow Systems, San Diego, CA).

2.6. *Measurement of LTB_4*

Cells were cultured in plates at 25×10^3 cells/well in 24-well plates for 24 h in DMEM supplemented with 10% FBS. After 24 h of serum starvation, cells were incubated for 48 h in 10% FBS medium in the presence of several products. An aliquot of culture supernatant medium was analysed by an enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, MI, USA) following the manufacturer's protocol.

2.7. *Measurement of annexin V binding*

RAW 264.7 macrophages were cultivated in media containing 10% FBS with treatments for 48 h at 37 °C. Thereafter, treatments were removed and cells were stained with fluorescein-isothiocyanate-labelled annexin V and propidium iodide following the manufacturer instructions (Roche Diagnostics, Mannheim, Germany). Representative samples were viewed with an inverted fluorescence microscope system (Nikon Eclipse TE200) linked to a CCD camera.

2.8. *Caspase activity determination*

RAW 264.7 macrophages were cultivated in media containing 10% FBS with treatments for 48 h at 37 °C. Thereafter, treatments were removed and cells were stained with FITC-VAD-FMK, which binds to activated caspases in apoptotic cells, following the manufacturer instructions (Medical and Biological Laboratories, Nagoya, Japan). Samples were viewed with an inverted fluorescence microscope system (Nikon Eclipse TE200) linked to a CCD camera, and caspase positive or negative cells were counted.

2.9. *Measurement of DNA fragmentation*

Degradation of chromosomal DNA was evaluated with TUNEL method. RAW 264.7 macrophages were cultivated in media containing 10% FBS with treatments for 48 h. Next, cells were fixed with 4% paraformaldehyde and permeabilized with 70% ethanol. Thereafter, 3′-OH DNA ends generated by DNA fragmentation were labelled with fluorescein-dUTP, mediated by terminal deoxynucleotidyl transferase, and were analysed on a Epics XL flow cytometer (Coulter Corporation, Hialeah, FL).

2.10. *Data analysis*

Results are expressed as mean ± S.E.M. Differences between control and treated cultures were tested using Student's *t*-test.

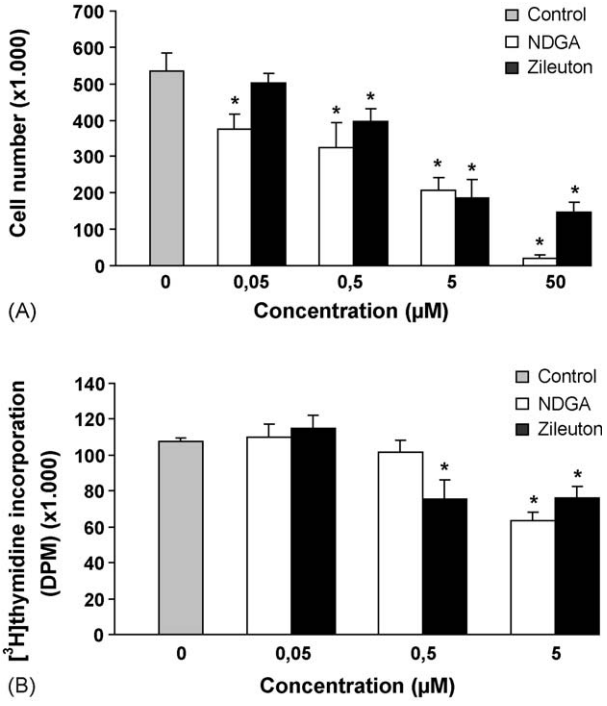

Fig. 1 – Effect of 5-LOX inhibitors on 10% FBS-induced macrophage proliferation and [^{3}H]-thymidine incorporation. Macrophages (25×10^3 cells/well) were plated and cultured in 10% FBS–DMEM. The next day, media were removed and free FBS–DMEM was added for 24 h. Cells were then incubated with 10% FBS–DMEM or with 10% FBS–DMEM containing NDGA or zileuton. After a 2-day treatment, cells were scraped off and counted (A), or the last 24 h [^{3}H]-thymidine (1 μCi/well) was added and radioactivity in cell lysates was determined in a scintillation analyser (B). Results are means ± S.E.M. from three experiments performed in triplicate. $^*P < 0.05$ compared with non-treated cells. Baseline levels of cell number and [^{3}H]-thymidine incorporation in serum-free medium were 56.256 ± 1.725 cells and 6.182 ± 167 dpm, respectively.

3. Results

3.1. Effect of LOX inhibitors on growth, DNA synthesis and cell cycle distribution of RAW 264.7 macrophages

To determine the role of 5-LOX metabolites in macrophage growth induced by FBS, cells were exposed to the specific 5-LOX inhibitor zileuton [18] (0.05–50 μM). We compared the effect of zileuton with a non-selective LOX inhibitor as NDGA [19] (0.05–50 μM). As shown in Fig. 1A, both compounds significantly inhibited cell growth in a concentration-dependent manner. Ethidium bromide/acridine orange staining and morphologic examination revealed that the LOX inhibitors did not affect cell structure or cell viability (data not shown). Only 50 μM NDGA showed an appreciable reduced cell viability.

The effect of LOX inhibitors on RAW 264.7 growth was also examined by [^{3}H]-thymidine uptake. Serum strongly increases the rate of [^{3}H]-thymidine incorporation in RAW 264.7 macrophage cultures. Zileuton and NDGA induced a concentration-dependent inhibition of [^{3}H]-thymidine uptake by macrophages after 48 h of exposure (Fig. 1B).

Finally, we quantified cell cycle changes induced by LOX inhibitors by flow cytometry. RAW 264.7 macrophage cultures that had been G_0/G_1 synchronised by 24 h serum starvation were incubated with 10% FBS-medium containing zileuton (5 μM) or NDGA (5 μM). Cells were then collected and stained with propidium iodide and cell cycle distribution was analysed. Zileuton raises the percentage of G_0/G_1 population and reduces the percentage of cells in S phase at 24 and 36 h (Table 1). Thus, 5-LOX inhibition appears to induce an appreciable cell cycle delay. In contrast, NDGA at 24 h increases the percentage of cells in G_2/M phase and decreases the number of cells in G_0/G_1 phase, while at 36 h, the percentage of cells in each cell cycle phase is closer to control values.

In parallel, we have confirmed that zileuton and NDGA inhibit LTB_4 production induced by 10% FBS in RAW 264.7 cell culture macrophages. Thus, control culture medium presents 209.9 ± 30.5 pg LTB_4/ml, whereas macrophages incubated with zileuton (5 μM) and NDGA (5 μM) present 15.2 ± 1.1 and 10.7 ± 1.5 pg LTB_4/ml, respectively.

Table 1 – Effect of LOX inhibitors and leukotriene receptor antagonists on cell cycle distribution in RAW 264.7 cultures

	Percentage of cells		
	G_0/G_1	S	G_2/M
Time 24 h			
Control	59.1 ± 0.38	31.7 ± 1.90	9.2 ± 1.58
Zileuton	$65.0 \pm 0.12^*$	27.0 ± 0.20	8.0 ± 0.32
NDGA	$47.9 \pm 2.26^*$	31.3 ± 0.57	$20.8 \pm 2.64^*$
U-75302	$63.5 \pm 0.10^*$	29.5 ± 0.13	7.0 ± 0.21
LY-171883	$67.2 \pm 0.35^*$	$25.5 \pm 0.55^*$	7.4 ± 0.25
Time 36 h			
Control	56.2 ± 1.09	37.2 ± 1.36	6.6 ± 0.85
Zileuton	58.1 ± 0.60	$30.5 \pm 1.10^*$	11.4 ± 1.70
NDGA	57.4 ± 0.85	30.9 ± 1.55	$11.8 \pm 0.70^*$
U-75302	$61.4 \pm 0.50^*$	33.7 ± 1.40	4.9 ± 0.95
LY-171883	$61.4 \pm 0.85^*$	$30.0 \pm 0.20^*$	8.7 ± 0.65

Cells were cultured with DMEM–FBS 10% in presence of zileuton 5 μM, NDGA 5 μM, U-75302 10 μM or LY-171883 100 μM for 24 or 36 h. Finally, they were harvested and fixed with ethanol, and DNA was stained with propidium iodide. DNA content was analysed by FACS. Data are expressed as percentage of cells in the G_0/G_1, S and G_2/M phases of cell cycle. Data are the means ± S.E.M. of three experiments performed in duplicate. $^*P < 0.05$ compared with non-treated cells.

3.2. Effect of leukotriene receptor antagonists on growth, DNA synthesis and cell cycle distribution of RAW 264.7 macrophages

To provide further evidences of the role of 5-LOX pathway on RAW 264.7 macrophage proliferation, we used leukotriene receptor antagonists such as LY-171883 and MK-571 as LTD_4 receptor antagonists [20,21], U-75302 as LTB_4 receptor antagonist [22] and REV-5901 as CysLTs receptor antagonist [23]. Treatment with these specific antagonists and Bay u9773, a CysLTR1 and CysLTR2 antagonist [24], reduced cell number in

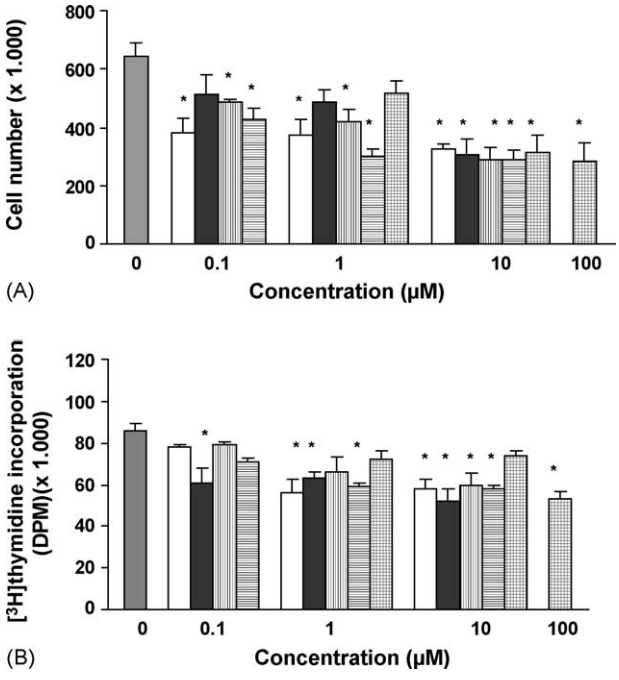

Fig. 2 – Effect of leukotriene receptor antagonists on 10% FBS-induced macrophage proliferation and [^{3}H]-thymidine incorporation. Cells (25×10^3 cells/well) were plated and cultured in 10% FBS–DMEM. The next day, media were removed and free FBS–DMEM was added for 24 h. Cells were then incubated with 10% FBS–DMEM or with 10% FBS–DMEM (grey bars) containing LY-171883 (square bars), MK-571 (black bars), U-75302 (white bars), REV-5901 (vertical line bars) and bay u9773 (horizontal line bars). After a 2-day treatment, cells were scraped off and counted (A) or [^{3}H]-thymidine (1 μCi/well) was added for the last 24 h and radioactivity in cell lysates was determined in a scintillation analyser (B). Results are means ± S.E.M. from three experiments performed in triplicate. $^*P < 0.05$ compared with non-treated cultures.

a concentration-dependent manner. Thus, the treatments produced a maximum growth inhibition around of 50% for LY-171883 (100 μM), MK-571 (10 μM), U-75302 (10 μM), REV-5901 (10 μM) and Bay u9773 (1 μM), respectively (Fig. 2A). Similar data were obtained with the leukotriene receptor antagonists on [^{3}H]-thymidine incorporation by macrophages, although the effect was not as strong as in cell growth inhibition (Fig. 2B). Macrophages were also exposed to the receptorial antagonists to determine the effect of treatments on cell cycle progression. Table 1 shows that LTD_4 receptor antagonist (LY-171883, 100 μM) and LTB_4 receptor antagonist (U-75302, 10 μM) at 24 and 36 h markedly increased the number of cells in G_0/G_1 phase and impaired the percentage of cells in S phase. Moreover, to demonstrate the specific effect of zileuton and leukotriene receptor antagonists on cell proliferation, we added exogenously the corresponding treatments in presence of LTB_4 or LTD_4. Thus, our results show that zileuton effects on cell growth and DNA synthesis were reverted by LTB_4 and LTD_4 (Fig. 3A). In a similar way, the inhibitory effect of U-75302 and

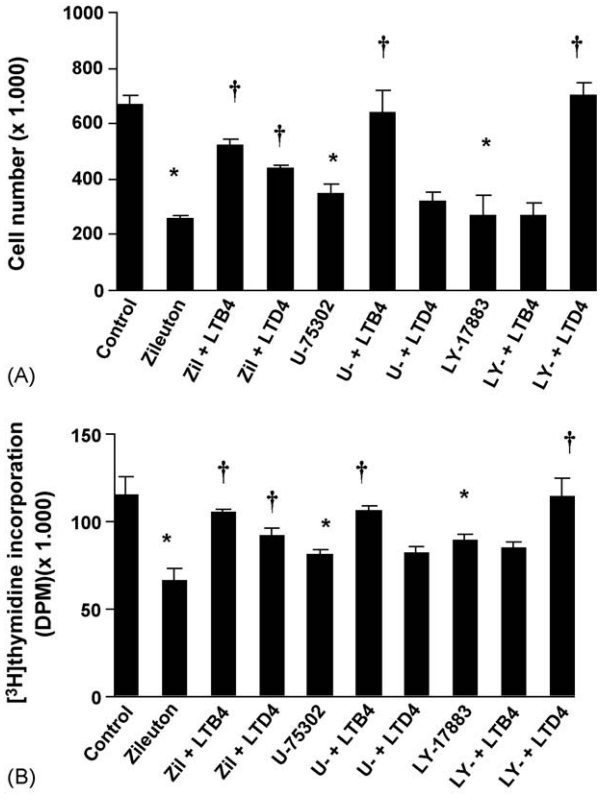

Fig. 3 – Effect of exogenous LTB_4/LTD_4 on cell proliferation and [^{3}H]-thymidine incorporation inhibited by zileuton or leukotriene receptor antagonists. Cells were treated for 48 h with zileuton (5 μM), U-75302 (1 μM) or LY-171883 (10 μM), or the inhibitor/antagonists in the presence of 0.01 μM LTB_4 or 0.1 μM LTD_4. Cells were scraped off and counted (A) or [^{3}H]-thymidine (1 μCi/well) was added to the cells for the last 24 h and radioactivity in cell lysates was determined in a scintillation analyser (B). Results are means ± S.E.M. from three experiments performed in triplicate. $^*P < 0.05$ compared with non-treated cultures. $^\dagger P < 0.05$ compared with cells treated only with the antagonist.

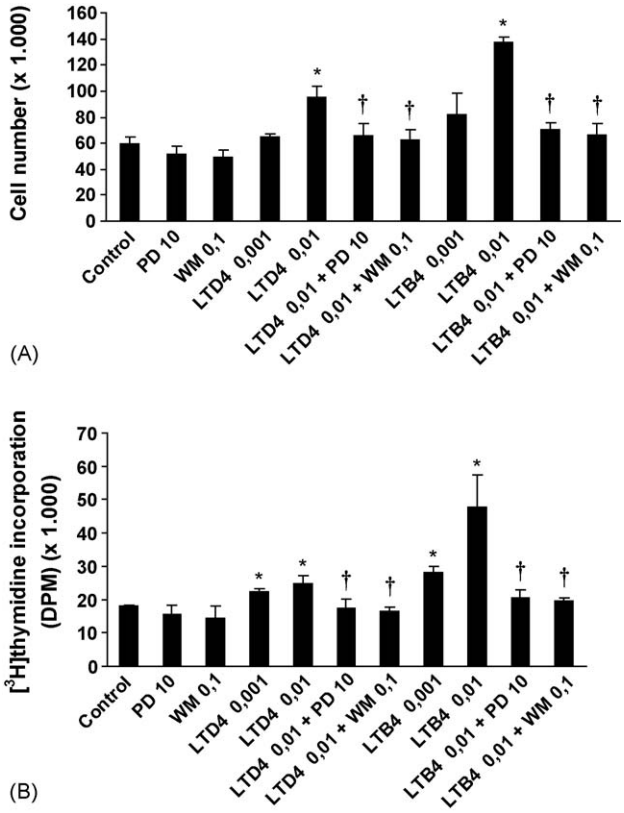

Fig. 4 – Mitogenic effects of LTD_4 and LTB_4 in absence of growth factors. Cells were incubated for 48 h with the leukotrienes at 0.001 and 0.01 μM in medium without fetal bovine serum, or leukotrienes at 0.01 μM and PD098059 (PD) 10 μM or wortmannin (WM) 0.1 μM. Cells were scraped off and counted (A) or [^{3}H]-thymidine (1 μCi/well) was added to the cells for the last 24 h and radioactivity in cell lysates were determined in a scintillation analyser (B). Data are means ± S.E.M. from three experiments performed in triplicate. $^*P < 0.05$ compared with the control group. $^\dagger P < 0.05$ compared with cells treated with LT at 0.01 μM.

LY-171883 on cell growth and [^{3}H]-thymidine uptake was also reverted by exogenous addition of LTB_4 or LTD_4, respectively (Fig. 3B).

The above results suggest that LTB_4 and CysLTs such as LTD_4 are involved in the signal transduction pathways induced by FBS, and that these eicosanoids can be regarded as co-mitogenic factors. To determine the mitogenic effect of 5-LOX pathway metabolites on RAW 264.7 macrophages, we performed the assays in absence of serum. LTB_4 and LTD_4 (0.001–0.01 μM) significantly increased [^{3}H]-thymidine uptake in our experimental conditions (Fig. 4B). LTB_4 was more mitogenic than LTD_4. Thus, LTB_4 (0.01 μM) doubled [^{3}H]-thymidine uptake. Since DNA synthesis can occur without cell division, the effects of LTs/CysLTs on RAW 264.7 growth were also determined. LTB_4 and LTD_4 also enhanced cell proliferation (Fig. 4A). Thus, LTB_4 (0.01 μM) approximately doubled cell numbers and LTD_4 (0.01 μM) also significantly increase cell growth although less than LTB_4. Next, we have checked the

Table 2 – Effect of LOX inhibitors and leukotriene receptor antagonists on apoptosis in RAW 264.7 macrophage cultures

Treatment	Apoptosis		
	Annexin V^+/PI^- (early apoptosis)	Annexin V^+/PI^+ (late apoptosis/necrosis)	Annexin V^-/PI^+ (necrosis)
Control	2.0 ± 0.26	3.6 ± 0.32	0.8 ± 0.14
Staurosporine (1 μM)	5.8 ± 0.84*	93.1 ± 0.99*	1.1 ± 0.37
NDGA (5 μM)	1.8 ± 0.58	7.5 ± 0.65*	1.3 ± 0.41
NDGA (50 μM)	12.1 ± 1.88*	81.1 ± 2.99*	6.8 ± 1.60*
Zileuton (5 μM)	3.5 ± 0.52	7.7 ± 1.37	2.3 ± 0.47
Zileuton (50 μM)	1.4 ± 0.17	6.8 ± 0.63*	5.4 ± 0.83*
REV-5901 (10 μM)	0.2 ± 0.14	0.7 ± 0.22	0.3 ± 0.11
MK-571 (10 μM)	2.0 ± 0.51	6.3 ± 1.63	2.0 ± 0.67
LY-171883 (100 μM)	1.7 ± 0.40	6.7 ± 0.62*	1.3 ± 0.22
U-75302 (10 μM)	1.0 ± 0.00	1.7 ± 0.11	0.1 ± 0.11

Cells were incubated for 48 h with DMEM–FBS 10% in the presence of NDGA, zileuton, REV-5901, MK-571, LY-171883 or U-75302. Apoptosis was measured by annexin-V and PI staining. Staurosporine was used as a positive control. Data are expressed in percentages and are means ± S.E.M. from three experiments performed in triplicate. *$P < 0.05$ compared with non-treated cells.

metabolic pathways involved on this mitogenic effect. Thus, Fig. 4A and B shows that the MAPK kinase inhibitor PD98059 [25] inhibits cell proliferation and [^{3}H]-thymidine incorporation induced by LTB_4 and LTD_4. In the same way, the PI3K inhibitor wortmannin [26] also reduced cell growth and [^{3}H]-thymidine uptake induced by LTB_4 and LTD_4 on RAW 264.7 (Fig. 4A and B).

3.3. *Effect of LOX inhibitors and leukotriene receptor antagonists on RAW 264.7 macrophage apoptosis*

Previous studies have shown that 5-LOX inhibitors induce apoptosis. To test whether 5-LOX inhibitors or leukotriene receptorial antagonists decrease RAW 264.7 proliferation by induction of apoptosis, we measured annexin V and propidium iodide staining in these cells. The inside–outside translocation of phosphatidylserine from the plasma membrane is accentuated in the early stage of apoptosis. Annexin V staining was performed to determine this translocation. Zileuton and LTs/CysLT receptor antagonists did not markedly raise annexin V staining (Table 2), whereas NDGA (50 μM) significantly increased the percentage of annexin V-positive or annexin V- and propidium iodide-positive cells, suggesting that it induces apoptosis. These effects were correlated with the caspase activation and the DNA fragmentation. Thus, zileuton and LTs/CysLT receptor antagonist did not induce caspase activation, whereas cell treated with NDGA showed marked caspase activation (Fig. 5). Finally, we observed that zileuton and LTs/CysLT receptor antagonists did not induce an appreciable DNA fragmentation, whereas NDGA induced a marked DNA fragmentation (Fig. 5).

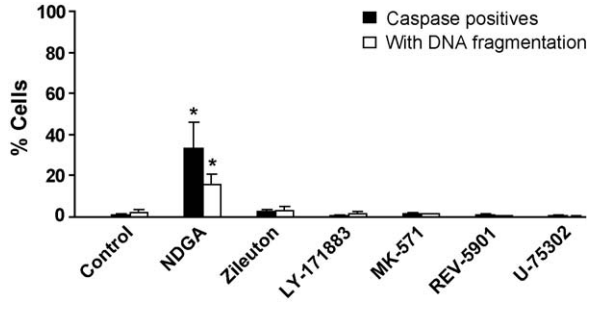

Fig. 5 – Effect of 5-LOX inhibitors and leukotriene receptor antagonists on caspase activation and DNA fragmentation in RAW 264.7 macrophage cultures. Macrophages were incubated for 48 h with DMEM–FBS 10% in the presence of NDGA 50 μM, zileuton 50 μM, LY-171883 100 μM, MK-571 10 μM, REV-5901 10 μM or U-75302 10 μM. Next, activated caspases were labelled and detected by fluorescence microscope. On the other hand, DNA ends derived from chromatin fragmentation were labelled and measured by flow cytometry. Results are means ± S.E.M. from three experiments performed in triplicate. *$P < 0.05$ compared with non-treated cultures.

4. Discussion

AA release and eicosanoid syntheses are early events of the mitogenic process induced by growth factors and subsequent cell proliferation. Thus, we observed that PGs and PGs interaction with its receptors are involved in the cell cycle progression and cell growth of 3T6 fibroblasts [27]. However, these cells failed to produce appreciable amounts of LTs. To gain more insight into the role of the 5-LOX pathway in the regulation of the mitogenic process, we examined the response of macrophages to 5-LOX inhibitors and leukotriene receptor antagonists, since these cells are able to produce LTB_4 and CysLTs [28].

There are four main structurally unrelated inhibitors of 5-LOX: redox inhibitors as NDGA, iron ligand inhibitors as BW 755C or BW A4C, AA analogs such as AA-861 or 5,8,11,14 eicosatetraynoic acid and N-hydroxyurea derivatives such as zileuton. NDGA is not selective to 5-LOX because inhibits 12-LOX [29] and 15-LOX [30] and other redox enzymes such as cyclooxygenases [31] and cytochromes P-450 [32] that are involved in AA metabolism. However, zileuton was not reported to affect these enzymes. Thus, zileuton appears a more specific 5-LOX inhibitor.

Our results shown that zileuton hinders cell growth and [^{3}H]-thymidine uptake, as well as LTB_4 synthesis in RAW 264.7 macrophage cultures. This treatment also appears to delay the RAW 264.7 macrophage cell cycle accumulating cells in G_0/G_1

phase. Thus, if a given response is inhibited by zileuton, the catalytic activity of 5-LOX pathway appears involved in RAW 264.7 macrophage proliferation. These results agree with Vargaftig and Singer [33], who suggested that zileuton reduces the fibroblast-like cell proliferation induced by interleukin-13.

Once secreted extracellularly, LTs act on specific receptors, and several studies have pointed to the presence of LTB_4 receptors [6] and CysLTRs [34] in macrophages. Thus, we aimed to elucidate the effect of LTs interaction with its receptors on the control of serum-induced RAW 264.7 macrophage progression through the cell cycle and subsequent proliferation.

REV-5901 inhibited RAW 264.7 macrophage proliferation and [^{3}H]-thymidine uptake without cytotoxicity at the concentrations and for the incubation times used. REV-5901 is a CysLTR antagonist but also a 5-LOX inhibitor. Thus, it can be argued that the effect of REV-5901 on RAW 264.7 growth was due to 5-LOX inhibition, as reported above for zileuton. To evaluate the role of CysLTRs in RAW 264.7 growth, we also used LY-171883 and MK-571, two selective antagonists of CysLTR1 that specifically blocked the binding of LTD_4 [20]. The effects of both compounds and the reversion of LY-171883 inhibition by exogenous LTD_4 addition clearly suggested that LTD_4 interaction with CysLTR1 is involved in FBS-induced RAW 264.7 proliferation. Moreover, we did not observed an additional macrophage growth inhibition when we used a dual CysLTR1 and CysLTR2 antagonist such as Bay u9773. These data suggest the main role of CysLTR1 versus CysLTR2 in the control of RAW 264.7 growth. On the other hand, a selective BLT1 receptor antagonist, U-75302 [22], inhibited FBS-induced RAW 264.7 proliferation, which was abolished by exogenous LTB_4. Thus, these results also implicate LTB_4 BLT1 interaction in RAW 264.7 growth. These findings agree with Vargaftig and Singer [33] who demonstrated that MK-571 reduces fibroblast-like cell proliferation, and Ciccarelli et al. [35] that reported the role of CysLTR1 in astrocyte growth. On the other hand, the LTD_4 receptor antagonist LY-171883 and the CysLTR antagonist REV-5901 have also been proposed as antipancreatic cancer agents because they inhibit thymidine incorporation and induce apoptosis in MiaPaCa-2 and AsPC-1 cells [36]. In contrast to our results, Przylipiak et al. [15] reported that LTD_4 inhibits thymidine incorporation in the human mammary carcinoma cell line MCF-7, which is reverted by LY-171883. On the other hand, Porreca et al. [37] showed that MK-571 inhibits the stimulatory effect of LTD_4 on thymidine incorporation in vascular smooth muscle cells in a dose-dependent manner. Here, inhibition of the 5-LOX pathway seriously hindered RAW 264.7 macrophage growth, whereas exogenous addition of 5-LOX pathway metabolites significantly reverted these effects, supporting the co-mitogenic effect of LTs/CysLTs.

Although LTB_4–BLT1 and LTD_4–CysLT1 interaction may be involved in FBS-induced RAW 264.7 proliferation, we cannot rule out the involvement of other receptors in the mechanisms of action of LTs/CysLTs in macrophage growth such as peroxisome proliferator-activated receptor-α.

Several authors reported the impairment of cell growth as consequence of apoptosis by 5-LOX inhibitors [12,17]. However, our results show that 5-LOX inhibitors such as zileuton and REV-5901 or LT/CysLTs antagonist such as U-45302, LY-171883 or MK-571 did not produce the detachment of macrophages, were not cytotoxic and did not induce a marked phosphatidylserine externalization, caspase activation or DNA fragmentation. Only NDGA induced annexin V staining and caspase activation and the subsequent DNA fragmentation. Recently, Romano et al. [38] observed that NDGA induces growth arrest of human malignant pleural mesothelial cells by an apoptotic process. Thus, although NDGA has been widely used as a 5-LOX inhibitor, the precise association between the inhibition of 5-LOX by NDGA and the events induced by NDGA described in this paper is not clear. We must consider that NDGA also inhibits 12-LOX and 15-LOX [29,30]. Furthermore, NDGA induces membrane alterations [39], increases intracellular calcium [40] and disrupts the filamentous actin cytoskeleton [41], events that may be related to cell detachment, the increase of cells in G_2/M phase and especially apoptosis. Thus, the inhibition of RAW 264.7 growth by NDGA may be consequence, at least in part, of the pro-apoptotic action, whereas the blockage of 5-LOX pathway by zileuton or LT/CysLT antagonists decrease RAW 264.7 macrophage proliferation without inducing apoptosis.

Panettieri et al. [42] noted that LTD_4 enhances EGF-induced human airway smooth muscle proliferation, but LTD_4 alone does not exert the same effect. In contrast, Paruchuri and Sjolander [43] demonstrated that LTD_4 increases the cell number and DNA synthesis of intestinal epithelial cells. In this way, Tong et al. [44] showed that LTB_4 stimulates thymidine incorporation in a concentration- and time-dependent manner and stimulate ERK1/2 phosphorylation in pancreatic cancer cells. In our study when LTD_4 or LTB_4 was added exogenously in absence of FBS, [^{3}H]-thymidine uptake and cell proliferation increased in RAW 264.7 cultures. It is noteworthy that the concentrations of exogenous LTs that evoke significant effects on RAW 264.7 proliferation are in a range similar to the amount released from macrophage cultures in our experimental conditions. Moreover, we have contributed suggesting that the pro-proliferative effect of LTB_4 and LTD_4 may be mediated by MAPK and PI3K cascades activation. Results in agreement with Klein et al. [45] who showed that LTB_4 can stimulate ERK and Akt phosphorylation in human neutrophils, and with Paruchuri et al. [46,47] who suggested that the mitogenic effect of LTD_4 is mediated by the activation of the MAPK ERK1/2 and Akt in intestinal epithelial cells.

To our knowledge, the effects of the 5-LOX-pathway on RAW 264.7 macrophage growth have not been described so far. In the present study, we provide several lines of evidence that support our hypothesis that the interaction of 5-LOX metabolites of AA (LTB_4 and LTD_4) with their plasma membrane receptors can influence RAW 264.7 macrophage cell cycle and proliferation.

Acknowledgments

D. Nieves was a recipient of a predoctoral fellowship from the Spanish Ministry of Science and Technology. This investigation was supported by a grant from Spanish Ministry of Science and Technology (BFI2001-3397) and Spanish Ministry of Education and Science (BFU2004-04960/BFI). FACS analyses were performed at the Serveis Científic-Tècnics, University of

Barcelona, Barcelona, Spain. We thank Robin Rycroft for valuable assistance in the preparation of the manuscript.

REFERENCES

[1] Funk CD. Prostaglandins and leukotrienes: advances in eicosanoid biology. Science 2001;294:1871–5.
[2] Bonney RJ, Humes JL. Physiological and pharmacological regulation of prostaglandin and leukotriene production by macrophages. J Leukocyte Biol 1984;35:1–10.
[3] Hong J, Bose M, Ju J, Ryu JH, Chen X, Sang S, et al. Modulation of arachidonic acid metabolism by curcumin and related beta-diketone derivatives: effects on cytosolic phospholipase A(2), cyclooxygenases and 5-lipoxygenase. Carcinogenesis 2004;25:1671–9.
[4] Kawajiri H, Makino I, Yoshimoto T. Intracellular activation and induction of 12-lipoxygenase in mouse peritoneal macrophages. Adv Exp Med Biol 2002;507:161–4.
[5] Christmas P, Fox JW, Ursino SR, Soberman RJ. Differential localization of 5- and 15-lipoxygenases to the nuclear envelope in raw macrophages. J Biol Chem 1999;274:25594–8.
[6] Tager AM, Luster AD. BLT1 and BLT2: the leukotriene B4 receptors. Prostag Leukotr Ess Fatty Acids 2003;69:123–34.
[7] Lötzer K, Spanbroek R, Hildner M, Urbach A, Heller R, Bretschneider E, et al. Differential leukotriene receptor expression and calcium responses in endothelial cells and macrophages indicate 5-lipoxygenase-dependent circuits of inflammation and atherogenesis. Arterioscler Thromb Vasc Biol 2003;23:E32–6.
[8] Peppelenbosch MP, Tertoolen LG, Hage WJ, de Laat SW. Epidermal growth factor-induced actin remodeling is regulated by 5-lipoxygenase and cyclooxygenase products. Cell 1993;74:565–75.
[9] Fiorucci S, Lanfrancone L, Santucci L, Calabro A, Orsini B, Federico B, et al. Epidermal growth factor modulates pepsinogen secretion in guinea pig gastric chief cells. Gastroenterology 1996;111:945–58.
[10] Antoniotti S, Pla AF, Pregnolato S, Mottola A, Lovisolo D, Munaron L. Control of endothelial cell proliferation by calcium influx and arachidonic acid metabolism: a pharmacological approach. J Cell Physiol 2003;197:370–8.
[11] Tang DG, Honn KV. Apoptosis of W256 carcinosarcoma cells of the monocytoid origin induced by NDGA involves lipid peroxidation and depletion of GSH: role of 12-lipoxygenase in regulating tumor cell survival. J Cell Physiol 1997;172:155–70.
[12] Ghosh J, Myers CE. Inhibition of arachidonic 5-lipoxygenase triggers massive apoptosis in human prostate cancer cells. Proc Natl Acad Sci USA 1998;95:13182–7.
[13] Walker JL, Loscalzo J, Zhang YY. 5-Lipoxygenase and human pulmonary artery endothelial cell proliferation. Am J Physiol Heart Circ Physiol 2002;282:H585–93.
[14] McMahon B, Mitchell D, Shattock R, Martin F, Brady HR, Godson C. Lipoxin, leukotriene, and PDGF receptors cross-talk to regulate mesangial cell proliferation. FASEB J 2002;16:1817–9.
[15] Przylipiak A, Hafner J, Przylipiak J, Kohn FM, Runnebaum B, Rabe T. Influence of 5-lipoxygenase on in vitro growth of human mammary carcinoma cell line MCF-7. Gynecol Obstet Invest 1998;46:61–4.
[16] Nishizawa Y, Nishii K, Kishimoto S, Matsumoto K, Sato B. Regulatory role of arachidonic acid-derived metabolites for proliferation of transformed murine Leydig cell in serum-free culture conditions. Anticancer Res 1990;10:317–22.
[17] Titos E, Clarià J, Planagumà A, López-Parra M, Villamar N, Párrizas M, et al. Inhibition of 5-lipoxygenase induces cell growth arrest and apoptosis in rat Kupffer cells: implications for liver fibrosis. FASEB J 2003;17:1745–7.
[18] Carter GW, Young PR, Albert DH, Bouska J, Dyer R, Bell RL, et al. 5-Lipoxygenase inhibitory activity of zileuton. J Pharmacol Exp Ther 1991;256:929–37.
[19] Tang DG, Chen YQ, Honn KV. Arachidonate lipoxygenases as essential regulators of cell survival and apoptosis. Proc Natl Acad Sci USA 1996;93:5241–6.
[20] Fleisch JH, Rinkema LE, Haisch KD, Swanson-Bean D, Goodson T, Ho PP, et al. LY171883, 1-less than 2-hydroxy-3-propyl-4-less than 4-(1H-tetrazol-5-yl) butoxy greater than phenyl greater than ethanone, an orally active leukotriene D4 antagonist. J Pharmacol Exp Ther 1985;233:148–57.
[21] Martin V, Sawyer N, Stocco R, Unett D, Lerner MR, Abramovitz M, et al. Molecular cloning and functional characterization of murine cysteinyl-leukotriene 1 (CysLT(1)) receptors. Biochem Pharmacol 2001;62:1193–200.
[22] 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 2000;192:421–31.
[23] Musser JH, Charkraborty UR, Sciortino S, Gordon RJ, Khandwala A, Neiss ES, et al. Substituted arylmethyl phenyl ethers. 1. A novel series of 5-lipoxygenase inhibitors and leukotriene antagonists. J Med Chem 1987;30:96–104.
[24] Nothacker HP, Wang Z, Zhu Y, Reinscheid RK, Lin SH, Civelli O. Molecular cloning and characterization of a second human cysteinyl leukotriene receptor: discovery of a subtype selective agonist. Mol Pharmacol 2000;58:1601–8.
[25] Pang L, Sawada T, Decker SJ, Saltiel AR. Inhibition of MAP kinase kinase blocks the differentiation of PC-12 cells induced by nerve growth factor. J Biol Chem 1995;270:13585–8.
[26] Powis G, Bonjouklian R, Berggren MM, Gallegos A, Abraham R, Ashendel C, et al. Wortmannin, a potent and selective inhibitor of phosphatidylinositol-3-kinase. Cancer Res 1994;54:2419–23.
[27] Sanchez T, Moreno JJ. Role of EP1 and EP4 PGE2 subtype receptors in serum-induced 3T6 fibroblast cycle progression and proliferation. Am J Physiol Cell Physiol 2002;282:C280–8.
[28] Lu MC, Peters-Golden M, Hostetler DE, Robinson NE, Derksen FJ. Age-related enhancement of 5-lipoxygenase metabolic capacity in cattle alveolar macrophages. Am J Physiol 1996;271:L547–54.
[29] Argentieri DC, Ritchie DM, Ferro MP, Kirchner T, Wachter MP, Anderson DW, et al. Tepoxalin: a dual cyclooxygenase/5-lipoxygenase inhibitor of arachidonic acid metabolism with potent anti-inflammatory activity and a favorable gastrointestinal profile. J Pharmacol Exp Ther 1994;271:1399–408.
[30] Hope WC, Welton AF, Fiedler-Nagy C, Batula-Bernardo C, Coffey JW. In vitro inhibition of the biosynthesis of slow reacting substance of anaphylaxis (SRS-A) and lipoxygenase activity by quercetin. Biochem Pharmacol 1983;32:367–71.
[31] Mikuni M, Yoshida M, Hellberg P, Peterson CA, Edwin SS, Bennstrom M, et al. The lipoxygenase inhibitor, nordihydroguaiaretic acid, inhibits ovulation and reduces leukotriene and prostaglandin levels in the rat ovary. Biol Reprod 1998;58:1211–6.
[32] Agarwal R, Wang ZY, Bik DP, Mukhtar H. Nordihydroguaiaretic acid, an inhibitor of lipoxygenase, also inhibits cytochrome P-450-mediated monooxygenase activity in rat epidermal and hepatic microsomes. Drug Metab Dispos 1991;19:620–4.
[33] Vargaftig BB, Singer M. Leukotrienes mediate murine bronchopulmonary hyperreactivity, inflammation, and part of mucosal metaplasia and tissue injury induced by

recombinant murine interleukin-13. Am J Respir Cell Mol Biol 2003;28:410–9.
[34] Ogasawara H, Ishii S, Yokomizo T, Kakinuma T, Komine M, Tamaki K, et al. Characterization of mouse cysteinyl leukotriene receptors mCysLT1 and mCysLT2. J Biol Chem 2002;277(21):18763–8.
[35] Ciccarelli R, D'Alimonte I, Santavenere C, D'Auro M, Ballerini P, Nargi E, et al. Cysteinyl-leukotrienes are released from astrocytes and increase astrocyte proliferation and glial fibrillary acidic protein via cys-LT1 receptors and mitogen-activated protein kinase pathway. Eur J Neurosci 2004;20:1514–24.
[36] Tong WG, Ding XZ, Witt RC, Adrian TE. Lipoxygenase inhibitors attenuate growth of human pancreatic cancer xenografts and induce apoptosis through the mitochondrial pathway. Mol Cancer Ther 2002;1:929–35.
[37] Porreca E, Di Febbo C, Di Sciullo A, Angelucci D, Nasuti M, Vitullo P, et al. Cysteinyl leukotriene D4 induced vascular smooth muscle cell proliferation: a possible role in myointimal hyperplasia. Thromb Haemostasis 1996;76: 99–104.
[38] Romano M, Catalana A, Nutini M, D'Urbano E, Crescenzi C, Clarià J, et al. 5-Lipoxygenase regulates malignant mesothelial cell survival: involvement of vascular endothelial growth factor. FASEB J 2001;15:2326–36.
[39] Fujiwara T, Misumi Y, Ikehara Y. Direct interaction of the Golgi membrane with the endoplasmic reticulum membrane caused by nordihydroguaiaretic acid. Biochem Biophys Res Commun 2003;301:927–33.
[40] Wang JL, Chang HJ, Tsung LL, Liu CP, Lee KC, Chou KJ, et al. Nordihydroguaiaretic acid elevates osteoblastic intracellular Ca^{2+}. Pharmacol Toxicol 2001;89:301–5.
[41] Seufferlein T, Seckl MJ, Schwarz E, Beil M, Wichert G, Baust H, et al. Mechanisms of nordihydroguaiaretic acid-induced growth inhibition and apoptosis in human cancer cells. Br J Cancer 2002;86:1188–96.
[42] Panettieri RA, Tan EML, Ciocca V, Luttmann MA, Leonard TB, Hay DWP. Effects of LTD4 on human airway smooth muscle cell proliferation, matrix expression, and contraction in vitro: differential sensitivity to cysteinyl leukotriene receptor antagonists. Am J Respir Cell Mol Biol 1998;19:453–61.
[43] Paruchuri S, Sjölander A. Leukotriene D4 mediates survival and proliferation via separate but parallel pathways in the human intestinal epithelial cell line Int 407. J Biol Chem 2003;278:45577–85.
[44] Tong WG, Ding XZ, Hennig R, Witt RC, Standop J, Pour PM, et al. Leukotriene B4 receptor antagonist LY293111 inhibits proliferation and induces apoptosis in human pancreatic cancer cells. Clin Cancer Res 2002;8:3232–42.
[45] Klein JB, Buridi A, Coxon PY, Rane MJ, Manning T, Kettritz R, et al. Role of extracellular signal-regulated linase and phosphatidylinositol-3 kinase in chemoattractant and LPS delay of constitutive neutrophil apoptosis. Cell Signal 2001;13:335–43.
[46] Paruchuri S, Hallberg B, Juhas M, Larsson C, Sjolander A. Leukotriene D4 activates MAPK through a Ras-independent but PKC?-dependent pathway in intestinal epithelial cells. J Cell Sci 2002;115:1883–93.
[47] Paruchuri S, Broom O, Dib K, Sjolander A. The pro-inflammatory mediator leukotriene D4 induces phosphatidylinositol 3-kinase and Rac-dependent migration of intestinal epithelial cells. J Biol Chem 2005;280:13538–44.