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Enantioselective effect of 12(S)-hydroxyeicosatetraenoic acid on 3T6 fibroblast growth through ERK 1/2 and p38 MAPK pathways and cyclin D_1 activation

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ABSTRACT

Hydroxyeicosatetraenoic acids (HETEs) have numerous physiological effects, including modulation of cell proliferation and differentiation. However, little is known about the selective effects of HETE enantiomers on cell proliferation and cell signalling pathways involved in the regulation of cell growth. Furthermore, information on epithelial and endothelial cells growth is controversial. Recently, we demonstrated that 5-, 12-, and 15-HETE are involved in the control of 3T6 fibroblast growth though serine/treonine Akt/PKB (Akt) pathway. Here we examined the participation of both enantiomers (S and R) of HETEs in the control of 3T6 fibroblast growth. Our results show that HETEs (5-, 12-, and 15-HETE) are enantioselective on protein and DNA synthesis and 3T6 fibroblast growth. Furthermore, we observed that 12(S)-HETE induces the enhancement of cAMP and intracellular calcium concentration, whereas 12(R)-HETE was uneffective. Our findings also demonstrated that 12(S)-HETE exerts these effects through enantiospecific interactions with a cellular element, probably a plasma membrane receptor coupling to a pertussis toxin-sensitive protein G. Moreover, these elements may be involved in the activation of mitogen-activated protein kinase pathways which induce the enhancement of cyclin D_1 levels.

1. Introduction

Arachidonic acid (AA) released from membrane phospholipids is the substrate for the production of hydroxyeicosatetraenoic acids (HETEs). Lipoxygenases (LOXs) insert a hydroxyperoxyl group into the AA, which is subsequently reduced to a stable hydroxyl group to produce HETEs. Thus, 5-LOX leads to the formation of 5-HETE whereas 12- and 15-LOX can form 12- and 15-HETE [1]. Cytochrome P-450 (CYP) also metabolise AA to produce HETEs by one or more of the following reactions: bis-allylic oxidation to generate 5-, 8-, 9-, 11-, 12-, and 15-HETE or ω/ω-1 hydroxylation to afford 16-, 17-, 18-, 19-, and 20-HETE. Thus, 5-, 12- and 15-HETE can be synthesized by LOXs and CYPs, whereas other HETEs such as 18-, 19- or 20-HETE appear to be synthesized exclusively by CYPs [2].

The presence of HETE enantiomers (R and S) is not attributable mainly to autooxidation. (R)-HETEs are considered the predominant enantiomers generated by the CYP pathway [3,4], whereas (S)-HETEs are produced by mammalian LOX. Thus, it was thought that (R)-HETEs were exclusively a product of CYP whereas (S)-HETEs were produced only by LOXs. However, the controversy on this issue increased when Boeglin et al. [5] characterised the origin of 12(R)-HETE from human epidermis, proving the existence of (R)-LOXs in mammals and the capacity of the LOX pathway to produce both HETE enantiomer. Moreover, Nieves and Moreno [6]

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recently reported that murine fibroblasts produce 12(S)-HETE as measured by immunoassay, an event inhibited by several CYP inhibitors. Thus, the contribution of LOX and CYP pathways to the formation of (S) and (R)-HETEs remains to be clarified.

HETEs have numerous physiological effects, including participation in the control of cell proliferation and differentiation. Thus, 5-, 12-, and 15-HETE are involved in the control of 3T6 fibroblast growth through AKT pathway activation [6]. However, little is known about the selective effects of HETE enantiomers on cell proliferation and cell signalling pathways involved in the control of cell growth. Furthermore, the information available on epithelial and endothelial cell growth is controversy. Thus, 12(S)-HETE but not 12(R)-HETE induces DNA synthesis in lens epithelial cell proliferation [7], whereas only 12(R)-HETE is proliferative in HT-29 colon carcinoma cells [8] and endothelial cells [9].

Here we examined the contribution of both enantiomers of HETEs to the control of 3T6 fibroblast growth. Our results demonstrated that HETEs are enantioselective in 3T6 fibroblast growth and suggest that (S)-HETEs exert these effects through enantiospecific interactions with a cellular element, probably a plasma membrane receptor coupling to a pertussis toxin (PTX)-sensitive protein G, and the subsequent activation of mitogen-activated protein kinase (MAPK) pathways.

2. Materials and methods

2.1. Materials

RPMI 1640 media, FBS, penicillin, streptomycin and trypsin-EDTA were purchased from BioWhittaker Europe (Verviers, Belgium). PD 98059, SB 202190, pertussis toxin from Bordetella pertussis, propidium iodide, Triton X-100, phenylmethylsulfonylfluoride (PMSF), Igepal CA-630, aprotinin, fura-II/AM, leupeptin, DTT, pluronic F-127, ribonuclease A from bovine pancreas, acridine orange and ethidium bromide were provided by Sigma Chemical Co. (St. Louis, MO, USA). 5(S)-, 5(R)-, 12(S)-, 12(R)-, 15(S)- and 15(R)-HETE as well as prostaglandin E_2 (PGE_2) were supplied by Cayman Chemical Co. (Ann Arbor, MI, USA). Stock solutions of eicosanoids were made in dimethylsulfoxide (DMSO) at 600 μM and were stored away from light exposure at −20 °C. Working dilutions were made directly in cell culture medium. Control conditions containing DMSO concentrations equivalent to those of present in eicosanoid conditions. The final concentration of DMSO was kept at no more than 0.1%. [Methyl-^{3}H]thymidine (20 Ci/mmol) was from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA) and [1-^{14}C] L-leucine (300 mCi/mmol) was obtained from DuPont-NEN (Boston, MA, USA). AH-6809 and AH-23848B were kindly provided by Glaxo Wellcome (Stevenage, Hertfordshire, UK).

2.2. Cell culture

Murine 3T6 fibroblasts (ATCC CCL96) were cultured in RPMI 1640 containing 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were harvested with trypsin/EDTA and seeded in 24-well plates (tissue-culture cluster 24; Costar Cambridge, MA, USA) or in 60-mm tissue-culture dishes (Costar) for experimental purposes. Cultures were maintained in a temperature- and humidity-controlled incubator at 37° with 95% air–5% CO_2 for 24 h.

2.3. Cell growth assay

The effect of the treatments was assessed on 3T6 fibroblast plates at 20×10^3 cells/well in 24-well plates. Cells were cultured for 24 h in RPMI 1640 supplemented with 10% FBS. After 24 h serum starvation cells were incubated for 48 h in the presence of various compounds. Finally, cells were washed, trypsinized and 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 3T6 fibroblasts in 24-well plates in RPMI 1640 with 10% FBS at a density of 20×10^3 cells/well. After 24 h serum starvation, cells were subjected to the treatments for 48 h and [^{3}H]thymidine (1 μCi/well) was added during 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, radioactivity present in the cell fraction was measured by scintillation counting, using a Packard Tri-Carb 1500 counter.

2.5. Protein synthesis assay

20×10^3 cells were loaded into each well on 24-well plates, allowed to attach overnight, then rendered quiescent in serum-free medium for 24 h. Cells were incubated with the treatments in the presence of 0.5 μCi of [^{14}C]leucine. After 24 h incubation, the culture media were aspirated, cells were rinsed twice with phosphate buffered saline and proteins were precipitated by two incubations with 10% TCA at room temperature. The TCA was removed and protein was solubilized in 300 μl lysis buffer (0.5 M NaOH, 1% Triton X-100) at room temperature for 15 min. The lysate was transferred to scintilation vials and radioactivity was measured.

2.6. cAMP levels

cAMP from cell was extracted with ethanol and measured following the instructions of the manufacturer of the radioimmunoassay kit (Amersham Life Science, Buckinghamshire, UK).

2.7. Measurement of intracellular calcium concentrations

Cells grown on glass slides were incubated with fura-II/AM (3 μM) and pluronic F-127 (0.02%, v/v) in RPMI at 37 °C for 45 min. Fluorescence was monitored on a spectrofluorometer with dual excitation wavelengths of 340 and 380 nm and an emission wavelength of 520 nm. Intracellular calcium concentrations (i[Ca^{2+}]) were calculated as described by Grynkiewicz et al. [10].

2.8. Western blot analysis of phosphorylated ERK 1/2 and p38 MAPK and cyclin D_1

Fibroblast cultures were starved of FBS for 24 h and incubated with HETEs. Total cellular fraction was obtained scraping off the cells in lysis buffer containing 200 mM Tris–HCl, 200 mM NaCl, 2% Igepal CA-630, 400 μM NaF, 20 μg/ml PMSF, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 200 μM DTT, and 400 μM Na_3VO_4, followed by incubation for 30 min at 4 °C. Immunoblot analyses were performed as follows: 20 μg of protein from cell lysates was separated on a 10% SDS-PAGE gel [11] and blotted for 1 h at a constant voltage of 100 V onto a polyvinylidene difluoride membrane (Immobilon-P, 0.45 μm, Millipore, Bedford, MA, USA) using a MiniProtean II system (Bio-Rad). Membranes were blocked with 5% nonfat milk powder in PBS-0.1% Tween 20 for 1 h. Monoclonal antibodies against diphosphorylated extracellular signal-regulated kinase 1/2 (ERK 1/2) (clone MAPK-YT) and diphosphorylated anti p38 MAPK (clone P38-TY) were applied in a 1:2000 dilution overnight. The blot was washed several times with PBS-0.1% Tween 20 and incubated with a horseradish peroxidase-conjugated goat anti-mouse antibody in a 1:2000 dilution for 1 h. The above antibodies were obtained from Sigma. A rabbit polyclonal antibody against cyclin D_1 was applied in a 1:500 dilution for 1 h. After the blot was washed and incubated with a goat anti-rabbit antibody in a 1:2000 dilution for 1 h. Both antibodies were from Santa Cruz (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). For β-actin immunobloting, stripped membranes were overlaid with monoclonal actin antibody (1:500) (Santa Cruz). Finally, blots were developed using an enhanced chemiluminescence with Supersignal West Dura Extended Duration Substrate from Pierce (Rockford, IL, USA) using a Bio Max Light-2 film. Total protein was measured by the Bradford method [12], using the Bio-Rad protein assay (Hercules, CA, USA) with bovine serum albumin as standard.

2.9. Statistics

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

3. Results

3.1. 12(S)-HETE but not 12(R)-HETE induced cell proliferation, DNA and protein synthesis

3T6 fibroblasts were cultured in the presence of the two 12-HETE enantiomers (0.001–1 μM) in order to determine their effect on proliferation. 12(S)-HETE induced significant 3T6 fibroblast growth. This effect was concentration-dependent and reached a plateau at 0.1 μM (Fig. 1A). However, no appreciable proliferative response was seen in presence of 12(R)-HETE. We also studied the effect of these two enantiomers on protein and DNA synthesis. Our results show that only 12(S)-HETE induced a significant [^{14}C]leucine uptake to cell proteins and [^{3}H]thymidine incorporation to DNA synthesis (Fig. 1B and C). Thus, these results indicate that only 12(S)-HETE showed mitogenic action on 3T6 fibroblast.

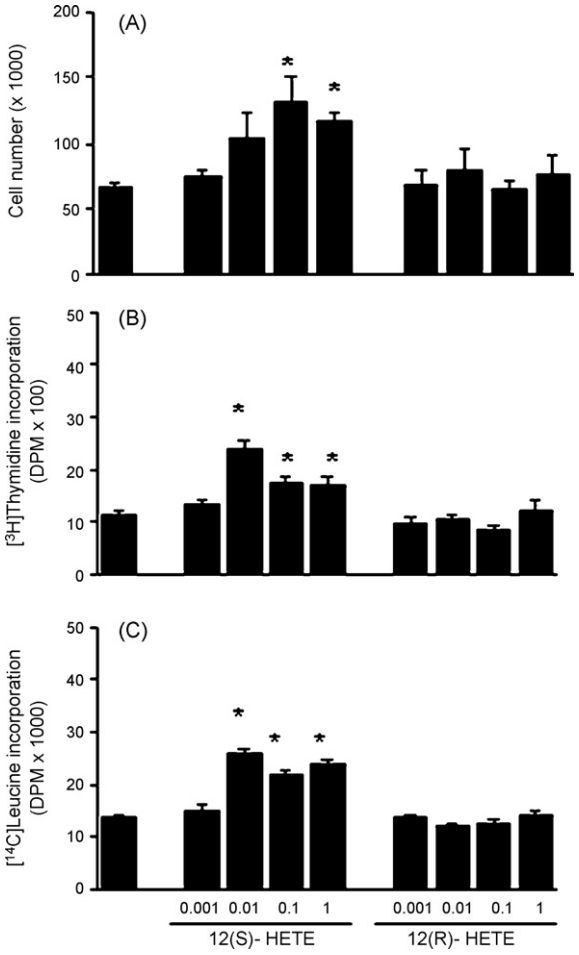

Fig. 1 – Effect of 12(S)-HETE enantiomers on cell growth and DNA and protein synthesis. 3T6 fibroblasts (20×10^3) were plated and cultured in 10% FBS-RPMI. The next day, media were removed and free FBS-RPMI was added for 24 h. Cells were then incubated for 2 days with RPMI containing 12(S)- or 12(R)-HETE (0.001–1 μM). (A) Cells were trypsinized and counted using microscopy. For the last 48 h of treatment, [^{3}H]thymidine (1 μCi/well) (B) or [^{14}C]leucine (2 μCi/well) (C) were added to the cells. Radioactivity in cell fraction was determined in a scintillation analyzer. Results are means ± S.E.M. from three experiments performed in triplicate. *$P < 0.05$ compared with control cells.

Previously, we observed that prostaglandins (PG) such as PGE_2 induce 3T6 fibroblast growth [13]. Our results here show that 12(S)-HETE also had effect on fibroblast proliferation. Interestingly, 12(S)-HETE and PGE_2, produced by 3T6 fibroblast in our culture conditions [6], showed a synergistic effect on fibroblast proliferation and DNA synthesis (Fig. 2), whereas 12(R)-HETE did not increase the effect of PGE_2.

To further elucidate the significance of G proteins in the mechanisms involved in the mitogenic actions of 12(S)-HETE, we examined the effects of PTX, a compound that inhibits ADP-ribosylate specific G-proteins [14] and has been used as a probe for G-protein involvement in cell signalling. Pre-treatment of 3T6 fibroblasts with PTX before stimulation with

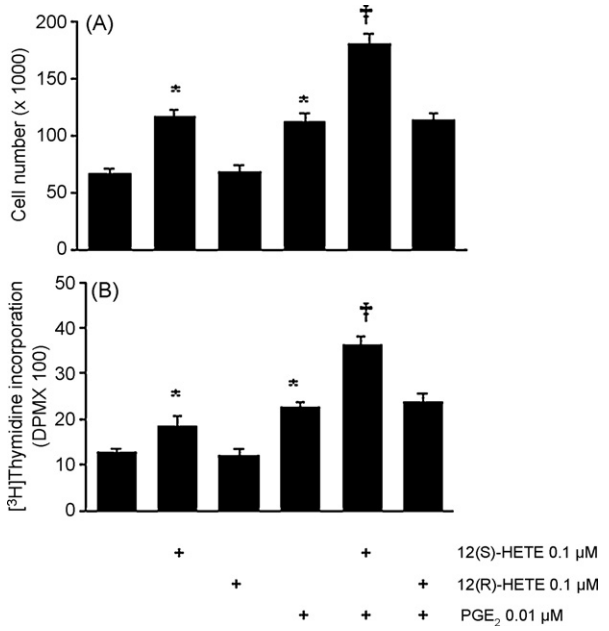

Fig. 2 – Synergistic effects of 12(S)-HETE and PGE_2 on cell growth. Cells (20×10^3) were plated and cultured in RPMI without FBS for 24 h. They were incubated for 2 days with RPMI containing 12(S)-HETE (0.1 μM), 12(R)-HETE (0.1 μM) and PGE_2 (0.01 μM). Finally, cell number (A) and [^{3}H]thymidine uptake (B) were determined. Results are means ± S.E.M. from three experiments performed in triplicate. $^*P < 0.05$ compared with control cells. $^\dagger P < 0.05$ compared with cells incubated with 12(S)-HETE or PGE_2 alone.

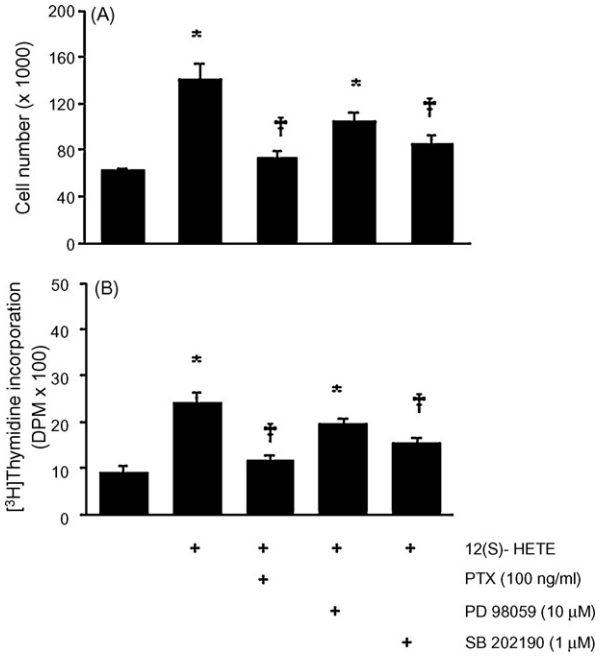

Fig. 3 – Role of MAPK pathway on proliferative effect of 12(S)-HETE. Cells (20×10^3) were plated and cultured in RPMI without FBS for 24 h. Then, cells were incubated for 2 days with RPMI with 12(S)-HETE (0.1 μM) in the absence or presence of pertussis toxin (PTX, 100 ng/ml), PD 98059 (10 μM) or SB 202190 (1 μM). Finally, cell number (A) and [^{3}H]thymidine uptake (B) were measured using 12(S)-HETE at 0.1 μM and 0.01 μM, respectively. Results are means ± S.E.M. from three experiments performed in triplicate. $^*P < 0.05$ compared with control cells. $^\dagger P < 0.05$ compared with cells treated with HETE alone.

12(S)-HETE blocked the mitogenic effects of this enantiomer (Fig. 3).

3.2. 5(S)- and 15(S)-HETE were also enantioselective on 3T6 fibroblast growth

We have recently observed that 5-HETE and 15-HETE also stimulate 3T6 fibroblast proliferation [6]. In the present study only (S) enantiomer of these two HETEs but not the respective (R) enantiomers induced cell growth and [^{3}H]thymidine incorporation in 3T6 fibroblast cultures (Table 1). Thus, our results demonstrate the enantioselective effect of 5(S)-, 12(S)-, and 15(S)-HETE on the growth of this cell line.

3.3. 12(S)-HETE increases i[Ca^{2+}] and cAMP levels in 3T6 fibloblast cultures

Once the mitogenic effect of 12(S)-HETE on 3T6 fibroblast proliferation was established, we examined the cell signalling pathway through which this eicosanoid acts. To this end, we studied the effect of 12(S)-HETE on intracellular calcium concentration (i[Ca^{2+}]) and cellular cAMP levels. 12(S)-HETE enhanced i[Ca^{2+}] and cAMP levels and these effects were increased by the incubation of 12(S)-HETE together with PGE_2 (Table 2). Moreover, the enhancement of i[Ca^{2+}] and cAMP levels induced by 12(S)-HETE was blocked by PTX. This observation suggests that PTX-sensitive G protein is a pivotal element of 12(S)-HETE-stimulated cell signalling. On the other hand, AH6899 and AH 23848B, EP_1/EP_2 and EP_4 antagonists [15,16], respectively, reverted the effect of PGE_2 on i[Ca^{2+}] and intracellular cAMP levels whereas did not affect the action of 12(S)-HETE. Data that suggest that 12(S)-HETE does not interact with these PGE_2 receptors.

Table 1 – Effects of HETE enantiomers on 3T6 fibroblast growth

	Cell number (×1000)	[^{3}H]Thymidine incorporation (DPM × 100)
Control	58.3 ± 3.7	12.6 ± 0.4
5(S)-HETE	112.6 ± 3.5*	21.2 ± 1.2*
5(R)-HETE	56.7 ± 2.9	13.9 ± 0.5
15(S)-HETE	136.4 ± 4.1*	24.8 ± 1.6*
15(R)-HETE	62.5 ± 3.4	13.7 ± 1.1

Cells were incubated with HETEs at 0.1 μM to determine cell proliferation and with HETEs at 0.01 μM to determine the effect on [^{3}H]thymidine incorporation. Data are means ± S.E.M. from three experiments performed in triplicate.
* $P < 0.05$ compared with control cells.

Table 2 – Effect of 12-HETE on i[Ca^{2+}] and cAMP

	i[Ca^{2+}]	cAMP
Control	367 ± 12	48 ± 2
12(S)-HETE	526 ± 14*	108 ± 4*
12(S)-HETE + PTX	387 ± 12†	49 ± 2†
12(S)-HETE + AH6809/AH23848B	531 ± 12*	111 ± 5*
PGE_2	585 ± 16*	134 ± 4*
PGE_2 + PTX	389 ± 11†	51 ± 3†
PGE_2 + AH6809/AH23848B	269 ± 9†	49 ± 3†
12(S)-HETE + PGE_2	674 ± 13*	168 ± 5*
12(S)-HETE + PGE_2 + PTX	402 ± 12†	54 ± 4†
12(R)-HETE	374 ± 11	52 ± 3

Cells were incubated with 12-HETEs (0.1 μM) and/or PGE_2 (0.01 μM). i[Ca^{2+}] (nM) and cAMP (pmol cAMP/mg protein) levels were measured 5 and 10 min after eicosanoids were added. Fibroblasts were incubated with PTX (100 ng/ml) 30 min before HETEs were added.

* $P < 0.05$ compared with control condition.

† $P < 0.05$ compared with eicosanoids without additional treatments.

3.4. 12(S)-HETE induces cyclin D_1 expression

Progression of the mammalian cell cycle is governed by cyclins together Cdks and CKIs [17]. The levels of cyclin D_1 were low in quiescent 3T6 fibroblasts. Exposure of the cells to 12(S)-HETE caused a marked increase in cyclin D_1 levels. In contrast, 12(R)-HETE was ineffective. Interestingly, cyclin D_1 expression induced by 12(S)-HETE was significantly reverted by PD 98059, a specific ERK 1/2 inhibitor [18] and by SB 202190, a highly selective p38 MAPK inhibitor [19] (Fig. 4). Thus, these findings

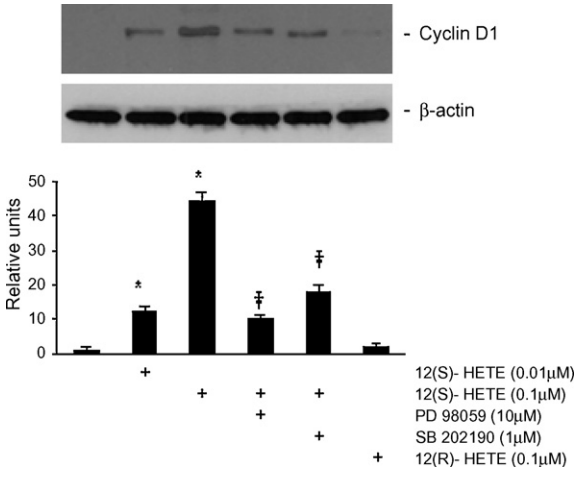

Fig. 4 – Effect of 12-HETE on cyclin D_1 expression in 3T6 fibroblast cultures. Cells were synchronized by serum starvation for 24 h. Then, 12(S)- or 12(R)-HETE (0.01–0.1 μM) were added in presence or absence of PD 98059 (10 μM) or SB 202190 (1 μM). Cells were harvested 6 h later to perform Western blot analysis. Western blot representative of 3 blots is shown. Cyclin expression were normalized to β-actin expression and is expressed as means of relative units ± S.E.M. *$P < 0.05$ compared with control cells. †$P < 0.05$ compared with cells treated with HETE alone.

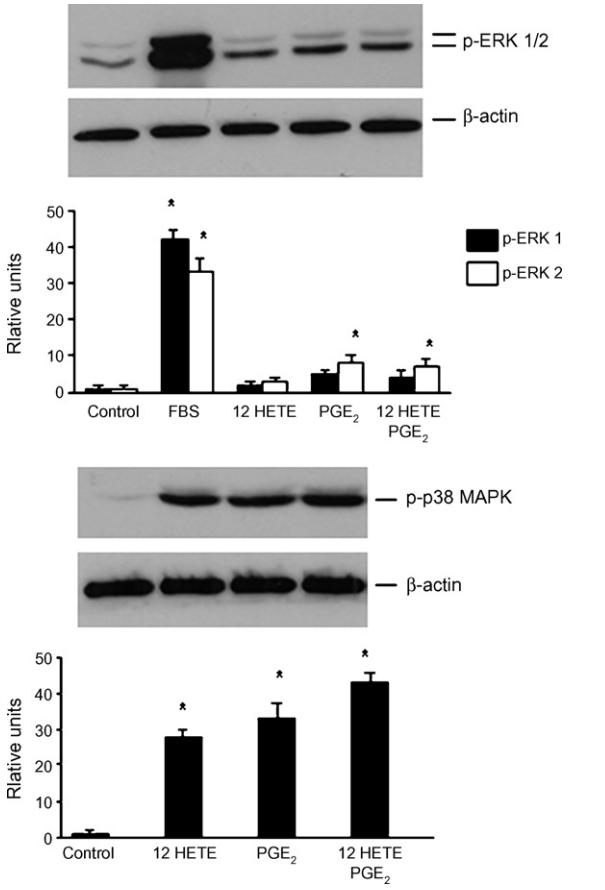

Fig. 5 – Western blot of phosphorylated ERK 1/2 and p38MAPK in cultured 3T6 fibroblast in presence of 12(S)-HETE. Cells were incubated with 12(S)-HETE (0.1 μM), PGE_2 (0.001 μM) or both for 15 min, and phosphorylated ERK 1/2 or p38MAPK were determined using specific antibodies. FBS (5%) was used as positive control. Phosphorylated kinases were normalized to β-actin expression and are expressed as means of relative units ± S.E.M. *$P < 0.05$ compared with control cells. †$P < 0.05$ compared with cells treated with HETE alone.

suggest that ERK 1/2 and p38 MAPK pathway are involved in the control of cyclin D_1 levels induced by 12(S)-HETE.

3.5. 12(S)-HETE activates ERK 1/2 and p38MAP kinases pathways

We recently observed that HETEs have the capacity to induce the phosphatidylinositol-3-kinase/AKT pathway [6]. This pathway, together with MAPK pathway, plays a crucial role in the control of cell growth and differentiation. Thus, we studied the contribution of ERK 1/2 and p38 MAPK phosphorylation to the mitogenic effect of 12(S)-HETE. PD 98059 and SB 202190 markedly decreased the enhanced growth of 3T6 fibroblast and DNA synthesis induced by 12(S)-HETE (Fig. 3). These results suggest that phosphorylation of ERK 1/2 and p38 MAPK are involved in the mitogenic effect of this enantiomer. To confirm this hypothesis, we performed the corresponding Western blot analysis. These experiments show that 12(S)-

HETE increased phosphorylated ERK 1/2 and p38 MAPK levels (Fig. 5) in a similar way to PGE_2. These observations support the role of these elements in the mitogenic signalling of these two eicosanoids. Moreover, our results showed a synergistic effect of 12(S)-HETE and PGE_2 on p38 MAPK phosphorylation. These findings can be correlated with the effect of 12(S)-HETE on proliferation/DNA synthesis and cyclin D_1 expression previously described.

4. Discussion

Eicosanoids have numerous physiological effects, including the induction of cell proliferation and differentiation. 3T6 fibroblast have the capacity to synthesize PGE_2 as well as 12(S)-HETE though COX and CYP pathways, respectively, and these two eicosanoids are involved in serum-induced growth as co-mitogenic factors [6]. Moreover, PGE_2 and 12(S)-HETE are mitogenic factors that induce 3T6 fibroblast growth and increase DNA synthesis in absence of another growth factor. Thus, both AA metabolites could be involved in wound repair as we proposed in previous studies [20,21]. Fibroblast and keratinocytes are the main cells in epidermal tissue. Although no information is available on fibroblast capacity to synthesize 12(R)-HETE, keratinocytes produce this 12-HETE enantiomer [5]. Thus, both enantiomers could be involved in fibroblast growth through a paracrine action.

Our data demonstrated that 12(S)-HETE is enantioselective on DNA synthesis, protein synthesis and cell growth in 3T6 fibroblast cultures. Our observations further demonstrated that only 12(S)-HETE presents a synergistic effect with PGE_2 on fibroblast proliferation. Interestingly, this enantioselective effect was also observed with 5(S)-HETE and 15(S)-HETE, whereas (R)-HETEs did not show appreciable effects on cell growth or DNA synthesis.

Understanding the mechanism by which (S)-HETEs contribute to cell growth may be a critical issue in cell growth/wound repair, cancer and lipid homeostasis. Although HETEs were first described several decades ago, relatively little is known about the mechanism of action by which they regulate cell proliferation. The fact that HETEs receptors have not been identified can be contributed to this disregard. However, subcellular 12(S)-HETE binding sites have been reported in lung carcinoma cells [22]. Consistent with this report, our results provide evidence that the effects of 12(S)-HETE on 3T6 fibroblast growth and DNA synthesis are associated with a PTX-sensitive G protein-coupled receptor, presents probably in plasma membrane.

We previously observed that eicosanoids such as PGE_2 control 3T6 fibroblast proliferation via the modulation of $i[Ca^{2+}]$ and cAMP levels [23]. Thus, PGE_2 interaction with EP_1 and EP_4 receptors induce the enhancement of $i[Ca^{2+}]$ and cAMP that regulate cyclin D and cyclin A expression, respectively, and consequently G_1 and S-phase progression. In the present study, we observed that 12(S)-HETE increases the intracellular levels of these two messengers while 12(R)-HETE was ineffective. These effects have been reported previously by Hasegawa et al. [24] using exclusively 12(S)-HETE in fibroblast TIG-1 cultures. However, to the best of our knowledge, our study is the first to report an enantioselective effect of 12(S)-HETE on calcium/cAMP levels. These two messengers are crucial at different stages of the cell cycle and both participate in the control of the cellular cyclin levels by eicosanoids such as PGE_2 [23], as we mentioned above. Thus, the enhancement of $i[Ca^{2+}]$ induced by 12(S)-HETE could be involved in the increase in cyclin D_1 levels and the subsequent cell growth. The role of cAMP in cell proliferation is controversial. In certain cell lines, such as fibroblasts, it behaves as a mitogenic stimulus [25], while in other cell types it induces cell cycle arrest [26]. Interestingly, our results show that only mitogenic eicosanoids such as 12(S)-HETE and PGE_2, but not 12(R)-HETE, increased cAMP levels. Thus, there may be a causal relation between cell growth and the enhancement of cAMP levels induced by 12(S)-HETE.

The biological actions of PGE_2 have been attributed to results from its interactions with cell surface prostanoid EP receptors [27]. The EP_1 receptor mediates PGE_2-induced elevation of $i[Ca^{2+}]$, whereas the EP_2 and EP_4 receptors mediate increase of cAMP by activation of adenylyl cyclase. At present, the precise receptor through which (S)-HETEs are coupled to their effects is still obscure and further studies are required to determine whether there are specific receptors to (S) and (R) enantiomers and the cellular expression of these receptors in distinct cells. However, our results show that the effects of 12(S)-HETE were not consequence of the interaction with these PGE_2 receptors.

Progression through the mammalian cell cycle is controlled at various levels by cyclins, cdks and cyclin-dependent kinase inhibitors [17]. Cyclin D family members regulate cell cycle exit [28] and are essential components of an early G_1 checkpoint [29]. Their expression depends on continues mitogenic stimulation. Thus, progression through G_1 phase is governed by cyclin D-Cdk 4/6 complexes in early G_1 phase [30]. The inhibition of baicalein-induced 12-HETE synthesis reduces cyclin D expression and prostate cancer cell growth [31]. Taking these observations together, we propose that the mechanism by which 12(S)-HETE induces 3T6 fibroblast growth is provided by the markedly stimulated increase in cyclin D_1 expression.

Several other potential pathways by which these AA metabolites may stimulate cellular growth have also been explored. Thus, 12(S)-HETE stimulates ERK 1/2 phosphorylation via a PTX-sensitive G protein-coupled receptor in PC3 cells [32]. In this regard, 12(S)-HETE stimulates ERK 1/2 via G proteins in A431 cells [28]. Unfortunately, these authors did not study the effects of 12(R)-HETE. Furthermore, racemic 12-HETE induces p38 MAPK, the transactivation of the transcription factor cAMP response element (CRE) binding protein [33] and the expression of c-fos/c-myc by proliferative epithelial cells [34]. Our results showed that 12(S) HETE, but not the (R) enantiomer, induces ERK 1/2 and p38 MAPK pathways, and that these actions are involved in the control of the 3T6 fibroblast growth induced by 12(S)-HETE.

MAPK family is an essential part of the signal transduction machinery in signal transmissions from cell surface receptors and environmental stimulation. This family includes three major MAPK subfamilies: ERK, p38 and JNK [35]. These have been proposed to serve as signal elements in fibroblast growth. Thus, the MEK/ERK pathway acts transcriptionally to induce the cyclin D_1 gene as well as

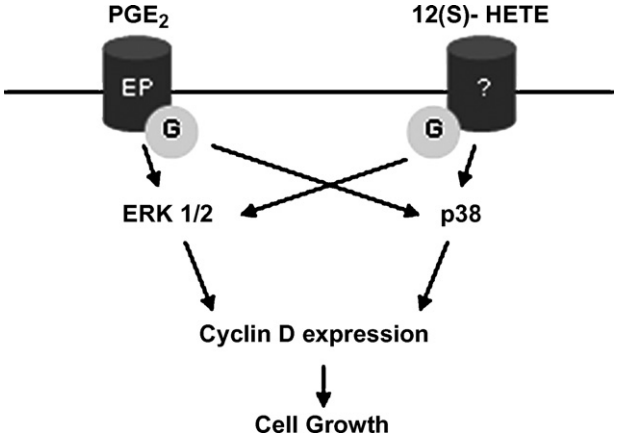

Fig. 6 – The mitogenic effects of 12(S)-HETE and PGE_2 are dependent on the ERK 1/2 and p38 MAPK pathways activation and the subsequent enhancement of cyclin D_1 levels.

posttranslationally to induce the cyclin D_1 assembly with cdk4 in murine fibroblast [36]. These events are of particular relevance, since our study provides evidence that the enhancement of cyclin D_1 induced by 12(S)-HETE is sensitive to ERK 1/2 and p38 MAPK pathway inhibitors, treatments that also inhibited 3T6 fibroblast growth. These findings suggest that ERK 1/2 and p38 MAPK pathways induced by 12(S)-HETE are involved in cyclin D_1 expression and consequently in the control of 3T6 fibroblast growth.

We conclude that the enantioselective mitogenic effect of 12(S)-HETE is dependent on the ERK 1/2 and p38 MAPK pathways as well as cyclin D_1 and propose that this effect is mediated through a PTX-sensitive G protein-coupled receptor (Fig. 6). Our results also show that 12(S)-HETE and PGE_2 share signal transduction pathways and present synergistic effects on cell growth.

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