Hydroxyeicosatetraenoic acids released through the cytochrome P-450 pathway regulate 3T6 fibroblast growth

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Abstract **Eicosanoids participate in the regulation of cellular proliferation. Thus, we observed that prostaglandin E_2 interaction with membrane receptors is involved in the control of 3T6 fibroblast growth induced by serum. However, our results suggested that another arachidonic acid pathway might be implicated in these events. Our results show that 3T6 fibroblasts synthesized hydroxyeicosatetraenoic acids (HETEs) such as 12-HETE through the cytochrome P-450 (CYP450) pathway. However, 3T6 fibroblasts did not produce leukotriene B_4 (LTB_4), and lipoxygenase inhibitors and LT antagonists failed to inhibit 3T6 fibroblast growth induced by FBS. In contrast, we observed that CYP450 inhibitors such as SKF-525A, 17-octadecynoic acid, 1-aminobenzotriazole, and 6-(2-propargyloxyphenyl)hexanoic acid reduced 12(*S*)-HETE levels, 3T6 fibroblast growth, and DNA synthesis induced by FBS. The impairment of DNA synthesis and 3T6 fibroblast growth induced by SKF-525A were reversed by exogenous addition of HETEs. Moreover, we report that 5-HETE, 12(*S*)-HETE, and 15(*S*)-HETE are mitogenic on 3T6 fibroblast in the absence of another growth factor, and this effect was dependent on the activation of the phosphatidylinositol-3-kinase pathway. In conclusion, our results show that HETEs, probably produced by CYP450, are involved in the control of 3T6 fibroblast growth.**—Nieves, D., and J. J. Moreno. **Hydroxyeicosatetraenoic acids released through the cytochrome P-450 pathway regulate 3T6 fibroblast growth.** ***J. Lipid Res.*** **2006.** 47: **2681–2689.**

Supplementary key words cell proliferation • cell cycle • Akt kinase

Free arachidonic acid (AA) can be oxidized by three major metabolic pathways: the cyclooxygenases (COXs), which produce prostaglandins (PGs) and thromboxanes; the lipoxygenases (LOXs), which form leukotrienes (LTs), hydroxyeicosatetraenoic acids (HETEs), and lipoxins; and the cytochrome P-450 monooxygenases (CYP450s) (1). The CYP450 proteins metabolized AA by one or more of the following reactions: bis-allylic oxidation (LOX-like reaction) to generate 5-, 8-, 9-, 11-, 12-, and 15-HETEs; ω/ω-1 hydroxylation gives 16-, 17-, 18-, 19-, and 20-HETEs; or olefin epoxidation, producing 5,6-, 8,9-, 11,12-, and 14,15-epoxyeicosatrienoic acids (EETs). Finally, the cytosolic epoxide hydrolases catalyze a rapid enzymatic hydration of the EETs to dihydroxyeicosatetraenoic acids (2).

Eicosanoids have numerous physiological effects (3), including cell proliferation and differentiation. Recent studies in our laboratory have suggested that the COX pathway is involved in serum-induced 3T6 fibroblast proliferation. On the one hand, we have demonstrated that COX-2 inhibition reduces 3T6 fibroblast proliferation in a concentration-dependent manner, producing an ~50% maximum effect (4). On the other hand, we have shown that EP_1 and EP_4 receptors of PGE_2 antagonists reduced 3T6 fibroblast growth in a concentration-dependent manner, but to almost complete inhibition (5). Furthermore, we have reported that phospholipase A_2 (PLA_2) inhibitors reduced serum-induced 3T6 fibroblast growth and [^{3}H]thymidine incorporation almost completely (6). These results suggested that other AA cascade pathways might be involved in these events (i.e., LOX or CYP450 pathway).

Little is known about LOX expression and LOX product biosynthesis in fibroblasts. Typically, LOX is expressed in platelets, leukocytes, neutrophils, monocytes, macrophages, endothelial cells, and smooth muscle cells (7–9). Kitzler and Eling (9) cloned, sequenced, and expressed 5-LOX cDNA from Syrian hamster embryo fibroblasts, although they did not detect 5-HETE. Furthermore, Newby and Mallet (10) found that human dermal fibroblasts produce 12-HETE and that without stimulation its concentration is higher than that of PGE_2. These authors also detected 15-HETE, whereas 5-HETE was not encountered. Other studies found that interleukin-1 increased the pro-

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Abbreviations: AA, arachidonic acid; ABT, 1-aminobenzotriazole; COX, cyclooxygenase; CYP450, cytochrome P-450; EET, epoxyeicosatrienoic acid; HETE, hydroxyeicosatetraenoic acid; LOX, lipoxygenase; LT, leukotriene; NDGA, nordihydroguaiaretic acid; 17-ODYA, 17-octadecynoic acid; PG, prostaglandin; PI3K, phosphatidylinositol-3-kinase; PLA2, phospholipase A2; PPOH, 6-(2-propargyloxyphenyl)-hexanoic acid.

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duction of 12- and 15-HETE in human dermal fibroblasts (11, 12). Additionally, it has been demonstrated that fibroblasts can respond to LOX products such as LTs or HETEs. For example, Hasegawa et al. (7) demonstrated that 12-HETE activates adenylyl cyclase via increasing intracellular Ca^2+ concentration in human normal fibroblast TIG-1 cells. Moreover, Chibana et al. (13) showed that human fetal lung fibroblast HFL-1 cells respond to LTC_4, and when they are stimulated with interleukin-13, cysteinyl LT receptor 1 mRNA and protein are upregulated.

With regard to CYP450s, several isoforms have been described in fibroblasts. Saeki et al. (14) demonstrated CYP450 1A1, 1B1, and 2E1 mRNA expression, whereas CYP450 1A2, 2A7, 2B6, and 3A4 mRNAs were not detected. Thus, CYP450s could release HETEs in fibroblast cultures. There are few studies about AA-CYP450 metabolites in fibroblasts, and the presence of EETs in fibroblasts has not been quantified. However, Fang et al. (15) demonstrated that human skin fibroblasts produce dihydroxyeicosatetraenoic acids when they are exposed to a relatively high concentration of EETs.

LOX and CYP450 pathway metabolites have been described as regulators of several functions, including cell proliferation. Thus, some studies show that LTD_4 and LTB_4 increase cell growth, inducing extracellular signal-regulated kinase 1/2 phosphorylation in intestinal epithelial cells and human pancreatic cancer cells, respectively (16, 17). It has also been demonstrated that 5-, 12-, 15-, and 20-HETEs stimulate cell proliferation through mitogen-activated protein kinase and Akt pathway activation in pancreatic cancer cells, human epidermoid carcinoma cells, and vascular smooth muscle cells (18–21). Finally, Chen et al. (22) found that all EETs stimulate [^{3}H]thymidine incorporation in renal epithelial cells, and their effects are mediated by the activation of the Src kinase and tyrosine kinase phosphorylation cascade.

Therefore, the aim of this study was to investigate the role of LOX and CYP450-dependent AA metabolism in the control of serum-induced 3T6 fibroblast proliferation.

MATERIALS AND METHODS

Materials

RPMI 1640 medium, FBS, penicillin, streptomycin, and trypsin-EDTA were purchased from BioWhittaker Europe (Verviers, Belgium). Nordihydroguaiaretic acid (NDGA), zileuton, 17-octadecynoic acid (17-ODYA), 1-aminobenzotriazole (ABT), 6-(2-propargyloxyphenyl)hexanoic acid (PPOH), wortmannin, *Penicillium funiculosum*, propidium iodide, Triton X-100, PMSF, Igepal CA-630, aprotinin, leupeptin, DTT, RNase A from bovine pancreas, acridine orange, and ethidium bromide were provided by Sigma Chemical Co. (St. Louis, MO). The LT antagonist receptors U-75302, LY-171883, and REV-5901 and the metabolites 5-, 12(*S*)-, 15(*S*), and 20-HETE and 11,12-EET were supplied by Cayman Chemical Co. (Ann Arbor, MI). Baicalein and SKF-525A hydrochloride (proadifen) were from Calbiochem (La Jolla, CA). [Methyl-^{3}H]thymidine (20 Ci/mmol) was from American Radiolabeled Chemicals, Inc. (St. Louis, MO). All chemicals were of the highest quality available commercially.

Cell culture

Murine 3T6 fibroblasts (CCL96; American Type Culture Collection) 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 on 24-well plates (Tissue-Culture Cluster 24; Costar, Cambridge, MA) or in Tissue-Culture 60 mm dishes (Costar) for experimental purposes. Cell cultures were maintained in a temperature- and humidity-controlled incubator at 37°C with 95% air and 5% CO_2 for 24 h.

Cell growth assay

The effect of the treatments was assessed on 3T6 fibroblast plates at 20×10^3 cells/well on 24-well plates cultured for 24 h in RPMI 1640 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, cells were washed, trypsinized, and counted with a microscope using ethidium bromide/acridine orange staining to assess viability.

Analysis of DNA synthesis

DNA synthesis was measured by a [^{3}H]thymidine incorporation assay, which involved culturing 3T6 fibroblasts on 24-well plates in RPMI 1640 with 10% FBS at a density of 20×10^3 cells/well. After 24 h of serum starvation, cells were incubated for 48 h with the treatments, and [^{3}H]thymidine (1 μCi/well) was added for the last 24 h. [^{3}H]thymidine-containing media were aspirated, and 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.

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

Fibroblasts were seeded in 60 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 ~80%. Cells were then cultured in 10% FBS RPMI containing the treatments. Thereafter, they were trypsinized, 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 analyzed on an Epics XL flow cytometer (Coulter Corp., Hialeah, FL). DNA was analyzed (ploidy analysis) on single fluorescence histograms using Multicycle software (Phoenix Flow Systems, San Diego, CA).

Assay of 12-HETE levels

Fibroblasts were seeded in 60 mm dishes at a density of 6×10^4 cells/dish and cultured for 24 h in RPMI 1640 supplemented with 10% FBS. Afterward, cells were incubated for 48 h in 10% FBS medium containing different compounds. Then, 12-HETE was extracted from culture supernatant medium through C_{18} reverse-phase extraction columns in ethyl acetate. Next, the organic phase was evaporated in a stream of nitrogen and 12(*S*)-HETE was measured using a 12(*S*)-HETE enzyme immunoassay kit (Assay Designs, Inc., Ann Arbor, MI) according to the manufacturer's protocol.

Measurement of PGE_2 and LTB_4

Cells were cultured at 25×10^3 cells/well on 24-well plates for 24 h in RPMI 1640 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 acidified with 1 ml of 1% formic acid. PGE_2 and LTB_4 were extracted in ethyl acetate, and the

organic phase was evaporated under a stream of nitrogen. Finally, PGE_2 and LTB_4 were determined using monoclonal enzyme immunoassay kits (Cayman Chemical Co., Ann Arbor, MI) according to the manufacturer's protocol.

Western blot analysis of Akt and phosphorylated Akt

Fibroblast cultures were FBS-starved for 24 h and incubated for 30 min with HETEs. The total cellular fraction was obtained by 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 (23) and blotted for 1 h with a constant voltage of 100 V onto a polyvinylidene difluoride membrane (Immun-Blot membrane, 0.2 μm; Bio-Rad, Hercules, CA) using a MiniProtean II system (Bio-Rad). Membranes were blocked with 5% nonfat milk powder in PBS-0.1% Tween 20 for 1 h. Rabbit polyclonal antibodies against Akt or phospho-Akt (serine 473) were applied in a 1:1,000 dilution overnight. The blot was washed several times with PBS-0.1% Tween 20 and incubated with a horseradish peroxidase-conjugated goat anti-rabbit antibody in a 1:2,000 dilution for 1 h. Finally, blots were developed using an enhanced chemiluminescence kit. Antibodies and the chemiluminescence kit were supplied by Cellular Signaling (Beverly, MA).

Statistics

Results are expressed as means ± SEM. Differences between control and treated cultures were tested using Student's *t*-test.

RESULTS

Effect of LOX inhibitors and LT receptor antagonists on the growth and DNA synthesis of 3T6 fibroblasts

First, we studied the role of the LOX pathway in the control of 3T6 fibroblast proliferation. We used several LOX inhibitors, such as zileuton, a specific 5-LOX inhibitor (24), the 5- and 12-LOX inhibitor baicalein (25), and the unspecific LOX inhibitor NDGA (26). As shown in **Fig. 1**, these treatments produced little growth inhibition and [^{3}H]thymidine incorporation inhibition compared with the COX inhibitor ketoprofen (27). We also studied the role of the LOX pathway on 3T6 fibroblast proliferation with LT receptor antagonists. We used U-75302, REV-5901, and LY-171883 as LTB_4, LTD_4, and CysLT receptor antagonists, respectively (28–30). All antagonists induced a low growth and [^{3}H]thymidine incorporation inhibition. On the other hand, we quantified the LTB_4 concentration in 3T6 fibroblast cultures stimulated with 10% FBS. **Table 1** shows that 3T6 fibroblasts produce a small quantity of this eicosanoid. Thus, our data indicate that the LOX pathway does not play a significant role in the control of 3T6 fibroblast growth.

Effect of CYP450 inhibitors on the growth, DNA synthesis, and cell cycle distribution of 3T6 fibroblasts

Once the LOX pathway was ruled out, we examined the role of CYP450 metabolites in the control of serum-induced 3T6 fibroblast growth. We used four structurally and mechanistically different CYP450 inhibitors. SKF-525A and 17-ODYA are nonselective CYP450 inhibitors that inhibit the ω-hydroxylation and epoxygenation of AA (31, 32). On the other hand, ABT is a specific ω-hydroxylation inhibitor (33), and PPOH is a potent and selective molecule that inhibits the conversion of AA into EETs (31). These compounds are reported as specific CYP450 enzyme inhibitors, but we checked to determine whether they have some effect on COX activity. Table 1 shows that some CYP450 inhibitors appreciably reduce PGE_2 production. Thus, ABT is the CYP450 inhibitor that has the greatest effect and reduces PGE_2 synthesis by ~50%. In parallel, we confirmed that CYP450 inhibitors significantly reduced 12(*S*)-HETE production induced by 10% FBS in 3T6 fibro-

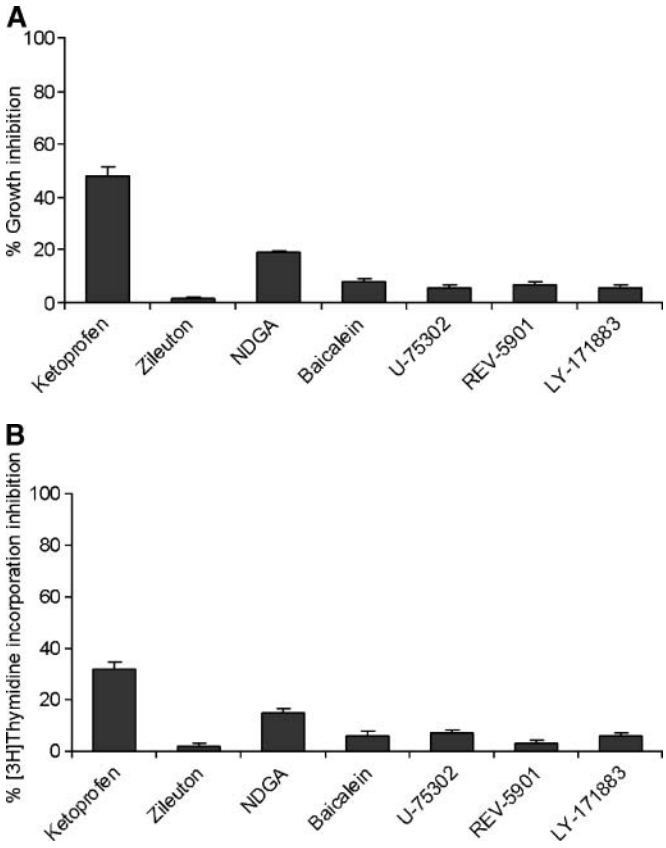

Fig. 1. Effect of lipoxygenase (LOX) inhibitors and leukotriene receptor antagonists on 10% FBS-induced 3T6 fibroblast proliferation and [^{3}H]thymidine incorporation. Fibroblasts (25×10^3 cells/well) were plated and cultured in 10% FBS-RPMI. The next day, media were removed and free FBS-RPMI was added for 24 h. Then, cells were incubated for 48 h with 10% FBS-RPMI containing ketoprofen (5 μM), zileuton (5 μM), nordihydroguaiaretic acid (NDGA; 5 μM), baicalein (30 μM), U-75302 (10 μM), REV-5901 (10 μM), or LY-171883 (100 μM). A: After a 2 day treatment, cells were trypsinized and counted using microscopy. B: For the last 24 h of treatment, [^{3}H]thymidine (1 μCi/well) was added to the cells. Radioactivity in cell lysates was determined in a scintillation analyzer. Cell number and [^{3}H]thymidine incorporation in control cells were 825,231 ± 79,254 cells and 176,344 ± 11,675 dpm, respectively. Results are means ± SEM from three experiments performed in triplicate.

TABLE 1. Effects of CYP450 inhibitors on PGE_2, LTB_4, and 12(*S*)-HETE production in 3T6 fibroblast cultures

Treatment	Concentration		
	PGE_2	LTB_4	12(*S*)-HETE
	pg/ml		
FBS, 10%	608 ± 13.20	18 ± 1.5	1,154 ± 23.4
Ketoprofen	52 ± 2.2	24 ± 2.1	1,572 ± 21.3
SKF-525A	511 ± 12.1	ND	528 ± 11.6
17-ODYA	508 ± 6.1	ND	394 ± 13.7
ABT	288 ± 9.8	ND	756 ± 9.8
PPOH	458 ± 10.1	ND	826 ± 24.5

ABT, 1-aminobenzotriazole; CYP450, cytochrome P-450; HETE, hydroxyeicosatetraenoic acid; LT, leukotriene; ND, not determined; 17-ODYA, 17-octadecynoic acid; PG, prostaglandin; PPOH, 6-(2-propargyloxyphenyl)hexanoic acid. Cells were incubated for 48 h with 5 μM SKF-525A, 50 μM 17-ODYA, 2 mM ABT, or 50 μM PPOH. Eicosanoid concentrations were assayed as described in Materials and Methods. Data are expressed in percentages and are means ± SEM from three experiments performed in triplicate.

blast cultures. In this way, the effects of SKF-525A and 17-ODYA were found to be higher than those of ABT or PPOH.

As shown in **Fig. 2**, CYP450 inhibitors inhibit 3T6 fibroblast growth and [^{3}H]thymidine incorporation in a concentration-dependent manner. Ethidium bromide/acridine orange staining and morphologic examination demonstrated that CYP450 inhibitors did not produce changes in cell structure and cell viability (**Table 2**). Thus, the effects on cell growth mentioned above do not appear to be a consequence of cytotoxicity.

Next, we chose the CYP450 inhibitor SKF-525A to examine cell cycle changes by flow cytometry. 3T6 fibroblast cultures were G_0/G_1 synchronized after 24 h serum starvation and incubated with 10% FBS medium containing SKF-525A (5 μM). Then, cells were collected and stained with propidium iodide and cell cycle distribution was analyzed. **Figure 3** provides data from a representative experiment and shows that SKF-525A produces a cell cycle delay. Thus, an increase in S population and a decrease in G_0/G_1 population were observed at 9 h in cultures incubated with 10% FBS, whereas these changes appeared at ~20 h in cells treated with SKF-525A (5 μM). This appreciable cell cycle delay could explain the inhibition of cell growth and [^{3}H]thymidine incorporation observed in cultures containing SKF-525A.

Effect of AA-CYP450 metabolites on 3T6 fibroblast growth and DNA synthesis

To elucidate the role of AA-CYP450 metabolism in cell growth, we exogenously added some AA-CYP450 metabolites to cells stimulated with 10% FBS containing the CYP450 inhibitor SKF-525A (5 μM). As shown in **Fig. 4A**, SKF-525A produced a growth inhibition of ~50%, and the addition of hydroxylated AA-CYP450 metabolites such as 5-, 12-, 15-, and 20-HETE reversed this inhibitory effect. In contrast, epoxygenated AA-CYP450 metabolites such as 11,12-EET did not reverse this cell growth inhibition. With regard to DNA synthesis, SKF-525A also significantly inhibited [^{3}H]thymidine uptake induced by 10% FBS, and

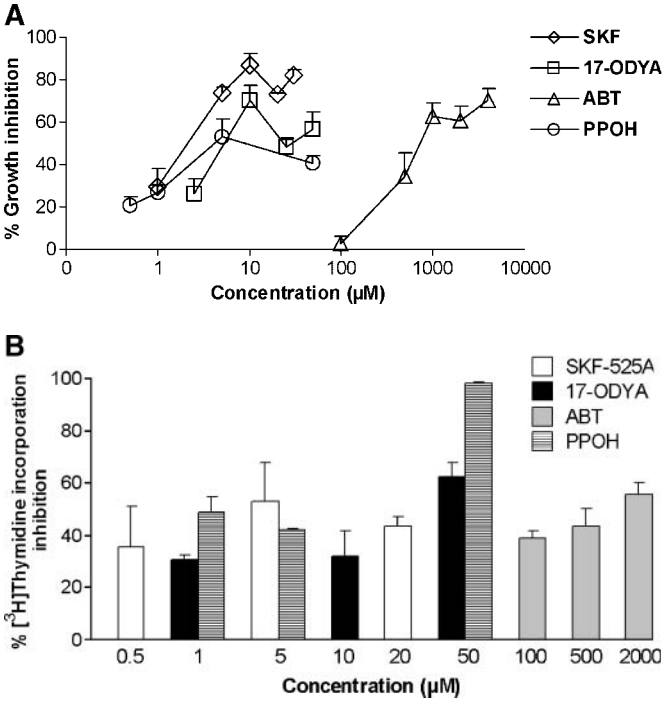

Fig. 2. Effect of cytochrome P-450 (CYP450) inhibitors on cell growth and [^{3}H]thymidine incorporation. 3T6 fibroblasts (25×10^3 cells/well) were plated and cultured in 10% FBS-RPMI. The next day, media were removed and free FBS-RPMI was added for 24 h. Then, cells were incubated for 48 h with 10% FBS-RPMI containing SKF-525A, 17-octadecynoic acid (17-ODYA), 1-aminobenzotriazole (ABT), or 6-(2-propargyloxyphenyl)hexanoic acid (PPOH) at the indicated concentrations. A: After a 2 day treatment, cells were trypsinized and counted using microscopy. B: For the last 24 h of treatment, [^{3}H]thymidine (1 μCi/well) was added to the cells. Radioactivity in cell lysates was determined in a scintillation analyzer. Cell number and [^{3}H]thymidine incorporation in control cells were 816,334 ± 69,474 cells and 155,251 ± 10,867 dpm, respectively. Results are means ± SEM from three experiments performed in triplicate.

5-, 12-, and 15-HETE reversed this effect, but 11,12-EET did not (Fig. 4B). Therefore, these results suggest that HETEs have comitogenic action together with growth factors present in FBS.

These data suggest than HETEs synthesized by CYP450 enzymes from AA are involved in the signal transduction pathways induced by FBS and consequently can be regarded as comitogenic factors. To determine the mitogenic effect of AA-CYP450 metabolites, we assayed the effect of HETEs on 3T6 fibroblast growth in the absence of growth factors (FBS). **Figure 5** shows that 5-, 12-, and 15-HETE significantly increase cell number. On the other hand, these HETEs also have a mitogenic effect on the [^{3}H]thymidine incorporation assay.

Role of the Akt pathway in 3T6 fibroblast growth and DNA synthesis stimulated by HETEs

Once the comitogenic and mitogenic effects of HETEs on 3T6 fibroblasts were determined, we investigated through which cell signaling pathways these eicosanoids

TABLE 2. Effects of CYP450 inhibitors on 3T6 fibroblast viability

Treatment	Concentration	Viability
	μM	%
SKF-525A	0.5	98.15 ± 0.61
	5	98.24 ± 1.42
	20	96.11 ± 0.31
17-ODYA	1	98.72 ± 0.40
	10	95.89 ± 2.04
	50	95.56 ± 1.65
ABT	100	99.04 ± 0.22
	500	97.45 ± 0.27
	2,000	97.00 ± 0.95
PPOH	1	96.40 ± 1.12
	5	95.77 ± 0.75
	50	96.20 ± 0.69

Cells were incubated for 48 h with SKF-525A, 17-ODYA, ABT, or PPOH at the concentrations indicated. Cells were washed, trypsinized, and counted with a microscope using ethidium bromide/acridine orange staining to assess viability. Data are means ± SEM from at least three experiments performed in triplicate.

act. For this purpose, we studied the classical phosphatidylinositol-3-kinase (PI3K)/Akt pathway that is activated by mitogens and growth factors and that plays an important role in the control of cell growth and differentiation.

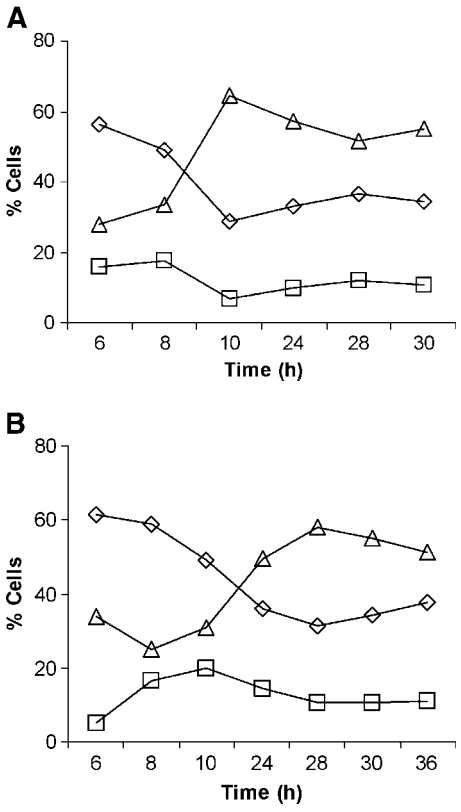

Fig. 3. Effect of the CYP450 inhibitor SKF-525A on cell cycle distribution in 3T6 fibroblast cultures. Cells were cultured with 10% FBS (A) or medium in the presence of 5 μM SKF-525A (B) for 6–36 h. Finally, cells were harvested and fixed with ethanol, and DNA was stained with propidium iodide. The DNA content was analyzed by fluorescence-activated cell sorting. Data are expressed as percentage of cells in the G_0/G_1 (diamonds), S (triangles), and G_2/M (squares) phases of the cell cycle. The results shown are representative of three independent experiments.

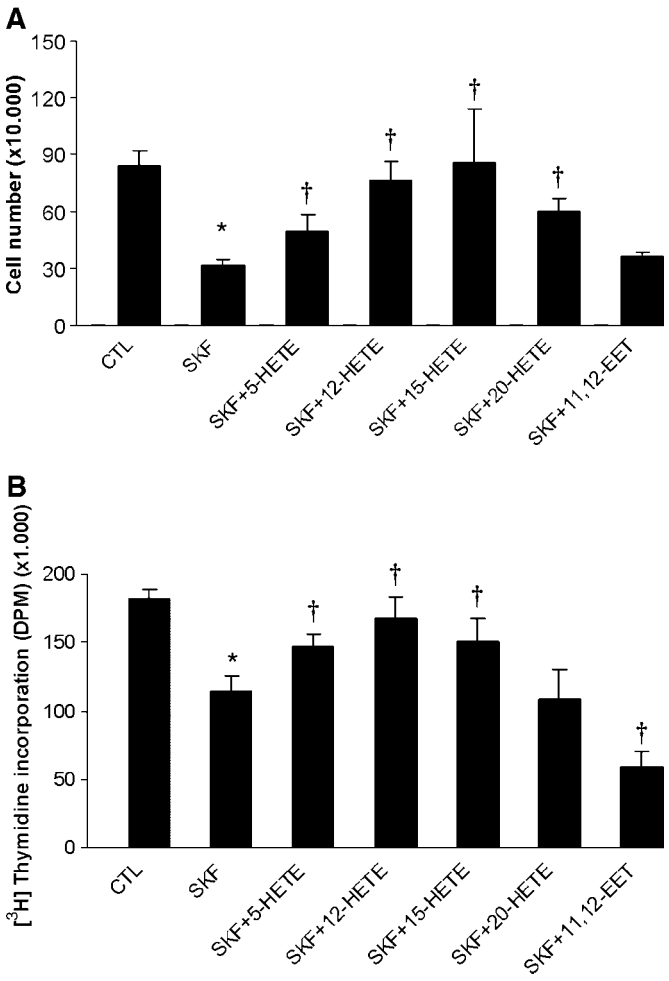

Fig. 4. Effect of exogenous hydroxyeicosatetraenoic acids (HETEs) on cell proliferation and [^{3}H]thymidine incorporation inhibited by SKF-525A. 3T6 fibroblasts (25×10^3 cells/well) were plated and cultured in 10% FBS-RPMI. The next day, media were removed and free FBS-RPMI was added for 24 h. Then, cells were incubated for 48 h with 10% FBS-RPMI containing 5 μM SKF-525A or the CYP450 inhibitor in the presence of 1 μM 5-HETE, 12(*S*)-HETE, 15(*S*)-HETE, 20-HETE, or 11,12-epoxyeicosatrienoic acid (EET). A: After a 2 day treatment, cells were trypsinized and counted using microscopy. B: For the last 24 h of treatment, [^{3}H]thymidine (1 μCi/well) was added to the cells. Radioactivity in cell lysates was determined in a scintillation analyzer. Results are means ± SEM from three experiments performed in triplicate. * $P < 0.05$ compared with nontreated cells; † $P < 0.05$ compared with SKF-525A-treated cells. CTL, control.

Our results show that wortmannin, a PI3K inhibitor (34), inhibited the increasing effect of 5-, 12-, and 15-HETE on 3T6 fibroblast growth (Fig. 5A). In a similar way, wortmannin reduced the [^{3}H]thymidine incorporation induced by 5-, 12-, and 15-HETE (Fig. 5). Thus, these results suggest that the mitogenic effect of HETEs was via PI3K/Akt. To confirm the effect of HETEs on the PI3K/Akt pathway, we performed Western blot analysis of Akt and phosphorylated Akt levels. Our results show that 5-, 12-, and 15-HETE increased phosphorylated Akt levels (**Fig. 6**), which supports the role of the PI3K/Akt pathway on the effects of HETEs in 3T6 fibroblast proliferation.

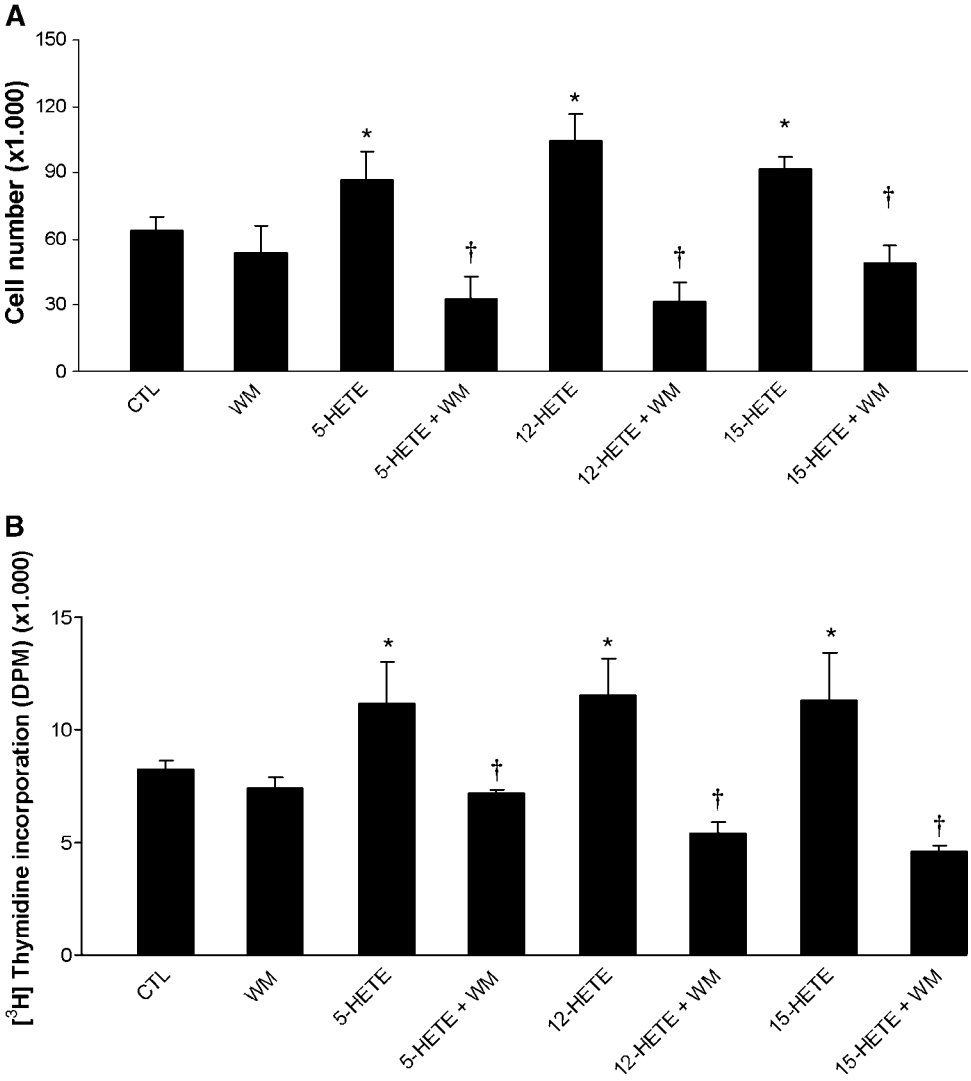

Fig. 5. Effect of exogenous HETEs on cell proliferation and [^{3}H]thymidine incorporation in 3T6 fibroblasts. Cells (25×10^3 cells/well) were plated and cultured in RPMI without FBS for 24 h. Then, cells were incubated for 2 days with RPMI without FBS containing 1 μM 5-HETE, 12(*S*)-HETE, or 15(*S*)-HETE in the absence or presence of 0.1 μM wortmannin (WM). A: Cells were trypsinized and counted using microscopy. B: For the last 24 h of treatment, [^{3}H]thymidine (1 μCi/well) was added to the cells. Radioactivity in cell lysates was determined in a scintillation analyzer. Results are means ± SEM from three experiments performed in triplicate. * $P < 0.05$ compared with nontreated cells. † $P < 0.05$ compared with cells treated with the corresponding HETE alone. CTL, control.

DISCUSSION

Calcium-independent cytosolic PLA_2 is involved in serum-induced 3T6 fibroblast proliferation (7), through AA release and the subsequent metabolism by the inducible form of COX-2, to synthesize PGs such as PGE_2 (5). The biological effects of PGE_2 have been attributed to its interaction with specific receptors. Thus, we provided evidence that PGE_2's interaction with EP_1 and EP_4 receptors is involved in the control of 3T6 fibroblast proliferation, which is associated with changes in D, E, and A cyclin levels (6). Recently, we also reported that the PGE_2-EP_3 receptor interaction may be involved in serum-induced 3T6 fibroblast growth as a result of their effects on cAMP levels and on the cell cycle machinery of the S phase (35). Thus, generation of and PGE_2 interaction with these receptors, in an autocrine or paracrine manner, may act as a necessary comitogenic signal.

When PG synthesis is abolished by COX inhibition, AA released after mitogenic stimuli by PLA_2 can be metabolized by LOXs and/or CYP450 enzymes. The mitogenic effect of other AA metabolites, which can be formed when the synthesis of PGs is blocked, may explain why COX inhibitors (36) have lower effects than PGE_2 antagonists (6) on 3T6 fibroblast growth. Considering these findings, the aim of this study was to elucidate the role of AA metabolism by LOX and CYP450 pathways in serum-induced 3T6 fibroblast growth.

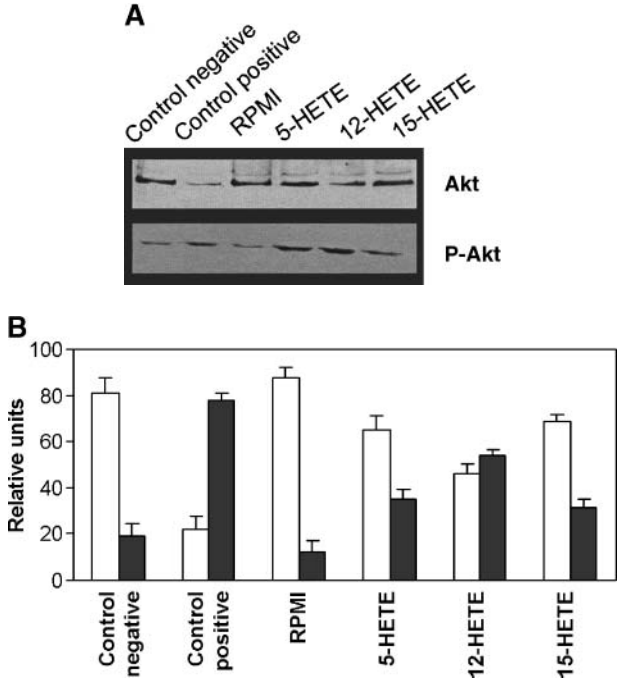

Fig. 6. A: Western blot of Akt and phosphorylated Akt (P-Akt) expression in cultured 3T6 fibroblasts. Cells were incubated with 1 μM 5-HETE, 12(*S*)-HETE, or 15(*S*)-HETE for 30 min, and Akt or phosphorylated Akt levels were determined using specific antibodies. Control negative was a LY-294002-treated Jurkat extract, and control positive was a calyculin A-treated Jurkat extract. Results are representative of three separate experiments. B: Akt (white bars) and phosphorylated Akt (black bars) values were normalized and are expressed as relative units. Results are means ± SEM from three experiments.

Our results show that serum induced PGE_2 and 12(*S*)-HETE synthesis by 3T6 fibroblasts but did not increase LTB_4 levels. Furthermore, we observed that COX inhibition by ketoprofen markedly enhanced 12(*S*)-HETE levels, suggesting that the inhibition of the COX pathway can upregulate the remaining pathways. Thus, we must consider that HETEs can be synthesized by LOXs or CYP450s. On the other hand, specific 5-LOX inhibitors (zileuton), 12-LOX inhibitors (baicalein), or LT antagonists (U-75302, REV-5901, and LY-171883) did not inhibit 3T6 fibroblast growth, whereas NDGA, a nonselective LOX inhibitor (25) and CYP450 inhibitor (37), had an appreciable effect on fibroblast growth. These preliminary results suggested that HETEs, probably produced through the CYP450 pathway, might be involved in the regulation of 3T6 fibroblast growth. To confirm this hypothesis, we measured the effect of CYP450 inhibitors such as SKF-525A, 17-ODYA, ABT, and PPOH on 12(*S*)-HETE synthesis and fibroblast proliferation. All inhibitors markedly reduced 12(*S*)-HETE levels, cell growth, and DNA synthesis. However, ABT and PPOH also reduced PGE_2 levels. Thus, their effects on cell proliferation could be a consequence of the impairment of both PG and HETE synthesis. In contrast, the effects of SKF-525A and 17-ODYA appear to be related to the inhibition of HETE synthesis.

To understand the effect of SKF-525A on 3T6 fibroblast growth, we investigated which point in the cell cycle is affected by the CYP450 inhibitor. Previously, Pidgeon et al. (38) demonstrated that the inhibition of 12(*S*)-HETE synthesis induced prostate cancer cell cycle arrest at G_0/G_1. In this study, we provide evidence that CYP450 inhibitors such as SKF-525A induce a cell cycle delay. Thus, serum induces entry into the cell cycle, and we observed the enhancement of S phase and the impairment of G_0/G_1 10 h later. However, these events were observed 24 h later, when SKF-525A was present. Therefore, it can be argued that the effect of SKF-525A on 3T6 fibroblast growth was the result of CYP450 inhibition and the subsequent impairment of HETE synthesis. The reversal of these SKF-525A effects by exogenous addition of HETEs confirms this hypothesis. Interestingly, 5-HETE, 12(*S*)-HETE, 15(*S*)-HETE, and 20-HETE but not 11,12-EET reversed the effects of SKF-525A. These data suggest that HETEs are involved in the signal transduction pathways induced by FBS and that HETEs can be regarded as comitogenic factors. These observations agree with the comitogenic effect of 20-HETE in proximal tubular cells (39), 12-HETE in microvascular endothelial cells (40), 5-, 12-, and 15-HETE in mammary epithelial cells (41), and 12-HETE in corneal epithelial cells (42).

On the other hand, several authors reported that HETEs could be mitogenic agents. Thus, Palmberg et al. (43) reported that 15-HETE but not 5-HETE induced DNA synthesis in arterial smooth muscle cells. 12-HETE also regulates DNA synthesis in human lens epithelial cells (44), microvascular endothelial cells (40), and artery smooth muscle cells (45). Additionally, Zeng et al. (46) recently reported that 5-HETE induced DNA synthesis in human microvascular endothelial cells. Our results show that 5(*S*)-, 12(*S*)-, and 15(*S*)-HETE are mitogenic factors that induce 3T6 fibroblast growth and increase DNA synthesis.

Understanding the mechanism by which HETEs are involved in cell growth may be a critical issue in cell growth/cancer and lipid homeostasis. However, these molecular mechanisms have not been fully elucidated. No cellular receptors for HETEs have yet been identified, but some potential pathways by which they may stimulate cellular activities have been explored. This work provides the first evidence that 5-, 12-, and 15-HETE are mitogenic in fibroblasts through PI3K/Akt, a metabolic pathway involved in the control of cell growth (47) and cell survival (48). Thus, wortmannin, a PI3K inhibitor, blocked 3T6 fibroblast growth and DNA synthesis induced by the three HETEs. Moreover, we observed that 5-, 12-, and 15-HETE increased phosphorylated Akt levels. Recently, it was shown that 5-, 12-, and 15-HETE activate Akt toward stimulating growth in microvascular endothelial cells (46), prostate cancer cells (38), and dermal microvascular endothelial cells (49), respectively. Our findings clearly provide evidence for the efficacy of HETEs on stimulating 3T6 fibroblast growth.

In conclusion, we provide evidence indicating that 5-HETE, 12(*S*)-HETE, and 15(*S*)-HETE, probably synthesized from AA by CYP450, are involved in the control of 3T6 fibroblast proliferation induced by serum. Moreover,

we observed that these HETEs are mitogenic on 3T6 fibroblasts and that this effect is dependent on activation of the PI3K pathway. Future studies are required to determine the receptors involved in these events and whether HETEs also regulate other pathways, such as mitogen-activated protein kinase, that are involved in fibroblast growth.

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