5-Lipoxygenase and human pulmonary artery endothelial cell proliferation

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Received 8 January 2001; accepted in final form 5 October 2001

5-Lipoxygenase (5LO) catalyzes the first two reactions in the biosynthesis of leukotrienes, which are potent mediators involved in inflammation and allergic reactions (20). A number of studies have also shown a role for 5LO in cell proliferation, although the effect of the enzyme on cell growth is cell-type specific: 5LO facilitates the growth of lung carcinoma (7, 38), adenocarcinoma of the prostate (5, 22, 25), pancreatic adenocarcinoma (17), adenocarcinoma of the colon (26), and chronic myelogenous leukemia (3), but it suppresses the growth of other cells, including glioma (21, 32), adenocarcinoma of the breast (36), and murine Leydig cell tumors (33). In addition, 5LO has been found to mediate cytokine-induced lymphocyte (30) and murine thymocyte (40) growth and to facilitate epidermal cell proliferation (12).

The mechanism of 5LO-associated cell proliferation is not yet completely understood. Some studies (7, 22) have shown that 5-hydroxy-eicosatetraenoic acid (5-HETE), a reduced derivative of the first product of 5LO, stimulates cell proliferation and reverses the effect of 5LO inhibition on cell growth. Other studies have demonstrated an apoptotic effect induced by 5LO inhibitors (2, 18, 22). A variety of 5LO inhibitors including MK-886, AA-861, nordihydroguaiaretic acid (NDGA), A-79175, BW-755c, BW-A4C, BW-B70C, zileuton, eicosatetraynoic acid, A-63162, SC-41661A,ICI-230487, cirsiol, L-651392, and L-651896 have been used to demonstrate the effect of 5LO on cell proliferation (4, 7, 9, 12, 17, 26, 37, 38, 42). Among them, MK-886 has been demonstrated by one report to cause apoptosis independent of 5LO inhibition (14, 15).

Increased expression of 5LO in pulmonary artery endothelial cells (PAECs) has been found in rats with pulmonary hypertension induced by chronic hypoxia (43), in patients with primary pulmonary hypertension (45), and in mice challenged with allergen (13). Although the mechanism is still unclear, the induction of 5LO expression in PAECs perhaps reflects altered endothelial cell function associated with these diseases. In pulmonary hypertension, abnormal endothelial cell proliferation is found in plexiform lesions of the remodeled pulmonary vasculature (44).

In a separate study, we have examined 5LO expression in cultured human PAECs (HPAEC). The study showed that the cells express minimal but detectable amounts of 5LO as determined by RT-PCR in combination with cDNA sequencing, Western blotting, and activity assay. Considering the role of 5LO in the proliferation of other types of cells, we tried to determine whether or not 5LO affects PAEC growth. To examine this question, we treated the cells with two 5LO inhibitors, AA-861 (46) and NDGA (39), and a...
5LO-activating protein (FLAP) inhibitor MK-886 (19, 23). In addition, 5LO antisense phosphorothioate oligodeoxynucleotide transfection was used as another more selective method for suppressing expression of 5LO. Using these reagents, we examined the effects of 5LO inhibition on PAEC proliferative responses.

EXPERIMENTAL PROCEDURES

Materials. HPAEC and EB2-MV media were obtained from Clonetics (Walkersville, MD). AA-861 was purchased from BioMol (Plymouth Meeting, PA). MK-886 and indomethacin were obtained from CalBiochem (San Diego, CA). NDGA, indomethacin, propidium iodide solution, and RNase I were purchased from Sigma (St Louis, MO). [3H]thymidine was obtained from New England Nuclear (Boston, MA). OptiMEM was purchased from Gibco (Grand Island, NY).

Cell culture and hypoxic treatment. Unless otherwise stated, HPAECs were grown in EGM2-MV medium, which contains basic growth medium (EBM-2) and antibiotics, ascorbic acid, vascular endothelial growth factor (VEGF), long-term insulin-like growth factor-1 (B3-IGF-1), human epidermal growth factor (hEGF), human fibroblast growth factor (hFGF-B), and 5% fetal bovine serum provided by the manufacturer. Cells were maintained in a 37°C incubator equilibrated with 5% CO2-95% air and 100% humidity.

[3H]thymidine incorporation. HPAECs were seeded on 24-well tissue culture plates at a density of 1 × 104 cells/well. After 24 h, the cell culture media was replaced with serum-free media, and after 48 h from seeding, a 5LO inhibitor or DMSO (vehicle control) was added. After 72 h from seeding, 5% fetal bovine serum was added back to the media, and after 48 h from seeding, a 5LO inhibitor or DMSO (vehicle control) was added. After 72 h from seeding, 5% fetal bovine serum was added back to the media along with 1 μCi/well [3H]thymidine, and the cells were incubated for an additional 6 h. Cells were then washed twice with PBS and incubated in 10% TCA overnight at 4°C. The cells were then washed twice with 100% ethanol and dried completely. Precipitated DNA and protein were solubilized with 1 N NaOH and then added to scintillation fluid. Radioactivity was counted in a liquid scintillation counter.

Cell counts. HPAECs were seeded to six-well plates. After 24 h, the cell culture media was replaced with serum-free media, and after 48 h from seeding, a 5LO inhibitor or DMSO (vehicle control) was added. After 72 h from seeding, 5% fetal bovine serum was added back to the media. The cells were incubated for an additional 6 or 24 h before being digested with trypsin, neutralized, and collected by centrifugation for 5 min at 500 rpm. The cells were resuspended in cold PBS (95 μl), and cold MeOH (405 μl) was slowly added to the cell suspension; the cells were allowed to settle in this solution at 4°C overnight. Cell suspension was subsequently centrifuged for 5 min at 500 rpm, and the cells were resuspended in 0.5 ml propidium iodide solution (35 μg/ml propidium iodide and 8 μg/ml RNase I in PBS). Cells were allowed to incubate for 1 h in the dark at room temperature. Samples were run on a fluorescence-activated cell sorting scanner (FACScan) (Flow Cytometer, Becton-Dickinson; San Jose, CA) and analyzed using WinMDI 2.8 software.

5LO activity assay. For analysis of 5LO activity in intact cells, HPAECs grown in 100-mm tissue culture plates were washed twice with D-PBS and incubated in 2.5 ml D-PBS (with or without inhibitors) for 30 min at 37°C. The activity assay was started by the addition of 2.5 ml of 10 μM A-23187 and 100 μM arachidonic acid in PBS, and carried out at 37°C for 20 min. An internal standard, prostaglandin B2 (PGB2) (0.25 nmol), was added at the end of incubation, and the culture plate was stored at −80°C until extraction. After thawing, 1 ml of methanol was added, and the medium was transferred to a 15-ml tube and centrifuged at 150 g for 15 min. The lipid in the supernatant was extracted with a C18 SepPak column (200 mg, Waters, Milford, MA) by consecutively passing the following solutions through the column: MeOH, H2O, sample, 0.1% acetic acid in water, and MeOH in volumes of 3, 3, 6, 3, and 3 ml, respectively. The final eluant was collected and dried under nitrogen. The lipids were dissolved in 250 μl solvent A (MeOH/H2O/acetic acid/NH4OH at 60:40:0:1:0:04), the solution was centrifuged at 20,000 g for 15 min, and 100 μl of the supernatant was injected into the HPLC column.

HPLC analysis was carried out using a C18 column (Radial-Pak model 5NYC1814μ, Waters, Milford, MA). After sample loading, the column was developed with a solvent gradient consisting of 38 min of solvent A, 1 min gradient to 30% of solvent B (MeOH/acetic acid at 100:1, vol/vol), 22 min of 30% of solvent B, 2 min gradient to 100% of solvent B, 4 min of 100% of solvent B, 1 min gradient to 100% of solvent A, and 18 min of solvent A. Eluate from the column was monitored by ultraviolet absorption at 234 and 280 nm, and quantitation of each compound was carried out by comparing its peak area with that of an internal standard, PGB2.

For analysis of activity in the presence of the three 5LO inhibitors, confluent plates of HPAECs were washed twice with D-PBS and then incubated with 2.5 ml of D-PBS with a corresponding concentration of inhibitor for 30 min at 37°C. The assay was then started by the addition of A-23187 and substrate as described above.

Antisense oligodeoxynucleotide transfection. An antisense phosphorothioate oligodeoxynucleotide to 5LO mRNA, 5’CAAGUCACGUGCUAAUGAUC3C’, was synthesized by Sequitur (Natick, MA), and a fluorescently labeled oligodeoxynucleotide with a random sequence was used as a control. HPAECs were plated on six-well tissue culture plates. After 24 h the cells had reached 80% confluence. Oligofectin I (6.6 μg/ml) and 5LO antisense or fluorescently labeled oligonucleotide (200 nM) was added to 2 ml of Opti-MEM and allowed to incubate with the cells for 15 min. The cells were washed twice with Opti-MEM, the media/Oligofectin/oligonucleotide mixture was added to the cells, and the cells were incubated for 5 h at 37°C. The transfection media were removed and replaced with 2 ml of EB2-MV with no serum. After 48 h, 5% fetal bovine serum and 1 μCi [3H]thymidine were added to each well, and then 50 μl of Opti-MEM and allowed to incubate with the cells for 15 min. The cells were washed twice with Opti-MEM, the media/Oligofectin/oligonucleotide mixture was added to the cells, and the cells were incubated for 5 h at 37°C. The transfection efficiency of the cells, HPAECs transfected with fluorescently labeled oligonucleotide were loaded with 1 μg CellTracker orange probe (Molecular Probes, Eugene, OR) for 30 min at 37°C. The cells were then assessed using an inverted microscope with fluorescein isothiocyanate and rhodamine filters.
COMET assay. Apoptosis in HPAECs was determined by the single-cell microgel electrophoresis assay (COMET assay) (27). Cells were treated with MK-886 for 48 h, after which they were embedded in situ in 1% agarose and exposed to alkaline lysis buffer (2.5 M NaCl, 1% sodium lauryl sarcosinate, 100 mM EDTA, 10 mM Tris base, 1% hydrogen peroxide, and carbonyl-free Triton X-100) for 30 min followed by 15 min of equilibration in electrophoresis buffer containing 300 mM NaOH, 10 mM EDTA, pH 10, 0.1% hydroxyquinoline, and 0.02% dimethyl sulfoxide. The nuclei were subsequently electrophoresed for 25 min at 2 V/cm, followed by staining with a fluorescent dye, YOYO-1 (3 μM/l), and visualized with a fluorescence microscope equipped with a fluorescein isothiocyanate filter. Comet formation was determined based on the appearance of the nucleus and the presence of a “tail” (8).

RESULTS

Effect of 5LO inhibitors on PAEC proliferation. [³H]thymidine incorporation was used to determine PAEC proliferation in the presence or absence of 5LO inhibitor. In this analysis, the cells were grown in standard culture medium for 24 h, in serum-free medium for 24 h, and in serum-free medium containing a 5LO inhibitor for 24 h. [³H]thymidine was then added to the medium together with 5% fetal bovine serum, and the incorporated radioactivity was measured 6 h later. As shown in Fig. 1, AA-861, MK-886, and NDGA all inhibited PAEC growth dose dependently with maximum inhibition of >90%. The IC₅₀ values for growth inhibition by these inhibitors were (in μM) 3.9, 0.48, and 1.8, respectively, and the IC₉₀ values were (in μM) 12.4, 0.75, and 2.6, respectively.
The effect of 5LO inhibitors on PAEC growth was also examined by cell number counting. In this analysis the same culturing scheme was followed, except that the [3H]thymidine was not added and the cell counts were taken at 24 h after serum replenishment. As shown in Fig. 2, the cell numbers decreased with the increasing concentrations of the inhibitors, and an abrupt decline of cell number was observed in the samples treated with 25 or 50 μM AA-861. Cell counts at 6 h after the serum replenishment did not show a significant difference between the untreated and inhibitor-treated PAECs, except for the samples treated with 25 and 50 μM AA-861 (Fig. 3). The decreased cell number in the two AA-861-treated samples suggested potential cytotoxicity of the compound at high concentrations (approximately two- and fourfold of its IC50 values for PAEC growth inhibition). The unchanged cell numbers in the remaining samples suggested that cell doubling had not begun by 6 h after serum replenishment.

To determine whether the 5LO inhibitors caused inhibition of cell proliferation by increasing any form of cell death, culture media from the inhibitor-treated PAECs were collected and analyzed for LDH activity. As shown in Fig. 4, no significant increase in LDH release was found in any of the inhibitor-treated samples except these treated with 25 or 50 μM AA-861.

Effect of 5LO inhibitors on cell cycles. Flow cytometric analysis for DNA content in the PAECs was carried out after fixation of the treated cells and staining with propidium iodide. As shown in Fig. 5, at 8 h after serum replenishment, the inhibitor-treated samples contained significantly less cells in S phase (DNA synthesis phase) than that of control ($P < 0.01$). Inhibitor-treated cells also showed trends toward an increase in the percentage of cells in G0/G1 (gap0/gap1 phase) and a decrease in the percentage of cells in G2/M (gap2/
mitosis phase), however, these did not reach significance. This suggested that the inhibitors prevented PAECs from entering S phase in response to serum replenishment.

**Effect of 5LO inhibitors on 5LO activity in PAECs.** Cultured HPAECs express a small amount of 5LO, and this endogenous enzyme activity could be detected when the cells were stimulated with calcium ionophore A-23187 in the presence of exogenous arachidonic acid. To attempt to correlate the effects of the 5LO inhibitors with cell proliferation and with 5LO activity, the cells were preincubated with AA-861, MK-886, or NDGA for 30 min before incubation with 10 μM calcium ionophore A-23187 and 50 μM arachidonic acid. Released lipid products were extracted and analyzed by HPLC. As shown in Fig. 6, 5LO activity in PAECs was inhibited by AA-861 and NDGA, and the dose-effect curves for inhibiting enzyme activity were comparable to those inhibiting cell growth (Fig. 1). MK-886, however, did not show an inhibitory effect on 5LO activity in this assay. Previous studies have demonstrated that utilizing exogenous substrate in an intact cell assay for 5LO activity greatly underestimates the potency of MK-886 (compare references 1, 19, 23), possibly due to the fact that 5LO is able to bind to its substrate without FLAP in the presence of abundant substrate.

**MK-886 and apoptosis.** Previous studies (2, 14, 18, 22) have shown that MK-886 induces apoptosis. We sought to examine whether MK-886 also has this effect on PAECs. At a concentration of 1.6 μM, a dose twice as high as its IC90 for inhibiting PAEC growth, MK-886 did not have a significant apoptotic effect on PAECs as determined by DNA ladder analysis. To detect mild apoptosis occurring in a small subpopulation of the MK-886-treated cells, we then used a more sensitive method, the COMET assay (8). This assay utilizes an alkaline agent to lyse individually embedded cells and visualizes endonucleosomal fragmentation by electrophoresis and DNA staining with YOYO-1. As shown in Fig. 7, ~9% of the MK-886-treated cells formed comet tails compared with only 1% of the untreated cells. These data indicated that high concentrations of MK-886 can produce a modest degree of apoptosis in PAECs. Owing to the concentration required to evoke this response, this apoptotic effect of MK-886 apparently does not explain its inhibitory effect on PAEC growth.

**Effect of growth factors in the culture medium.** The HPAECs used in this study were cultured in the presence of ascorbic acid and several growth factors, such as human VEGF (hVEGF), R3-IGF-1, and hEGF, and FGF-B. To determine whether or not the 5LO inhibitors were acting through these components of the medium, we compared the effect of AA-861 on the growth of the PAECs in different media. As shown in Fig. 8, eliminating the growth factors or growth factors plus ascorbic acid reduced cell growth by 27% and 40%, respectively. However, the inhibition of cell growth by AA-861 was not significantly altered (93.2, 94.3, and 97.1%, respectively), suggesting inhibition was not mediated through the specific integrity or effects of growth factors.

**Effect of indomethacin.** Inhibition of 5LO in PAECs would be expected to shunt the substrate arachidonic acid to cyclooxygenase. We, therefore, examined the effect of the cyclooxygenase inhibitor indomethacin on PAEC growth. As shown in Fig. 9, indomethacin had no effect on PAEC proliferation in the concentration range of 0.25–5.0 μM.

**Effect of 5LO antisense oligodeoxynucleotide on PAEC growth.** Introducing a 5LO antisense oligodeoxynucleotide into PAECs blocks the translation of
5LO mRNA into protein and thus provides an alternative method for inhibiting 5LO activity in PAECs. Because primary human endothelial cells are difficult to transfect, we initially examined various transfecting reagents and the doses of oligodeoxynucleotide in combination with the reagents. The highest transfection efficiency achievable in our experiments was 60% using 6.6 g/ml Oligofectin I, a cationic lipid transfecting reagent, combined with 200 nM of oligonucleotide in a total volume of 2 ml for transfecting one well of PAECs in a six-well plate (Fig. 10A). For evaluating the effect of 5LO antisense oligodeoxynucleotide on PAEC growth, this transfection method was used, and a sequence-unrelated, fluorescent-labeled oligodeoxynucleotide was employed as a transfection control. As shown in Fig. 10B, 5LO antisense oligodeoxynucle-
otide-transfected PAECs incorporated 40 ± 26% less [3H]thymidine than the control oligodeoxynucleotide-transfected cells, indicating that suppressing 5LO expression also inhibits PAEC proliferation.

DISCUSSION

To understand the role of 5LO in endothelial cell proliferation, we examined the effect of 5LO inhibition on PAEC growth. Four agents (AA-861, NDGA, MK-886, and a 5LO antisense oligonucleotide) were used to inhibit 5LO in PAECs, and four methods (thymidine incorporation, cell number counting, LDH release, and DNA content analysis) were employed to assess the growth inhibition. In addition, a 5LO activity assay was carried out to confirm the inhibitory effect of the agents on 5LO in PAECs. The data show that each of the agents inhibited PAEC proliferation. In the case of AA-861, NDGA, and MK-886, the inhibition of cell growth was not caused by increased cell death but, rather, by preventing the cells from entering the S phase. Dose-effect curves for AA-861 and NDGA on 5LO activity in PAECs was found to be similar to those for PAEC growth.

The reason for using multiple agents and methods to test a single effect of 5LO inhibition on PAEC growth is to minimize possible nonspecific effect(s) of the inhibitors. These agents could inhibit cell growth by simply killing the cells or by interacting with other molecules instead of 5LO. AA-861 and NDGA are quinone and phenol compounds, respectively. They inhibit lipoxygenase activity via redox effect (20). Reported IC50 for AA-861 is 1–10 μM for inhibiting 5LO activity (6, 37, 46), and that for NDGA is 0.3–2 μM (10, 39). At higher concentrations, AA-861 has been shown to inhibit activities of 12-lipoxygenases from porcine leukocytes and rat lung (100 μM IC50) (46); and NDGA inhibits activities of 12- and 15-lipoxygenases (30 μM IC50) and cyclooxygenase (100 μM IC50) in human leukocyte and platelet suspensions (39). In the present study, the IC50 values of AA-861 and NDGA for inhibiting PAEC proliferation were found to be 3.9 and 1.8 μM, respec-

Fig. 9. Effect of indomethacin, a nonselective cyclooxygenase inhibitor, on HPAEC growth. Cells were seeded and treated as in Fig. 1, except that indomethacin (0, 0.31, 0.63, 1.25, 2.5, or 5.0 μM) was added to the serum-free media. Each data point is presented as a percent control from the average of three experiments, each performed in quadruplicate.

Fig. 10. Effect of 5LO antisense oligodeoxynucleotide transfection on cell proliferation. A: transfection efficiency of a fluorescently labeled sequence-unrelated (control) oligodeoxynucleotide is compared with the total number of HPAECs stained with 1 μM CellTracker orange. B: effect of the antisense oligodeoxynucleotide transfection on HPAEC proliferation was determined by [3H]thymidine incorporation. Each column represents the percent control from the average of four experiments.
5-LO is an indole compound and interacts with FLAP (31); it inhibits 5LO activity in intact cells but has no effect on purified 5LO or 5LO in cell homogenate (23). Studies have shown that FLAP is an arachidonic acid-binding protein and suggest that it activates 5LO by presenting substrate (arachidonic acid) to the enzyme (29). In our assay system, exogenous substrate was required to detect 5LO activity in HPAECs. However, using excess substrate also minimized the effect of FLAP on activation, because 5LO is able to bind to its substrate without FLAP when the substrate is provided exogenously or in high concentrations. Thus the lack of effect of MK-886 on 5LO activity in PEAECs determined here may not be conclusive. Whether inhibition of PAEC proliferation by MK-886 was due to the inhibition of 5LO activity remains to be determined. Several reports have shown that MK-886 induces apoptosis in various cell types (2, 14, 18, 22). Two recent studies reported that the apoptotic effect of MK-886 is not due to the inhibition of 5LO (15) or even of FLAP (14). In the present study, no significant apoptotic effect of MK-886 was observed in PEAECs. Therefore, the specific effect of MK-886 apparent in other cell types may not be applicable to PEAECs.

The mechanism by which 5LO affects cell proliferation is not known, but it has long been suspected that it is related to the nuclear localization of the enzyme (11). 5LO may influence DNA transcription or synthesis by several possible mechanisms. One mechanism may involve its catalytic activity and the effect of downstream leukotrienes on gene transcription or DNA synthesis. A recent study has shown that leukotriene B4 binds peroxisome proliferator-activated receptor-α (PPARα), a nuclear hormone receptor or transcription factor, and upregulates PPARα-targeted genes (16). A second possibility may involve its binding to other proteins. 5LO has been shown to contain a Src homology 3-binding motif and to bind to growth factor receptor-bound protein 2 (28). Recent screening of a human lung cdNA library with a yeast two-hybrid system found that 5LO binds to transforming growth factor β-receptor-associated protein (35). Binding to these proteins could allow 5LO to influence correlated growth factor signaling and, therefore, cell growth. A third possibility involves 5LO binding to DNA directly or forming a DNA-binding complex with other nuclear factors, thereby affecting DNA transcription. The exact mechanism remains to be clarified.

Under normal in vivo conditions, adult vascular endothelial cells do not proliferate and remain quiescent for many years. Demonstrating a role of 5LO in PAEC proliferation, therefore, would have implications for specific pathophysiological conditions where endothelial cell proliferation and vascular remodeling occur in conjunction with 5LO expression e.g., primary pulmonary hypertension.

The authors express their gratitude to Stephanie Tribuna for excellent secretarial assistance.

This study was supported by National Heart, Lung, and Blood Institute Grants HL-55993, HL-58976, and HL-61795.
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5-LIPOXYGENASE INHIBITION AND ENDOTHELIAL CELL GROWTH


