Expression of 5-lipoxygenase in pulmonary artery endothelial cells

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Increased expression of 5-lipoxygenase (5LO) in pulmonary artery endothelial cells (PAECs) has been observed in disease states such as pulmonary hypertension and allergen challenge. To understand the function of endothelial 5LO, we examined the expression of this enzyme in normally cultured human PAECs and its characteristics when overexpressed. A small amount of 5LO message and protein was detected by reverse-transcriptase-mediated PCR (RT–PCR) and Western blotting in PAECs. Sequencing of the RT–PCR products that overlapped the entire coding region of 5LO mRNA indicated that the sequence of PAEC 5LO was identical with that of leucocyte 5LO. Incubation of the PAECs with A23187 and arachidonic acid led to a small production of 5-hydroxyeicosatetraenoic acid (5-HETE) (46–98 pmol/4×10^6 cells) but no leukotrienes. Overexpression of 5LO in PAECs by adenovirus-mediated gene transfer revealed

that the enzyme was localized in the nucleus. Incubation of the transduced cells with A23187 (5 μM) caused the production of both 5LO products and downstream leukotrienes. The proportions of the produced leukotriene A_4 (LTA_4) hydrolates (sum of 6-*trans*-LTB_4 and 12-epi-6-*trans*-LTB_4), LTB_4 and cysteinyl leukotriene were approx. 17:14:10. cGMP production in the 5LO-transduced PAECs was decreased by $33 \pm 14\%$ on stimulation with A23187. These results show that cultured PAECs express a minimal amount of 5LO, which can generate some 5-HETE, but not leukotrienes. However, increased expression of 5LO in PAECs can lead to the production of all downstream leukotrienes, which could potentially cause endothelial dysfunction in the pulmonary vasculature.

Key words: adenovirus, cGMP, leukotrienes.

INTRODUCTION

5-Lipoxygenase (5LO) catalyses two consecutive reactions, which convert arachidonic acid first into 5-hydroperoxyeicosatetraenoic acid (5HPETE) and then into 6-oxido-eicosatetraenoic acid [leukotriene A_4 (LTA_4)]. In the presence of downstream enzymes, LTA_4 can be converted into LTB_4, a potent chemotactic agent [1], or into LTC_4, LTD_4 and LTE_4 [cysteinyl leukotrienes (Cys-LTs)], a group of potent smooth-muscle constrictors, which induce constriction of bronchi [2,3] and pulmonary vessels [4–8]. In addition, 5LO is also involved in cell proliferation [9–13], tyrosine kinase signalling [14] and F-actin polymerization [14–16].

5LO has been studied primarily in leucocytes, macrophages and mast cells, where it is expressed predominantly and constitutively. 5LO is also expressed in other cells and tissues, including keratinocytes [17], neurons and brain tissue [18–21] and cancer cells and malignant tissue [9,22–25]. The expression level of 5LO in these cells is generally lower than that found in leucocytes and seems to be related to the state of cell proliferation and differentiation. Reports show that, in the vasculature, 5LO is expressed in intrapulmonary arteries and veins of perinatal lambs (examined by activity assay and Western blotting of isolated vessels) [26] and in isolated rat hepatic sinusoidal endothelial cells [analysed by activity assay or reverse-transcriptase-mediated PCR (RT–PCR)] [27,28], but not in human umbilical vein [29] or pig aortic endothelial cells [30] (determined by activity assay).

Increased expression of 5LO in pulmonary artery endothelial cells (PAECs) has been reported in patients with primary pulmonary hypertension [31], in rats subjected to chronic hypoxia

[32] and in mice challenged with antigen [33]. These expression patterns were detected by immunohistochemical staining of 5LO protein and/or by *in situ* hybridization of 5LO mRNA in lung tissue. Although the mechanism is still unknown, the induction of 5LO expression perhaps reflects an altered endothelial cell function associated with these diseases. Abnormal PAEC proliferation and vascular remodelling are associated with the development of pulmonary hypertension.

To understand better the functional consequences of the expression of 5LO in PAECs, the present study examined the mRNA sequence, protein and activity of the endogenously expressed 5LO in cultured human PAECs. Adenovirus-mediated gene transfer was employed to overexpress 5LO in PAECs, which facilitated the determination of the subcellular location and translocation of 5LO in PAECs, as well as the profiles of downstream leukotrienes generated after 5LO activation.

EXPERIMENTAL

Materials

Human PAECs and EBM2-MV medium were obtained from Clonetics (Walkersville, MD, U.S.A.). HEK-293 cells were obtained from American Type Culture Collection (Manassas, VA, U.S.A.). AA861 was purchased from Biomol (Plymouth Meeting, PA, U.S.A.). MK886 and indomethacin were obtained from CalBiochem (San Diego, CA, U.S.A.). LIPOFECTAMINE™ and goat serum were purchased from Gibco (Grand Island, NY, U.S.A.). Calcium ionophore A23187, fibronectin, heparin (grade IA from pig intestine), 3-isobutyl-1-methylxanthine and FITC-

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Abbreviations used: Cys-LTs, cysteinyl leukotrienes; DPBS, Dulbecco's phosphate-buffered saline; FLAP, 5-lipoxygenase-activating protein; GFP, green fluorescent protein; HETE, hydroxyeicosatetraenoic acid; hGM-CSF, human granulocyte/macrophage colony-stimulating factor; 5HPETE, 5-hydroperoxyeicosatetraenoic acid; 5LO, 5-lipoxygenase; LT, leukotriene; MOI, multiplicity of infection; PAECs, pulmonary artery endothelial cells; PGB_2, prostaglandin B_2; RT–PCR, reverse-transcriptase-mediated PCR.
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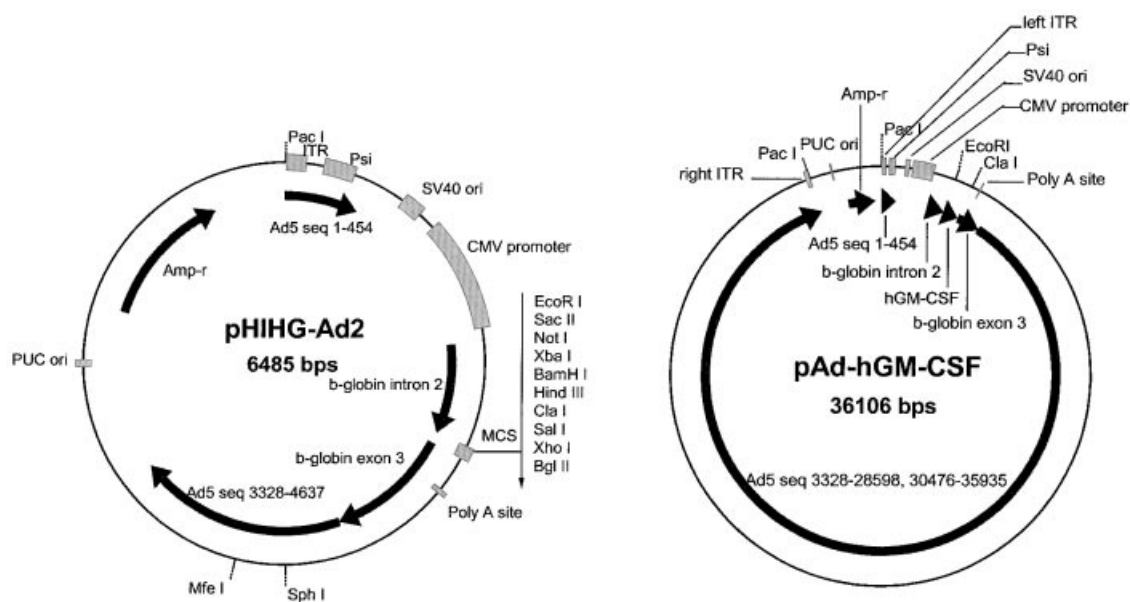
Figure 1   Vectors used for construction of the 5LO-expressing adenovirus vector pAd-5LO

conjugated goat anti-rabbit IgG antibody were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). Anti-5LO antiserum was produced by injecting purified 5LO into rabbits. Socumb-6GR was obtained from Butler (Columbus, OH, U.S.A.).

PAEC culture

Human PAECs were grown in EGM2-MV medium, containing basic growth medium (EBM-2) and antibiotics, ascorbic acid, human vascular endothelial growth factor, long R insulin-like growth factor 1, human epidermal growth factor, human fibroblast growth factor B and 5 % (v/v) fetal bovine serum provided by the manufacturer. Cells were maintained at 37 °C in an incubator equilibrated with air/CO_2 (19:1) at 100 % humidity.

Pig PAECs were harvested from pigs, which were killed by injection with 5 ml of Socumb-6GR administered via the ear vein. The pulmonary artery was excised and placed in ice-cold L-15 medium containing 3 % (v/v) fetal bovine serum, 300 i.u./ml of penicillin and 300 μg/ml of streptomycin. Extraneous tissue was carefully removed and the artery was incised vertically. After the artery had been washed several times in L-15 medium, endothelial cells were gently scraped from the artery wall and placed in growth medium containing M199, 15 % (v/v) fetal bovine serum, 100 i.u./ml penicillin, 100 μg/ml of streptomycin, 2 mM glutamine and 10 μg/ml heparin. The cells were then seeded in growth medium and cultured at 37 °C in air/CO_2 (19:1). Cultures exhibited typical endothelial cell morphology and stained for factor VIII-related antigen; cells were used up to passage 8.

cDNA sequencing

Total RNA was extracted from human PAECs with RNeasy kits (Qiagen, Valencia, CA, U.S.A.). RT–PCR was subsequently conducted for 33 cycles with a GeneAmp PCR core kit (Perkin Elmer, Branchburg, NJ, U.S.A.). The PCR product was purified with Wizard PCR preps (Promega, Madison, WI, U.S.A.) and sequenced by a fluorescence dideoxy termination method with an

automated sequencer (Model ABI 377–96; Big Dyes). DNA sequencing was performed by the Molecular Genetics Core at Boston University School of Medicine. The primer pairs used for obtaining 5LO cDNA fragments were as follows: 5′-CAAATG-CCACAAGGATTTACCCCG-3′ and 5′-CGTATTTTGCATC-CGAAGGGAGGA-3′, 5′-GCGCCATGCCCTCCTACACGG-TC-3′ and 5′-GAGCTGCTCACGGGCCTTGGTGTT-3′, and 5′-CTGCTGTGCACCCCATTTTCAAG-3′ and 5′-TGTGCA-AGGAATTGGACTAAGCACA-3′.

Western blotting

Human PAECs were grown in P100 tissue culture plates to a density of 4×10^6 cells per plate. The cells were washed, digested and resuspended in PBS containing 2 mM EDTA, 100 mM PMSF, 50 μg/ml soybean trypsin inhibitor, 1 μg/ml leupeptin, 1.25 μg/ml pepstatin and 10 mM 2-mercaptoethanol. SDS/PAGE loading buffer was added and the suspension was boiled for 10 min. Protein (70 μg) was loaded on a 10 % (w/v) polyacrylamide gel and electrophoresed. The protein was transferred to a nitrocellulose membrane, blocked and incubated overnight with anti-5LO antibody. The membrane was then incubated with horseradish-peroxidase-conjugated goat anti-rabbit antibody (Sigma) and developed with enhanced chemiluminescence reagents (ECL®, Amersham). Purified 5LO was used as a standard [34].

Construction of adenoviral-5LO expression vector

Human 5LO cDNA was excised from pT3–5LO [35] by *Eco*RI–*Hin*dIII digestion and inserted into a shuttle vector, pHIHG-Ad2 (generously provided by John Gray, Harvard Gene Therapy Initiative, Cambridge, MA, U.S.A.) (Figure 1). The resulting plasmid was digested with *Pac*I and *Mfe*I; the fragment containing 5LO cDNA was used to transform *Escherichia coli* BJ5183 together with a *Cla*I-linearized adenovirus vector, pAd-

hGM-CSF (in which hGM-CSF stands for human granulocyte/macrophage colony-stimulating factor) (Figure 1). Homologous recombination of the two DNA fragments in BJ5183 [36] produced a new adenoviral vector, pAd-5LO, in which hGM-CSF in the original vector was replaced by 5LO. pAd-5LO was extracted from BJ5183 and transferred to *E. coli* DH5α for large-scale plasmid preparation [36]. The sequence of pAd-5LO was confirmed by DNA restriction digestion (with *Hin*dIII) and by transfection of HEK-293 cells followed by Western blotting and activity assay of the expressed 5LO protein.

Production of recombinant adenovirus Ad-5LO

pAd-5LO was linearized with *Pac*I and transfected into HEK-293 cells by a LIPOFECTAMINE™ method. In brief, 4 μg of linearized pAD-5LO was incubated with 0.5 ml of Opti-MEM I and 20 μl of LIPOFECTAMINE™ for 30 min at 25 °C and added to a 60 mm tissue culture plate containing HEK-293 cells (80% confluent) in 2.5 ml of Opti-MEM I. Transfection was performed at 37 °C for 4 h; the cells were maintained in growth medium [DMEM low-glucose medium containing 10% (v/v) fetal bovine serum, 100 i.u./ml of penicillin and 100 μg/ml of streptomycin] until viral maturation. The virus, Ad-5LO, was harvested from the cells by four cycles of freezing and thawing in PBS containing 10% (v/v) glycerol. Further amplification of Ad-5LO was performed by several rounds of infection of HEK-293 cells; the titre of the Ad-5LO produced was determined by viral plaque assay.

Immunochemical staining

PAECs were seeded on glass chamber slides and infected with recombinant adenovirus. At 48 h after infection, cells were washed twice with Dulbecco's phosphate-buffered saline (DPBS) and incubated in DPBS with or without the calcium ionophore A23187 at 37 °C for specified durations. DPBS was then removed and the cells were fixed with 4% (w/v) paraformaldehyde in DPBS for 15 min and permeabilized for 10 min at 25 °C with 0.2% (v/v) Triton X-100 in DPBS. After blocking with 5% (w/v) BSA in DPBS for 30 min and 5% (v/v) goat serum in PBS for a further 30 min, the cells were incubated overnight at 4 °C with rabbit anti-5LO antiserum [1:1000 dilution in 5% (v/v) goat serum/PBS]. The cells were then washed with DPBS three times (5 min each) and incubated for 1 h at 25 °C with FITC-conjugated goat anti-rabbit IgG antibody. After three final washes with DPBS, the slides were air-dried, mounted with slow fade (Molecular Probes) and sealed with nail polish.

Confocal microscopy

The immunochemically stained slides were examined with a LSM 510 confocal microscopy system (Zeiss Instruments) with the use of an argon laser (488 nm) and a 40× objective lens. Photographs were taken at the midsection of the cells (the optical slide thickness was 0.9 μm).

5LO activity assay

For analysis of 5LO in cell homogenates, PAECs were washed twice with DPBS without calcium, then scraped into 0.5 ml of sonication buffer [100 mM Tris/HCl (pH 7.5)/2 mM EDTA/60 μg/ml soybean trypsin inhibitor/1 mM PMSF]. The cells were transferred to a 1.5 ml tube, cooled on ice and sonicated twice (5 s each). Protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA, U.S.A.) and the cell homogenate was diluted further with 100 mM Tris/HCl (pH 7.5)/2 mM EDTA. The assay mixture contained 0.1 mM arachidonic acid, 2.5 mM CaCl$_2$, 2 mM EDTA, 100 mM Tris/HCl, pH 7.5, and cell homogenate protein. The reaction was performed at 25 °C for 10 min and stopped by the addition of 0.3 ml of acetonitrile containing 0.4% (v/v) acetic acid and 1 nmol of 17-hydroxydocosatetraenoic acid as an internal standard. The reaction solution was then centrifuged at 4 °C for 15 min; the supernatant was analysed by HPLC as described previously [37].

For analysis of 5LO in intact cells, PAECs were washed twice with DPBS and incubated for 15 min in 2.5 ml of DPBS at 37 °C. The activity assay was started by the addition of 2.5 ml of 10 μM A23187 with or without 100 μM arachidonic acid in PBS and performed at 37 °C for 20 min. An internal standard, prostaglandin B$_2$ (PGB$_2$) (0.25 nmol), was added at the end of incubation; the culture dish 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 C$_{18}$ SepPak column (200 mg; Waters, Milford, MA, U.S.A.) by consecutively passing the following solutions through the column: 3 ml of methanol, 3 ml of water, 6 ml of sample, 3 ml of water containing 0.1% (v/v) acetic acid, and 3 ml of methanol. The final eluate was collected and dried under nitrogen. The lipids were dissolved in 250 μl of solvent A (methanol/water/acetic acid/NH$_4$OH, 60:40:0.1:0.04, by vol.) and centrifuged at 20000 *g* for 15 min; 100 μl of the supernatant was injected into the HPLC column.

HPLC analysis was performed with a C$_{18}$ column (Radial-Pak, 5NVC184μ; Waters). After sample loading, the column was developed with a solvent gradient consisting of 38 min of solvent A, 1 min of gradient to 30% (v/v) solvent B (methanol/acetic acid, 100:0.1, v/v), 22 min of 30% (v/v) B, 2 min of gradient to 100% B, 4 min of 100% B, 1 min of gradient to 100% A, and 18 min of A. The eluate from the column was monitored by UV absorption at 234 and 280 nm; quantification of each compound was performed by comparing its peak area with that of an internal standard, PGB$_2$.

Liquid chromatography–tandem MS analysis

Data from HPLC-coupled tandem MS were acquired with an LCQ (Finnigan MAT, San José, CA, U.S.A.) quadruple-ion-trap MS system equipped with an electrospray ionization probe as described elsewhere [38]. In brief, samples were suspended in mobile phase and injected into the HPLC component, consisting of a P4000 quaternary gradient pump (ThermoQuest, San José, CA, U.S.A.), a LUNA C$_{18}$-2 (150 mm × 2 mm, 5 μm particle size) column (Phenomenex, Torrance, CA, U.S.A.) and a UV2000 UV/visible absorbance detector (ThermoQuest). The column was eluted isocratically at 0.2 ml/min with methanol/water/acetic acid (65:34.99:0.01, by vol.) for 20 min followed by a linear gradient to methanol/acetic acid (99.99:0.01, v/v) over 20 min. The spray voltage was set to 5 kV and the heated capillary to 250 °C. LTB$_4$, LTE$_4$ and 5-hydroxyeicosatetraenoic acid (5-HETE) were identified by selected ion monitoring for analyte molecular anions (e.g. $[M-H]^- = m/z$ 335.5 for LTB$_4$, m/z 438.7 for LTE$_4$ and m/z 319.5 for 5-HETE). Product ion mass spectra (MS–MS) were also acquired for definitive identification of the compounds.

cGMP measurement

PAECs were grown in six-well plates and infected with Ad-5LO or Ad-GFP (a recombinant adenovirus expressing green fluores-
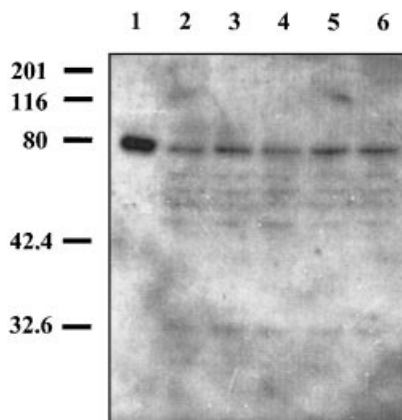
cent protein) for 48 h. The cells were then equilibrated for 30 min at 37 °C in Hepes-buffered physiological salt solution (PSS) containing 22 mM Hepes, pH 7.4, 124 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 0.16 mM HPO_4^{2-}, 0.4 mM $H_2PO_4^-$, 5 mM $NaHCO_3$, 5.6 mM glucose, 200 μM L-arginine and 200 μM isobutylmethylxanthine. Equilibrated cells were then stimulated with 5 μM A23187 for 3 min and lysed by the addition of 6% (w/v) ice-cold trichloroacetic acid. The cell lysate was subjected to centrifugation at 10000 *g* for 10 min and the supernatant and pellet were stored at −70 °C until analysis. Determination of cGMP was performed with a commercially available ELISA kit (Cayman, Ann Arbor, MI, U.S.A.) in accordance with the manufacturer's instructions. The protein content of cell pellets was determined by the bicinchoninic acid assay (Pierce, Rockford, IL, U.S.A.) after solubilization with 1 M NaOH.

RESULTS
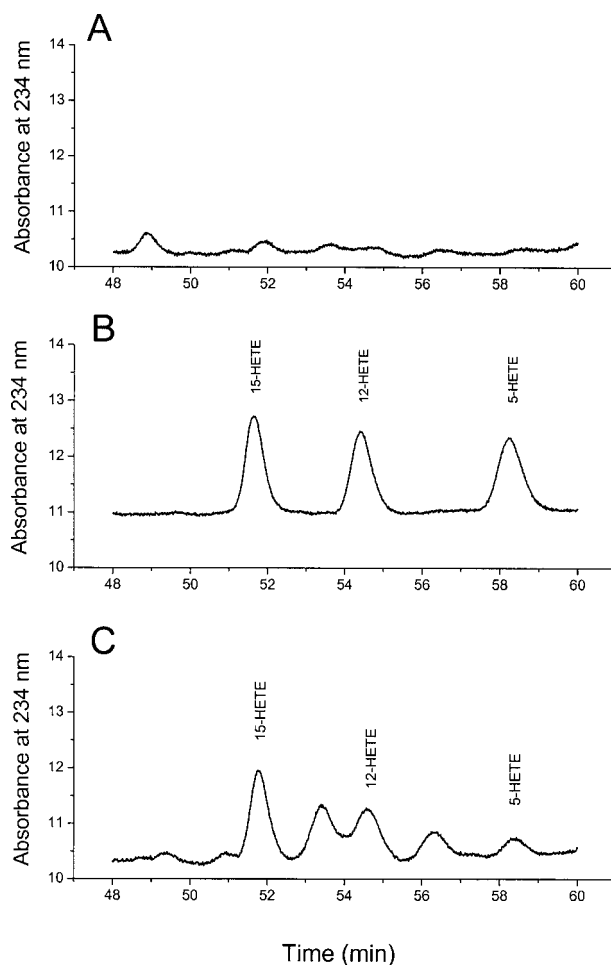
Detection of 5LO expression in cultured PAECs

The expression of 5LO in cultured human PAECs was examined by RT–PCR, Northern blotting, Western blotting and 5LO activity assay. A 5LO signal was most easily detected in PAECs by RT–PCR but was not detected by Northern blotting (when 20 μg of total RNA was loaded on an agarose gel). With the use of a primer pair that covered the 5LO cDNA region 518–1083, a single band was detected after RT–PCR. To confirm that the PCR product was indeed derived from 5LO mRNA, the cDNA was extracted and sequenced (from both the 3' end and the 5' end) with the same set of primers as used for RT–PCR. Sequencing showed that the cDNA fragment had the same sequence as that of published 5LO cDNA.

To examine whether the 5LO mRNA was truncated or spliced differently in PAECs, we used several different sets of primers for RT–PCR to obtain overlapping 5LO cDNA fragments that encompassed the entire coding region of 5LO mRNA, as well as part of the 3' and 5' untranslated regions. The 5LO cDNA sequence obtained from PAECs was 99% identical with the published sequence of human 5LO cDNA (GenBank® accession number J03600), indicating that 5LO mRNA in PAECs was


Figure 3   HPLC chromatogram analysing 5LO activity in human PAECs

A P-100 tissue culture plate containing no cells (**A**) and a sample plate containing 4×10^6 human PAECs (**C**) were each washed with PBS and incubated with 10 μM A23187/50 μM arachidonic acid for 20 min. Lipids were extracted from the incubation medium and analysed by HPLC as described in the Experimental section. (**B**) Chromatogram of lipid standards.

Figure 2   Western blot of 5LO in human PAECs

Human PAECs were harvested and subjected to Western blotting analysis as described in the Experimental section. Lane 1 was loaded with purified 5LO protein; lanes 2–6 were loaded with human PAEC samples (70 μg of protein per well) from separate culture plates. The positions of molecular mass markers are indicated (in kDa) at the left.

spliced in the same way as that in leucocytes. The minute differences between our sequencing data and the published data were probably an artifact of the PCR or sequencing methodology.

The 5LO protein in PAECs was examined by Western blotting with the use of an enhanced chemiluminescence detection method (Figure 2). The level of 5LO protein in PAECs was routinely found to be very low and varied between culturing periods. Because the culture medium used in this study was provided by the manufacturer, it is unclear whether the fluctuation was due to a minor variation of a component of the medium, such as that in different lots of fetal bovine serum, or to other culturing conditions, such as the passage number or density of the cells.

The activity of 5LO in PAECs was analysed by incubation of the cells with 10 μM A23187 and 50 μM arachidonic acid and examining the lipid products on HLPC. As shown in Figure 3, a small amount of 5-HETE (0.052 nmol/4×10^6 cells) was produced by the cells, which was approx. 29% of the 15-HETE and 40% of the 12-HETE produced by the cells. No leukotrienes were detected in the assay. The production of 5-HETE varied between 0.046 and 0.098 nmol/4×10^6 cells during routine analy-
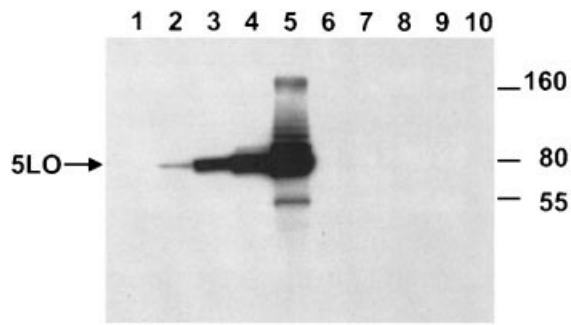
Figure 4 Western blotting of 5LO-transduced PAECs

Human PAECs were infected with Ad-5LO at 0, 0.1, 1, 10 or 100 MOI (lanes 1–5) or with Ad-GFP at 0, 0.1, 1, 10 or 100 MOI (lanes 6–10). At 48 h after infection, cells were washed, harvested and sonicated. Protein (10 μg) was loaded in each well and subjected to Western blotting analysis with anti-5LO antibody. The positions of the corresponding molecular masses are indicated (in kDa) at the right.

sis. Stimulation of the cells with A23187 alone did not lead to 5-HETE production.

Expression of 5LO in PAECs with adenovirus-mediated gene transfer

To facilitate an understanding of 5LO behaviour in PAECs, we first attempted to transfect the cells with expression vectors containing 5LO cDNA. However, primary PAECs were difficult to transfect, as found previously for human umbilical endothelial cells [39]. In the present study we used adenovirus-mediated gene transfer to overcome the difficulty. Construction of the adenoviral expression vector, pAd-5LO, was performed by inserting 5LO cDNA first into a shuttle vector, pHIHG-Ad2, and then into an adenoviral vector, pAD-hGM-CSF, by bacterial homologous recombination. The adenoviral vector expresses replication-deficient human adenovirus 5, with E1 and E3 genes in the viral genome deleted. Expression of 5LO by this vector is controlled by a CMV promoter and facilitated by a β-globin intronic sequence. Production of the recombinant adenovirus particles (Ad-5LO) was performed in HEK-293 cells, and the recombinant virus was titrated by a standard plaque assay.
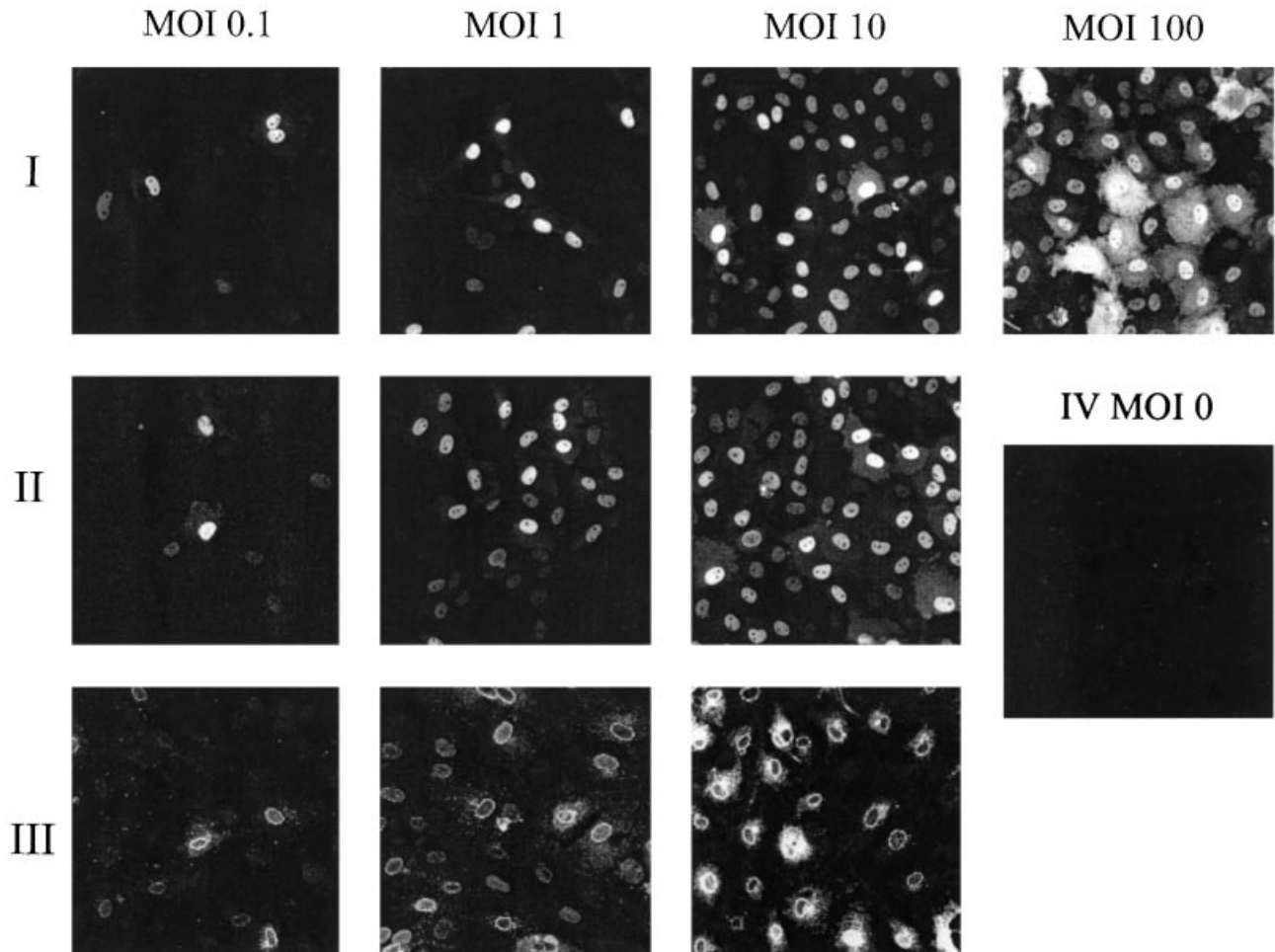
Figure 5 Subcellular localization of 5LO in PAECs

Pig PAECs were infected with Ad-5LO at the doses indicated and grown for 48 h. Cells were then washed with DPBS and incubated with DPBS for 20 min (**I**), 5 μM A23187 in DPBS for 20 min (**II**) or 20 μM A23187 in DPBS for 45 min (**III**) before fixation for immunochemical staining; (**IV**) shows an uninfected control. 5LO protein was detected by anti-5LO antiserum and the slides were examined by confocal microscopy at the midsection of the cells in the Z-series.
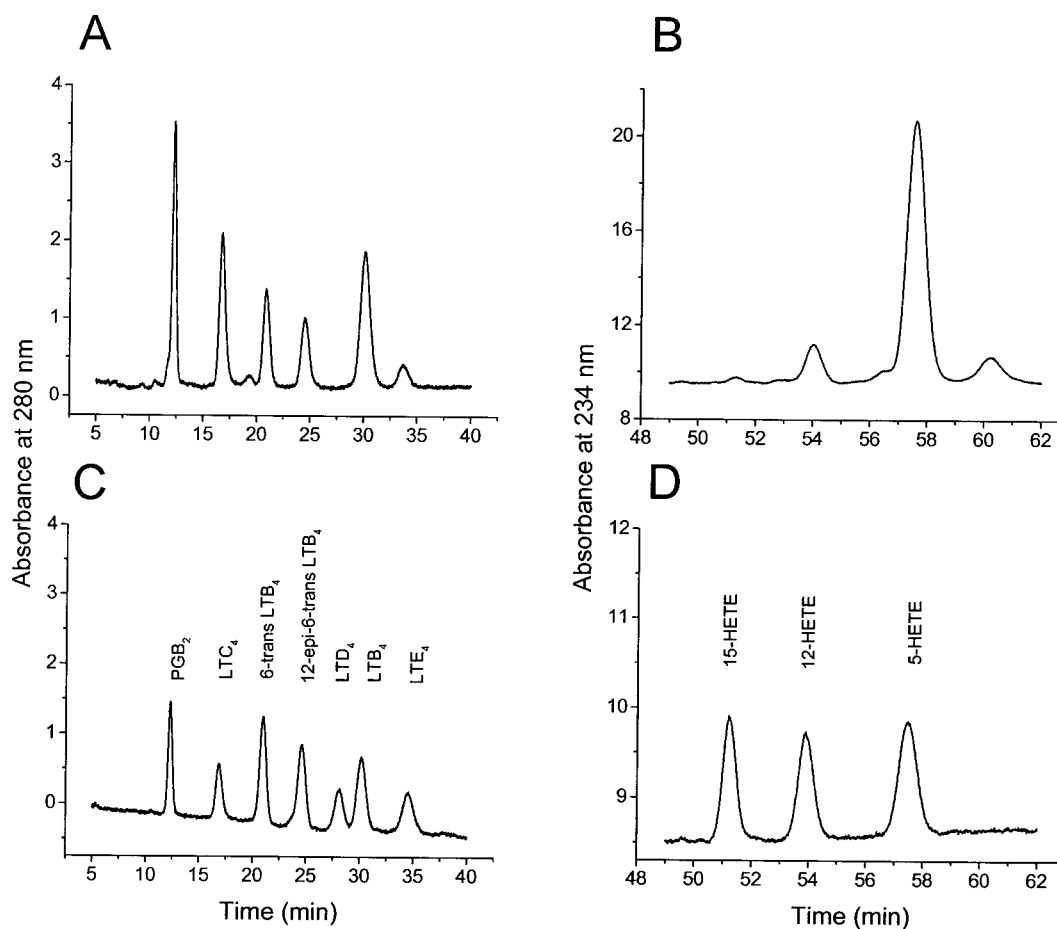
Figure 6   HPLC elution profile of the leukotrienes generated in 5LO-transduced PAECs

Pig PAECs were infected with Ad-5LO at 20 MOI. The cells were then treated with 5 μM A23187 in DPBS; the released lipid products were extracted by solid-phase extraction and separated on a C_{18} HPLC column (see the Experimental section for details). (**A**, **B**) Separation profiles of the generated leukotrienes (eluted between 5 and 40 min; monitored at 280 nm) (**A**) and HETEs (eluted between 49 and 62 min; monitored at 234 nm) (**B**). (**C**, **D**) Separation profiles of leukotriene (**C**) and HETE (**D**) standards.

Western blotting and immunofluorescence staining evaluated the efficiency of the transduction of PAECs with Ad-5LO. In these studies, Ad-5LO was added to cultured PAECs at various virus particle-to-cell ratios [multiplicity of infection (MOI)] for 40–48 h before the final assessment. As shown in Figure 4, increased 5LO expression was detected in PAECs infected with increasing doses of Ad-5LO, but not in PAECs infected with Ad-GFP, suggesting that adenovirus infection itself did not induce the expression of endogenous 5LO. Note that the basal level expression of 5LO in PAECs is not shown in this Figure. Significantly less protein was loaded on the gel and a shorter exposure time was used after ECL staining than in the Western blot in Figure 2. Immunofluorescence staining (Figure 5, panels I) revealed individual cells expressing 5LO at 0.1 MOI, which comprised less than 1 % of the total population. At an infection dose of 10 MOI, 80–90 % of the cells expressed 5LO protein. At 100 MOI, all cells expressed 5LO, although some of them showed membrane 'ruffling', which is consistent with cell activation.

Subcellular distribution of 5LO

The subcellular distribution of 5LO in PAECs was studied by immunofluorescence staining of the 5LO-transduced cells in combination with confocal microscopic examination. As shown in Figure 5 (panels I), the expressed 5LO was localized to the cell nucleus at infective doses of 10 MOI or less. At 100 MOI, 5LO in some of the transduced cells was found in both the nucleus and the cytosol.

Incubation of the 5LO-transduced PAECs with 5 μM A23187 for 20 min did not cause apparent translocation of 5LO (Figure 5, panels II). However, incubation of the cells with a higher concentration of A23187 (20 μM) for a longer duration (45 min) led to the translocation of most of the 5LO to the nuclear membrane (Figure 5, panels III).

Leukotriene generation by 5LO-transduced PAECs

To examine whether the 5LO-transduced PAECs can synthesize leukotrienes, the cells were stimulated with calcium ionophore (5 μM A23187 in DPBS for 20 min) in the absence of exogenous substrate. The released lipid products were isolated by solid-phase extraction and analysed by HPLC. The column eluates were monitored by UV absorption at 280 and 234 nm; the products were identified by their retention times in comparison with those of lipid standards. Confirmation of the identification was made by HPLC-coupled tandem MS analysis. As shown in Figure 6, stimulation of the 5LO-transduced PAECs with A23187
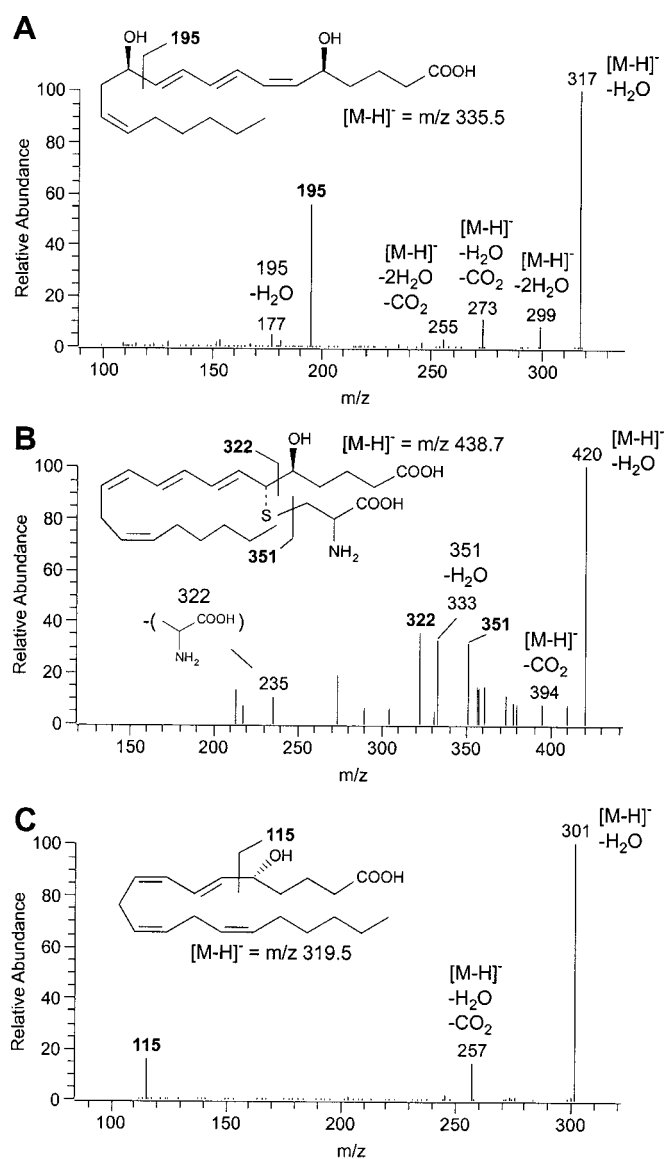
Figure 7 MS–MS spectra of LTB$_4$ (A), LTE$_4$ (B) and 5-HETE (C) generated in 5LO-transduced PAECs

5LO-transduced pig PAECs were treated with 5 μM A23187 and the released lipid products were extracted with a solid-phase column before being separated on a C$_{18}$ HPLC column linked to a mass spectrometer (see the Experimental section for details). LTB$_4$, LTE$_4$ and 5-HETE were initially identified by selected ion monitoring for analyte molecular anions with m/z of 335.5 (LTB$_4$), 438.7 (LTE$_4$) and 319.5 (5-HETE). Product ion mass spectra (MS–MS) were subsequently obtained for definitive identification of the compounds.

resulted in the production of compounds with the same retention times as those of the lipid standards 6-*trans*-LTB$_4$ and 12-epi-6-*trans*-LTB$_4$ (from the hydrolysis of LTA$_4$), LTB$_4$, LTC$_4$ and LTE$_4$, as well as 15-HETE, 12-HETE and 5-HETE. LTD$_4$ was not detected, possibly owing to the high activity of cysteinyl-glycine dipeptidase in PAECs. LTB$_4$, LTE$_4$ and 5-HETE were also identified on the basis of their product ion mass spectra (MS–MS) shown in Figure 7. The molecular anion of LTB$_4$ ([M−H]$^-$ = m/z 335.5) fragmented in its MS–MS spectrum (Figure 7A) via (1) neutral loss of H$_2$O and CO$_2$ to yield the product ions m/z 317 ([M−H]$^-$ −2H$_2$O), 273 ([M−H]$^-$ −H$_2$O, −CO$_2$) and 255 ([M−H]$^-$ −2H$_2$O, −CO$_2$) and (2) cleavage of the 11,12 C–C bond to yield the product ion m/z 195 [[M−H]$^-$ −CHOCH$_2$CHCH(CH$_2$)$_4$CH$_3$]. The molecular anion of LTE$_4$ ([M−H]$^-$ = m/z 438.7) fragmented in its MS–MS spectrum (Figure 7B) via (1) neutral loss of H$_2$O and CO$_2$ to yield the product ions m/z 420 ([M−H]$^-$ −H$_2$O) and m/z 394 ([M−H]$^-$ −CO$_2$), (2) cleavage of the carbon–sulphur bond of the cysteinyl moiety to yield the fragment m/z 351, and (3) cleavage of the 5,6 C–C bond to yield the product ion m/z 322 (loss of the carboxylate end of the molecule, C-1 to C-5). The molecular anion of 5-HETE ([M−H]$^-$ = m/z 319.5) fragmented in its MS–MS spectrum (Figure 7C) via (1) neutral loss of H$_2$O and CO$_2$ to yield the product ions m/z 301 ([M−H]$^-$ −H$_2$O) and 257 ([M−H]$^-$ −H$_2$O −CO$_2$) and (2) cleavage of the 5,6 C–C bond to yield the product ion m/z 115 [CHO(CH$_2$)$_3$COO$^-$].

## Quantification of 5LO products and downstream leukotrienes

To estimate the relative amounts of products generated by the leukotriene biosynthetic pathway in PAECs, the cells were infected with increasing doses of Ad-5LO. The 5LO activities were analysed under two stimulation conditions: with 5 μM A23187 alone, or with 5 μM A23187 plus 50 μM arachidonic acid. As shown in Figure 8, without the provision of exogenous substrate (Figure 8A), total 5LO activity (the sum of the production of 5-HETE and leukotrienes) in the PAECs increased with infective doses up to 40 MOI, reaching a plateau between 40 and 80 MOI. Provision of exogenous substrate (50 μM arachidonic acid) (Figure 8C) did not increase product formation beyond 40 MOI, indicating that the plateau was probably caused by inactivation of the excessively expressed 5LO protein rather than by exhaustion of endogenous substrate. At 10, 20 and 40 MOI, total 5LO activity in the presence of exogenous substrate was respectively 2.5-fold, 2.8-fold and 2.6-fold higher than that in the absence of exogenous substrate. Thus the endogenous substrate provided by phospholipase A$_2$ and 5-lipoxygenase-activating protein (FLAP) supported more than one-quarter of the maximally expressed 5LO activity in PAECs.

The production of 5-HETE and leukotrienes is also plotted separately in Figures 8(A) and 8(C). Without exogenous substrate (Figure 8A), approx. 40% of 5-HPETE was converted to LTA$_4$ (5[S],6-diHETEs were not measured). In the presence of exogenous substrate (Figure 8C), approx. 25–30% of 5-HPETE was converted into LTA$_4$.

The extent of conversion of LTA$_4$ into downstream leukotriene products is plotted in Figures 8(B) and 8(D). In the absence of exogenous arachidonic acid substrate (Figure 8B), the proportions of LTA$_4$ hydrolates (i.e. the sum of 6-*trans*-LTB$_4$ and 12-epi-6-*trans*-LTB$_4$), LTB$_4$ and Cys-LTs (LTC$_4$ and LTE$_4$) were 17:14:10 (average of activities at MOI 10, 20, 40 and 80). In the presence of exogenous substrate (Figure 8D), the proportions were 55:13:10 (average of activities at MOI 10, 20, 40 and 80). The absolute amount of LTA$_4$ produced was increased 4-fold in the presence of exogenous substrate (from 0.48 to 2.4 nmol) but only by 0.4-fold in LTB$_4$ and Cys-LTs production (LTB$_4$, from 0.33 to 0.46 nmol; Cys-LTs, from 0.27 to 0.42 nmol). The comparatively modest increase in LTB$_4$ and Cys-LT production in the presence of exogenous substrate suggests that LTA$_4$ hydrolase and LTC$_4$ synthase were operating nearly at substrate saturation with the 5LO products generated from endogenous substrate.
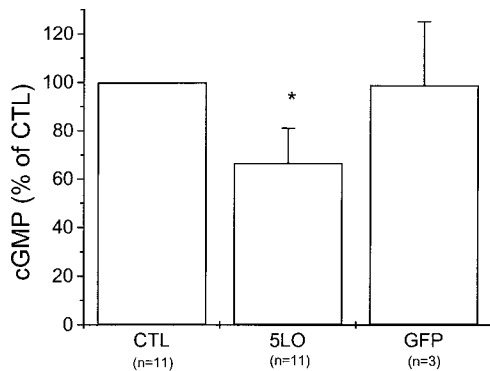
cGMP production in 5LO-transduced PAECs

Stimulation of PAECs with calcium ionophore also activates endothelial nitric oxide synthase and initiates the production of NO. NO activates guanylate cyclase and causes cGMP pro-
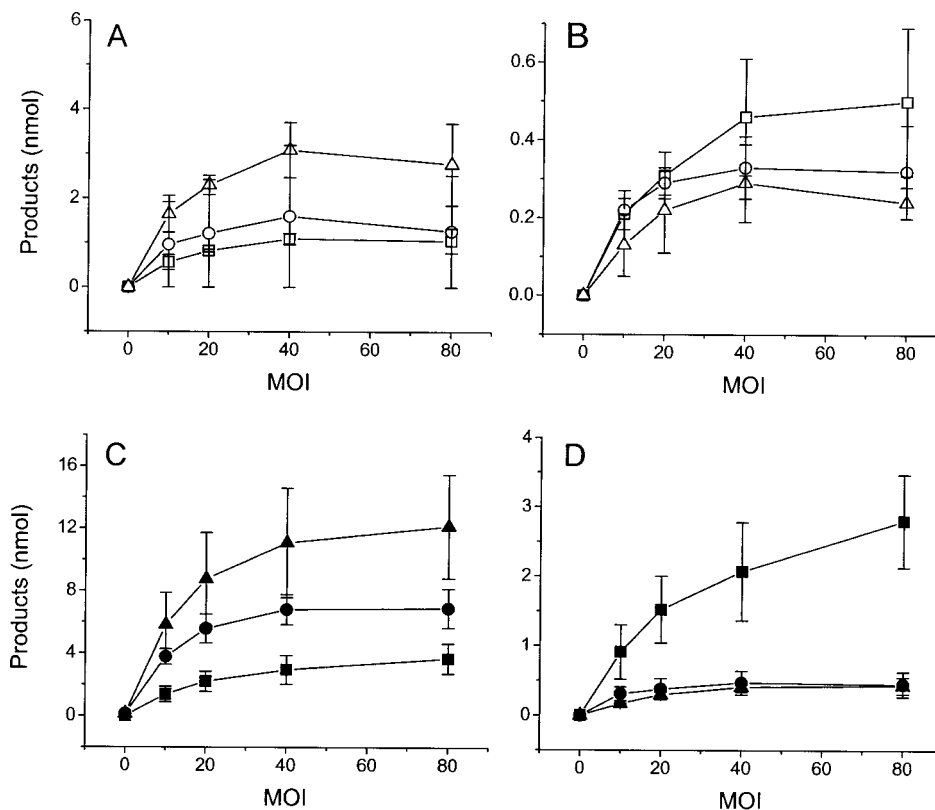
Figure 8   Relative amounts of 5-HETE and leukotriene products generated in 5LO-transduced PAECs

Pig PAECs were infected for 48 h with Ad-5LO at various doses, then incubated with either 5 μM A23187 (**A**, **B**) or 5 μM A23187 plus 50 μM arachidonic acid (**C**, **D**) before extraction for lipid analysis. (**A**, **C**) Production of 5-HETE ($\bigcirc$,$\bullet$), total leukotrienes ($\square$,$\blacksquare$) and 5-HETE plus total leukotrienes ($\triangle$,$\blacktriangle$) in one 100 mm culture plate (the cell number was 3×10^6 at the time of infection). (**B**, **D**) Re-plots of (**A**) and (**C**) showing the production of LTA_4 (sum of 6-*trans*-LTB_4 and 12-epi-6-*trans*-LTB_4) ($\square$,$\blacksquare$), LTB_4 ($\bigcirc$,$\bullet$) and peptidoleukotrienes ($\triangle$,$\blacktriangle$) separately. Data points are means $\pm$ S.D. for four experiments run in duplicate.

Figure 9   cGMP production in PAECs with and without 5LO transduction

Pig PAECs were infected with 20 MOI of recombinant adenovirus expressing 5LO or GFP, or were uninfected (CTL). The cells were grown for 48 h and analysed for cGMP production with an ELISA kit as described in the Experimental section. *$P < 0.05$. Results are means $\pm$ S.D. for the numbers of experiments shown.

duction. To examine whether 5LO activation affected NO activity, cGMP production was compared in PAECs transduced with Ad-5LO or Ad-GFP or without transduction. As shown in Figure 9, 5LO-transduced cells produced $33 \pm 14\%$ less cGMP than normal PAECs or PAECs transduced with Ad-GFP ($P < 0.05$).

DISCUSSION

This study showed that cultured human PAECs expressed a minimal but detectable amount of 5LO. The mRNA sequence of the PAEC 5LO was identical with that of leucocyte 5LO; activation of the PAECs resulted in the production of a small amount of 5-HETE but no leukotrienes. To determine whether an increased expression of 5LO could lead to leukotriene biosynthesis in PAECs, the present study employed an adeno-virus-mediated gene transfer system. The results showed that 5LO-transduced PAECs are capable of production of all types of leukotrienes on activation.

A low level of expression of 5LO in PAECs of normal human lung tissue has previously been shown by immunohistostaining [31]. Our finding of a basal-level expression of 5LO in cultured human PAECs is consistent with this observation. However, it should be noted that the growth conditions of cultured human PAECs are significantly different from those *in vivo*. Whereas endothelial cells remain quiescent for many years in the adult vasculature under normal conditions, they are stimulated to proliferate under culturing conditions. In the present study several growth factors, as well as vitamin C and hydrocortisone,

were used to maintain the human PAEC culture. Whether these factors facilitate the 5LO expression in PAECs is unknown. A previous study has shown that dexamethasone increases the expression of 5LO in human monocytes and THP-1 cells [40].

Cultured human PAECs produced a very small amount of 5-HETE and no leukotrienes. It is therefore unlikely that this amount of 5LO could mediate any inflammatory or vasoconstrictive responses under normal conditions. However, the localization of 5LO in the cell nucleus might facilitate the binding of 5LO products to nuclear factors. If such a binding occurred, a very small amount of 5LO products might be sufficient to influence gene transcription. There has been no evidence so far of any interaction between 5LO products and nuclear factors; however, LTB_4 has been found to bind to peroxisome-proliferator-activated receptor α, a nuclear hormone receptor mediating pleiotropic responses to peroxisome proliferators [41]. Thus 5LO could potentially exert some nuclear function, even with the small amount of expression observed under normal conditions.

To examine downstream leukotriene biosynthesis in PAECs, we initially overexpressed 5LO in cultured human PAECs by infection of the cells with Ad-5LO. Surprisingly, the transduced cells did not generate 5LO products or any leukotrienes when stimulated with calcium ionophore alone. Incubation of the cells with A23187 and arachidonic acid led to a significant production of 5-HETE and leukotrienes. This finding suggests that the endogenous substrate for 5LO was somehow unavailable. We next transduced human PAECs with both Ad-5LO and Ad-FLAP; however, the same phenomenon was observed (i.e. there was no evidence of 5LO activity unless arachidonic acid was added). One possibility for this observation is the presence of hydrocortisone in the culture medium. Hydrocortisone might inhibit the activity of phospholipase A_2. Because pig PAECs do not require additional factors for cell culture, the remainder of the study was conducted on these cells.

Transduction of pig PAECs with Ad-5LO caused a full spectrum of leukotriene production by the cells on stimulation with A23187. This observation implies that PAECs contain not only the upstream enzymes/proteins needed to provide substrate to 5LO but also a full set of downstream leukotriene synthesis enzymes. These results suggest that when 5LO expression in PAECs is increased under certain disease states, the enzyme can have a significant role in inflammatory and vasoconstrictive responses. There are conflicting reports regarding whether or not endothelial cells can generate LTB_4 on receiving the exogenous 5LO product, LTA_4. LTB_4 synthesis has been found in cultured primary endothelial cells in one study [42], but not in others [30,43]. In the present study, the LTB_4 production in PAECs was comparable to Cys-LT generation, indicating that LTA_4 hydrolase, the enzyme that catalyses LTB_4 formation, is constitutively active in PAECs.

The expressed 5LO was found in the cell nucleus of PAECs, a location similar to that of 5LO in alveolar macrophages and mast cells [44,45]. Interestingly, stimulation of the transduced PAECs with 5 μM A23187 did not cause a significant translocation of 5LO to the nuclear membrane, but activated the enzyme. This observation might be due to the reversible membrane binding of 5LO that has been reported previously [46–48]. These studies found that the translocation of 5LO to the membrane compartment is transient and reversible when the cells are activated by a weak stimulator (IgE/antigen) [46], low concentrations of A23187 [48] or Zileuton (a reversible 5LO inhibitor) plus A23187 [47].

Lipid peroxide radicals generated by lipoxygenase react with and inactivate NO [49–51]. Because calcium influx activates both 5LO and endothelial nitric oxide synthase, we examined the

effect of 5LO overexpression on cGMP production. The results showed that 5LO-transduced PAECs generated one-third less cGMP than control PAECs after stimulation with calcium. This finding suggests that the overexpression of 5LO can affect endothelial function by impairing cGMP-mediated vasorelaxation and inhibition of platelet aggregation. The generation of 5LO products and downstream leukotrienes in PAECs might also affect other aspects of endothelial function that have not been examined in the present study. Examining these effects in the future might help to improve our understanding of the importance of endothelial 5LO activity in various vascular disorders.

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