Cytosolic 85-kDa Phospholipase A2-mediated Release of Arachidonic Acid Is Critical for Proliferation of Vascular Smooth Muscle Cells*

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Recent evidence suggests that arachidonic acid (AA) may be involved in regulating cellular proliferation. The predominant mechanism of AA release from cellular phospholipids is via phospholipase A2 (PLA2) hydrolysis. The purpose of this study was to examine the roles of the distinct 14-kDa and 85-kDa PLA2 enzymes in human coronary artery vascular smooth muscle cell (hCAVSMC) proliferation. Cultured hCAVSMCs proliferate in the presence of growth medium with a typical doubling time of 30–40 h, grow at a slower proliferative rate upon reaching confluence (day 8), and eventually undergo contact inhibition of growth (day 10). Neither Type II 14-kDa PLA2 activity nor mass changed over a 10-day culture period. In contrast, 85-kDa PLA2 protein activity and mRNA decreased as time in culture progressed. This reduction in 85-kDa PLA2 correlated with reductions in DNA synthesis and suggested a possible association between 85-kDa PLA2 and proliferation. To directly evaluate the role of the 85-kDa PLA2 in proliferation, we examined the effects of an 85-kDa PLA2 inhibitor (AACOCF3) and 85-kDa PLA2 antisense oligonucleotides on proliferation. Both reagents dose dependently inhibited proliferation, whereas a 14-kDa PLA2 inhibitor (SB203347), a calcium-independent PLA2 inhibitor (HELSS), an 85-kDa sense oligonucleotide, and a nonrelevant scrambled control oligonucleotide had no effect. The mechanism by which 85-kDa PLA2 influences cellular proliferation remains unclear. Inhibition of 85-kDa PLA2 activity produced neither phase-specific cell cycle arrest nor apoptosis (fluorescence-activated cell sorter analysis). Addition of AA (20 μM) attenuated the effects of both AACOCF3 and 85-kDa antisense oligonucleotides implicating AA as a key mediator in cellular proliferation. However, although prostaglandin E2 (PGE2) was present in the culture medium, it peaked early (day 3) in culture, and indomethacin had no effect on cellular proliferation indicating that hCAVSMC proliferation was not mediated through PGE2. These data provide the first direct evidence that PLA2 is involved in control of VSMC proliferation and indicate that 85-kDa PLA2-mediated liberation of AA is critical for cellular proliferation.

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1 The abbreviations used are: AA, arachidonic acid; PL, cellular phospholipids; PLA2, phospholipase A2; VSMC, vascular smooth muscle cell; hCAVSMC, human coronary artery VSMC; PGE2, prostaglandin E2; rh, its metabolites may be involved in regulating cellular proliferation (1–7). Arachidonic acid is released from cellular phospholipids (PL) via phospholipase A2 (EC 3.1.1.4, PLA2) hydrolysis of the acyl bond at the sn-2 position (8). Multiple forms of mammalian PLA2 have been identified. The type IIa 14-kDa PLA2 is well characterized and known to exist in both an extracellular form in inflammatory fluids (9, 10) and in a cell-associated form (11–14). The cytosolic 85-kDa PLA2 is structurally distinct and, unlike the 14-kDa PLA2, exhibits a preference for AA in the sn-2 position of PL and is regulated by physiological intracellular Ca2+ concentrations and phosphorylation (15–17). An 80-kDa calcium-independent cytosolic PLA2 first identified in P388D1, macrophages (18) and recently cloned from these cells (19) and Chinese hamster ovary (CHO) cells (20) possibly serves as a housekeeping enzyme involved in the remodeling of membrane phospholipids (21). We and others have shown that both the 14- and 85-kDa enzymes are induced by inflammatory cytokines and growth factors (22–29) and that both enzymes influence cellular AA release and subsequent eicosanoid production in a variety of cell types (8, 22, 30–32). However, the contribution of these distinct enzymes in regulating cellular proliferation has not been examined directly.

Proliferation of vascular smooth muscle cells (VSMCs) is implicated in the pathogenesis of hypertension, primary atherosclerosis, and restenosis following interventional revascularization procedures such as balloon angioplasty, arterial stenting, and by-pass surgery (33–35). Evidence suggestive of a role for PLA2 in VSMC proliferation includes: 1) that exogenous administration of AA increases expression of the early response genes c-myc (36), c-fos (36), and c-jun (37), as well as activity of mitogen-activated protein kinase (38); and 2) that these effects as well as serum- and growth factor-induced proliferation are inhibited by nonselective PLA2 inhibitors such as mepacrine (1, 37, 39).

VSMCs exhibit both 14-kDa and high molecular mass cytosolic PLA2 activities (24, 40). Recent descriptions of hydrogen peroxide-stimulated DNA synthesis and expression of proliferation-associated early response genes (36–38, 41), and hydrogen peroxide- (42) and angiotensin II-mediated (43) phosphorylation of the 85-kDa PLA2 infer that this enzyme may participate in AA release during cellular proliferation. Other data (44, 45) demonstrate that vascular smooth muscle cells possess high affinity Type I PLA2-specific binding sites and that 14-kDa phospholipase A2 is present in atherosclerotic plaques (46, 47), suggesting that the 14-kDa PLA2 serves a function in processes related to vascular disease. But, whether the 14-kDa PLA2 plays a role in cellular proliferation is not known. The purpose of this study was to directly examine the recombinant human; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FACS, fluorescence-activated cell sorter; ELISA, enzyme-linked immunosorbent assay; TUNEL, terminal transferase-mediated biotin dUTP nick end labeling.

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roles of the distinct 14- and 85-kDa PLAr enzymes in VSMC proliferation. Herein is presented the first direct evidence that PLAr activity is associated with VSMC proliferation. Neither the 14-kDa PLAr nor prostaglandin E2 (PGE2) appears to mediate VSMC proliferation. In contrast, the 85-kDa enzyme appears to serve a selective role in the initial production of arachidonic acid, which, through direct action or via action of metabolites other than PGE2, is critical for VSMC proliferation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**

Human coronary artery vascular smooth muscle cells (hCAVSMCs, Clonetics, San Diego, CA) were plated at 3 × 10^6 cells/cm^2 and grown in monolayer in the optimized culture medium recommended by the manufacturer (Clonetics SmGM2: modified MCD131 containing fetal bovine serum (5%), insulin (5 mg/ml), fibroblast growth factor (2 ng/ml), epidermal growth factor (0.5 mg/ml), gentamycin (50 mg/ml), and amphotericin B (50 ng/ml) at 37 °C in an atmosphere of 5% CO2, 95% air and 95% humidity. Cells were passaged when 70–80% confluent via a 4-min treatment (37 °C) with trypsin-EDTA (0.01:0.02%) in calcium-magnesium free Hanks’ balanced salt solution and 95% humidity. Cells were resuspended in 24-well plates, 150- or 600-cm² culture flasks at 3 × 10^6 cells/cm^2. The medium was changed every third day, and these asynchronous cultures of randomly dividing cells were allowed to grow for 14 days, which, based on prior experience in our laboratory, was expected to span the entire proliferative (4-h pulse) into trichloroacetic acid precipitable material as described previously (48). [3H]Thymidine incorporation was adjusted from percent hydrolysis values.

**Smooth Muscle Subcellular Fractionation**

Preconfluent (day 3; i.e. actively proliferating), near or at confluence (~day 8), and postconfluent (days 10–14) cells were harvested by trypsinization and centrifugation. The smooth muscle cell pellet (2.6–5.7 × 10^6 cells) was resuspended to 1 × 10^6 cells/ml of homogenization buffer containing 0.34 M sucrose, 10 mM HEPES, pH 7.4, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 200 μM leupeptin, 20 μM/μl soybean trypsin inhibitor, and 20 μM/μl aprotinin at 4 °C. Inclusion of EGTA localized the 85-kDa PLAr predominantly in the cytosolic fraction (50, 51). The cell suspension was disrupted by nitrogen cavitation (450 p.s.i. for 15 min at 4 °C), and the homogenate was centrifuged at 400 × g for 10 min at 4 °C to remove unbroken cells and debris. The resulting supernatant fraction was centrifuged at 100,000 × g for 60 min at 4 °C to obtain a supernatant (cytosolic) and particulate (microsomal) fraction. The microsomal pellet was resuspended in homogenization buffer, and both fractions were flash frozen with liquid N2, and stored at −70 °C until analysis.

**Preparation of Purified Human Phospholipase A2 Enzymes**

Recombinant human Type II 14-kDa PLAr (rh Type II 14-kDa PLAr) was cloned from a human placenta library, expressed, and purified as described previously (52, 53). Recombinant human cPLAr (rh 85-kDa PLAr) was prepared using a U937 85-kDa PLAr cDNA subcloned into the baculovirus vector pAcEL29 and expression in Spodoptera frugiperda (SF21) cells as described previously (54).

**Phospholipase A2 Enzyme Assay**

Phospholipase A2 activity of hCAVSMC subcellular fractions (50–100 μg of protein/assay) was measured by the acylhydrolysis of 1,2-diHAA-Escherichia coli (NEN Life Science Products) or 1,2-di[14C]Palmitoyl-2-arachidonyl phosphatidylcholine ([14C]AA-PC, Amersham Corp.) as described previously (14). The assay was initiated by addition of substrate, and assays were incubated at 37 °C for a time predetermined to be on the linear portion of a time versus hydrolysis plot. Specific activity (picomoles of fatty acid hydrolyzed/minute/milligram) was determined from percent hydrolysis values.

**Immunoblot Analysis**

Human CAVSMC fractions (20–50 μg of protein as indicated) and recombinant enzymes used as standards were analyzed by SDS-polyacrylamide gel electrophoresis (10% gels, Bio-Rad). Proteins were transferred to nitrocellulose paper, incubated with rabbit polyclonal antiserum (NS1; 1:500–1:1000) against rh 85-kDa PLA2 (30, 54), and then incubated with donkey anti-rabbit IgG conjugated to horseradish peroxidase (1:5000) (Boehringer Mannheim). Immunoreactive bands were detected via chemiluminescence (ECL Western blotting system, Amersham International, Arlington Heights, IL).

**Quantity of Type II 14-kDa PLAr by ELISA**

Mouse anti-rh Type II 14-kDa PLAr, monocalonal antibodies were prepared as described previously (30, 31) and demonstrated no cross-reactivity with either type I pancreatic 14-kDa PLAr, 85-kDa PLAr₂, albumin, or various inflammatory mediators such as tumor necrosis factor, interleukin-1, or platelet activating factor. Microtiter plates (Nunc Immuno Plate Maxisorp F96, Roskilde, Denmark) were coated with the monovalent antibody SK088–3C-6.2 (Biometallics Inc., Princeton, NJ) in 50 mM sodium phosphate, 150 mM NaCl, 0.02% NaN₃, pH 7.4, for 1 h at 4 °C. The plates were washed 4 times with 10 mM Tris, 150 mM NaCl, 0.02% NaN₃, pH 7.4, for 5 min at 37 °C. Smooth muscle subcellular fractions and the corresponding standards (50 μl) were diluted in assay medium (SmGM2) and coincubated with 50 μl of conjugate (2 μg/ml biotinylated monoclonal antibody SK097–18S.5.2) for 1 h at 37 °C. The plates were washed 4 times with wash buffer, followed by the addition of 100 μl per well of the streptavidin-alkaline phosphatase conjugate (diluted 1:2000 in streptavidin buffer, 0.5% bovine gamma globulin, 50 mM Tris, 150 mM NaCl, 0.02% NaN₃, 1 mM MgCl₂, pH 7.4) and incubated for 30 min at 37 °C. The plates were washed again, followed by the addition of 100 μl/well of substrate (p-nitrophenyl phosphate, 1 mg/ml) and incubated for 30 min at 37 °C after which the plate was read at 405 nm (M, 7000 plate reader) (Dynatech Laboratories Inc., Chantilly, VA). Purified rh type II 14-kDa PLAr was used to generate a standard curve (0.1–4 ng/50 μl). Standard curves and results were calculated using Delta Soft v2.12 (Biometallics Inc., Princeton, NJ).
RNA Isolation and Northern Blot Analysis

Total RNA was isolated from 150-cm² flasks by the method of Chomczynski and Sacchi (55) and resuspended in diethyl pyrocarbonate-treated sterile water. RNA concentrations were determined by spectrophotometry (A₅₂₀), and samples were stored at −80 °C. Northern blots were prepared by capillary transfer and UV fixation of electrophoretically separated (1.2% agarose/formaldehyde gel) RNA (10 µg total RNA per lane) to nylon hybridization membranes (Amersham Hybond N). Blots were hybridized (10% dextran sulfate, 1 m NaCl, 0.1% SDS, 0.1 mg/ml denatured herring sperm DNA, 65 °C) overnight with 10⁶ cpm/ml of the indicated probe and washed sequentially as follows: 20 min at room temperature in 1 × SSC, 1× EDTA, 0.1% SDS, 0.01 m NaHPO₄; 20 min at room temperature in 0.2 × SSC, 1 m EDTA, 0.1% SDS, 0.01 m NaH₂PO₄, 20 min at 65 °C in 0.1 × SSC, 1 m EDTA, 0.1% SDS, 0.01 m NaHPO₄. The washed blots were exposed to phosphor screens (Molecular Dynamics, Sunnyvale, CA) overnight. Probes were labeled by random priming (Promega, Madison, WI) and incorporation of radiolabel (Redivue [³H]dCTP, 3000 Ci/mmol, Arlington Heights, IL) to a specific activity of ≥1 × 10⁶ cpm/µg of cDNA. The 85-kDa PLA₂ probe was the full-length cDNA (2.87 kilobases). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was generated by CLONTECH (Palo Alto, CA) PCR primers.

Flow Cytometry

Apoptosis was measured by TUNEL (56, 57), using the ApopTag kit from Oncor (Gaithersburg, MD). In brief, the enzyme terminal deoxyribonucleotidyltransferase extends the DNA fragments with digoxigenin-containing nucleotides, which are then detected with an anti-digoxigenin antibody carrying fluorescein to allow detection by fluorescence (494-nm excitation, 523-nm emission). Propidium iodide was used as a counterstain to measure total DNA content and to determine distribution of cells in G₀/G₁, S, and G₂/M phases of the cell cycle. Flow cytometric analysis was performed on a Becton Dickinson FACScan instrument using CellQuest software.

Eicosanoid Measurement

PGE₂, PGF₂α, leukotriene B₄, and leukotriene C₄ levels in cell-free medium were directly measured using enzyme immunoassay (Cayman Chemical Co.). Sample or standard dilutions were made with SmGM2 and analyzed in triplicate.

Protein Determination

All protein concentrations were determined by Bradford protein analysis kits (Bio-Rad).

Data Analysis

Data are expressed as mean ± S.D. or standard error of the mean (S.E.) as indicated. Each data point represents n = 2–3 unless otherwise stated. Individual statistical comparisons of paired data were evaluated by Student’s t test with p < 0.05 representing significance.

RESULTS

Normal Growth of Human Coronary Artery Vascular Smooth Muscle Cells

Cells were plated at 3 × 10⁴ cells/cm² (6000 cells/well) on day 0 and maintained in normal growth medium as described under “Experimental Procedures.” On days 3 (spare), 8 (near confluent), 11 (confluent), and 14 (postconfluent) cells were harvested for determination of cell number. As shown in Fig. 1, absolute cell number increased as a function of time. Analysis of the data revealed that cell number increased 2.2-fold, 2.5-fold, and 1.2-fold between days 0 and 3, 8 and 11, and 11 and 14, respectively, reflecting the expected reduction in cellular proliferative rate in postconfluent cells. Parallel wells were pulsed with [³H]thymidine to measure relative rates of DNA synthesis. Incorporation of the radiolabeled thymidine was normalized to the number of cells present during the radioactive pulse. [³H]Thymidine incorporation was highest in sparsely seeded, robustly proliferating cells (day 3) (Fig. 1) and decreased with time in culture, with little or no [³H]thymidine incorporation evident in postconfluent (day 14) cells. Thus, as expected, DNA synthesis decreased prior to the reduction in cellular proliferative rate, and ultimately proliferation slowed in postconfluent cells. These observations are consistent with postconfluent contact-inhibited reduction in cellular growth. Subsequent studies were done using hCAVSMCs cultured to these same timepoints unless otherwise stated.

Eicosanoid Measurements

The hCAVSMCs used in these studies produced PGE₂ and PGF₂α as determined by its presence in the culture medium (ELISA). PGE₂ levels increased from day 0 to day 3, but did not change over days 3–14 of the culture period: pg/ml/cell, 2.08 ± 0.25 (n = 2, day3); 2.18 ± 1.01 (n = 2, day 8); and 1.76 (n = 1, day 10). Indomethacin (1 µM) produced 87.3 ± 2.04 percent reduction in PGE₂ synthesis (mean ± S.E., n = 3 in duplicate). The levels of PGF₂α were comparable to the levels of PGE₂ detected in the same samples. Given that PGE₂ synthesis was inhibited by indomethacin, one can assume that indomethacin had equivalent effects on PGF₂α synthesis as has been previously described in a number of cell systems (58). These cells did not produce detectable levels of leukotriene B₄ or leukotriene C₄ (data not shown).

Analysis of 85-kDa PLA₂ and 14-kDa PLA₂ as a Function of Culture Period

Measurement of Enzyme Activity—Human CAVSMCs were harvested and homogenized, and subcellular fractions were made from preconfluent (day 3), near or at confluent (days 7–8), and postconfluent (days 14–15) cultures as described under “Experimental Procedures.” Cell fractions were assayed for acylhydrolytic activity of 85-kDa PLA₂ activity via using [1-¹⁴C]palmitoyl-2-arachidonyl phosphatidylcholine as described under “Experimental Procedures.” As shown in Fig. 2, cytosolic 85-kDa PLA₂ activity was highest on day 3 commensurate with the highest proliferative rate. Activity steadily decreased from day 3 to day 14 coincident with the reduction in the relative rate of DNA synthesis over this culture period. The 85-kDa PLA₂ activity in the smooth muscle microsomal fraction was very low and remained constant over the length of the culture period, indicating that the reduced cytosolic 85-kDa PLA₂ activity was not due to migration of protein to microsomes.

Western and Northern Analysis of 85-kDa PLA₂—To examine if the reduction in 85-kDa PLA₂ activity resulted from lower protein levels, the microsomal and cytosolic fractions were sub-
85-kDa PLA2 Is Critical for Cellular Proliferation

85-kDa PLA2 activity of smooth muscle cell subcellular fractions collected after different periods of culture. Cell fractions were assayed for activity using [1-14C]palmitoyl-2-arachidonyl phosphatidylcholine (see “Experimental Procedures”). Data expressed as specific activity (pmol of free fatty acid hydrolyzed/min/mg). The bars represent mean ± S.D. values of three determinations. Activity of cell fractions was measured over 60 min.

Fig. 2. sn-2 Acylhydrolytic (85-kDa PLA2) activity of smooth muscle cell subcellular fractions collected after different periods of culture. Cell fractions were assayed for activity using [1-14C]palmitoyl-2-arachidonyl phosphatidylcholine (see “Experimental Procedures”). Data expressed as specific activity (pmol of free fatty acid hydrolyzed/min/mg). The bars represent mean ± S.D. values of three determinations. Activity of cell fractions was measured over 60 min.

Fig. 3. Western analysis of microsomal and cytosolic fractions of human VSMCs cultured 0–14 days is shown in panels A and B. Smooth muscle subcellular fractions were subjected to SDS-polyacrylamide gel electrophoresis (10%), transferred to nitrocellulose, and blotted with rabbit antiserum NS1 made against the 85-kDa PLA2; duplicate independent experiments run on different hCAVSMC cell lines. S indicates the rh U937 85-kDa PLA2 used as a standard (0.86 M) and further incubated for 3 days. Relative to the vehicle (0.03% ethanol) control cells, this compound dose dependently increased on day 3. Levels subsequently decreased by day 10 in agreement with the data shown in Fig. 3, A and B. Northern analysis of hCAVSMCs in parallel culture dishes is shown in Fig. 4. RNA from two independent studies was fractionated on the same gel. Hybridization indicates that the 85-kDa PLA2 message is present at day 3 and that it decreases with increased time in culture. This supports that the reduction in 85-kDa PLA2 protein level from day 3 to day 14 was due, at least in part, to a decrease in 85-kDa PLA2 message.

Assessment of 14-kDa PLA2 Mass by ELISA—Analysis of the subcellular fractions by ELISA indicated the presence of low levels of type II 14-kDa PLA2 immunoreactive protein in the cytosol and approximately 10-fold greater levels in the particulate fraction, but no significant change in the 14-kDa PLA2 protein level increased between 3 h and day 1, and was further increased on day 3. Levels subsequently decreased by day 10 in agreement with the data shown in Fig. 3, A and B. Northern analysis of hCAVSMCs in parallel culture dishes is shown in Fig. 4. RNA from two independent studies was fractionated on the same gel. Hybridization indicates that the 85-kDa PLA2 message is present at day 3 and that it decreases with increased time in culture. This supports that the reduction in 85-kDa PLA2 protein level from day 3 to day 14 was due, at least in part, to a decrease in 85-kDa PLA2 message.

Effect of PLA2 Modulation on hCAVSMC Proliferation

The Effect of PLA2 Inhibitors—To further examine the nature of the association of 85-kDa PLA2 with cellular proliferation and to examine whether 85-kDa PLA2 may have an active role in regulation of VSMC proliferation, we evaluated the effects of 85-kDa PLA2, 14-kDa PLA2, and PLA2 inhibitors on cellular proliferation. AACOCF3, a trimethyl ketone analogue of arachidonic acid recently shown to inhibit human recombinant 85-kDa PLA2 and cell-associated 85-kDa PLA2 (59, 60) was added to day 3 cultures at different concentrations (0–10 μM) and further incubated for 3 days. Relative to the vehicle (0.03% ethanol) control cells, this compound dose dependently
inhibited hCAVSMC proliferation (Fig. 5). Recall that these cells were seeded at a density of 6000 cells/well on day 0 and that by day 3, the cell number had increased 2.2-fold to 13,209 ± 200 cells/well (Fig. 1). Absolute cell number following the 3-day incubation period with 10 μM AACOCF3 was 12,472 ± 788.2 (mean ± S.D.) indicating that this concentration of AACOCF3 completely inhibited cellular proliferation, and that the apparent IC50 of AACOCF3 is ≈1 μM (Fig. 5). In contrast, neither the selective 14-kDa PLA2 inhibitor, SB203347 (0–10 μM) (59, 61), nor the cyclooxygenase inhibitor, indomethacin (1 μM), had significant effects on hCAVSMC proliferation. The long doubling time of these cells required that long incubation times be used to ensure a sufficient window over which to observe changes in proliferation. A possible concern is that the lack of an effect of the SB compound on hCAVSMC proliferation may be due in part to a possible breakdown of the drug over the 3-day incubation period used in these studies. However, 1 and 2 days of exposure to SB203347 (10 μM) produced no reduction in cell number (97.9 and 93.3% of control, respectively). Thus, the lack of an effect of SB203347 reported in Fig. 5 is not likely due to compound instability. Morphologic examination (Fig. 6) demonstrated that the various drug treatments produced no obvious changes in cell structure, and trypan blue exclusion methodology determined that cell viability in AACOCF3- and SB203347-treated cells (10 μM) was >95% and was equivalent to vehicle (ethanol) control cells. Thus, AACOCF3-mediated reduction of cellular growth was not due to cellular toxicity. Higher (30 μM) concentrations of AACOCF3 were toxic as suggested by gross morphological changes and lack of trypan blue exclusion. Evaluation of a possible role of the iPLA2 in hCAVSMC proliferation was performed in a separate series of experiments via examination of the effect of the potent and selective iPLA2 inhibitor HELSS (21) in hCAVSMC proliferation. HELSS (0–3.0 μM) had no effect on proliferation as assessed by cell counts via hemacytometer. Expressed as percent of control, cell number was 102.0 ± 2.25, 98.1 ± 1.20, and 98.9 ± 2.65 (mean ± S.E.) for 0.3 μM, 1.0 μM, and 3.0 μM, respectively (n = 2; each in triplicate). Observed gross morphological changes indicated apparent cellular toxicity with 10 μM HELSS (data not shown).

Effects of 85-kDa PLA2 Antisense and Exogenous Arachidonic Acid on hCAVSMC Proliferation—Previous work in this laboratory utilized 85-kDa PLA2 initiation site-directed antisense oligonucleotides in human monocytes (31, 59) and human synovial fibroblasts (49, 62) to assess the role of this enzyme in prostanoid and leukotriene production. To more specifically examine the nature of the regulation of cellular proliferation by 85-kDa PLA2, hCAVSMCs were seeded (day 0), allowed to adhere overnight and then incubated (18–24 h, 37 °C) in growth factor- and antibiotic-free medium (SmGM2) was added. For comparison, some cells received AACOCF3, 3 μM (C) and 10 μM (D), and the 14-kDa PLA2 inhibitor SB203347, 3 μM (E) and 10 μM (F).

TABLE I
Quantification of cell-associated type II 14-kDa PLA2 from cultured hCAVSMC homogenates following 3, 8, and 14 days in culture

| Culture time | Type II 14-kDa PLA2 |
|--------------|--------------------|
|              | Microsomal         | Cytosolic          |
| Day 3        | 2.44 ± 0.43        | 0.34 ± 0.21        |
| Day 8        | 3.64 ± 1.91        | 0.25 ± 0.20        |
| Day 14       | 3.21 ± 1.18        | 0.32 ± 0.03        |

FIG. 5. Effects of selective PLA2 inhibitors on hCAVSMC proliferation. Drugs were added with fresh culture medium on day 3 after passage and left to incubate for 3 days prior to determination of cell counts and viability. Data represents averaged cell counts (hemacytometer) ± S.E. for three experiments each done in triplicate and is expressed as percent of control. *, p < 0.05 versus vehicle control.

FIG. 6. PLA2 inhibitors did not effect cell morphology. Representative photographs of hCAVSMCs after 3 days of continual incubation with smooth muscle cell growth medium containing vehicle (ethanol) (A); indomethacin, 1 μM (B); 85-kDa PLA2 inhibitor AACOCF3, 3 μM (C) and 10 μM (D); and the 14-kDa PLA2 inhibitor SB203347, 3 μM (E) and 10 μM (F).
oligonucleotides had no effect (Table II). Addition of 20 μM AA itself directly and significantly stimulated cellular proliferation (Table II). Exogenous AA attenuated the anti-proliferative effect of SB7111 as evidenced by only 10 and 20% reductions in cell number in the presence of both AA and SB7111 (0.1 and 1.0 μM, respectively) versus 37 and 55% reductions in the presence of SB7111 alone. In agreement with other data presented herein (Fig. 5) AACOCF3 (3.0 μM) inhibited growth factor-induced proliferation by 47%. This was significantly attenuated by addition of AA as indicated by only 9% inhibition of proliferation during co-incubation of 20 μM AA and AACOCF3. Trypan blue exclusion and close visual inspection of cell morphology indicated that the observed decreases in cell number following exposure to SB7111 or AACOCF3 were not due to cytotoxicity.

**Flow Cytometry**

Fluorescence-activated cell sorter (FACS) analysis was performed to evaluate the effect of AACOCF3 on the cell cycle. Cells incubated with 10 μM AACOCF3 (the highest concentration used in the proliferation studies described above) showed no evidence of morphological changes and no overt cytotoxicity but had reduced cell number as expected (42% of non-AA-AACOCF3-treated controls). Cells were collected and stained with propidium iodide and TUNEL after 1, 2, and 3 days of exposure to the drug. Table III provides data from one representative of two experiments that shows 10 μM AACOCF3 caused no remarkable change in the distribution of cells in G0/G1, S, and G2/M phases over 3 days. The left panel of Fig. 7 shows the counts versus propidium iodide intensity and graphically depicts the numerical data for the cell cycle stages given in Table III; again, no difference was observed in the distribution of the cells in AACOCF3-treated cells. The right panel of Fig. 7 shows that 3 days of AACOCF3 exposure produced no DNA fragmentation (e.g., no apoptosis) as evidenced by no movement of signal into quadrant R3. A shift of cells into the R3 quadrant is typically observed in our laboratory with anti-FAS antibody-induced apoptosis in Jurkat cells and is routinely reported for other apoptotic cells (57).

**DISCUSSION**

Although previous studies demonstrated that nonselective PLA2 inhibitors reduce VSMC proliferation (36–39, 42, 43), the present study represents the first direct examination of the two distinct PLA2 enzymes in VSMC proliferation. Moreover, it is the first study that clearly demonstrates in any cell type that the 85-kDa PLA2 and not the 14-kDa PLA2 is critical for cellular proliferation. Herein it is confirmed that vascular smooth muscle cells possess both the 14-kDa PLA2 and 85-kDa PLA2 enzymes. By taking advantage of contact inhibition of cellular growth despite continual presence of growth medium, it was first demonstrated that 85-kDa PLA2 activity, protein level and mRNA expression (but not 14-kDa PLA2 activity or mass) correlated with DNA synthesis. 85-kDa PLA2 levels were highest early in culture and rose to a maximum level and activity at day 3, which corresponds with the peak in [3H]thymidine uptake, indicating an association between 85-kDa PLA2 and cellular proliferation and suggesting an important role of this protein in robustly proliferating hCAVSMCs.

To further and more directly evaluate the role of 85-kDa PLA2 in hCAVSMC proliferation, PLA2 inhibitors and antisense oligonucleotides were used. In both AACOCF3- and SB7111-treated cells, replication assessed by cell counts was concentration dependently reduced relative to vehicle control. Indeed, at the highest concentration of SB7111 cell numbers at day 3 did not vary from the initial seeding density suggesting that SB7111 produced complete cessation of cell cycle activity and thereby completely inhibited cellular proliferation. Again, neither SB203347, HELSS, nor SB9030 affected proliferation. Taken together, these data further support an important role of the 85-kDa PLA2 but not the 14-kDa PLA2 in hCAVSMC proliferation.

FACS scan analysis suggests that the reduction in cell number following inhibition of 85-kDa PLA2 results from generalization of total cellular proliferative rate rather than phase-specific cell cycle arrest. Furthermore, the AACOCF3-treated cells showed no indication of entering programmed cell death. Similar results were observed in HUVEC cells exposed to AACOCF3 (63). The present data may offer an explanation for a previous observation (64); increased PLA2 activity is readily observed following stimulation of proliferating cultures of murine embryoid palate mesenchymal cells with the calcium ionophore A23187, but confluent cultures showed no such response. Based on our findings, we hypothesize that the confluent murine embryoid palate mesenchymal cell cultures treated with AACOCF3 failed to respond in a proliferative manner but instead, differentiated towards a more quiescent state.

**Table II**

**85-kDa PLA2 antisense dose dependently reduces proliferation of hCAVSMCs**

| Condition | % control (−AA) | −AA | +AA |
|-----------|----------------|-----|-----|
| Control   | 100            | 109.0 ± 4.10a | 112.6 ± 5.95ab |
| Lipoferin | 95.9 ± 1.88    | 95.8 ± 2.59    | 105.2 ± 6.15 |
| SB7111    | 20 μM          | 62.7 ± 7.45a   | 89.5 ± 7.40b |
|           | 0.01 μM        | 45.1 ± 5.24a   | 80.6 ± 6.65b |
|           | 0.10 μM        | 102.4 ± 2.10a  | ND |
|           | 1.00 μM        | 95.3 ± 1.21a   | ND |
|           | 1.00 μM        | 85.2 ± 7.30    | ND |
|           | Scrambled      | 104.3 ± 7.40a  | ND |
|           | 1.00 μM        | 53.1 ± 6.39a   | 91.0 ± 1.53b |

a) p < 0.05 vs. control (no AA).
b) p < 0.05 vs. paired data in the absence of AA. ND, not determined.

**Table III**

**The 85-kDa PLA2 inhibitor AACOCF3 (10 μM) produces no phase-specific arrest of the cell cycle**

| Cell cycle phase | Day 1 | Day 2 | Day 3 |
|------------------|-------|-------|-------|
|                  | Control | AACOCF3 | Control | AACOCF3 | Control | AACOCF3 |
| G0/G1            | 92.2   | 92.4   | 83.9   | 84.4   | 82.1   | 86.4   |
| S                | 8.3    | 8.4    | 10.6   | 8.7    | 11.5   | 8.0    |
| G2/M             | 2.8    | 2.3    | 4.4    | 5.8    | 2.5    | 1.3    |
85-kDa PLA2 Is Critical for Cellular Proliferation

The mechanism by which 85-kDa PLA2 influences cellular proliferation may be related to previous demonstrations that inhibition of the lipoxygenase-cytochrome P450 system reduces VSMC proliferation or proliferation-associated events (1, 37–39) and/or that 85-kDa PLA2 is activated by oncogenic ras (65, 66). Alternatively, or additionally, recent reports indicate that the release of AA by PLA2 is accompanied by the formation of biologically active lipid derivatives, the glycerophosphoinositides, which show evidence of being accumulated specifically in ras-transformed cells. Moreover, these lipids serve as precursors to a new class of biologically active lipid derivatives, the lysolipids, which are known to be potent mitogens in a variety of cell types (67–71). Furthermore, these lipids may influence cellular proliferation largely through their generation of free AA. Interestingly, 20 μM AA by itself stimulated hCAVSMC proliferation, increasing the cell number 9% above untreated controls. The exact role of AA is not known, but as discussed above, conversion to PGE2, LTB4, and LTC4 does not seem to be obligatory for proliferation to proceed. This raises the possibility that AA is itself an intracellular signal and/or that it is converted to an unidentified eicosanoid product important in regulation of cellular proliferation. It is possible that the present demonstration of a direct proliferative effect of exogenous administration of AA to hCAVSMCs and attenuation of AAOCF2, and 85-kDa PLA2 antisense-mediated reductions in proliferation by AA may be related to the effect of AA to stimulate c-fos, c-jun, and/or mitogen-activated protein kinase (36–39, 42, 43).

The mechanism by which 85-kDa PLA2 influences cellular proliferation is not mediated through PGE2. This is consistent with the coordinate early rise in 85-kDa PLA2 protein during the initial phase of proliferation. However, indomethacin had no effect on cellular proliferation under the conditions of our experiments, indicating that the 85-kDa PLA2 dependent effect on VSMC proliferation is not mediated through PGE2. This is in agreement with previous reports that cyclooxygenase inhibition by indomethacin had no effect on VSMC proliferation (1, 39), AA-, or hydrogen peroxide-induced c-jun expression in VSMCs (37) or AA activation of mitogen-activated protein kinase (38). When cells were treated with exogenous AA, the reduction in cell number produced by either AAOCF2 or 85-kDa PLA2 antisense oligonucleotides (SB7111) was prevented or significantly attenuated, suggesting that the 85-kDa PLA2 may influence cellular proliferation largely through its generation of free AA. Interestingly, Cov variables appear to be obligatory for proliferation to proceed. This raises the possibility that AA is itself an intracellular signal and/or that it is converted to an unidentified eicosanoid product important in regulation of cellular proliferation. It is possible that the present demonstration of a direct proliferative effect of exogenous administration of AA to hCAVSMCs and attenuation of AAOCF2, and 85-kDa PLA2 antisense-mediated reductions in proliferation by AA may be related to the effect of AA to stimulate c-fos, c-jun, and/or mitogen-activated protein kinase (36–39, 42, 43).

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