Interferon-γ Induces Secretory Group IIA Phospholipase A2 in Human Arterial Smooth Muscle Cells

INVolvement of cell differentiation, STAT-3 activation, and modulation by other cytokines*

Received for publication, April 3, 2000, and in revised form, May 10, 2000
Published, JBC Papers in Press, May 12, 2000, DOI 10.1074/jbc.M002783200

Helena Peilot, Birgitta Rosengren, Göran Bondjers, and Eva Hurt-Camejo‡
From the Wallenberg Laboratory for Cardiovascular Disease, Sahlgrenska University Hospital, Göteborg 413 45, Sweden

Increased expression of secretory non-pancreatic phospholipase A2 (sPLA2-IIA) could be part of the inflammatory reaction in atherosclerosis. However, the factors controlling sPLA2-IIA production in human vascular cells are unknown. We investigated regulation of sPLA2-IIA expression and secretion by human arterial smooth muscle cells in culture (HASMC). S PLA2-IIA was induced after 3–14 days of culture in non-proliferating conditions. S PLA2-IIA was co-expressed with heavy caldesmon, a cytoskeleton protein, and p27, a G1 cyclin inhibitor, proteins characteristically expressed by differentiated cells. Further incubation with 50–500 units/ml of interferon (IFN)-γ significantly increased sPLA2-IIA mRNA and secretion. IFN-γ-induced sPLA2-IIA was found to be active in cell media and associated with cell membrane proteoglycans. IFN-γ induced sPLA2-IIA expression was antagonized by tumor necrosis factor (TNF)-α and interleukin (IL)-10. TNF-α added individually induced a significant but transient (4 h) increase in sPLA2-IIA secretion. IL-10 by itself did not affect sPLA2-IIA expression and secretion. IFN-γ-stimulated sPLA2-IIA transcription involved STAT-3 protein. Interestingly, IL-6 but not IFN-γ up-regulated the sPLA2-IIA expression in HepG2 cells, thus sPLA2-IIA induction by IFN-γ response appears to be cell specific. In summary, conditions leading to cell differentiation induced sPLA2-IIA expression in HASMC and further exposure to IFN-γ can up-regulate sPLA2-IIA transcription and secretion. This IFN-γ stimulatory effect can be modulated by other cytokines.

Group IIA secretory non-pancreatic phospholipase A2 (sPLA2-IIA)1 catalyzes hydrolysis of fatty acids from the sn-2 position of glycerophospholipids yielding nonesterified free fatty acids and lysophospholipids (1). These products may either act as intracellular second messengers or can be further metabolized into proinflammatory and mitogenic lipid mediators including eicosanoids, platelet activating factor, and lysophosphatidic acid (1). Lysophosphatidylcholine is a mediator of a broad range of cellular processes on vascular and inflammatory cells (2–4). Many of these lipid mediators accumulate during atherosclerotic lesion development (5, 6). S PLA2-IIA appears to be involved in several physiological and pathological processes. It contributes to membrane remodeling and removal of oxidized phospholipids (7). In addition, sPLA2-IIA has bactericidal and anti-tumorigenic properties, participates in TNFα-induced activation of nuclear transcription factor and expression of cell-adhesion molecules (8–10). Regarding pathological situations, sPLA2-IIA can induce acute inflammatory changes when injected in vivo (11). Furthermore, there is a correlation between elevated levels of sPLA2-IIA and inflammatory conditions, such as rheumatoid arthritis, septic shock, acute respiratory distress syndrome, and coronary artery disease (12, 13). Atherosclerosis has characteristics of an inflammatory process (14). Furthermore, a high incidence of atherosclerosis and high mortality from cardiovascular diseases have been reported in patients with chronic inflammatory diseases that have prolonged periods of high extracellular sPLA2-IIA activity (15). Additionally, transgenic mice overexpressing human sPLA2-IIA with high levels of the enzyme in plasma and in the aortic intima/media develop more aortic atherosclerosis than non-transgenic littermates (16). In humans it was recently reported that in patients with coronary artery disease the plasma level of sPLA2-IIA was an independent risk factor. Furthermore, a high level of sPLA2-IIA was also a significant predictor of new coronary events during a 2-year follow up period (13).

Earlier immunohistochemistry studies from our group and others indicate that smooth muscle cells are the main source of sPLA2-IIA in human arteries (17–19). Our data from electron microscopy-immunogold examination revealed that the major- ity of sPLA2-IIA in human atherosclerotic lesions is localized extracellularly associated with collagen fibers and in close contact with extracellular lipid droplets. Intracellular sPLA2-IIA was observed in electron-dense vesicles in the cytosol (20). These observations suggest that sPLA2-IIA may be involved in the pathogenesis of atherosclerosis. One mechanism by which sPLA2-IIA may be atherogenic is by modifying lipoproteins and releasing inflammatory lipid mediators at places of lipoprotein retention in the arterial wall (21). To evaluate the possible biological and pathological function(s) of sPLA2-IIA in the arterial wall it is necessary to clarify the mechanisms regulating its expression and secretion by vascular cells. Inflammatory
cytokines that are present in atherosclerotic lesions can regulate genes in cells of the vascular wall cells and macrophages (22). Several in vitro studies indicate that cytokines, such as IL-1β, IL-6, and TNF-α, can stimulate different cell systems to release sPLA2-IIA (23). However, little is known regarding the effect of IFN-γ on sPLA2-IIA expression. IFN-γ is a potent activator of smooth muscle cells modulating different cell functions related with pathological conditions of the arterial wall (24). Furthermore, mRNA transcripts of sPLA2-IIA, TNF-α, IL-1β, and IFN-γ are present in human atherosclerotic lesions together with microbial agents (25).

Arterial vascular smooth muscle cells can change their phenotype between contractile/non-proliferating and synthetic/proliferating forms. These phenotypic changes are accompanied by changes in gene pattern expression and are associated with the pathological arteriosclerotic process (26). The aims of the present study were two. First, to study the expression of sPLA2-IIA at mRNA and protein level in an in vitro model of phenotypic modulation of HASMC. Second, to investigate the effect of IFN-γ and other pro- and anti-inflammatory cytokines in the transcription and secretion of sPLA2-IIA by HASMC.

MATERIALS AND METHODS

Reagents—Collagen-I (rat-tail) was purchased from Collaborative Biomedical, Becton Dickinson, Labware, Bedford, MA. Waymouth’s cell culture medium and Dulbecco’s phosphate-buffered saline with and without calcium and magnesium were purchased from Life Technologies, Inc., Grand Island NY. Human recombinant interferon-γ (IFN-γ), interleukin 1β (IL-1β), IL-6, IL-10, and tumor necrosis factor-α (TNF-α) were purchased from 1st Immunokontakt, Switzerland. Monoclonal mouse antibodies against p27 and p21 proteins were from Transduction Laboratory, Lexington, KY; monoclonal mouse anti-sPLA2-IIA was from Cayman Chemicals (Ann Arbor, MI); biotin-SP-conjugated affinity pure donkey anti-rabbit IgG (H+L) and peroxidase-conjugated streptavidin were from Jackson Immunoresearch, Laboratories, Inc. Bromodeoxyuridine ELISA kit for non-radioactive immunodetection of cell proliferation was purchased from Boehringer Mannheim, Germany. Biotin-DIHN (10 ng/ml) and IFN-γ (100 units/ml) were present in human atherosclerotic lesions (26). Several studies indicate that cytokines, such as TNF-α, IL-1β, IFN-γ and other pro- and anti-inflammatory cytokines (27). After 2 days the medium was removed, cells washed 3 times with Dulbecco’s phosphate-buffered saline, and then cultured in Waymouth’s medium containing the supplements indicated above. Cells were stimulated to differentiate in serum-free Waymouth’s medium containing the other supplements indicated above (serum-free medium).

HASMC were isolated from inner media of human uterine arteries and cultured in Waymouth’s medium, supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 4 mM glutamine at 37 °C in a 5% CO2, 95% air atmosphere. The medium was changed every 3 days, and cells were detached by treatment with 0.05% trypsin, 0.02% EDTA solutions. Studies were conducted on HASMC between passages 4 and 9. HASMC were seeded at 5 × 10^4 cells/cm² in 25- and 80-cm² cell culture bottles coated with collagen-I in growing medium (27). After 2 days the medium was removed, cells washed 3 times with Dulbecco’s phosphate-buffered saline, and then cultured in Waymouth’s medium containing the supplements indicated above but with only 0.5% FBS (serum-poor medium) in order to synchronize the cells by arresting proliferation. After 3 days the medium was removed, cells washed 3 times with Dulbecco’s phosphate-buffered saline, and cultured in growing medium for 3–4 days until the cells were confluent. At confluence, cells were stimulated to differentiate in serum-free Waymouth’s medium containing the other supplements indicated above (serum-free medium). HASMC were cultured in this medium up to 21 days. Human aortic and pulmonary cells (Clonetics Corp.) were cultured under similar conditions as HASMC. HepG2 liver tumor cells (ATCC, Manassas, VA) were culture in Eagle’s modified essential medium containing 10% FBS and supplemented as the growing medium described above. Cells were free of mycoplasma. Endotoxin levels were regularly tested in cell culture medium and cell culture reagents with Coatest/endotoxin, Chromogenix AB (Molndal, Sweden). Levels detected were below 0.01 units/ml.

Incubation with Cytokines—Most experiments were performed after the cells were 7 days in serum-free medium in order to guaranty the expression of both mRNA and protein for sPLA2-IIA. Some experiments with IFN-γ were performed with HASMC preincubated 1 or 3 days in serum-free conditions. HASMC were incubated with cytokines for 4 or 24 h in serum-free medium. The following cytokines concentrations were used: IFN-γ (1.7–500 units/ml), IL-6; 10 ng/ml; IL-1β, 10 ng/ml; TNF-α, 500 ng/ml; IL-10 (10 ng/ml).

Immunoblot Analysis—HASMC were extracted with 1 ml/25-cm² bottle of electrophoresis sample buffer, 0.325 M Tris, pH 6.8, 0.1% SDS, containing 0.1% bromophenol blue, 5 μM AEBSF, 0.1 mM leupeptin, 5 μM aprotinin, and 1 μM soybean trypsin inhibitor. SDS-polyacrylamide gel electrophoresis and Western blot were performed as described (17). Membranes were incubated with monoclonal antibodies against p27 or p21 from Transduction Laboratories, Lexington, KY (1:2500 and 1:500 dilutions, respectively), and then incubated with goat-anti-mouse IgG conjugated to horseradish peroxidase (1:1000). Immunoreactive bands were detected via chemiluminescence, ECL Western blotting system, Amersham Pharma Biotech. Western blot detection of STAT-3 phosphorylation was performed using the PhosphoPlus Stat (Tyr) Antibody Kit from New England BioLabs, Inc. (Beverly, MA).

RNA Preparation and RT-PCR Procedure—Total cellular RNA was isolated from HASMC and CHO-cell line expressing human sPLA2-IIA (28) using a single step, acid guanidinium thiocyanate/choroform extraction method (17). RT-PCR with 0.1 or 0.5 μg of total RNA was performed using a GeneAmp kit from Perkin-Elmer. Oligonucleotide conditions for amplification of cDNA were as designed. The oligonucleotides designed as the determined cDNA sequence for human sPLA2-IIA (29) amplified a 444-base pair product and the sequences were: sense primer, ATGAGGACCTCTTCACTGTT, and antisense primer, AAGCAGTTATACACTAC (17). The human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer set amplified a 953-base pair product and the sequences were: sense primer, TGGAGCTGGACTACAAGGGA and antisense primer, CATGTGGGCATGAGGTCTGA (30). The high molecular weight caldesmon primer set amplified a 530-base pair product and the sequences were: sense primer, AAACACTGGAACCGAGG and antisense primer, GCTGCTGGTACGTTACTG (31). All incubations were done in a Biometra, TRIO-Thermocycler. The PCR products were then separated on a 4% Nusieve GTG agarose gel, FMC Bioproducts Corp., Rockland, ME.

Analysis and Quantification of the cDNA—Fluorescent labeled PCR-amplified products. PCR products were subjected to a semiquantitative analysis following the fluorescent labeling method described in ABI PRISM 377, DNA Sequencer GeneScan (32) Chemistry Guide, Perkin-Elmer. PCR products were labeled on one strand using fluorescent detection system. After purification of the PCR product was separated and detected by running the gel in the ABI Prism 377 DNA sequencer, Perkin-Elmer essentially as described by the manufacturer. The relative quantity of the amplified fragments was determined by dividing the peak areas corresponding to the fluorescence of the sPLA2-IIA fragment against the peak area of the GAPDH fragment. GAPDH and sPLA2-IIA fragments were amplified simultaneously in separate PCR reaction tubes. The results presented represent average and standard deviation of three separate GeneScan analyses.

Quantification of sPLA2-IIA by ELISA—Cell-associated and secreted sPLA2-IIA were measured by enzyme-linked capture antibody immunoassay (32). The antibodies used were: monoclonal antibody against human sPLA2-IIA (1 μg/ml); polyclonal antibody (IgG fraction) against human recombinant sPLA2-IIA developed at the lab with demonstrated no cross-reactivity with PLA2 type V or antigen; and biotin-conjugated AffiniPure donkey anti-rabbit IgG (H+L). Microtiter plates (Sero-Wel, Bibby Sterilin Ltd. Stone, Staffs, United Kingdom) were coated with the monoclonal antibody against sPLA2-IIA (50 μl/well, 5 μg/ml) in 15 mM carbonate buffer, pH 9.6, for 18 h in a humid chamber at room temperature. The plates were washed 3 times with 50 mM Tris, 150 mM NaCl pH 7.6 (TBS buffer), containing 0.05% Tween 20 (washing buffer). HASMC in a 25-cm² flask were extracted with 0.5 ml of phosphate-buffered saline, pH 7.4, containing 0.5% Triton X-100, 5 μM AEBSF, 0.1 mM leupeptin, 5 μM aprotinin, and 1 μM soybean trypsin inhibitor. For the ELISA, 50 μl of cell medium or 50 μl of cell extract were added to the wells by quadruplicate and incubated for 1 h at room temperature. In total 100 μl of cell extract or media were applied in each well. The plates were washed 3 times with washing buffer, followed by the addition of 1:5000 dilution of polyclonal antibody (IgG fraction) against human recombinant sPLA2-IIA, and incubated 1 h at 37 °C. The plates were then washed 3 times and...
incubated with 1:5000 dilution of biotin-SP-conjugated donkey anti-rabbit IgG for 1 h at 37 °C. All the antibodies were diluted in TBS buffer containing 1% bovine serum albumin and 0.05% Tween 20 (incubation buffer). The plates were washed 3 times with washing buffer followed by the addition of 50 μl/well of the peroxidase-conjugated streptavidin diluted 1:2000 in incubation buffer and incubated 1 h at 37 °C. Then the plates were washed again, followed by the addition of 50 μl/well of substrate azino diethylbenzthiazoline sulfonate (22 mg/ml) and incubated for 30 min at 37 °C after which the plates were read at 405 nm (Spectra MAX Plus Microplate Spectrophotometer System, Molecular Devices, Sunnyvale, CA). Purified human recombinant sPLA2-IIA (28) was used to generate a standard curve (62.5–2000 pg/50 μl/well).

Assay of sPLA2-IIA Activity—HASMC cell media were concentrated 10 times with Centricron-10 concentrates (Amicon, Inc., Beverly, MA) and incubated 4 h with 1-3-phosphatidylycholine, 1-palmitoyl-2-(1-14C)oleoyl as substrate (50–25 mCi/mmol; Amersham Pharmacia Biotech). PLA2 activity was assayed essentially as described (33). A calibration curve with different concentrations of human recombinant sPLA2-IIA was performed in parallel. Based in this curve, PLA2 activity from HASMC was expressed as the concentration of sPLA2-IIA (ng/ml) in non-concentrated cell media responsible for the release of 14C-labeled nonesterified fatty acids NEFA.

Preparation of Nuclear Protein Extracts and Electrophoretic Mobility Shift Assay—HASMC were incubated with or without cytokines as described above. After 4 and 24 h incubation cell nuclear extracts were prepared. Nuclear extract preparation, annealing, and purification of oligonucleotides and electrophoretic mobility shift assay were performed as described (34). Oligonucleotides were synthesized by Life Technology, Ltd., Paisley, UK. Double-stranded oligonucleotides identical to the sequences from −563 to −535 and −240 to −208 in the human sPLA2-IIA promoter, containing a NF-kB and STAT-binding site, respectively, were used. In some experiments for identification of nuclear transcription factors in the EMSA assay, rabbit polyclonal antibodies (Santa Cruz Biotechnology Inc.) against the p65 subunit of NF-κB and against STAT 1, 3, 5, and 6 proteins were added to the EMSA binding reactions.

Protein Determination—Protein concentrations were determined by Bradford protein analysis kits (Bio-Rad).

Data Analysis—Data are expressed as mean ± S.D. Each experiment was performed at least 3 times. The results obtained were reproducible between experiments. Variation in the total levels of sPLA2-IIA expression, mRNA, secretion, and cell associated, could be observed sometimes between batches of HASMC. Each data point represent n = 3 or 4. Individual statistical comparisons of paired data were evaluated by Student’s t test with p ≤ 0.05 representing significance. Standard curves and statistical analysis of the results were performed using GraphPad Prism, v2.0 (GraphPad Software, San Diego, CA).

RESULTS

Phenotypic Changes during Differentiation of Cultured Human Arterial Smooth Muscle Cells—To investigate the regulation of sPLA2-IIA expression in HASMC, we used an in vitro model of phenotypic modulation or differentiation in culture shown in Fig. 1 and described under “Experimental Procedures.” We studied the following parameters: 1) growth of the cells by determination of cell number and DNA synthesis; 2) cell morphology; 3) expression of the cell differentiation markers, cyclin-dependent kinase inhibitors of mammalian cell cycle p27 and p21 and heavy caldesmon, a marker of human smooth muscle cell differentiation. As shown in Fig. 2A after 3 days of cell synchronization in 0.5% FBS (serum-poor medium) there was a marked reduction in DNA synthesis with little change in cells numbers. These results reflect the expected reduction in proliferation rate of confluent cells cultured in the absence of serum. Phase-contrast microscopy showed no morphological changes between HASMC maintained 1, 3, 7, and 14 days in serum-free medium (Fig. 2B). These HASMC cultures were judged as normal dense cultures of fully confluent spindle shaped cells.

The expression of p27 and p21 cyclin-dependent kinase (Cdk2) inhibitors, as markers of cell differentiation, was evaluated by Western blot analysis of cell extracts. As shown in Fig. 3A p27 protein was not detected in HASMC after 2 days in 10% FBS when proliferation was as highest, whereas the same cells after 1 or 14 days in defined serum-free medium (non-proliferating conditions) showed as expected a markedly increase in the expression of p27. The p21 protein was detected under proliferating and non-proliferating conditions, however, its expression was increased after 1-day culture in serum-free medium. We also analyzed the mRNA level for heavy caldesmon, a marker of smooth muscle cell differentiation found in the vascular wall, in parallel to analysis of mRNA for sPLA2-IIA and GAPDH. PCR-amplified fragments showed the corresponding size of the different fragments and only one well defined band was amplified (Fig. 3B). These results shown that post-confluent HASMC expression of sPLA2-IIA and heavy caldesmon mRNA increased significantly after 7 and 14 days in culture in serum-free medium. In contrast, the levels of GAPDH mRNA remained constant during culture. These observations indicate that the in vitro protocol used for culture of HASMC lead the cells to a quiescent, differentiated state that promotes expression of sPLA2-IIA. Similar results were observed with human smooth muscle cells from aorta and pulmonary artery (data not shown).

Modulation of sPLA2-IIA mRNA, Cell-secreted and Cell-associated sPLA2-IIA during in Vitro Differentiation of HASMC—Analysis of mRNA content by semiquantitative RT-PCR showed that subconfluent proliferating cells cultured in growing medium (3-day 10% FBS) and confluent cells after 1 day in defined serum-free medium (1-day 0% FBS), exhibited a low constitutive expression of sPLA2-IIA (Fig. 4A). When these cells were switched to defined serum-free medium, we observed a time-dependent increase in the mRNA content of sPLA2-IIA after 3 days that remained constant up to 14 days in culture. The results of sPLA2-IIA protein levels presented in Fig. 4, B and C, indicate that similar to the mRNA levels, culture of confluent cells in defined serum-free medium induced a time-dependent increase in the cell-secreted and cell-associated protein content of sPLA2-IIA. Despite an increase in sPLA2-IIA mRNA levels no sPLA2-IIA protein could be detected in cell media or associated to the cells after 3 days in serum-free media (3-day 0%). A significant increase in sPLA2-IIA cell-associated protein was observed after 7 days (2–2.5-fold) that continued up to 14 days of culture in serum-free medium, where after, sPLA2-IIA protein levels started to decrease. The amount and period of sPLA2-IIA secretion varied between different batches of HASMC. In some cultures, secretion was
highest after 7 days in serum-free medium. To further investigate if this increase in sPLA2-IIA mRNA and cell-associated protein levels in culture was due to the absence of serum, cells expressing sPLA2-IIA after 5 or 7 days in defined serum-free medium were switched to 10% FBS growing medium. In these cells sPLA2-IIA mRNA, secreted and cell-associated sPLA2-IIA protein levels decreased (Fig. 4, A, B, and C). In contrast, an increase in the sPLA2-IIA mRNA and cell-associated protein levels were observed when the same cells were switched back to defined serum-free medium (data not shown). These results together with the results presented in Figs. 2 and 3 indicate that sPLA2-IIA mRNA and sPLA2-IIA protein levels correlate with the degree of cell proliferation in cultures of HASMC. To search for the mechanisms of sPLA2-IIA induction, proliferating HASMC nonexpressing sPLA2-IIA and 1-day post-confluent HASMC were incubated for 1 or 3 days with conditioned media from 3 and 7 days post-confluent HASMC. However, these conditioned media from HASMC did not induce or enhance the expression of sPLA2-IIA at mRNA or protein levels (data not shown).

**Effect of Interferon-γ on sPLA2-IIA Expression**—Incubation of 7 days post-confluent HASMC with IFN-γ for 4 or 24 h stimulated the transcription of sPLA2-IIA inducing a dose-dependent (50–500 units/ml) increase of sPLA2-IIA mRNA and also of cell associated and cell secreted sPLA2-IIA (Fig. 5, A, B, and C). IFN-γ at 500 units/ml, the highest concentration used, increased the mRNA levels significantly but the sPLA2-IIA protein levels decreased. Highest induction of sPLA2-IIA protein levels was observed with 100 units/ml. The IFN-γ-sPLA2-IIA up-regulation remained constant for 48 h (data not shown). The induction of sPLA2-IIA expression by IFN-γ was also observed in postconfluent HASMC after 1 or 3 days culture in serum-free medium (data not shown).

The induction of sPLA2-IIA expression by IFN-γ significantly increased the amount of active sPLA2-IIA secreted in the cell medium (Fig. 6). To investigate if secreted sPLA2-IIA could be found associated to extracellular membrane proteoglycans, HASMC were pretreated with chondroitinase ABC (0.1 units/ml) and heparitinase I (0.01 units/ml) for 2 h at 37°C before collecting the cell media. As shown in Fig. 6, the degradation of cell surface proteoglycans increased the amount of sPLA2-IIA released in the cell medium. This indicated that part of the sPLA2-IIA secreted by HASMC is associated with cell membrane proteoglycans.

**Modulation of sPLA2-IIA Expression by Different Cytokines**—Fig. 7 shows the results obtained when studying the effect of different cytokines on the sPLA2-IIA mRNA level (I) as measured by semiquantitative RT-PCR and the amount of sPLA2-IIA protein secreted into the cell media (II). The RT-PCR results (Fig. 7I) showed that IFN-γ (B) was the most potent cytokine inducing a 14-fold increase in the mRNA level after 4 and 24 h incubation time compared with the other cytokines used such as IL-1β (G), IL-6 (H), and TNF-α (I) at similar concentrations. This difference between cytokines was especially clear after 24 h incubation. Furthermore, incubation with IL-1β (C), IL-6 (H), TNF-α (I), and IL-10 (D) when individually added only induced a slight increase in the sPLA2-IIA level of mRNA after 4 h incubation. The combination of IFN-γ and IL-6 showed that IL-6 had no additional effect on the IFN-γ-induced increase in sPLA2-IIA mRNA (C). After 24 h incubation IL-1β (D), TNF-α (E), and IL-10 (F) antagonized the IFN-γ-induced sPLA2-IIA mRNA expression; however, the mRNA levels were still higher compared with the control cells without cytokine stimulation (A). Interestingly, this down-regulation or antago-
nizing effect was also observed when TNF-α and IL-10 were added simultaneously (P). This effect became stronger at secretion level (Fig. 7I, F). Thus, suggesting a possible synergistic effect between these cytokines.

The amount of cell-secreted and cell-associated sPLA2-IIA protein after 4 and 24 h incubation with cytokines were measured in parallel to the mRNA levels. The levels of secreted sPLA2-IIA are presented in Fig. 7III. Results from cell-associated sPLA2-IIA protein level are not shown. These results suggest that TNF-α appears to regulate sPLA2-IIA translation and secretion. This TNF-α stimulatory effect on sPLA2-IIA secretion was antagonized when TNF-α was added together with IFN-γ (E), IL-1β (M), or IL-6 (N). The strongest antagonizing effect was observed with IL1-β (M).

Levels of sPLA2-IIA mRNA and sPLA2-IIA secretion after 4 and 24 h incubation with different combinations of IL-1β, IL-6, IL-10, and TNF-α remained similar to or under control levels (without cytokines). There was synergistic effect between some of these cytokines in down-regulating sPLA2-IIA mRNA accumulation. For example, IL-6 plus IL-1β (K) and TNF-α plus IL-1β (N). No effect on sPLA2-IIA mRNA or protein expression was observed when HASMC were incubated with TGF-β (0.1–10 ng/ml) or IL-4 (data not shown).

Levels of sPLA2-IIA mRNA and sPLA2-IIA secretion after 4 and 24 h incubation with different combinations of IL-1β, IL-6, IL-10, and TNF-α remained similar to or under control levels (without cytokines). There was synergistic effect between some of these cytokines in down-regulating sPLA2-IIA mRNA accumulation. For example, IL-6 plus IL-1β (K) and TNF-α plus IL-1β (N). No effect on sPLA2-IIA mRNA or protein expression was observed when HASMC were incubated with TGF-β (0.1–10 ng/ml) or IL-4 (data not shown).

Levels of sPLA2-IIA mRNA and sPLA2-IIA secretion after 4 and 24 h incubation with different combinations of IL-1β, IL-6, IL-10, and TNF-α remained similar to or under control levels (without cytokines). There was synergistic effect between some of these cytokines in down-regulating sPLA2-IIA mRNA accumulation. For example, IL-6 plus IL-1β (K) and TNF-α plus IL-1β (N). No effect on sPLA2-IIA mRNA or protein expression was observed when HASMC were incubated with TGF-β (0.1–10 ng/ml) or IL-4 (data not shown).

Levels of sPLA2-IIA mRNA and sPLA2-IIA secretion after 4 and 24 h incubation with different combinations of IL-1β, IL-6, IL-10, and TNF-α remained similar to or under control levels (without cytokines). There was synergistic effect between some of these cytokines in down-regulating sPLA2-IIA mRNA accumulation. For example, IL-6 plus IL-1β (K) and TNF-α plus IL-1β (N). No effect on sPLA2-IIA mRNA or protein expression was observed when HASMC were incubated with TGF-β (0.1–10 ng/ml) or IL-4 (data not shown).
HASMC, HepG2 cells did not respond to IFN-γ. In addition, IL-6 was more potent stimulating sPLA2-IIA mRNA accumulation (Fig. 8) in HepG2 liver cells than in HASMC.

SPLA2-IIA Up-regulation and NF-κB and STAT Activity in Human Arterial Smooth Muscle Cells Induced by Cytokines—EMSA were performed to explore if the nuclear transcription factor NF-κB and the family of STAT transcription factors were involved in the regulation of sPLA2-IIA transcription. Nuclear extracts were prepared from HASMC incubated with or without cytokines incubated for 4 and 24 h as described above in the experiments shown in Fig. 7. Two different sequences, homologous to STAT and NF-κB-binding sites, were chosen from the sPLA2-IIA promoter. Nuclear extracts from HASMC stimulated with IL-1β and TNF-α for 4 or 24 h resulted as expected in one major complex with NF-κB-labeled oligonucleotide (Fig. 9). NF-κB activation was observed whether cells were incubated with IL-1β or TNF-α individually or in combination with other cytokines. For identification of the protein-DNA complex we also used an antibody against the p65 subunit of NF-κB (data not shown). Stimulation of HASMC with IFN-γ for 4 or 24 h resulted in increased binding of STAT proteins to the DNA oligo. STAT activation was observed in all incubations whether IFN-γ was added individually or in combination with other cytokines (Fig. 9). Unstimulated (controls) HASMC showed no NF-κB or STAT complex formation with labeled DNA oligonucleotides. Thus indicating the absence of activation of NF-κB or STAT by lipopolysaccharide contaminants and that the cytokines used were functionally active.

There are several known mammalian STAT proteins. In order to identify the STAT proteins involved in IFN-γ stimulation of sPLA2-IIA expression we used antibodies against STAT1, STAT2, STAT3, and STAT5. Fig. 10A shows that only the antibody against STAT3 protein blocked the binding of STAT3 to the labeled oligo. Non-immune rabbit IgG did not affect STAT-DNA complex formation. As phosphorylation of STAT3 at Tyr705 is essential for dimerization and DNA binding, phosphorylation at this site is a marker of STAT3 activity. Fig. 10B show the presence of active phosphorylated STAT 3 protein in an immunoblot analysis of cell extracts from HASMC after incubation with IFN-γ for 15 and 35 min. In control cells the STAT3 band is unphosphorylated. These results together indicate that in HASMC the up-regulation of sPLA2-IIA expression by IFN-γ involved activation of STAT3 nuclear transcription factor but not NF-κB activation.

**DISCUSSION**

We believe that this is the first study investigating the control of the sPLA2-IIA expression at mRNA and protein level by human arterial smooth muscle cells in culture. We found that the expression of sPLA2-IIA by HASMC is induced once cells are exposed to non-proliferating culture conditions that promote cell differentiation. Taking advantage of continual culture of HASMC in serum-free medium, we demonstrated that sPLA2-IIA mRNA, cell-associated and cell-secreted protein levels are found in non-proliferating, quiescent HASMC, expressing cyclin-dependent kinase inhibitor p27 (Fig. 3). This is a marker of cell differentiation found in vascular smooth
IFN-γ Induces Secretory Group IIA Phospholipase A2 Expression

Fig. 7. Effect of specific individual cytokines and combination of cytokines in mRNA levels (I) and secreted levels (II) of sPLA2-IIA by HASMC after 4 and 24 h. A, total RNA (0.1 μg) was analyzed by semi-quantitative RT-PCR for sPLA2-IIA and GAPDH. The units represent relative fluorescence of the amplified fragment for sPLA2-IIA against GAPDH measured as described under “Experimental Procedures.” Representative results from one study are shown, data are expressed as mean ± S.D. of three GenScan analyses. II, sPLA2-IIA amount secreted into the cell media and measured by ELISA technique. Data represent mean ± S.D of quadruplicate values. Letters in I and II correspond to: A, control (without cytokines); B, IFN-γ; C, IFN-γ + IL-6; D, IFN-γ + IL-1β; E, IFN-γ + TNF-α; F, IFN-γ + IL-10; G, IL-1β; H, IL-6; I, TNF-α; J, IL-10; K, IL-6 + IL-1β; L, IL-6 + IL-1β + IL-10; M, TNF-α + IL-1β; N, TNF-α + IL-6; O, TNF-α + IL-6 + IL-10; P, IFN-γ + IL-10 + TNF-α (concentration used are described under “Experimental Procedures”).

Fig. 8. Effect of IFN-γ and IL-6 on the expression of sPLA2-IIA by HepG2 liver tumor cells. HepG2 cells were incubated with IL-6, IFN-γ, and IL-6 plus IFN-γ in Eagle’s modified essential medium containing 10% FBS. After 24 h the cell media were collected and cell harvested for isolation of total RNA. RT-PCR analysis of total RNA (0.1 M) was analyzed by semi-quantitative RT-PCR for sPLA2-IIA and GAPDH. The units represent relative fluorescence of the amplified fragment for sPLA2-IIA against GAPDH measured as described under “Experimental Procedures.” Data are expressed as mean ± S.D. of three GenScan analyses.

muscle cells in vitro and in vivo (35, 36). This was supported by the presence of heavy caldesmon mRNA a specific marker for HASMC differentiation (37). No large differences in the total levels of p21 were found (Fig. 3), probably because its inhibitory action is achieved by changes in the ratio of p21 free to cyclin-Cdk bound complexes without affecting its total intracellular levels (38). The presence of serum, a source of growth factors, induces cell proliferation and de-differentiation suppressing both sPLA2-IIA mRNA and protein expression (Fig. 4). The results indicate that sPLA2-IIA expression by HASMC in culture requires conditions that lead to a phenotypic change from proliferating, de-differentiated cells, toward quiescent (non-proliferating) cells (30). Pulmonary artery smooth muscle cells showed similar behavior. These results agree with previous data reported by Anderson and co-workers (39) showing that the expression of sPLA2-IIA by confluent human coronary artery smooth muscle cells is not changed over a 10-day culture period in the presence of serum. Together these results support our previous immunohistochemistry work showing the presence of sPLA2-IIA associated with α-actin positive, spindle-shaped differentiated smooth muscle cells in intima and media of human arteries (17, 20). Inflammatory cytokines are reported to up-regulate the expression and secretion of sPLA2-IIA in different cell systems (23). Our previous electron microscopy study indicates that human atherosclerotic lesions contain more extracellular sPLA2-IIA than adjacent non-atherosclerotic regions in the same coronary artery (20). Although, the exact mechanism responsible for this increase in extracellular sPLA2-IIA is not established, one possibility is that proinflammatory cytokines stimulate sPLA2-IIA synthesis and secretion by arterial smooth muscle. IFN-γ, IL-1β, and TNF-α are proinflammatory cytokines present in atherosclerotic lesion (40). The results presented here showed that IFN-γ was the most potent of all cytokines studied in stimulating sPLA2-IIA mRNA and protein levels (Fig. 5). IFN-γ already after 4 h incubation induced a significant increase in mRNA and cell-associated and cell-secreted protein levels of sPLA2-IIA in HASMC, this effect was sustained for 48 h. The secreted sPLA2-IIA was catalytically active as shown in Fig. 6. Furthermore, treatment of HASMC with enzymes that degrade glycosaminoglycans increased the amount of active sPLA2-IIA released into the cell medium supporting our previous suggestions that pericellular glycosaminoglycans are an important compartment of active enzyme. The role of IFN-γ in stimulation of sPLA2-IIA expression was supported by EMSA and immunoblot analysis showing the presence of activated STAT3, a characteristic transcription factor of the IFN-γ intracellular signaling pathway in HASMC (41). IFN-γ is a potent inhibitor of HASMC proliferation (42). Our results indicate that expression of sPLA2-IIA in HASMC is stimulated under non-proliferating conditions. Therefore, one may speculate that up-regulation sPLA2-IIA expression by IFN-γ may also be a cellular
sPLA2-IIA promoter. An equal protein amount of NE was added to each binding reaction. The identified transcription factors NF-

response related to the anti-mitogenic property of IFN-γ.

Arterial smooth muscle cells in vitro and in vivo respond markedly to IFN-γ by expressing class II major histocompati-

bility antigens such as HLA-DR (42, 43). These genes are up-regulated in smooth muscle cells in human atherosclerotic plaques, probably induced by secretion of IFN-γ by a subset of T cells present in the arterial wall (44). In addition, extracellu-

lar sPLA2-IIA was reported to increase T-lymphocyte response (45). Taken together, these results suggest that IFN-γ signaling may promote atherogenesis by stimulating sPLA2-IIA expression and creating a positive feedback mechanism, sustaining chronic inflammation at places of lipid deposition in the arterial wall.

Several studies previously reported that IL-1β and TNF-α induce sPLA2-IIA expression and secretion over a long period in different animal cell systems, including rat vascular smooth muscle cells (46–48). Our results show that incubation of HASMC with IL-1β induced a moderate increase of sPLA2-IIA mRNA and sPLA2-IIA protein secretion that was stable during 24 h of incubation. However, TNF-α induced a strong and transit sPLA2-IIA mRNA accumulation and sPLA2-IIA secretion by HASMC after 4 h incubation, decreasing to the levels observed in nonstimulated cells (control) in the case of mRNA, and below those control levels in the case of the amount of secreted protein, after 24 h incubation (Fig. 7, I and II). TNF-α was the most potent of all cytokines studied in stimulating secretion of sPLA2-IIA with cytokines after 4 h incubation. Unexpectedly and interestingly, incubation of HASMC for 4 h with the combination of IL-1β and TNF-α, decreased the secre-

tion by HASMC after 4 h incubation, decreasing to the levels observed in nonstimulated cells (control) in the case of mRNA, and below those control levels in the case of the amount of secreted protein, after 24 h incubation (Fig. 7, I and II). TNF-α was the most potent of all cytokines studied in stimulating secretion of sPLA2-IIA with cytokines after 4 h incubation. Unexpectedly and interestingly, incubation of HASMC for 4 h with the combination of IL-1β and TNF-α, decreased the secretion of sPLA2-IIA bellow the control levels, indicating that these cytokines antagonized each other when present together. On the other hand, EMSA results showed the presence of active NF-κB binding using α-32P-labeled oligonucleotide probes bearing an NF-κB-binding site (left side) or a STAT-binding site from the human sPLA2-IIA promoter. An equal protein amount of NE was added to each binding reaction. The identified transcription factors NF-κB and STAT are indicated with an arrow. Unbound oligonucleotide is at the bottom of the lanes.

![FIG. 9. Effect of cytokines on nuclear translocation of NFκB and STAT in HASMC.](image)

Nuclear extracts (NE) were prepared from untreated cells (A) and from cells treated 24 h (NF-κB) or 4 h (STAT) with cytokines as indicated under "Experimental Procedures" and shown in Figs. 6, 7, and 8A. A, control without cytokines; B, IFN-γ; C, IFN-γ + IL-6; D, IFN-γ + IL-1β; E, IFN-γ + TNF-α; F, IFN-γ + IL-10; G, IL-1β; H, IL-6; I, TNF-α; J, IL-10; K, IL-6 + IL-1β; L, IL-6 + IL-1β + IL-10; M, TNF-α + IL-1β; N, TNF-α + IL-6; O, TNF-α + IL-6 + IL-10; P, IFN-γ + IL-10 + TNF-α (concentrations are indicated under "Experimental Procedures"). NE from HASMC were analyzed with electrophoretic mobility shift assay to evidence NF-κB binding using α-32P-labeled oligonucleotide probes bearing an NF-κB-binding site (left side) or a STAT-binding site from the human sPLA2-IIA promoter. An equal protein amount of NE was added to each binding reaction. The identified transcription factors NF-κB and STAT are indicated with an arrow. Unbound oligonucleotide is at the bottom of the lanes.

![FIG. 10. Identification of the STAT family members involved in the transcription of sPLA2-IIA.](image)

A, nuclear extracts (NE) from HASMC incubated 24 h with IFN-γ (200 units/ml) were analyzed for binding to the α-32P-labeled oligonucleotide probe bearing a STAT-binding site from the human sPLA2-IIA promoter in the presence or absence of specific antibodies against STAT 1, 3, 5, and 6 protein. Lane 1, α-32P-labeled oligonucleotide probe without NE; lane 2, α-32P-labeled oligonucleotide probe without NE and with specific antibody against STAT 1, 3, 5, or 6; lane 3, NE plus α-32P-labeled oligonucleotide; lane 4, NE plus α-32P-labeled oligonucleotide probe with specific antibody against STAT 1, 3, 5, or 6 transcription factor. B, Western blot analysis of STAT-3 phosphorylation. Cell extracts from HASMC incubated with or without IFN-γ were run in a polyacrylamide gel electrophoresis 10% system and then immunoblot for detection of STAT3-phosphorylated proteins as described under “Experimental Procedures.” a, HASMC culture in growing medium; b and d, HASMC incubated 15 min with IFN-γ (200 units/ml) in serum-free medium; c and e, HASMC incubated 35 min with IFN-γ (200 units/ml) in serum-free medium; f and g, HASMC incubated without IFN-γ for 15 and 35 min, respectively; molecular weight standard (MW St).
cells, produce IL-6, usually as a response to IL-1β, TNF-α, or lipopolysaccharide stimulation. In addition, IL-6 is required for the induction of acute phase reaction proteins such as C-reactive protein (50). SPLA2-IIA is classified as an acute phase protein and therefore IL-6 may also be responsible for its synthesis by the liver (51). However, whether IL-6 has a pro- or anti-inflammatory function in local or systemic responses remains to be established (50, 52). Previously, IL-6 has been shown to stimulate gene expression and secretion of sPLA2-IIA in HepG2 liver hepatoma cells (51). In the present work we compared the effect of IL-6 in the expression and secretion of sPLA2-IIA by HepG2 cells and HASMC. Our results showed that incubation of HASMC for 4 h with IL-6 induced a significant increase in secretion of sPLA2-IIA accompanied by an increase in mRNA accumulation after 4 h incubation (Fig. 7). This stimulation was transient and it was also lower than the secretion induced by TNF-α. In combination with other cytokines, IL-6 antagonized sPLA2-IIA secretion induced by IFN-γ, IL-β, and TNF-α in HASMC. Interestingly, experiments with HepG2 cells (Fig. 8) indicated that these cells responded to IL-6 but not to IFN-γ by increasing both sPLA2-IIA mRNA accumulation and sPLA2-IIA secretion after 24 h of incubation. These results suggest that sPLA2-IIA induction by IFN-γ and IL-6 cytokines are cell specific. In addition, these experiments suggest that the pro- or anti-inflammatory properties of IL-6 may be modulated by the presence of other proinflammatory cytokines. Our results support previous data by Crow and coworkers (51) suggesting that the main source of sPLA2-IIA in plasma may be hepatic cells stimulated by IL-6. However, IL-6 or IFN-γ stimulated vascular smooth muscle cells may also contribute to increase plasma sPLA2-IIA concentration by transit leakage from the vascular wall that may take place during acute inflammatory conditions. Circulating levels of IL-6, C-reactive protein, and sPLA2-IIA are increased in patients with cardiovascular disease and are associated with poor prognostic outcome (13, 53). However, the mechanism for this association is unknown. Increased levels of circulating IL-6 is reported to exacerbate early atherosclerosis (54). Furthermore, low density lipoprotein modification by PLA2 activity in total plasma induces atherogenic small, dense low density lipoprotein particles with high affinity for arterial proteoglycans (55). Taken together, these results suggest that IL-6 may contribute to atherosclerosis by stimulating hepatic cells and arterial smooth muscle cells to secrete sPLA2-IIA and as a consequence increase the extracellular activity of sPLA2-IIA in plasma and arterial wall.

There is ample support for the participation of proinflammatory cytokines in atherosclerosis. However, the potential contribution of anti-inflammatory cytokines in the modulation of atherosclerosis is less documented. IL-10 is an anti-inflammatory cytokine, expressed in atherosclerotic lesions and is reported to be an anti-atherosclerotic cytokine (56). Our results showed that IL-10 by itself did not affect the expression or secretion of sPLA2-IIA. However, IL-10 was able to block IFN-γ-induced sPLA2-IIA expression and secretion (Fig. 7). IL-10 markedly inhibited IFN induced sPLA2-IIA transcription despite a strong STAT3 activation observed with EMSA (Fig. 9). Inhibitor effect of IL-10 on IFN-γ and TNF-α induced gene expression are reported previously in the literature (57). Our results showed that the anti-inflammatory or protective function of IL-10 in atherosclerosis may be partially due to its capacity to decrease sPLA2-IIA expression in the presence of proinflammatory cytokines.

Our results showed that sPLA2-IIA binds to cell surface proteoglycans (Fig. 6). It has been proposed that this binding is important in the hydrolysis of cell phospholipids for the generation of arachidonic acid (58). While this hypothesis has been challenged (59), results from our group indicate that sPLA2-IIA activity can be modulated by its interaction with different types of glycosaminoglycans (60). Cytokines are reported to differentially influence synthesis and composition of proteoglycans in different cell systems (38). Therefore, one may expect that the levels of sPLA2-IIA secretion after cytokine stimulation may be affected by the direct effect on sPLA2-IIA secretion and changes in cell membrane proteoglycan composition influencing binding of sPLA2-IIA.

In summary, the results presented here showed that differentiated HASMC constitutively express sPLA2-IIA. SPLA2-IIA is mainly cell associated in unstimulated cells. Exposure to proinflammatory cytokines can stimulate sPLA2-IIA transcription and secretion of intracellular storage or de novo synthesized sPLA2-IIA in a transient or sustained form. This may have physiological relevance for the triggering of an acute or chronic inflammatory response in the arterial wall. Interestingly, a recent report shows the simultaneous presence of mRNA transcripts for sPLA2-IIA, IFN-γ, IL-1β, and TNF-α in human atherosclerotic lesion (25). However, further work is required to elucidate the molecular regulatory mechanisms controlling transcription, translation, and secretion of sPLA2-IIA by HASMC. These results suggest that changes in the balance of different pro- and anti-inflammatory cytokines present in the arterial wall may regulate the levels of extracellular sPLA2-IIA and its potential contribution to atherogenesis.

Acknowledgment—We are grateful to Prof. Germán Camejo for critically reading the manuscript.

REFERENCES

1. Dennis, E. A. (1997) Trends Biochem. Sci. 22, 1–2
2. Durante, W., Liao, L., Peyman, K. J., and Schader, A. I. (1997) J. Biol. Chem. 272, 30154–30159
3. Wong, J. T., Tran, K., Pierce, G. N., Chan, A. C., Karmin, O. K., and Choy, P. C. (1998) J. Biol. Chem. 273, 6830–6836
4. Wu, R., Huang, Y. H., Elinder, L. S., and Frostegard, J. (1998) Arterioscler. Thromb. Vasc. Biol. 18, 626–630
5. Subhanagounder, G., Leitinger, N., Shih, P. T., Faull, K. F., and Berliner, J. A. (1999) Circ. Res. 85, 311–318
6. Siess, W., Zhang, K. J., and Essler, M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6931–6936
7. Eckery, R., Menshchikowski, M., Lattke, P., and Jarous, W. (1997) Atherosclerosis 132, 163–176
8. Weintrach, Y., Abod, C., Liang, N.-S., Lowry, S. F., and Weiss, J. (1998) J. Clin. Invest. 102, 633–638
9. Cormier, R. T., Hong, K. C., Hallberg, R. B., Hawkins, T. L., Richardson, P., Mullerkar, R., Dove, W. F., and Lander, E. S. (1997) Nat. Genet. 17, 88–91
10. Thoosen, L., Sjursen, W., Gavsk, K., Hansen, W., Brekke, O.-L., Skattebol, L., Holmeide, A. K., Espetvik, T., Johansen, B., and Lasagre, A. (1998) J. Immunol. 161, 3421–3427
11. Vadás, P., Pruzanski, W., Kim, J., and Fornasi, V. (1989)Am. J. Pathol. 134, 807–811
12. Nevalainen, T. J., and Gronroos, J. M. (1997) in Phospholipase A2: Basic and Clinical Aspects in Inflammatory Diseases (Uhl, W., Nevalainen, T. J., and Buchler, M. W., eds) Vol. 24, pp. 104–109, KARGER, Basel
13. Kugiyama, K., Ota, Y., Takage, K., Matiyama, Y., Kawano, H., Miyao, Y., Sakamoto, T., Szejima, H., Ogawa, H., Doe, H., Sugiyama, S., and Yasue, H. (1999) Circulation 100, 1280–1284
14. Ross, R. (1999) N. Engl. J. Med. 340, 115–126
15. Wallberg-Jansen, M., Ohman, M.-L., and Dahlbrot, S. R. (1997) J. Rheuma- tol. 24, 445–451
16. Ivanidc, B., Castellini, L. W., Wang, X.-P., Qiao, J.-H., Mehrabian, M., Navah, M., Fogelman, A. M., Gross, D., Swanson, M. E., de Beer, M. C., de Beer, F., and Lusis, A. J. (1999) Arterioscler. Thromb. Vasc. Biol. 19, 1284–1290
17. Hurt-Camejo, E., Anderssen, S., Standal, R., Rosengren, B., Sartipi, P., Stadberg, E., and Johansen, B. (1997) Arterioscler. Thromb. Vasc. Biol. 17, 300–309
18. Elinder, L. S., Dumretrescu, A., Larsson, P., Hedun, I., Fostergard, J., and Claesson, H.-E. (1997) Arterioscler. Thromb. Vasc. Biol. 17, 2257–2263
19. Schiering, A., Menschikowski, M., Mueller, E., and Jarous, W. (1999) Atherosclerosis 144, 73–78
20. Romano, M., Romano, E., Bjerkerud, S., and Hurt-Camejo, E. (1998) Arterioscler. Thromb. Vasc. Biol. 18, 519–525
21. Hurt-Camejo, E., and Camejo, G. (1997) Atherosclerosis 132, 1–8
22. Libby, P., and Gallis, Z. S. (1995) Ann. N. Y. Acad. Sci. 748, 158–170
23. Petersilger, J., Walker, G., Kunz, D., Pignat, W., and Bosch, H. v. d. (1997) in Phospholipase A2 Basic and Clinical Aspects in Inflammatory Diseases (Uhl, W., Nevalainen, T. J., and Buchler, M. W., eds) Vol. 24, pp. 31–37, Karger, Basel
24. Hansson, G. (1997) J. Clin. Invest. 8, 301–311
25. Menschikowski, M., Rosner-Schiering, A., Ecken, R., Mueller, E., Koch, R., and Jarosz, W. (2000) *Atherosclerosis. Thromb. Vasc. Biol.* **20**, 751–762

26. Shanahan, C. M., and Weissberg, P. L. (1999) *Curr. Opin. Lipid* **10**, 507–513

27. Hurt-Camejo, E., Rosengren, B., Camejo, G., Sartipy, P., Fager, G., and Bondjers, G. (1995) *Atherosclerosis. Thromb. Vasc. Biol.* **15**, 1456–1465

28. Sartipy, P., Johansen, B., Rosengren, B., Bondjers, G., and Hurt-Camejo, E. (1996) *J. Biol. Chem.* **271**, 26307–26314

29. Kramer, R. M., Hession, C., Johansen, B., Hayes, G., McGray, P., Chow, E. P., Tizard, R., and Pepinsky, R. B. (1989) *J. Biol. Chem.* **264**, 5768–5775

30. Duplaà, C., Couffinhal, T., Dufourcq, P., Llanas, B., Moreau, C., and Bennet, J. (1997) *Circ. Res.* **80**, 159–169

31. Humphrey, M. B., Herrera-Sosa, H., Gonzalez, G., Lee, R., and Bryan, J. (1992) *Gene (Amst.)* **112**, 197–204

32. Crowther, J. R. (1995) *Methods Mol. Biol.* **42**, pp. 161–175, Human Press, Totowa

33. Wu, T., Levine, S. J., Lawrence, M. G., Logun, C., Angus, C. W., and Shellhammer, J. H. (1994) *J. Clin. Invest.* **93**, 571–577

34. Ohlsson, B. G., Englund, M. C. O., Karlsson, A.-L. K., Knutsen, E., Erixon, C., Skribeck, H., Liu, Y., Bondjers, G., and Wiklund, O. (1996) *J. Clin. Invest.* **98**, 78–89

35. Tanner, F. C., Yang, Z.-Y., Duckers, E., Gordon, D., Nabel, G. J., and Nabel, E. G. (1998) *Circ. Res.* **82**, 396–403

36. Sherr, C. J., and Roberts, J. M. (1995) *Genes Dev.* **9**, 1149–1163

37. Glukhova, M. A., Frid, M. G., and Koteliansky, V. E. (1990) *J. Biol. Chem.* **265**, 13042–13046

38. Wight, T. N. (1996) in *Atherosclerosis and Coronary Artery Disease* (Fuster, V., Ross, R., and Topol, E. J., eds) Vol. 1, pp. 421–440, Lippincott-Raven, Philadelphia, PA

39. Anderson, K. M., Roshak, A., Winkler, J. D., McCord, M., and Marshall, L. A. (1990) *J. Biol. Chem.* **265**, 30504–30511

40. Sukhova, G. K., Schinbeek, U., Rabkin, E., Schoen, F. J., Poole, A. R., Billington, R. C., and Libby, P. (1999) *Circulation* **99**, 2503–2509

41. Caldenhoven, E., Buitenhuys, M., van, T. B. D., Raaijmakers, J. A., Lammers, J. W., Koenderman, L., and Groot, R. P. d. (1999) *J. Leukoc. Biol.* **65**, 391–396

42. Hurt-Camejo, E., Rosengren, B., Sartipy, P., Elsfberg, R., Camejo, G., and Svensson, L. (1999) *J. Biol. Chem.* **274**, 18967–18964

43. Tellides, G., Tereb, D. A., Kirkles-Smith, N. C., Kim, R. W., Wilson, J. H., Schechner, J. S., Lorber, M. I., and Pober, J. S. (2000) *Nature* **403**, 207–211

44. Zhou, X., Paulsson, G., Stemme, S., and Hansson, G. K. (1998) *J. Clin. Invest.* **101**, 1717–1725

45. Asaoka, Y., Yoshida, K., Sasaki, Y., Nishizuka, Y., Murakami, M., Kudo, I., and Inoue, K. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 716–719

46. Pfeilschifter, J., Schulzkij, C., Briner, V. A., and Bosch, H. V. d. (1993) *J. Clin. Invest.* **92**, 2516–2523

47. Nakano, T., Ohara, O., Teraoka, H., and Arita, H. (1990) *FEBS Lett.* **251**, 171–174

48. Couturier, C., Brouillet, A., Couiraud, C., Koumanov, K., Béréziat, G., and Andreani, M. (1999) *J. Biol. Chem.* **274**, 23085–23093

49. Kishimoto, T., Akira, S., and Taga, T. (1992) *Science* **258**, 593–597

50. Xing, Z., Gauldie, J., Cox, G., Baumann, H., Jordana, M., Lei, X.-F., and Rezau, G. K. (1998) *J. Biol. Chem.* **273**, 18957–18964

51. Crowl, R. M., Stoller, T. J., Conroy, R. R., and Stoner, C. R. (1991) *J. Biol. Chem.* **266**, 2647–2651

52. Boe, A., Baiocchi, M., Carbonatto, M., Papiain, R., and Serluosi-Crescenzi, O. (1990) *Cytokine* **11**, 1057–1064

53. Biasucci, L., Vitelli, A., Liuzzi, G., Altamura, G., Monaco, C., Rebuffi, A., and Caliberto, G., and Maseri, A. (1996) *Circulation* **94**, 874–877

54. Huber, S. A., Sakkinen, P., Conde, D., Hardin, N., and Tracy, R. (1999) *Atherosclerosis. Thromb. Vasc. Biol.* **19**, 2364–2367

55. Sartipy, P., Camejo, G., Svensson, L., and Hurt-Camejo, E. (1999) *J. Biol. Chem.* **274**, 25913–25920

56. Mallat, Z., Besnard, S., Duriez, M., Deleuze, V., Emmanuel, F., Bureau, M. F., Soubrier, F., Espoto, B., Dueau, H., Fiervet, C., Staels, B., Duverger, N., Scherman, D., and Tedgui, A. (1999) *Circ. Res.* **85**, 1–8

57. Schoehelius, A. J. G., Mayo, M. W., Sartor, R., and Baldwin, A. S., Jr. (1999) *Circ. Res.* **85**, 25813–25820

58. Murakami, M., Kambe, T., Shimbra, S., Yamamoto, S., Kudo, I., and Kudo, K. (1999) *J. Biol. Chem.* **274**, 29927–29936

59. Bezzine, S., Koduri, R. S., Valentin, E., Murakami, M., Kudo, I., Ghomashchi, F., Sadilek, M., Lambiot, G., and Gelb, M. H. (2000) *J. Biol. Chem.* **275**, 3179–3191

60. Sartipy, P., Bondjers, G., and Hurt-Camejo, E. (1998) *Atherosclerosis. Thromb. Vasc. Biol.* **18**, 1934–1941