

Atherogenic properties of LDL particles modified by human group X secreted phospholipase A2 on human endothelial cell function

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ABSTRACT Increasing evidence suggests that secreted phospholipases A2 (sPLA2s) play an important role in the pathophysiology of atherosclerosis. Among sPLA2s, the human group X (hGX) enzyme has the highest catalytic activity toward phosphatidylcholine, one of the major phospholipid species of cell membranes and low-density lipoprotein (LDL). Our study examined the presence of hGX sPLA2 in human atherosclerotic lesions and investigated the ability of hGX modified LDL to alter human endothelial cell (HUVEC) function. Our results show that hGX sPLA2 is present in human atherosclerotic lesions and that the hydrolysis of LDL by hGX sPLA2 results in a modified particle that induces lipid accumulation in human monocyte-derived macrophages. Acting on endothelial cells, hGX-modified LDL activates the MAP kinase pathway, which leads to increased arachidonic acid release, increased expression of adhesion molecules on the surface of HUVEC, and increased adhesion of monocytes to HUVEC monolayers. Together, our data suggest that LDL modified by hGX, rather than hGX itself may have strong proinflammatory and proatherogenic properties, which could play an important role in the propagation of atherosclerosis.—Karabina, S. A., Brochériou, I., Le Naour, G., Agrapart, M., Durand, H., Gelb, M., Lambeau, G., Ninio, E. Atherogenic properties of LDL particles modified by human group X secreted phospholipase A2 on human endothelial cell function. *FASEB J.* 20, E000–E000 (2006)

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ATHEROSCLEROSIS IS CHARACTERIZED by lipid accumulation and foam cell formation caused by the uptake of modified forms of low-density lipoprotein (LDL; ref 1). Modification of LDL occurs as a consequence of either oxidation or the action of enzymes such as secreted phospholipases A2 (sPLA2; refs 2–5).

sPLA2s represent a diverse family of structurally

related calcium-dependent enzymes that hydrolyze the sn-2 position of glycerophospholipids present in cell membranes and plasma lipoproteins (6–8). The hydrolysis products of these enzymes are lysophospholipids and free fatty acids, which are important second messengers in cell signaling and signal transduction. When the fatty acid released is arachidonic acid (AA), it can be converted through the cyclooxygenase and lipoxygenase (LOX) pathways into prostaglandins, thromboxanes, and leukotrienes, which are well-known pleiotropic secondary mediators that target the vessel wall (9). Extracellular levels of sPLA2s are increased in both plasma and inflammatory fluids in various inflammatory diseases (10, 11), indicating that sPLA2s are released systemically as well as at sites of tissue inflammation. As the accompanied production of biologically active lipid mediators is related to inflammatory diseases, knowledge of how sPLA2 expression is regulated is an important step toward understanding the pathogenesis of atherosclerosis where inflammation plays an important role.

Among the functional sPLA2s that have been identified in humans, three sPLA2 subtypes [human group IIA, V, and X (hGIIA, hGV, and hGX)] have been proposed to play a role in atherosclerosis (4, 12, 13). hGIIA sPLA2 may exert proatherogenic effects in the arterial wall mainly by increasing the binding affinity of apolipoprotein B containing lipoproteins to proteoglycans, making them susceptible to further modifications, such as oxidation. GV sPLA2 has been detected in human and mouse atherosclerotic lesions, and hydrolysis of LDL by mouse GV PLA2 induced particle aggregation and lipid accumulation in mouse peritoneal macrophages *in vitro* (12). A number of studies have demonstrated that the lipolytic effect of sPLA2

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results in physicochemical changes in LDL particles (14–17).

hGX sPLA2 has been cloned by Cupillard *et al.* (18), and its expression in thymus, spleen, and leukocytes suggests involvement in the immune system and/or in inflammation. Among the different sPLA2s, hGX sPLA2 binds with the highest affinity to phosphatidylcholine (PC)-rich membranes *in vitro* (19, 20) and nanomolar concentrations of exogenously added hGX sPLA2 are sufficient to release AA that can lead to cyclooxygenase-2 (COX-2)-dependent prostaglandin E2 generation in several types of mammalian cells (21–23). Comparative studies on LDL hydrolysis by GIB, GIIA, GV, and GX sPLA2s showed that hGX sPLA2 induces the most potent release of linoleic and AA, which is accompanied by a large production of lysophosphatidylcholine (LPC; ref 24). Since the “oxidation hypothesis of atherosclerosis” (25, 26) still remains inconclusive, as clinical trials with antioxidants in humans have not been successful (27), it becomes apparent that in addition to oxidation, alternative mechanisms, such as the enzymatic modification of LDL by sPLA2, may contribute to the onset and propagation of atherosclerosis.

The present study was therefore undertaken to examine whether hGX sPLA2 is present in human atherosclerotic lesions and whether LDL particles that have been lipolytically modified by hGX can have proinflammatory effects on endothelial cells. As AA is a key player in inflammation and the expression of adhesion molecules is crucial for monocyte transmigration in the subendothelial space during atherogenesis, we examined the AA releasing capacity and the expression of adhesion molecules in primary human umbilical vein endothelial cells (HUVEC) after treatment with hGX sPLA2 modified LDL (hGX-LDL).

Our results demonstrate that 1) hGX sPLA2 is expressed in the intima of human atherosclerotic plaques and colocalizes with foam cells and smooth muscle cells resembling myofibroblasts. 2) Treatment of LDL with hGX sPLA2 results in a modified particle that induces foam cell formation in human monocyte-derived macrophages *in vitro*. 3) hGX-LDL activates the MAPK kinase pathway, inducing AA release and increased expression of adhesion molecules on the surface of HUVEC, which in turn results in an increased adhesion of THP-1 cells to HUVEC monolayers. Our data suggest a strong atherogenic property of hGX sPLA2 in modifying LDL into a proinflammatory and proatherogenic particle that may contribute to the initiation and propagation of atherosclerosis.

MATERIALS AND METHODS

Recombinant hGIIA, recombinant hGX sPLA2, and the active site mutant H48Q were prepared as described previously (21). [³H]AA (218 Ci/mmol) was from Perkin Elmer (Boston, MA, USA); antibodies against human intracellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM)-1 were from R&D (Minneapolis, MN, USA) and

Santa Cruz Biotechnology (Santa Cruz, CA, USA) respectively and the MAPK antibodies from Cell Signaling (Beverly, MA, USA). Cell tracker green CMFDA was from Invitrogen (Paris, France), PD 98059 and SB 203580 were from Calbiochem (San Diego, CA, USA). Rabbit polyclonal anti-hGX were produced as described previously (28). The synthesis of the hGX-sPLA2 inhibitor LY 329722 [3-(3-aminooxalyl-1-benzyl-2-ethyl-6-methyl-1*H*-indol-4-yl)-propionic acid] was carried as reported (29). The synthesis of IV PLA2 inhibitor Wyeth-1 (4-(3-(1-benzhydryl-5-chloro-2-[2-(3,4-dichlorophenylmethanesulfonylamino)-ethyl]-1*H*-indol-3-yl)-propyl)-benzoic acid) was carried as published by Kew, John C., Tam, Steven Y., Lee, Katherine L., Chen, Lihren, Thakker Pares, Sum, Fuk-Wah, Behnke, Mark, Hu, Baihua, and Clark, James D., (2004) *US Patent 6,797,708*.

Double immunofluorescence staining in tissue sections

Full-thickness resections of common carotid arteries and aortas were collected from patients ($n=7$) undergoing surgery in the Department of Vascular Surgery at the Pitié-Salpêtrière Hospital (Paris, France). The sections were characterized as intact vessels composed of well-defined intima, media, and adventitia layers. Atherosclerotic plaques were localized in both intima and media and were composed of a necrotic core adjacent to cholesterol cleft, covered by a fibrous cap. Selected specimens showed neither plaque rupture nor thrombus formation. The plaques were fibroatherosclerotic and did not contain bifurcations. After resection, the specimens were fixed in 10% buffered neutral formalin, processed to paraffin blocks, and stored at room temperature. Serial sections of 3 μ m fixed on slides were deparaffinized, rehydrated, and then treated in a microwave oven (2 \times 5 min at 900 W in citrate buffer). For double staining, the slides were incubated for 30 min at room temperature with anti-hGX rabbit polyclonal antibody (pAb) at 1:700 dilution [the antibody (Ab) has previously been shown to be specific for the hGX sPLA2 antigen with no cross-reactivity with other sPLA2s; ref 28] and with one of the monoclonal mouse anti-human antibodies directed either to CD68 at 1:500 dilution (KP-1 clone, Dako, Copenhagen, Denmark) or to α -actin smooth muscle 1:1000 dilution (1A4 clone, Sigma, St. Louis, MO) or to anti-CD3 at 1:100 dilution (F7.2.38 clone, Dako). Subsequently, the sections were incubated for 35 min with an Alexa 594 conjugated goat anti-rabbit IgG at a 1:200 dilution in 15% goat serum/PBS and with an Alexa 488-conjugated goat anti-mouse IgG at a 1:200 dilution for the detection of CD68, CD3, or α -actin. Negative controls were performed by replacing primary antibodies by mouse IgG1. To verify the specificity of the signal, sections were also incubated with the primary Ab in the presence of an excess of hGX sPLA2 antigen (1 μ M recombinant hGX). After three washes with PBS, samples were cover slipped with immuno-mount (Shandon, CML, Nemours, France). Sections were analyzed using a Leica TCS, SP2 confocal laser scanning microscope (Leica Microsystems, Bucks, UK).

LDL isolation and treatment with hGX sPLA2

LDL was isolated from frozen plasma by density gradient ultracentrifugation, as described previously (30) and was free of HDL as detected by agarose gel electrophoresis and laser nephelometry for apoA1 (<0.05g/l). Its protein content was determined by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL). Freshly prepared filter (0.45 μ m) sterile LDL, 1 mg/ml of protein in buffer containing 1 mM CaCl₂, 12.5 mM Tris-HCl (pH 8.0), 0.25 M NaCl, and 0.0125% BSA was incubated with 100 nM hGX sPLA2 for 3 h, unless otherwise

specified and denoted as hGX-LDL. The reaction was stopped with 5 mM EDTA that was tested to be sufficient to block the activity of hGX sPLA2. LDL treated as above but devoid of hGX sPLA2, is denoted as LDL and served as control. In some experiments, LDL treated with hGX sPLA2 was reisolated by an additional ultracentrifugation step and extensively dialyzed against PBS. Treatment of LDL with hGX sPLA2 or mock treatment as well as the subsequent reisolation did not result in oxidative modification of the particle as determined by the lack of production of conjugated dienes at 234 nm, a typical index of oxidation (31). Aggregated LDL was prepared by being vortexed 1 mg/ml LDL for 1 min.

Electron microscopy

To determine the particle size of LDL after treatment with hGX sPLA2, LDL or hGX-LDL particles were deposited onto 200 mesh Formvar carbone-coated copper grids and stained with acid phosphotungstate (negative staining). The images were photographed with a Philips CM100 electron microscope (LEP) at $\times 100,000$ magnification. Morphometric analysis of images was made with Leica Qwin software (Leica Microsystems), which allowed a precise examination of the size distribution of the LDL particles.

Oil Red O staining

Human monocyte-derived macrophages prepared as described previously (32) were cultured for 24 h in the presence of 200 $\mu\text{g}/\text{ml}$ of either LDL or hGX-LDL or aggregated LDL. After the incubation period, the cells were fixed in 10% formalin for 10 min and washed with 60% isopropanol. Cells were stained with Oil Red O for 30 min and washed with 60% isopropanol. Hematoxylin was used for nuclear staining. Cells were visualized with a Nikon ECLIPSE.E 800 equipped with a Nikon digital camera DXM 1200 at $\times 40$ magnification and were analyzed with Lucia V 4.60 software.

HUVEC isolation and cell culture

HUVEC were isolated by the method of Jaffe *et al.* (33). Cells were maintained at 37°C and 5% CO₂ in a humidified incubator and were grown in MCDB 131 medium (Sigma) supplemented with 20% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin-streptomycin, 10 ng/ml human epidermal growth factor (EGF), and 1 $\mu\text{g}/\text{ml}$ hydrocortisone. Cells were used at passages 2 or 3.

[³H]AA release from HUVEC

Eighty percent confluent HUVEC cells seeded in 24-well plates were labeled with 0.1 μCi of [³H]AA in 1 ml of complete medium and cultured for 18–20 h. The cells were then washed three times with PBS-0.05% BSA. When specified, cells were preincubated in serum-free medium for 30 min with 10 μM of either the MAPK inhibitors (PD 98059 or SB 203580) or 10 μM of the cytosolic PLA2 inhibitor, Wyeth-1. The cells were then treated with 100 $\mu\text{g}/\text{ml}$ LDL or hGX-LDL, or 100 nM hGX sPLA2 for 1 h, except otherwise specified. After incubation, the supernatant was collected and centrifuged at 1,200 *g* for 10 min to pellet any detached cells. The cells were washed with PBS –0.05% BSA, and the pellets were dissolved in 1N NaOH. Supernatants and cell lysates were counted, and the percentage of [³H]AA released into cell supernatant was calculated as [(dpm in supernatant) $\times 100$ / (total incorporated dpm)].

Reverse transcription and quantitative-polymerase chain reaction

Confluent HUVEC cells were changed to serum-free medium for 6 h before treatments for 3 or 6 h with 100 $\mu\text{g}/\text{ml}$ LDL or hGX-LDL, or 100 nM hGX sPLA2. RNA was isolated using RNA easy kit (Qiagen, Valencia, CA). Five micrograms of total RNA were reverse transcribed to cDNA using Superscript II RNase H reverse transcriptase and random primers (Invitrogen, Carlsbad, CA). In all assays, 20 ng of cDNA from each sample was amplified using a standardized program (3 min JumpStart™ *Taq* Polymerase activation step; 40 cycles of 30 s at 95°C and 1 min at 60°C; a 1 min step at 95°C and dissociation steps: 36 cycles of 30 s between 60°C –95°C) in a MX 4000 real-time polymerase chain reaction system (Stratagene, La Jolla, CA) with a SYBR Green detection kit (Sigma). mRNA expression data were normalized to the housekeeping gene YWAZ (NM_003406; ref 34). The relative level of expression between sample 1 (control) and sample 2 (assay of interest) was calculated using the formula: $2^{(Ct1 - CtYWAZ1) - (Ct2 - CtYWAZ2)}$.

SDS/PAGE and Western blot analysis for ICAM, VCAM, and MAPK kinases

After starvation of confluent HUVEC cells for 6 h in serum-free medium, cells were treated for 3 or 6 h with 100 $\mu\text{g}/\text{ml}$ LDL, or hGX-LDL, or 100 nM hGX sPLA2. Cells were lysed in SDS sample buffer (62.5 mM Tris-HCl pH, 6.8, 2% SDS 10% glycerol, 50 mM DTT and 0.01% bromphenol blue). Equal amounts of cell lysate protein were analyzed by 10% SDS/PAGE gels.

Static adhesion assays

For the adhesion assays under static conditions, HUVEC were seeded in 24-well plates and allowed to reach confluence in complete medium. Cells were subsequently cultured in serum-free media for 3 h in the presence of various concentrations (50–200 $\mu\text{g}/\text{ml}$) of LDL or hGX-LDL, or 100 nM hGX PLA2. THP-1 monocytes were labeled with the fluorescent dye, CMFDA. HUVEC monolayers were rinsed twice with warmed medium and 5×10^6 fluorescent THP-1 cells were added to each well and allowed to adhere for 60 min. After incubation, the cells were washed three times with PBS. A 200 μl aliquot of fresh PBS was added to each well, and the fluorescence was measured in a Packard Fusion spectrofluorometer at excitation and emission wavelengths of 492 nm and 517 nm, respectively.

Cytotoxicity assay

Cytotoxicity in the presence of the different LDL treatments, hGX sPLA2 inhibitors, or MAPK inhibitors was performed using the MTT kit (Sigma).

Statistical analysis

Data are mean \pm SE of duplicate or triplicate determinations of 2 to 10 separate experiments. Differences between means were determined by Student's *t* test. A *P* < 0.05 was considered as significant.

RESULTS

Expression of hGX sPLA2 in human atherosclerotic lesions

The expression of hGX sPLA2 in atherosclerotic lesions was analyzed by double immunofluorescence staining of human carotid artery and aorta specimens with a specific Ab against hGX sPLA2 (28) and antibodies against markers specific for either macrophages (CD68), T-lymphocytes (CD3), or smooth muscle cells (α -actin). By confocal microscopy analysis, hGX sPLA2 was detected in all specimens tested ($n=7$), in the intima where it colocalized with the majority of foam cells (Fig. 1A), and in phenotypically dedifferentiated smooth muscle cells (SMC) resembling myofibroblasts (35; Fig. 1B). hGX sPLA2 was detected neither in T-lymphocytes nor in the lesion-free areas (data not shown). Interestingly, although hGX sPLA2 was detected in the extracellular matrix (ECM) of media (Fig. 1C), we did not observe colocalization with SMC, raising the question of the origin of the enzyme in this area. The signal was specific for hGX sPLA2 because preincubation of the primary Ab with 1 μ M recombinant hGX abolished the fluorescence (Fig. 1D).

Physicochemical properties of hGX-LDL

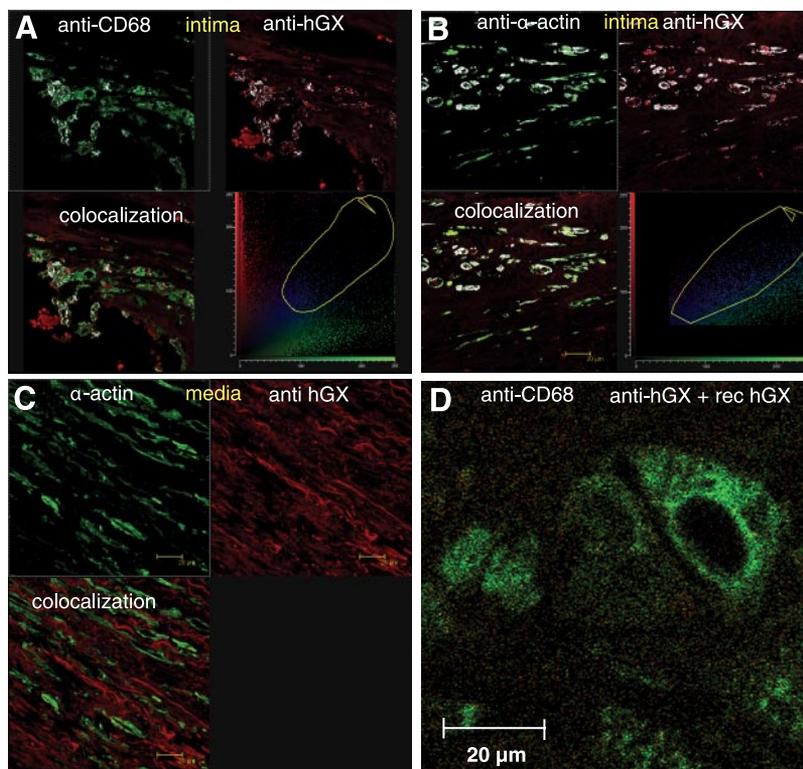
Among the different sPLA2s, hGX has the highest catalytic activity toward PC vesicles (20) and can efficiently hydrolyze PC major phospholipid at the surface of LDL particles (13, 24, 36, 37). In accordance with previous work (13), hGX-modified LDL showed in-

creased electrophoretic mobility on agarose gel [relative electrophoretic mobility (REM) = 2.9 ± 0.1 , $P < 0.05$] as compared to native LDL. The REM value of hGX-LDL was higher than that measured for Cu^{2+} -oxidized LDL (REM = 2.1 ± 0.2 , $P < 0.05$). In accordance with the fact that hGX sPLA2 can hydrolyze PC much more efficiently than hGIIA (20, 24), the electrophoretic mobility of LDL did not change when LDL was treated for 3 h with 100 nM hGIIA sPLA2 (REM = 1.1 ± 0.1). Electron microscopy showed that treatment of LDL (3 independent preparations) with hGX sPLA2 generates a particle of significantly smaller diameter (19.35 ± 4.35 nm, $n=3$) as compared to untreated particles (21.87 ± 4.65 nm, $n=3$).

hGX-LDL induces human macrophage foam cell formation

Previous work has shown that mouse macrophage foam cell formation occurs following treatment of LDL with hGX sPLA2 (13). To verify the ability of hGX-LDL to also induce foam cell formation in human macrophages, human monocyte-derived macrophages were cultured in the presence of LDL, hGX-LDL, or aggregated LDL. In contrast to untreated (Fig. 2A) or LDL-treated cells (Fig. 2B), numerous intracellular lipid droplets were detected under light microscopy in macrophages incubated with hGX-LDL, as indicated by Oil Red O staining (Fig. 2D). A similar, yet weaker, staining was observed with aggregated LDL (Fig. 2C), which was used as a positive control (38). This result clearly indicates that hGX-LDL is a modified particle that can induce macrophage-derived foam cell formation, one of the fundamental steps in atherosclerosis.

Figure 1. Cellular localization of hGX sPLA2 in human carotid arteries containing atherosclerotic plaques. Sections of arteries were stained with anti-CD68 (green, A and D), anti-hGX sPLA2 (red, A–D) and anti α -actin (green, B and C). Double immunofluorescence confocal analysis was performed and colocalized regions are shown in white. In intima (A and B), hGX sPLA2 expression is colocalized with foam cells (A) and smooth muscle cells resembling myofibroblasts (B). Inserts represent colocalization plots between hGX sPLA2 (red) and specific cell markers cluster of differentiation (CD) 68, or α -actin, (green). Blue dots represent colocalization points. In media (C), hGX sPLA2 expression is localized to ECM. No colocalization with smooth muscle cells (stained with α -actin, green) of media was observed. In intima (D) incubation of sections with 1 μ M recombinant hGX sPLA2 (rec hGX) abolished the hGX sPLA2 labeling.



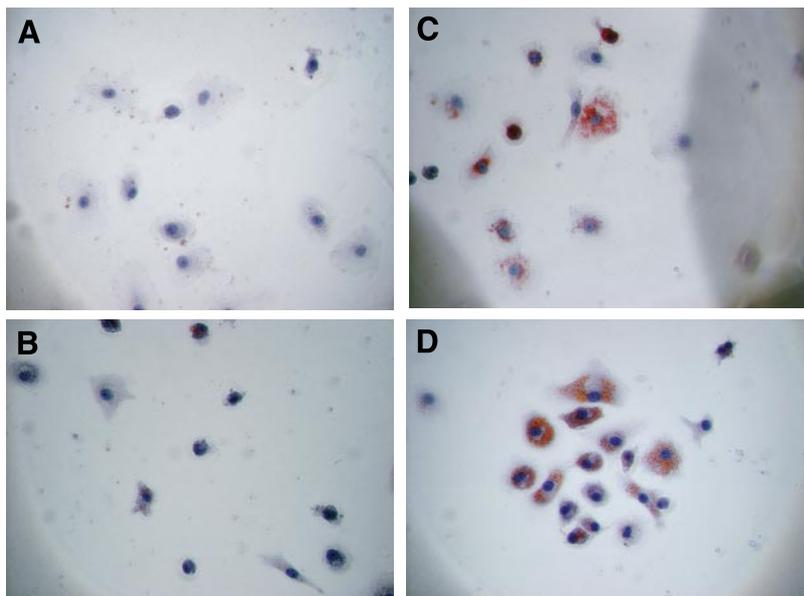


Figure 2. hGX-LDL induces monocyte derived foam cell formation. Human monocyte derived macrophages were incubated for 24 h in the presence of medium alone (A), 200 µg/ml LDL (B), 200 µg/ml aggregated LDL (C), or 100 nM hGX-LDL (D). Subsequently, cells were fixed and stained with Oil Red O and hematoxylin (magnification $\times 40$).

AA release from HUVEC exposed to hGX-LDL

Since endothelial cells are the first barrier that lipoproteins encounter before entering the subendothelial space, and the release of AA is tightly associated with inflammation, we first studied the effect of hGX-LDL on AA release from endothelial cells. Importantly the ability of exogenously added sPLA2s to liberate AA from mammalian cells has always been studied in the presence of fetal calf serum (21, 23, 39). Since serum contains large amounts of lipoproteins that could mask the effect of our treatments either by having a direct effect on cells or by being substrates for hGX sPLA2, it was critical to perform all of the cell culture experiments in serum-free medium. As shown in Fig. 3A, addition of hGX sPLA2 alone in the absence of fetal calf serum can induce a significant release of AA from HUVEC. Further addition of BSA led to a more sustained release of AA, which is probably due to the fact that BSA trapped the labeled AA released from hydrolyzed cell membranes (Fig. 3A). LDL particles modified with hGX led to a potent release of AA, which appears stronger than that produced by either hGX alone or LDL alone, showing that the modification of the LDL particle is important for the release. In contrast to the AA release induced by hGX sPLA2, the AA release induced by hGX-LDL was not influenced by further addition of BSA (Fig. 3A), probably because our LDL preparations already contained albumin (a common contaminant of LDL prepared by ultracentrifugation).

Treatment of HUVEC with hGX-LDL induced a significant time- and dose-dependent release of AA as compared to cells treated with either LDL or hGX sPLA2 alone (Fig. 3B and C), suggesting that the modified-LDL particle *per se* contributes significantly to AA release, besides the release triggered by hGX sPLA2 on its own.

To address the role of hGX sPLA2 catalytic activity on AA release induced by modified LDL, we used the

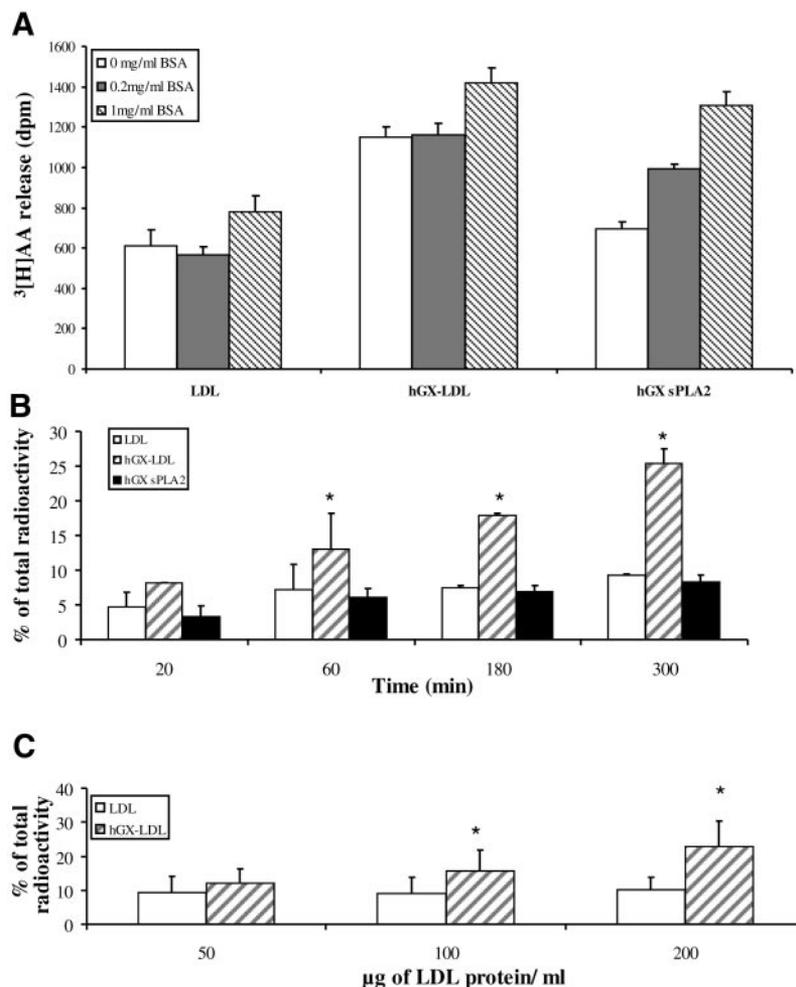
H48Q active site mutant of hGX, which has $<1\%$ of the wild-type (WT) enzymatic activity (not shown). As expected, no AA release was observed when HUVEC were exposed to the H48Q hGX mutant alone ($2\pm 0.5\%$, $P < 0.05$ of the hGX sPLA2 AA release). Treatment of LDL with the H48Q hGX mutant did not result in significant AA release (Fig. 4A).

LY 329722, a potent inhibitor of hGX sPLA2 (20, 29), was used to further discriminate between the roles that hGX sPLA2 could have either by acting directly on cells or by modifying LDL particles. Addition of LY329722 to LDL before treatment with hGX sPLA2 fully inhibited AA release (Fig. 4A). Conversely, addition of 10 µM LY329722 after the modification of LDL by hGX sPLA2 had no significant effect on the hGX-LDL-induced AA release (Fig. 4A), indicating that AA release is essentially due to modified LDL and not to hGX sPLA2 acting on labeled cells. Treatment of hGX sPLA2 (100 nM) with 10 µM LY329722 inhibited the release of AA from HUVEC ($5.2\pm 0.5\%$ of total without LY329722 and $2.1\pm 0.5\%$ of total with LY329722, $P < 0.05$), confirming that treatment of hGX with this inhibitor is efficient under our conditions. Finally, addition of LY 329722 to LDL alone had no effect on AA release (not shown).

Extensive dialysis of LDL after treatment with hGX sPLA2 and before addition to cells did not affect AA release (not shown). Additionally, the reisolation of LDL by ultracentrifugation followed by extensive dialysis, after the treatment with hGX sPLA2, did not affect the capability of hGX-LDL to induce AA release either in the presence or absence of the hGX sPLA2 inhibitor (Fig. 4B). Our results demonstrate that hGX sPLA2 can release AA from HUVEC on its own but this enzyme can also modify LDL in a form that by itself triggers an additional release of AA.

Among the multiple PLA2s found in mammalian tissues, the 85 kDa group IVA PLA2 (cPLA2-IVA) has been shown to play a key role in agonist-induced

Figure 3. Release of [^3H]AA from HUVEC cells by hGX-LDL particles. *A*) HUVEC were pre-labeled with [^3H]AA and the release of AA in the cell medium was measured on addition of either 100 $\mu\text{g/ml}$ LDL alone, or 100 $\mu\text{g/ml}$ hGX-LDL, or 100nM hGX sPLA2 alone. Cells were treated for 60 min in serum-free medium in either the absence or presence of BSA. Radioactivity associated with released [^3H]AA in the supernatant is shown. A representative experiment of 2 independent experiments with similar results performed in duplicate is shown. *B*) Time-dependent release of [^3H]AA in serum-free medium. HUVEC were preincubated with [^3H]AA in complete medium and then exposed to 100 $\mu\text{g/ml}$ LDL, hGX-LDL or 100 nM hGX sPLA2 in serum-free medium for the times indicated. $P < 0.05$ as compared to LDL or hGX sPLA2. Results are mean \pm SE. of at least 4 independent experiments performed in duplicate. *C*) Concentration dependent release of [^3H]AA in serum-free medium. HUVEC were preincubated with [^3H]AA in complete medium and then exposed to 50, 100, or 200 $\mu\text{g/ml}$ LDL, or hGX-LDL in serum-free medium for 60 min. $P < 0.05$ as compared to LDL. Results are mean \pm SE. of at least 4 independent experiments performed in duplicate.



release of AA (39, 40). To explore the possible implication of cPLA2-IVA in hGX-LDL induced AA release, HUVEC cells were pretreated with Wyeth-1, a specific and potent inhibitor c-PLA2-IVA. Addition of 10 μM of Wyeth-1 did not influence the hGX-LDL induced AA release (10.1 ± 0.9 vs. $10.9 \pm 0.6\%$ of total without and with inhibitor respectively, $P > 0.05$); conversely, it reduced AA release induced by the calcium ionophore A23187 (10.2 ± 0.8 of release vs. $16.5 \pm 1.6\%$, $P < 0.05$ with and without inhibitor respectively), suggesting that the cPLA2-IVA is not involved in the AA release by hGX-LDL.

Mitogen-activated protein kinase inhibitors and AA release

Since mitogen-activated protein (MAP) kinases are involved in AA release in HUVEC (41, 42) and as several sPLA2s can activate the ERK1/2 pathway (43), we analyzed whether or not the AA release induced by hGX-LDL is dependent on MAP kinases. HUVEC were pretreated for 30 min with either 10 μM PD 98059, an inhibitor of the ERK1/2 pathway, or with 10 μM SB 203580, an inhibitor of the p38 pathway, before addition of hGX-LDL, LDL, or hGX sPLA2 in serum-free medium supplemented with 0.2 mg/ml BSA. None of

the inhibitors were cytotoxic for the cells as assessed by MTT assay. The inhibitor SB 203580 had a modest, but not statistically significant effect, on AA release induced by hGX-LDL (Fig. 5A). In contrast, PD 98059 significantly inhibited AA release induced by HUVEC exposed to hGX-LDL (Fig. 5A). Interestingly as shown in Fig. 5A, both PD 98059 and SB 203580 had a more pronounced inhibitory effect on the hGX-sPLA2 evoked AA release from HUVEC, as compared to that of hGX-LDL. Furthermore, treatment of cells with hGX-LDL showed a rapid phosphorylation of ERK1/2 that persisted up to 60 min (Fig. 5B). The activation of ERK1/2 was blocked by PD 98059 (data not shown), which is in agreement with an implication of the ERK1/2 pathway in hGX-LDL induced AA release. In contrast, a minor effect on ERK1/2 phosphorylation was found when cells were treated with hGX sPLA2 or LDL alone (Fig. 5B).

Effect of LPC on AA release

One of the main products of LDL hydrolysis by hGX sPLA2 is LPC (13, 24). It has been shown that exogenously added LPC stimulates AA release in HUVEC through ERK1/2 (44). These results were confirmed in our study in which LPC in the range of 5–25 $\mu\text{g/ml}$ also

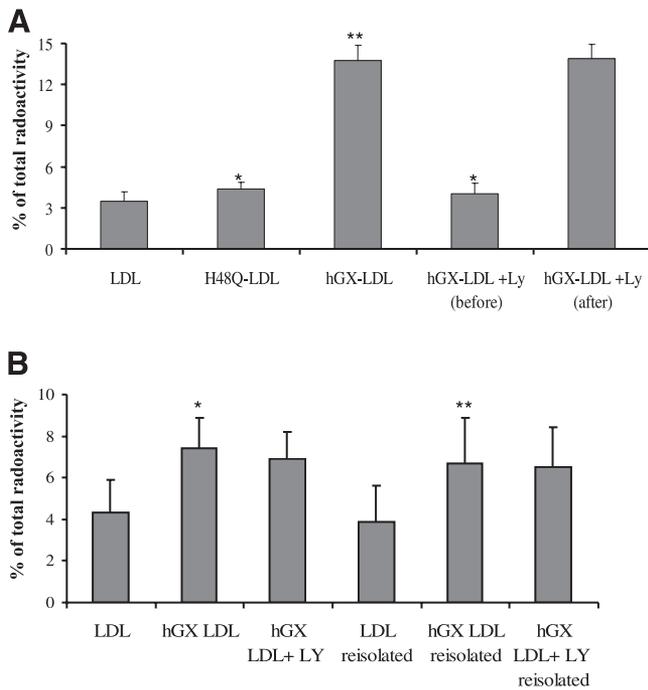


Figure 4. Release of [³H]AA triggered by hGX-LDL. Effects of an hGX sPLA2 active site mutant a specific hGX sPLA2 inhibitor and reisolated hGX-LDL. **A)** LDL was treated for 3h in the presence of 100 nM hGX sPLA2 or its active site mutant H48Q as described in Materials and Methods. In selected experiments, the specific inhibitor LY 329722 was added to LDL before addition of hGX sPLA2 (denoted as hGX-LDL+LY before) or after the 3 h treatment of LDL with hGX sPLA2 (denoted as hGX-LDL+LY after). Mixture was then added to HUVEC monolayers preincubated with [³H]AA and release of AA in serum-free medium was measured after 60 min. **P* < 0.05 as compared to hGX-LDL, ***P* < 0.05 as compared to LDL. Results are mean values ± SE. of at least 3 independent experiments performed in triplicate. **B)** LDL was treated for 3 h with 100 nM hGX sPLA2. The reaction was stopped by the addition of EDTA (5 mM final) and then divided into 4 aliquots. 10 μM of the hGX sPLA2 inhibitor was added to 1 aliquot and the modified LDL was kept at 4°C (denoted as hGX-LDL+LY). Another aliquot was kept at 4°C with no addition of the inhibitor (denoted as hGX-LDL); 10 μM of the hGX sPLA2 inhibitor was added to a third aliquot and the hGX modified LDL with inhibitor was resubmitted to ultracentrifugation followed by overnight dialysis (denoted as hGX-LDL+LY reisolated). Finally, the last aliquot was reisolated by ultracentrifugation and overnight dialysis without addition of hGX sPLA2 inhibitor (denoted as hGX-LDL reisolated). LDL subjected to same conditions but with omission of hGX sPLA2 (LDL) was also reisolated (LDL reisolated). Subsequently, HUVEC were preincubated with [³H]AA and the release of AA in serum-free medium was measured 60 min after of the addition of 100 μg/ml of LDL, hGX-LDL, hGX-LDL + LY, LDL reisolated, hGX-LDL reisolated, hGX-LDL + LY reisolated. **P* < 0.05 as compared to LDL, ***P* < 0.05 as compared to LDL reisolated. Results mean ± SE. of at least 2 independent experiments performed in triplicate

induced AA release. Typically, when HUVEC were treated with 25 μg/ml LPC, AA release was 36.4 ± 4.7% of total AA. The presence of 10 μM PD 98059 reduced AA release to 23.9 ± 1.7 (*P* < 0.05), indicating that the ERK1/2 kinase pathway is in part implicated in LPC induced AA release.

Analysis of mRNA levels and functional expression of adhesion molecules

To further examine the effects of hGX modified LDL on endothelial cell function, we examined the expression of genes coding for the major adhesion molecules involved in monocyte adhesion to endothelial cells, including VCAM-1 (ICAM-1), P- and E-selectins and platelet-endothelial cell adhesion molecule-1 (PECAM-1). With the use of quantitative-polymerase chain reaction, a significant increase in ICAM-1 and VCAM-1 expression was observed after 3 and 6h of treatment with hGX-LDL, as compared to LDL or hGX sPLA2 alone (Fig. 6A). A significant increase in the mRNA level of E-selectin was also apparent after 6 h, whereas there was no significant change in the expression of P-selectin and PECAM (Fig. 6A). ICAM-1 and VCAM-1 mRNA up-regulation was accompanied by a time-dependent increase in protein expression as shown by Western blot analysis for these molecules (Fig. 6B). Increased expression of these adhesion molecules was functionally relevant since we observed a significantly higher adhesion of fluorescent THP-1 cells to HUVEC treated with hGX-LDL as compared to LDL or hGX sPLA2 alone (Fig. 6C). The addition of the hGX sPLA2

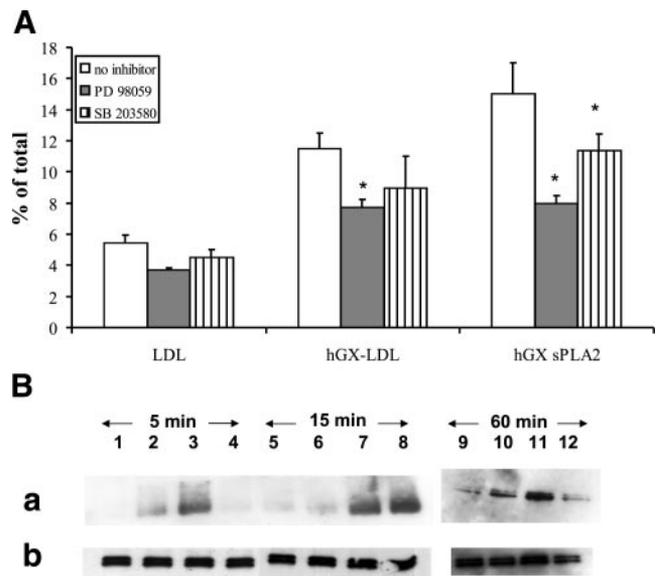
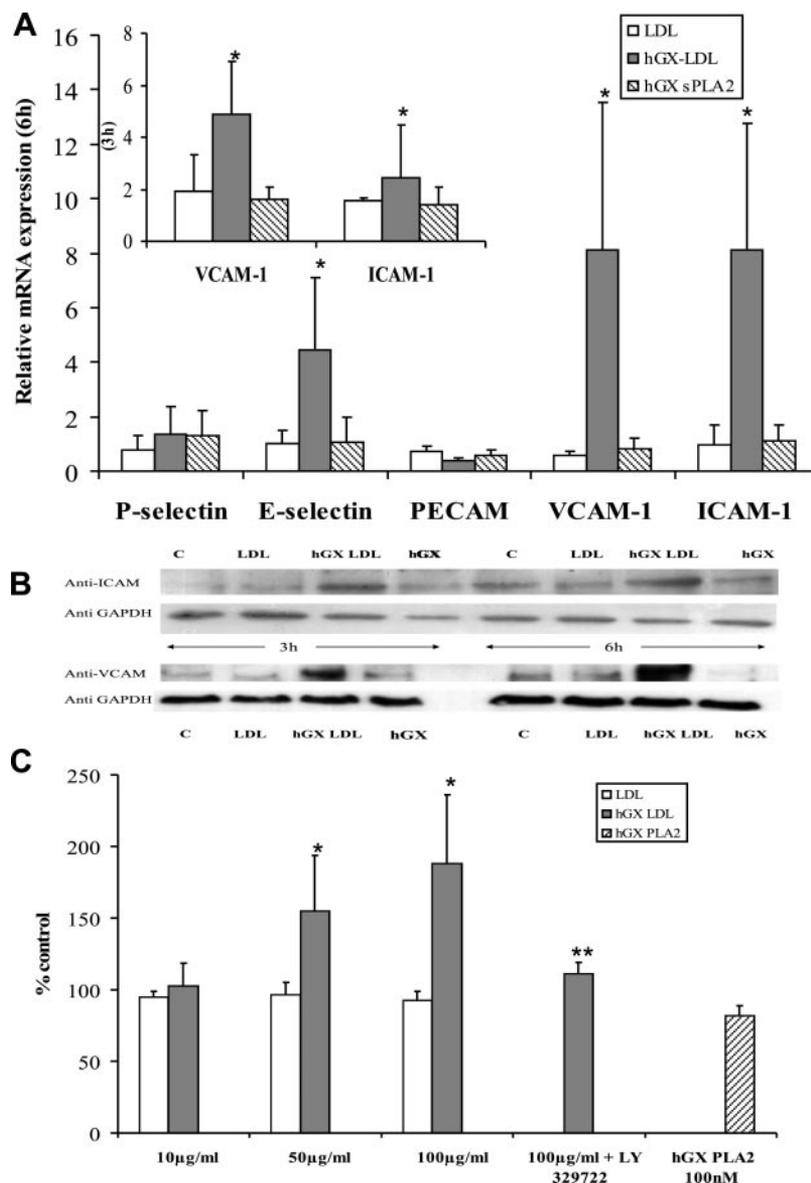


Figure 5. MAPK implication in hGX-LDL induced AA release. **A)** [³H]AA labeled HUVEC cells were preincubated with either 10 μM PD 98059 or SB 203580. Cells were subsequently treated with 100 μg/ml LDL or hGX-LDL or 100 nM hGX-sPLA2 for 1 h and AA release was measured in serum-free medium containing 0.2 mg/ml BSA. Results are mean ± SE. of 3 independent experiments performed in duplicate (**P* < 0.05 without the inhibitor). **B)** Cells were treated with medium alone (lines 1, 5, 9) or 100 μg/ml LDL (lines 2, 6, 10), or hGX-LDL (lines 3, 7, 11) or 100 nM hGX sPLA2 (lines 4, 8, 12) for 5, 15 and 60 min respectively. ERK1/2 activation was then examined in cell lysate by Western blot analysis using (a) a rabbit polyclonal phospho-ERK1/2 Ab or (b) a rabbit polyclonal ERK1/2 Ab to verify equal loading. Representative blots of 4 independent experiments in different gels are shown.

Figure 6. hGX-LDL induced expression of adhesion molecules. *A*) HUVEC cells were exposed for 3 h (inset) or 6 h to 100 $\mu\text{g}/\text{ml}$ LDL, 100 $\mu\text{g}/\text{ml}$ hGX-LDL or to 100 nM hGX sPLA2 in serum-free medium. Total RNA was isolated and Q-PCR was performed as described in Materials and Methods. Results are mean \pm SE. of 4 independent experiments performed in duplicate ($*P < 0.05$ as compared to LDL). *B*) HUVEC cells were exposed for 3 and 6 h to 100 $\mu\text{g}/\text{ml}$ LDL, 100 $\mu\text{g}/\text{ml}$ hGX-LDL or to 100nM hGX sPLA2 in serum-free medium. Same amount of cell lysate was used for Western blot analysis of ICAM-1 and VCAM-1 and GAPDH as described in Materials and Methods. Results represent 4 independent experiments performed in different gels with similar results. *C*) HUVEC were pretreated for 3 h with increasing concentrations of hGX-LDL, LDL, or 100 nM hGX sPLA2 and the adhesion of fluorescent THP-1 cells was measured after 60 min incubations as described in Materials and Methods. In selected experiments, LY 329722, a specific hGX sPLA2 inhibitor was added to LDL before modification by hGX sPLA2. Results (mean \pm SE) represent 4–6 independent experiments performed in duplicate. $*P < 0.05$ as compared to LDL, $**P < 0.05$ as compared to hGX-LDL.



inhibitor LY 329722 before treatment of LDL with the sPLA2 completely blocked the effect, demonstrating that the modification of LDL is a prerequisite step for adhesion of THP-1 cells to HUVEC monolayers. The inhibitor had no effect on THP-1 adhesion when added to either LDL or hGX sPLA2 alone or when it was added after the treatment of LDL with hGX sPLA2.

DISCUSSION

Much evidence suggests that certain sPLA2s are implicated in inflammatory diseases such as atherosclerosis (45–47). By immunohistochemical methods, GIIA, GV, and GX sPLA2s have been detected in human or murine arterial wall in regions of lipid accumulation (12, 13, 48–50). Our study is the first to demonstrate that hGX sPLA2 is expressed in the intima of human atherosclerotic lesions where it colocalizes with foam cells and phenotypically dedifferentiated smooth mus-

cle cells (Fig. 1A–B). Presently, we are not aware whether or not the presence of hGX sPLA2 in the lesions is due to local biosynthesis in the vascular wall or deposition from plasma lipoproteins after their cellular internalization. The detection of hGX sPLA2 in the ECM of the media (Fig. 1C), however, raises the possibility that its local production could originate from smooth muscle cells of the media. The physiological relevance of previously detected sPLA2s in human atherosclerotic plaques is still debated. The low protein homology and different tissue localization of sPLA2s suggest that their roles in arterial wall and more specifically in foam cell formation are distinct. GIIA sPLA2 modifies LDL by increasing its affinity for proteoglycans through conformational changes in apolipoprotein B-100 (51). On the other hand, mouse GV sPLA2 promotes LDL particle aggregation and foam cell formation in a scavenger receptor-independent manner that involves cellular proteoglycans (52). In contrast to GIIA and GV sPLA2, hGX sPLA2 does not

bind to proteoglycans and does not promote LDL particle aggregation, yet it efficiently hydrolyzes it. Here we show that the lipolytic effect of hGX on LDL produces a modified particle that induces human macrophage foam cell formation *in vitro* (Fig. 2). For the above reasons, we hypothesize that the cellular pathways implicated in the uptake of hGX-LDL are different from those for GIIA- and GV-modified LDL. The precise structural alterations of hGX-LDL that lead to such enhanced lipid accumulation *in vitro* remain to be elucidated.

Our results demonstrating that the lipolytic action of hGX renders the LDL particles smaller in diameter are important, as the smaller the size of the LDL particle the higher its atherogenic potential (53). A common feature of small dense LDL and sPLA2 modified LDL is the reduced phospholipid content of their surface (54). As hGX is the most active sPLA2 toward PC (24) the major phospholipid constituent of lipoproteins, it would be of interest to examine the possible involvement of this enzyme in lipoprotein metabolism and small dense LDL formation.

The release of AA upon addition of hGX sPLA2 from several types of mammalian cells has already been described, and recently Pruzanski *et al.* (24) showed that there is a preferential release of AA when hGX sPLA2 (as compared to GV sPLA2) acts on LDL. In our study, we showed that hGX sPLA2 as well as hGX-LDL was able to induce a time- and concentration-dependent release of AA from HUVEC in serum-free medium (Fig. 3). AA release was not observed when hGX-LDL was pretreated either with the potent hGX sPLA2 inhibitor LY 329722 or with the hGX active site mutant, H48Q, indicating that the enzymatic modification of LDL by hGX is prerequisite for cell activation (Fig. 4A). Additionally, when hGX-LDL was reisolated by ultracentrifugation and extensively dialyzed to remove all loosely bound molecules, it retained the ability to release AA from HUVEC cells even in the presence of the hGX sPLA2 inhibitor (Fig. 4B). We can not, however, exclude, that products of the hydrolytic action of hGX sPLA2 on LDL, namely LPC and/or free fatty acids act alone or in synergy with the modified LDL to induce AA release.

The 85 kDa c-PLA2 has been shown to play a key role in various agonist-induced release of AA (40). The AA release due to hGX-LDL appeared to be independent from cPLA2-IVA activation, as shown here by the use of a specific cPLA2-IVA inhibitor; the result is in accordance with Hanasaki *et al.* (23). Moreover, in cPLA2 knockout mice, the amount of AA released by hGX sPLA2 from spleen cells was not significantly altered by cPLA2 deficiency (55). Whether or not other intracellular and/or Ca²⁺ independent forms of PLA2s might participate in the AA release observed in this study needs further investigation. Interestingly, AA release was dependent on ERK1/2 activation (Fig. 5), suggesting that such PLA2s might indeed be implicated.

Our results demonstrate that the lipolytic effect of hGX sPLA2 on LDL results in the formation of an

atherogenic LDL particle that increases both the mRNA and protein expression of major adhesion molecules, including ICAM-1 and VCAM-1, which was accompanied by increased adherence of monocytes to endothelial cell monolayers (Fig. 6). Such observation is of prime importance, as the expression of adhesion molecules is increased during inflammation and contributes to the progression of vascular dysfunction. An activated endothelium is prerequisite for monocyte adherence and recruitment into the subendothelial space and is one of the major events not only in atherosclerosis but also in systemic inflammation and in tumor invasion (56).

The "oxidation hypothesis of atherosclerosis" (25, 26) still remains inconclusive, and oxidation alone cannot explain the accumulation of large amounts of lipids and LPC in foam cells and fatty streak lesion formation (57). Our current studies on hGX sPLA2 and studies by others on hGIIA and hGV show that in addition to oxidation, other forms of LDL modification, such as the hGX sPLA2 lipolytic modification of LDL, participate into the progression of atherosclerosis. In the arterial wall, hGX sPLA2 may exert proatherogenic actions by inducing the release of lipid mediators such as LPC that can affect functions and properties of vascular cells at sites of lipoprotein accumulation. In addition, hGX sPLA2 may also modify lipoprotein particles by hydrolyzing PC in a way that promotes foam cell formation independently of LDL oxidation. Our results clearly indicate that the action of hGX on LDL leads to a modified particle that can act and alter cell function independently from the hGX sPLA2. The questions of how the expression and activation of this potent sPLA2 is regulated and whether or not the enzyme is active in blood and/or associated with lipoproteins or other proteins remain to be answered.

In conclusion, our data suggest a strong atherogenic property of hGX sPLA2 in modifying LDL into a proinflammatory and proatherogenic particle that may contribute to the initiation and propagation of atherosclerosis. EJ

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