Phosphatidylinositol turnover during stimulation of plasminogen activator inhibitor-1 secretion induced by oxidized low density lipoproteins in human endothelial cells

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Abstract In a previous study (Latron et al. 1991. *Arterioscler. Thromb.* **11:** 1821–1829) we have shown that oxidized low density lipoproteins (ox-LDL) stimulated the synthesis and secretion of plasminogen activator inhibitor-1 (PAI-1) by human umbilical vein endothelial cells (HUVEC) in culture. The present study is intended to give insight into the intracellular process responsible for this stimulation. The HUVEC lipids were labeled for 16 h with [^{3}H]arachidonate and incubated either with native LDL (n-LDL) or ox-LDL for various times (15, 30, 60 min). Compared with unstimulated cells (no LDL added), ox-LDL induced a significant increase in the intracellular level of unesterified [^{3}H]arachidonate, concomitantly with a significant decrease of the phosphatidylinositol fraction. The most marked effect was observed at 30 min and was significantly much less with n-LDL. Phospholipase inhibitors (4-bromophenacylbromide and mepacrine) added to the culture medium completely prevented the ox-LDL-induced stimulation of phosphatidylinositol degradation, [^{3}H]arachidonate release, and PAI-1 secretion. HUVEC possess both phospholipase C and A activities and a high lysophospholipase activity, the phospholipase A pathway being in vitro more sensitive to inhibition by 4-bromophenacylbromide than the phospholipase C pathway. These results suggest that the stimulation of PAI-1 secretion by ox-LDL is mediated by the hydrolysis of membrane phosphatidylinositol through the activation of phospholipase A.—**Chautan, M., Y. Latron, F. Anfosso, M-C. Alessi, H. Lafont, I. Juhan-Vague, and G. Nalbone.** Phosphatidylinositol turnover during stimulation of plasminogen activator inhibitor-1 secretion induced by oxidized low density lipoproteins in human endothelial cells. *J. Lipid Res.* 1993. **34:** 101–110.

Supplementary key words phospholipases A and C • arachidonic acid • atherothrombosis

It is now acknowledged that low density lipoproteins (LDL) are one of the factors that contributes to endothelium injury or dysfunction, leading to atherosclerotic lesion formation. Clinical and epidemiological studies have clearly shown that the LDL fraction of plasma cholesterol is highly involved in atherogenesis, notably lipid accumulation in the arterial wall (1, 2).

It is now well established that the atherogenicity of LDL is due in part to their oxidative status (3) which may concern the lipid and protein fractions (4). Lipid peroxidation in LDL particles is a phenomenon that occurs in vivo, as oxidized LDL (ox-LDL) have been recovered from atherosclerotic vessel lesions in humans and rabbits (5, 6), and autoantibodies against ox-LDL have been detected in the plasma of healthy subjects (7). It is also recognized that the resistance of LDL to peroxidative injury is strongly dependent on their antioxidant content, mainly α-tocopherol (8). The atherosclerotic process is complex and involves a multitude of cellular events such as cellular proliferation, monocyte adhesion to the endothelium and subsequent transformation of monocytes into macrophage foam-cells, activation of endothelial cells, etc. Among these events, the impairment of the fibrinolytic system may be of major importance because thrombus formation is the ultimate step in the atherosclerotic process. In the latter, endothelial cells play a pivotal role through the release of various components regulating fibrinolysis. It

Abbreviations: HUVEC, human umbilical vein endothelial cells; n-LDL, native low density lipoprotein; ox-LDL, oxidized low density lipoprotein; PLA, phospholipase A; PI-PLC, phospholipase C specific for phosphatidylinositol; PAI-1, plasminogen activator inhibitor-1; PL, phospholipid; PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; TG, triacylglycerol; DG, diacylglycerol; CE, cholesteryl ester; UV, ultraviolet; PGI_2, prostaglandin I_2; TLC, thin-layer chromatography; TBARS, thiobarbituric acid reactive substances; 4-BPB, 4-bromophenacylbromide; MDA, malondialdehyde.

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was recently demonstrated that defects in plasma fibrinolysis are most often due to an increased plasma concentration of an inhibitor of the fibrinolytic system called plasminogen activator inhibitor-1 (PAI-1) (9), which is now recognized as a risk factor for atherothrombosis (9–11).

It was recently shown that some atherogenic lipoproteins were able to stimulate the synthesis and secretion of PAI-1 by human umbilical vein endothelial cells (HUVEC) in culture. This was demonstrated for VLDL from hypertriglyceridemic patients which stimulates the secretion of PAI-1 (12), for the atherogenic lipoprotein[a] which enhances PAI-1 expression in HUVEC (13), and for LDL oxidized by 2 h of ultraviolet (UV) radiation, which stimulates the synthesis and the secretion of PAI-1 by HUVEC in culture (14). However, the degree of LDL peroxidation seems determinant in this stimulation, as copper-induced LDL peroxidation (a treatment known to drastically alter both lipid and protein fractions) (12, 13) or prolonged exposure (longer than 2 h) of LDL to UV (14) did not induce PAI-1 secretion. In our study (14), we also observed that this ox-LDL-induced PAI-1 stimulation suggested a mechanism that involved the internalization of the particles via a receptor different from the classical apoB/E receptor as initially characterized (2).

Receptor-mediated signal transduction often involves arachidonic acid which can be released from intracellular stores via different routes, among which phospholipase A (PLA) and phospholipase C (PLC) activities are often found to be involved (15–17). For example, in human and animal vascular endothelial cells, various compounds, such as bradykinin, thrombin, hydrogen peroxides, ionophore, etc., all elicit the hydrolysis of membrane phospholipids through an activation of a PLA_2 (18–22). Thus, it seemed to be of interest to investigate whether the stimulation of PAI-1 secretion by ox-LDL could be mediated by the hydrolysis of membrane phospholipids.

In this study, we demonstrate in cultured HUVEC that the enhanced secretion of PAI-1 by ox-LDL is mediated by the liberation of arachidonic acid from membrane phosphatidylinositols (PI), probably through the activation of a PLA.

MATERIALS AND METHODS

Cell culture

HUVEC were isolated (23) and cultured (14) as previously described. Cells from either the second or third passages were used in these experiments. Before incubation with lipoproteins, cell monolayers were incubated for 16 h in serum-poor medium (containing 5% calf serum) followed, for the indicated incubation period, by the addition of either 50 μg/ml of n-LDL protein or ox-LDL protein. In some experiments, cells were collected after trypsinization and homogenized in phosphate-buffered saline (PBS) at 4°C with a glass Potter-Elvehjem homogenizer. Homogenates were stored in aliquots at −70°C.

Labeling of HUVEC with [^{3}H]arachidonic acid

Cells were grown to confluency in culture flasks (25 cm^2), then plated into 2.9-mm wells and incubated for 16 h (24) with 1.9 μCi of [5,6,8,9,11,12,14,15-^{3}H]arachidonate (NEN-DuPont, Paris, France) previously solubilized under nitrogen in the culture medium. About 65 ± 5.0% of the radioactivity was recovered in the cells. The cells were rinsed three times with PBS, and stimulated with n-LDL or ox-LDL for indicated times. Values at time 0 were obtained from cells in which no LDL was added. After the incubation period, the medium was quickly removed and the cells were immediately collected after trypsinization and lipids were extracted with chloroform–methanol 2:1 (v/v) according to Folch, Lees, and Sloane Stanley (25). An aliquot of the medium was also submitted to lipid extraction. Twenty μl of human plasma was added to the extract to serve as a cold carrier of radioactive lipids. The lipid extract was concentrated under nitrogen and analyzed by thin-layer chromatography (TLC) on a silica gel plate (F-1500, Schleicher et Schuell, Basel, Switzerland). The solvent system used to separate phospholipids (26) consisted of chloroform–methanol–acetic acid–water 50:30:11:4 (v/v); heptane–diethylether–acetic acid 90:60:4 (v/v) allowed the separation of neutral lipids (27). The developed plates were dried and spots of lipids were revealed by iodine vapor. Spots were identified with known 99% pure standards (Sigma, l'Isle d'Abeau Chesne, France), then scraped and counted. Counts were corrected for a recovery of 100%, a calculation that included the loss of radioactivity during lipid extraction, concentration, TLC loading, and scraping.

Preparation and peroxidation of LDL

LDL (1.019–1.063 g/ml) were isolated from the plasma of healthy normolipidemic donors by sequential preparative ultracentrifugation as described (28). Protein LDL content was determined by the method of Lowry et al. (29) with bovine serum albumin as the standard. The LDL were peroxidized as described (14) under ultraviolet (UV) radiation (254 nm) for 2 h, according to the method of Dousset et al. (30). Lipid peroxidation was determined by quantification of thiobarbituric acid reactive substances (TBARS) which were measured by spectrofluorometry (31).

Studies with PLA inhibitors

In some experiments, the effect of phospholipase inhibitors, mepacrine and 4-bromophenacyl bromide (4-BPB) (Sigma), on PAI-1 secretion by HUVEC was investigated. The inhibitor was added to the culture medium at the indicated concentration and HUVEC were incubated with the inhibitor for 1 h. Then, 50 μg/ml of ox-LDL protein

was added and left for 24 h. PAI-1 levels were measured in the medium as described below. To investigate the effects of phospholipase inhibitors on lipid metabolism of HUVEC stimulated with ox-LDL, the same protocol as above was applied to [^{3}H]arachidonic acid-labeled HUVEC, using inhibitor concentrations of 3 μM and 12 μM for 4-BPB and mepacrine, respectively. Cells were incubated with ox-LDL for 30 min. The distribution of the radioactivity in the phospholipid and neutral lipid classes was analyzed as described above.

Studies with products issued from the hydrolysis of PI by PLA

In some experiments, either arachidonic acid (Sigma, 99% pure) at a final concentration of 1, 10, 100 μM, or 1-acyl-3-glycerophosphoinositol (lysoPI, Sigma 99% pure) at a final concentration of 1, 10, 100 μM (both previously solubilized in the culture medium containing 5% serum) was added to HUVEC pre-incubated for 16 h with serum-poor medium. After 24 h, supernatants were collected and assayed for PAI-1 as described below.

Cell-free assay of phospholipase A (PLA), phosphatidylinositol-specific phospholipase C (PI-PLC), and lysophospholipase activities

The activity of PLA of HUVEC on exogenous substrate was assayed according to a previously described method (27). Briefly, 1-palmitoyl-2[1-^{14}C]linoleoyl-*sn*-glycero-3-phosphocholine or 1-stearoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphoinositol (NEN-DuPont) with specific radioactivities of 60 Bq/nmol and 30 Bq/nmol, respectively, was dispersed into unilamellar liposomes (in 100 mM Tris-HCl or cacodylate buffer at the desired pH and at a concentration of 0.4 mM) by sonication for 5 min under nitrogen. Standard assays of PLA were carried out with 40 μg of total protein of cell homogenates preincubated at 4°C with 5 mM EDTA. The reaction was initiated by the addition of the radioactive substrate (final concentration: 0.2 mM) and the mixture (final volume: 0.2 ml) was incubated at 37°C for 15 min at the indicated pH. The radioactive fatty acid released was isolated and counted as described (27). Assays were done in triplicate. The values were corrected for a blank without proteins, treated under the same conditions.

PI-PLC was assayed according to a classical method based on the water extraction of the reaction product after a lipid–water partition of the incubation mixture (32). Briefly, radioactive PI (myoinositol-2-[^{3}H]phosphatidylinositol) (NEN-DuPont), with a specific radioactivity of 60 Bq/nmol was dispersed for 5 min into unilamellar liposomes buffered with 100 mM sodium cacodylate or Tris-HCl. Standard assays were performed with 40 μg of protein at pH 5.0 with 5 mM calcium. The reaction was initiated by the addition of the radioactive substrate (final concentration: 0.2 mM) and the mixture (final volume: 0.2 ml) was incubated at 37°C for 10 min. The reaction was stopped with 4 ml of chloroform–methanol 2:1 (v/v). Then, 0.85 ml 0.01 N HCl was added and the mixture was vigorously stirred. After separation of the phases, [^{3}H]inositol phosphate was recovered in the aqueous phase, an aliquot of which was counted. Values were corrected for a blank without protein.

Lysophospholipase activity was assayed as already described (27) using 1-[1-^{14}C]palmitoyl lysophosphatidylcholine as substrate (NEN-DuPont), at a final concentration of 0.2 mM with a specific radioactivity of 20 Bq/nmol. As our purpose was to estimate the lysophospholipase activity only under PLA assay conditions, these latter conditions (pH, time, EDTA) were used for lysophospholipase.

PAI-1 assay

The concentrations of PAI-1 in 24-h conditioned medium were assayed with specific ELISA as previously described (33). Under our conditions, almost all PAI-1 measured was in the latent form and we have determined that neither n- nor ox-LDL treatment had any effect on the proportion of the latent form.

RESULTS

Effect of n-LDL and ox-LDL on lipid metabolism in [^{3}H]arachidonic acid-prelabeled HUVEC

To determine the effect of n-LDL and ox-LDL treatments on HUVEC membrane phospholipid metabolism, cells were first incubated with [^{3}H]arachidonic acid for 16 h and then treated with 50 μg/ml of either n-LDL or ox-LDL protein. Values for TBARS in LDL were in the range of those obtained previously (14): 1.20 and 7.32 nmol MDA/mg protein for n-LDL and ox-LDL, respectively. The protein assay in cell homogenates revealed that the treatments did not induce any change in protein contents. They were (in mg) control, 0.46 ± 0.06; 15 min with n-LDL, 0.53 ± 0.07; 30 min with n-LDL, 0.46 ± 0.03; 60 min with n-LDL, 0.53 ± 0.02; 15 min with ox-LDL, 0.48 ± 0.02; 30 min with ox-LDL, 0.49 ± 0.04; and 60 min with ox-LDL, 0.53 ± 0.01. The basal incorporation of [^{3}H]arachidonic acid into the various classes of lipids is represented by the amount of radioactivity present at time 0 (unstimulated cells) in the lipids (Figs. 1 and 2). The greatest incorporation was seen in triglyceride (TG), 30.6%; followed by phosphatidylinositol (PI), 19.9%; phosphatidylethanolamine (PE), 18.6%; and phosphatidycholine (PC), 17.6%. The basal level of unesterified [^{3}H]arachidonic acid represented about 4% of the total radioactivity recovered. Incorporation into cholesteryl esters (CE) represented 3.6% (data not shown). The time-dependent level of [^{3}H]arachidonate into phospholipids (PL) of HUVEC stimulated by LDL

is shown in **Fig. 1.** In PE, no significant changes in the level of [^{3}H]arachidonate were induced by n-LDL or ox-LDL treatment. In PC, a slight increase in the [^{3}H]arachidonate level was observed at 30 min with both n-LDL and ox-LDL but the difference did not reach the level of significance compared with time 0. In PS, the [^{3}H]arachidonate level tended to increase with time of treatment with n-LDL but not with ox-LDL. The increase became significant at 60 min with n-LDL (+31%). Also at this time, the levels of [^{3}H]arachidonate in PS of cells treated with n-LDL were significantly higher than cells treated with ox-LDL. With n-LDL-treated cells, the [^{3}H]arachidonate level in PI tended to decrease at 30 min and then return to the basal level at 60 min. The decrease at 30 min was found significant only when compared at time 15 min. With ox-LDL-treated cells, the decrease in [^{3}H]arachidonate in PI could be detected at 15 min and became significant ($P < 0.01$) at 30 min (−25%) and 60 min (−30%) when compared with time 0. Also, the [^{3}H]arachidonate level in PI of ox-LDL-treated cells was significantly lower than that of n-LDL-treated cells at times 30 and 60 min.

The time-dependent level of [^{3}H]arachidonate into neutral lipids is shown in **Fig. 2.** In DG and TG, the level was lower at 30 min and returned to the basal level at 60 min, but due to relatively elevated standard devia-

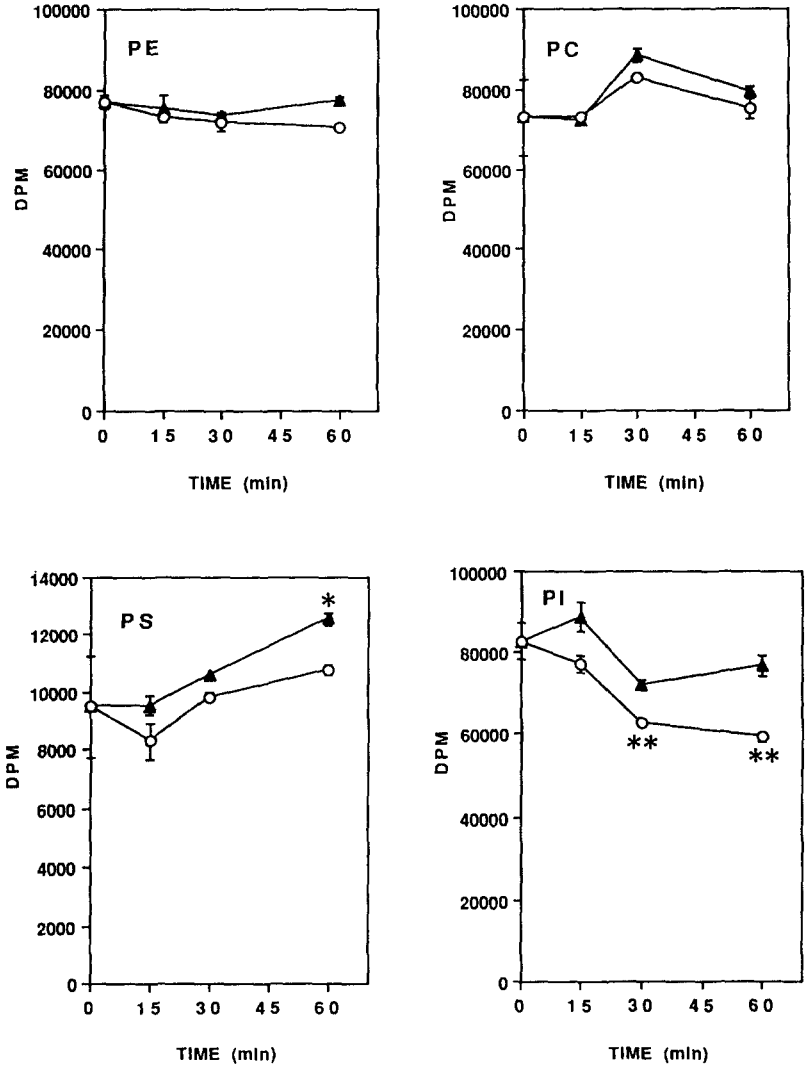

Fig. 1. Time-dependent effects of incubation of n-LDL and ox-LDL on [^{3}H]arachidonate distribution into the various classes of HUVEC phospholipids. Cells were labeled as described in Experimental Procedures and incubated for various times with n-LDL (closed triangles) or ox-LDL (open circles). Results are the mean of two separate experiments (two different LDL and HUVEC preparations), each performed in duplicate. Asterisks indicate a significant difference when compared with control cells at time 0 ($P = 0.05$*, $P = 0.01$**) using ANOVA test and Sheffe's comparison method). Student's *t*-test indicated that [^{3}H]arachidonate level was significantly lower in PI at 30 min ($P = 0.007$) and 60 min ($P = 0.03$) with ox-LDL when compared with n-LDL (significance not shown in the figure).

tions, the differences were not significant. It must be pointed out that in TG and DG, the position(s) labeled by [^{3}H]arachidonate and those preferentially cleaved by HUVEC lipase are not known. Thus, any change in the [^{3}H]arachidonate level in these neutral lipids may not be systematically accompanied by a corresponding change in unesterified [^{3}H]arachidonate levels. Unesterified [^{3}H]arachidonate levels started to increase at 15 min both with n-LDL and ox-LDL. Then, with n-LDL, the [^{3}H]arachidonate level plateaued at 30 min and returned to the basal level at 60 min. A very different pattern was obtained with ox-LDL, where it continued to increase at 30 min (+100%, $P < 0.001$) and was still 54% higher at 60 min ($P < 0.01$). Also, the unesterified [^{3}H]arachidonate levels were significantly higher in ox-LDL-treated cells than in n-LDL-treated cells at 30 min ($P < 0.05$) and 60 min ($P < 0.01$). The release of unesterified [^{3}H]arachidonate in the culture medium was also examined (Fig. 2). Up to 30 min, the release was hardly detectable in the culture medium. At 60 min, about 18 times more unesterified [^{3}H]arachidonate was released in the medium of ox-LDL-treated cells compared with n-LDL-treated cells.

Effects of PLA inhibitors on PAI secretion and [^{3}H]arachidonic acid turnover in ox-LDL-treated HUVEC

Previous results have shown that ox-LDL stimulated the synthesis and secretion of PAI-1 by HUVEC (13). The pres-

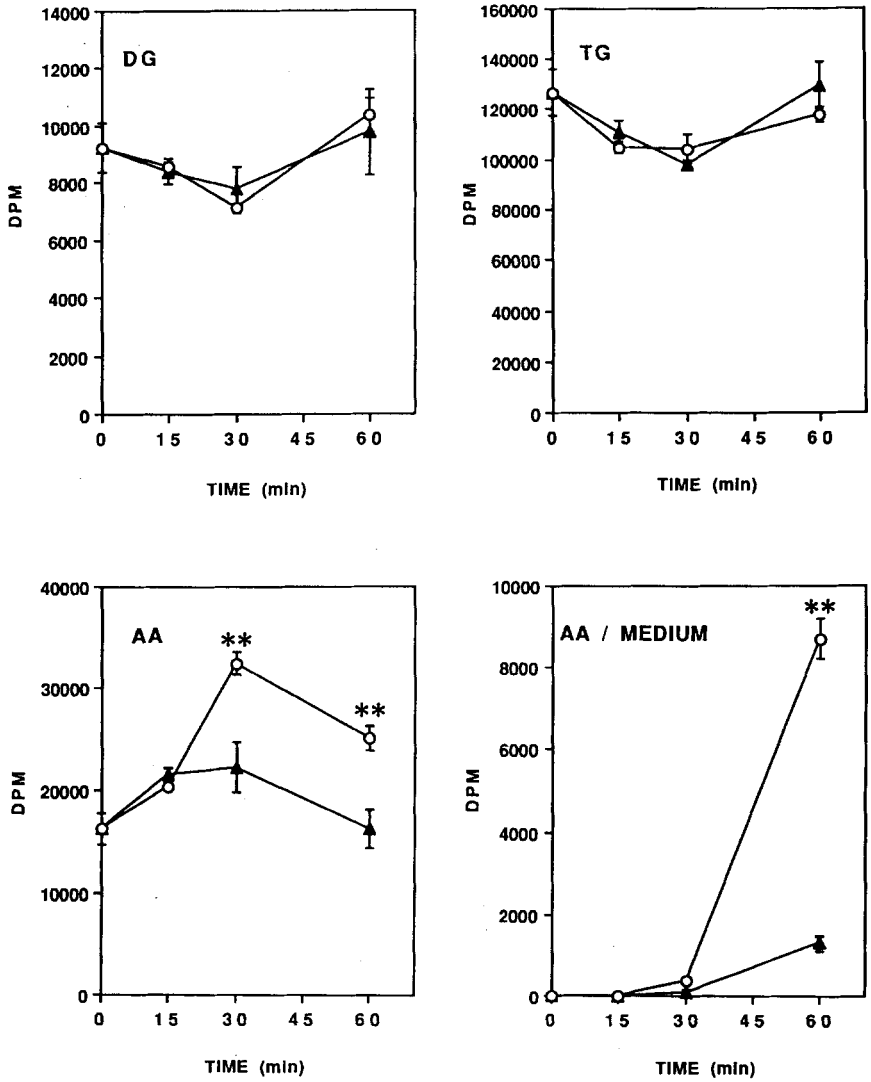

Fig. 2. Time-dependent effects of incubation of n-LDL and ox-LDL on [^{3}H]arachidonate distribution into the various classes of HUVEC neutral lipids. Cells are the same as in Fig. 1. Asterisks indicate a significant difference when compared with control cells at time 0 ($P = 0.01$*, $P = 0.001$** using ANOVA test and Sheffe's comparison method). Also, for unesterified [^{3}H]arachidonate (AA) in ox-LDL-treated cells, differences were significant ($P = 0.01$) between 15 and 30 min, and between 30 min and 60 min. Student's *t*-test indicated that the level of unesterified [^{3}H]arachidonate was significantly higher in ox-LDL-treated cells (open circles) at time 30 min ($P = 0.05$) and time 60 min ($P = 0.01$) than in n-LDL-treated cells (closed triangles) (significance not shown in the figure).

ent results show that the stimulation of HUVEC by ox-LDL results in an enhanced accumulation of [^{3}H]arachidonic acid concomitant with a reduction in the PI fraction. In order to establish whether the increase in [^{3}H]arachidonic acid was due to a defect of reacylation or to the hydrolysis of PI and whether a link existed between the ox-LDL-induced PI turnover and ox-LDL-induced PAI-1 secretion, the effects of phospholipase inhibitors on PI and arachidonate turnover and on PAI-1 secretion were investigated. To this end, HUVEC were first incubated for 1 h with two well-known phospholipase inhibitors at various concentrations, ranging between 1.5–12.0 μM and 0.35–3.0 μM for mepacrine and 4-BPB, respectively. These concentrations were selected for their failure to induce cytotoxicity.

The effect of phospholipase inhibitors on [^{3}H]arachidonic acid turnover in HUVEC was examined in the PL and neutral lipid fractions using the concentration of inhibitors that induced the maximum inhibition of PAI-1 secretion. As shown in **Fig. 3,** the decrease in PI (−21%) under ox-LDL stimulation was almost abolished by the phospholipase inhibitors, suggesting that they prevented the ox-LDL-induced degradation of PI. Other lipids were not significantly affected by inhibitors (data not shown). Furthermore, the increase in the intracellular release of unesterified [^{3}H]arachidonic acid observed with ox-LDL (+82%) was totally suppressed by 4-BPB, whereas the increase fell from 82 to 23% with mepacrine. As shown in **Fig. 4,** ox-LDL induced about 100% stimulation of PAI-1 secretion, which confirms our previous results (14). It is worthy to note that this stimulation was impaired by phospholipase inhibitors as it was totally suppressed at their highest concentration. This inhibition appears more specifically dose-dependent for 4-BPB than for mepacrine. The fact that, for the highest concentrations of inhibitors, PAI-1 secretion returned to its basal level under ox-LDL stimulation strongly indicates that the combination of phospholipase inhibitor and ox-LDL was not cytotoxic within the duration of the incubation. In control HUVEC (no LDL treatment), the phospholipase inhibitors did not affect the basal secretion of PAI-1, even at the highest concentrations (data not shown).

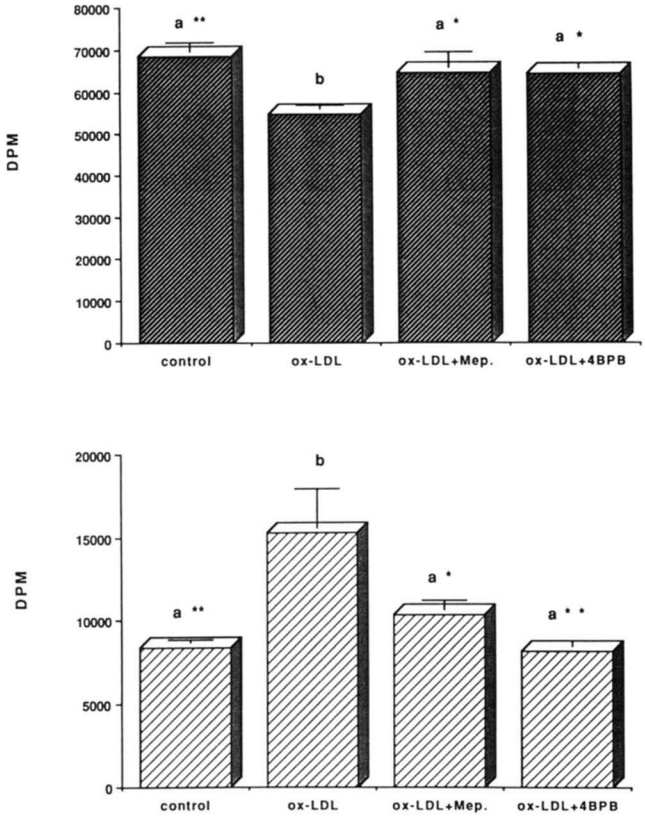

Fig. 3. Effects of phospholipase inhibitors on PI level (upper panel) and [^{3}H]arachidonate release (lower panel) in [^{3}H]arachidonate-labeled HUVEC incubated for 30 min with ox-LDL. "Control" are labeled cells without incubation with LDL. "Ox-LDL" are cells incubated for 30 min with 50 μg/ml of ox-LDL. Protocols of inhibitor studies are detailed in Experimental Procedures. The final concentration of mepacrine (Mep) in the medium was 12 μM and 4-BPB was 3.0 μM. Two separate experiments (two different LDL and HUVEC preparations) were performed in duplicate. Bars having different superscript letters are statistically different at $P = 0.01^{**}$ and $P = 0.05^{*}$.

Effects of PI degradation products on PAI-secretion

The above results suggest that the hydrolysis of PI elicited by ox-LDL is responsible for the increase in PAI-1 secretion. Therefore, to examine if the hydrolysis products of PI were able to induce an increase in PAI-1 secretion, we incubated HUVEC with 1, 10, or 100 μM of either arachidonic acid or lysoPI for 24 h. We checked that the highest concentration of lysoPI did not damage the HUVEC membranes by measuring lactate dehydrogenase (LDH) release in the culture medium. No significant increase in PAI-1 secretion was observed with either arachidonic acid or lysoPI treatment.

Effects of 4-BPB on HUVEC PLA and PI-PLC activities

We carried out in vitro assays of PLA using HUVEC homogenates as the enzyme source and radioactive PC or PI, labeled on the *sn*-2 position, as substrate. For both, PLA activity was determined with 40 μg of protein for 15 min, which was in the linear range of protein and time-dependence (data not shown). As shown in **Fig. 5,** optimum activity was found at pH 7.4 and 7.0 on PC and PI, respectively.

The positional specificity of PLA could not be determined because of the high lysophospholipase activity present in HUVEC. This activity (63.0 ± 2.5 nmol/h per mg), which is much higher than that of PLA, is not rate-limiting and is thus more than sufficient to remove all the lyso-compounds formed during PLA action (27). Using di[1-^{14}C]oleoylPC as substrate, no significant radioactivity could be detected after TLC in the lysoPC area,

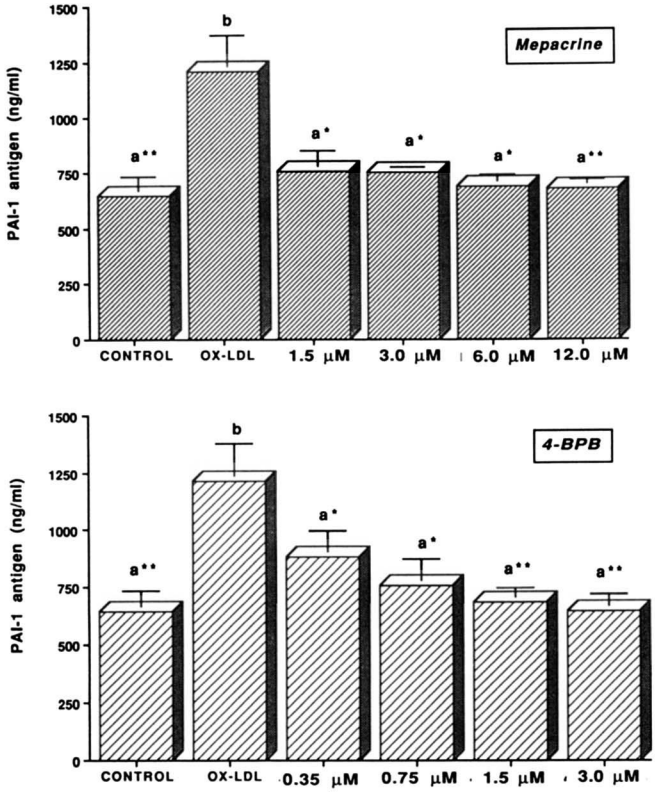

Fig. 4. Dose-dependent effects of various concentrations of phospholipase inhibitors, mepacrine (upper panel), and 4-BPB (lower panel), on PAI-1 secretion in the culture medium. Protocols of inhibitor studies are detailed in Experimental Procedures. Two separate experiments (two different LDL and HUVEC preparations) were performed in duplicate. Bars having different superscript letters are statistically different: at $P = 0.01^{**}$ and $P = 0.05^{*}$.

which confirmed that all the lysoPC formed were rapidly degraded. Attempts (effects of pH, detergent, calcium) to limit the lysophospholipase activity, without affecting PLA activity during its assay, were unsuccessful. If it is not the same enzyme that bears the two activities (PLA + lysophospholipase) as demonstrated for an intestinal brush-border phospholipase (34), separation of these two activities after a partial purification is thus necessary to determine the positional specificity. The data presented in **Table 1** illustrate the effect of calcium and 4-BPB on PLA activity. It appeared that the PLA of HUVEC did not require calcium for its full activity as 5 mM EDTA gave the highest specific activity. This was demonstrated with PC as substrate. This could not be demonstrated with PI as at pH 7.0 and with 5 mM calcium we cannot distinguish between the releases of the radioactive arachidonate coming from the PLA pathway and from the PI-PLC plus diglyceride lipase pathway (see below). The PLA of HUVEC was inhibited by 4-BPB. The inhibition attained 60 and 37% at 10 μM on PC and PI, respectively, and was quite complete at 1 mM of the inhibitor on PC and PI. Inasmuch as the [1-^{14}C]linoleic acid released from the *sn*-2 position may come from PLA_1 plus lysophospholipase activities, we investigated whether 4-BPB might also inhibit the lysophospholipase. As Table 1 shows, 4-BPB inhibited the enzyme as at 100 μM, 70% inhibition was observed under conditions of pH and EDTA concentration identical to those used for PLA assay. These results allowed us to conclude that 4-BPB was able to inhibit the PLA pathway, but it cannot be ascertained whether it is via a PLA_2 or a PLA_1 plus lysophospholipase pathway.

The PI-PLC activity was determined with 40 μg of protein and for 10 min which was in the linear range of protein and time-dependence. Optimum pH was 5.0 and 40% of the activity was still present at pH 7.0 (not shown). As indicated in Table 1, PI-PLC activity is dependent on the presence of calcium in the incubation as 5 mM EDTA completely abolished the activity. The PI-PLC activity was hardly affected by 4-BPB as 10 μM inhibited the activity by 19% and 1 mM by 56%.

DISCUSSION

The aim of this work was to obtain insights into the intracellular mechanisms leading to the stimulation of PAI-1 synthesis and secretion induced by ox-LDL in HUVEC. The labeling of HUVEC lipids by [^{3}H]arachidonic acid allowed us to demonstrate that upon addition of ox-LDL, a significant accumulation of unesterified [^{3}H]arachidonic acid occurred which was concomitant with a decrease in PI content. This may be the result of either a defect of reacylation or of enhanced degradation.

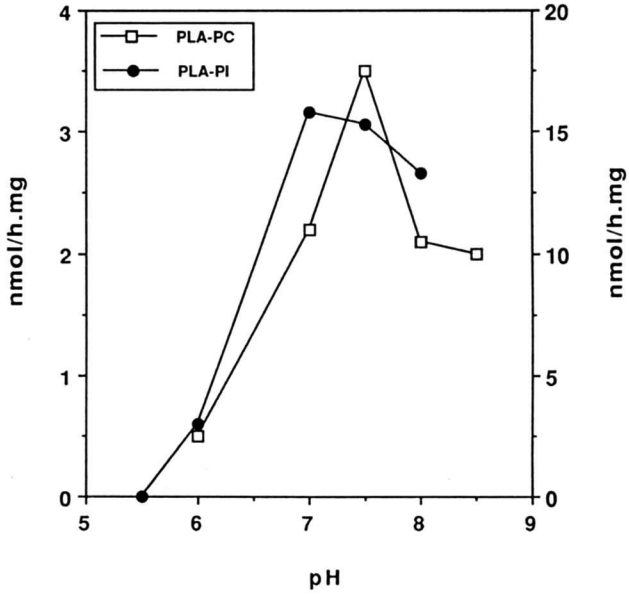

Fig. 5. Effect of pH on PI (filled circles, right scale) and PC (open squares, left scale) hydrolysis by PLA. Substrate concentration was 0.2 mM. Between pH values of 5.5 and 6.5, pH was adjusted with sodium cacodylate, and with Tris-HCl between pH 7.0 and 8.5.

TABLE 1. Effect of $CaCl_2$ and 4-BPB on PLA, PI-PLC, and lysophospholipase activities of HUVEC

Conditions	Specific Activity			
	PLA			
	PC	PI	PI-PLC	LPLA
	%			
EDTA 5 mM[a]	100	100	0	71
$CaCl_2$ 5 mM[a]	57		100	100
$CaCl_2$ 10 mM[a]	44		100	n.d.
4-BPB 10 μM[b] + EDTA 5 mM	40	63		55
4-BPB 100 μM[b] + EDTA 5 mM	8	36		28
4-BPB 1 mM[b] + EDTA 5 mM	8	7		n.d.
4-BPB 10 μM[b] + $CaCl_2$ 5 mM	20		81	n.d.
4-BPB 100 μM[b] + $CaCl_2$ 5 mM			61	n.d.
4-BPB 1 mM[b] + $CaCl_2$ 5 mM			44	n.d.

Enzyme activities were assayed as described in Experimental Procedures. PLA was assayed either on PC or PI as substrate. LPLA, lysophospholipase; n.d., not determined.

[a]Calcium and EDTA were pre-incubated with HUVEC homogenate (40 μg protein) at 4°C before initiating the reaction at 37°C (10 min, pH 7.4) with the substrate.

[b]The 4-BPB, solubilized in ethanol (final ethanol concentration in the assay 2.5%), was pre-incubated with HUVEC homogenate at 4°C for 60 min before starting the reaction. To take into account the effect of ethanol on enzyme activity, control HUVEC homogenates were pre-incubated under the same conditions of ethanol concentration. Values are from two separate HUVEC preparations.

However, the fact that phospholipase inhibitors clearly prevented the [^{3}H]arachidonic acid release as well as the degradation of PI supports the idea that phospholipase activity is involved in both [^{3}H]arachidonate release and PI degradation. This result is compatible with others, showing that in endothelial cells, some stimuli such as thrombin, histamine, or bradykinin trigger PLA_2 activation and that PI was, with PE, an important source of arachidonic acid release (19, 20). An inhibition of [^{3}H]arachidonic acid release by phospholipase inhibitors has also been reported in bradykinin-stimulated porcine aortic endothelial cells (18), in hydrogen peroxide-stimulated HUVEC (35), and in melittin-stimulated hydrolysis of trophoblast PI (36). Several pathways may contribute to [^{3}H]arachidonic acid release in stimulated HUVEC. Among them, a direct release from membrane PL by PLA_2, PLA_1 followed by a lysophospholipase, or PLC followed by a diglyceride lipase are the most likely candidates. The metabolism of PI and PC has been thoroughly investigated in bovine aortic pulmonary endothelial cells by Martin et al. (37, 38). Two pathways for arachidonic acid release from PI have been identified. One is the PLA_1 plus lysophospholipase pathway (pH optimum 7.7) that is calcium-independent and the other a phospholipase C plus diglyceride lipase pathway that is calcium-dependent and active at pH 5.0 and 7.0. In these studies (37, 38), no PLA_2 activity could be detected. Using PC as substrate, the authors found a PLA activity at pH 7.5, calcium-independent and totally inhibited by 140 μM of 4-BPB. We obtained quite similar results with HUVEC. The PI-PLC was fully active at pH 5.0 with 5 mM calcium but we did not find a second peak at pH 7.0. The PLA, measured on PC or PI, was fully active at pH 7.4 with EDTA and inhibited by 4-BPB. The results we obtained from in situ experiments, using [^{3}H]arachidonic acid pre-labeled cells did not allow us to distinguish which pathway was involved in the ox-LDL-induced stimulation of PI degradation. However, the fact that the HUVEC PLA pathway was demonstrated in vitro to be much more sensitive to 4-BPB than PI-PLC and that 4-BPB prevented the ox-LDL-induced degradation of PI strongly suggests that, within the time-range of stimulation studied, a PLA (PLA_1 or PLA_2) was involved in the activation process. Recently, Hirata, Akita, and Yokoyama (39) showed that LDL peroxidized by copper elicited the release of inositol phosphate 1 and 2 when incubated for 30 min with bovine aortic endothelial cells. Therefore, to unequivocally prove which kind of phospholipase is involved under our experimental conditions, characterization and measurement of the respective activities under stimulated conditions are needed. This is under current investigation.

We tried to establish whether a link existed between PI hydrolysis and PAI-1 secretion. Using PLA inhibitors under ox-LDL treatment, we demonstrated that they strongly abolished the stimulation of both PAI-1 secretion and PI degradation, suggesting a link between both. But this could be also the result of an independent simultaneous action of the inhibitors on PLA and PAI-1 secretion. It does not seem to be the case as under control conditions (no LDL stimulation), PLA inhibitors did not affect the basal secretion of PAI-1. This suggests that *i)* the effect of inhibitors on the stimulation of PAI-1 secretion under ox-LDL treatment was causally related to the inhibition of the stimulation of PI hydrolysis; and *ii)* that a PI-PLA hydrolysis product (arachidonate or lysoPI) could be an intracellular second messenger in activating PAI-1 synthesis and secretion. However, we were unable to mimic the effect of PI-PLA activation by incubating HUVEC with PI-PLA hydrolysis products. This may simply indicate that adding a second messenger extracellularly will not give the same response as that observed when this second messenger is generated intracellularly. This is because the second messenger must be liberated at a precise intracellular site to be efficient for the expected response. This is particularly the case for lipid second messengers that can be metabolized by the cell before reaching their site of action. For example, arachidonate can be rapidly incorporated into membrane PL and lysoPI can be either reacylated by an acyltransferase or degraded by a lysophospholipase. We have demonstrated the latter very active in HUVEC. Although stronger evidence of a link between PI hydrolysis and PAI-1 secretion is needed, we can hypothesize that *i)* under basal conditions (unstimulated

HUVEC), PAI-1 secretion is not linked to PI hydrolysis; and *ii)* under ox-LDL binding and internalization, an activation pathway would be triggered, involving a product of PLA-induced PI degradation, leading to an increased pathological secretion of PAI-1.

It should be noted that at 30 min, n-LDL tended to stimulate [^{3}H]arachidonic acid release together with a slight degradation of PI. This may be in accordance with previous results showing that native LDL may contribute to endothelial PGI_2 production, even if this contribution is modest (40); this could be explained by either the low level of TBARS initially present in n-LDL or those generated during oxidative modification of n-LDL once internalized in HUVEC (41). This low level of TBARS may be sufficient to slightly stimulate PI degradation, but not high enough to lead to an enhanced PAI-1 expression and secretion. For example, it has recently been demonstrated that native LDL increased the 3.4 kb mRNA species of PAI-1 mRNA but not the 2.4 kb species, so that PAI-1 functional activity was not expressed (13). On the other hand, Lp[a] which was able to increase the two mRNA species, stimulated the production of active PAI-1 (13).

A good candidate for inducing the signal for PI degradation could be the lipid peroxides carried by LDL. Several studies support this hypothesis. In endothelial cells, peroxides were found to stimulate PGI_2 production (21, 35, 42, 43). A 30-min incubation of pulmonary porcine endothelial cells with a hydroperoxide of linoleic acid elicited the degradation of PI both by PLA and PLC (21). Interestingly, it has been shown that ox-LDL were able to stimulate PGI_2 production in endothelial cells (44) and peritoneal macrophages (45). Also, we have tested the effect of different concentrations (0.1–10 μg/ml) of fucoidan (a scavenger receptor ligand) on PAI-1 release. We could not detect any modification in PAI-1 secretion after a 24-h incubation (M. Chautan, unpublished data). All these data underline the importance of peroxide-LDL content in the stimulation process described herein.

In conclusion, our results allow us to suggest that the ox-LDL-induced stimulation of PAI-1 production is mediated by a phospholipase, probably a PLA, acting on PI. As it appears that pathologic situations contributing to an increased level of peroxide (LDL of atherosclerotic patients, VLDL of diabetic patients) may contribute to the development of thrombogenic events at the surface of the endothelium (11–13, 46), it remains to be established whether atherogenic lipoproteins isolated from plasma also trigger PLA activation in endothelial cells. This could provide new insights into the pathological vascular process of atherothrombosis.

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