Lysophosphatidylcholine and 7-oxocholesterol modulate Ca^{2+} signals and inhibit the phosphorylation of endothelial NO synthase and cytosolic phospholipase A_2

Elisabeth MILLANVOYE-VAN BRUSSEL*, Gökçe TOPAL*, Annie BRUNET*, Thuc DO PHAM*, Valérie DECKERT†, Francine RENDU* and Monique DAVID-DUFILHO*¹

*UMR 7131 CNRS, Hôpital Broussais, Paris, France, and †Laboratoire de Biochimie des Lipoprotéines, INSERM U498, Faculté de Médecine, Dijon, France

The oxidation of plasma LDLs (low-density lipoproteins) is a key event in the pathogenesis of atherosclerosis. LPC (lysophosphatidylcholine) and oxysterols are major lipid constituents of oxidized LDLs. In particular, 7-oxocholesterol has been found in plasma from cardiac patients and atherosclerotic plaque. In the present study, we investigated the ability of 7-oxocholesterol and LPC to regulate the activation of eNOS (endothelial nitric oxide synthase) and cPLA₂ (cytosolic phospholipase A₂) that synthesize two essential factors for vascular wall integrity, NO (nitric oxide) and arachidonic acid. In endothelial cells from human umbilical vein cords, both 7-oxocholesterol (150 μM) and LPC (20 μM) decreased histamine-induced NO release, but not the release activated by thapsigargin. The two lipids decreased NO release through a PI3K (phosphoinositide 3-kinase)-dependent pathway, and decreased eNOS phosphorylation. Their mechanisms of action were, however, different. The NO release reduction was dependent on superoxide anions in LPC-treated cells and not in 7-oxo-

cholesterol-treated ones. The Ca^{2+} signals induced by histamine were abolished by LPC, but not by 7-oxocholesterol. The oxysterol also inhibited (i) the histamine- and thapsigargin-induced arachidonic acid release, and (ii) the phosphorylation of both cPLA₂ and ERK1/2 (extracellular-signal-regulated kinases 1/2). The results show that 7-oxocholesterol inhibits eNOS and cPLA₂ activation by altering a Ca^{2+} -independent upstream step of PI3K and ERK1/2 cascades, whereas LPC desensitizes eNOS by interfering with receptor-activated signalling pathways. This suggests that 7-oxocholesterol and LPC generate signals which cross-talk with heterologous receptors, effects which could appear at early stage of atherosclerosis.

Key words: cytosolic phospholipase A₂, endothelial cell, extracellular-signal-regulated kinases 1/2 (ERK1/2), 7-oxocholesterol, lysophosphatidylcholine, nitric oxide.

INTRODUCTION

Endothelial dysfunction represents a key early step in the development of atherosclerosis and is responsible for plaque progression and atherosclerotic complications [1]. Endothelial dysfunction is characterized by an imbalance between relaxing and contracting factors, pro-coagulant and anti-coagulant substances, and between pro-inflammatory and anti-inflammatory mediators [2]. Nitric oxide (NO) is a potent vasodilator essential in the maintenance of vascular wall integrity. It is synthesized from L-arginine by NOS (NO synthase) and it inhibits platelet aggregation and adhesion, leucocyte migration and adhesion, and smooth muscle cells migration and proliferation [3,4]. In addition, NO plays a fundamental role in the development of atherosclerosis by stimulating or inhibiting lipid peroxidation reactions and lipid or lipoprotein oxidation, according to the underlying oxidative status of the vascular tissue [5,6]. Peroxidation of membrane lipids affects the activity of the cPLA₂ (cytosolic phospholipase A₂) that releases arachidonic acid by hydrolysing phospholipids [7]. Arachidonic acid is the precursor of eicosanoids, especially prostacyclin and thromboxane A₂ that exert opposite action on vascular tone and platelet aggregation [8].

Clinical studies have shown that the elevation of plasma LDLs (low-density lipoproteins) and their oxidation are associated with impairment of endothelium-mediated vasodilation

in the earliest stage of atherosclerosis [9,10]. Oxidation of LDL result in extensive conversion of phosphatidylcholine to LPC (lysophosphatidylcholine) and formation of cholesteryl ester core aldehydes, which are further converted into free- and apolipoprotein-B-bound oxysterols, and, in particular, 7-oxocholesterol [11,12]. Although it is well documented that LPC has inflammatory activities and activates signalling molecules, such as Ca^{2+} , arachidonic acid or tyrosine kinases [13–15], the specific cell-surface receptor by which it mediates signals at the vessel wall has not yet been clearly characterized in endothelial cells. In addition to LPC, oxysterols may also contribute to the development of atherosclerosis [16]. Increased plasma levels of β -epoxycholesterol and 7-oxygenated oxysterols have been observed in patients with coronary artery diseases [17,18].

In a previous study, we demonstrated that cholesterol derivatives oxidized at the 7 position reduce agonist-activated NO release in human endothelial cells [19]. In the present study, we investigated the ability of 7-oxocholesterol and LPC to regulate the signalling pathways involved in eNOS (endothelial NOS) and cPLA₂ activation. The results suggest that 7-oxocholesterol inhibits eNOS and cPLA₂ activation by altering a Ca^{2+} -independent step upstream of PI3K (phosphoinositide 3-kinase) and ERK1/2 (extracellular-signal-regulated kinases 1/2) cascades, whereas LPC desensitizes eNOS by interfering with the signalling pathways activated by histamine.

Abbreviations used: BHT, butylated hydroxytoluene; $[\text{Ca}^{2+}]_i$, cytosolic Ca^{2+} concentration; cPLA₂, cytosolic phospholipase A₂; ERK1/2, extracellular-signal-regulated kinases 1/2; GPCR, G-protein-coupled receptor; HUVEC, human umbilical vein endothelial cell; LDL, low-density lipoprotein; LPC, lysophosphatidylcholine; MEK, MAPK/ERK kinase; NOS, nitric oxide synthase; eNOS, endothelial NOS; OSBP, oxysterol-binding protein; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; p38 MAPK, p38 mitogen-activated protein kinase.

¹ To whom correspondence should be addressed (e-mail monique.dufilho@bbs.ap-hop-paris.fr).

EXPERIMENTAL

Materials

Culture medium and products were purchased from Invitrogen (Cergy-Pontoise, France). fura-2/AM (fura 2 acetoxymethyl ester) was obtained from Molecular Probes (Eugene, OR, U.S.A.). The polyclonal antibodies against phospho-Ser¹¹⁷⁷-eNOS, phospho-Ser⁵⁰⁵-cPLA₂, phospho-ERK1/2, phospho-p38 MAPK (p38 mitogen-activated protein kinase) and p38 MAPK, and monoclonal antibody against ERK1/2, were from Cell Signaling Technology (Beverly, MA, U.S.A.). The monoclonal antibodies against eNOS and cPLA₂ were from BD Transduction Laboratory (Heidelberg, Germany) and Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) respectively. The [1-¹⁴C]arachidonic acid was obtained from PerkinElmer (Boston, MA, U.S.A.). All other products were purchased from Sigma-Aldrich Chimie (St Quentin Fallavier, France) or Bio-Rad Laboratories (Hercules, CA, U.S.A.).

LDL was isolated by sequential ultracentrifugation from fresh citrated plasma from normolipidaemic donors, and the oxidative modification of LDL was performed as described previously [20]. Briefly, 1.2 mg of protein/ml LDL was incubated for 24 h at 37 °C in PBS containing 5 µM CuSO₄, and oxidation was stopped by adding 200 µM EDTA and 20 µM BHT (butylated hydroxytoluene). Native LDL was maintained for 24 h at 4 °C in PBS containing EDTA and BHT. Both native and oxidized LDL were dialysed overnight against PBS. The samples we used contained 158 and 38 µg/ml LPC, 8.62 and 1.28 µg/ml 7α-hydroxycholesterol, 20.43 and 1.39 µg/ml 7β-hydroxycholesterol, and 173 and 2.6 µg/ml 7-oxocholesterol for oxidized and native LDL respectively.

Cell culture and treatments

Endothelial cells were isolated from segments of human umbilical cord vein by collagenase digestion. HUVECs (human umbilical vein endothelial cells) were cultured in medium 199 (40 %) and RPMI 1640 (40 %) supplemented with 20 % fetal calf serum as described previously [21]. At confluence (4–5 days), cells were detached by incubation for 1–2 min at 22 °C with 0.01 % trypsin and 0.004 % EDTA, washed and grown until subconfluence to be used at the first passage. HUVECs were cultured on to glass coverslips (9 mm × 35 mm) for [Ca²⁺]_i (cytosolic Ca²⁺ concentration) determinations, or on to 35- or 60-mm plastic culture dishes for NO, arachidonic acid measurements or Western blot analysis. Three different cell treatments were performed: (1) incubation for 2 h at 37 °C in culture medium with either 50 µg of protein/ml native or oxidized LDL or with 155 µM (60 µg/ml) cholesterol and 20 µM BHT to prevent cholesterol oxidation [19] or with 150 µM (60 µg/ml) 7-oxocholesterol (50 mM stock solutions in ethanol); (2) incubation for 15 min at 37 °C in respective reaction buffers with 20 µM LPC (20 mM stock solution in ethanol); (3) incubation for 30 min in culture medium in the dark with inhibitors PD98059 or LY294002 (20 µM stock solution in DMSO). After treatment, cells were washed and then incubated in the respective reaction buffers. The amount of proteins per dish, measured as described previously [22], was not altered by the treatment.

Determination of [Ca²⁺]_i

HUVECs were firstly equilibrated for 30 min with the reaction buffer (136 mM NaCl, 5 mM KCl, 2 mM Na₂PO₄, 0.4 mM MgSO₄, 4 mM NaHCO₃, 1 mM CaCl₂, 8 mM glucose, 2 mM gluta-

mine, 25 mM Hepes, and a cocktail of amino acids, pH 7.4) containing 20 % fetal calf serum and loaded with 2 µM fura-2 acetoxymethyl ester as described previously [21]. Fluorescence intensities were recorded on a spectrofluorimeter SPEX CMIII (ISA-Jobin-Yvon, Longjumeau, France). Each measurement was systematically corrected for autofluorescence of unloaded cells and the calibration parameters of fura-2 signals were determined as described previously [21].

Measurements of NO

Cells were incubated for 15 min at 37 °C in PBS containing 5 mM glucose, 0.5 mM MgCl₂ and 1 mM CaCl₂ (PBS-MgCa). NO concentration was measured at the surface of HUVECs by differential pulse amperometry with a three-electrode potentiostatic Biopulse system (Tacussel-Radiometer, Lyon, France) and a porphyrinic NO-selective microsensor, as described previously [23]. NO sensor was calibrated at both the beginning and the end of measurements in the presence of cells, by the addition of NO standard solutions as described previously [24]. The calibration curves were similar for control and treated cells. The electrode sensitivity was 1.5 ± 0.2 pA/nM NO (*n* = 17). NO release was determined as the maximum of the agonist-induced oxidation current.

Quantification of arachidonic acid release

Arachidonic acid release, that reflects cPLA₂ activation under our experimental conditions, was determined as reported previously [22]. HUVECs labelled for 20 h with 0.06 µCi of [1-¹⁴C] arachidonic acid were washed, treated with cholesterol or 7-oxocholesterol, and then stimulated by histamine or thapsigargin for 10 min in PBS-MgCa. Cholesterol, 7-oxocholesterol or kinase inhibitors did not modify arachidonic acid release in unstimulated cells.

Immunoblotting

Cells, incubated in PBS-MgCa, were washed and harvested in ice-cold buffer containing 150 mM sodium chloride, 50 mM Tris, 1 mM EDTA, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin and 1 % (v/v) Nonidet P-40. The lysates were cleared by centrifugation at 12 000 *g* for 15 min at 4 °C. The different samples containing equal amounts of protein were resolved by SDS/PAGE (8 % gel). The separated proteins were transferred electrophoretically on to nitrocellulose membranes and non-specific binding sites were blocked in Tris-buffered saline containing 0.1 % Tween 20 and 5 % non-fat dried milk. Membranes were incubated overnight with primary antibodies against phospho-ERK, -p38 MAPK, -cPLA₂ or -eNOS, or against corresponding non-phosphorylated enzymes. The washed membranes were then incubated with horseradish-peroxidase-conjugated secondary antibody. The immunoreactive bands were detected by an enhanced chemiluminescence system and quantified by densitometry using National Institutes of Health Image 1.61 software.

Statistical analysis

Results are expressed as the means ± S.E.M. for *n* independent experiments. Multiple comparisons and time-dependent effect were examined by one-way analysis of variance with post hoc Fisher's test, with *P* < 0.05 considered as statistically significant.

Figure 1 Effects of oxidized LDL constituents on NO and arachidonic acid release

NO production (**A, B, C** and **D**), and arachidonic acid release (**E** and **F**) were measured in HUVECs pretreated for 2 h with either vehicle (control), or 50 μ g of protein/ml native LDL (nLDL) or oxidized LDL (oxLDL), 150 μ M cholesterol (chol) or 7-oxocholesterol (7-keto) in culture medium, or for 15 min with 20 μ M LPC in PBS-MgCa. NO was detected by differential pulse amperometry. Basal [14 C]arachidonic acid release measured in unstimulated cells incubated for 10 min at 37 °C in PBS-MgCa ($1.8 \pm 0.3 \times 10^3$ d.p.m./mg of protein) was subtracted from that determined in cells stimulated for 10 min by histamine (**E**) or thapsigargin (**F**). Results are expressed as the means $\pm$ S.E.M for 6 independent experiments. ** $P < 0.01$ and *** $P < 0.001$ when compared with control cells.

RESULTS

Effects of LDL and its lipid constituents on NO and arachidonic acid release

The stimulation of HUVECs by histamine, an agonist of the GPCR (G-protein-coupled receptor) H₁, or by thapsigargin, a selective inhibitor of endoplasmic reticulum Ca²⁺-ATPase, activated both NO and arachidonic acid release (Figure 1). Histamine induced a transient NO production that progressively returned to basal levels (Figure 1A). The cell treatment with 7-oxocholesterol or LPC inhibited the histamine-induced NO release by 36 ± 4 or 50 ± 7 % respectively ($n = 6$, $P < 0.001$ or $P < 0.01$ respectively), whereas that with cholesterol had no effect (95 ± 14 %) (Figure 1A). Similarly, oxidized LDL decreased NO release by 44 ± 12 %, whereas native LDL had no effect (Figure 1B). In contrast with histamine, thapsigargin progressively increased the NO concentrations to a plateau that remained stable during 3–4 min. Cholesterol, 7-oxocholesterol or LPC treatment did not affect the thapsigargin-activated NO release (Figures 1C and 1D). The result indicates that both 7-oxocholesterol and LPC require the receptor-dependent activation of eNOS to reduce NO release.

Histamine and thapsigargin also activated cPLA₂, leading to arachidonic acid release (Figures 1E and 1F). As expected, pretreatment of HUVECs by cholesterol had no effect on the arachidonic acid release, whereas 7-oxocholesterol was a strong inhibitor (over 63 % inhibition). The LPC effects were not studied, since it has been previously demonstrated that this phospholipid activates the arachidonic acid release [14]. Native and

Figure 2 Regulation of histamine-induced eNOS phosphorylation and Ca²⁺ signals by 7-oxocholesterol and LPC

Time-course of phosphorylation of Ser¹¹⁷⁷ on eNOS induced by 10 μ M histamine (**A**) ($n = 3$) is measured as described in the Experimental section. Proteins from cell lysates were resolved by SDS/PAGE and immunoblots were probed with anti-(phospho-Ser¹¹⁷⁷-eNOS) or anti-eNOS antibodies (p-eNOS, phospho-eNOS). Histamine-induced Ca²⁺ signals (**B**) ($n = 4-9$) and eNOS phosphorylation (**C**) ($n = 4-7$) were measured in HUVECs pretreated for 2 h with either vehicle (control) or 150 μ M 7-oxocholesterol (7-keto) or for 15 min with 20 μ M LPC. Results and graph from densitometric analysis represent the means $\pm$ S.E.M. * $P < 0.01$ when compared with untreated histamine-stimulated cells.

oxidized LDLs induced non-significant increases in arachidonic acid release in histamine- (25 ± 18 and 24 ± 16 % respectively) and thapsigargin-stimulated cells (35 ± 18 and 29 ± 15 % respectively). Of note, 7-oxocholesterol alone inhibited the arachidonic acid release, although LDLs tend to increase the arachidonic acid release.

It is generally admitted that oxidation of LDLs results in a decreased NO availability by increasing O₂⁻ (superoxide anions) production. In order to examine the participation of extracellular O₂⁻ in the 7-oxocholesterol and LPC effects, HUVECs were stimulated by histamine in the presence of superoxide dismutase to scavenge O₂⁻. In the presence of 200 units/ml superoxide dismutase, the inhibition of NO release by 7-oxocholesterol was maintained (10 ± 1 versus 23 ± 3 nM in control, $n = 6$), whereas that by LPC was suppressed (26 ± 5 versus 23 ± 3 nM, $n = 6$). These results show that the two major lipid constituents of oxidized LDL act differently, since the LPC effect was dependent of extracellular O₂⁻, not that of 7-oxocholesterol.

7-Oxocholesterol and LPC effects on Ca²⁺ signals and eNOS phosphorylation

To study further the different signalling pathways which LPC and 7-oxocholesterol could effect, we studied eNOS activation. eNOS is activated by intracellular Ca²⁺ signals and by phosphorylation cascades. Histamine induced a transient phosphorylation of Ser¹¹⁷⁷ on eNOS and a biphasic Ca²⁺ signal (Figure 2). The eNOS phosphorylation was maximal between 1 and 2 min, and progressively returned to basal levels within 20 min. (Figure 2A). The Ca²⁺ peak, corresponding to Ca²⁺ mobilization from internal stores, is reached 30 s after histamine addition and is followed by a sustained plateau reflecting Ca²⁺ entry from the extracellular

Figure 3 Role of PI3K in histamine-induced eNOS activation

Dose-dependent effects of PI3K inhibitor LY294002 on histamine-activated NO release (**A**) and phosphorylation of Ser¹¹⁷⁷ on eNOS (**B**) (p-eNOS, phospho-eNOS) were measured in cells treated for 30 min with 10 μ M histamine before stimulation. Results are expressed as a percentage of the control, i.e. untreated cells ($n=4-8$). The effects of 7-oxocholesterol and LPC (**C**) were determined in cells pretreated either for 90 min with vehicle (control) or 150 μ M 7-oxocholesterol (7-keto), and further incubated for 30 min with vehicle or 0.5 μ M LY294002, or for 15 min with vehicle or 0.5 μ M LY294002 and further incubated for 15 min with 20 μ M LPC. Cells were then stimulated by 10 μ M histamine ($n=4$). All results are expressed as the means $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$ compared with control cells in the absence of LY294002.

space (Figure 2B). Cholesterol treatment did not modify basal $[Ca^{2+}]_i$ or the histamine-induced Ca^{2+} signals and phosphorylation (results not shown). Both 7-oxocholesterol and LPC treatments increased the basal $[Ca^{2+}]_i$ from 117 ± 15 nM to 177 ± 18 nM and 243 ± 47 nM ($P < 0.05$ and $P < 0.01$) respectively. However, the two lipids altered the Ca^{2+} response to histamine differently. The Ca^{2+} signals were increased by $86 \pm 22\%$ by 7-oxocholesterol, whereas they were totally inhibited by LPC (Figure 2B). In contrast, both lipids reduced the histamine-induced eNOS phosphorylation (Figure 2C). The results suggest that 7-oxocholesterol decreases NO release by inhibiting the eNOS phosphorylation through a Ca^{2+} -independent mechanism, whereas LPC inhibits both Ca^{2+} signals and eNOS phosphorylation.

Since eNOS is phosphorylated independently of Ca^{2+} through the PI3K-Akt pathway [25], we studied the effect of the PI3K inhibitor, LY294002. The latter dose dependently inhibited the histamine-activated NO release with a biphasic effect of an apparent IC_{50} of 0.1 μ M and 80% reduction at 20 μ M (Figure 3A). From 50 nM to 50 μ M, however, LY294002 reduced the histamine-induced eNOS phosphorylation at Ser¹¹⁷⁷ by only 30% (Figure 3B), indicating that other residues than Ser¹¹⁷⁷ were phosphorylated on eNOS through the PI3K cascade. Cell treatment by LY294002 following that with 7-oxocholesterol or preceding that with LPC did not further decrease the NO release (Figure 3C). Furthermore, the histamine-activated NO release did not depend on ERK1/2 pathways, since the MEK1/2 (MAPK kinase) inhibitor PD98059 (from 5 to 50 μ M) had no effect on it (16 ± 3 versus 14 ± 2 nM in control cells, $n=5$). Altogether the results suggest that the two lipids alter the PI3K-dependent eNOS activation.

Figure 4 Effects of 7-oxocholesterol on histamine-induced phosphorylation of ERKs, p38 MAPK and cPLA₂

Phosphorylation was measured in histamine-stimulated HUVECs by Western-blot analysis using antibodies directed against phospho-ERKs (p-ERK1/2), -p38 MAPK (p-p38) or -Ser⁶⁰⁵ cPLA₂ (p-cPLA₂). Levels of ERK1/2, p38 MAPK or cPLA₂ were assessed using antibodies against the non-phosphorylated forms. (**A**) Time course of ERK1/2, p38 MAPK or cPLA₂ phosphorylation in 10 μ M histamine-stimulated HUVECs. Densitometric data are expressed as the means $\pm$ S.E.M. for 3 independent experiments. (**B**) Effects of 7-oxocholesterol on ERK1/2, p38 MAPK or cPLA₂ phosphorylation in HUVECs pretreated for 2 h with either vehicle (control) or 150 μ M 7-oxocholesterol (7-keto) and stimulated for 10 min by 10 μ M histamine. Densitometric data are expressed as the means $\pm$ S.E.M. for 3–7 independent experiments. * $P < 0.05$ compared with control cells.

7-Oxocholesterol effects on ERK1/2 and cPLA₂ phosphorylation

We have investigated the ability of 7-oxocholesterol to regulate the kinase pathways involved in the cPLA₂ activation, the ERK1/2 and p38 MAPK cascades. As shown in Figure 4(A), histamine activated the phosphorylation of ERK1/2 (left-hand panel), p38 MAPK (middle panel) and cPLA₂ (right-hand panel) with similar time courses. Phosphorylation was maximal at 10 min. Cell treatment by 7-oxocholesterol inhibited histamine-induced phosphorylation of ERK1/2 and cPLA₂ by 27 ± 5 and $33 \pm 13\%$ respectively. In contrast, it had no effect on phospho-p38 MAPK (Figure 4B).

Figure 5(A) shows that thapsigargin, which acts independently of a receptor, activated the phosphorylation of ERK1/2 (left-hand panel), p38 MAPK (middle panel) and cPLA₂ (right-hand panel). The phosphorylation of ERK1/2 and cPLA₂ was intense and maximal at 10 min, and phosphorylation of p38 MAPK was slow and weak. The 7-oxocholesterol treatment resulted in 40% decrease in ERK1/2 and cPLA₂ phosphorylation, but had no effect on that of p38 MAPK (Figure 5B), indicating that the oxysterol decreases the cPLA₂ activity by inhibiting the ERK1/2 phosphorylation. Thapsigargin induced a progressive $[Ca^{2+}]_i$ increase which remained stable over several minutes (Figure 5C). The 7-oxocholesterol treatment suppressed the thapsigargin-induced Ca^{2+} signals.

To investigate whether the inhibitory effect of 7-oxocholesterol on ERK1/2 phosphorylation was direct or occurred at an upstream step, we used the MEK inhibitor PD98059. The inhibitor inhibited dose-dependently the ERK1/2 phosphorylation activated by histamine and thapsigargin with a total inhibition at 10 μ M (Figure 6A, left-hand panels). At this concentration, PD98059 reduced the phosphorylation of cPLA₂ in histamine- and thapsigargin-stimulated HUVECs by only 34 ± 2 and $26 \pm 13\%$ respectively (Figure 6A, right-hand panels). In accordance with this

Figure 5 Effects of 7-oxocholesterol on thapsigargin-induced phosphorylation of ERKs, p38 MAPK and cPLA₂, and Ca²⁺ signals

Phosphorylation was measured in thapsigargin-stimulated HUVECs by Western-blot analysis, using antibodies directed against phospho-ERKs (p-ERK1/2), -p38 MAPK (p-p38) or -Ser⁵⁰⁵ cPLA₂ (p-cPLA₂). ERK1/2, p38 MAPK or cPLA₂ were assessed using antibodies against the non-phosphorylated forms. (A) Time-course of ERK1/2, p38 MAPK or cPLA₂ phosphorylations in 1 μ M thapsigargin-stimulated HUVECs. Densitometric data are expressed as the means $\pm$ S.E.M. for 3 independent experiments. (B) Effects of 7-oxocholesterol on ERK1/2, p38 MAPK or cPLA₂ phosphorylations in HUVECs treated for 2 h with either vehicle (control) or 150 μ M 7-oxocholesterol (7-keto) and stimulated for 10 min by 1 μ M thapsigargin. Densitometric data are expressed as the means $\pm$ S.E.M. for 3–7 independent experiments. * P < 0.05 compared with control cells. (C) Time course of Ca²⁺ signals induced by 1 μ M thapsigargin in control and 7-oxocholesterol-treated cells and determined in fura-2-loaded cells as described in the Experimental section. Traces are representative of 7 independent experiments.

Figure 6 Implication of MEK kinase in cPLA₂ phosphorylation and activity

(A) Dose-dependent effects of the MEK inhibitor, PD98059, on phosphorylation of ERKs and cPLA₂. HUVECs pretreated for 30 min with increasing concentrations of PD98059 were stimulated by 10 μ M histamine or 1 μ M thapsigargin (n = 3). (B) Effects of PD98059 on histamine- or thapsigargin-activated arachidonic acid release in cells pretreated for 90 min with either vehicle (control) or 150 μ M 7-oxocholesterol (7-keto) and incubated for a further 30 min with 20 μ M PD98059. Results are expressed as the percentage of the control values (1.9 ± 0.2 10^3 d.p.m./mg of protein) measured in untreated cells (n = 3–5). * P < 0.05, ** P < 0.01 versus controls in the absence of PD98059.

observation, PD98059 inhibited the histamine- and thapsigargin-induced arachidonic release by 40 ± 5 and 35 ± 5 % respectively (Figure 6B). Interestingly, the 7-oxocholesterol inhibitory effect was greater than that of PD98059 (61 versus 40 % and 64 versus 35 % in histamine- and thapsigargin-activated cells respectively). Addition of PD98059 following 7-oxocholesterol treatment did not decrease further the histamine- or thapsigargin-induced arachidonic acid release. However, the effects of 7-oxocholesterol and PD98059 were not cumulative. Furthermore, histamine and thapsigargin activated arachidonic acid release independently of the PI3K cascade, since the PI3K inhibitor LY294002, even at the high concentration of 20 μ M, had no effect (14.0 ± 2.5 versus 17.7 ± 3.5 and 18.1 ± 0.1 versus 19.7 ± 6.6 10^3 d.p.m./mg of protein respectively). The results show that the 7-oxocholesterol inhibits cPLA₂ activity through the ERK1/2 signalling pathway.

DISCUSSION

The membrane eNOS and the cPLA₂ are two essential enzymes for endothelial function, and in particular the regulation of vascular tone, homeostasis and integrity. The present study shows that two major constituents of oxidized LDL, LPC and 7-oxocholesterol, reduced NO and arachidonic acid release, and did so through distinct mechanisms of action. LPC desensitizes eNOS by interfering with the signalling pathways of the histamine receptor. The cholesterol oxide, 7-oxocholesterol, inhibits eNOS and cPLA₂ activity by altering the PI3K- and ERK1/2-dependent phosphorylation respectively.

The eNOS activation by GPCR agonists depends on calmodulin and Ca²⁺ and on phosphorylation/dephosphorylation events that control calmodulin binding to the enzyme and the flow of electrons for NADPH oxidation [25]. The kinase cascades involved in the above processes depend on the stimulus applied; eNOS is phosphorylated either by protein kinase A, protein kinase B, AMP-activated protein kinase or PKC (protein kinase C). The cPLA₂ activation following a GPCR stimulation depends on Ca²⁺ signal, phosphorylation by ERK1/2 and p38 MAPK, and translocation from cytosol to either the perinuclear region, including the nuclear envelope, the Golgi apparatus and the endoplasmic endoplasmic reticulum or the plasma membrane [26,27].

In the present study, the LPC treatment of HUVECs increases the basal [Ca²⁺]_i values and suppresses the Ca²⁺ response to histamine. This agrees with previous studies demonstrating that LPC induced Ca²⁺ signals in a phospholipase C-dependent manner [28], and that basal [Ca²⁺]_i values did not return to their initial levels after LPC removing [13]. Indeed, LPC activates Ca²⁺ signals through the GPCR GPR4 [29], which is expressed in human endothelial cells [30]. Through its binding to GPR4, LPC may desensitize the Ca²⁺ response to histamine. In rabbit aorta, LPC inhibited the acetylcholine-induced Ca²⁺ signals and endothelium-dependent relaxation [31]. In the present study, LPC had no effect on the receptor-independent activation of NO release induced by thapsigargin, but reduced the histamine-activated NO release through a mechanism dependent on extracellular O₂^{•−}. It has been demonstrated that LPC activated O₂^{•−} synthesis by NAD(P)H oxidase [32] and elicited NO-dependent cGMP release [33]. The O₂^{•−} is rapidly dismutated to H₂O₂, which has been shown to activate both NO synthesis and phosphorylation of Ser¹¹⁷⁷ on eNOS through a Ca²⁺- and PI3K-sensitive pathway [34]. We observed that LPC decreased the level of phosphorylated Ser¹¹⁷⁷ on eNOS and reduced NO release through a PI3K-dependent pathway. LPC could inhibit the histamine-induced eNOS activation by eliciting enzyme desensitization and/or its translocation from plasma membrane to perinuclear region.

Various GPCR agonists, indeed, induced the eNOS translocation [35,36].

The cholesterol oxide 7-oxocholesterol reduced the histamine-induced NO release through a mechanism different from that of LPC, since its effect was independent of extracellular O_2^- . These results are in agreement with previous observations relating an impairment of endothelium-dependent relaxation by 7-oxocholesterol independently of extracellular O_2^- [37]. It has been recently demonstrated that 7-oxocholesterol accumulates preferentially in lipid rafts of the plasma membrane and decreases their cholesterol content [38]. Caveolin-1, a structural protein of the lipid raft caveolae, negatively regulates eNOS activity [39]. 7-Oxocholesterol may reduce the NO release by disturbing the local environment of eNOS in caveolae, which is involved in intracellular cholesterol transport. We observed that cholesterol had no effect on $[Ca^{2+}]_i$ and on NO and arachidonic acid release. In contrast, 7-oxocholesterol increased the basal $[Ca^{2+}]_i$ and the agonist-induced Ca^{2+} signals, whereas it inhibited the eNOS and cPLA₂ activity. Previous studies have shown that incubation of cultured human endothelial cells with plasma containing high levels of 7-oxygenated oxysterols increased Ca^{2+} entry into the cells [17,18]. In the present study, the increase in basal $[Ca^{2+}]_i$ induced by 7-oxocholesterol depended on the presence of external Ca^{2+} (results not shown), confirming an activation of Ca^{2+} influx. The pre-stimulation of cells by increasing $[Ca^{2+}]_i$ led to the suppression of thapsigargin-evoked Ca^{2+} signals, but had no effect on NO release. This strengthens our previous observation that 7-oxocholesterol did not alter the receptor-independent NO synthesis [19], and supports the proposal that it inhibited eNOS and cPLA₂ activity through Ca^{2+} -independent mechanisms.

The inhibition of NO and arachidonic acid release by 7-oxocholesterol was associated with a decreased phosphorylation of Ser¹¹⁷⁷ on eNOS and Ser⁵⁰⁵ on cPLA₂. The fact that the oxysterol inhibited only the histamine-induced NO production, while it decreased both receptor-dependent and -independent arachidonic acid release, could be due to the different localization of eNOS and cPLA₂ and to the different kinase cascades involved in their activation. Phosphorylation of eNOS at Ser¹¹⁷⁷ is down-regulated by PKC signalling, which attenuates the eNOS activation [40]. Interestingly, 7-oxocholesterol does increase PKC activity [37]. In the present study, 7-oxocholesterol, by increasing the cytosolic Ca^{2+} , may activate a Ca^{2+} -dependent PKC isoform that would prevent the phosphorylation of Ser¹¹⁷⁷ on eNOS by histamine. The participation of PI3K in the eNOS activation was indicated by the reduction of eNOS phosphorylation and the strong inhibition of NO release elicited by the PI3K inhibitor LY294002. The effects of the latter were, however, annihilated in 7-oxocholesterol-treated cells, suggesting that the oxysterol and the PI3K inhibitor act on a common target. The phosphorylation of caveolin-2, which activates the caveolin-1-dependent formation of caveolae, was also inhibited by LY294002 [41]. 7-Oxocholesterol could inhibit eNOS phosphorylation by altering the caveolar structure.

Histamine and thapsigargin phosphorylated cPLA₂, ERK1/2 and p38 MAPK with similar time courses as we have described previously for arachidonic acid release [22]. This agrees with previous studies demonstrating the stimulation of ERK1/2 by histamine in HUVECs [42,43]. Interestingly, 7-oxocholesterol decreased the phosphorylation of cPLA₂ and ERK1/2, but did not modify that of p38 MAPK in both histamine- and thapsigargin-treated cells. However, at a concentration that abolished the ERK1/2 phosphorylation, the MEK inhibitor PD98059, had a less marked inhibitory effect than 7-oxocholesterol on arachidonic acid release. The oxysterol may prevent the full activation of cPLA₂ by histamine or thapsigargin by modulating upstream

steps of ERK1/2 cascade. In HUVECs, the association of Ca^{2+} -independent PKC δ with the upstream activator of MEK, Raf-1, controlled the ERK1/2 activation [44]. Through the activation of a Ca^{2+} -dependent PKC isoform, 7-oxocholesterol could down-regulate the Raf-MEK pathway by involving another PKC isoform. OSBP (oxysterol-binding protein) is a cytosolic receptor for different oxysterols, including 7-oxocholesterol, that interacts with proteins involved in cellular signalling processes and, in particular, the anchoring protein of PKC [45]. 7-Oxocholesterol could mediate its effects on ERK1/2 cascade through binding to OSBP, but the expression of this protein remains to be demonstrated in human endothelial cells.

Our present study highlights a modulation by two oxidized LDL constituents of the signalling events activating eNOS. The enzyme desensitization by LPC and the potentiation of Ca^{2+} signalling by 7-oxocholesterol could be explained by the new concepts of cross-talk or transactivation/transinactivation between heterologous receptors [46,47]. These findings obtained *in vitro* provide new insights on the regulation mechanisms of eNOS and cPLA₂, which may participate in endothelial dysfunction in the early stage of atherosclerosis.

G. T. is a recipient of support from the Société Française d'Athérosclérose (SFA) and Comité Français de Coordination des Recherches sur l'Athérosclérose et le Cholestérol (ARCOL). We thank Mrs Schaller, Mrs Joutel and colleagues from Maternités of Robert Debré Hospital and Notre Dame de Bon Secours Hospital for collecting umbilical cords.

REFERENCES

- Bonetti, P. O., Lerman, L. O. and Lerman, A. (2003) Endothelial dysfunction: a marker of atherosclerotic risk. *Arterioscler Thromb. Vasc. Biol.* **23**, 168–175
- Shimokawa, H. (1999) Primary endothelial dysfunction: atherosclerosis. *J. Mol. Cell. Cardiol.* **31**, 23–37
- Vallance, P. and Chan, N. (2001) Endothelial function and nitric oxide: clinical relevance. *Heart* **85**, 342–350
- Gewaltig, M. T. and Kojda, G. (2002) Vasoprotection by nitric oxide: mechanisms and therapeutic potential. *Cardiovasc. Res.* **55**, 250–260
- Bloodsworth, A., O'Donnell, V. B. and Freeman, B. A. (2000) Nitric oxide regulation of free radical- and enzyme-mediated lipid and lipoprotein oxidation. *Arterioscler. Thromb. Vasc. Biol.* **20**, 1707–1715
- Patel, R. P., Levonen, A., Crawford, J. H. and Darley-Usmar, V. M. (2000) Mechanisms of the pro- and anti-oxidant actions of nitric oxide in atherosclerosis. *Cardiovasc. Res.* **47**, 465–474
- Nigam, S. and Schewe, T. (2000) Phospholipase A₂s and lipid peroxidation. *Biochim. Biophys. Acta* **1488**, 167–181
- MacIouf, J., Folco, G. and Patrono, C. (1998) Eicosanoids and iso-eicosanoids: constitutive, inducible and transcellular biosynthesis in vascular disease. *Thromb. Haemostasis* **79**, 691–705
- Dart, A. M. and Chin-Dusting, J. P. (1999) Lipids and the endothelium. *Cardiovasc. Res.* **43**, 308–322
- Holvoet, P., Harris, T. B., Tracy, R. P., Verhamme, P., Newman, A. B., Rubin, S. M., Simonsick, E. M., Colbert, L. H. and Kritchevsky, S. B. (2003) Association of high coronary heart disease risk status with circulating oxidized LDL in the well-functioning elderly: findings from the Health, Aging, and Body Composition study. *Arterioscler. Thromb. Vasc. Biol.* **23**, 1444–1448
- Meyer, D. F., Nealis, A. S., Macphree, C. H., Groot, P. H., Suckling, K. E., Bruckdorfer, K. R. and Perkins, S. J. (1996) Time-course studies by synchrotron X-ray solution scattering of the structure of human low-density lipoprotein during Cu^{2+} -induced oxidation in relation to changes in lipid composition. *Biochem. J.* **319**, 217–227
- Kawai, Y., Saito, A., Shibata, N., Kobayashi, M., Yamada, S., Osawa, T. and Uchida, K. (2003) Covalent binding of oxidized cholesterol esters to protein: implications for oxidative modification of low density lipoprotein and atherosclerosis. *J. Biol. Chem.* **278**, 21040–21049
- Chaudhuri, P., Colles, S. M., Damron, D. S. and Graham, L. M. (2003) Lysophosphatidylcholine inhibits endothelial cell migration by increasing intracellular calcium and activating calpain. *Arterioscler. Thromb. Vasc. Biol.* **23**, 218–223
- Wong, J. T., Tran, K., Pierce, G. N., Chan, A. C., O, K. and Choy, P. C. (1998) Lysophosphatidylcholine stimulates the release of arachidonic acid in human endothelial cells. *J. Biol. Chem.* **273**, 6830–6836

- 15 Rikitake, Y., Kawashima, S., Takahashi, T., Ueyama, T., Ishido, S., Inoue, N., Hirata, K. and Yokoyama, M. (2001) Regulation of tyrosine phosphorylation of PYK2 in vascular endothelial cells by lysophosphatidylcholine. *Am. J. Physiol. Heart Circ. Physiol.* **281**, H266–H274
- 16 Brown, A. J. and Jessup, W. (1999) Oxysterols and atherosclerosis. *Atherosclerosis* **142**, 1–28
- 17 Zhou, Q., Wasowicz, E., Handler, B., Fleischer, L. and Kummerow, F. A. (2000) An excess concentration of oxysterols in the plasma is cytotoxic to cultured endothelial cells. *Atherosclerosis* **149**, 191–197
- 18 Kummerow, F. A., Cook, L. S., Wasowicz, E. and Jelen, H. (2001) Changes in the phospholipid composition of the arterial cell can result in severe atherosclerotic lesions. *J. Nutr. Biochem.* **12**, 602–607
- 19 Deckert, V., Brunet, A., Lantoin, F., Lizard, G., Millanvoe-van Brussel, E., Monier, S., Lagrost, L., David-Dufilho, M., Gamber, P. and Devynck, M. A. (1998) Inhibition by cholesterol oxides of NO release from human vascular endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **18**, 1054–1060
- 20 Deckert, V., Persegol, L., Viens, L., Lizard, G., Athias, A., Lallemand, C., Gamber, P. and Lagrost, L. (1997) Inhibitors of arterial relaxation among components of human oxidized low-density lipoproteins. Cholesterol derivatives oxidized in position 7 are potent inhibitors of endothelium-dependent relaxation. *Circulation* **95**, 723–731
- 21 Iouzal, L., Devynck, M. and David-Dufilho, M. (1995) Isradipine affects histamine-induced cytosolic Ca²⁺ movements in human endothelial cells. *Eur. J. Pharmacol.* **289**, 189–195
- 22 Millanvoe-Van Brussel, E., David-Dufilho, M., Pham, T. D., Iouzal, L. and Aude Devynck, M. (1999) Regulation of arachidonic acid release by calcium influx in human endothelial cells. *J. Vasc. Res.* **36**, 235–244
- 23 Lantoin, F., Iouzal, L., Devynck, M. A., Millanvoe-Van Brussel, E. and David-Dufilho, M. (1998) Nitric oxide production in human endothelial cells stimulated by histamine requires Ca²⁺ influx. *Biochem. J.* **330**, 695–699
- 24 Lantoin, F., Trévin, S., Bédoui, F. and Devynck, J. (1995) Selective and sensitive electrochemical measurement of nitric oxide in aqueous solution: discussion and new results. *Electroanal. Chem.* **392**, 85–89
- 25 Fleming, I. and Busse, R. (2003) Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **284**, R1–R12
- 26 Kudo, I. and Murakami, M. (2002) Phospholipase A₂ enzymes. Prostaglandins Other Lipid Mediat. **68–69**, 3–58
- 27 Zhou, H., Das, S. and Murthy, K. S. (2003) Erk1/2- and p38 MAP kinase-dependent phosphorylation and activation of cPLA2 by m3 and m2 receptors. *Am. J. Physiol. Gastrointest. Liver Physiol.* **284**, G472–G480
- 28 Yokoyama, K., Ishibashi, T., Ohkawara, H., Kimura, J., Matsuoka, I., Sakamoto, T., Nagata, K., Sugimoto, K., Sakurada, S. and Maruyama, Y. (2002) HMG-CoA reductase inhibitors suppress intracellular calcium mobilization and membrane current induced by lysophosphatidylcholine in endothelial cells. *Circulation* **105**, 962–967
- 29 Zhu, K., Baudhuin, L. M., Hong, G., Williams, F. S., Cristina, K. L., Kabarowski, J. H., Witte, O. N. and Xu, Y. (2001) Sphingosylphosphorylcholine and lysophosphatidylcholine are ligands for the G protein-coupled receptor GPR4. *J. Biol. Chem.* **276**, 41325–41335
- 30 Lum, H., Qiao, J., Walter, R. J., Huang, F., Subbaiah, P. V., Kim, K. S. and Holian, O. (2003) Inflammatory stress increases receptor for lysophosphatidylcholine in human microvascular endothelial cells. *Am. J. Physiol. Heart Circ. Physiol.* **285**, H1786–H1789
- 31 Miwa, Y., Hirata, K., Kawashima, S., Akita, H. and Yokoyama, M. (1997) Lysophosphatidylcholine inhibits receptor-mediated Ca²⁺ mobilization in intact endothelial cells of rabbit aorta. *Arterioscler. Thromb. Vasc. Biol.* **17**, 1561–1567
- 32 Heinloth, A., Heermeier, K., Raff, U., Wanner, C. and Galle, J. (2000) Stimulation of NADPH oxidase by oxidized low-density lipoprotein induces proliferation of human vascular endothelial cells. *J. Am. Soc. Nephrol.* **11**, 1819–1825
- 33 Konopatskaya, O., Whatmore, J. L., Tooke, J. E. and Shore, A. C. (2003) Insulin and lysophosphatidylcholine synergistically stimulate NO-dependent cGMP production in human endothelial cells. *Diabet. Med.* **20**, 838–845
- 34 Thomas, S. R., Chen, K. and Keaney, Jr, J. F. (2002) Hydrogen peroxide activates endothelial nitric-oxide synthase through coordinated phosphorylation and dephosphorylation via a phosphoinositide 3-kinase-dependent signaling pathway. *J. Biol. Chem.* **277**, 6017–6024
- 35 Marrero, M. B., Venema, V. J., Ju, H., He, H., Liang, H., Caldwell, R. B. and Venema, R. C. (1999) Endothelial nitric oxide synthase interactions with G-protein-coupled receptors. *Biochem. J.* **343**, 335–340
- 36 Wagner, S., Groschner, K., Mayer, B. and Schmidt, K. (2002) Desensitization of endothelial nitric oxide synthase by receptor agonists. *Biochem. J.* **364**, 863–868
- 37 Deckert, V., Duverneuil, L., Poupon, S., Monier, S., Le Guern, N., Lizard, G., Masson, D. and Lagrost, L. (2002) The impairment of endothelium-dependent arterial relaxation by 7-ketocholesterol is associated with an early activation of protein kinase C. *Br. J. Pharmacol.* **137**, 655–662
- 38 Gaus, K., Kritharides, L., Schmitz, G., Boettcher, A., Drobnik, W., Langmann, T., Quinn, C. M., Death, A., Dean, R. T. and Jessup, W. (2004) Apolipoprotein A-1 interaction with plasma membrane lipid rafts controls cholesterol export from macrophages. *FASEB J.* **18**, 574–576
- 39 Sowa, G., Pypaert, M. and Sessa, W. C. (2001) Distinction between signaling mechanisms in lipid rafts vs. caveolae. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 14072–14077
- 40 Michell, B. J., Chen, Z., Tiganis, T., Stapleton, D., Katsis, F., Power, D. A., Sim, A. T. and Kemp, B. E. (2001) Coordinated control of endothelial nitric-oxide synthase phosphorylation by protein kinase C and the cAMP-dependent protein kinase. *J. Biol. Chem.* **276**, 17625–17628
- 41 Sowa, G., Pypaert, M., Fulton, D. and Sessa, W. C. (2003) The phosphorylation of caveolin-2 on serines 23 and 36 modulates caveolin-1-dependent caveolae formation. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 6511–6516
- 42 Gudmundsdottir, I. J., Halldorsson, H., Magnusdottir, K. and Thorgeirsson, G. (2001) Involvement of MAP kinases in the control of cPLA₂ and arachidonic acid release in endothelial cells. *Atherosclerosis* **156**, 81–90
- 43 Keogh, R., Houlston, R. and Wheeler-Jones, C. (2002) Thrombin-stimulated Pyk2 phosphorylation in human endothelium is dependent on intracellular calcium and independent of protein kinase C and Src kinases. *Biochem. Biophys. Res. Commun.* **294**, 1001–1008
- 44 Gliki, G., Abu-Ghazaleh, R., Jezequel, S., Wheeler-Jones, C. and Zachary, I. (2001) Vascular endothelial growth factor-induced prostacyclin production is mediated by a protein kinase C (PKC)-dependent activation of extracellular signal-regulated protein kinases 1 and 2 involving PKC-δ and by mobilization of intracellular Ca²⁺. *Biochem. J.* **353**, 503–512
- 45 Lehto, M. and Olkkonen, V. M. (2003) The OSBP-related proteins: a novel protein family involved in vesicle transport, cellular lipid metabolism, and cell signalling. *Biochim. Biophys. Acta* **1631**, 1–11
- 46 Wetzker, R. and Bohmer, F. D. (2003) Transactivation joins multiple tracks to the ERK/MAPK cascade. *Nat. Rev. Mol. Cell Biol.* **4**, 651–657
- 47 Werry, T. D., Wilkinson, G. F. and Willars, G. B. (2003) Mechanisms of cross-talk between G-protein-coupled receptors resulting in enhanced release of intracellular Ca²⁺. *Biochem. J.* **374**, 281–296

Received 12 January 2004/16 February 2004; accepted 1 March 2004

Published as BJ Immediate Publication 1 March 2004, DOI 10.1042/BJ20040069