

Specific monocyte adhesion to endothelial cells induced by oxidized phospholipids involves activation of cPLA₂ and lipoxygenase

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Department of Vascular Biology and Thrombosis Research,* Medical University of Vienna, Vienna, Austria; Department of Medicine/Pathology,[†] University of California Los Angeles, Los Angeles, CA; and Department of Endocrinology and Metabolism[§] and Department of Pharmacology and Robert M. Berne Cardiovascular Research Center,** University of Virginia, Charlottesville, VA

Abstract Oxidized phospholipids stimulate endothelial cells to bind monocytes, but not neutrophils, an initiating event in atherogenesis. Here, we investigate intracellular signaling events induced by oxidized phospholipids in human umbilical vein endothelial cells (HUVECs) that lead to specific monocyte adhesion. In a static adhesion assay, oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine and one of its components, 1-palmitoyl-2-oxoalderoyl-*sn*-glycero-3-phosphorylcholine, stimulated HUVECs to bind U937 cells and human peripheral blood monocytes but not HL-60 cells or blood neutrophils. Monocyte adhesion was dependent on protein kinases A and C, extracellular signal-regulated kinase 1/2, p38 mitogen activated protein kinases (MAPKs), and cytosolic phospholipase A₂ (cPLA₂). Inhibition of 12-lipoxygenase (12-LOX), but not cyclooxygenases, blocked monocyte adhesion, and addition of 12-hydroxyicosatetraenoic acid (12-HETE) mimicked the effects of oxidized phospholipids. Peroxisome proliferator-activated receptor α (PPAR α) was excluded as a possible target for 12-HETE, because monocyte adhesion was still induced in endothelial cells from PPAR α null mice. Together, our results suggest that oxidized phospholipids stimulate HUVECs to specifically bind monocytes involving MAPK pathways, which lead to the activation of cPLA₂ and 12-LOX. Further analysis of signaling pathways induced by oxidized phospholipids that lead to specific monocyte adhesion should ultimately lead to the development of novel therapeutic approaches against chronic inflammatory diseases.—Huber, J., A. Fürnkranz, V. N. Bochkov, M. K. Patricia, H. Lee, C. C. Hedrick, J. A. Berliner, B. R. Binder, and N. Leitinger. **Specific monocyte adhesion to endothelial cells induced by oxidized phospholipids involves activation of cPLA₂ and lipoxygenase.** *J. Lipid Res.* 2006. 47: 1054–1062.

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One of the earliest steps in the development of the atherosclerotic lesion is the adhesion of monocytes to endothelial cells of the vessel wall (1). Previously, it was demonstrated that, if stimulated with minimally modified low density lipoprotein (MM-LDL), endothelial cells are activated to specifically bind monocytes but not neutrophils (2). This specificity toward mononuclear cells was subsequently observed with the activation of endothelial cells by oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (OxPAPC) (3), implicating lipid oxidation products as culprits in chronic inflammation. One biologically active oxidized component phospholipid in OxPAPC is 1-palmitoyl-2-oxoalderoyl-*sn*-glycero-3-phosphorylcholine (POVPC), which essentially mimics the actions of MM-LDL and OxPAPC. POVPC has also been found in MM-LDL, atherosclerotic lesions of animals and humans, and membranes of apoptotic cells (4–7).

Monocyte binding induced by oxidized phospholipids in human aortic endothelial cells (HAECs) was shown to be independent of nuclear factor- κ B (NF- κ B) activation or increased expression of E-selectin, vascular cell adhesion molecule-1 (VCAM-1), or intercellular adhesion molecule-1 (ICAM-1) (8–10), but it involves the activation of an

Abbreviations: CDC, cinnamoyl-3,4-dihydroxy- α -cyanocinnamate; cPLA₂, cytosolic phospholipase A₂; CS-1, connecting segment-1; ERK 1/2, extracellular signal-regulated kinase 1/2; ETYA, 5,8,11,14-eicosatetraenoic acid; HAEC, human aortic endothelial cell; 12-HETE, 12-hydroxyicosatetraenoic acid; HUVEC, human umbilical vein endothelial cell; ICAM-1, intercellular adhesion molecule-1; IL-8, interleukin 8; 12-LOX, 12-lipoxygenase; LPS, lipopolysaccharide; M199, medium 199; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein-1; MEK, mitogen-activated ERK kinase; MM-LDL, minimally modified low density lipoprotein; NF- κ B, nuclear factor- κ B; OxPAPC, oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine; PKA, protein kinase A; PKC, protein kinase C; POVPC, 1-palmitoyl-2-oxoalderoyl-*sn*-glycero-3-phosphorylcholine; PPAR α , peroxisome proliferator-activated receptor α ; STAT3, signal transducer and activator of transcription 3; VCAM-1, vascular cell adhesion molecule-1.

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$\alpha_5\beta_1$ integrin and increased deposition of the connecting segment-1 (CS-1) domain of fibronectin on the luminal surface of endothelial cells (11). Moreover, lipopolysaccharide (LPS)-induced expression of E-selectin was inhibited by OxPAPC via cAMP and protein kinase A (PKA) (12). Activation of $\alpha_5\beta_1$ integrins and monocyte-endothelium adhesion by OxPAPC and POVPC was shown to be dependent on cAMP and R-Ras activation (13). β_1 integrins are rapidly activated by oxidized phospholipids within 15 min (11). However, maximal β_1 integrin activation and monocyte adhesion are observed after 4 h and require new protein synthesis (11).

Lipoxygenase activity and related arachidonate metabolites were shown to be necessary for MM-LDL to increase monocyte binding to HAECs (14). Recently, we demonstrated that OxPAPC induces tissue factor expression in human umbilical vein endothelial cells (HUVECs) by activating signaling pathways involving protein kinase C (PKC), extracellular signal-regulated kinase 1/2 (ERK 1/2), mitogen-activated protein kinase (MAPK), and increased intracellular calcium, ultimately activating the transcription factors endothelial growth related-1 (EGR-1) and nuclear factor of activated T-cells (NFAT) (9). Induction of interleukin 8 (IL-8) and monocyte chemotactic protein-1 (MCP-1) by OxPAPC in endothelial cells has been suggested to involve peroxisome proliferator-activated receptor α (PPAR α) (15). In addition, OxPAPC-induced endothelial IL-8 expression was independent of the NF- κ B signaling pathway (10) and involved the activation of c-Src and signal transducer and activator of transcription 3 (STAT3) (10). Activation of this pathway was mainly induced by 1-palmitoyl-2-(5,6-epoxyisoprostane E2)-sn-glycero-3-phosphorylcholine, which is another component phospholipid of OxPAPC (10).

In this study, we examined the signaling pathways induced by OxPAPC and POVPC that result in the specific adhesion of monocytes to HUVECs. We report that multiple signaling pathways involving PKA, PKC, ERK 1/2, and p38 MAPK need to be activated, resulting in the activation of cytosolic phospholipase A₂(cPLA₂) and the release of free arachidonic acid that is subsequently used by lipoxygenase, producing 12-hydroxyicosatetraenoic acid (12-HETE), which acts as a signal mediator.

MATERIALS AND METHODS

Reagents

1-Palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine, DMSO, butylated hydroxytoluene, medium 199 (M199), and *O*-phenylene diamine were purchased from Sigma (St. Louis, MO). A multi-test *Limulus* amoebocyte lysate was from BioWhittaker (Walkersville, MD). The mitogen-activated ERK kinase (MEK) 1/2 inhibitor (PD098059), p38 inhibitor (SB203580), and PKC inhibitor (bisindolylmaleimide I) were obtained from Calbiochem (La Jolla, CA). 12(*S*)-HETE [12(*S*)-hydroxyicoso-5Z, 8Z, 10E, 14Z-tetraenoic acid], cinnamoyl-3,4-dihydroxy- α -cyanocinnamate (CDC), and the cPLA₂ inhibitor arachidonyltrifluoromethyl ketone were from Biomol; 5,8,11,14-eicosatetraenoic acid (ETYA) was from Cayman Chemical; arachidonic acid [5,6,8,9,11,12,14,15-

³H(N)] was from Perkin-Elmer (Boston, MA). Tumor necrosis factor- α was from Boehringer Mannheim (Germany); anti-human E-selectin, ICAM-1, and VCAM-1 antibodies (all IgG) were from R&D Systems (Minneapolis, MN). Peroxidase-conjugated secondary anti-rabbit and anti-mouse antibodies were from Amersham Pharmacia Biotech (Uppsala, Sweden). Polyclonal antibodies against nonphosphorylated and phosphorylated ERK1/2 and LumiGLO were purchased from New England Biolabs (Beverly, MA). U937 cells and HL-60 cells were from the American Type Culture Collection (Manassas, VA), and supplemented calf serum was from HyClone (Logan, UT).

Tissue culture

HUVECs were prepared and cultured as described previously (16). Cells were used for experiments at passages three to five.

Phospholipid preparation

OxPAPC and POVPC were prepared as described previously (4, 5). Lipids were tested for endotoxin content by *Limulus* test (from BioWhittaker). Only preparations containing <0.03 U/ml endotoxin were used for experiments.

Leukocyte adhesion assays

Adhesion assay of leukocytes to HUVECs. Adhesion assays were performed as described elsewhere (2, 17). In a previous publication (8), we demonstrated the similar behavior of monocyte-like U937 cells with respect to freshly isolated human peripheral blood mononuclear cells in adhesion assays. Briefly, confluent HUVECs on 48-well plates were incubated with agonists in M199 containing 10% supplemented calf serum for 4 h at 37°C. In some experiments, HUVECs were preincubated in the presence or absence of inhibitors dissolved in ethanol, DMSO, or solvent only, and then lipids dissolved in M199 or M199 only (control) were added for another 4 h. After incubation, HUVECs were washed and a suspension of unstimulated leukocytes (2×10^5 cells/well) was added for 15 min. Nonadherent leukocytes were removed by washing, and adherent cells were counted using an inverted phase-contrast microscope.

Adhesion assay of a mouse monocytic cell line (WEHI 78/24) to mouse aortic endothelial cells. Mouse aortic endothelial cells isolated from normal and PPAR α null C57BL/6 mice were cultured as described previously (18). After confluence in 48-well dishes, mouse aortic endothelial cells were stimulated with OxPAPC, LPS, or medium alone for 4 h at 37°C. After incubation, a suspension of unstimulated WEHI 78/24 cells was added for 15 min (19). Nonadherent leukocytes were removed by washing, and adherent cells were counted using an inverted phase-contrast microscope.

Western blotting

After stimulation, HUVECs were lysed in Laemmli buffer and proteins were separated by electrophoresis on a 10% SDS-polyacrylamide gel. Proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA), blocked with 5% dry milk/0.1% Tween-20, and incubated with primary antibodies in the same solution. Peroxidase-conjugated anti-IgG was used as a secondary antibody and detected by chemiluminescence (LumiGLO).

Cell ELISA

Confluent HUVECs on 96-well plates were treated with agonists for 4 h at 37°C. The assay was then performed as

described previously (20). In all leukocyte adhesion assays and ELISA experiments, tumor necrosis factor- α (20–50 U/ml) or LPS (2 ng/ml) was used as a positive control and yielded a reproducible 3- to 5-fold increase compared with cells treated with medium alone or containing a solvent vehicle, which is indicated as control.

Measurement of arachidonic acid release

HUVECs were radiolabeled with 1 μ Ci/ml [3 H]arachidonate culture M199 containing 10% fetal calf serum as described previously (21). After labeling, HUVECs were washed with HEPES-buffered saline containing 0.025% essentially fatty acid-free BSA and then stimulated with OxPAPC (150 μ g/ml) or incubated with buffer alone (control) for the indicated time periods. The arachidonate released from cells was determined as described previously (21). Briefly, the supernatant was first collected and acetified with glacial acetic acid. Then, after lipid extraction, unlabeled arachidonic acid was added to the extraction mixture as a fatty acid carrier. The free fatty acid fraction was resolved by TLC with a solvent system consisting of hexane-diethyl ether-acetic acid (70:30:1, v/v) and was visualized by iodine vapor. Radioactivity was determined by liquid scintillation counting.

Construction of 12-lipoxygenase ribozyme adenovirus vector and monocyte adhesion assays

A hammerhead ribozyme was designed to cleave the porcine leukocyte-type 12-lipoxygenase (12-LOX) RNA. The structure of the 12-LOX-hammerhead ribozyme has been described previously (22–24). Transfections of porcine aortic endothelial cells with ribozyme and monocyte adhesion assays were performed according to Patricia and coworkers (25).

Statistical analysis

Results are expressed as means \pm SEM. Statistical analysis was performed using one-way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

OxPAPC and POVPC induce monocyte but not neutrophil adhesion to HUVECs

HUVECs were stimulated with OxPAPC or POVPC, and the binding of monocyte-like U937 cells was determined. OxPAPC and POVPC stimulated HUVECs to bind monocytes (Fig. 1A), which is in accordance with previously published data on HAECs (4, 12, 26). In contrast, OxPAPC did not induce the binding of neutrophil-like HL-60 cells to endothelial cells (Fig. 1A). To confirm these findings for human peripheral blood cells, we isolated monocytes and neutrophils from human blood. Consistently, OxPAPC stimulated the binding of human mononuclear cells, but not neutrophils, to HUVECs (Fig. 1B). Based on these results, we used monocyte-like U937 cells for further adhesion experiments.

We have previously shown that OxPAPC did not up-regulate the inflammatory adhesion molecules E-selectin, VCAM-1, or ICAM-1 but in contrast upregulated CS-1 fibronectin surface expression in HAECs (12). Accordingly, treatment of HUVECs for 5 and 8 h did not result in the

upregulation of E-selectin, VCAM-1, or ICAM-1 (Fig. 1C). However, adhesion of monocytes to OxPAPC-treated HUVECs was mediated by CS-1 fibronectin, because an antibody directed against CS-1, but not an irrelevant IgM, inhibited monocyte binding (Fig. 1D).

OxPAPC and POVPC were shown to increase endothelial cAMP levels (12, 27), which contributes to the induction of monocyte-endothelium interactions (13). Inhibition of PKA, which is downstream of cAMP, by H89 reduced monocyte adhesion induced by OxPAPC in a concentration-dependent manner (Fig. 1E). These results show that the effects of OxPAPC on HUVECs are similar and comparable to those on HAECs.

Roles of PKC and MAPK-dependent pathways in OxPAPC- and POVPC-induced monocyte adhesion to HUVECs

We have shown previously that treatment of HUVECs with OxPAPC induced the phosphorylation of ERK 1/2 within 20 min of stimulation, which was sustained for up to 8 h (9). To determine whether POVPC, one component lipid of OxPAPC, contributes to this effect, HUVECs were stimulated for 20 min with POVPC and phosphorylation of ERK 1/2 was detected by Western blotting. As shown in Fig. 2A, both OxPAPC and POVPC stimulated the phosphorylation of ERK 1/2 (Fig. 2A).

Activation of ERK 1/2 requires phosphorylation by its upstream kinase MEK 1/2 (28), which can be activated by a PKC-dependent pathway. To investigate the role of the PKC/MEK 1/2 and ERK 1/2 pathways in OxPAPC- and POVPC-induced endothelial-monocyte interactions, HUVECs were pretreated with a specific MEK 1/2 or PKC inhibitor (PD098059 or bisindolylmaleimide I, respectively) before addition of lipids. U937 cell binding induced by OxPAPC and POVPC was blocked by PD098059 (Fig. 2B, C) and bisindolylmaleimide I (Fig. 2D, E). Treatment of HUVECs with inhibitors alone or vehicle did not affect the basal level of adherent U937 cells (data not shown). Together, these results indicate that activation of HUVECs leading to monocyte-like U937 cell adhesion is dependent on PKC/MEK 1/2- and ERK 1/2-dependent pathways.

Role of p38 MAPK in OxPAPC-induced monocyte adhesion

In addition, we found that OxPAPC-induced U937 cell binding to HUVECs was blocked by the p38 inhibitor SB203580 (Fig. 3A). Addition of SB203580 or vehicle to untreated HUVECs had no effect on the level of U937 cell binding compared with control levels (data not shown). Moreover, phosphorylation of p38 was induced by OxPAPC (Fig. 3B). To examine whether the activation of p38 MAPK occurs independently from the PKC/ERK 1/2 pathway, HUVECs were incubated with specific inhibitors of PKC (bisindolylmaleimide I) and ERK 1/2 (PD098059) before stimulation with OxPAPC. Phosphorylation of p38 was determined subsequently by Western blot analysis. As shown in Fig. 3B, phosphorylation of p38 MAPK by OxPAPC was inhibited by neither bisindolylmaleimide I nor PD098059, whereas the p38 inhibitor

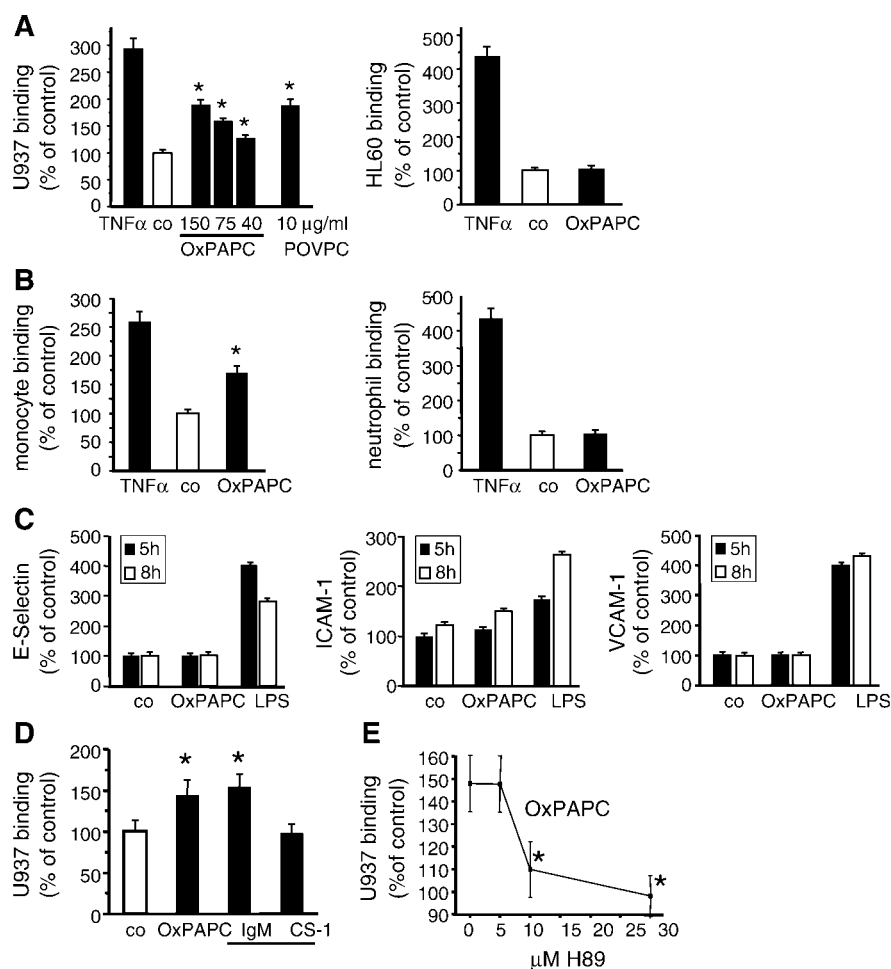


Fig. 1. Oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (OxPAPC) stimulates human umbilical vein endothelial cells (HUVECs) to specifically bind human peripheral blood mononuclear cells and monocyte-like U937 cells. **A:** HUVECs were treated with OxPAPC or 1-palmitoyl-2-oxoaleryl-*sn*-glycero-3-phosphorylcholine (POVPC) at the indicated concentrations, and the binding of unstimulated monocyte-like U937 cells or neutrophil-like HL-60 cells was determined. co, control; TNF α , tumor necrosis factor- α . **B:** HUVECs were treated with OxPAPC (150 μ g/ml), and the binding of unstimulated human peripheral blood mononuclear cells or human peripheral blood neutrophils was determined. **C:** HUVECs were treated with OxPAPC, medium only (control), or lipopolysaccharide (LPS) as a positive control for the indicated time points. Expression of indicated adhesion molecules was detected by ELISA. ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1. **D:** HUVECs were treated with OxPAPC (150 μ g/ml) with or without the addition of anti-connecting segment-1 (CS-1)/fibronectin antibody or an irrelevant isotype-matched control antibody, and binding of monocyte-like U937 cells was determined. **E:** Preincubation of HUVECs with a specific inhibitor of protein kinase A (H89) dose-dependently inhibits monocyte-like U937 cell binding stimulated by OxPAPC. Adhesion assays were performed as described in Materials and Methods. Data are expressed as means \pm SEM and are representative of three independent experiments. * P < 0.05 compared with control (A, B, D) or compared with OxPAPC only (E).

SB203580 abolished the OxPAPC-induced phosphorylation of p38.

Roles of cPLA₂ and arachidonic acid in OxPAPC- and POVPC-induced monocyte-like U937 cell adhesion

Among the targets that are phosphorylated directly and thereby activated by ERK 1/2 and p38 MAPK is cPLA₂ (29–31). Thus, we asked whether cPLA₂, which plays an important role in arachidonate release, was involved in monocyte adhesion induced by oxidized phospholipids. Pretreatment of HUVECs with the cPLA₂ inhibitor

arachidonyltrifluoromethyl ketone significantly reduced monocyte-like U937 cell adhesion induced by OxPAPC and POVPC (Fig. 4A, B). Treatment of HUVECs with the inhibitor alone did not affect the basal level of adherent U937 cells (data not shown). Activation of cPLA₂ would result in the release of arachidonate from phospholipids. Indeed, treatment of HUVECs with OxPAPC increased levels of free arachidonic acid in a time-dependent manner (Fig. 4C). However, treatment of HUVECs with arachidonic acid up to 60 μ M did not induce monocyte-like U937 adhesion (Fig. 4D), leading us to hypothesize

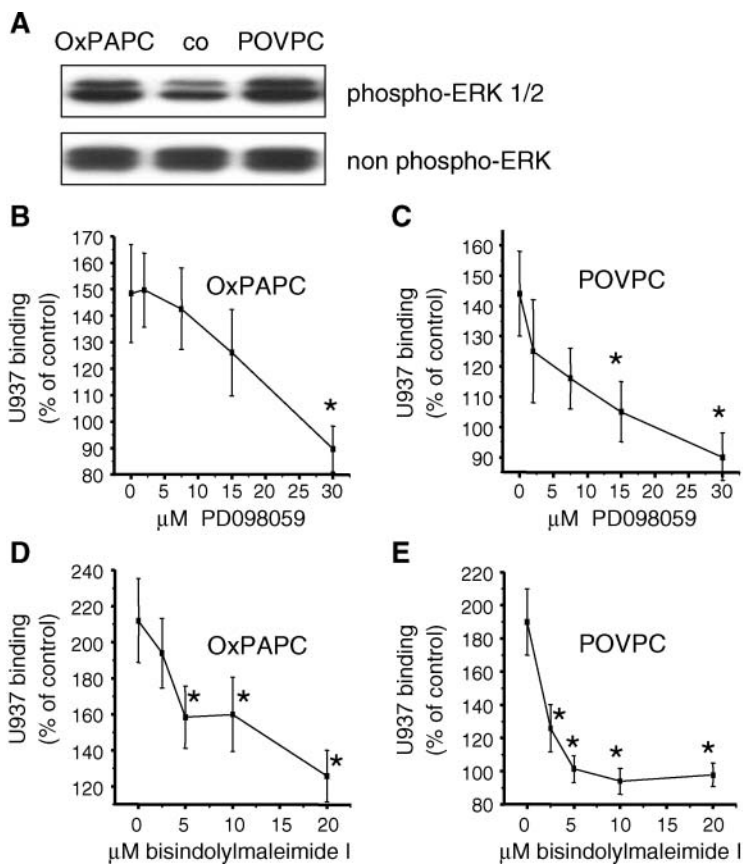


Fig. 2. A: OxPAPC and POVPC stimulate extracellular signal-regulated kinase 1/2 (ERK 1/2) phosphorylation. HUVECs were treated with OxPAPC (150 $\mu\text{g/ml}$) or POVPC (10 $\mu\text{g/ml}$) for 1 h. Cells were harvested and processed for Western blotting with antibodies to phosphorylated ERK 1/2 or nonphosphorylated ERK to confirm equal loading. Data are representative of three separate experiments. co, control. B–E: Preincubation of HUVECs with specific inhibitors of mitogen-activated ERK kinase (MEK) (PD098059) or protein kinase C (bisindolylmaleimide) dose-dependently inhibits monocyte-like U937 cell binding stimulated by OxPAPC (150 $\mu\text{g/ml}$) or POVPC (10 $\mu\text{g/ml}$). Data are expressed as means \pm SEM (percentage of control) and are representative of three independent experiments. * $P < 0.05$ compared with OxPAPC and POVPC without inhibitors.

that released arachidonic acid needs to be metabolized to exert biological activity.

Roles of lipoxygenase and cyclooxygenase in OxPAPC- and POVPC-induced monocyte-like U937 cell adhesion

The release of arachidonate from phospholipids is the rate-limiting step in the synthesis of bioactive eicosanoids (32). In a previous publication, we demonstrated that MM-LDL but not native LDL increases the metabolism of arachidonic acid and the production of lipoxygenase products in HAECs. Moreover, addition of lipoxygenase inhibitors blocked monocyte adhesion to HAECs stimulated with MM-LDL (14).

To determine the role of enzymes metabolizing arachidonic acid, HUVECs were preincubated with the lipoxygenase inhibitor CDC or the nonselective cyclooxygenase 1 and 2 inhibitor indomethacin. After 45 min, OxPAPC and POVPC were added to HUVECs for 4 h at 37°C. The lipoxygenase inhibitor CDC but not the cyclooxygenase inhibitor indomethacin blocked U937 cell adhesion induced by OxPAPC and POVPC (Fig. 5A, B). Another lipoxygenase inhibitor, ETYA, also inhibited monocyte adhesion to HUVECs induced by OxPAPC and POVPC (data not shown). Treatment of HUVECs with the inhibitors alone or vehicle did not affect the basal level of adherent U937 cells (data not shown).

Pharmacological inhibitors that reduce lipoxygenase activity, such as ETYA and CDC, cannot distinguish the

different isoforms and may have unwanted side effects (33). Increased production of 12-LOX eicosanoids was shown to accelerate monocyte-endothelium interactions (34) and aortic lesion formation (35) in diabetic mice. To determine the role of 12-LOX in OxPAPC-induced signaling, we used adenoviruses encoding a ribozyme against porcine leukocyte 12-LOX or β -galactosidase, as described (25). Porcine aortic endothelial cells were then infected with these adenoviruses, and the effects of OxPAPC and LPS on monocyte adhesion were determined. OxPAPC-induced monocyte adhesion to porcine aortic endothelial cells was blocked by the ribozyme against 12-LOX but not by that against β -galactosidase (Fig. 5C). In contrast, LPS-induced monocyte adhesion was affected by neither the 12-LOX nor the β -galactosidase ribozyme (Fig. 5C). Adenovirus-transfected cells that were treated with culture medium alone bound monocytes at low basal levels (Fig. 5C).

Metabolites generated by 12-LOX include 12(*S*)-HETE and 12(*R*)-HETE. Treatment of HAECs with 12(*S*)-HETE but not 12(*R*)-HETE was shown to induce monocyte adhesion (25). Accordingly, stimulation of HUVECs with 12(*S*)-HETE resulted in increased binding of monocyte-like U937 cells in a concentration-dependent manner (Fig. 5D).

These data indicate that OxPAPC- and POVPC-induced monocyte-like U937 cell adhesion to HUVECs requires the release of arachidonate by cPLA₂ and further metaboliza-

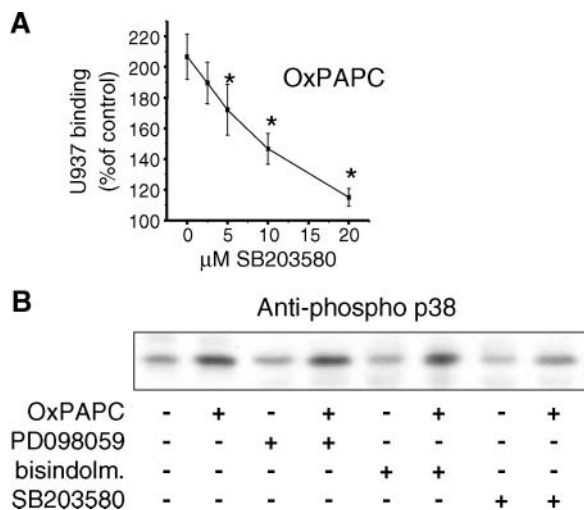


Fig. 3. p38 mitogen-activated protein kinase (MAPK) mediates OxPAPC-stimulated monocyte-endothelium interactions. **A:** Preincubation of HUVECs with a specific inhibitor of p38 MAPK (SB203580) dose-dependently inhibits monocyte-like U937 cell binding stimulated by OxPAPC (150 $\mu\text{g}/\text{ml}$). Data are expressed as means \pm SEM (percentage of control) and are representative of three independent experiments. * $P < 0.05$ compared with OxPAPC without inhibitor preincubation. **B:** OxPAPC-induced phosphorylation of p38 MAPK was inhibited by preincubation with SB203580, but not PD098059 or bisindolylmaleimide I. HUVECs were preincubated with inhibitors for 45 min before addition of OxPAPC (150 $\mu\text{g}/\text{ml}$), and phosphorylated p38 was determined by Western blot analysis.

tion by 12-LOX to generate 12(S)-HETE. To investigate whether the activation of 12-HETE is downstream of ERK 1/2 and p38, we treated HUVECs with 12-HETE up to a concentration of 2 μM and tested for phosphorylation of ERK 1/2 and p38. No phosphorylation of ERK 1/2 or p38 was observed (data not shown), indicating that the generation of 12-HETE was downstream of MAPK.

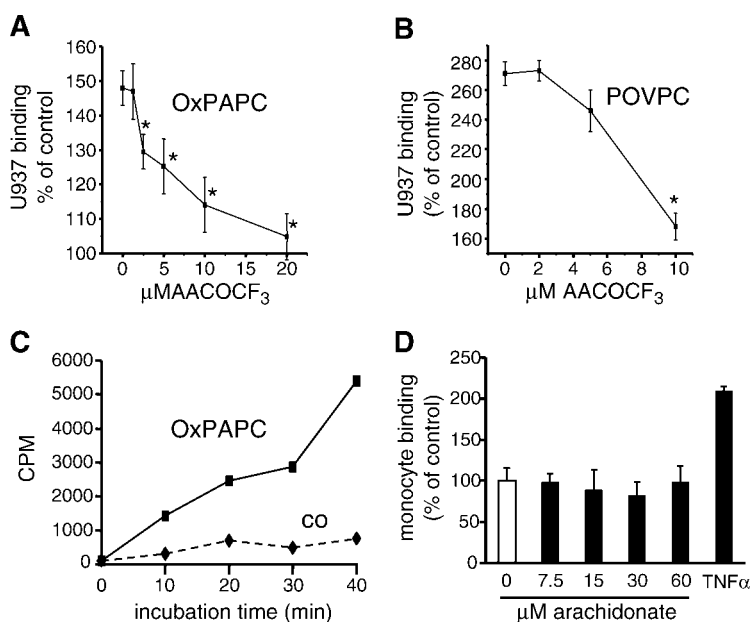


Fig. 4. Cytosolic phospholipase A₂(cPLA₂) and arachidonic acid are involved in OxPAPC-stimulated monocyte-endothelium interactions. **A, B:** Preincubation of HUVECs with a specific inhibitor of cPLA₂ [arachidonyltrifluoromethyl ketone (AACOCF₃)] for 3 h inhibits U937 cell binding stimulated by OxPAPC or POVPC. Data are expressed as means \pm SEM (percentage of control) and are representative of two separate experiments. * $P < 0.05$ compared with OxPAPC and POVPC without inhibitor preincubation. **C:** OxPAPC induces arachidonic acid release from HUVECs. After labeling with [³H]arachidonic acid, HUVECs were washed and then stimulated with OxPAPC (150 $\mu\text{g}/\text{ml}$) or incubated with buffer alone [control (co)] for the indicated time periods. ³H-labeled arachidonic acid was determined in cell supernatant by liquid scintillation counting. Data are representative of four independent experiments. **D:** Incubation of HUVECs with up to 60 μM arachidonic acid does not induce U937 cell binding. HUVECs were treated with arachidonate for 4 h at the indicated concentrations, and U937 cell adhesion was determined. Data are expressed as means \pm SEM (percentage of control) and are representative of three independent experiments. TNF α , tumor necrosis factor- α .

PPAR α activation is not involved in OxPAPC-induced monocyte adhesion

OxPAPC- and POVPC-mediated IL-8 and MCP-1 production in HAECs was shown to involve the activation of PPAR α (15). To investigate the role of PPAR α activation in OxPAPC-induced monocyte binding, HUVECs were treated with the PPAR α agonist fenofibrate for 4 h at 37°C, and monocyte binding assays were then performed. Fenofibrate did not stimulate HUVECs to bind monocyte-like U937 cells (Fig. 6A) at concentrations shown to exert various effects on endothelial cells (36). PPAR α is expressed in HUVECs in sufficient amounts (as indicated by quantitative RT-PCR) to induce PPAR α ligand-induced gene expression (data not shown).

To confirm that PPAR α was not involved in OxPAPC-induced monocyte adhesion, we isolated mouse aortic endothelial cells from PPAR α null and wild-type C57BL/6 mice, as described previously (18). Mouse aortic endothelial cells were stimulated with OxPAPC, and WEHI 78/24 cells, a mouse monocytic cell line, were then added for adhesion assays. Both OxPAPC and LPS, which was used as a positive control, stimulated mouse aortic endothelial cells from wild-type and PPAR α null C57BL/6 mice to bind increased levels of WEHI 78/24 cells (Fig. 6B), thus excluding a possible role for PPAR α .

DISCUSSION

The adhesion of monocytes to the endothelium of the vessel wall is considered an important step in the initial stage of atherosclerosis. There is considerable evidence that oxidized phospholipids present in oxidized LDL and apoptotic cells play an important role in the recruitment of monocytes into the vessel wall (3, 37). However, the molecular mechanisms that govern the activation of endothelial cells by oxidized phospholipids are still poorly understood.

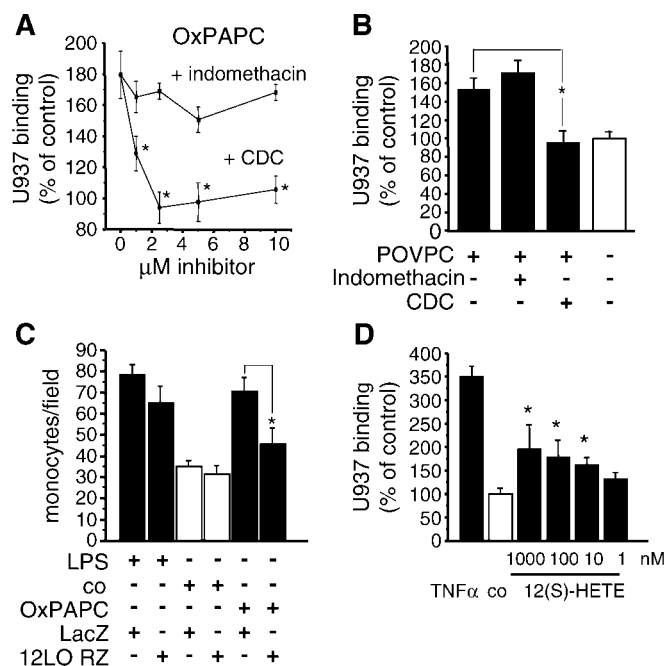


Fig. 5. Role of eicosanoid metabolites in OxPAPC-stimulated monocyte-endothelium interactions. A, B: The lipoxygenase inhibitor cinnamoyl-3,4-dihydroxy- α -cyanocinnamate (CDC) but not the cyclooxygenase inhibitor indomethacin blocks monocyte-endothelium interactions induced by OxPAPC or POVPC. A: HUVECs were pretreated for 30 min with inhibitors at the indicated concentrations or before stimulation with OxPAPC (150 μ g/ml) for 4 h and determination of U937 cell adhesion. B: HUVECs were preincubated with indomethacin (10 μ M) or CDC (5 μ M) for 30 min before stimulation with POVPC (10 μ g/ml) for 4 h. Binding of unstimulated U937 cells to HUVECs was then determined. Data are expressed as means \pm SEM (percentage of control) and are representative of four separate experiments. $*P < 0.05$ compared with OxPAPC and POVPC only. C: Inhibition of OxPAPC-induced monocyte adhesion to porcine aortic endothelial cells by a 12-lipoxygenase (12-LOX) ribozyme. Monocyte (human peripheral blood) binding assay to porcine aortic endothelial cells treated with OxPAPC (100 μ g/ml) or LPS (1 ng/ml) was performed 72 h after infection with an adenoviral vector containing a ribozyme against 12-LOX (12LO RZ) or β -galactosidase (LacZ), which was used as a control vector. Data are representative of three independent experiments and are expressed as mean monocytes per field \pm SEM. $*P < 0.01$, control. D: 12(S)-Hydroxyeicosatetraenoic acid [12(S)-HETE] dose-dependently stimulates HUVECs to bind monocyte-like U937 cells. HUVECs were treated with 12(S)-HETE for 4 h at the indicated concentrations, and monocyte-like U937 adhesion was determined. Data are expressed as means \pm SEM (percentage of control). Similar results were obtained in another two separate experiments. $*P < 0.01$ compared with control. TNF α , tumor necrosis factor- α .

In acute inflammation, cytokines or bacterial products activate the NF- κ B pathway in endothelial cells, resulting in the expression of various inflammatory adhesion molecules, including E-selectin and VCAM-1, and the recruitment of neutrophils, T-cells, and monocytes. In chronic inflammation, in which monocyte accumulation is predominant, presumably alternative signaling pathways are induced to provide monocyte specificity. We could show that activation of MAPK pathways, rather than the

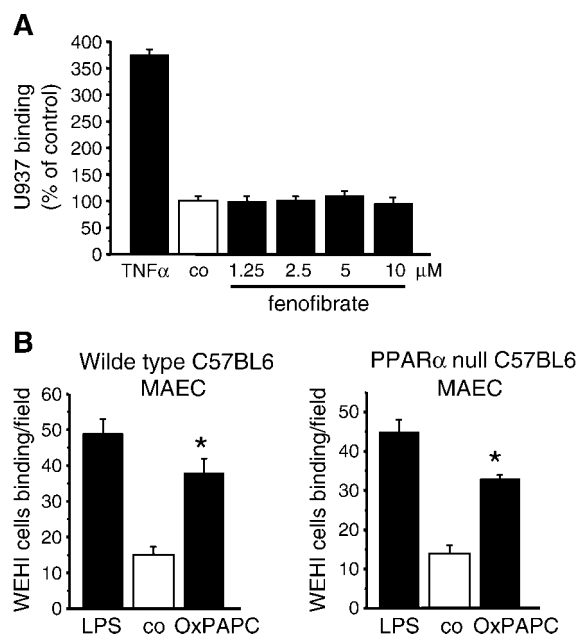


Fig. 6. Role of peroxisome proliferator-activated receptor α (PPAR α) in OxPAPC induced monocyte-endothelium interactions. A: HUVECs were treated with the indicated concentrations of the PPAR α agonist fenofibrate for 4 h, and binding of monocyte-like U937 cells was determined. Data are expressed as means \pm SEM (percentage of control). Similar results were obtained in another two separate experiments. co, control; TNF α , tumor necrosis factor- α . B: Mouse aortic endothelial cells (MAEC) from wild-type C57BL/6 mice and PPAR α null mice were treated with OxPAPC (40 μ g/ml) for 4 h, and adhesion of monocytic WEHI cells was determined. Data are expressed as means \pm SEM (WEHI cells per field) and are representative of two separate experiments. $*P < 0.05$ compared with control.

classical NF- κ B pathway, plays a crucial role in the signaling events in HUVECs stimulated by lipid oxidation products (8, 9, 17). Recently, a role for c-Src kinase leading to the activation of STAT3 was demonstrated in OxPAPC-mediated IL-8 expression (38). Moreover, OxPAPC increased levels of endothelial cAMP and induced monocyte-endothelium interactions by a cAMP/Ras-dependent signaling pathway in HAECs (12, 13). In this study, we demonstrate that OxPAPC-induced activation of HUVECs to bind monocytes was dependent on PKA. These data confirm that previously described mechanisms in HAECs are also active in HUVECs.

We show here that activation of HUVECs to bind monocytes was dependent on ERK 1/2 and p38 MAPK pathways. In addition to their ability to modify transcription, ERK 1/2 and p38 MAPK have been demonstrated to play a role in the activation of cPLA₂, phosphorylating the enzyme at serine 505 (39, 40). Full activation of cPLA₂ requires both cPLA₂ phosphorylation and increased cytosolic Ca²⁺, the latter initiating the translocation of cPLA₂ to the plasma membrane (40). Given the evidence that OxPAPC induces both the activation of ERK 1/2 and p38 MAPK and an increase in intracellular Ca²⁺ levels in HUVECs (9), our observation that a specific inhibitor of cPLA₂ abrogates OxPAPC-induced monocyte adherence


to endothelial cells suggests a role of cPLA₂ acting downstream of the MAPK.

The release of arachidonic acid from membrane phospholipids is the rate-limiting step in the synthesis of eicosanoid mediators (32), including lipoxygenase products. 12-LOX has been shown to play an important role in atherosclerosis: atherosclerotic lesions were considerably reduced in LDL-receptor-deficient and 12/15-LOX-deficient double knockout mice compared with LDL-receptor null mice (41); disruption of the 12/15-LOX gene in apolipoprotein E-deficient mice significantly retarded atherosclerosis (42, 43). MM-LDL has been shown to increase arachidonic acid and 12-HETE levels in HAECs, involving lipoxygenase but not cyclooxygenase. Lipoxygenase inhibitors blocked enhanced monocyte adhesion to HAECs upon MM-LDL stimulation (14). Here, we show that the same holds true for HUVECs stimulated with OxPAPC. When disrupting 12-LOX activity by the use of an adenovirus containing a ribozyme against 12-LOX, we found that OxPAPC-induced monocyte adhesion was significantly inhibited. Moreover, 12(S)-HETE stimulates HUVECs to bind monocytes, in accordance with previous reports on HAECs (25). However, other biologically active lipoxygenase products may also be generated and thus involved in endothelial activation leading to monocyte adhesion (44).

Possible targets for HETEs are the lipid-dependent transcription factors the PPARs. Both PPAR α and PPAR γ have been linked to signaling events induced by lipids and inflammatory mediators, and a role in atherosclerosis was implicated (15, 36). PPAR α null mice exhibit reduced atherosclerosis in an apolipoprotein E null background (45). Oxidized LDL-mediated PPAR α activation in endothelial cells was shown to involve cPLA₂ (46). MM-LDL, OxPAPC, and POVPC activated HAECs to synthesize IL-8 and MCP-1 by activating PPAR α , the most abundant member of the PPAR family in HAECs (15). In contrast, PPAR γ or PPAR δ were not activated significantly by MM-LDL, OxPAPC, or POVPC (15). However, murine arterial endothelial cells lacking PPAR α still could be stimulated by OxPAPC to bind a mouse monocytic cell line. In addition, fenofibrate, a known agonist of PPAR α , failed to induce increased levels of monocyte adhesion to HUVECs. These data suggest that PPAR α does not play a role in OxPAPC-induced monocyte adhesion to HUVECs. Thus, the downstream effectors recruited by 12(S)-HETE, which ultimately lead to enhanced monocyte adhesion, may involve other nuclear receptors that remain to be determined.

In this study, effects on monocyte adhesion were seen at 40 μ g/ml OxPAPC and 10 μ g/ml POVPC. We determined previously that homogenates of aortas from rabbits fed an atherogenic diet contained 116 ng/mg wet weight POVPC. This is equivalent to 116 μ g/ml (5). Thus, the amount of POVPC used was approximately one-tenth of that found in the aortas.

In conclusion, we show that multiple signaling pathways need to be activated for the induction of monocyte-endothelium interactions by oxidized phospholipids. Monocyte binding is mediated by adhesion molecules on the endothelial cell surface, released monocyte activators,

and possibly molecules at the endothelial junctions that mediate monocyte migration but may also mediate firm adhesion (47). The molecular dissection of the upstream and downstream components of the vascular regulatory pathways of OxPAPC leading to specific monocyte adhesion remains to be undertaken, but it should ultimately lead to the development of novel therapeutic approaches against atherosclerosis and/or chronic inflammatory diseases. 

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