Objective—Previous studies have demonstrated the importance of endothelial apical expression of connecting segment-1 (CS-1) fibronectin in mediating the entry of monocytes into atherosclerotic lesions and other sites of chronic inflammation. We previously demonstrated that oxidized PAPC (OxPAPC) increases monocyte-specific binding to arterial endothelium by causing deposition of CS-1 fibronectin on apical α5β1 integrin. The present studies identify important signal transduction components regulating this pathway.

Methods and Results—Using endothelial cells in culture, we demonstrate that activation of R-Ras is responsible for CS-1–mediated monocyte binding. Although few natural activators of R-Ras have been demonstrated, OxPAPC activated endothelial R-Ras by 2.5-fold but decreased levels of activated H-Ras. The importance of R-Ras/H-Ras balance in regulating monocyte binding was shown by overexpression studies. Constitutively active R-Ras enhanced monocyte adhesion, whereas coexpression with constitutively active H-Ras was inhibitory. Elevated cAMP, mediated by OxPAPC and specific components POVPC and PEIPC, was responsible for R-Ras activation, and dibutyryl cAMP and pertussis toxin were also effective activators of R-Ras. Using inhibitor and dominant-negative constructs, we demonstrated that phosphatidylinositol 3-kinase (PI3K) was a key downstream effector of R-Ras in this pathway.

Conclusions—OxPAPC, POVPC, and PEIPC induce a cAMP/R-Ras/PI3K signaling pathway that contributes to monocyte/endothelial cell adhesion and potentially atherosclerosis. (Arterioscler Thromb Vasc Biol. 2003;23:lll-lll.)

Key Words: PLEASE ■ SUPPLY ■ KEY ■ WORDS ■ XXXX

Monocytes have been demonstrated to play an important role in chronic inflammatory diseases including atherosclerosis, indicating the importance of determining the mechanisms regulating monocyte/endothelial cell (EC) interactions. Two endothelial membrane proteins, vascular cellular adhesion molecule-1 (VCAM-1) and connecting segment-1 (CS-1) fibronectin, have been demonstrated to bind monocyte integrin α5β1, causing firm adhesion, and there is evidence that both VCAM-1 and CS-1 play an important role in the entry of monocytes into human atherosclerotic lesions. VCAM-1 expression is increased in small-vessel but not large-vessel ECs of human atherosclerotic lesions. CS-1 fibronectin was demonstrated to be increased in large vessel endothelium overlying human atherosclerotic lesions. Blockade of CS-1/monocyte interactions inhibited atherosclerosis in mice. Studies by other groups demonstrated the importance of VCAM-1 in mouse atherosclerosis. Together, these studies suggest an important role for both VCAM-1 and CS-1 in monocyte entry into atherosclerotic lesions of both mice and humans. The present studies examine the mechanism of EC CS-1 regulation in response to phospholipid oxidation products.

The phospholipid component of minimally modified (mildly oxidized) low density lipoprotein (MM-LDL), 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (OxPAPC), as well as its component phospholipids 1-palmitoyl-2-oxovaleroyl-sn-glycero-3-phosphorylcholine (POVPC) and 1-palmitoyl-2-(5,6-epoxysiospropane E2)-sn-glycero-3-phosphorylcholine (PEIPC), activate human aortic ECs (HAECs) to bind monocytes. These lipids were demonstrated by mass spectrometry to accumulate in rabbit atherosclerotic lesions at concentrations that could activate monocyte binding to ECs. For MM-LDL, OxPAPC, and POVPC, monocyte binding to HAECs was shown to be mediated by CS-1 fibronectin. Activation of α5β1 integrin expressed on the apical surface of ECs played a critical role in CS-1...
deposition on the apical surface of cells. The present studies address the intracellular mechanism leading to α5β1 integrin activation and deposition of CS-1 on the EC surface.

Recent studies have determined that the Ras family of small GTPases are key cellular molecules regulating integrin/ligand interaction. R-Ras and H-Ras were shown to act in concert to modulate integrin-mediated cell adhesion of the basal cell surface to matrix. We present evidence that R-Ras/H-Ras balance may also play an important role in causing deposition of CS-1 fibronectin on the apical surface of aortic endothelium in response to oxidized phospholipids, thus contributing to inflammation.

Methods

Lipids and Other Reagents

PAPC was purchased from Avanti Polar Lipids, and oxidized PAPC (OxPAPC) was prepared in our laboratory and analyzed by mass spectrometry to confirm the lipid profile described previously. POVPC and 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphorylcholine (PGPC) were prepared by ozonolysis of PAPC. PEIPC was purified from OxPAPC as reported previously. Ras binding domain–bound agarose was then incubated for 90 minutes while rocking. Ras binding domain–bound agarose was mixed with glutathione-agarose (Sigma) and incubated at 4°C for 90 minutes while rocking. Ras binding domain–bound agarose was then incubated with 700 μL of control media or various concentrations of oxidized lipids as indicated. Cells were then rinsed twice with 300 μL of PBS, fixed with 4% paraformaldehyde for 30 minutes, and stored at 4°C in PBS until assay. Levels of CS-1–containing fibronectin, VCAM-1, and HUTS-21 binding were measured on the surface of HAECs as described previously. Nonoxidized PAPC, administered at concentrations up to 150 μg/mL, had no effect on cell-surface levels of CS-1 and VCAM-1 compared with control media, suggesting that the lipids themselves do not alter the ELISA assay.

Statistical Analysis

Data were analyzed using one-way ANOVA. Levels of significance were calculated using StatView (Abacus Concepts, Inc). P<0.05 was considered statistically significant.

Results

OxPAPC, POVPC, and PEIPC Induce CS-1-Dependent/VCAM-1-Independent Monocyte Adhesion to HAECs

Dose-response curves demonstrate that OxPAPC, POVPC, and, most potently, PEIPC increase the binding of monocytes to ECs (Figure 1A). Concentrations of lipid that stimulated significant levels of monocyte/EC adhesion were well below levels causing toxicity to HAECs. We next examined the monocyte binding molecules induced by these lipids. PEIPC, POVPC, and OxPAPC induced the expression of CS-1 fibronectin but not VCAM-1 (Figure 1B). In contrast, PGPC elevated VCAM-1 levels but not CS-1 fibronectin levels, consistent with our previous demonstration of PGPC as an inducer of monocyte as well as neutrophil adhesion to HAECs.

OxPAPC Induces Activation of R-Ras But Not H-Ras in HAECs

We previously demonstrated that deposition of CS-1 fibronectin depended on activation of α5β1 integrin. Because it was previously demonstrated by others that R-Ras and H-Ras could act in concert to modulate integrin activity, we next...
sought to determine whether oxidized phospholipids could activate R-Ras or H-Ras in HAECs. Treatment of HAECs with OxPAPC for 1 hour induced the GTP-bound (active) form of R-Ras by 2- to 3-fold when normalized to total R-Ras levels (Figure 2A). Activation was time-dependent (peak activation at 30 to 90 minutes), because levels of GTP-bound R-Ras were not increased compared with control at 15 minutes or 3 hours (data not shown). In contrast with the observed increase in OxPAPC-induced R-Ras activity, H-Ras activity in HAECs was decreased by OxPAPC compared with media (control) at 1 hour (Figure 2B). This decrease in H-Ras activity was also observed at other times tested, including 30 minutes and 3 hours (data not shown). Nonoxidized PAPC had no effect on R-Ras activity (Figure 2C) or H-Ras activity (data not shown).

R-Ras Directly Stimulates \( \alpha_5\beta_1 \)-Dependent Monocyte Binding to Aortic Endothelium

To examine the significance of altered levels of GTP-H-Ras and GTP-R-Ras in the induction of monocyte binding,
expression constructs for activated R-Ras and H-Ras were transfected into BAECs, which are easily transfected and responsive to OxPAPC. Expression of the constructs was verified by Western analysis and immunofluorescence (data not shown). BAECs were transfected with either empty vectors (EVs), constitutively active (V38) R-Ras, constitutively active (G12) H-Ras, or V38 R-Ras in combination with G12 H-Ras, and monocyte binding was measured. Expression of V38 R-Ras but not G12 H-Ras significantly enhanced monocyte adhesion (Figure 3A). Furthermore, the ability of V38 R-Ras to stimulate monocyte binding was attenuated by cotransfection with G12 H-Ras (Figure 3A). To verify the opposite roles of R-Ras and H-Ras in oxidized phospholipid-induced monocyte/endothelial cell adhesion, cells expressing constitutively active R-Ras or H-Ras were stimulated with OxPAPC, PEIPC, or nonoxidized PAPC (control) for 4 hours before assay for monocyte adhesion. OxPAPC and PEIPC increased monocyte adhesion to EV-transfected BAECs (Figure 3B). Importantly, all V38 R-Ras–transfected BAECs bound significantly more monocytes than BAECs transfected with EV and treated with nonoxidized PAPC (Figure 3B). However, G12 H-Ras expression inhibited the ability of OxPAPC and PEIPC to induce monocyte binding (Figure 3B). We next tested whether expression of constitutively active R-Ras could cause activation/occupation of β1 integrin (detected by the HUTS-21 antibody). As shown in Figure 3C, cells expressing active (V38) R-Ras bound significantly more HUTS-21 antibody compared with cells expressing G12 H-Ras. Together, these findings support a role for R-Ras stimulation and H-Ras inhibition in OxPAPC-induced monocyte adhesion to aortic ECs and demonstrate the importance of R-Ras activation in modulating endothelial β1 integrin to bind fibronectin, the key event in OxPAPC-induced monocyte-specific adhesion to endothelium.

Role of cAMP in R-Ras Activation and Stimulation of Monocyte Adhesion by OxPAPC, POVPC, and PEIPC

Inhibition of cAMP activity was previously shown to block MM-LDL–induced monocyte adhesion to ECs.27 It was also demonstrated that OxPAPC and POVPC increased endothelial cAMP levels.8,28 In this study, we performed studies to determine if PEIPC, like POVPC, could elevate cAMP levels. In voltage clamp experiments, Xenopus oocytes were used to measure current across a cAMP-dependent chloride channel. The fold increase in conductance caused by exposure of the oocytes to PEIPC was similar to that of POVPC: 40.4±2.3-fold over baseline (mean±SD from 4 oocytes). However, the amount of PEIPC required to cause this increase was approximately 10-fold lower (0.5 μmol/L) than that of POVPC, suggesting that the PEIPC component of OxPAPC is a potent cAMP-elevating phospholipid.

To analyze the role of cAMP in oxidized phospholipid-induced R-Ras signaling, the effect of Rp-cAMPS (a nonhydrolyzable diastereomer of cAMP29,30) on the actions of OxPAPC as well as POVPC, PGPC, and PEIPC was tested. HAECs were pretreated with media alone or Rp-cAMPS alone for 1 hour and subsequently treated with either nonoxidized PAPC (control), Rp-cAMPS, OxPAPC, POVPC, PGPC, or each lipid in combination with Rp-cAMPS for an additional 1 hour. OxPAPC, POVPC, and PEIPC (known to increase cAMP) significantly enhanced R-Ras activation (GTP-R-Ras levels) (Figure 4A). Notably, activation of R-Ras by OxPAPC, POVPC, and PEIPC was strongly inhibited by Rp-cAMPS (Figure 4A). Basal levels of activated R-Ras were also decreased in the presence of Rp-cAMPS (PAPC versus Rp-cAMPS), suggesting that cAMP is required for maintenance of activated R-Ras levels in untreated HAECs. PGPC, which does not elevate cAMP levels, did not activate R-Ras (data not shown). Rp-cAMPS also inhibited monocyte adhesion induced by OxPAPC, PEIPC, and POVPC (Figure 4B). LPS treatment was used as a positive control treatment for inducing monocyte binding, and the effect of LPS was not altered by Rp-cAMPS.

To confirm the importance of cAMP in activating R-Ras, HAECs were exposed to the cell-permeable cAMP analog dibutylryl cAMP or pertussis toxin, which elevates cAMP levels by inhibiting Gaq. Treatment of HAECs with pertussis toxin resulted in a modest but significant and repeatable 30% increase in levels of active (GTP-bound) R-Ras (Figure 4C). More notably, treatment with dibutylryl cAMP enhanced R-Ras activity by approximately 3-fold (Figure 4C). Figure 4 demonstrates that cAMP plays an important role in R-Ras...
activation and monocyte/EC adhesion induced by oxidized phospholipids.

**PI3K Mediates OxPAPC and R-Ras Stimulation of β1 Integrin–Dependent Monocyte Adhesion to Aortic ECs**

Because PI3K is a known downstream mediator of R-Ras, we next tested whether inhibition of PI3K activity would alter the effectiveness of OxPAPC in inducing monocyte binding to HAECs. The PI3K inhibitor Ly-294002 strongly inhibited the marked increase in monocyte binding induced by OxPAPC and PEIPC compared with nonoxidized PAPC (Figure 5A). To confirm that activated R-Ras could directly promote monocyte adhesion to ECs in a PI3K-dependent fashion, R-Ras and PI3K expression constructs were used.

**Figure 4.** Role of cAMP in oxidized phospholipid activation of R-Ras and monocyte/EC adhesion. A, HAECs were pretreated with media alone or with the cAMP inhibitor Rp-cAMPS (11 μmol/L) for 1 hour and subsequently treated with either nonoxidized PAPC (35 μg/mL, control), OxPAPC (35 μg/mL), POVPC (10 μg/mL), PEIPC (1 μg/mL), PGPC (10 μg/mL), or each lipid in combination with Rp-cAMPS for an additional 1 hour. Levels of total and activated R-Ras were measured and quantified and displayed as described in Figure 2. B, HAECs were pretreated with or without Rp-cAMPS (11 μmol/L) for 1 hour, subsequently treated with nonoxidized PAPC (35 μg/mL, control), OxPAPC (35 μg/mL), POVPC (10 μg/mL), PEIPC (1 μg/mL), or LPS (2 ng/mL) in the presence [Rp(−)] or absence [Rp(+)] of Rp-cAMPS for an additional 4 hours, and then assayed for monocyte adhesion. C, HAECs were untreated (Con) or treated with pertussis toxin (PT, 800 ng/mL) or dibutylryl cAMP (Db-cAMP, 10 mmol/L) for 10 minutes. Levels of total and activated R-Ras were measured and quantified and displayed as described in Figure 2. A through C, Values are mean ± SEM for at least 4 experiments. *P<0.05 compared with treatments without Rp-cAMPS [Rp(−)] (A), corresponding treatments with Rp-cAMPS (B), or control (C). **P<0.01 compared with control (C).

**Figure 5.** OxPAPC-induced, PEIPC-induced, and R-Ras–induced monocyte binding is PI3K-dependent. A, HAECs were pretreated with media alone or with Ly-294002 (20 μmol/L) for 30 minutes and subsequently incubated with nonoxidized PAPC (Con), 50 μg/mL OxPAPC, or 1 μg/mL PEIPC in the presence [Ly(−)] or absence [Ly(+)] of 20 μmol/L Ly-294002 for 4 hours. B, BAECs were transfected with EV or V38 R-Ras in the presence or absence of Ly-294002 or transfected with EV or V38 R-Ras in combination with dominant-negative PI3K (p85DN). Appropriate levels of EV were included with all constructs to make the total amount of DNA equal for each transfection group. Monocyte adhesion was then measured (A and B). Values are mean ± SEM from 3 to 5 independent experiments. *P<0.05 between the indicated groups.
Transfection of BAECs with constitutively active (V38) R-Ras caused a significant increase in monocyte binding, which was inhibited by Ly-294002 (Figure 5B). As an additional method to inhibit cellular PI3K activity, BAECs were transfected with dominant-negative p85DN (a mutated PI3K subunit) alone or in combination with V38 R-Ras. Cotransfection of p85DN with V38 R-Ras prevented the increase in monocyte binding observed after V38 R-Ras transfection alone (Figure 5B). The findings, together with Figure 5, demonstrate that PI3K, a major downstream effector of activated R-Ras, is an important mediator of OxPAPC-induced monocyte-specific binding to aortic ECs.

Discussion

We present evidence that activation of EC R-Ras by oxidized phospholipids plays an important role in mediating the ability of these lipids to induce monocyte binding to CS-1 fibronectin. OxPAPC, PEIPC, and POVPc, all of which elevate cAMP, increased EC surface levels of CS-1 (Figure 1) and induced R-Ras activation (Figures 2 and 4). Confirming the importance of R-Ras in inducing monocyte binding, transient expression of constitutively active (V38) R-Ras enhanced monocyte adhesion and induced the ligand (fibronectin)-occupied conformation of \( \beta_{\text{c}} \) integrin (Figure 3). These data are consistent with previous observations demonstrating that V38 R-Ras expression increased \( \alpha_{\text{c}} \beta_{\text{c}} \)-dependent adhesion of 3D cells to fibronectin and increased fibronectin matrix assembly by CHO263a cells. Because the regulation of integrins by Ras-like molecules has been studied mainly in hematopoietic cells and cell lines, these studies extend the importance of R-Ras to regulating the assembly of fibronectin at the apical surface of ECs. Few physiological stimulators of R-Ras have been reported. The presented studies demonstrate for the first time that naturally occurring phospholipid oxidation products of MM-LDL, namely OxPAPC, PEIPC, and POVPc, are potent activators of R-Ras in HAECs and that this activation is responsible for increasing monocyte/endothelial interactions.

The current studies also demonstrate that inhibition of H-Ras activity is important in the induction of monocyte binding by OxPAPC. OxPAPC was shown to inhibit H-Ras activity while increasing R-Ras activity (Figure 2). Furthermore, overexpression of G12 H-Ras reversed the effect of V38 R-Ras on monocyte binding (Figure 3A) and prevented OxPAPC- and PEIPC-induced monocyte adhesion (Figure 3B). The idea that a cell activator may modulate integrin activity by differentially affecting R-Ras and H-Ras is supported by the reports of several investigators. Correlating with the negative effects of H-Ras on integrin activation discussed above, activation of H-Ras inhibited integrin activation in a CHO cell line. Activation of integrins by R-Ras, however, was determined to be the result of R-Ras’s ability to overcome H-Ras/Raf-1–mediated integrin inhibition. Therefore, OxPAPC-induced activation of R-Ras and inhibition of H-Ras is consistent with the hypothesis that OxPAPC-induced monocyte binding is mediated by its effect on each molecule.

We have identified cAMP as an important mediator of R-Ras activation by OxPAPC and component phospholipids POVPc and PEIPC. Previous studies reported that treatment of ECs with MM-LDL, OxPAPC, and POVPc but not PGPC increased cellular levels of cAMP. Monocyte binding to ECs was mimicked by cAMP elevating agents, and the effect of MM-LDL on monocyte binding was inhibited by H8, an inhibitor of cyclic nucleotide-dependent protein kinases, including protein kinase A. The present studies showed that PEIPC, the most active phospholipid component of OxPAPC, also increases cAMP. They also demonstrate that activation of R-Ras by OxPAPC, PEIPC, and POVPc is critically dependent on elevated levels of cAMP (Figures 4A and 4B). Confirming a role for cAMP in activation of R-Ras, treatment of cells with dibutylryl cAMP caused an elevation of R-Ras activity (Figure 4C). We therefore hypothesize that cAMP may modulate R-Ras activity through a yet to be identified R-Ras–specific cAMP-responsive GEF. An alternate mechanism for R-Ras activation by cAMP is through activation of PKA. Additional investigation is needed to determine a direct role for PKA or a cAMP-responsive GEF in R-Ras–specific signaling.

Inhibition of PI3K decreased OxPAPC–induced monocyte binding as well as V38 R-Ras–stimulated monocyte binding (Figure 5). Supporting the concept that R-Ras activity is mediated by PI3K, V38 R-Ras–induced adhesion to fibronectin was shown to be markedly inhibited by the dominant-negative mutant of PI3K. Although the mechanism by which PI3K causes increased deposition of apical surface fibronectin is not yet clear, our previous findings suggest an involvement of aggregated \( \alpha_{\text{c}} \beta_{\text{c}} \) integrin, because \( \alpha_{\text{c}} \beta_{\text{c}} \) was observed to be colocalized with CS-1 fibronectin in patches at the apical surface of HAECs after OxPAPC treatment. PI3K activity has been shown to promote interaction of talin with the \( \beta_{\text{c}} \) cytoplasmic tail, an event believed to lead to clustering and activation of integrins.

In summary, these studies have shown that OxPAPC, POVPc, and PEIPC induction of monocyte/EC adhesion is mediated by a cAMP-dependent R-Ras/PI3K signaling pathway that leads to deposition of CS-1–containing fibronectin on apical surface \( \alpha_{\text{c}} \beta_{\text{c}} \) integrins of HAECs and subsequent binding of monocytes. A novel finding of this study is the demonstration that R-Ras/H-Ras regulation of integrin function, previously shown to be important in adhesion of cells to matrix, also plays an important role in the inflammatory response to oxidized phospholipids. Monocyte entry into the artery wall plays an important role in the disease process of atherosclerosis, and macrophage accumulation in coronary vessels has been shown to be an important determinant of unstable angina. Because previous studies have identified increased levels of CS-1 fibronectin in human atherosclerotic lesions where oxidized phospholipids also accumulate, the signaling pathway reported here may be of therapeutic value in patients at risk for acute coronary events.

Acknowledgments

This research was supported by Giannini Family Foundation (A.L.C.), American Heart Association 441357 (D.K.V.), and NIH HL30568 and HL64731 (J.A.B.).
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Oxidized Phospholipid-Induced Endothelial Cell/Monocyte Interaction Is Mediated by a cAMP-Dependent R-Ras/PI3-Kinase Pathway
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Arterioscler Thromb Vasc Biol. published online June 12, 2003;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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