Phosphatidylcholine hydroperoxide-induced THP-1 cell adhesion to intracellular adhesion molecule-1[S]

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Abstract The accumulation of phosphatidylcholine hydroperoxide (PCOOH), a primary oxidation product of phosphatidylcholine (PC), in blood plasma and tissues has been observed in various pathological conditions, including atherosclerosis. However, the biological roles of PCOOH in these conditions remain unknown. To estimate the atherogenicity of PCOOH, we evaluated the effect of PCOOH on THP-1 monocytic cell adherence to immobilized vascular endothelial cell adhesion molecules. THP-1 cell adhesion to intracellular adhesion molecule-1 (ICAM-1) was dose-dependently increased by addition of PCOOH. Phosphatidylcholine hydroxide (a hydroxyl analog of PCOOH) also induced THP-1 cell adhesion to ICAM-1, whereas nonoxidized PC, *sn*-2 truncated PCs, and other hydroperoxide compounds did not affect the adhesion. In the PCOOH-treated cells, obvious protruding F-actin-rich membrane structures were formed, and lymphocyte function-associated antigen-1 (LFA-1) was localized to the protruding structures. Cytochalasin D, an actin polymerization inhibitor, suppressed the PCOOH-induced cell adhesion to ICAM-1 and the membrane protrusions. These results indicate that PCOOH evokes LFA-1-mediated cell adhesion to ICAM-1 via actin cytoskeletal organization, and the mechanism may participate in monocyte adherence to the arterial wall in the initiation of atherosclerosis.—Asai, A., F. Okajima, K. Nakagawa, D. Ibusuki, K. Tanimura, Y. Nakajima, M. Nagao, M. Sudo, T. Harada, T. Miyazawa, and S. Oikawa. **Phosphatidylcholine hydroperoxide-induced THP-1 cell adhesion to intracellular adhesion molecule-1. *J. Lipid Res.* 2009.** 50: **957–965.**

Supplementary key words lipid oxidation • phospholipid • atherosclerosis • monocyte • integrin • cell adhesion molecule • actin cytoskeleton

Oxidative lipid modifications play important roles in the pathogenesis of atherosclerosis (1–4). Among oxidatively modified lipids, phosphatidylcholine hydroperoxide (PCOOH), a primary oxidation product of phosphatidylcholine (PC), was observed to be accumulated in arterial walls and blood plasma in atherosclerotic rabbits (5). PCOOH was also identified in human atherosclerotic lesions (6, 7). Furthermore, PCOOH accumulation in plasma has been shown in human subjects with pathological conditions such as hyperlipidemia (8, 9), diabetes (10), uremia (9), and alcoholism (11). Because hyperlipidemia and diabetes are strongly associated with increased incidence of atherosclerosis (12–14), higher plasma PCOOH conditions in these patients may be involved in atherogenesis.

In the course of phospholipid oxidation, PC is primarily oxidized to PCOOH. Further oxidative modification of PCOOH yields various secondary oxidation products. To date, a number of the secondary oxidation products have been reported to mediate several atherogenic processes (15, 16). For instance, a series of oxidized PCs with a truncated *sn*-2 acyl group (*sn*-2-truncated PCs), such as 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphocholine (POVPC) and 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphocholine, activate aortic endothelial cell binding to monocytes (17–19). The *sn*-2-truncated PCs with a terminal aldehyde group (PC core aldehydes), such as POVPC, are also postulated to bind to lysine residues of proteins and, consequently, to

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Abbreviations: HNE, 4-hydroxy-2-nonenal; H(p)ETE, hydro(pero)xyeicosatetraenoic acid; H(p)ODE, hydro(pero)xyoctadecadienoic acid; ICAM-1, intracellular adhesion molecule-1; LFA-1, lymphocyte function-associated antigen-1; LysoPC, lysophosphatidylcholine; mAb24, monoclonal antibody 24; PAPC, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine; PAPCO(O)H, PAPC hydro(pero)xide; PC, phosphatidylcholine; PCOH, phosphatidylcholine hydroxide; PCOOH, phosphatidylcholine hydroperoxide; PKC, protein kinase C; PLA_2, phospholipase A_2; PLPC, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine; PLPCO(O)H, PLPC hydro(pero)xide; POVPC, 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphocholine; ROS, reactive oxygen species; VCAM-1, vascular cell adhesion molecule-1.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of four figures.

modify the protein activities (16). Members of another series of *sn*-2-truncated PCs, which incorporate a terminal γ-hydroxy(or oxo)-α,β-unsaturated carbonyl, such as 1-palmitoyl-2-(4-keto-2-dodecenedioyl)-*sn*-glycero-3-phosphocholine and 1-palmitoyl-2-(4-keto-2-octenedioyl)-*sn*-glycero-3-phosphocholine, serve as ligands for CD36 (termed $oxPC_{CD36}$) and promote macrophage foam cell formation (20, 21). In addition, lipid hydroperoxide-derived short-chain aldehydes, such as 4-hydroxy-2-nonenal (HNE), are reported to modulate proatherogenic responses (22). Lysophosphatidylcholine (LysoPC) and hydro(pero)xy fatty acids, such as hydro(pero)xyoctadecadienoic acid [H(p)ODE] and hydro(pero)xyeicosatetraenoic acid [H(p)ETE], have been reported to induce expression of cell adhesion molecules in endothelial cells (23–25). LysoPC and hydro(pero)xy fatty acids might be generated in atherosclerotic lesions by certain phospholipase A_2 (PLA_2) enzymes (26, 27). These findings indicate that PCOOH could contribute to the development of atherosclerosis as a precursor of these bioactive compounds.

In contrast to the proatherogenic properties of the secondary oxidation products, little is known regarding the biological functions of PCOOH, a primary oxidation product of PC. To estimate the effect of PCOOH on monocyte adherence to the arterial wall, we performed a cell adhesion assay using THP-1 cells with immobilized cell adhesion molecules in vitro. Although various oxidized lipids have been reported to induce cell adhesion molecule expressions in vascular endothelial cells, the direct interaction of oxidized lipids with circulating monocytes has not been fully investigated to date.

EXPERIMENTAL PROCEDURES

Materials

Fc chimeric recombinant proteins of intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin were purchased from R and D Systems (Minneapolis, MN). Anti-human IgG (Fc specific) [anti-IgG(Fc)], 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (PLPC), 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PAPC), LysoPC (1-palmitoyl-*sn*-glycero-3-phosphocholine), PLA_2 (from *Naja mossambica mossambica*), PMA, α-tocopherol, and BSA were from Sigma-Aldrich (St. Louis, MO). 1-Palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphocholine, POVPC, 1-palmitoyl-2-(4-keto-2-dodecenedioyl)-*sn*-glycero-3-phosphocholine, 1-palmitoyl-2-(4-keto-2-octenedioyl)-*sn*-glycero-3-phosphocholine, cholesteryl linoleate hydroperoxide, H(p)ODE, H(p)ETE, and HNE were purchased from Cayman Chemical (Ann Arbor, MI). 1-Palmitoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine and 1-palmitoyl- 2-(9-oxononanoyl)-*sn*-glycero-3-phosphocholine were from Avanti Polar Lipids (Alabaster, AL). Cumene hydroperoxide, *tert*-butyl hydroperoxide, hydrogen peroxide (H_2O_2), and butylhydroxytoluene were from Wako Pure Chemical (Osaka, Japan). Monoclonal antibodies for human CD11a (clone 38), CD11b (ICRF44), and CD18 (IB4), and isotype control IgG_1 (MOPC 31C) and IgG_{2a} (RPC 5) were from Ancell (Bayport, MN). Anti-CD11c (clone 3.9) was purchased from BioLegend (San Diego, CA). Monoclonal antibody 24 (mAb24), which recognizes β_2-integrin in high affinity conformation (28), was from Abcam (Cambridge, UK). Monoclonal antibodies for human protein kinase Cα (PKCα) (clone 3) and PKCδ (clone 14) were from BD Biosciences (Franklin Lakes, NJ). Cytochalasin D was purchased from Merck (Darmstadt, Germany).

PCOOH was enzymatically synthesized from PLPC or PAPC using soybean lipoxygenase-1 and chromatographically purified to yield an essentially pure compound (29) (see supplementary Fig. I). Phosphatidylcholine hydroxide (PCOH), a hydroxyl analog of PCOOH, was obtained by reducing PCOOH with $NaBH_4$ and purified chromatographically as above (see supplementary Fig. I). The predominant *sn*-2 residues of the purified PCO(O)H derived from PLPC [PLPCO(O)H] and that derived from PAPC [PAPCO(O)H] were 13-H(p)ODE and 15-H(p)ETE, respectively (see supplementary Fig. I). Purified PCO(O)H was stored at −80°C as methanol solution, and the concentrations were routinely measured using a molar extinction coefficient of 23,000 $M^{-1}cm^{-1}$ at 234 nm for conjugated diene (30). PCO(O)H-containing medium used in this study did not contain detectable amounts of endotoxin (<0.01 EU/ml), measured with a *Limulus* amebocyte lysate chromogenic test kit (Toxicolor LS; Seikagaku, Tokyo, Japan). PLA_2 treatment of PCO(O)H was performed as described by Obinata et al. (31). In this study, PLPCOOH was used for all experiments as a representative of PCOOH molecular species unless otherwise stated because PLPCOOH is a predominant PCOOH molecule in human blood plasma (11, 32) and is a major oxidized lipid molecule in the early phase of LDL oxidation (33). PAPCOOH was also used in some experiments because a series of secondary oxidation products derived from PAPC has been reported to possess various proatherogenic properties (15, 16).

Cell culture

The monocytic cell line THP-1 was obtained from Dainippon Sumitomo Pharma (Osaka, Japan). The cells were grown in RPMI 1640 medium supplemented with L-glutamine (Invitrogen, Carlsbad, CA), penicillin/streptomycin (Invitrogen), and 10% heat-inactivated fetal calf serum (Tissue Culture Biologicals, Tulare, CA). Before performing the assays described below, THP-1 cells were incubated overnight in fetal calf serum-free RPMI 1640 medium containing 0.1% BSA (0.1%BSA-RPMI).

Cell adhesion assay

A polystyrene high-bind 96-well plate (Corning Inc., Corning, NY) was used for the adhesion assay. The wells were coated with 100 μl/well of anti-IgG(Fc) at 5 μg/ml (for ICAM-1) or 0.625 μg/ml (for VCAM-1 and E-selectin) in 50 mM sodium carbonate buffer (pH 9.2) overnight at 4°C. The anti-IgG(Fc) concentrations were defined such as to yield moderate cell adhesion (~10% of loaded cells) without any stimulant. The wells were then washed three times with 200 μl/well of PBS, and nonspecific binding sites were blocked with 0.5% BSA in PBS (150 μl/well) for 30 min at 37°C. After washing three times with PBS (200 μl/well), the wells were coated with 1 μg/ml of Fc chimeric protein of ICAM-1, VCAM-1, or E-selectin in PBS containing 0.1% BSA (50 μl/well) for 2 h at room temperature. The wells were then washed three times with 200 μl/well of HBSS. Immediately after THP-1 cells were suspended at a concentration of 5×10^5 cells/ml in 0.1%BSA-RPMI with stimulants, e.g., PCOOH, the cells were incubated in the adhesion molecule-immobilized wells [100 μl (5×10^4 cells)/well] at 37°C for 15 min, unless otherwise stated. DMSO was used as vehicle at a final concentration of 0.4%. After the incubation, nonadherent cells were removed by centrifugation (top side down) at 40 *g* for 5 min. Adherent cells were then fixed with 5% glutaraldehyde for 30 min. The fixed cells were rinsed three times with distilled water and stained for 20 min with 100 μl/well of 0.1% crystalviolet in 0.2 M 2-morpholinoethanesulfonic acid

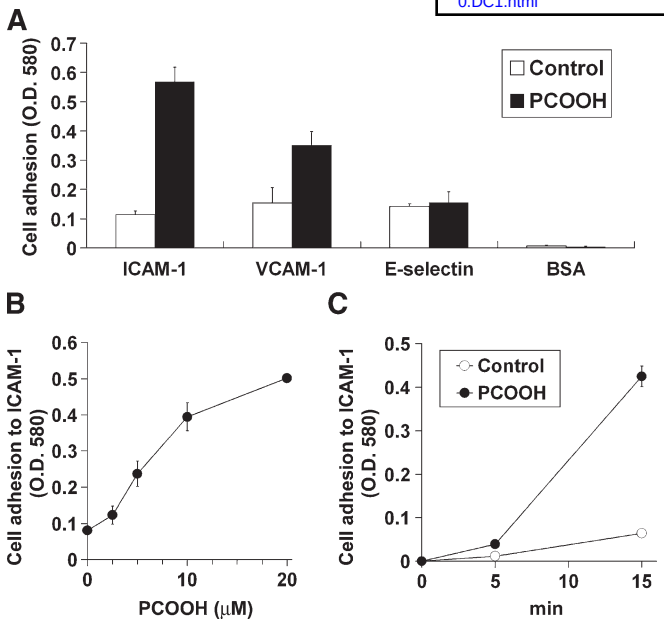

Fig. 1. Effect of PCOOH on THP-1 cell adhesion to immobilized cell adhesion molecules. A: THP-1 cells were incubated in the presence (closed bars) or absence (open bars) of 10 μM PCOOH for 15 min in ICAM-1-, VCAM-1-, or E-selectin-coated wells. BSA indicates nonspecific cell attachment. B: THP-1 cells were incubated with 0–20 μM PCOOH for 15 min in ICAM-1-coated wells. C: THP-1 cells were incubated in the presence (closed circles) or absence (open circles) of 10 μM PCOOH for 5 or 15 min in ICAM-1-coated wells. Results are representative of at least three independent experiments. Data represent the mean ± SD of quadruplicate wells.

(pH 6.0). After excess dye was washed out three times with distilled water, the cell-bound dye was solubilized with 100 μl/well of 10% acetic acid (34). The absorbance at 580 nm was recorded with a microplate reader (FLUOstar OPTIMA; BMG Labtechnologies, Offenburg, Germany). The absorbance/cell number relation was linear up to an optical density of 0.9, which represented $\sim 5 \times 10^4$ cells/well (100% of loaded cells).

Intracellular reactive oxygen species

The intracellular production of reactive oxygen species (ROS) was evaluated using dichlorofluorescein as a fluorescent probe. In brief, THP-1 cells were treated with 5 μM 2′,7′-dichlorofluorescein diacetate (Invitrogen) in PBS at 37°C for 30 min and washed twice with PBS. The cells were then resuspended in 0.1%BSA-RPMI and incubated with stimulants as described above. The cellular fluorescence intensity was measured with a FACSCan flow cytometer using CellQuest software (BD Biosciences).

Flow cytometric analysis of β_2-integrin

To analyze the cell surface expression of β_2-integrin subtypes, i.e., lymphocyte function-associated antigen-1 (LFA-1; CD11a/CD18; $\alpha_L\beta_2$), Mac-1 (CD11b/CD18; $\alpha_M\beta_2$), and p150/95 (CD11c/CD18; $\alpha_X\beta_2$), cells were treated with anti-CD11a, -CD11b, -CD11c, or -CD18. In brief, after PCOOH-treated cells were washed with PBS containing 0.1% BSA and 0.05% NaN_3 (labeling buffer), the cells were then treated with each antibody in the labeling buffer for 30 min at 4°C. The cells were washed again and further treated with phycoerythrin-conjugated anti-mouse IgG (BD Biosciences) in the labeling buffer for 30 min at 4°C. Flow cytometric analysis was performed as described above. For the analysis of β_2-integrin in high-affinity conformation, mAb24 and phycoerythrin-conjugated anti-mouse IgG were sequentially added to the cell suspension without washing and incubated for 10 min each at 37°C to minimize the possible conformational changes by the mechanical and physical stresses during the sample preparation.

Analysis of actin polymerization

After cells were fixed with 3.7% formaldehyde in PBS, filamentous actin (F-actin) was stained with 10 U/ml of Alexa Fluor 488-conjugated phalloidin (Invitrogen) in PBS containing LysoPC

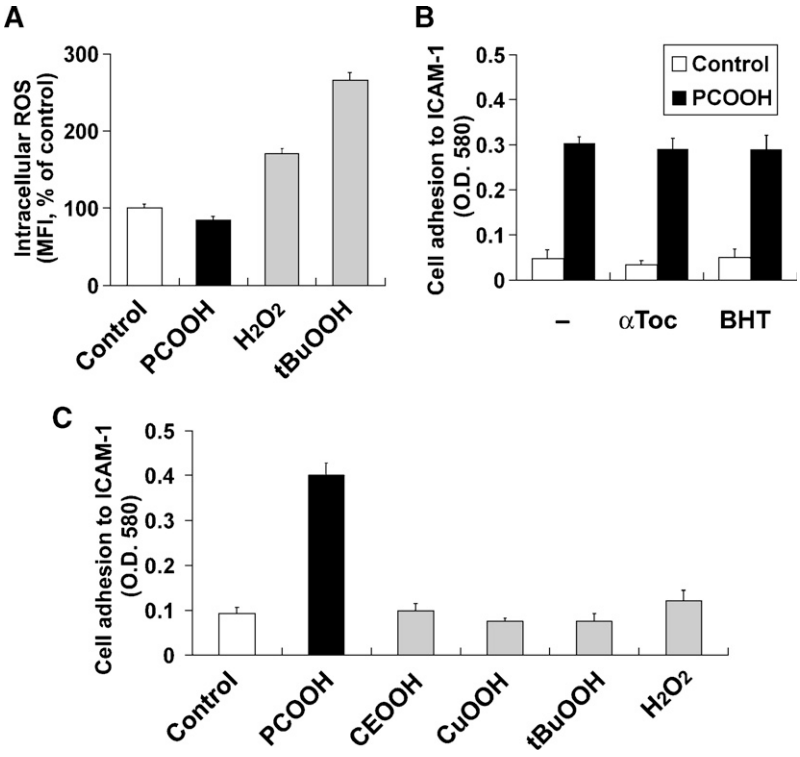

Fig. 2. PCOOH-induced THP-1 cell adhesion to ICAM-1 was not mediated by intracellular ROS production. A: Intracellular ROS levels were evaluated as dichlorofluorescein fluorescence intensity after dichlorofluorescein-treated THP-1 cells were incubated with PCOOH (10 μM), H_2O_2 (100 μM), *tert*-butyl hydroperoxide (tBuOOH; 100 μM), or vehicle alone (Control) for 10 min (mean ± SD of triplicate measurements). B: THP-1 cells were subjected to the adhesion assay on ICAM-1 in the presence (closed bars) or absence (open bars) of 10 μM PCOOH after the cells were preincubated with α-tocopherol (α-Toc; 50 μM), butylhydroxytoluene (BHT; 50 μM), or vehicle alone (−) for 16 h. C: THP-1 cells were subjected to the adhesion assay on ICAM-1 in the presence of hydroperoxide molecules at 10 μM. CEOOH, cholesteryl linoleate hydroperoxide; CuOOH, cumene hydroperoxide. Results are representative of three independent experiments. Data represent the mean ± SD of triplicate measurements (A) or quadruplicate wells (B, C).

(100 μg/ml) to permeabilize the membrane. The intracellular distribution of F-actin was analyzed by fluorescence microscopy (IX71; Olympus, Tokyo, Japan). Images were acquired and analyzed with Aquacosmos software (Hamamatsu Photonics, Hamamatsu, Japan). The cellular fluorescence intensity was also measured by flow cytometry to estimate the intracellular F-actin content. To determine the cell surface distribution of LFA-1, cells were probed with anti-CD11a followed by Alexa Fluor 555-conjugated anti-mouse IgG (Invitrogen) before the fixation. To avoid mechanical disruption of the cell morphology during the sample preparation, the antibodies were sequentially added to the cell suspension as described above.

Western blot analysis of PKC translocation

Cells were sonicated in ice-cold isolation buffer [50 mM Tris-HCl (pH 7.5) containing 2 mM EDTA, 2 mM EGTA, and a protease inhibitor cocktail (Complete; Roche Diagnostics, Mannheim, Germany)] (35). After the homogenates were centrifuged at 9,000 *g* for 10 min at 4°C, the supernatant was further centrifuged at 105,000 *g* for 60 min at 4°C. The resultant supernatant was taken as cytosolic fraction, and the precipitate dissolved in 1% Triton X-100 in the isolation buffer was taken as the corresponding membrane fraction. Equal amounts (corresponding to 10^5 cells) of each fraction were separated on 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane. The blots were probed with monoclonal antibody for PKCα or PKCδ and detected using an ECL Plus detection kit with horseradish peroxidase-conjugated anti-mouse IgG (GE Healthcare, Buckinghamshire, UK).

RESULTS

PCOOH-induced THP-1 cell adhesion to ICAM-1

THP-1 cell adhesion to ICAM-1 was increased ~4-fold by 10 μM PCOOH, whereas the adhesion to VCAM-1 was increased to a lesser extent (~2-fold), and that to E-selectin was not affected by PCOOH (**Fig. 1A**). The PCOOH-dependent cell adhesion to ICAM-1 was increased in a dose- and time-dependent manner (Fig. 1B, C). In this range of PCOOH concentrations (≤20 μM), no cytotoxic effect was observed even when the incubation time was prolonged up to 24 h (see supplementary Fig. II).

PCOOH-induced THP-1 cell adhesion was not mediated by ROS production

Treatment with 10 μM PCOOH did not increase intracellular ROS levels in THP-1 cells, whereas the ROS levels were increased in cells treated with 100 μM H_2O_2 or *tert*-butyl hydroperoxide (positive controls) (**Fig. 2A**). Moreover, pretreatment of THP-1 cells with antioxidants (α-tocopherol or butylhydroxytoluene) did not inhibit the PCOOH-induced cell adhesion (Fig. 2B). In addition, none of the other hydroperoxide compounds tested (cholesteryl linoleate hydroperoxide, *tert*-butyl hydroperoxide, cumene hydroperoxide, and H_2O_2) induced the cell adhesion (Fig. 2C). These results suggested that the PCOOH-induced THP-1

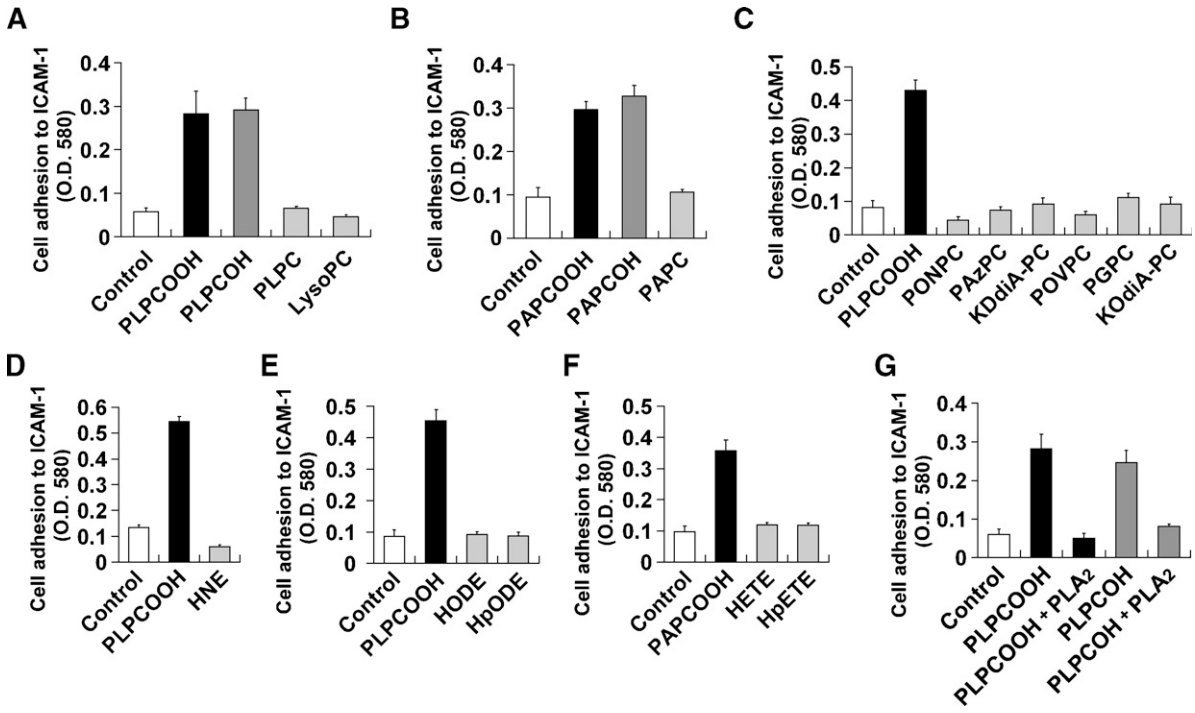

Fig. 3. PCOOH- and PCOH-specific induction of THP-1 cell adhesion to ICAM-1. THP-1 cells were subjected to the adhesion assay on ICAM-1 in the presence (10 μM each) of PLPCO(O)H, nonoxidized PLPC, or LysoPC (A); PAPCO(O)H or nonoxidized PAPC (B); *sn*-2-truncated PCs (C); HNE (D); or nonesterified hydro(pero)xy fatty acids (E, F). PONPC, 1-palmitoyl-2-(9-oxononanoyl)-*sn*-glycero-3-phosphocholine; PAzPC, 1-palmitoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine; KDdiA-PC, 1-palmitoyl-2-(4-keto-2-dodecenedioyl)-*sn*-glycero-3-phosphocholine; KOdiA-PC, 1-palmitoyl-2-(4-keto-2-octenedioyl)-*sn*-glycero-3-phosphocholine. G: The adhesion assay was performed in the presence of PLPCO(O)H or its PLA_2-treated products (+PLA_2) [10 μM equivalent of PLPCO(O)H]. Results are representative of three independent experiments. Data represent the mean ± SD of quadruplicate wells.

cell adhesion to ICAM-1 was not mediated by intracellular ROS production.

PCOOH and PCOH specifically induced THP-1 cell adhesion to ICAM-1

PLPCOOH and its hydroxyl analog (PLPCOH) at the same concentrations increased THP-1 cell adhesion to ICAM-1 to a similar extent, whereas native (nonoxidized) PLPC and LysoPC did not affect the adhesion (**Fig. 3A**) (see supplementary Fig. III). PAPCOOH, another PCOOH molecule, and its hydroxyl analog (PAPCOH) also increased the cell adhesion (Fig. 3B). On the other hand, *sn*-2-truncated PCs and HNE did not induce the adhesion (Fig. 3C, D). In addition, nonesterified hydro(pero)xy fatty acids [H(p)ODE and H(p)ETE] did not affect the adhesion (Fig. 3E, F). The PLPCO(O)H-induced cell adhesion was also abolished by pretreatment of the lipids with PLA_2 (Fig. 3G). These results indicated that the THP-1 cell adhesion to ICAM-1 was stimulated by PCOOH itself or its reduced product (PCOH) but not by their further metabolized secondary oxidation products.

LFA-1 mediated THP-1 cell adhesion to ICAM-1

Because β_2-integrin is a monocytic counterreceptor for ICAM-1, we examined this property to determine which β_2-integrin subtype contributes to the PCOOH-induced THP-1 cell adhesion to ICAM-1. Pretreatment of THP-1 cells with anti-CD11a (LFA-1 α-subunit) and anti-CD18 (common β_2-subunit) almost completely abrogated the cell adhesion (**Fig. 4A**). In contrast, anti-CD11b (Mac-1 α-subunit), anti-CD11c (p150/95 α-subunit), and isotype control IgGs did not affect the adhesion (Fig. 4A). These results indicate the THP-1 cell adhesion to ICAM-1 is exclusively mediated by LFA-1 (CD11a/CD18). However, the PCOOH-induced cell adhesion to ICAM-1 may not be due to the increased cell surface expression of LFA-1 because PCOOH did not affect the cell surface expressions of CD11a and CD18 (Fig. 4B).

PCOOH-induced membrane protrusion and its involvement in LFA-1-mediated cell adhesion

Phorbol esters have been reported to activate β_2-integrin via PKC activation (36–38). Consistently, PMA induced the cell surface expression of β_2-integrin in high-affinity conformation (mAb24 epitope) and consequently increased THP-1 cell adhesion to ICAM-1 (**Fig. 5A, B**). However, the PCOOH-induced cell adhesion mechanism appears to be different from the PMA-induced adhesion mechanism. The longer time-course analysis indicated that PCOOH-induced cell adhesion was at least partially reversible, whereas PMA induced irreversible adhesion (differentiation to macrophage-like cells) (see supplementary Fig. IV). In contrast to the PMA-treated cells, PCOOH-treated cells showed much lower conformational activation of β_2-integrin (Fig. 5A) and did not indicate apparent PKC activation (Fig. 5C). On the other hand, PCOOH-treated cells, but not PMA-treated cells, showed obvious protruding membrane structures with actin polymerization (Fig. 5D, E). In addition, LFA-1

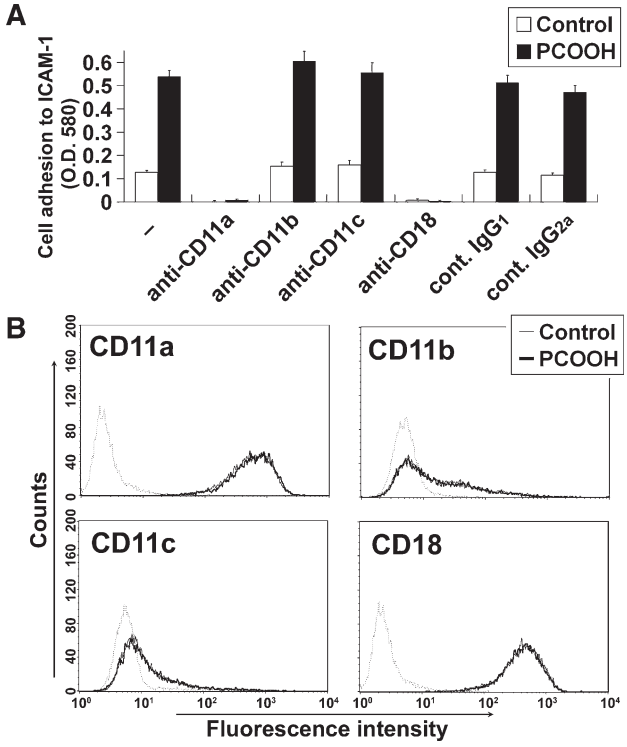

Fig. 4. LFA-1 (CD11a/CD18)-mediated THP-1 cell adhesion to ICAM-1. A: THP-1 cells were subjected to the adhesion assay on ICAM-1 in the presence (closed bars) or absence (open bars) of 10 μM PCOOH after the cells were incubated with the blocking antibody for each β_2-integrin subunit or isotype control IgG. Data represent the mean ± SD of quadruplicate wells. B: Cell surface expression of each β_2-integrin subunit was analyzed after THP-1 cells were incubated in the presence (bold line) or absence (thin solid line) of 10 μM PCOOH for 10 min. Dotted lines indicate negative controls (isotype control IgG). Results are representative of three independent experiments.

was localized to the protruding membrane structures in PCOOH-treated cells (Fig. 5F). As well as the protrusion formation with actin polymerization, PCOOH-induced THP-1 cell adhesion to ICAM-1 was suppressed by cytochalasin D, an actin polymerization inhibitor (**Fig. 6A–D**), whereas cytochalasin D did not affect the conformational activation of β_2-integrin (Figs. 5A and 6E). These results indicated that intracellular actin cytoskeletal remodeling was required for the PCOOH-induced THP-1 cell adhesion to ICAM-1.

DISCUSSION

With respect to the involvement of phospholipid modifications in the pathogenesis of atherosclerosis, much attention has been focused on the proatherogenic properties of the secondary oxidation products derived from PC. When LDL particles are retained in the arterial wall, they are thought to become susceptible to oxidative modifications. In this case, in the course of LDL oxidation, various oxidative reactions may lead to the accelerated production of the secondary metabolites, such as *sn*-2-truncated PCs in

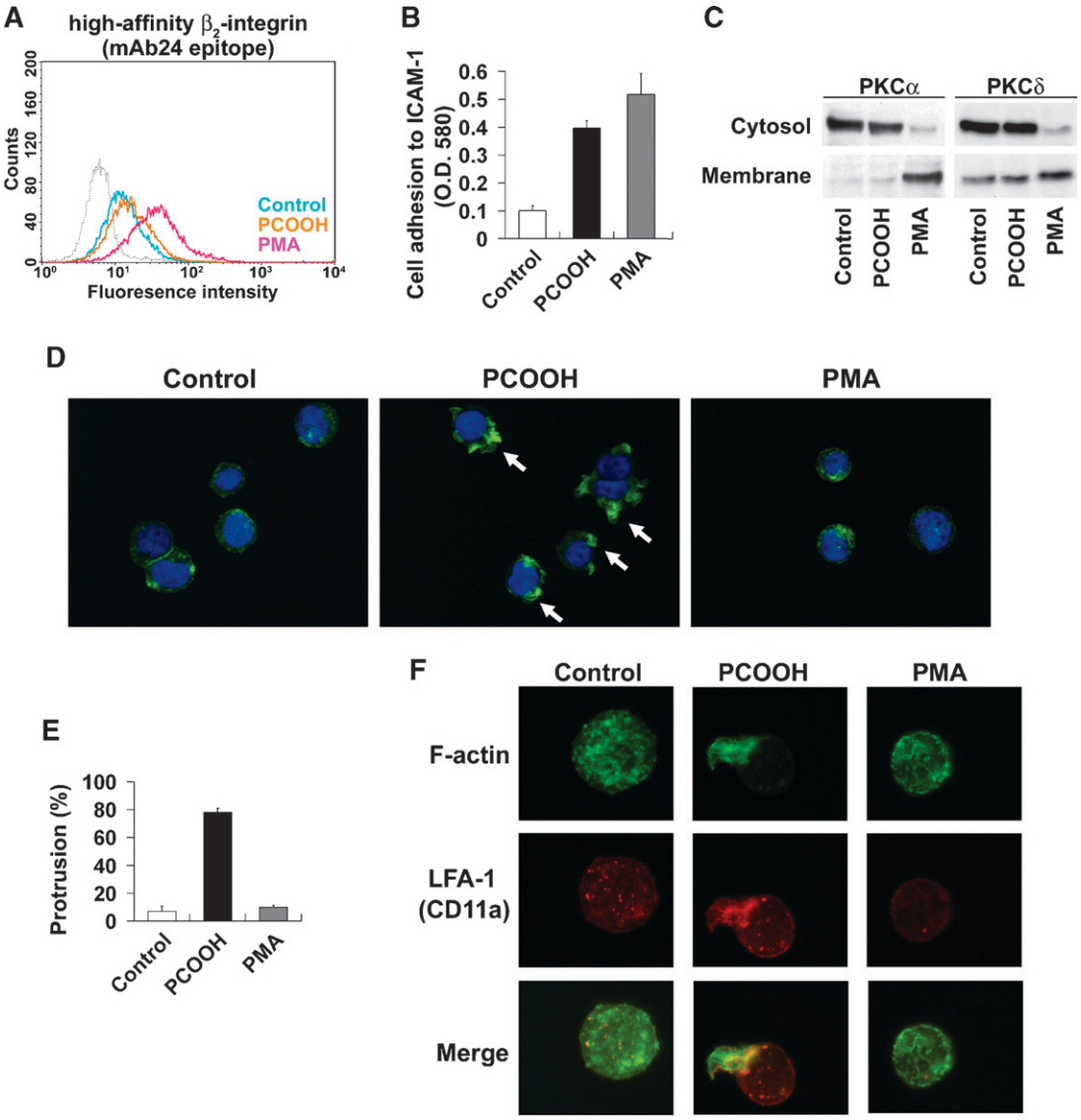

Fig. 5. Involvement of F-actin-rich membrane protrusion formation in PCOOH-induced THP-1 cell adhesion to ICAM-1. A: The cell surface expression of β_2-integrin in high-affinity conformation (mAb24 epitope) was analyzed after THP-1 cells were treated with PCOOH (10 μM), PMA (1 μM), or vehicle alone (Control) for 10 min. Dotted line indicates negative control (isotype control IgG). B: THP-1 cell adhesion assay on ICAM-1 was performed in the presence of PCOOH (10 μM), PMA (1 μM), or vehicle alone (Control). Data represent the mean ± SD of quadruplicate wells. C: PKC distributions in cytosolic and membrane fractions were analyzed after THP-1 cells were treated with PCOOH (10 μM), PMA (1 μM), or vehicle alone (Control) for 3 min. D: F-actin-stained fluorescence (green) images after THP-1cells were treated with PCOOH (10 μM), PMA (1 μM), or vehicle alone (Control) for 10 min. Obvious membrane protrusion formations (arrows) were observed in PCOOH-treated cells. Nuclei were costained with 4′,6-diamidino-2-phenylindole (blue). E: The percentage of the cells with the protruding structures was calculated from the fluorescence images shown in D (>100 cells for each measurement; mean ± SD of triplicate measurements). F: F-actin (green) and CD11a (red) were costained after THP-1cells were treated with PCOOH (10 μM), PMA (1 μM), or vehicle alone (Control) for 10 min. Results are representative of three independent experiments.

the arterial subendothelium. LysoPC and hydro(pero)xy fatty acids would be also produced by certain PLA_2 enzymes. Therefore, PCOOH may participate in the development of atherosclerosis at least as a common precursor of these proatherogenic secondary oxidation products.

In contrast to the oxidation-prone state in the subendothelial compartment, lipoproteins are thought to be protected from severe oxidative modifications in blood circulation where various plasma components act as antioxidants. Hence, the oxidative modifications of plasma lipoproteins may be limited to a lesser extent; however, the oxidized LDL levels in plasma have been reported to correlate with the severity of cardiovascular diseases (39–42). In addition, PCOOH has been identified as a major oxidized lipid in the early phase of LDL oxidation (33). In this context, our previous observations of higher plasma PCOOH concentrations in subjects with hyperlipidemia (8) and diabetes (10) prompted the hypothesis that PCOOH may initiate the atherosclerotic lesion formation by increasing monocyte adherence to the arterial wall because both hyperlipidemia and diabetes are strongly associated with an increased incidence of atherosclerosis (12–14).

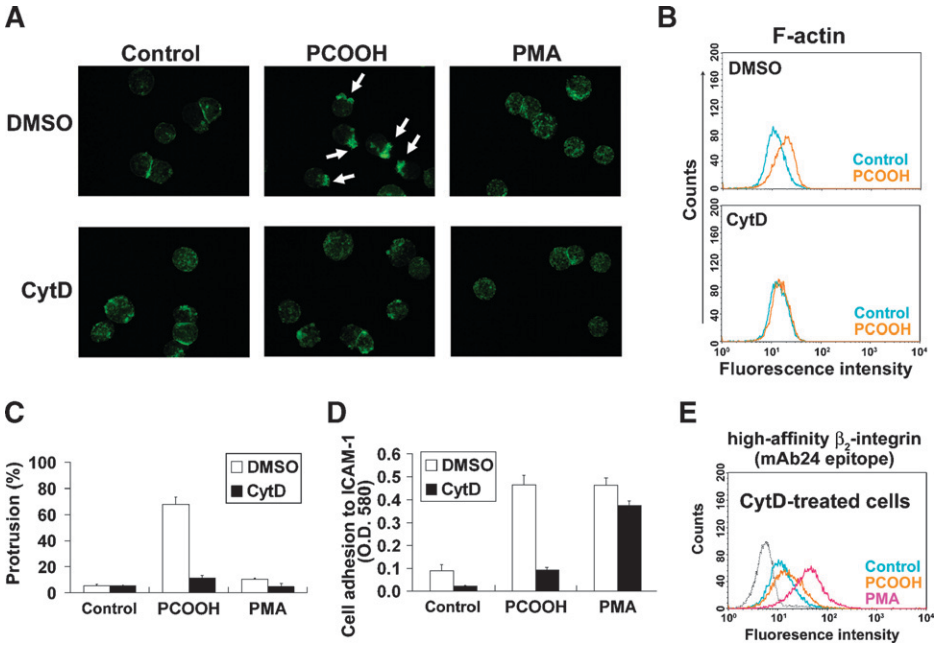

Fig. 6. Cytochalasin D suppressed F-actin-rich membrane protrusion formation and cell adhesion to ICAM-1 in PCOOH-treated THP-1 cells. THP-1 cells were preincubated with 1 μM cytochalasin D (CytD) or vehicle (DMSO) for 30 min before the cells were treated with PCOOH (10 μM), PMA (1 μM), or vehicle alone (Control) for 10 min. A–C: THP-1 cells were preincubated with 1 μM cytochalasin D or vehicle (DMSO) for 30 min. After that, the cells were treated with PCOOH (10 μM), PMA (1 μM), or vehicle alone (Control) for 10 min. F-actin-stained fluorescence images (A), intracellular actin polymerization levels (F-actin fluorescence intensity) (B), and the percentage of the cells with the protruding structures (C) are shown. Arrows in A indicate the protruding structures. Data in C represent the mean ± SD of triplicate measurements. D: After THP-1 cells were treated with cytochalasin D as above, the cell adhesion assay on ICAM-1 was performed in the presence of PCOOH (10 μM), PMA (1 μM), or vehicle alone (Control). Data represent the mean ± SD of quadruplicate wells. E: The cell surface expression of β_2-integrin in high-affinity conformation (mAb24 epitope) was analyzed after the cytochalasin D treatment. Dotted line indicates negative control (isotype control IgG). Results are representative of three independent experiments.

In this study, we examined the effect of PCOOH on the adherence of THP-1 cells and found that the cell adhesion to ICAM-1 was induced by PCOOH (Fig. 1). Further experiments indicated that the PCOOH-induced cell adhesion was not mediated by intracellular ROS production or secondary oxidation metabolites (Figs. 2 and 3). Hence, PCOOH itself may play a role in the initial step of atherogenesis by directly modulating the adherence of circulating monocytes. In addition to lipoprotein particles in the circulation, PCOOH has been reported to be present in the arterial wall, especially in the oxidation-prone atherosclerotic lesions (5–7). Therefore, topical PCOOH production in the lesions may also facilitate the monocyte recruitment that would eventually accelerate the lesion development.

Interestingly, PCOH, a hydroxyl analog of PCOOH, also induced THP-1 cell adhesion to ICAM-1 (Fig. 3). Although the metabolism of PCOH has not been fully revealed in vivo, several mechanisms (both enzymatic and nonenzymatic) have been proposed to generate PCOH from PCOOH (43). In general, the reduction of PCOOH into PCOH would contribute to preventing the formations of ROS and proatherogenic *sn*-2-truncated PCs from PCOOH. However, PCOH might still play roles in atherogenesis, not only as a precursor of LysoPC and hydroxy fatty acids but also by increasing monocyte adherence. Also in this study, PCOH generated from PCOOH might contribute in part to the induction of adherence in PCOOH-treated cells.

The THP-1 cell adhesion to ICAM-1 was exclusively mediated by LFA-1 (CD11a/CD18) (Fig. 4A). However, the PCOOH-induced cell adhesion was not mediated by the cell surface expression level of LFA-1 (Fig. 4B). The lack of involvement of other types of β_2-integrin [Mac-1 (CD11b/CD18) and p150/95 (CD11c/CD18)] may be due to the lower cell surface expression in THP-1 cells (Fig. 4B) (35, 44) or the lower affinity to ICAM-1 (45, 46) than those of LFA-1.

The morphological changes in THP-1 cells clearly indicate that the induction of the F-actin-rich membrane protrusion formation participates in the PCOOH-induced cell adhesion (Figs. 5 and 6). In general, the gross strength of cell adherence is governed by the affinity of the individual receptor-ligand bond and by the density of receptor and ligand on the adhesion site. In the leukocyte adhesion, the conformational activation and the cell surface localization of LFA-1 have been recognized to modulate the cell adherence (47). In this study, PCOOH-treated cells showed apparent LFA-1 localization on the protruding membrane structures (Fig. 5). Moreover, not only the protrusion for-

mation but also the cell adhesion to ICAM-1 was suppressed by cytochalasin D in PCOOH-treated cells (Fig. 6). These results indicate that the cell surface localization, rather than the individual affinity regulation, of LFA-1 chiefly contributes to the PCOOH-induced cell adhesion. Previous reports also demonstrated the involvement of intracellular actin cytoskeletal remodeling in the cell surface trafficking of LFA-1 and other integrins (47–50).

To date, several lipid mediators that act via specific receptors (e.g., sphingosine-1-phosphate, lysophosphatidic acid, and eicosanoids) are reported to exert their biological actions at nanomolar concentrations. However, low micromolar concentrations of PCOOH were required to induce THP-1 cell adhesion in this study. Although it remains to be established, the difference in the effective dose to exert biological actions in cell culture may be due to the different signaling mechanisms. In this study, it is likely that PCOOH perturbs membrane homeostasis since the introduction of another polar group into PC molecule (i.e., hydroperoxy group in PCOOH) changes the physical property in lipid bilayers. If so, the membrane perturbation may contribute to the biological action of PCOOH.

In summary, these results indicate that PCOOH induces THP-1 cell adhesion to ICAM-1 via actin cytoskeletal polymerization and subsequent localization of LFA-1. In addition, the reduction of PCOOH to PCOH could not abrogate the induction of the cell adhesion. These findings reveal a novel aspect of the proatherogenic properties of oxidatively modified phospholipids. These results support the hypothesis that higher plasma PCOOH conditions in hyperlipidemic and diabetic patients participate in the increased incidence of atherosclerosis. However, at present, it remains to be established whether the induction of adherence observed in THP-1 monocytic cells is pathophysiologically relevant; i.e., PCOOH actually contributes the adhesion of circulating monocytes to arterial wall in vivo. It also remains uncertain how PCOOH triggers actin polymerization and subsequent LFA-1 localization. Further research on these issues is needed for a better understanding of the role of PCOOH in the pathogenesis of atherosclerosis.

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