Transmembrane signaling pathway mediates oxidized low-density lipoprotein-induced expression of plasminogen activator inhibitor-1 in vascular endothelial cells

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Sangle GV, Zhao R, Shen GX. Transmembrane signaling pathway mediates oxidized low-density lipoprotein-induced expression of plasminogen activator inhibitor-1 in vascular endothelial cells. Am J Physiol Endocrinol Metab 295: E1243–E1254, 2008.—Atherosclerotic cardiovascular disease is the number one cause of death for adults in Western society. Plasminogen activator inhibitor-1 (PAI-1), the major physiological inhibitor of plasminogen activators, has been implicated in both thrombogenesis and atherogenesis. Previous studies demonstrated that copper-oxidized low-density lipoprotein (C-oLDL) stimulated production of PAI-1 in vascular endothelial cells (EC). The present study examined the involvement of lectin-like oxidized LDL receptor-1 (LOX-1) and Ras/Raf-1/ERK1/2 pathway in the upregulation of PAI-1 in cultured EC induced by oxidized LDLs. The results demonstrated that C-oLDL or FeSO₄-oxidized LDL (F-oLDL) increased the expression of PAI-1 or LOX-1 in human umbilical vein EC (HUVEC) or coronary artery EC (HCAEC). Treatment with C-oLDL significantly increased the levels of H-Ras mRNA, protein, and the translocation of H-Ras to membrane fraction in EC. LOX-1 blocking antibody, Ras farnesylation inhibitor (FTI-277), or small interference RNA against H-Ras significantly reduced C-oLDL or LDL-induced expression of H-Ras and PAI-1 in EC. Incubation with C-oLDL or F-oLDL increased the phosphorylation of Raf-1 and ERK1/2 in EC compared with LDL or vehicle. Treatment with Ras inhibitor blocked Raf-1 phosphorylation and the elevation of PAI-1 mRNA level in EC induced by C-oLDL or LDL. Treatment with PD-98059, an ERK1/2 inhibitor, blocked C-oLDL or LDL-induced ERK1/2 phosphorylation or PAI-1 expression in EC. The results suggest that LOX-1, H-Ras, and Raf-1/ERK1/2 are implicated in PAI-1 expression induced by oxidized LDLs or LDL in cultured EC.

oxidized lipoproteins; fibrinolytic inhibitor; lectin-like oxidized low-density lipoprotein receptor-1; H-Ras; Raf-1

ATHEROSECROTIC CARDIOVASCULAR DISEASE, particularly coronary artery disease (CAD), is a common cause of morbidity and mortality in Western society. Thrombosis at atherosclerotic lesions is the key underlying mechanism for acute coronary syndrome (31). Imbalance between coagulation and fibrinolysis leads to thrombosis. Plasmin is the key enzyme of the fibrinolytic system. The generation of plasmin is regulated by tissue (tPA) or urokinase plasminogen activator (uPA). Plasminogen activator inhibitor-1 (PAI-1) is the major physiological inhibitor of tPA and uPA (4). Elevated PAI-1 activity in plasma has been considered as a nontraditional risk factor for CAD (45).

Several lines of evidence suggest that an increased level of plasma low-density lipoprotein (LDL) cholesterol constitutes a major modifiable risk factor for CAD. Oxidation of LDL and interactions between oxidized LDL and vascular endothelial cells (EC) are key steps in atherogenesis (42, 43). Previous studies by our group or others demonstrated that copper-oxidized LDL (C-oLDL) is a potent agonist for the production of PAI-1 from cultured human umbilical vein EC (HUVEC) or human coronary artery EC (HCAEC) (28, 35, 36, 46). C-oLDL increased the generation of reactive oxygen species (ROS) and functional activities of multiple antioxidant enzymes in EC (55). Oxidative modification by copper, ultraviolet, or long exposure to EC enhanced the effect of LDL on the generation of PAI-1 from EC (40). PAI-1 has been implicated in inflammation, endothelial dysfunction, and extracellular matrix remodeling (6). Increased levels of PAI-1 protein and mRNA were detected in atherosclerotic or thrombotic lesions in vascular (3, 52).

Transmembrane signaling pathway for oxidized LDL-induced PAI-1 production in EC remains unclear. Previous studies demonstrated that LDL receptor (LDLR) blocking antibody had no effect on LDL or oxidized LDL-induced PAI-1 release from EC (46). Our previous study indicates that the activation of protein kinase C-β is involved in oxidized LDL-induced PAI-1 overproduction in EC (36). A recent study demonstrated that high glucose-induced PAI-1 expression in EC is mediated via Rho/Rho-kinase and nuclear factor-κB pathway (21). Lectin-like oxidized LDL receptor-1 (LOX-1) mediates oxidized LDL-induced cellular events in EC (10, 15, 29, 30). The role of LOX-1 in oxidized LDL-induced PAI-1 expression in EC remains unknown.

Small G proteins function as biological switches that modulate signaling between multiple membrane receptors and downstream signal transduction systems (44). Several groups demonstrated close interactions between oxidative stress and H-Ras (16, 27). One of best-characterized downstream effectors of Ras is Raf-1 (48). ROS enhance interactions between H-Ras and several of its downstream effectors, including Raf-1 and ERK1/2 (12, 16). Previous studies demonstrated that LDL increased H-Ras abundance in the membrane fraction of EC...
The involvement of LOX-1, H-Ras, Raf-1, or ERK1/2 in oxidized LDL-induced PAI-1 expression in EC has not been documented.

The present study examined the roles of LOX-1, H-Ras, and Raf-1/ERK1/2, and interactions between those signaling effectors and activators in oxidized LDL-induced PAI-1 upregulation in cultured vascular EC.

MATERIALS AND METHODS

Isolation and modification of lipoproteins. Plasma was freshly separated from blood of healthy donors by centrifugation (2,000 g) for 15 min at 4°C. LDL (density 1.019–1.063) was isolated from plasma using sequential flotation density ultracentrifugation. The modification of C-oLDL was achieved through dialysis against 5 μM CuSO4 for 24 h at 22°C (35), and that of FeSO4-modified LDL (F-oLDL) was generated through dialysis against 6 μM FeSO4 for 24 h at 22°C (49). Free copper or iron ions in oxidized LDLs were removed via dialysis. The oxidation of LDL was confirmed using thioarbituric acid-reactive substance assay and nondenatured gel electrophoresis. The levels of malondialdehyde in oxidized LDLs were 10-fold greater than that in corresponding batches of LDL. Lipoprotein preparations containing endotoxin levels that in corresponding batches of LDL. Lipoprotein preparations containing endotoxin levels >0.05 ng/ml measured using E-Toxate kits (Sigma) were excluded from experiments. Lipoproteins were stored in sealed tubes at 4°C in the dark under a layer of nitrogen to prevent autooxidation (35).

Cell culture. Seed HUVEC were obtained from American Type Culture Collection (Manassas, VA). Cells were grown in F-12K medium (Invitrogen) and fed with 10% of fetal bovine serum, 0.1 mg/ml of heparin, and EC growth supplements (Sigma) (37). The initial batch of HCAEC was received from Clonetics (San Diego, CA). EC were used within eight passages from seed cells.

Cell treatment. EC were treated with LDL, F-oLDL, or C-oLDL in dosages and time intervals as indicated. Identical volume of vehicle was added to control cultures for the matching period. Anti-human LOX-1 antibody (a gift from Dr. T. Sawamura, National Cardiovascular Center Research Institute, Osaka, Japan) at 10 μg/ml and LDLR blocking antibody (R & D Systems) at 5 μg/ml were used. Ras farnesyltransferase inhibitor, FTI-277 (10 μM; Calbiochem), Raf-1 inhibitor (1 μM; Calbiochem), or ERK1/2 inhibitor, PD-90859 (10 μM; Sigma), was used. For experiments using blocking antibody or signal transduction inhibitors, 30 min of preincubation was applied. Endothelial cytotoxicity to lipoproteins was determined by cell morphology or leucine incorporation assay (56). No measurable cytotoxicity was detected in EC treated with LDL or oxidized LDLs at experimental conditions.

Western blotting. Western blotting analysis was performed using total cellular lysate, as previously described (37, 56). Proteins were assessed using polyclonal antibodies against LOX-1, LDLR (R & D Systems), H-Ras, phosphorylated (p)Raf-1 (Santa Cruz Biotechnology), ERK1/2, H-Ras (Cell Signaling), or monoclonal antibodies PAI-1, β-actin, or nonspecific mouse IgG from Santa Cruz Biotechnology or Sigma. Second antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology) and enhanced chemiluminescence reagents (Amersham) were used to visualize targeted antibodies on nitrocellulose membranes. Relative intensities of protein bands were visualized using ChemiDoc system or autoradiography and assessed using Quantity One software (Bio-Rad Laboratories). The protein bands were normalized with internal controls such as β-actin or total ERK1/2 in corresponding samples.

Measurement of hydrogen peroxide. The levels of hydrogen peroxide (H2O2) in postculture media of EC were measured using PeroxiDetect kit from Sigma, as described previously (55).

PAI-1 antigen measurements. The levels of PAI-1 antigen in media of cultured EC were measured using human PAI-1 enzyme-linked immunosorbent assay (ELISA) kits from American Diagnostic, as described previously (13).

RT-PCR. Total RNA was isolated from cultured EC using TRI reagent (MRC, 동리에 대체) with the use of the Promega kit. The levels of PAI-1 or H-Ras mRNA were examined using RT-PCR. Primers for H-Ras mRNA (sense: 5’-GAATCTGGAGGCTCAGTCTG-3’; antisense: 5’-CAGACCAAGGAGCTCTCCAC-3’), PAI-1 mRNA (sense: 5’-CAAGGAGGAGCTCCAC-3’; antisense: 5’-ATCACT-TGACCCATGAAAG-3’), and β-actin gene (sense: 5’-GTGTTGGCCGCCCTTAGGCACCA-3’; antisense: 5’-TTGGGCTTAGGTTGTCAGGGGG-3’). cDNAs were amplified using the Expand RT-PCR system and Quantifier One software. Each specific mRNA band was justified with the intensity of β-actin mRNA.

Detection of translocation of H-Ras. After experimental treatment, HUVEC were harvested by a rubber policeman and homogenized in a Dounce homogenizer. Cellular membrane from cell lysate was separated using Beckman TLX-100 table top ultracentrifugation at 100,000 g at 4°C for 1 h (36, 57). H-Ras expression in membrane fraction was detected using Western blotting and polyclonal antibody against human H-Ras (Santa Cruz Biotechnology).

Gene silence. Small interference RNA (siRNA) targeting H-Ras mRNA was obtained from Santa Cruz Biotechnology. H-Ras siRNA was transfected to EC in serum-free medium using Silence siPort Lipid kit (Ambion), as described previously (56). SiRNA for β-actin or negative control siRNA (Ambion) was transfected in parallel cultures to verify the methodology.

Statistics. Data were expressed as means ± SE and analyzed by one-way ANOVA followed by Student’s t-test. The level of significance was defined as P < 0.05.

RESULTS

Effects of oxidized LDLs on cell-associated PAI-1 protein and mRNA. Previous studies from our laboratory demonstrated that C-oLDL significantly increased the release of PAI-1 from HUVEC or HCAEC (36). The present study characterized the effects of C-oLDL (50–150 μg/ml) for 12–48 h on PAI-1 protein and mRNA levels in HUVEC. The maximal increase of PAI-1 protein or mRNA was detected in HUVEC treated with 100 μg/ml of C-oLDL for 24 h (Fig. 1, A–C). Incubation with C-oLDL at 100 μg/ml for 24 h induced a significantly greater increase in cell-associated PAI-1 in HUVEC or HCAEC compared with F-oLDL, a type of oxidized LDL modified by a physiologically relevant condition (49) or unmodified LDL (P < 0.05). The increased expression of cell-associated PAI-1 in HCAEC after LDL, C-oLDL, or F-oLDL treatment was comparable with that in HUVEC (Fig. 1D).

Involvement of LOX-1 in oxidized LDL-induced increase of PAI-1 in EC. The involvement of LOX-1 in oxidized LDL-induced upregulation of PAI-1 in EC was examined using blocking antibody against LOX-1. HUVEC were pretreated with LOX-1 antibody (10 μg/ml) for 30 min and then exposed to C-oLDL or LDL (100 μg/ml) for 24 h. C-oLDL or F-oLDL significantly increased the abundance of LOX-1 in HUVEC or HCAEC compared with vehicle (P < 0.05; Fig. 2A). LOX-1 antibody prevented the effect of C-oLDL on the upregulation of PAI-1 in EC (P < 0.05) and partially

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Fig. 1. Dose and time dependence of oxidized LDLs on the plasminogen activator inhibitor-1 (PAI-1) protein or mRNA in human umbilical vein endothelial cells (HUVEC). A and B: HUVEC were treated with 100 µg/ml copper-oxidized LDL (C-oLDL) or vehicle (control) for 12–48 h for measurements of PAI-1 protein (A) and mRNA (B). C: dose response of C-oLDL (50–100 µg/ml) on PAI-1 protein. D: HUVEC or human coronary artery EC (HCAEC) were treated with vehicle, 100 µg/ml LDL, C-oLDL, or FeSO₄-modified LDL (F-oLDL) for 24 h. The abundance of PAI-1 and β-actin in total cellular proteins was examined using Western blotting (A, C, and D). The mRNA level of PAI-1 and β-actin was measured using RT-PCR (B). Integrative data were expressed in the %controls after normalization with β-actin (means ± SE; n = 3 experiments). * and **P < 0.05 or 0.01 vs. control; #P < 0.05 vs. LDL; ##P < 0.05 vs. F-oLDL.
inhibited LDL-induced increase of PAI-1 in EC (Fig. 2C). LOX-1 antibody did not change the basal level of PAI-1. The results suggest that LOX-1 is implicated in cross-membrane signaling of oxidized LDL-induced increase of PAI-1 in EC.

The effect of LDLR blocking antibody on LDL- or C-oLDL-induced increase in PAI-1 abundance in EC was examined. HUVEC were pretreated with LDLR blocking antibody (5 μg/ml) for 30 min and then exposed to C-oLDL or LDL (100 μg/ml) for 24 h. LDLR blocking antibody did not significantly
Fig. 3. Involvement of H-Ras in oxidized LDL-induced PAI-1 in EC. A: HUVEC were pretreated with vehicle or FTI-277 (10 μM) for 30 min and then incubated with 100 μg/ml LDL or C-oLDL or vehicle for 24 h. PAI-1 and β-actin in cellular proteins were determined using Western blotting. B: HUVEC were treated with vehicle or 100 μg/ml C-oLDL for 20 min. H-Ras and β-actin in membrane fractions and whole cell lysate were determined by Western blotting. C: HUVEC were treated with FTI-277 and LDL or C-oLDL as described in A. The levels of PAI-1 antigen in the postcultural medium were measured using ELISA assay kits. D: HUVEC were treated with FTI-277 and LDL or C-oLDL as described in A. H-Ras and β-actin in cellular proteins were determined by Western blotting. E: HUVEC were treated with 100 μg/ml LDL, C-oLDL, or vehicle [control (CTL)] for 24 h. The mRNA levels of H-Ras and β-actin were measured using RT-PCR. Values are expressed in %controls after normalization with β-actin (means ± SE; n = 3 experiments). * and **P < 0.05 or 0.01 vs. control; + and ++P < 0.05 or 0.01 vs. LDL; #P < 0.05 vs. C-oLDL.
Involvement of H-Ras in oxidized LDL-induced PAI-1 production. To determine whether H-Ras is involved in oxidized LDL-induced increase in PAI-1, HUVEC were preincubated with FTI-277 (10 μM), an H-Ras inhibitor, for 30 min and then exposed to 100 μg/ml of LDL or C-oLDL for 24 h. Cell-associated PAI-1, H-Ras, and β-actin were measured using Western blotting, and the levels of PAI-1 antigen were examined in postcultural medium of HUVEC. FTI-277 blocked C-oLDL- or LDL-induced cell-associated PAI-1 in EC (P < 0.05; Fig. 3A). The level of PAI-1 was significantly increased in the media of EC treated with C-oLDL compared with LDL or vehicle. Treatment with FTI-277 prevented C-oLDL- or LDL-induced increase of PAI-1 antigen in the media of EC (P < 0.05; Fig. 3C).

Several studies demonstrated that LDL significantly increased H-Ras abundance in the membrane fraction of EC (57, 58). To explore whether oxidized LDL affects Ras translocation within EC, HUVEC were exposed to C-oLDL (100 μg/ml) for 20 min. H-Ras abundance in membrane fraction was significantly increased by C-oLDL compared with control (P < 0.05; Fig. 3B). We did not detect obvious change in H-Ras expression in the whole cell lysate at similar condition. FTI-277 inhibited LDL- or C-oLDL-induced H-Ras expression in HUVEC (P < 0.05; Fig. 3D). To determine the effect of C-oLDL on H-Ras mRNA level, HUVEC were treated with 100 μg/ml of LDL or C-oLDL for 24 h. The level of H-Ras mRNA was significantly increased in EC treated with C-oLDL (P < 0.05; Fig. 3E).

Effect of H-Ras siRNA on oxidized LDL-induced upregulation of PAI-1. The involvement of H-Ras in oxidized LDL-induced upregulation of PAI-1 in EC was verified using siRNAs targeting H-Ras mRNA. H-Ras siRNA blocked C-oLDL- or LDL-induced increases of H-Ras and PAI-1 protein in HUVEC (P < 0.05; Fig. 4). In HUVEC transfected with H-Ras siRNA but without an addition of lipoproteins, the abundance of both H-Ras and PAI-1 was inhibited (P < 0.05). H-Ras siRNA evidently did not affect the abundance of β-actin in EC with or without lipoprotein treatment, and siRNA for β-actin or negative control siRNA evidently did not affect the level of PAI-1 in EC. The results demonstrate that H-Ras is required for oxidized LDL-induced PAI-1 expression in EC.

Effect of LOX-1 antibody on oxidized LDL-induced H-Ras expression. To determine relationship between LOX-1 and H-Ras expression induced by oxidized LDL, HUVEC were pretreated with LOX-1 blocking antibody (10 μg/ml) for 30 min and then exposed to 100 μg/ml of C-oLDL or LDL for 24 h. LOX-1 antibody partially, but significantly, inhibited the effect of C-oLDL or LDL on the expression of H-Ras in HUVEC (P < 0.05; Fig. 5). The results demonstrate that

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**Fig. 4.** Effect of H-Ras small interference RNA (siRNA) on oxidized LDL-induced H-Ras and PAI-1 expression in HUVEC. HUVEC transfected with siRNA against H-Ras gene for 48 h (1st serum-free medium and remaining time in the presence of 10% serum) were stimulated with vehicle (CTL) or with an addition of 100 μg/ml LDL or C-oLDL for 24 h. H-Ras, PAI-1, and β-actin in cellular proteins were detected by Western blotting. Values are expressed in %controls after normalization with β-actin (means ± SE; n = 3 experiments). * and **P < 0.05 or 0.01 vs. control; + and +++P < 0.05 or 0.01 vs. LDL; #P < 0.05 vs. C-oLDL.

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**Fig. 5.** Effect of LOX-1 antibody on oxidized LDL-induced H-Ras expression. To determine relationship between LOX-1 and H-Ras expression induced by oxidized LDL, HUVEC were pretreated with LOX-1 blocking antibody (10 μg/ml) for 30 min and then exposed to 100 μg/ml of C-oLDL or LDL for 24 h. LOX-1 antibody partially, but significantly, inhibited the effect of C-oLDL or LDL on the expression of H-Ras in HUVEC (P < 0.05; Fig. 5). The results demonstrate that...
activation of LOX-1 is involved in the upregulation of H-Ras in EC induced by oxidized LDL.

*Effects of oxidized LDLs on Raf-1 phosphorylation in EC.* Raf-1 is one of the best-characterized downstream effectors of H-Ras involved in the cell signaling pathway. The effects of a physiologically relevant concentration of LDL or C-oLDL (100 µg/ml) (18) on Raf-1 phosphorylation in HUVEC were determined through incubations for 2–120 min. The peak of pRaf-1 was detected in HUVEC treated with C-oLDL for 10 min compared with baseline or LDL at the same condition (P < 0.05). LDL-induced a delayed increase of pRaf-1 expression at 60 min compared with baseline (P < 0.05; Fig. 6A). F-oLDL at 100 µg/ml for 10 min significantly increased Raf-1 phosphorylation in EC compared with control or LDL (P < 0.05; Fig. 6C). HUVEC were preincubated with Raf-1 inhibitor (1 µM) for 30 min and then exposed to 100 µg/ml of C-oLDL for 10 min or LDL for 60 min. Raf-1 phosphorylation induced by C-oLDL or LDL was significantly suppressed by Raf-1 inhibitor (P < 0.05; Fig. 6, B and D). The findings suggest that oxidized LDLs accelerate the phosphorylation of Raf-1 compared with LDL.

*Effect of Raf-1 inhibitor on oxidized LDL-induced PAI-1 mRNA.* Previous studies from our laboratory demonstrated that C-oLDL significantly increased the steady state of mRNA level of PAI-1 or the release of PAI-1 from EC (32, 36). To determine whether Raf-1 inhibitor affects C-oLDL-induced

![Fig. 6. Effect of oxidized LDLs on Raf-1 phosphorylation (pRaf-1) in EC. A: time course of LDL- or C-oLDL-stimulated Raf-1 phosphorylation in HUVEC. HUVEC were treated with 100 µg/ml LDL or C-oLDL for 2–120 min. C: HUVEC or HCAEC were incubated with LDL, C-oLDL, or F-oLDL (100 µg/ml) for 10 min. B and D: HUVEC were pretreated with Raf-1 inhibitor (Raf-1i; 1 µM) for 30 min and then exposed to 100 µg/ml LDL or C-oLDL for 10 min (B) and LDL for 60 min (D). The abundance of pRaf-1 or β-actin in total cellular proteins was analyzed by Western blotting. Values are presented in %controls after normalization with β-actin (means ± SE; n = 3 experiments). * and **P < 0.05 or 0.01 vs. control; + and ++P < 0.05 or 0.01 vs. LDL; #P < 0.05 vs. C-oLDL; *P < 0.05 vs. F-oLDL.

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PAI-1 at mRNA level, HUVEC were preincubated with Raf-1 inhibitor (1 μM) for 30 min and then stimulated with 100 μg/ml of C-oLDL or LDL for 24 h. Raf-1 inhibitor significantly reduced the level of PAI-1 mRNA induced by C-oLDL or LDL (P < 0.05; Fig. 7). The results suggest that Raf-1 activation is required for oxidized LDL or LDL-induced PAI-1 production in EC at mRNA level.

*Involvement of ERK1/2 in oxidized LDL-induced increase of PAI-1.* ERK1/2 is a common downstream signaling effector of Raf-1 and MEK (51). The activation of ERK1/2 was detected in vascular cells treated with oxidized LDL. To examine the time dependence of ERK1/2 activation in EC induced by oxidized LDL, HUVEC were treated with C-oLDL or LDL (100 μg/ml) for 5–120 min. C-oLDL-induced ERK1/2 phosphorylation reached a peak at 20 min. LDL significantly increased ERK1/2 phosphorylation after 60 min of incubation (Fig. 8A). Treatment with F-oLDL increased ERK1/2 phosphorylation in a pattern similar to that of C-oLDL (P < 0.05; Fig. 8B). Treatment with an ERK1/2 inhibitor, PD-98059 (10 μM), blocked LDL- or C-oLDL-induced (100 μg/ml for 24 h) PAI-1 expression in EC (P < 0.05; Fig. 8C). ERK1/2 phosphorylation induced by 100 μg/ml C-oLDL (20 min) or LDL (60 min) was significantly inhibited by PD-98059 (P < 0.05; Fig. 8, D and E).

**Effect of antioxidant on oxidized LDL-induced H_{2}O_{2} release.** Previous studies in our group demonstrated that 80 μmol/l butylated hydroxytoluene (BHT), a potent antioxidant, prevented glycated LDL (glyLDL)-induced generation of H_{2}O_{2} and PAI-1 in EC (56). The effect of BHT on C-oLDL-induced H_{2}O_{2} release from HUVEC was examined in the present study. The levels of H_{2}O_{2} in the postculture medium of EC treated with BHT-C-oLDL were significantly lower than that in cultures treated with C-oLDL without BHT treatment (P < 0.05; Fig. 9).

**DISCUSSION**

Major novel findings generated from the present study include the following: 1) LOX-1 mediates oxidized LDL-induced PAI-1 expression in cultured vascular EC, 2) the activation and expression of H-Ras are implicated in oxidized LDL-induced PAI-1 upregulation in EC, and 3) increased phosphorylations of Raf-1 and ERK1/2 are required for oxidized LDL-induced expression of PAI-1 in EC.

Several types of membrane receptors are capable of internalizing oxidized LDL, including scavenger receptor (SR)-AI/II, SR-BI, LOX-1, CD36, and macrosialin-CD68 (17). The expressions of both LOX-1 and PAI-1 are increased in atherosclerotic tissues (3, 8, 23, 52). Kakatani et al. (22) reported that LOX-1 antibody reduced arterial thrombus formation in rats. The results of the present study demonstrated that oxidized LDL increased abundance of LOX-1 in vascular EC, which is consistent with a previous report by Sawamura et al. (39) in the same type of cells. The present study originally demonstrated that LOX-1 antibody efficiently blocked oxidized LDL-induced PAI-1 expression. The results suggest that LOX-1 is involved in oxidized LDL-induced PAI-1 expression in cultured vascular EC. The findings suggest a new role of LOX-1 in atherogenic lipoprotein-induced thrombotic process.

The receptor responsible for LDL-induced PAI-1 expression in EC has not been identified. The present study demonstrated that the expression of LDLR was downregulated by LDL, C-oLDL, or F-oLDL, which may reflect a negative feedback of increased intracellular cholesterol, as described previously (7). LDLR antibody failed to prevent LDL or oxidized LDL-induced PAI-1 expression in EC, which is consistent with an earlier report by Tremoli et al. (46). Our previous study demonstrated that treatment of EC with LDL increased lipid peroxidation in LDL, and antioxidants inhibited LDL-induced PAI-1 generation or lipid peroxidation (37). LDL-induced PAI-1 expression in EC may result from EC-mediated oxidation of LDL (37, 40). The present study demonstrated that LOX-1 blocking antibody partially inhibited LDL-induced PAI-1 expression in EC. The findings suggest that increased PAI-1 expression in EC induced by previously unmodified LDL is regulated partially via LOX-1, which is possibly following cell-mediated oxidation of LDL in cultured EC.

Membrane-associated small G proteins, such as Ras and Rho, often mediate the activation of membrane receptors (34, 38). H-Ras is linked closely with oxidative stress (16). H-Ras proteins exist in two interconvertible forms, GDP-bound inactive cytosolic form or GTP-bound active membrane form. By cycling between the two forms, H-Ras mediates various signal transduction pathways that emanate from the activation of membrane receptors. Farnesylation is essential for the translocation of Ras from cytosol to plasma membrane (53). Previous studies by Zhu et al. (57) demonstrated that LDL increased the membrane translocation and activation of H-Ras in human EC. The results of the present study demonstrated that incubation of EC with C-oLDL rapidly increased translocation of H-Ras to cell membrane. Prolonged incubation with C-oLDL significantly increased mRNA level of H-Ras in EC. We further
Fig. 8. Involvement of ERK1/2 phosphorylation in oxidized LDL-induced PAI-1 expression in EC. A: time course of LDL- or C-oLDL-stimulated ERK1/2 phosphorylation in HUVEC. HUVEC were treated with vehicle or 100 μg/ml LDL or C-oLDL for 5–120 min. B: HUVEC or HCAEC were incubated with vehicle, LDL, C-oLDL, or F-oLDL (100 μg/ml) for 20 min. C: HUVEC were pretreated with vehicle or PD-98059 (10 μM) and then exposed to vehicle or 100 μg/ml LDL or C-oLDL for 24 h. PAI-1 and β-actin in cellular proteins were determined by Western blotting. D and E: HUVEC were pretreated with vehicle or PD-98059 (10 μM) and then exposed 100 μg/ml C-oLDL for 20 min or LDL for 60 min. The abundances of pERK1/2 and ERK1/2 in total cellular proteins were analyzed by Western blotting. Values are presented in %controls after normalization with total ERK1/2 or β-actin (means ± SE; n = 3 experiments). * and **P < 0.05 or 0.01 vs. control; + and ++P < 0.05 or 0.01 vs. LDL; #P < 0.05 vs. C-oLDL.
demonstrated that LOX-1 blocking antibody reduced C-oLDL-induced H-Ras expression in EC. Farnesylation inhibitor FTI-277 prevented oxidized LDL-induced PAI-1 expression and release from EC, which was associated with a reduction of the intracellular abundance of H-Ras. H-Ras siRNA effectively inhibited LDL- or C-oLDL-induced upregulation of H-Ras or PAI-1 in EC. The combination of findings suggests that the farnesylation and expression of H-Ras are required for oxidized LDL-induced upregulation of PAI-1 in cultured EC. H-Ras also mediates oxidized LDL-induced PAI-1 expression in other types of cells, such as mesangial cells, as described previously (41). The activation of LOX-1 by oxidized LDL stimulates the activation of H-Ras in EC.

A large body of evidence indicates that intracellular ROS production triggers Ras activation (16, 20, 25). The uptake of oxidized LDL via LOX-1 may increase oxidative stress in EC, which may activate H-Ras. Previous studies by our group and others demonstrated that oxidized LDL induced a quick production of ROS in EC (9, 55). ROS increased interaction between H-Ras and its downstream effectors (12, 16, 25). The present study indicated that BHT, a potent antioxidant, inhibited oxidized LDL-induced H2O2 production in EC. Our recent study demonstrated that BHT inhibited PAI-1 production in EC induced by glyLDL (56) or oxidized LDL (Zhao R, Ma X, and Shen GX, unpublished observation). Relationships between ROS and various cell-signaling effectors related to oxidized LDL-induced PAI-1 production remain to be further investigated in subsequent study. A recent report demonstrated that Rho mediates high-glucose-induced PAI-1 expression in EC (21), which suggests that the upregulation of PAI-1 in EC by metabolic factors may be modulated via different small G proteins.

Numerous lines of evidence demonstrate close links between H-Ras and Raf-1 (25) or other MAP kinases such as ERK, JNK, and p38 kinase (16, 50). Activation of H-Ras recruits Raf-1 from cytosol to cell membrane, leading to the phosphorylation of Raf-1, which in turn phosphorylates and activates MEK and subsequently ERK1/2 (25, 48). Chen et al. (11) reported that increased expression of PAI-1 was associated with elevated phosphorylation of Raf-1 in vascular smooth muscle cells. The present study demonstrated that the phosphorylation of Raf-1 was increased in EC when incubated with C-oLDL or F-oLDL. Raf-1 inhibitor blocked oxidized LDL-induced upregulation of PAI-1 and phosphorylation of Raf-1 in EC. The finding provides new evidence for the modulatory role of Raf-1 in oxidized LDL-induced thrombotic disorders in vasculature.

The present study demonstrated that the phosphorylation of ERK1/2 was rapidly increased in EC exposed to C-oLDL or F-oLDL, which is consistent with previous studies in smooth muscle cells or mesangial cells (26, 19). The results of the present study demonstrated that PD-98059 significantly inhibited oxidized LDL-induced increase in PAI-1 expression, suggesting that the activation of ERK1/2 is required for oxidized LDL-induced PAI-1 expression in EC.

The present study suggests that oxidized LDL increased H-Ras, Raf-1, and ERK1/2 following 10–30 min of incubation. Our previous studies showed that oxidized LDL significantly increased ROS production within 30 min and reached a peak around 2 h (55). Oxidative stress activates heat shock factor-1 (HSF1) (1). GlyLDL or oxidized VLDL (oxVLDL) increased HSF1 expression as early as 2 h and peaked at 6 h. HSF1 mediates oxVLDL- or glyLDL-induced PAI-1 expression in EC through its binding to a homolog of HSF1 in PAI-1 promoter (54, 56). Preliminary studies in our laboratory demonstrated that C-oLDL activated HSF1 in a pattern similar to that of glyLDL or oxVLDL (Zhao R, Ma X, and Shen GX, unpublished data). The results of the present study indicated that oxidized LDL increased the levels of protein and mRNA of PAI-1, which was initiated at 12 h and peaked at 24 h. Oxidized LDL-induced earlier activation of Ras/Raf/ERK may be involved in the regulation of ROS production through an unidentified mechanism, which further activates HSF1 and increases PAI-1 transcription and expression in EC at later time intervals. Previous studies demonstrated that oxidized lipids, which are biologically active components of oxidized LDL, increased LOX-1 expression (24). Oxidized phospholipid-induced monocyte binding to EC was modulated via the activation of cAMP-dependent R-Ras/phosphatidylinositol 3-kinase pathway (14). Oxidized phospholipids also induced inflammatory responses in pulmonary EC through the activation of ERK1/2 cascade (5). Internalized oxidized lipids via LOX-1 potentially contribute to intracellular signaling for the upregulation of PAI-1 in EC. This possibility requires further investigations in subsequent studies.

In summary, the findings of the present study indicate that LOX-1, H-Ras, and Raf-1/ERK1/2 modulate oxidized LDL-induced PAI-1 expression in an interactive way in cultured vascular EC. Further studies in atherosclerotic animal models may verify the findings in vivo. Pharmacological interventions on the suggested signaling mediators potentially help to prevent atherogenic lipoprotein-induced intravascular thrombosis.

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REFERENCES


37. Song CY, Kim BC, Lee HS. Lovastatin inhibits oxidized low-density lipoprotein-induced plasminogen activator inhibitor and transforming growth factor-beta expression via a decrease in Ras/extracellular


