Involvement of NADPH oxidase in oxidized LDL-induced upregulation of heat shock factor-1 and plasminogen activator inhibitor-1 in vascular endothelial cells

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Zhao R, Ma X, Xie X, Shen GX. Involvement of NADPH oxidase in oxidized LDL-induced upregulation of heat shock factor-1 and plasminogen activator inhibitor-1 in vascular endothelial cells. Am J Physiol Endocrinol Metab 297: E104–E111, 2009. First published April 28, 2009; doi:10.1152/ajpendo.91023.2008.—Plasminogen activator inhibitor-1 (PAI-1) is implicated in thrombogenesis, inflammation, and extracellular matrix remodeling. Previous studies indicated that oxidized low-density lipoprotein (LDL) stimulated the generation of PAI-1 in vascular endothelial cells (EC). The present study demonstrated that LDL oxidized by copper, iron, or 3-morpholinosydnonimine increased the expression of NADPH oxidase (NOX) 2, PAI-1, and heat shock factor-1 (HSF1) in human umbilical vein EC or coronary artery EC compared with LDL or vehicle. Diphenyleniodon, a NOX inhibitor, prevented the increases of the expression of HSF1 and PAI-1 in EC induced by oxidized LDLs. Small-interference RNA (siRNA) for p22phox, an essential subunit of NOX, prevented oxidized LDL-induced expression of NOX2, HSF1, and PAI-1 in EC. HSF1 siRNA inhibited oxidized LDL-induced expression of PAI-1 and HSF1, but not NOX2, in EC. The binding of HSF1 to PAI-1 promoter and the activity of PAI-1 promoter in EC were enhanced by oxidized LDL. Butylated hydroxytoluene, a potent antioxidant, inhibited oxidized LDL-induced release of hydrogen peroxide (H2O2) and the expression of NOX2, HSF1, and PAI-1 in EC. Treatment with H2O2 increased the abundance of NOX2, HSF1, and PAI-1 in EC. The results of the present study indicate that oxidized LDL-induced expression of NOX may lead to the elevated release of reactive oxygen species, the activation of HSF1, and the enhancement of the transcription of PAI-1 gene in cultured vascular EC.

Elevated low-density lipoprotein (LDL)-cholesterol is a classical risk factor for CAD (3). Oxidation enhances the atherogeneity of LDL (25). Increased levels of biomarkers for oxidized LDL were detected in blood circulation of CAD patients (10, 29). Results of previous studies indicate that oxidized LDL is a potent agonist for the generation of PAI-1 from cultured vascular endothelial cells (EC) (19, 20, 28). Previous studies in our laboratory demonstrated that oxidized LDL activated PAI-1 promoter in EC, which was associated with an increase of PAI-1 expression in EC (9). Our group recently demonstrated that lectin-like oxidized LDL receptor, H-Ras, and Raf/extracellular signal-regulated kinase pathway mediated oxidized LDL-induced PAI-1 production in EC (23). Oxidized LDL stimulated the generation of reactive oxygen species (ROS) from EC (34). NADPH oxidase (NOX) is activated by oxidized LDL, and it increases the generation of ROS in EC (6). Relationship between NOX and oxidized LDL-induced PAI-1 production in EC remains uncharacterized.

Environmental stresses enhance the expression of heat shock proteins (Hsp) and mediate the posttranslational modifications of stress response-related proteins. Exposure to oxidized LDL augmented the expression of Hsp-70 in EC (34). The expression of Hsp is modulated by heat shock factor (HSF). HSF1 is the most widely distributed form of HSF in human tissues (14). HSF1 may be activated by heat shock, shearing stress, prooxidants, or hormones. The activation of HSF1 was detected during embryo growth (2) or in atherosclerotic lesions (12). Our recent study demonstrated that HSF1 was involved in glyicated LDL or oxidized very low density lipoprotein (VLDL)-induced transcription of PAI-1 gene in EC through its binding to a homolog of HSF1-responsive element (HSE) in a distal region of PAI-1 promoter. Oxidation contributes to glyicated LDL-induced HSF1 or PAI-1 expression in EC (33, 35). The present study aims to investigate the effects of oxidized LDLs on the expression of NOX2 (gp91phox) and its relationship with HSF1, PAI-1, and ROS in EC.

METHODS

Isolation and modification of lipoproteins. LDL (density 1.019–1.063) was isolated from plasma of healthy donors using sequential density floatation ultracentrifugation. Copper-oxidized LDL (CLDL) was prepared through dialysis against 5 μM CuSO4 for 24 h at 22°C (19). Iron (FeSO4)-oxidized LDL (FLDL) was prepared through dialysis against 6 μM FeSO4 for 24 h or 60 h at 22°C as previous described (30). 3-Morpholinosydnonimine (SIN-1) is a source of peroxynitrite, and the latter is a type of reactive nitrogen species existing in vivo. SIN-1-oxidized LDL (SLDL) was prepared as previous described (4). The oxidation of LDL was verified using thiorbarbituric acid reactive substance assay and nondenatured gel elec-
trophoresis. Lipoproteins were excluded from experiments if the level of endotoxin in lipoproteins was >0.05 ng/ml measured using the E-Toxate kit (Sigma, St. Louis, MO). Lipoproteins were stored in sealed tubes under a layer of nitrogen at 4°C in the dark to prevent auto-oxidation (20).

Cell culture. Seed human umbilical vein ECs (HUVECs) were obtained from American Type Culture Collections (Manassas, VA). Cells were grown in F12K medium (GIBCO Canada, Burlington, ON, Canada) containing 10% FCS, heparin, and EC growth supplements (Sigma) as previously described (20). Seed human coronary artery ECs (HCAECs) and required medium or supplements were received from Clonetics (San Diego, CA). Cells within eight passages from seed cells were used in the following experiments. Endothelial cytotoxicity of lipoproteins was assessed using morphological or leucine incorporation assay as previously described (20).

Western blotting analysis. Targeted proteins in cell lysates were analyzed using Western blotting analysis as previously described (8).

PAI-1 antigen and activity. The levels of PAI-1 in media EC were analyzed using human PAI-1 enzyme-linked immunosorbent assay kits (American Diagnostica, Stamford, CT). PAI activity in the media of EC was measured using PAI activity assay kits from American Diagnostica.

Real-time PCR. Total RNA was isolated from EC using TriReagent (Molecular Research Center, Cincinnati, OH), and 1 μg of RNA from each sample was reversely transcripted to cDNA using the QuantiTect Reverse Transcription kit (Qiagen, Duesseldorf, Germany). Real-time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems or ABI, Austin, TX) and the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA).

Monoclonal antibodies against human NOX2, p22phox, HSF1, PAI-1, β-actin, or nonspecific mouse IgG were obtained from Santa Cruz (Santa Cruz, CA) or Sigma. Corresponding secondary antibody conjugated with horseradish peroxidase (Santa Cruz) and enhanced chemiluminescence reagents (Amersham, Piscataway, NJ) was applied to visualize targeted antigens on nitrocellulose membranes. The abundance of antigens was assessed using the Chemi-Doc system and Quantity One software (Bio-Rad, Hercules, CA) and normalized with the abundance of β-actin in corresponding samples.

**Fig. 1.** Effect of oxidized low-density lipoproteins (LDLs) on the abundance of NADPH oxidase (NOX2) in endothelial cells (EC). Human umbilical vein ECs (HUVECs) were treated with 50–150 μg/ml of copper-oxidized LDL (CLDL) or vehicle control (C) for 1 h (B) or 100 μg/ml of the lipoproteins for 0.25–24 h (A). HUVECs (C) or human coronary artery ECs (HCAECs; D) were treated with vehicle, 100 μg/ml of LDL, CLDL, FeSO₄-oxidized LDL (FLDL), or 3-morpholinosydnonimine (SIN-1)-oxidized LDL (SLDL) for 1 h. The abundance of NOX2 or β-actin in EC was analyzed using Western blotting. NOX2 mRNA levels from HUVEC treated with 100 μg/ml of LDL, CLDL, or vehicle control for 1 h were analyzed using real-time PCR (E). The integrative data of NOX2 protein or mRNA were expressed in folds of control after normalization with β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (mean ± SD, n = 3 experiments). P < 0.05 or 0.01 vs. control (¢ and ¢¢) and vs. LDL (+ and ++).
Time PCR system (95°C for 5 min, 95°C for 15 s, 60°C for 20 s, and 72°C for 40 s for 45 cycles). Primers for HSF1, NOX2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were designed using Primer Express software (version 1.4; PE Biosystems, Foster City, CA). The sequences of primers used in the present study were as follows: HSF1 mRNA (sense: 5’-CCGTGTCCTGGTTGCACCCACTCCTTGTT; anti-sense: 5’-CTGTGCTGGTCCGTCGCCCACTCCA), NOX2 mRNA (sense: 5’-AGGGTCAAGAACAGGCTAAGGA; anti-sense: 5’-TCTCTCACCTCACAACCCTTT), and GAPDH mRNA (sense: 5’-CACCCACTCCTCCACCTTTTG; anti-sense: 5’-GTCCTTCCTCTTGCTGTGCTTGGT). Primers for PAI-1 mRNA (sense: 5’-AAGTACTCCCCGACCTC, anti-sense: 5’-GGGCGTGTTGAACCTCAGTAG) were synthesized as previously reported sequences (3).

Relative quantification of specific mRNA was assessed from cycle threshold and normalized with GAPDH mRNA in same samples.

RNA silence. Small-interference RNA (siRNA) for HSF1 was obtained from Ambion (Austin, TX). siRNA for human p22phox (catalog no. sc-36149) was purchased from Santa Cruz. siRNA for β-actin and negative control siRNA were obtained from Ambion. siRNA was transfected into EC using the Silence siPort Lipid kit (Ambion) as previously described (35).

Transfection assay. PAI-1 promoter (−1,197/+55 bp, −1,105/+55 bp)/luciferase reporter vectors were constructed as previously described (35). Calcium phosphate-precipitated promoter vector was transfected to HUVEC as described (18). Chlromaphenicol acetyltransferase expression vector was cotransfected with PAI-1 promoter reporter gene vector as an internal control.

Electrophoratic mobility shift assay and supershift. Nuclear proteins were extracted from EC as previously described (23). Double-strand oligonucleotide corresponding to the −1,141/−1,126 bp (AATAGAATAAAGCAG) region of PAI-1 promoter (GenBank no. J03764) was synthesized as a probe for electrophoretic mobility shift assay. The probe was labeled with [32P]dNTP at single end and incubated with nuclear extracts at 4°C for 15 min. DNA-protein complexes were analyzed using 5% nondenatured acrylamide gel electrophoresis and visualized under autoradiography (35). Blocking antibody against HSF1 (Santa Cruz) or nonspecific IgG was applied in supershift assay.

Analysis of hydrogen peroxide. Hydrogen peroxide (H2O2) in media of EC was measured using the PeroxyDetect kit from Sigma as previously described (34).

Statistics. One-way ANOVA was used for assessing the probability of data from more than two groups. The level of significance was defined as P < 0.05.

RESULTS

Effects of oxidized LDLs on NOX2 expression in EC. The dose and time dependence of oxidized LDLs on the abundance of NOX2, the catalytic core of NOX complex, was characterized in HUVEC treated with physiologically relevant concentrations of CLDL (50–150 μg protein/ml) for up to 24 h compared with vehicle control. The maximal effect of CLDL on NOX2 expression was detected in HUVEC treated with 100 μg/ml of oxidized LDL for 1 h (Fig. 1, A and B). CLDL, FLDL, or SLDL (100 μg/ml for 1 h) increased NOX2 abundance in HUVEC or HCAEC to similar extents compared with LDL or control (P < 0.05, Fig. 1, C and D). Treatments with oxidized LDLs or LDL did not evidently affect the abundance of β-actin in EC compared with control. Real-time PCR analysis demonstrated that CLDL (100 μg/ml for 1 h) signif-

Fig. 3. Effect of oxidized LDLs on the production of plasminogen activator inhibitor-1 (PAI-1) in EC. HUVECs were treated with 50–150 μg/ml of CLDL or vehicle (control) for 24 h (A) or 100 μg/ml of the lipoproteins for 12–48 h (B). HUVECs (C) or HCAECs (D) were treated with vehicle or 100 μg/ml of LDL, CLDL, FLDL, or SLDL for 24 h. The abundance of PAI-1 mRNA or β-actin in EC was analyzed using Western blotting. E: HUVECs were treated with vehicle or 100 μg/ml of LDL or CLDL for 24 h. PAI-1 antigen in medium was measured using enzyme-linked immunosorbent assay (ELISA). F: HCAECs were treated with vehicle or 100 μg/ml of LDL or CLDL for 24 h. PAI activity in medium was measured using a PAI activity kit. PAI-1 mRNA levels from HUVEC treated with 100 μg/ml of LDL CLDL or control for 24 h were analyzed using real-time PCR (G). The integrative data of PAI-1 protein, activity, or mRNA were expressed in folds of control after normalization with β-actin, total cellular proteins, or GAPDH mRNA (mean ± SD, n = 3 experiments). P < 0.05 (*) or 0.01 (**) vs. control. P < 0.05 (+) or 0.01 (+ +) vs. LDL.
Effects of oxidized LDLs on NOX2 expression in EC. We previously reported that CLDL increased the release of PAI-1 from cultured HUVEC or HCAEC (21). The influence of other types of oxidized LDLs on PAI-1 expression, or that of CLDL on cell-associated PAI-1 abundance in EC, remains uncharacterized. The present study demonstrated that CLDL treatment at 100 μg/ml for 24 h induced a peak increase of the abundance of PAI-1 in HUVEC (P < 0.05, Fig. 3, A and B). CLDL, FLDL, and SLDL (100 μg/ml for 24 h) significantly increased cell-associated PAI-1 in HUVEC or HCAEC compared with LDL (P < 0.05, Fig. 3, C and D). CLDL (100 μg/ml for 24 h) increased the release of PAI-1 from HUVEC compared with LDL or vehicle control (P < 0.01, Fig. 3E). CLDL at the optimized condition also significantly increased PAI activity in the media of HCAEC compared with LDL or control (P < 0.05 or 0.01, Fig. 3F). CLDL (100 μg/ml for 24 h) significantly increased PAI-1 mRNA in HUVEC compared with LDL or control (P < 0.01, Fig. 3G).

Effect of NOX inhibitor on oxidized LDL-induced expression of NOX2, HSF1, and PAI-1 in EC. To determine the involvement of NOX in oxidized LDL-induced expression of HSF1 and PAI-1 in EC, HUVECs were pretreated with a NOX inhibitor, 10 μM diphenyleneiodonium (DPI). Cells were further treated with CLDL, SLDL, or LDL in optimized conditions as described above to examine the expression of NOX2, HSF1, or PAI-1 in EC. DPI treatment blocked the increases of NOX2, HSF1, and PAI-1 in EC induced by CLDL, SLDL, or LDL (Fig. 4). The findings suggest that NOX is involved in oxidized LDL-induced upregulation of PAI-1 and HSF1 in EC.

Impact of p22phox siRNA on oxidized LDL-induced expression of NOX2, HSF1, and PAI-1 in EC. To confirm the involvement of NOX in oxidized LDL-induced expression of HSF1 and PAI-1 expression, NOX was downregulated by siRNA against p22phox, an essential component of NOX complex, in EC. siRNA against p22phox substantially decreased the abundance of p22phox, NOX2, HSF1, and PAI-1 in HUVEC induced by CLDL, SLDL, or LDL. In HUVEC transfected with p22phox, but without an addition of lipoprotein, the abundance of NOX2, HSF1, and PAI-1 was partially prevented (Fig. 5).
Effects of oxidized LDL on the activation of PAI-1 promoter and DNA binding of HSF1. Our recent studies demonstrated that glycated LDL or oxidized VLDL increased the binding of HSF1 to PAI-1 promoter through a homolog of HSE located at −1,137/−1,128 bp of PAI-1 promoter (32, 34). The effect of oxidized LDL on the binding of HSF1 to the targeted region of PAI-1 promoter has not been documented. The results of the present study demonstrated that CLDL increased the activation of −1,197/+55 bp, but not −1,105/+55 bp, PAI-1 promoter fragment transiently transfected in HUVEC (Fig. 6A), which suggests that oxidized LDL activated a response element located between −1,197 and −1,105 bp of the PAI-1 promoter. Treatment with CLDL for 24 h enhanced the binding of a nuclear protein to a radioactively labeled PAI-1 promoter fragment (−1,141/−1,126 bp) compared with LDL or control. Excess amount (200-fold) of unlabeled probe (the −1,141/−1,126 bp fragment of PAI-1 promoter) suppressed CLDL-induced DNA-protein interaction to an undetectable level. Treatment with antibody against human HSF1 induced an upward shift of the DNA-protein complex (Fig. 6B). Nonspecific mouse IgG did not shift the migration of complex (data not shown). The results suggest that HSF1 is involved in oxidized LDL-induced activation of PAI-1 promoter.

Impact of HSF1 siRNA on oxidized LDL-induced expression of PAI-1 and NOX2 in EC. The impact of HSF1 in oxidized LDL-induced upregulation of PAI-1 or NOX2 in EC was examined using siRNA against HSF1 mRNA. HSF1 siRNA inhibited the levels of HSF1 and PAI-1 protein in EC induced by 100 μg/ml of CLDL or LDL. HSF1 siRNA did not evidently alter the abundance of NOX2 in EC induced by CLDL or LDL. Transfection with HSF1 siRNA, but without an addition of lipoproteins, partially inhibited the abundance of HSF1 and PAI-1 in EC (Fig. 7). Negative control siRNA or β-actin siRNA did not affect the expression of the targeted proteins (data not shown).

Effect of antioxidant on oxidized LDL-induced expression of NOX2, HSF1, and PAI-1. Our previous studies demonstrated that butylated hydroxytoluene (BHT), a potent antioxidant, at 80 μM prevented glycated LDL-induced PAI-1 production in EC (20). The effect of BHT on oxidized LDL-induced expression of NOX2, HSF1, and PAI-1 in HUVEC, and the levels of H2O2 in conditioned media, were examined in the present study. BHT treatment normalized CLDL-induced NOX2, HSF1, and PAI-1 expression in EC (P < 0.01) (Fig. 8A). The levels of H2O2 released from EC were significantly increased by treatment with CLDL in a condition (100 μg/ml for 2 h) optimized previously (32) compared with vehicle or LDL. Treatment with BHT blocked the CLDL-induced increase of H2O2 (P < 0.01, Fig. 8B). The results indicate that oxidative stress appeared to be a key factor in the induced expression of genes associated with EC dysfunction.
stress plays a role in the expression of NOX, HSF1, and PAI-1 induced by oxidized LDL in EC.

**Effect of exogenous H2O2 on the abundance of PAI-1, HSF1, and NOX2 in HUVEC.** To determine whether H2O2 can directly upregulate the levels of PAI-1, HSF1, and NOX2 in EC, HUVECs were treated with exogenous H2O2 at 100 μM as previously described (16) for up to 24 h. The results indicate that H2O2 significantly increased the abundance of PAI-1, HSF1, and NOX2 in HUVEC (P < 0.05 or 0.01). The maximal increases of NOX2, HSF1, and PAI-1 were detected in HUVEC after treatment with 100 μM H2O2 for 30 min (NOX2), 2 h (HSF1), or 12 h (PAI-1) (Fig. 9).

**DISCUSSION**

The major novel findings generated from the present study are the following: 1) NOX is implicated in oxidized LDL-induced upregulation of HSF1 and PAI-1 in EC; 2) HSF1 expression and DNA binding are required for the increased transcriptional regulation of PAI-1 in EC induced by oxidized LDL; and 3) ROS plays an important role in oxidized LDL-induced expression of HSF1 and PAI-1 in EC.
Oxidized LDL is a potent stimulant of pro-oxidants. Our previous studies demonstrated that oxidized LDL increased the release of \( \text{H}_2\text{O}_2 \) from EC which was started at 30 min and with a peak effect after 2 h of incubation (34). The results of the present study indicated that the increase of \( \text{H}_2\text{O}_2 \) release from EC may partially result from the activation of NOX. The maximal expression of NOX2 induced by oxidized LDL was at 1 h and that directly induced by exogenous \( \text{H}_2\text{O}_2 \) was at 30 min. The sequence of the elevations of levels of NOX2 and \( \text{H}_2\text{O}_2 \) suggest that NOX2 may induce an additional increase of ROS in EC after the initial burst of ROS in EC exposed to oxidized LDL. \( \text{H}_2\text{O}_2 \) plays an important role in signal transduction for cell responses induced by oxidative stress partially because of its relative stability compared with other forms of ROS. Previous studies indicated that \( \text{H}_2\text{O}_2 \) activates HSF1 (37). In addition, the results of the present study demonstrated that BHT inhibited oxidized LDL-induced expression of NOX2, HSF1, and PAI-1 in EC, which was associated with a reduction in the release of \( \text{H}_2\text{O}_2 \). The combination of the findings suggest that the increase of oxidative stress induced by oxidized LDL, possibly through NOX, may play an interactive role in oxidized LDL-induced HSF1 and PAI-1 upregulation in EC.

The results of the present study demonstrated that oxidized LDL modified by metal ions (CLDL and FLDL) or biological reactive nitrogen species (SLDL) enhanced the expression of NOX-2, HSF1, and PAI-1 in EC. Our previous studies indicated that the LDL-induced moderate increase in PAI-1 generation from EC may be attenuated by an antioxidant (21), which is an indication that cell-mediated oxidative modification contributes to PAI-1 upregulation in EC induced by LDL. The combination of the present and previous findings suggests that oxidative modification plays critical roles in LDL or its modified forms induced upregulation of HSF1 and PAI-1 in cultured vascular EC.

The levels of PAI-1 are elevated in stress-related situations, such as acute infection (5) or recurrent myocardial infarction (32). PAI-1 not only prevents the clearance of fibrin clots but also mediates the processes of tissue remodeling and inflammation, which may attribute to the development of restenosis, atherosclerosis, and wound healing after myocardial infarction (24). Results from previous studies on the relationship between PAI-1 and heat shock response or its mediators were controversial. Rüber et al. (22) reported that local heat shock increased PAI-1 expression in arterioles from rat skeletal muscle. Uchiyama et al. (30) described that adenovirus vector-mediated overexpression of HSF1 gene reduced PAI-1 expression in arterial EC; however, their results were not verified using a gene knockdown approach or knockout animal model. The results of the present study support the report from Rüber et al. (22). HSF1-mediated stress response is considered as an important chain reaction for a self-defense mechanism of the body. The upregulation of HSF1 and PAI-1 in EC may be a part of the defensive mechanism against oxidative and metabolic stress. The acute elevation of PAI-1 expression and its activity in vasculature may be beneficial for the body to defend acute stress, such as wound or bleeding, through maintaining tissue integrity by stabilizing intravascular fibrin clots or extracellular matrix. However, a chronic increase of PAI activity in vasculature leads to hypofibrinolytic and thrombotic tendency. For patients with chronic cardiovascular risks, such as diabetes and hypercholesterolemia, circulatory levels of PAI-1 may be substantially attenuated by a cholesterol-lowering agent (8).

In conclusion, the results of the present study indicate that the expression and activation of NOX and HSF1 are required for the upregulation of PAI-1 in vascular EC induced by oxidized LDLs. Increased ROS generation, possibly via the activation of NOX by oxidized LDL, may contribute to the upregulation of HSF1 and PAI-1 in EC. The findings of the present study provide a new linkage between hypercholesterolemia, oxidative stress, stress response mediators, and intravascular hypofibrinolysis.

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