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Involvement of NADPH oxidase in up-regulation of plasminogen activator inhibitor-1 and heat shock factor-1 in mouse embryo fibroblasts induced by oxidized LDL and in apolipoprotein E-deficient mice

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Abstract

The present study demonstrated that oxidized LDL (oLDL) increased the generation of superoxide and hydrogen peroxide (H_2O_2), the abundances of NADPH oxidase (NOX)4, NOX2, p22-phox and lectin-like oLDL receptor-1 (LOX-1) in wild-type or heat shock factor-1 (HSF1)-deficient mouse embryo fibroblasts (MEF). LOX-1 antibody inhibited LDL or oLDL-induced expression of NOX components in MEF. Abundance of HSF1 or plasminogen activator inhibitor-1 (PAI-1) was increased by oLDL in wild-type, but not in HSF1-deficient MEF. Diphenyleneiodonium or siRNA for NOX or p22-phox inhibited oLDL-induced increases of HSF1, PAI-1 and H_2O_2 in MEF. Increased NOX4, NOX2, LOX1, HSF1 and PAI-1 were detected in aortae and hearts of apolipoprotein E-knockout (apoE-KO) mice compared to controls, which were associated with increased serum cholesterol or plasma PAI-1. The results suggest that NOX is required for oLDL-induced HSF1 or PAI-1 expression in MEF, which was supported by the up-regulation of NOX, LOX-1, HSF1 and PAI-1 in apoE-KO mice.

Keywords: *NADPH oxidase, plasminogen activator inhibitor-1, heat shock factors-1, oxidized LDL, mouse embryo fibroblasts, apolipoprotein E-knockout mice*

Abbreviations: *apoE-KO, apolipoprotein E-knockout; CAD, coronary artery disease; CAT, Chlormaphenicol acetyltransferase; EC, endothelial cells; ELISA, enzyme-linked immunosorbant assay; HSF1, heat shock factor-1; LDL: low density lipoprotein; LOX-1, lectin-like LDL receptor-1; MEF, mouse embryo fibroblasts; NOX, NADPH oxidase; oLDL, oxidized LDL; PAI-1, plasminogen activator inhibitor-1; ROS, Reactive oxygen species; siRNA, small-interference RNA; tPA, tissue plasminogen activators; uPA, urokinase plasminogen activators; VLDL, very low density lipoproteins.*

Introduction

Elevated low density lipoproteins (LDL)-cholesterol in blood circulation is a classic risk factor for atherosclerotic cardiovascular diseases [1]. Oxidation enhances the atherogenecity of LDL [2]. Thrombosis was often detected at sites of cholesterol-rich atherosclerotic lesions in patients with acute coronary syndrome [3]. Intravascular thrombosis results from imbalance between coagulation and fibrinolysis in local vasculature. Attenuated fibrinolytic activity was detected in patients with coronary artery disease (CAD) [4]. The major biological activator of fibrinolytic system is plasmin. The generation of plasmin is regulated by tissue and urokinase plasminogen activators (tPA and uPA). Plasminogen activator inhibitor-1 (PAI-1) is the physiological inhibitor of tPA and uPA. PAI-1 is also involved in wound healing, atherosclerosis, obesity and inflammation [5]. Oxidized LDL (oLDL) or very low density lipoproteins (VLDL) induced the expression of PAI-1 in cultured smooth muscle cells [6], endothelial cells (EC) [7] or fibroblasts [8]. Increased level of PAI-1 in plasma has been considered as a non-traditional risk factor of CAD [9].

Heat shock factor-1 (HSF1), the key mediator for stress response, is wildly expressed in most types of cells and tissues [10]. Previous studies by our group

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demonstrated that the binding of HSF1 to PAI-1 promoter is critical for glycated LDL or oLDL-induced PAI-1 transcription in EC [11,12]. Fibroblasts are one of most abundant types of cells in hearts or vascular wall and they play important roles in the construction and homeostasis of the cardiovascular system. HSF1-deficient mouse embryo fibroblasts (MEF) provide a powerful tool for studying the role of HSF1 in cellular processes. Our group reported that oxidized VLDL increased the abundance of PAI-1 in wild-type, but not HSF1-deficient MEF [8]. HSF1 may be activated by oxidative stress [10]. Increased levels of biomarkers of oLDL were detected in CAD patients [13] and in apolipoprotein E-knockout (apoE-KO) mice [14]. Lectin-like oLDL receptor-1 (LOX-1) mediates the transmembrane signalling of oLDL-induced PAI-1 expression in human EC [15]. Reactive oxygen species (ROS) induced by oLDL from vascular cells [16] may be generated from multiple sources [17]. NADPH oxidase (NOX) is one of the major intracellular source of ROS in EC [18]. Previous studies suggest that NOX promotes angiotensin II-induced myofibroblast migration [19]. NOX activation is required for oLDL-induced PAI-1 expression in EC [12]. The effect of oLDL on the expression of NOX complex components or the role of NOX in oLDL-induced PAI-1 in fibroblasts has not been known. Relationships between HSF1 and oLDL-induced NOX expression in mouse models remain unclear.

The present study examined effects of oLDL on the expression of NOX, LOX-1, PAI-1 and their relationship with HSF1 using MEF from wild-type and HSF1-KO mice. Correlations between contents of NOX, LOX-1, HSF1 and PAI-1 in cardiovascular tissue and serum cholesterol in apoE-KO and control mice were investigated.

Materials and methods

Cell culture

MEF from male HSF1-KO mice and matching C57BL/6 control mice were generously provided by Dr Ivor Benjamin in the University of Utah. Cells were cultured in medium containing 10% foetal bovine serum, 1 mM sodium pyruvte and 0.1 mM 2-mecaptoethanol as previously described [20].

Animal experiments

Male apoE-KO and C57BL/6 control mice (4 weeks of age) were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in an air-conditioned room and received high cholesterol/high fat diet as previously described [21]. Abdominal aortae, hearts and blood were harvested from apoE-KO and control mice at 24 weeks of age. Segments of hearts containing aortic valves were fixed in 10% buffered formalin. Aortic roots were sectioned and stained with haematoxylin and eosin. Atherosclerotic lesion areas were analysed as previously described [21]. Protocols of animal experiments were approved by the Animal Protocol Management and Review Committee at the University of Manitoba.

Isolation and modification of lipoproteins

LDL (density 1.019–1.063) was isolated from plasma of healthy donors using sequential density floatation ultracentrifugation. LDL was oxidized through dialysis against 5 μM $CuSO_4$ for 24 h at 22°C [7]. The oxidation of LDL was verified using thiobarbituric acid reactive substance assay and non-denatured gel electrophoresis. Lipoproteins were stored in sealed tubes under a layer of nitrogen at 4°C in the dark to prevent auto-oxidation.

Western blotting analysis

Tissue specimens or cellular proteins were analysed using Western blotting as previously described [11]. Antibodies against mouse HSF1, NOX4, NOX2, PAI-1, p22-phox, LOX-1 and *β*-actin were received from Santa Cruz (Santa Cruz, CA), Oxford Biomedical Research (Oxford, UK), Abcam (Cambrige, MA) or R&D Systems (Minneapolis, MN).

Measurement of PAI-1 antigen

Mouse PAI-1 enzyme-linked immunosorbant assay (ELISA) kits were obtained from Oxford Biomedical Research for measuring PAI-1 in mouse plasma or in conditioned medium of cultured MEF.

Gene silence

Small interference RNAs (siRNA) targeting mouse p22-phox mRNA (sc-36150), NOX2 mRNA (sc-35504) and NOX4 mRNA (sc-41587) were obtained from Santa Cruz, siRNA for *β*-actin mRNA and scramble siRNA were obtained from Ambion (Austin, TX). The siRNAs were transfected to MEF in serum-free medium using Silence siPort Lipid kit (Ambion) as described [11].

Measurement of hydrogen peroxide (H_2O_2)

The levels of H_2O_2 in media of MEF were measured using the reagents of PeroxiDetect™ kits from Sigma as previously described [16].

Intracellular superoxide detection

Levels of superoxide in MEF were detected by detecting lucigenin chemoluminescence in the presence of

superoxide anion as previously described [22]. Following experimental treatment, cells were homogenized in lysis buffer (50 mM KH_2PO_4 pH 7.0, 1 mM EGTA, 10 μg/ml of aprotinin, 0.5 μg/ml of leupeptin, 1 μg/ml of pepstatin and 0.5 mM PMSF). Aliquots of 50 μg cellular proteins were added in an assay buffer (50 mM KH_2PO_4 pH 7.0, 1 mM EGTA, 150 mM sucrose and 100 μM NADPH). The reaction was started by the addition of 25 μM lucigenin. Chemoluminescence was measured with a photon counter (Lumat LB 9507) for 30 min.

Transfection assay

The proximal promoter (1.1 kb)/luciferase reporter vector of rat NOX4 (−1018/+113 bp) was constructed as previously described [23]. The construct was kindly provided by Dr Isabel Fabregat (University of Barcelona, Spain). Calcium phosphate-precipitated promoter vector was transfected to MEF as described [11]. Chloramphenicol acetyltransferase (CAT) expression vector was co-transfected as an internal control.

Statistical methods

One-way ANOVA analysis was performed for comparing the probability of quantitative data among multiple groups. Student's *t*-test was used for comparisons between two groups. The level of significance was defined as $p < 0.05$.

Results

Effect of oLDL on the abundances of NOX4, NOX2 and p22-phox in MEF

NOX4 plays an important role in oxidative stress and fibrosis in mouse hearts [24]. The effect of physiologically relevant concentrations of oLDL (50–150 μg/ml for up to 24 h) on the abundance of NOX4 was characterized in wild-type MEF using Western blotting. The maximal stimulation of oLDL on the abundance of NOX4 was detected in cells treated with 100 μg/ml of oLDL for 6 h (Figures 1A and B). Significant increase in NOX4 content was detected in wild-type MEF treated with 100 μg/ml of oLDL for 6 h compared to LDL or vehicle control ($p < 0.05$). Treatment with oLDL (100 μg/ml for 6 h) significantly increased the activity of NOX4 promoter in MEF ($p < 0.01$, Figure 1C). NOX2 is a common isoform of NOX in cardiovascular cells. p22-phox is a sub-unit of NOX complex and an essential component for NOX4 or NOX2 complex. The abundances of NOX2 and p22-phox were increased by 100 μg/ml of LDL or oLDL for 6 h in wild-type MEF compared to vehicle ($p < 0.05$, 0.01). Treatment with oLDL induced significantly greater increase of NOX2 or p22-phox in MEF

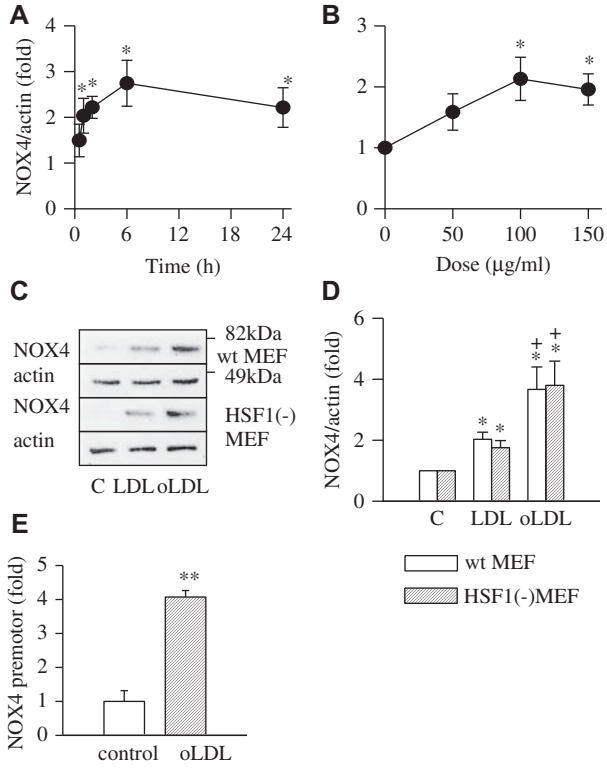

Figure 1. Effects of oxidized LDL (oLDL) on NOX4 expression in cultured wild-type (wt) and heat shock factor 1-deficient [(HSF1(−)] mouse embryo fibroblasts (MEF). (A) and (B) MEF from wild-type mice were treated with vehicle (control) or 100 μg/ml of oLDL for 0.5–24 h (time-course) or 50–150 μg/ml of oLDL for 6 h (dose–response). (C) MEF were transiently transfected with NOX4 promoter/luciferase reporter gene vector and chloramphenicol acetyltransferase (CAT) gene vector, then treated with vehicle or 100 μg/ml of oLDL for 6 h. (D) Wild-type (wt) and HSF1(−)MEF were treated with 100 μg/ml of LDL, oLDL or vehicle (control) for 6 h. NOX4 and *β*-actin in cell lysates were analysed using Western blotting. Luciferase activity was normalized with CAT activity. Values were expressed in folds of control after normalization with *β*-actin (mean ± SD, $n = 3$ independent experiments). $^{*,**}p < 0.05$ or 0.01 vs control; $^{+}p < 0.05$ vs LDL.

($p < 0.05$, Figure 2). The effects of LDL and oLDL on the abundance of NOX4, NOX2 or p22-phox in HSF1-deficient MEF were not significantly different from that in wild-type MEF (Figures 1D and 2), which suggests that oLDL-induced expressions of NOX4, NOX2 and p22phox in MEF are independent from stress response regulation.

Effect of oLDL on ROS production in MEF

To determine the effect of oLDL on the production and specificity of ROS and the relationship with stress response, wild-type or HSF1-deficient MEF were incubated with 100 μg/ml of LDL or oLDL for 6 h. Treatment with oLDL significantly increased the levels of superoxide and H_2O_2 in wild-type or HSF1-deficient MEF compared to LDL or vehicle ($p < 0.05$, Figure 3). An addition of 100 units/ml of superoxide dismutase (SOD) reversed oLDL or LDL-induced superoxide

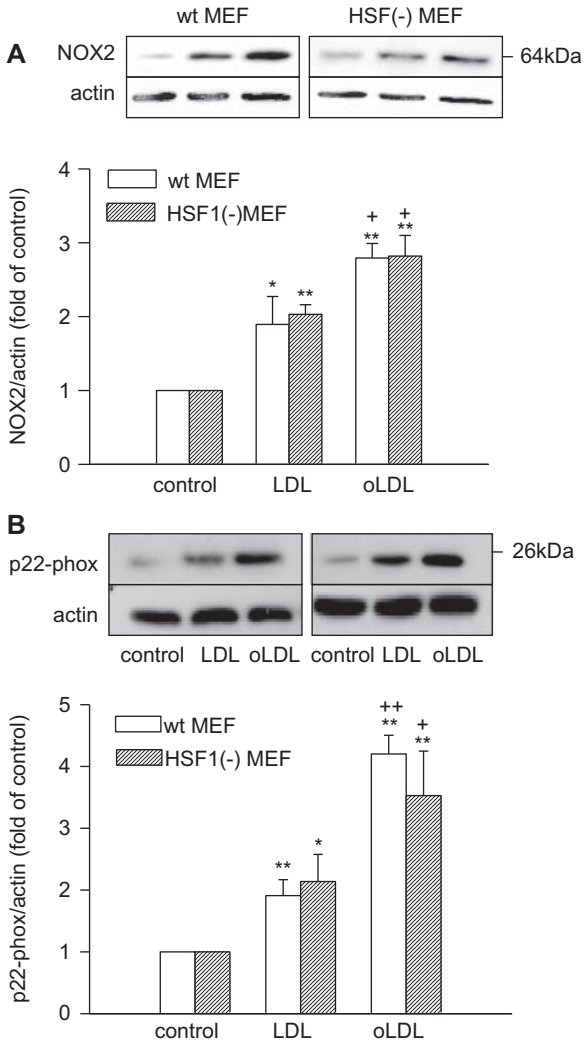

Figure 2. Effect of oLDL on NOX2 and p22-phox expression in MEF. Wild-type and HSF1(−)MEF were treated with vehicle (control), LDL or oLDL (100 μg/ml, for 6 h). NOX2, p22-phox and β-actin were detected in cellular proteins using Western blotting. Values were expressed in mean ± SD in folds of control ($n = 3$ independent experiments). $^{*, **}p < 0.05$ or 0.01 vs control; $^{+, ++}p < 0.05$ or 0.01 vs LDL.

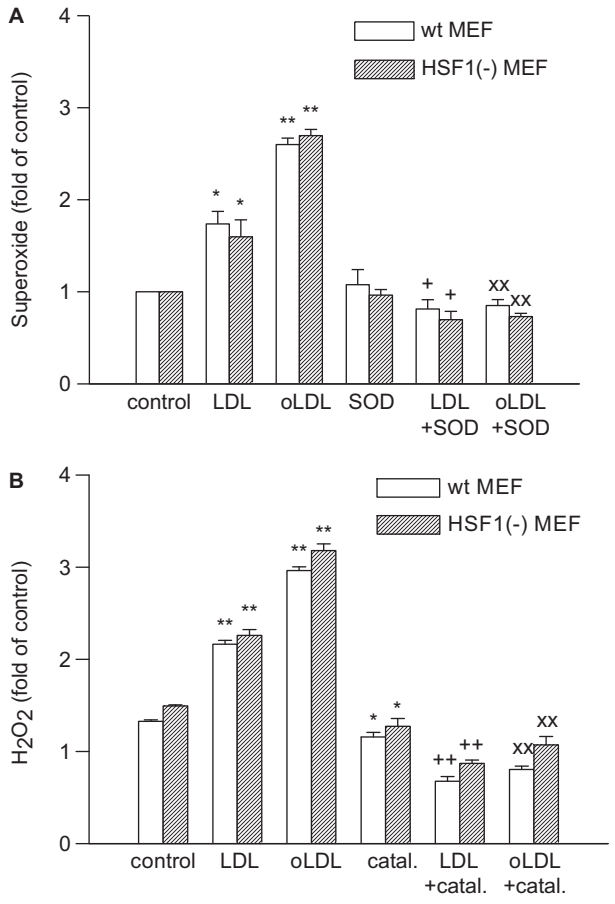

Figure 3. Effects of superoxide dismutase (SOD) and catalase on oLDL-induced ROS production in MEF. Wild-type and HSF1(−) MEF were treated with vehicle (control), LDL or oLDL (100 μg/ml, for 6 h) with and without the addition of 100 units/ml of SOD (A) or 200 units of catalase (B). (A) Superoxide in cells was measured using a lucigenin method. (B) H_2O_2 in conditioned media was detected using biochemical method. Values were expressed in mean ± SD in folds of control ($n = 3$ independent experiments). $^{*, **}p < 0.05$ or 0.01 vs control; $^{+, ++}p < 0.05$ or 0.01 vs LDL; $^{xx}p < 0.01$ vs oLDL.

levels in both types of cells ($p < 0.05$ or 0.01). Treatment with 200 units/ml of catalase normalized LDL or oLDL-induced H_2O_2 from wild-type or HSF1-deficient MEF ($p < 0.01$, Figure 3). The results suggest that LDL, particularly its oxidized form, increases the generation of superoxide and H_2O_2 in HSF1-deficient or wild-type MEF, which may be specifically reversed by addition of SOD or catalase.

Role of LOX-1 in oLDL-induced NOX4 and p22-phox in MEF

LOX-1 is a receptor for oLDL in multiple types of cells. The effect of oLDL on LOX-1 expression in fibroblasts remains unclear. The present study examined the abundances of LOX-1 in wild-type and HSF1-deficient MEF treated with 100 μg/ml of LDL or oLDL for 6 h using Western blotting. The abundance of LOX-1 was significantly increased by LDL or oLDL in both types of cells ($p < 0.01$), and oLDL induced significantly greater increase in LOX-1 compared to LDL ($p < 0.05$, Figure 4A). The involvement of LOX-1 in oLDL-induced NOX4 or p22-phox was examined using blocking antibody against LOX-1 (LoxAb, Santa Cruz, CA) in wild-type MEF. MEF were pre-treated with LoxAb (10 μg/ml) for 30 min then exposed to oLDL or LDL (100 μg/ml) for 6 h. LoxAb prevented the increase of NOX4 or p22-phox induced by oLDL or LDL in MEF ($p < 0.01$, Figure 4B). LoxAb did not significantly change the basal levels of NOX4 or p22-phox in MEF. The results indicate that LOX-1 mediates cross-membrane signalling for oLDL-induced NOX4 or p22-phox in MEF.

Effects of oLDL on PAI-1 and HSF1 in MEF

Treatment with 100 μg/ml of LDL for 24 h moderately, but significantly, increased the release of PAI-1 from

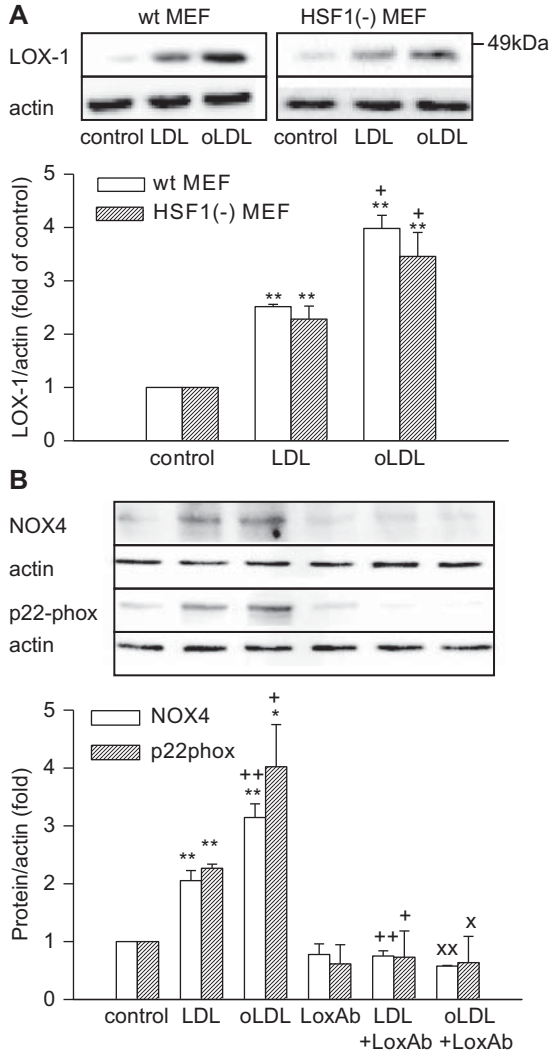

Figure 4. Effects of oLDL on LOX-1 expression and LOX-1 antibody on oLDL-induced NOX4 and p22-phox in MEF. (A) The abundance of LOX-1 and β-actin in cell lysates of wt or HSF1(−) MEF treated with vehicle (control), 100 μg/ml of LDL or oLDL for 24 h were analysed using Western blotting. (B) The role of LOX-1 in oLDL-induced NOX4 or p22-phox in MEF was examined using blocking antibody against LOX-1 (LoxAb). MEF were pre-treated with LoxAb (10 μg/ml) for 30 min then exposed to oLDL or LDL (100 μg/ml) for 6 h. Values were expressed in folds of control after normalization with β-actin (mean ± SD, $n = 3$ independent experiments). $^{*,**}p < 0.05$, 0.01 vs control; $^{+,++}p < 0.05$ or 0.01 vs LDL; $^{x,xx}p < 0.05$, 0.01 vs oLDL.

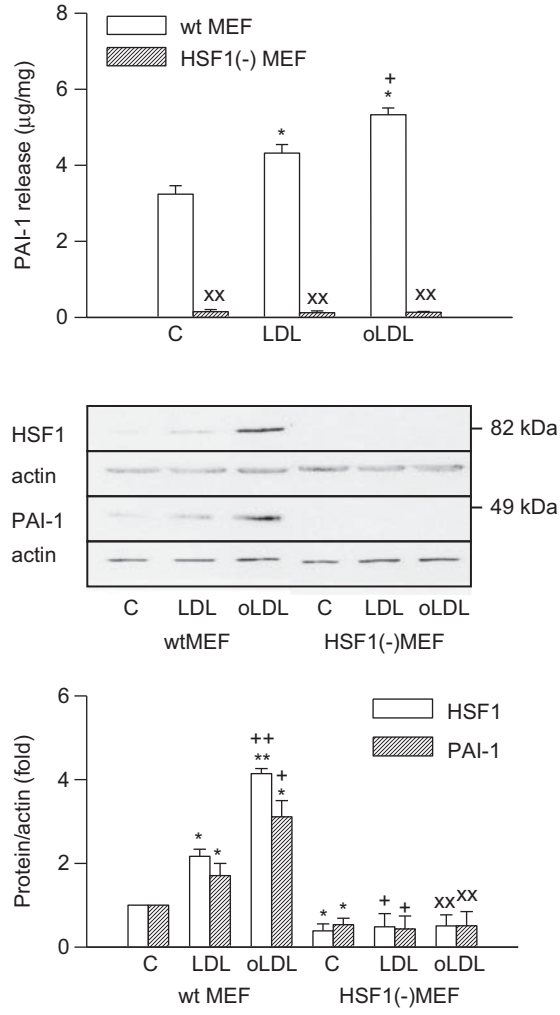

Figure 5. Effects of oLDL on PAI-1 and HSF1 in MEF. (A) Levels of PAI-1 in conditional media of wt or HSF1(−)MEF treated with vehicle (control or C), 100 μg/ml of LDL or oLDL for 24 h were measured using mouse PAI-1 ELISA kits. (B) Abundances of HSF1 and PAI-1 in cell lysates of MEF were analysed using Western blotting. Values were expressed in mean ± SD folds of control or μg/mg after normalization with β-actin or total cellular proteins ($n = 3$ independent experiments). $^{*,**}p < 0.05$ or 0.01 vs C of wild-type MEF; $^{+,++}p < 0.05$ or 0.01 vs LDL; $^{xx}p < 0.01$ vs oLDL.

wild-type MEF ($p < 0.05$). Treatment with oLDL (at the same condition) enhanced the release of PAI-1 from wild-type MEF compared to LDL ($p < 0.01$). The basal level of PAI-1 in the medium of HSF1-deficient MEF was undetectable. The release of PAI-1 from HSF1-deficient MEF was not significantly altered by LDL or oLDL treatment (Figure 5A). Abundances of HSF1 and PAI-1 in cell lysates of HSF1-deficient and wild-type MEF were examined after treatment with 100 μg/ml of LDL or oLDL for 24 h (for detecting PAI-1) or 6 h (for HSF1) (Figure 5B). in unstimulated conditions, the abundance of HSF1 or PAI-1 in HSF1-deficient MEF was significantly lower than that in wild-type MEF. The abundance of HSF1 or PAI-1 in LDL-treated wild-type MEF was significantly greater than that in control cultures ($p < 0.05$). Treatment with oLDL significantly increased abundances of PAI-1 and HSF1 in wild-type MEF compared to LDL ($p < 0.05$ or 0.01). No evident increase in the abundance of HSF1 or PAI-1 was detected in HSF1-deficient MEF treated with LDL or oLDL compared to baseline levels (Figures 5B and C).

Effect of NOX inhibitor on oLDL-induced PAI-1, HSF1, NOX4 and H2O2 in MEF

To determine whether NOX inhibitor affects oLDL-induced PAI-1 or HSF1 expression, wild-type MEF

were pre-treated with 10 μM diphenyleneiodonium (DPI), a NOX inhibitor for 4 h and then incubated with 100 μg/ml of LDL or oLDL for optimized time to examine NOX4, HSF1 (for 6 h) or PAI-1 (for 24 h). DPI inhibited the levels of NOX4, but not *β*-actin, at basal or stimulated conditions in wild-type or HSF1-deficient MEF ($p < 0.05$, Figure 6A). DPI prevented LDL or oLDL-induced increase in the abundance of PAI-1 or HSF1 in wild-type MEF ($p < 0.05$ or 0.01, Figure 6B). The increase of the release of H_2O_2 reached the maximal level in wild-type MEF treated with oLDL for 2–4 h (Figure 6C). The release of H_2O_2 from wild-type MEF treated with oLDL (100 μg/ml for 2 h) was increased compared to LDL or vehicle ($p < 0.01$). Effects of LDL and oLDL on H_2O_2 releases from HSF1-deficient MEF were similar to that from wild-type MEF treated with corresponding lipoprotein. DPI inhibited the releases of H_2O_2 from wild-type or HSF1-deficient MEF induced by LDL or oLDL ($p < 0.01$, Figure 6D). The results suggest that NOX activation is required for oLDL-induced ROS production and the expression of PAI-1 or HSF1 in MEF.

Impact of siRNA for NOX2, NOX4 or p22-phox on oLDL-induced expression of HSF1 and PAI-1 in MEF

Transfection of siRNA against NOX2, NOX4 or p22-phox mRNA effectively blocked LDL or oLDL-induced increases of HSF1 and PAI-1 in wild-type MEF ($p < 0.01$, Figures 7 and 8). NOX2 or NOX4 siRNA inhibited the expression of HSF1 ($p < 0.05$), but not PAI-1 in wild-type MEF without an addition of lipoproteins (Figure 7). In wild-type MEF transfected with p22-phox siRNA without an addition of lipoprotein, the contents of PAI-1, HSF1, p22-phox and NOX4 were

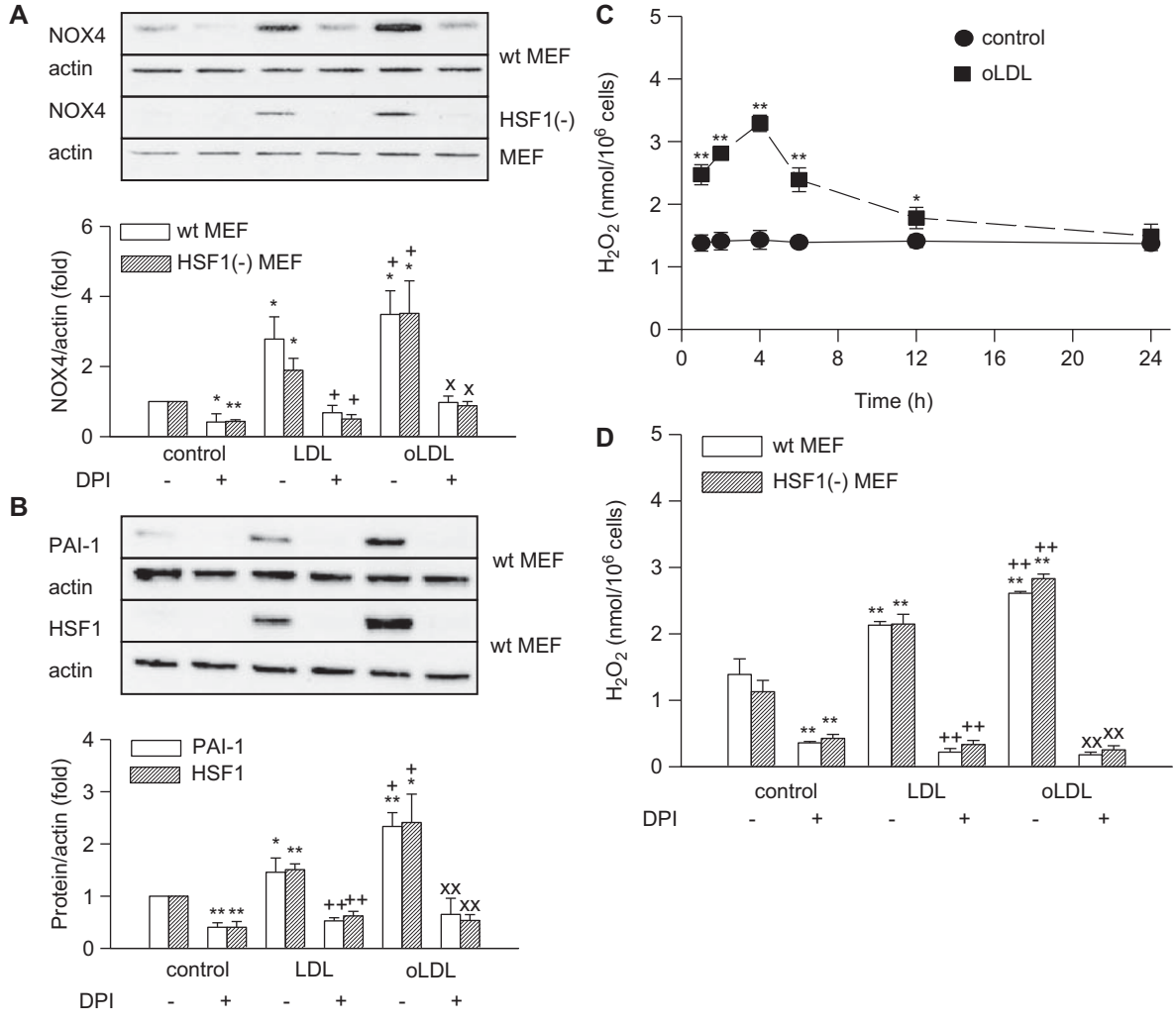

Figure 6. Effects of NOX inhibitor on oLDL-induced increase of PAI-1, HSF1 and NOX4 and release of H_2O_2 in MEF. Wild-type or HSF1(−)MEF were pre-treated with 10 μM diphenyleneiodonium (DPI, a NOX inhibitor) for 4 h, then were treated with LDL or oLDL (100 μg/ml, for 6 h or 24 h) or vehicle (control). Abundances of NOX4, PAI-1, HSF1 and *β*-actin in cell lysates were analysed using Western blotting. H_2O_2 levels in media were determined as described in the Methods. (A) NOX4; (B) PAI-1 and HSF1; (C) and (D) H_2O_2. Integrative data are presented in mean ± SD folds of control or nmol $H_2O_2/10^6$ cells after normalization with *β*-actin or cell numbers ($n = 3$ independent experiments). $^{*, **}p < 0.05$ or 0.01 vs control without DPI; $^{+, ++}p < 0.05$ or 0.01 vs LDL without DPI; $^{x, xx}p < 0.05$ or 0.01 vs oLDL without DPI.

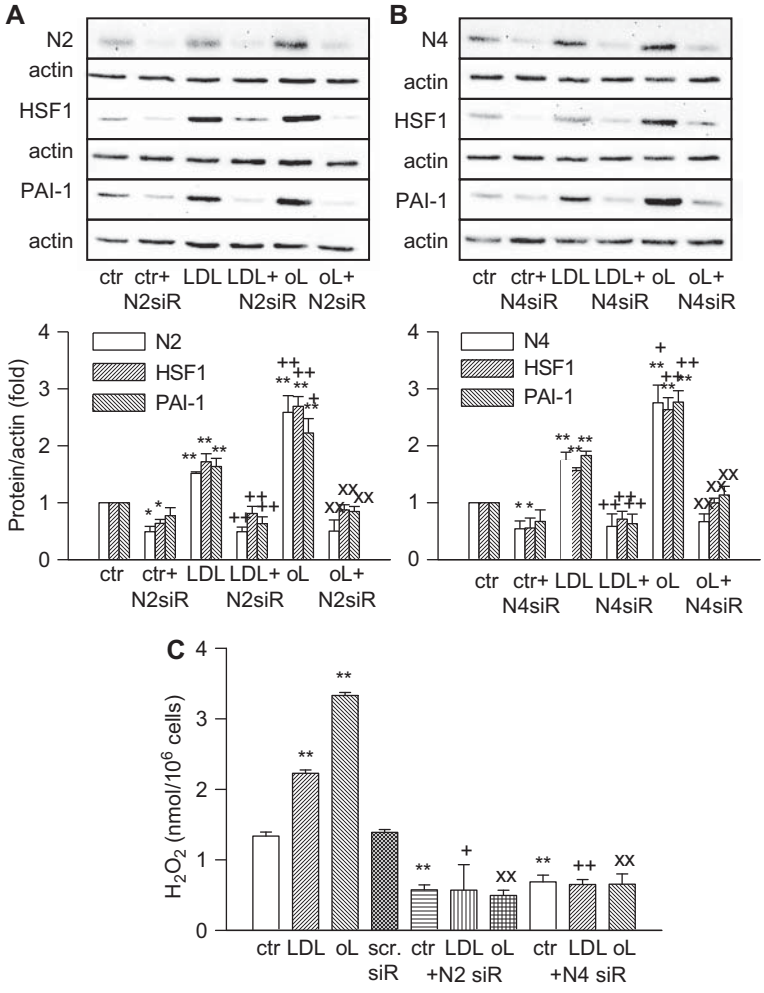

Figure 7. Effects of siRNAs against NOX2 or NOX4 on the expression of HSF1 and PAI-1 and superoxide in MEF. Wild-type MEF were transfected with siRNA (siR) against NOX2 (N2), NOX4 (N4) or scrambled siRNA then treated with LDL, oLDL (100 μg/ml, for 6 h or 24 h) or vehicle or control (ctr). Abundance of N2, N4, HSF1, PAI-1 and β-actin were detected using Western blotting. Values were presented in mean ± SD folds of control after normalization with β-actin ($n = 3$ independent experiments). $^{*}, ^{**}p < 0.05$ or 0.01 vs control without siR; $^{+}, ^{++}p < 0.05$ or 0.01 vs LDL without siR, $^{xx}p < 0.01$ vs oLDL without siR.

partially, but significantly, reduced compared to control ($p < 0.05$). Gene silence of p22-phox did not evidently affect the abundance of β-actin in MEF with or without lipoprotein treatment (Figure 8). Scramble siRNA did not significantly affect the level of NOX components, HSF1 or PAI-1 (data not shown). NOX2 or NOX4 siRNA neutralized oLDL or LDL-induced generation of H_2O_2 from wild-type MEF. Scramble siRNA did not inhibit H_2O_2 release MEF at baseline condition (Figure 8). The findings confirmed that NOX2, NOX4 or p22-phox play crucial roles in the regulation of PAI-1 or HSF1 expression in addition to ROS production in MEF induced by LDL or oLDL.

Abundance of LOX-1, NOX, PAI-1 and HSF1 in cardiovascular tissue of apoE-KO mice

Increased serum total cholesterol was detected in apoE-KO mice compared to control mice ($p < 0.01$, Figure 9A) as expected. ApoE-KO mice developed atherosclerotic lesions in aortic roots. No atherosclerotic lesion was detected in control wild-type mice. Atherosclerotic lesion areas in aortae of apoE-KO mice were significantly greater than that in controls ($p < 0.01$, Figure 9B). Plasma levels of PAI-1 in apoE-KO mice were significantly higher than that of controls ($p < 0.01$, Figure 9C). Abundances of LOX-1, NOX4, NOX2, HSF1 and PAI-1 in hearts (Figures 10A and C) and aortae (Figures 10B and D) were significantly increased in apoE-KO mice compared to control mice ($p < 0.05$ or 0.01). The levels of PAI-1 in plasma of apoE-KO and control mice positively correlated with serum total cholesterol ($r = 0.92$, $n = 10$, $p < 0.01$). Abundances of HSF1, PAI-1, NOX2, NOX4 and LOX-1 in aortae or hearts of the mice positively correlated with each other ($r = 0.71$–0.97, $n = 10$, $p < 0.05$ or 0.01) and with the levels of plasma PAI-1 or serum total cholesterol ($r = 0.70$–0.99, $n = 10$, $p < 0.05$ or 0.01, Table I).

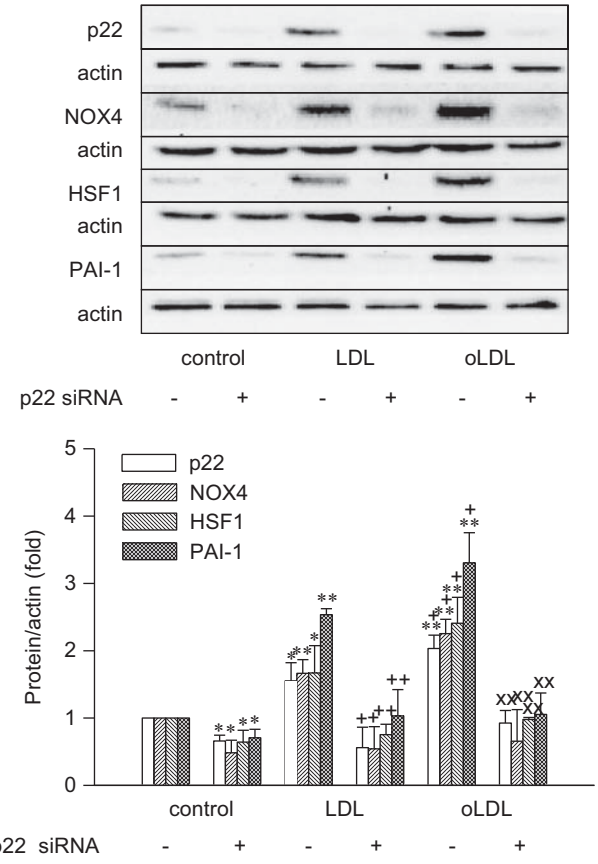

Figure 8. Effects of siRNAs against p22-phox on the expression of HSF1 and PAI-1 and superoxide in MEF. Wild-type MEF were transfected with siRNA against p22-phox and then treated with LDL, oLDL (100 μg/ml, for 6 h or 24 h) or vehicle control. Abundance of p22-phox, NOX4, HSF1, PAI-1 and β-actin were detected using Western blotting. Values were presented in mean ± SD folds of control after normalization with β-actin ($n = 3$ independent experiments). $^{*,**}p < 0.05$ or 0.01 vs control without siRNA; $^{+,++}p < 0.05$ or 0.01 vs LDL without siRNA; $^{xx}p < 0.01$ vs oLDL without siRNA.

Discussion

Novel findings generated from the present study include the following: (a) treatment with oLDL substantially increased the activity of NOX, the transcriptional activity of NOX4 gene and abundances of NOX4, NOX2 and p22-phox in wild-type or HSF1-deficient MEF; (b) oLDL augmented PAI-1 and HSF1 contents in wide-type MEF, but not in HSF1-deficient MEF; (c) oLDL elevated the abundance of LOX-1 in wild-type or HSF1-deficient MEF and LoxAb inhibited oLDL-induced expression of NOX4 complex components in MEF; (d) DPI or siRNA for NOX4, NOX2 and p22-phox neutralized the increases of HSF1 and PAI-1 contents in wild-type MEF induced by oLDL; (e) the abundances of LOX-1, NOX4, NOX2, HSF1 and PAI-1 were increased in hearts or aortae of apoE-KO mice compared to control mice, which were positively correlated with total cholesterol or PAI-1 in their blood circulation.

Previous studies demonstrated that elevated expression of NOX components induced by radiation-induced oxidative stress was associated with increased PAI-1 generation in rat brain EC [25]. HSF1 mediates oLDL-induced PAI-1 expression in EC [12]. Relationship between HSF1 in oLDL-induced NOX in fibroblasts has not been documented. The present study demonstrated that oLDL increased the production of superoxide, the abundance of NOX4, NOX2 and p22-phox in wild-type or HSF1-deficient MEF, which suggests that oLDL enhanced the expression of NOX complex components and NOX activity through an HSF1-independent pathway.

DPI inhibited oLDL-induced HSF1 and PAI-1 expression in wild-type MEF, but the inhibitor is not specific to NOX. Therefore, we examined the effects for NOX4, NOX2 or p22-phox siRNA on HSF1 and PAI-1 expression in wild-type MEF as described [26]. The results of the present study indicated that NOX4, NOX2 or p22-phox siRNA inhibited the expression of HSF1 or PAI-1 induced by oLDL in MEF. The findings provide original evidence that NOX plays a critical role in the upstream regulation of oLDL-induced HSF1 and PAI-1 in mouse fibroblasts.

Previous studies demonstrated that ROS was involved in cyclic strain-induced PAI-1 expression in EC [27]. ROS activated HSF1 in rat C6 glioma cells [28]. Our group demonstrated that antioxidant inhibited LDL-induced PAI-1, NOX2 and HSF1 in EC [12]. The present study demonstrated that NOX4, NOX2 or DPI normalized oLDL-induced increase in H_2O_2, that was associated with reduction in HSF1 and PAI-1 expression in MEF. The findings suggest that NOX-induced increase of the generation of ROS via NOX

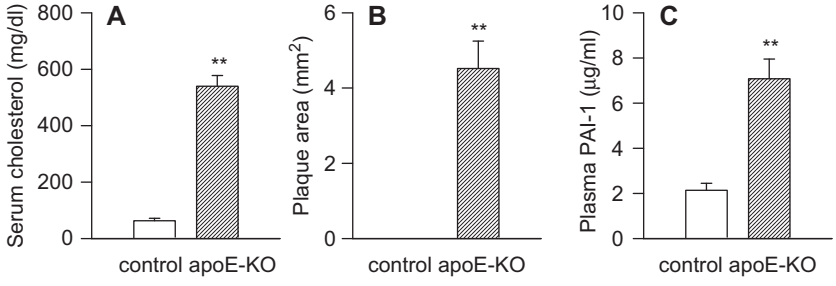

Figure 9. Serum cholesterol, areas of atherosclerotic plaque and plasma PAI-1 in apoE KO mice. (A) serum total cholesterol; (B) areas of atherosclerotic plaque lesions in aortic roots; (C) plasma PAI-1. Values were expressed in mean ± SD ($n = 5$/group). $^{**}p < 0.01$ vs control mice.

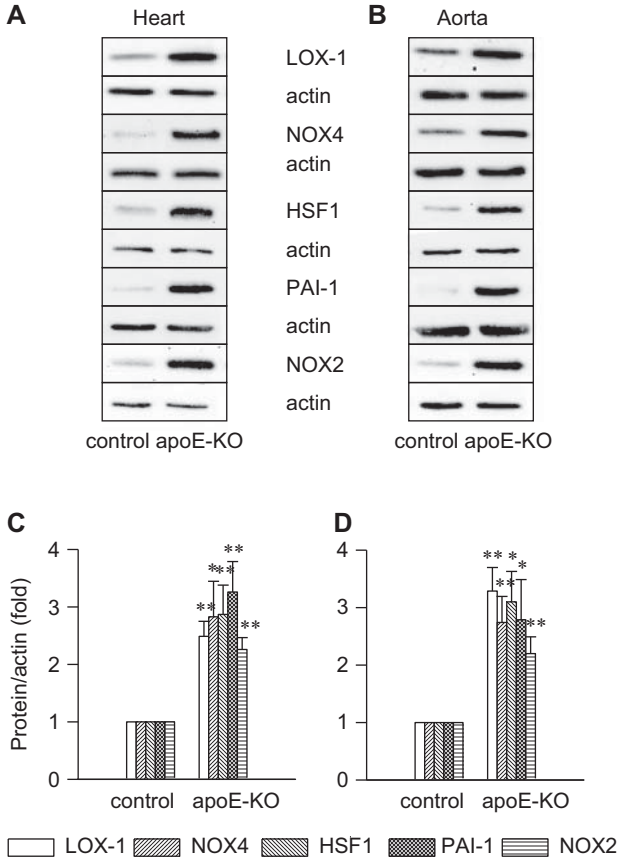

Figure 10. LOX-1, NOX2, NOX4, HSF1 and PAI-1 in tissue of apoE-KO mice. Abundances of LOX-1, NOX2, NOX4, HSF1 and PAI-1 in aortae and hearts of apoE-KO and C57BL/6 control mice were measured using Western blotting. Values were expressed in mean ± SD folds of control ($n = 5$/group). $^{*,**}p < 0.05$ or 0.01 vs control mice.

may contribute to the up-regulation of HSF1 or PAI-1 in MEF induced by oLDL.

A recent study from Judkins et al. [29] demonstrated that the levels of NOX4 were not altered in 12 or 19 weeks old apoE-KO mice [29]. The expression of NOX components in apoE-KO mice older than 20 weeks remains unclear. Increased levels of oLDL were detected in the circulation of apoE-KO mice with a peak at 20 weeks of age and gradually returned to baseline after 40 weeks of age [14], which implies that the levels of oLDL are expected to be elevated in the blood of apoE-KO mice ~24 weeks of age as it was observed in the present study. Increased expression of NOX [30,31], elevated PAI-1 levels and thrombotic tendency were detected in apoE-KO mice [32]. Limited information was available for HSF1 or its relationship with NOX, HSF1 or PAI-1 in apoE-KO mice. The present study originally demonstrated that the abundance of HSF1 in hearts or aortae of apoE-KO mice was substantially elevated, which was associated with increases in the contents of NOX4, NOX2 and PAI-1 in cardiovascular tissues. The results suggest that stress responses are increased in apoE-KO mice, which may be part of an adaptive response of the body to metabolic disorders induced by apoE-deficiency.

Kataoka et al. [33] reported that the expression of LOX-1 is increased in atherosclerotic tissues. Previous studies in our laboratory demonstrated that multiple types of oLDL increased the expression of LOX-1 in human umbilical vein EC (HUVEC) or coronary artery EC (HCAEC) [15]. The present study demonstrated that oLDL stimulated the expression of LOX-1 in MEF. LoxAb not only reduced oLDL-induced expression of NOX4 and p22-phox, but also inhibited LDL-induced NOX4 or p22-phox in MEF, which suggests that EC-mediated oxidation may be implicated in LDL-induced PAI-1 or NOX expression in EC. Abundances of LOX-1 in aortae or hearts were increased in apoE-KO mice compared to wild-type mice. The levels of LOX-1 in cardiovascular tissue correlated with that of NOX4, HSF1 or PAI-1 in the mice. The results suggest that LOX-1 plays a critical role in EC or copper ion-modified oLDL-induced activation of NOX, HSF1 and PAI-1 in mouse fibroblasts and cardiovascular tissue.

The results of the present study demonstrated that NOX activation in MEF exposed to oLDL was associated with increased ROS generation. ROS is a known agonist of HSF1 [34]. Previous studies suggest

Table I. Correlation between NOX2, NOX4, HSF1, PAI-1 and cholesterol in apoE-KO and control mice.

	Chol	pPAI-1	aHSF1	aPAI-1	aNOX2	aNOX4	aLOX-1	hHSF1	hPAI	hNOX2	hNOX4
pPAI-1	0.92**										
aHSF1	0.96**	0.84**									
aPAI-1	0.78**	0.77**	0.80**								
aNOX2	0.94**	0.96**	0.86**	0.97**							
aNOX4	0.91**	0.70*	0.82**	0.86**	0.98**						
aLOX-1	0.99**	0.92**	0.90**	0.80**	0.91**	0.93**					
hHSF1	0.95**	0.90**	0.91**	0.83**	0.87**	0.86**	0.92**				
hPAI-1	0.84**	0.80**	0.71*	0.81**	0.94**	0.72*	0.78**	0.89**			
hNOX2	0.97**	0.81**	0.89**	0.86**	0.90**	0.94**	0.98**	0.89**	0.81**		
hNOX4	0.96**	0.82**	0.90**	0.84**	0.97**	0.94**	0.95**	0.88**	0.75*	0.99**	
hLOX-1	0.98**	0.86**	0.95**	0.73*	0.90**	0.94**	0.97**	0.92**	0.76*	0.92**	0.93**

p, plasma; h, heart; Chol, total serum cholesterol; a, aorta; Data in the tables are *r* values ($n = 10$). $^{*,**}p < 0.05$ or 0.01.

HSF1 mediates the transcription of genes involved in wound healing beside heat shock proteins [35]. Our group reported that HSF1 binds to a distal responsive element in the PAI-1 promoter and up-regulates the transcription of PAI-1 gene in EC [11]. The increase of PAI-1 production in the cardiovascular system may be a part of the stress response of the body [11]. PAI-1 not only inhibits clot lysis, but also prevents degradation of the extracellular matrix, which contributes to wound healing. Vascular fibroblasts may be exposed to oLDL during endothelial injury or interact with oLDL incorporated by macrophages in atherosclerotic plaques. Increased PAI-1 expression in fibroblasts exposed to oLDL may contribute to extracellular matrix remodelling in the vasculature.

In summary, the present study demonstrated that oLDL increased the NOX expression production and activity in MEF via a LOX-1-dependent, but HSF1-independent, pathway. NOX and HSF1 are essential for oLDL-induced PAI-1 expression. The increases of NOX components in cardiovascular tissue in apoE-KO mice were associated with LOX-1, HSF1, PAI-1 and serum cholesterol. The results suggest that fibroblasts are one type of targets of oxidatively modified LDL, which lead a series of cellular responses regarding oxidative stress, stress response and hypofibrinolysis.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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