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**Involvement of Heat Shock Factor-1 in Glycated Low Density Lipoprotein-Induced Upregulation of Plasminogen Activator Inhibitor-1 in Vascular Endothelial Cells**

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Running title: HSF1 in glycated LDL-induced PAI-1

**Abstract:** Coronary artery disease is the predominant cause of death in diabetic patients. Plasminogen activator inhibitor-1 (PAI-1) is the major physiological inhibitor of plasminogen activators. Heat shock protein (Hsp) was upregulated in uncontrolled diabetic patients. Our previous studies demonstrated that glycated low density lipoproteins (gly-LDL) stimulated the generation of PAI-1 from vascular endothelial cells (EC). The present study examined the effect of gly-LDL on the expression of heat shock factor-1 (HSF1), a physiological transcription factor of Hsp, and the involvement of HSF-1 in gly-LDL-induced production of PAI-1 in cultured human umbilical vein EC (HUVEC) and coronary artery EC (HCAEC). Treatment with gly-LDL increased the expression of HSF1 and Hsp-70 compared to LDL in subconfluent HCAEC or HUVEC, and that was associated with an increase of PAI-1 expression. The transfection of HSF1 gene enhanced the expression of PAI-1 in EC. Small interference RNA against HSF1 prevented gly-LDL-induced upregulation of PAI-1 in HCAEC or HUVEC. Gly-LDL increased the binding of a nuclear protein to the PAI-1 promoter. The nuclear protein-DNA complex was supershifted by HSF1 antibody. The presence of an antioxidant, butylated hydroxytoluene, during the glycation of LDL, prevented gly-LDL-induced increases of the expression of HSF1 or PAI-1 in EC. The results suggest that HSF-1 is involved in gly-LDL-induced upregulation of PAI-1 in subconfluent vascular EC through the binding of HSF1 to PAI-1 promoter. Glyco-oxidation may contribute to gly-LDL-induced expression of HSF1 and PAI-1 in EC.

## Introduction

The incidence of diabetes mellitus (DM) in North America has been rapidly increased during last three decades, and the trend is expected to continue (1). The most common cause of death in diabetic patients is coronary artery disease (CAD). Acute coronary syndrome is often associated with thrombosis at the lesions of atherosclerotic plaques (2, 3). Thrombogenesis depends on imbalance between coagulation and fibrinolysis in local blood circulation. Attenuated fibrinolytic activity has been detected in peripheral circulation of type 1 or type 2 DM patients (4, 5). Plasminogen activator inhibitor-1 (PAI-1) is the major physiological inhibitor for fibrinolysis, which modulates the activity of tissue and urokinase plasminogen activators on the formation of plasmin. PAI-1 is also implicated in inflammation, endothelial dysfunction, and extracellular matrix remodeling (5). An elevated level of PAI-1 in plasma has been considered as a non-traditional risk factor for CAD and a marker of endothelial dysfunction (7).

Hyperglycemia and dyslipoproteinemia are two major biochemical markers of diabetes. Elevated low density lipoprotein (LDL) is a classical risk factor for atherosclerotic cardiovascular disease. LDL clearance via the LDL receptor is attenuated by glycation (8). Elevated levels of small, dense LDL and glycated LDL (gly-LDL) were frequently detected in diabetic patients (9-11). Previous studies in our laboratory demonstrated that gly-LDL increased the production of PAI-1 in cultured venous or arterial endothelial cells (EC). LDL isolated from diabetic patients or gly-LDL modified *in vitro* enhanced the activity of PAI-1 promoter in EC (12-15). Gly-LDL stimulated the

generation of reactive oxygen species (ROS) and decreased the abundance of reduced glutathione in EC (16). The findings imply that gly-LDL may induce oxidative stress in vasculature. Heat shock, mechanical shear or oxidative stress induces stress responses in cells, which is mediated by heat shock proteins (Hsp). The transcription of Hsp is mediated by heat shock factor (HSF) (17). HSF1 is the most widely distributed form of HSF in human body (19, 20). The activation of HSF1 is detected during embryo growth (21) or in diet-induced atherosclerotic animal models (22). The levels of Hsp-70 were increased in the peripheral circulation of diabetic patients with ketoacidosis (23). The impact of gly-LDL on HSF1, or the relationship between HSF1 and PAI-1, has not been documented. The present study investigated the effect of gly-LDL on HSF1 expression, and the involvement of HSF1 in gly-LDL-induced PAI-1 production in cultured human arterial and venous EC.

## Materials and Methods

### Isolation and modification of LDL

LDL (density 1.019-1.063) was isolated from the plasma of healthy donors using sequential floatation density ultracentrifugation. LDL was glycated by incubation with 50 mM glucose and 50 mM sodium cyanoborohydride for 2 weeks at 37°C in dark overlaid with nitrogen as previously described (12). In a parallel preparation, LDL was glycated with the presence of 80 μM butylated hydroxytoluene (BHT-gLDL) (16). Free glucose was removed from gly-LDL through dialysis. The extent of glycation in gly-LDL was estimated using trinitrobenzenesulfonic acid assay (24). Approximately 60% of lysine residues were glycated in the preparations of gly-

LDL used in following experiments. Endotoxin level in lipoproteins was monitored using E-Toxate kit with a threshold of 0.05 ng/ml (Sigma, St Louis, MO). LDL and its modified forms were stored in sealed tubes under a layer of nitrogen at 4°C in dark to prevent auto-oxidation (25).

### Cell culture

Seed human umbilical vein endothelial cells (HUVECs) were obtained from American Type Culture Collections (Manassas, VA). Cells were grown in F12K medium (Gibco Canada, Burlington, ON) containing 10% fetal calf serum, 0.1 mg/ml of heparin and 30 µg/ml of EC growth supplements (Sigma) (15). Seed human coronary arterial EC (HCAEC) and cultured supplements were obtained from Clonetics (San Diego, CA). Subconfluent EC cultures within 8 passages from the seed cells were treated with or without an addition of lipoproteins or serum as indicated. No obvious cytotoxicity was detected in EC treated with LDL or gly-LDL in tested conditions using morphological observation or leucine incorporation assay (25).

### Western blotting assay

Western blotting analysis was performed as previously described (25) using monoclonal antibodies against human HSF1, Hsp-70, PAI-1, β-actin, non-specific mouse IgG or anti-mouse IgG antibody conjugated with horse radish peroxidase obtained from Santa Cruz (Santa Cruz, CA) or Sigma. Enhanced chemilluminence reagents (Amersham, Piscataway, NJ) were used for detecting targeted antigens on nitrocellulose membrane. The densities of the antigens were detected using Chemi-Doc system and Quantity One software (BioRad, Hercules, CA). The abundance of targeted

proteins was normalized with the level of β-actin in corresponding samples.

### PAI-1 antigen and activity measurements

The levels of PAI-1 antigen and activity in experimental medium of EC cultures were analyzed using PAI-1 enzyme-linked immunosorbent assay (ELISA) or PAI activity assay kits (Am. Diagnostic Inc., Stamford, CT) as previously described (26).

### Measurement of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)

The levels of H<sub>2</sub>O<sub>2</sub> in the conditioned media of EC were analyzed using PeroxiDetect™ kit (Sigma) as previously described (16).

### Reverse transcription-polymerase chain reaction (RT-PCR)

The levels of HSF1 and PAI-1 mRNA were assessed using RT-PCR and justified with the level of β-actin mRNA in corresponding samples. Primers for HSF1 mRNA (sense: 5'-GACATAAAGATCCGCACGGA, antisense: 5'-CTGCACCAGTGAGATCAGGA) was designed according to the sequence of HSF1 cDNA from GenBank (NM\_005526). Primers for PAI-1 mRNA (sense: 5'-CAGACCAAGAGCCTCTCCAC, antisense: 5'-ATCACTTGGCCCATGAAAAG) and β-actin gene (sense: 5'-CGTGGGCCGCCCTAGGCACCA, antisense: 5'-TTGGCCTTAGGGTTCAGGGGGG) were synthesized according to their reported cDNA sequences (27, 28). PCR was performed at 95°C, 60°C, and 72°C for 1, 2, and 3 minutes for 35 cycles. HSF1 (210 bp), PAI-1 (202 bp) and β-

actin (300 bp) mRNA fragments were visualized on ethidium bromide-stained 1% agarose gel, and were semi-quantified using the Chemi-Doc system.

### Gene silence

Small interference RNA (siRNA) targeting HSF1 mRNA (5'-GGAAAGUGACCAGUGUGUCTt) was obtained from Ambion Inc. (Autsin, TX). HSF1 siRNA was transfected to EC in serum-free medium using Silence siPort Lipid kit (Ambion). SiRNA for  $\beta$ -actin or negative control siRNA (Ambion) was transfected in parallel cultures to verify the methodology.

### Overexpression of HSF1 gene

The full length HSF1 gene in pCMVHuHSFB plasmid (a kind gift from Dr. Carl Wu, National Cancer Institute, Bethesda, MD) (29) was excised using EcoRI and BglII. The insert was amplified via PCR and the product was inserted into pcDNA3.1 vector /V5-His-TOPO<sup>®</sup> expression vector (Invitrogen, San Diego, CA). The sequence of HSF1 gene was confirmed by DNA sequencing. HCAEC were transfected with HSF1/pcDNA3.1 or empty vector using CaCl<sub>2</sub> (125 mM) as previously described (30).

### Transfection assay

PAI-1 promoter (-1528/+55)/luciferase reporter (Luc) vector was constructed for transfection assay as previously described (13). Two additional PAI-1 promoter fragments (-1197/+55 bp, -1105/+55 bp) were generated through 5'-deletion. The products were inserted into the pXP1/Luc vector to generate pPAI-1(-1197/+55)/Luc and pPAI-1(-1105/+55)/Luc vector. PAI-1 promoter/reporter constructs were precipitated with HEPES buffer (pH 7.05) containing 125 mM CaCl<sub>2</sub> for 20 min. Cells were incubated with calcium

phosphate-precipitated DNA as previously described (13). Chlormaphenicol acetyltransferase (CAT)/pcDNA3 expression vector (31) was co-transfected as an internal control.

### Point mutagenesis

Point mutagenesis of the PAI-1 promoter within -1139/-1126 bp region was generated using QuickChange<sup>™</sup> Site-Directed Mutagenesis kits (Stratagene, La Jolla, CA) and the -1528/+55 PAI-1 promoter/Luc reporter gene vector was used as the template. The sequences of mutants were verified through DNA sequencing.

### Electrophoretic mobility shift assay (EMSA) and supershift

Nuclear proteins were extracted from EC as previously described (32). A double-strand oligonucleotide corresponding to the -1141/-1126 bp (AATAGAAATAAAGCAC) of the PAI-1 promoter (GenBank No. J03764) was synthesized as a probe for EMSA. The probe was labelled with <sup>32</sup>P-dNTP at single-end. The labelled probe was incubated with nuclear extracts at 4°C for 15 min. DNA-protein complexes were analyzed using 5% non-denatured acrylamide gel electrophoresis and visualized using autoradiography. Monoclonal antibody against HSF1 (0.5  $\mu$ g/ $\mu$ l, Santa Cruz) was used in supershift assay. Non-specific mouse IgG was used as an antibody control for the supershift assay.

### Statistics

Student's t-test was used for the determination of probabilities between two groups. One-way ANOVA analysis was performed for comparisons among multiple groups. The level of significance was defined as p<0.05.

## Results

### Effects of gly-LDL on HSF1 and Hsp-70 in cultured venous and arterial EC

The effect of gly-LDL on cell-associated HSF1 was characterized using Western blotting in HUVEC treated with physiologically relevant concentrations of gly-LDL or LDL (50-150  $\mu\text{g}$  protein/ml) for up to 24 h compared to vehicle control. The maximal effect of gly-LDL or LDL on HSF1 expression was detected in HUVEC treated with 100  $\mu\text{g}/\text{ml}$  of gly-LDL or LDL for 6 h (Fig. 1A, 1B). The stimulating effect of gly-LDL (100  $\mu\text{g}/\text{ml}$  for 6 h) on HSF1 expression in HCAEC was similar to that in HUVEC (Fig. 1C, 1D). The abundance of Hsp-70 was examined in HUVEC and HCAEC exposed to 100  $\mu\text{g}/\text{ml}$  of gly-LDL or LDL for 6 h. Significant increase in cell-associated Hsp-70 was detected in HUVEC or HCAEC treated with gly-LDL compared to LDL ( $p < 0.05$ , Fig. 1E, 1F).

### Effect of gly-LDL on PAI-1 protein and activity

Previous studies in our laboratory demonstrated that gly-LDL increased the mRNA and protein release of PAI-1 from EC (12). The effect of gly-LDL on cell-associated PAI-1 in EC has not been characterized. The present study examined the effect of gly-LDL and LDL on abundance of PAI-1 in HUVEC exposed to 50-150  $\mu\text{g}/\text{ml}$  of the lipoproteins for 12-72 h. The maximal increase of PAI-1 was detected in HUVEC treated with 100  $\mu\text{g}/\text{ml}$  of gly-LDL or LDL for 24 h (Fig. 2A, 2B). The effect of 100  $\mu\text{g}/\text{ml}$  of gly-LDL or LDL for 24 h on cell-associated PAI-1 in HCAEC was similar to that in HUVEC. Gly-LDL induced significantly greater expression of PAI-1 than LDL in HCAEC or HUVEC ( $p < 0.05$ , Fig. 2C, 2D). The levels of PAI-1 antigen and

activity released from EC were examined in post-cultural medium of HCAEC treated with 100  $\mu\text{g}/\text{ml}$  of gly-LDL or LDL for up to 72 h. The maximal increase of PAI-1 antigen or activity was detected in the media of HCAEC exposed to gly-LDL or LDL for 48 h. The level of PAI activity, but not PAI-1 antigen, was attenuated after 72 of incubation with gly-LDL, LDL or vehicle (Fig. 2E, 2F).

### Impact of the overexpression of HSF1 gene on PAI-1 expression

Potential relationship between HSF1 and PAI-1 expression was determined in HCAEC transiently transfected with HSF1 gene. The abundance of HSF1 was apparently increased in EC transfected with HSF1 gene (Fig. 3A). The overexpression of HSF1 significantly increased the abundance of cell-associated Hsp-70 and PAI-1 in HCAEC compared to cells transfected with empty pcDNA3.1 vector (vector) or untransfected EC treated with vehicle control (Fig. 3B). The transfection of HSF1 did not substantially alter the abundances of  $\beta$ -actin in corresponding cells (Fig. 3A). The transfection of HSF1 gene also increased the expression of HSF1, Hsp-70 and PAI-1 in HUVEC (data not shown). The results indicate that the overexpression of HSF1 gene enhances the expression of PAI-1 in vascular EC.

### Impact of HSF1 siRNA on gly-LDL-induced PAI-1 expression

We hypothesize that HSF1 mediates the increase of the expression of PAI-1 induced by gly-LDL in EC. The hypothesis was examined using siRNA against HSF1 mRNA in both venous and arterial EC. HSF1 siRNA efficiently blocked gly-LDL or LDL-induced increases of HSF1 and PAI-1 protein or

mRNA in HUVEC (Fig. 4). HSF1 siRNA also effectively prevented gly-LDL or LDL-induced increase in cell-associated HSF1 or PAI-1 in HCAEC (Fig. 5). In EC transfected with HSF1 siRNA but without an addition of the lipoproteins, the expression of HSF1 and PAI-1 was partially inhibited. HSF1 siRNA did not evidently affect the abundance of  $\beta$ -actin protein or mRNA in EC with or without lipoprotein treatment (Fig. 4, 5). Negative control siRNA or siRNA against  $\beta$ -actin did not noticeably alter the expression of HSF1 or PAI-1 protein or mRNA in EC (data not shown). The results suggest that the expression of HSF1 is required for the upregulation of PAI-1 expression in arterial or venous EC induced by gly-LDL.

#### **Location of responsive element in PAI-1 promoter**

We speculate that a responsive element within the PAI-1 promoter may indirectly mediate gly-LDL-induced activation of the PAI-1 promoter. The hypothesis was examined using transfection assay in HUVEC transiently infected with a battery of 5'-deletion PAI-1 promoter fragment/reporter gene vectors. Treatment with gly-LDL or LDL (100  $\mu$ g/ml for 24 h) significantly increased the activity of -1528/+55 bp and -1197/+55 bp PAI-1 promoter, but not that of -1105/+55 bp PAI-1 promoter, in comparison to control ( $p < 0.05$  or  $0.01$ ). The effects of gly-LDL on the activation of the PAI-1 promoter were significantly greater than LDL at comparable conditions ( $p < 0.05$ , Fig. 6A). The transfection of empty vector did not substantially alter in PAI-1 promoter activity compared to no transfection cultures (data not shown). The results suggest that a responsive element activated by gly-LDL or LDL locates between -1197 bp and -1105 bp of the

PAI-1 promoter. A homologue of heat shock responsive element (HSE) was detected within -1140/-1127 bp region of the PAI-1 promoter (TAGAAATAAAGCA) using a transcription factor searching tool (<http://www.cbrc.jp/research/tfsearch.html>). The results of point mutagenesis assay within the region confirmed that the -1137/-1128 bp region of the PAI-1 promoter was required for the activation of PAI-1 promoter induced by gly-LDL or LDL (Fig. 6B).

#### **Effect of gly-LDL on the binding of nuclear protein to the PAI-1 promoter**

HSF1 activates the transcription of multiple stress response-related genes through its binding to HSE in the promoters of target genes (16). The effect of gly-LDL on the binding of nuclear proteins to the targeted region of PAI-1 promoter was investigated using EMSA. Treatment with gly-LDL or LDL visibly enhanced the binding of a nuclear protein to the labelled -1141/-1126 bp PAI-1 promoter fragment compared to control. Gly-LDL induced considerably greater binding of the nuclear protein to the PAI-1 promoter fragment compared to LDL. The addition of 50- or 200-fold (50x, 200x) of the unlabeled -1141/-1126 bp PAI-1 promoter fragment blocked the binding of the nuclear protein to undetectable level (Fig. 7A). Antibody against HSF1 induced a visible upward shift in the migration of the DNA-protein complex induced by gly-LDL, LDL or at basal condition (Fig. 7B). Non-specific mouse IgG did not affect the migration of the nuclear protein (data not shown). The results were reproduced in 4 experiments. The findings suggest that gly-LDL increased the binding of HSF1 to a putative HSE in the PAI-1 promoter.

### **Effect of antioxidant on gly-LDL-induced HSF1 and PAI-1 expression**

Previously studies in our group demonstrated that 80  $\mu$ M BHT, a potent antioxidant, prevented gly-LDL-induced PAI-1 release from HUVEC or HCAEC (15). The effect of BHT on gly-LDL-induced HSF1 and PAI-1 expression in HCAEC was examined in the present study. BHT-gLDL significantly reduced gly-LDL-induced HSF1 and PAI-1 expression in HCAEC ( $p < 0.05$  or  $0.01$ , Fig. 8A). The levels of PAI-1 antigen or  $H_2O_2$  in the conditioned media of HUVEC treated with BHT-gLDL were significantly lower than that treated with gly-LDL without BHT treatment ( $p < 0.05$  or  $0.01$ , Fig. 8B, 8C).

### **Discussion**

The major findings of the present study include that: a) gly-LDL stimulated the expression of HSF1 and Hsp-70 in cultured arterial or venous EC; b) the overexpression of HSF1 gene upregulated the expression of PAI-1 in EC; c) HSF1 siRNA blocked gly-LDL-induced PAI-1 expression in EC; d) gly-LDL enhanced the binding of HSF1 to a PAI-1 promoter fragment containing a HSE homologue. The findings suggest that HSF1 is involved in the upregulation of PAI-1 induced by gly-LDL in vascular EC.

Previous studies reported that proliferating EC responded to oxidized LDL on the expression of Hsp-70 was more active than non-proliferating EC (33). The results provided additional evidence that gly-LDL increased the expression of HSF1 in subconfluent human arterial or venous EC. The stimulating effect of gly-LDL on HSF1 was weaker in confluent EC compared to that in subconfluent EC (data not shown). The collection of the findings suggests

that growing vascular EC may actively respond to hyperglycemia or oxidative stress-associated hyperbetalipoproteinemia on the expression of stress-response mediators.

Previous studies demonstrated that  $H_2O_2$  activated HSF1 (34).  $H_2O_2$  is derived from superoxide under the influence of superoxide dismutase (SOD) in cells.  $H_2O_2$  is more stable than other ROS; therefore, it may function as an intracellular signal of oxidative stress (35, 36). Our recent studies demonstrate that gly-LDL is a potent agonist for the generation of superoxide and  $H_2O_2$  from EC compared to LDL, which implies that gly-LDL, a diabetes-associated lipoprotein, may lead to endothelial oxidative stress. The effect of gly-LDL on  $H_2O_2$  generation from EC reached a peak after 2 h of incubation, which was associated with an elevated activity of SOD in EC (16). The results of the present study demonstrate that the expression of HSF1 in EC is significantly increased by gly-LDL following 4-6 h of incubation. The presence of antioxidant during the glycation suppressed the generation of  $H_2O_2$  and the expression of HSF1 or PAI-1 in EC induced by gly-LDL. The combination of findings suggests that glyco-oxidation in gly-LDL and EC-derived ROS may contribute to gly-LDL-induced expression of HSF1 and PAI-1 in EC.

The increased expression of HSF1 was detected in human atherosclerotic lesions (37). The levels of circulating Hsp-72, an important member of inducible 70 kDa Hsp family, were significantly higher in type 1 DM patients with ketoacidosis compared to age-matching type 1 DM patients without ketoacidosis (22). The results of the

present study indirectly support the hypothesis that diabetes-associated metabolic disorders may enhance stress responses in vascular EC. The upregulation of HSF1 in EC by gly-LDL may contribute to the upregulation of Hsp detected in EC or in the circulation of uncontrolled diabetic patients.

PAI-1 is an acute phase reactant (38). Increased levels of PAI-1 have been observed in patients with sepsis (39) or recurrent myocardial infarction (40). During certain stresses including wounding or bleeding, the increased generation of PAI-1 from EC may play a protective role through maintaining fibrin clots or the integrity of ECM. Rucker et al. (41) demonstrated that heat shock increased the expression of PAI-1 in vascular tissue of rat muscle. Uchiyama et al. (42) reported that adenovirus vector-mediated overexpression of HSF1 gene reduced PAI-1 expression in cultured arterial EC, but the finding was not verified using any gene knock-down approach. The present study provided multiple lines of evidence for the involvement of HSF1 in gly-LDL-induced upregulation of PAI-1 in EC, including the inhibition of PAI-1 expression in arterial or venous EC by HSF1 siRNA and the suppression of PAI-1 promoter activity via mutagenesis of a HSE homologue in the PAI-1 promoter. The results suggest that HSF1 upregulate PAI-1 production through the binding of HSF1 to the PAI-1 promoter. The sequence of the -1137/-1128 bp of the PAI-1 promoter partially, but not completely, matches the authentic HSE. Considerable variations of HSE homologues have been described (43). HSF1 may interact with a homologue of HSE, the -1137/-1128 bp of

PAI-1 promoter, which further enhances the transcription of the PAI-1 gene in EC.

The results of the present study demonstrated that LDL moderately increased the expression of HSF1 and cell-associated PAI-1 in EC. LDL may be oxidatively modified by a prolonged incubation with EC (44). A previous study in our group demonstrated that antioxidants reduced the abundance of lipid peroxides in LDL and prevented LDL-induced release of PAI-1 from EC (15). The effects of LDL on HSF1 and PAI-1 after a prolonged incubation with EC may result, at least in part, from cell-mediated oxidative modification on LDL.

In conclusion, gly-LDL stimulates the expression of HSF1 in vascular EC. HSF1 mediates gly-LDL-induced expression of PAI-1 in EC through enhancing the binding of HSF1 to the PAI-1 promoter. Glyco-oxidation of LDL and EC-derived ROS may play crucial roles in the upregulation of HSF1 and PAI-1 induced by gly-LDL. The findings from the present study provide a potential linkage between gly-LDL, stress response and hypofibrinolysis. The results of the present study are generated from cultured EC. Subsequent studies in animal models potentially provide additional useful information on interrelationship between diabetes-associated lipoproteins and stress-response- or fibrinolysis-related proteins.

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## Figures

Fig.1

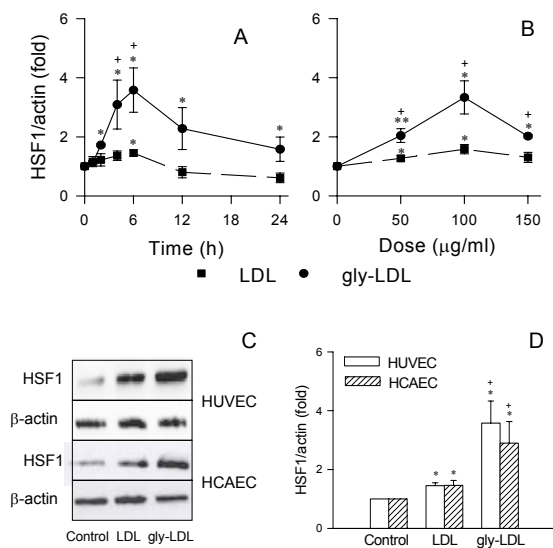


Fig.1a

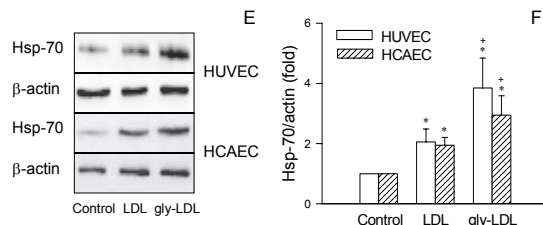


Fig. 1b

### Effect of gly-LDL on the expression of HSF1 and HSp-70 in arterial and venous EC.

A and B: Subconfluent HUVECs were treated with 50-150  $\mu\text{g/ml}$  of gly-LDL, LDL or vehicle (control) for 1-24 h. HSF1 and  $\beta$ -actin in cellular proteins were determined using Western blotting. A: time course; B: dose-response. C-F: Subconfluent HUVECs or HCAECs were treated with 100  $\mu\text{g/ml}$  of gly-LDL, LDL or vehicle (control) for 6 h. C and D: Western blots and integrative data for HSF1. E and F: Western blots and integrative data for Hsp-70. Values are expressed in the folds of control after normalization with  $\beta$ -actin (mean  $\pm$  SD, n = 3 experiments). \*, \*\*: p < 0.05 or 0.01 versus control; +: p < 0.05 versus LDL.

Fig. 2

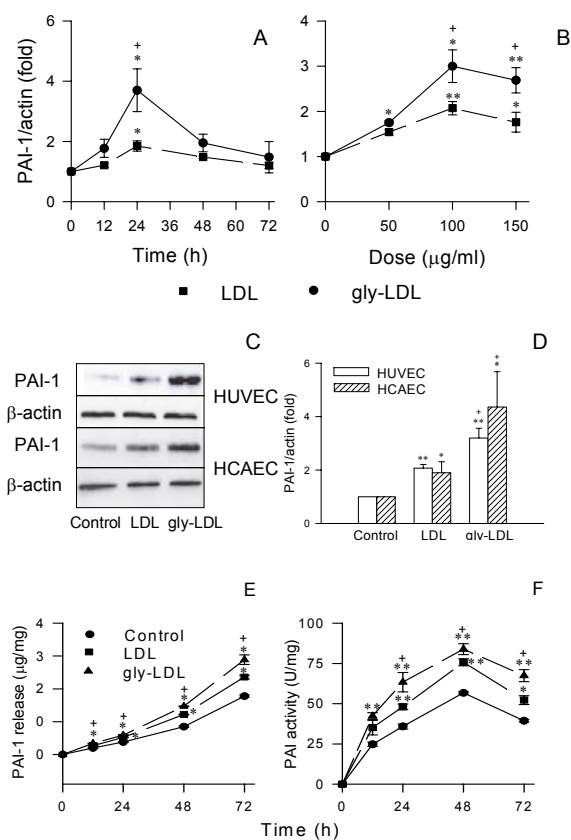


Fig.2

**Effect of gly-LDL on PAI-1 protein and activity.** A and B: Subconfluent HUVECs were treated with medium without addition (control), 50-150  $\mu\text{g/ml}$  of gly-LDL or LDL for 12-72 h. PAI-1 and  $\beta$ -actin in cellular proteins were determined using Western blotting. A: time course; B: dose-response. C and D: Subconfluent HUVECs or HCAECs were treated with 100  $\mu\text{g/ml}$  of gly-LDL, LDL or vehicle (control) for 24 h. Values are expressed in the folds of control after normalization with  $\beta$ -actin (mean  $\pm$  SD,  $n = 3$  experiments). E and F: Subconfluent HCAECs were treated with vehicle (control), 100  $\mu\text{g/ml}$  of gly-LDL or LDL for 12-72 h. The levels of PAI-1 and its activity in the post-cultural media were measured using ELISA or activity assay kits. E: PAI-1 antigen; F: PAI activity. Values are expressed in  $\mu\text{g/mg}$  or Unit/mg total cellular proteins (mean  $\pm$  SD,  $n = 3$  experiments). \*, \*\*:  $p < 0.05$  or  $0.01$  versus control; +:  $p < 0.05$  versus LDL.

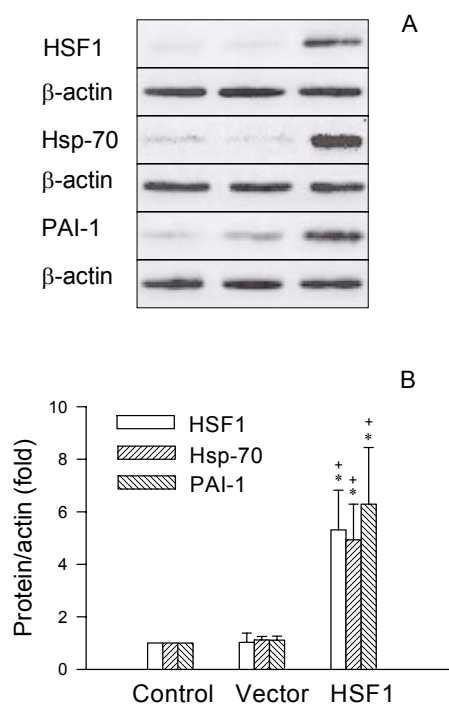
**Fig. 3**

Fig.3

**Impact of overexpression of HSF1 gene on PAI-1 expression.** Subconfluent HCAECs were transfected with empty pcDNA3.1 vector (vector) or HSF1/pcDNA3.1 expression vector (HSF1) for 6 h (for HSF1 or Hsp-70) or 24 h (for PAI-1). Total cellular proteins from EC without transfection (control), transfected with HSF1 or empty vector were analyzed using Western blotting with antibodies against HSF1, Hsp-70, PAI-1 or β-actin. A: Western blots; B: integrative data. Values were presented in the folds of control after normalization with β-actin (mean ± SD, n = 3 experiments).\*: p<0.05 versus control; +: p<0.05 versus vector.

Fig.4

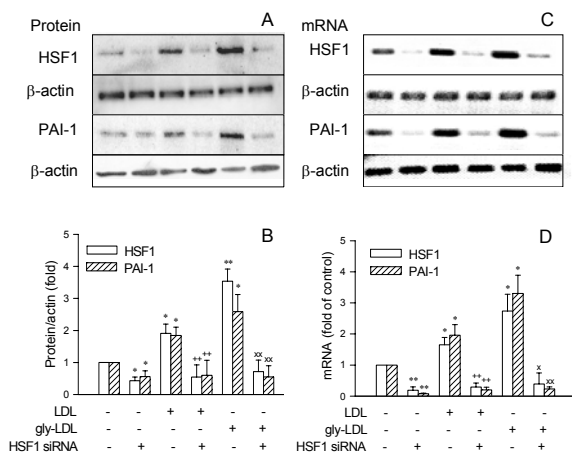


Fig. 4

**Effect of HSF1 siRNA on gly-LDL-induced HSF1 and PAI-1 protein and mRNA in HUVEC.** Subconfluent HUVECs transfected with siRNA against HSF1 gene for 48 h (first 4 h in serum-free medium, in the presence of 10% serum during the rest of incubation) were treated with vehicle (control), or with an addition of 100  $\mu\text{g/ml}$  of LDL or gly-LDL for 6 h (for HSF1) or 24 h (for PAI-1). A: Western blots for HSF1, PAI-1 and  $\beta$ -actin; B: integrative data of Western blotting; C: RT-PCR for HSF1, PAI-1 and  $\beta$ -actin mRNA; D: integrative data of mRNA. Values are presented in the folds of control after normalization with  $\beta$ -actin protein or mRNA (mean  $\pm$  SD, n = 3 experiments). \*, \*\*: p<0.05 or 0.01 versus control without HSF1 siRNA; ++: p<0.01 versus LDL without HSF1 siRNA; x, xx: p<0.05 or 0.01 versus gly-LDL without HSF1 siRNA.

Fig.5

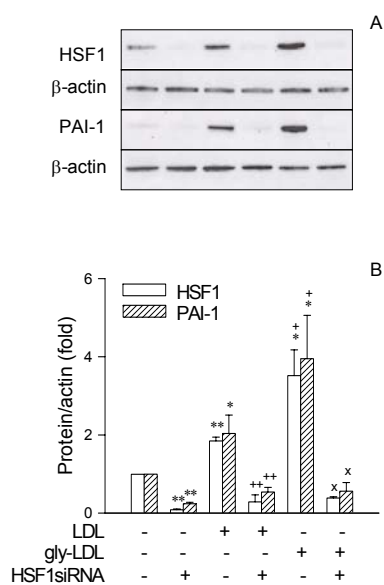


Fig.5

**Effect of HSF1 siRNA on gly-LDL-induced HSF1 and PAI-1 protein in HCAEC.** Subconfluent HCAECs were transfected with siRNA against HSF1 gene then treated with gly-LDL, LDL or vehicle as described in the legend of Fig. 4. The abundance of HSF1, PAI-1 or  $\beta$ -actin protein was detected using Western blotting. A: Western blots; B: integrative data. Values are presented in the folds of control after normalization with  $\beta$ -actin (mean  $\pm$  SD, n = 3 experiments). \*, \*\*: p<0.05 or 0.01 versus control without HSF1 siRNA; +, ++: p<0.05 or 0.01 versus LDL without HSF1 siRNA; x: p<0.05 versus gly-LDL without HSF1 siRNA.

Fig. 6

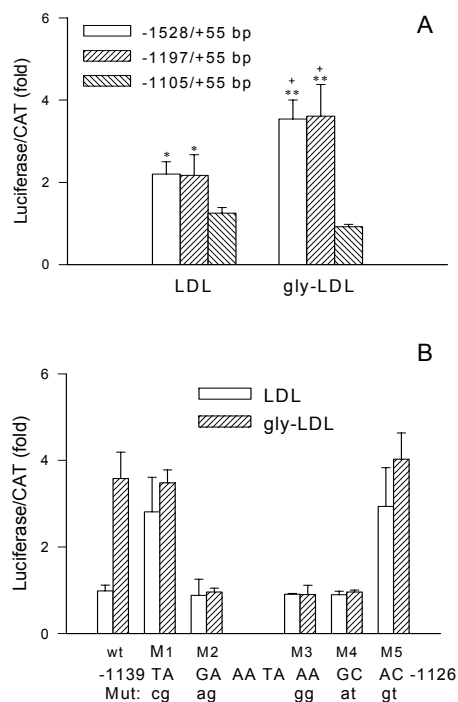


Fig. 6

**Location of responsive element in the PAI-1 promoter activated by gly-LDL.** Subconfluent HUVECs were transfected with 5'-depletion PAI-1 promoter/reporter gene vector (-1528/+55, -1197/+55 or -1105/+55 bp), empty vector, wild type (wt, -1528/+55 bp) or mutant PAI-1 promoter vectors (-1139/-1126 bp, sequence as indicated). Chlormaphenicol acetyltransferase (CAT) gene expression vector was co-transfected to the cells. Four hours after the transfection, the cells were treated with 100  $\mu$ g/ml of LDL or gly-LDL, or vehicle (control) for 24 h. The luciferase activities of PAI-1 promoter were normalized with CAT activity and expressed in the folds of controls (mean  $\pm$  SD, averages of duplicates from 3 experiments). A: 5' depletion transfection assay; B: mutagenesis. M, mut: mutants. Capital letter: wild-type sequence; small letters: mutant sequence. \*, \*\*:  $p < 0.05$  or  $0.01$  versus controls; +:  $p < 0.05$  versus LDL.

Fig. 7

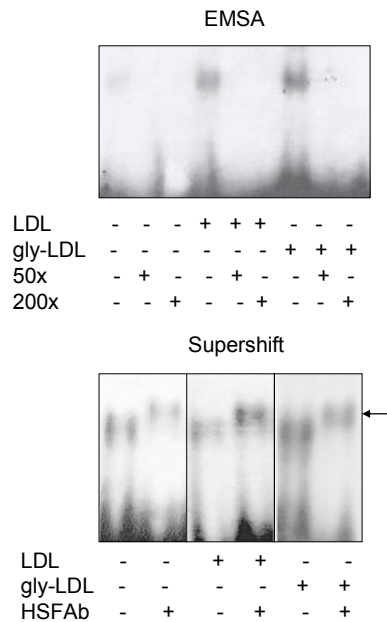


Fig. 7

**Binding of nuclear protein to the PAI-1 promoter induced by gly-LDL.** Subconfluent HUVEC cultures were treated with vehicle (control), 100  $\mu\text{g}/\text{ml}$  of LDL or gly-LDL for 24 h. A: Nuclear proteins extracted from LDL or gly-LDL-treated or control EC were incubated with  $^{32}\text{P}$ -dNTP labelled -1141/-1126 bp of the PAI-1 promoter in the absence or presence of 50-fold (50x) or 200-fold (200x) excess unlabeled probe using electrophoretic migration shift assay (EMSA). DNA-protein complexes were analysed on 5% non-denatured acrylamide gel electrophoresis. B: Nuclear proteins extracted from LDL or gly-LDL-treated or control cells were incubated with the labelled probe in the absence and presence of 0.5  $\mu\text{g}/\text{ml}$  of anti-HSF1 blocking antibody (HSFAB) or non-specific mouse IgG (Ig G). Arrow indicates shifted DNA HSF1-antibody complex.

Fig. 8

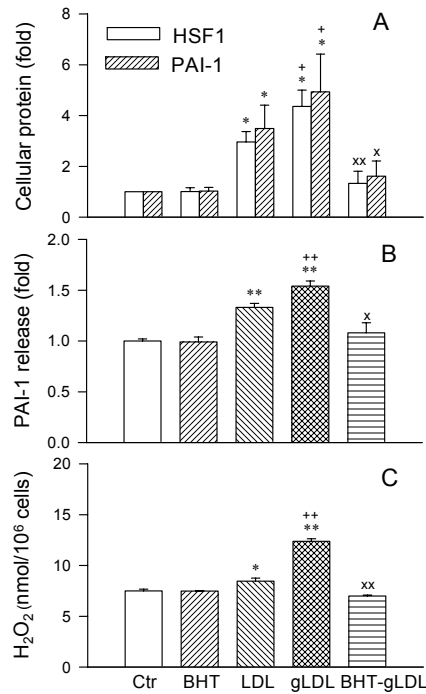


Fig. 8

**Effect of butylated hydroxytoluene (BHT) on the expression of HSF1 and PAI-1 expression, and the release of PAI-1 or H<sub>2</sub>O<sub>2</sub>.** A. Subconfluent HCAECs were treated with vehicle (Ctr), 80  $\mu$ M BHT, 100  $\mu$ g/ml of LDL, gly-LDL (gLDL), or 100  $\mu$ g/ml of BHT-gLDL (glycated with the presence of 80  $\mu$ M BHT) for 6 h (for HSF1) or 24 h (for PAI-1). The abundance of HSF1, PAI-1 and  $\beta$ -actin was analyzed using Western blotting. Values are presented in mean  $\pm$  SD after normalization with  $\beta$ -actin (n =3 experiments). B and C: HUVECs were treated with lipoproteins as described in Fig. 8A for 48 h (for PAI-1) or for 2 h (for H<sub>2</sub>O<sub>2</sub>). B: PAI-1 levels in post-cultural media. C: H<sub>2</sub>O<sub>2</sub> levels in post-cultural media. Values were presented in fold of control (mean  $\pm$  SD, n =3 wells) after normalization with total cellular proteins (for PAI-1 antigen) or cell numbers (for H<sub>2</sub>O<sub>2</sub>). \*, \*\*: p<0.05 or 0.01 versus control; +, ++: p<0.05 or 0.01 versus LDL, x, xx: p<0.05 or 0.01 versus gly-LDL.