Involvement of RAGE, NADPH Oxidase, and Ras/Raf-1 Pathway in Glycated LDL-Induced Expression of Heat Shock Factor-1 and Plasminogen Activator Inhibitor-1 in Vascular Endothelial Cells

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Atherothrombotic cardiovascular diseases are the predominant causes of mortality of diabetic patients. Plasminogen activator inhibitor-1 (PAI-1) is the major physiological inhibitor for fibrinolysis, and it is also implicated in inflammation and tissue remodeling. Increased levels of PAI-1 and glycated low-density lipoprotein (glyLDL) were detected in patients with diabetes. Previous studies in our laboratory demonstrated that heat shock factor-1 (HSF1) is involved in glyLDL-induced PAI-1 overproduction in vascular endothelial cells (EC). The present study investigated transmembrane signaling mechanisms involved in glyLDL-induced HSF1 and PAI-1 up-regulation in cultured human vascular EC and streptozotocin-induced diabetic mice. Receptor for advanced glycation end products (RAGE) antibody prevented glyLDL-induced increase in the abundance of PAI-1 in EC. GlyLDL significantly increased the translocation of V-Ha-Ras Harvey rat sarcoma viral oncogene homologue (H-Ras) from cytoplasm to membrane compared with LDL. Farnesyltransferase inhibitor-277 or small interference RNA against H-Ras inhibited glyLDL-induced increases in HSF1 and PAI-1 in EC. Treatment with diphenyleneiodonium, a nicotinamide adenine dinucleotide phosphate oxidase (NOX) inhibitor, blocked glyLDL-induced translocation of H-Ras, elevated abundances of HSF1 and PAI-1 in EC, and increased release of hydrogen peroxide from EC. Small interference RNA for p22phox prevented glyLDL-induced expression of NOX2, HSF1, and PAI-1 in EC. GlyLDL significantly increased V-raf-1 murine leukemia viral oncogene homolog 1 (Raf-1) phosphorylation. Treatment with Raf-1 inhibitor blocked glyLDL-induced increase of PAI-1 mRNA in EC. The levels of RAGE, H-Ras, NOX4, HSF1, and PAI-1 were increased in hearts of streptozotocin-diabetic mice and positively correlated with plasma glucose. The results suggest that RAGE, NOX, and H-Ras/Raf-1 are implicated in the up-regulation of HSF1 or PAI-1 in vascular EC under diabetes-associated metabolic stress. (Endocrinology 151: 4455–4466, 2010)
Hyperglycemia promotes nonenzymatic glyco-oxidation of proteins and lipids and the later further form advanced glycation end products (AGEs) (6). Increased level of plasma low-density lipoprotein (LDL)-cholesterol is a classical risk factor for coronary artery disease (7). High levels of glucose increase nonenzymatic glycation at lysine residues of apolipoprotein-B or phospholipids in the lipid core of LDL. Elevated levels of glycated LDL (glyLDL) were detected in diabetic patients (8, 9). Previous studies in our laboratory demonstrated that glyLDL or diabetes-associated LDL increased PAI-1 production in cultured human umbilical vein endothelial cells (EC) (HUCEC) or human coronary artery EC (HCAEC) (10, 11). Heat shock factor-1 (HSF1) is involved in glyLDL-induced transcription of PAI-1 gene in EC (12). Stress responses induced by heat shock, environmental, or oxidative stress are mainly mediated by heat shock proteins (Hsp). The transcription of Hsp genes is regulated by HSF (13). Treatment with glyLDL increased Hsp70, HSF1, and PAI-1 expression in EC. Treatment with antioxidant reduced glyLDL-induced reactive oxygen species (ROS) release, HSF1, and PAI-1 expression in EC (12). PAI-1 is implicated in the development of vascular disease in diabetes. Several groups detected excessive PAI-1 in atherosclerotic plaques in diabetic patients (14, 15). The findings suggest that glyLDL-induced oxidative stress may be implicated in the up-regulation of PAI-1 in EC and the development of diabetic cardiovascular complications. GlyLDL increased the generation of ROS and activities of multiple antioxidant enzymes in EC (16). Transmembrane signaling mechanism for glyLDL-induced PAI-1 production and the source of ROS in EC remains unclear.

Increased expression of receptor for AGEs (RAGE), a multiligand receptor on surfaces of vascular cells, is associated with diabetic vascular complications (17). Our recent study indicates that lectin-like oxidized LDL (ox-LDL) receptor-1 is implicated in PAI-1 expression induced by ox-LDL (18). The membrane receptor involved in glyLDL-induced PAI-1 expression in EC remains uncharacterized.

Small G proteins act as key regulators of signaling cascades that modulate interactions between multiple membrane receptors and downstream effector proteins (19). Diabetes increases oxidative stress (20). Several groups demonstrated close interactions between oxidative stress and V-Ha-Ras Harvey rat sarcoma viral oncogene homologue (H-Ras) (21, 22). Rho/Rho-kinase and nuclear factor-κB (NF-κB) are involved in high-glucose-induced PAI-1 expression in EC (23). AGEs/RAGE-induced oxidative stress activates H-Ras and a cascade of MAPKs, resulting in the activation of NF-κB in smooth muscle cells (24). V-raf-1 murine leukemia viral oncogene homolog 1 (Raf-1), one of the components of MAPK, is considered as the best characterized downstream effector of H-Ras (25). ROS affects interactions between H-Ras and several of its downstream effectors, including Raf-1 (26). The role of Ras/Raf-1 in glyLDL-induced HSF1 or PAI-1 in EC has not been described.

Nicotinamide adenine dinucleotide phosphate oxidase (NOX) is one of the major sources of ROS in EC (27). The engagement of RAGE by AGEs triggers the generation of ROS via the activation of NOX (28). NOX catalyzes the reduction of molecular O2 through donating an electron from reduced nicotinamide adenine dinucleotide phosphate to generate superoxide (27). NOX plays an important role in hyperglycemia-induced ROS release. Increased vascular NOX activity has been detected in diabetic patients (29). The role of NOX in glyLDL-induced HSF1 or PAI-1 in EC remains to be determined.

The present study investigated the involvement of RAGE, NOX, H-Ras, and Raf-1 in glyLDL-induced HSF1 and PAI-1 up-regulation in cultured EC. Relationships between blood glucose and the abundance of RAGE, H-Ras, NOX, HSF1, or PAI-1 in heart tissue were examined in streptozotocin (STZ)-induced diabetic mice.

Materials and Methods

Isolation and modification of lipoproteins
Plasma was freshly separated from blood of healthy donors by centrifugation (2000 × g) for 15 min at 4 C. LDL (density, 1.019–1.063) was isolated from fresh human plasma using sequential floatation ultracentrifugation. LDL was glycated by incubation with 50 mmol/liter glucose and 50 mmol/liter sodium cyanoborohydride in the presence of 0.01% EDTA for 2 wk at 37 C as previously described (10). Free glucose or chemicals in glyLDL preparation was removed via dialysis. Approximately 60% of lysine residues was glycated in the preparations of glyLDL used in following experiments assessed using trinitrobenzenesulfonic acid assay (30). The level of endotoxin 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 at 4 C in dark under a layer of nitrogen to prevent autooxidation (12).

Cell culture
Seed cells of HUVEC were obtained from American Type Culture Collections (Manassas, VA). Cells were grown in F12K medium (Life Technologies, Inc., Burlington, Ontario, Canada) supplemented with 10% of fetal bovine serum, 0.1 mg/ml of heparin, and 30 μg/ml EC growth supplements (Sigma) as previously described (31). Seed HCAEC and required medium or supplements were received from Clonetics (San Diego, CA). Cytotoxicity was determined by cell morphology or leucine incorporation assays (12). No detectable cytotoxicity was found in EC treated with lipoproteins at tested conditions.
Cell treatment
EC were treated with physiological concentrations of glyLDL (25–150 µg/ml) or LDL (100 µg/ml) for various lengths of time as indicated. Equal volume of vehicle was added to control cultures. Polyclonal RAGE-blocking antibody (a gift from A. M. Schmidt; Columbia University, New York, NY) and control polyclonal goat IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used at 10 µg/ml with a 30 min pretreatment. Farnesyltransferase inhibitor (FTI)-277 (20 µM with a 30-min preincubation; Calbiochem, San Diego, CA), diphenyleneiodonium (DPI) (10 µM with a 4-h preincubation; Sigma), or Raf-1 inhibitor (1 µM with a 30-min preincubation; Calbiochem) were used in following experiments.

Western blot analysis
Targeted proteins in total cellular lysates were determined using Western blot analysis as previously described (12, 18). Polyclonal antibodies against human RAGE, NOX2 (gp91phox), H-Ras, phosphorylated Raf-1 (pRaf-1), p22phox, mouse H-Ras, HSF1 (Santa Cruz Biotechnology, Inc.), or monoclonal antibodies against human HSF1, PAI-1, β-actin, or nonspecific mouse IgG were obtained from Sigma or Santa Cruz Biotechnology, Inc. Monoclonal antibodies against mouse PAI-1 or mouse β-actin were received from Oxford Biomedical Research (Oxford, UK) or Abcam (Cambridge, MA). Corresponding second antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Inc.) and enhanced chemiluminescence reagents (Amersham, Piscataway, NJ) were used for detecting antigens on nitrocellulose membrane. Relative densities of protein bands were visualized using Chemi-Doc system or autoradiography and analyzed using Quantity One software (Bio-Rad, Hercules, CA). The abundance of targeted proteins was normalized with β-actin in corresponding samples.

Measurement of hydrogen peroxide (H₂O₂)
The levels of H₂O₂ in postculture media of EC were measured using PeroxiDetect kit from Sigma as previously described (16).

RT-PCR
The levels of PAI-1 mRNA were assessed using RT-PCR and justified with the level of β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in corresponding samples. Total RNA was isolated from cultured EC or heart tissues of STZ-diabetic mice using TRI Reagent (MRC, Cincinnati, OH) or TRIZOL Reagent (Invitrogen, Burlington, Ontario, Canada). Primers for PAI-1 (sense, 5′-CAGACAAAGGCTCTTCCAC; antisense, 5′-ATCACCTGGCCATGAAAG) and β-actin gene in EC (sense, 5′-CTGGCCGCCTGACTGAAC; antisense, 5′-TTGGCCCTAGGGTTAGGGG) were prepared as previously reported sequences (32, 33). PCR for PAI-1 and β-actin in EC was performed at 95, 60, and 72 C for 1, 2, and 3 min, respectively, with 35 cycles. Primers for PAI-1 (sense, GAGTG-GCTGTAGGAATCCTATCC; antisense, GACCTTGCG AAGGTGATGCTTGGCAAC) and GAPDH gene in mouse tissues (sense, ATGTTCCGATATGACTCCACTCAG; antisense, GAAGACACCAGTACCCAGGACA) were generated as previously reported (34). PCR for PAI-1 mRNA in mouse hearts was performed at 95, 61, and 72 C for 1, 1, and 2, respectively, min with 35 cycles. In the case of GAPDH mRNA, PCR was performed at 95, 60, and 72 C for 1, 1, and 2 min, respectively, with 35 cycles. Amplified mRNA fragments were visualized on 1% agarose gel stained with ethidium bromide and semiquantified using Chemi-Doc system plus Quantity One software. The abundance of specific mRNA was normalized with the intensity of β-actin or GAPDH mRNA in same samples.

Detection of translocation of H-Ras
HUVEC treated with LDL or glyLDL were harvested using a rubber policeman and homogenized in a Dounce homogenizer. Membrane proteins from cell lysate were separated using Beckman TLX-100 table top ultracentrifugation at 100,000 x g at 4 C for 1 h (35, 36). Expression of H-Ras in membrane fraction was detected using Western blot analysis and polyclonal antibody against human H-Ras (Santa Cruz Biotechnology, Inc.).

Gene silence
Small interference RNA (siRNA) for H-Ras and human p22phox was obtained from Santa Cruz Biotechnology, Inc. Small interference RNA was transfected in EC in serum-free medium using Silence siPort Lipid kit (Ambion, Austin, TX) as previously described (18). Small interference RNA for β-actin or scramble siRNA (Ambion) was transfected in parallel cultures to verify the methodology.

STZ-diabetic mice
Male C57BL/6J (C57) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were placed in stainless cage in an air-conditioned room and received regular rodent chow after arrival at 4 wk of age. Diabetes was induced with STZ (Sigma) injection (150 mg/kg body weight ip) at 9 wk of age. Control mice received equal volume of sodium citrate (pH 4.5) via the identical route. Hearts and blood were collected after 3 wk of STZ injection from diabetic and control mice (n = 5 animals/group). Tissue specimens were frozen immediately after harvest and stored at −70 C. Targeted proteins in heart homogenate were determined using Western blot analysis. Plasma was freshly isolated for PAI-1 and glucose analysis. The protocol of the animal experiments was approved by the Animal Protocol Management and Review Committee in the University of Manitoba.

Statistical analysis
Data were presented as mean ± SD (in vitro experiments) or SE (in vivo experiments). Comparisons among multiple treatment groups were done with the use of one-way ANOVA followed by post hoc Newman-Keuls test. Student’s t test was applied for analyzing probabilities between two groups. All statistical analyses were performed using GraphPad Prism 4 (GraphPad Software, San Diego, CA), with P < 0.05 taken as significant.

Results
RAGE mediates glyLDL-induced increase of PAI-1 in EC
Previous studies in our laboratory demonstrated that glyLDL stimulated the generation and up-regulation of PAI-1 in EC, which reached a peak at 100 µg/ml for 24 h (12, 31). To examine the effect of glyLDL on the abundance of RAGE, HUVEC or HCAEC were treated with
glyLDL (100 μg/ml) for 24 h. GlyLDL significantly increased the abundance of RAGE in EC compared with LDL or vehicle (HUVEC: glyLDL, 2.1 ± 0.6; LDL, 1.0 ± 0.1; HCAEC: glyLDL, 2.2 ± 0.5; LDL, 1.1 ± 0.1-fold of control; P < 0.05) (Fig. 1A). To determine whether glyLDL induces PAI-1 levels via RAGE, HUVEC were pretreated with RAGE-blocking antibody or control goat IgG (10 μg/ml) in the absence and presence of glyLDL (100 μg/ml) for 24 h. RAGE antibody prevented glyLDL-induced up-regulation of PAI-1 in EC (P < 0.05) (Fig. 1B). Goat IgG had no effect on glyLDL-induced PAI-1 expression in EC (Fig. 1C). The results suggest that RAGE is implicated in glyLDL-induced increase of PAI-1 in EC.

**FIG. 1.** Involvement of RAGE in glyLDL-induced PAI-1 up-regulation in EC. A, HUVEC or HCAEC were incubated for 24 h with vehicle or control (CTL), 100 μg/ml of LDL or glyLDL (B and C). HUVEC were pretreated with RAGE-blocking antibody (RAGE-ab) (B) or control goat IgG (C) at 10 μg/ml for 30 min and then exposed to vehicle or 100 μg/ml of glyLDL for 24 h. The abundance of RAGE, PAI-1, or β-actin in cellular protein was assessed using Western blot analysis. Values are expressed in percentage of controls after normalization with β-actin (mean ± SD, n = 3 experiments). *, P < 0.05 or **, P < 0.01 vs. control; +, P < 0.05 vs. LDL; #, P < 0.05 vs. glyLDL; x, P < 0.05 vs. goat IgG.

H-Ras regulates glyLDL-induced HSF1 or PAI-1 production

Previous studies demonstrated that ox-LDL or LDL increased the translocation of H-Ras in EC (18, 36). To examine whether glyLDL affects H-Ras translocation, HUVEC were treated with glyLDL (100 μg/ml) for 5–60 min (Fig. 2A). The translocation of H-Ras to membrane was detected as early as 5 min and reached a peak at 15 min. The abundance of H-Ras in membrane fraction was significantly increased by glyLDL compared with LDL or control after 15 min (glyLDL, 2.3 ± 0.7; LDL, 1.1 ± 0.5-fold of control; P < 0.05) (Fig. 2B). No evident difference in H-Ras was detected in whole-cell lysates of EC treated with glyLDL or LDL for 15 min. Furthermore, we
compared effects of glyLDL on the abundance of H-Ras in HUVEC and HCAEC treated with LDL or glyLDL (100 μg/ml) for 24 h. GlyLDL significantly increased the abundance of H-Ras protein in both HUVEC and HCAEC compared with LDL or control after a 24 h of treatment (P < 0.05) (Fig. 2C). The results suggest that glyLDL rapidly increases H-Ras translocation and elevates the content of H-Ras in EC after a prolonged incubation in EC.

Our previous studies demonstrated that glyLDL increased the abundance of HSF1 in EC with a peak at 100 μg/ml for 6 h (12). To examine whether H-Ras is involved in glyLDL-induced increase of HSF1 or PAI-1, HUVEC were preincubated with FTI-277 (20 μM), a H-Ras inhibitor, for 30 min, and then exposed to 100 μg/ml of LDL or glyLDL for 24 h for PAI-1 analysis or 6 h for HSF1 detection as optimized previously (12). FTI-277 inhibited glyLDL or LDL-induced increase in cell-associated HSF1 or PAI-1 in EC (P < 0.05) (Fig. 2D). The involvement of H-Ras in glyLDL-induced increases of HSF1 or PAI-1 in EC was verified using H-Ras-specific siRNA. HUVEC were transfected with H-Ras siRNA and then exposed to LDL or glyLDL for 6 h (HSF1) or 24 h (PAI-1). H-Ras siRNA prevented glyLDL or LDL-induced increases of HSF1 and PAI-1 in EC (P < 0.05) (Fig. 2E). In HUVEC transfected with H-Ras siRNA without an exposure to LDL or glyLDL, the levels of cell-associated HSF1 and PAI-1 were inhibited (P < 0.05). H-Ras siRNA did not evidently alter the level of HSF1 or PAI-1 in EC. H-Ras siRNA did not evidently alter the abundance of Α-actin in EC with or without lipoprotein treatment. Scramble siRNA did not evidently alter the level of HSF1 or PAI-1 in EC. The results suggest that H-Ras is required for the regulation of glyLDL-induced HSF1 and PAI-1 expression in EC.

Requirement of NOX in glyLDL-induced H-Ras translocation and up-regulation of PAI-1, HSF1, or ROS production

Previous studies demonstrated that interactions between AGEs and RAGE activated NOX in EC (28). The dose and time dependence of glyLDL (25–150 μg/ml) on the expression of NOX2 was characterized in EC for up to 2 h compared with vehicle control. The maximal increase in the abundance of NOX2 was detected in HUVEC at 1 h after the start of glyLDL (100 μg/ml) incubation (P < 0.05) (Fig. 3, A and B). GlyLDL signifi-
cantly increased the abundance of NOX2, a catalytic core of NOX complex, in HUVEC or HCAEC compared with LDL or control (HUVEC: glyLDL, 2.5 ± 0.5; LDL, 1.7 ± 0.2-fold of control; HCAEC: glyLDL, 3.1 ± 0.2; LDL, 2.0 ± 0.4-fold of control; \( P < 0.05 \) (Fig. 3C).

Previous studies demonstrated that the H-Ras activated NOX (21). To determine the role of NOX2 on the activation of H-Ras, we examined the effect of a NOX inhibitor (DPI) on the translocation of H-Ras in HUVEC induced by glyLDL. The results demonstrated that DPI significantly reduced the membrane translocation of H-Ras in EC induced by glyLDL (\( P < 0.05 \)) (Fig. 3D). To determine whether NOX is involved in glyLDL-induced ROS production, HUVEC were preincubated with DPI and then exposed to 100 \( \mu \)g/ml of LDL or glyLDL for 2 h as previously optimized (16). DPI blocked the increase of levels of \( \text{H}_2\text{O}_2 \) in the postculture media of EC induced by LDL or glyLDL (\( P < 0.05 \)) (Fig. 3E). To determine the involvement of NOX in glyLDL-induced expression of HSF1 and PAI-1, HUVEC were treated with DPI in the absence or presence of LDL or glyLDL at optimized condition (12). Treatment with DPI significantly reduced the abundance of NOX2, HSF1, and PAI-1 in EC induced by glyLDL or LDL (\( P < 0.05 \)) (Fig. 3F).

**Impact of p22phox gene silence on glyLDL-induced expression of NOX2, HSF1, and PAI-1 in EC**

The involvement of NOX in glyLDL-induced up-regulation of HSF1 and PAI-1 in EC was verified using p22phox siRNA, an essential component of NOX complex. Small interference RNA against p22phox blocked the increase of the abundance of p22phox, NOX2, HSF1, and PAI-1 in HUVEC induced by glyLDL or LDL. In EC transfected with p22phox siRNA, but without an addition of lipoprotein, the abundances of NOX2, HSF1, and PAI-1 were partially inhibited (\( P < 0.05 \)) (Fig. 4A). Scramble siRNA did not evidently affect the expression of the targeted proteins (Fig. 4B).

**Involvement of Raf-1 in glyLDL-induced PAI-1 production**

The effect of glyLDL (100 \( \mu \)g/ml) on Raf-1 phosphorylation in EC was examined through incubations up to 60 min. Increase in the abundance of pRaf-1 was first detected at 2 min and peaked at 5 min after the start of glyLDL treatment (Fig. 5A). HUVEC were preincubated with Raf-1 inhibitor (1 \( \mu \)M) for 30 min and then exposed to 100 \( \mu \)g/ml of
glyLDL or LDL for 5 min. LDL did not significantly change the level of pRaf-1 at 5 min. Raf-1 phosphorylation induced by glyLDL was significantly suppressed by Raf-1 inhibitor (∗, P < 0.05) (Fig. 5B). The findings suggest that glyLDL accelerates the phosphorylation of Raf-1 in EC compared with LDL.

GlyLDL (100 μg/ml for 24 h) significantly increased PAI-1 mRNA in HUVEC compared with LDL or vehicle as supported by previous studies (10). To investigate whether the phosphorylation of Raf-1 is involved in glyLDL-induced increase in PAI-1 abundance by up-regulating PAI-1 mRNA, the effect of Raf-1 inhibitor on glyLDL-induced PAI-1 mRNA was determined using RT-PCR. HUVEC were pre-incubated with Raf-1 inhibitor (1 μM) for 30 min and then stimulated with 100 μg/ml of glyLDL or LDL for 24 h. Raf-1 inhibitor significantly reduced the level of PAI-1 mRNA induced by glyLDL or LDL (P < 0.05) (Fig. 5C). The results suggest that the Raf-1 activation is required for glyLDL-induced PAI-1 up-regulation in EC.

Effect of STZ-induced diabetes on signaling mediators and PAI-1 in mice

Significant increases in plasma glucose were detected in STZ-diabetic mice compared with control mice (P < 0.05) (Fig. 6A and Table 1). To verify the above in vitro findings, we analyzed the abundance of RAGE, H-Ras, NOX, HSF1, and PAI-1 in the hearts of STZ-diabetic mice. Abundances of RAGE, H-Ras, NOX, HSF1, and PAI-1 in the hearts of diabetic mice were significantly increased compared with that in control mice (∗, P < 0.05 vs. control; #, P < 0.05 vs. LDL; xx, P < 0.01 vs. SsiRNA). Significant increase in PAI-1 mRNA expression was detected in hearts of STZ-diabetic mice compared with control mice (P < 0.05) (Fig. 6C and Table 1). Plasma levels of PAI-1 were significantly increased in diabetic mice compared with control mice. The levels of glucose positively correlated with RAGE, NOX, H-Ras, HSF1, and PAI-1 in hearts of the mice (r = 0.72–0.96, P < 0.05 or 0.01) (Table 2).

Discussion

The major findings of the present study include the following: 1) RAGE is involved in glyLDL-induced PAI-1 up-regulation in cultured vascular EC; 2) NOX is implicated in glyLDL-induced HSF1 and PAI-1 up-regulation in EC; 3) H-Ras activation and Raf-1 phosphorylation are required for glyLDL-induced PAI-1 up-regulation in EC;
findings indicate that RAGE mediates the transmembrane signaling of glyLDL-induced PAI-1 in EC.

Small G proteins, such as Ras and Rho, are often associated with the activation of membrane receptors (42). Hyperglycemia increases oxidative stress and H-Ras activation (24). H-Ras exists in either an inactive GDP-bound cytosolic form or an active GTP-bound membrane-associated form. By cycling between the two forms, H-Ras activates downstream effectors after the activation of membrane receptors. Farnesylation mediates the activation or translocation of Ras from cytosol to plasma membrane (43). Previous studies, including ours, demonstrated that ox-LDL or LDL increased the membrane translocation of H-Ras in human EC (18, 36). Kowluru et al. (20) reported that the protein and mRNA levels of H-Ras were increased in EC exposed to high glucose. The results of the present study suggest that a short incubation (15 min) of EC with glyLDL increased the abundance of H-Ras in EC exposed to high glucose. The results of the present study also demonstrated an increased expression of H-Ras in the hearts of STZ-diabetic mice, which may be a chronic response to hyperglycemia and hyperlipidemia in diabetic condition. FTI-277 blocked glyLDL-induced HSF1 and PAI-1 expression in EC. H-Ras siRNA effectively inhibited LDL or glyLDL-induced up-regulation of HSF1 and PAI-1 in EC. A recent study suggests that small G proteins, Ras and Rac, mediated cyclic strain stress-induced HSF1 activation and Hsp70 expression in vascular smooth muscle cells (44). Lander et al. (24) reported that AGES/RAGE interaction-induced ROS activated p21Ras/MAPK pathway, which resulted in nuclear translocation of NF-κB. The combination of findings from the present and previous studies suggests that glyLDL activates H-Ras, which is required for glyLDL-induced expression of HSF1 and PAI-1 in vascular EC.

Previous studies by our group demonstrated that glyLDL induced a quick generation of ROS from EC (16).
Multiple lines of evidence suggest that intracellular ROS may activate H-Ras (21). NOX is one of the major sources of ROS in vascular cells. The expression of NOX has been detected in EC (27). NOX is involved in AGEs-induced ROS production in human EC (28). The present study indicated that DPI blocked glyLDL-induced H_{2}O_{2} production in EC, which is consistent with results from a previous study using glyco-oxidized high-density lipoprotein in EC (45). The findings suggest the involvement of NOX in glycated lipoproteins induced ROS production in EC. p22phox, the only membrane-associated subunit beside catalytic core in NOX complex, is essential for the activation of NOX. Our recent study demonstrated that siRNA against p22phox suppressed the expression of NOX2 in EC induced by ox-LDL (46). The results of the present study demonstrated that p22phox siRNA blocked glyLDL-induced NOX2, HSF1, and PAI-1 expression in EC. DPI blocked glyLDL-induced H-Ras translocation as well as ROS production in EC. These findings suggest that NOX-mediated ROS is directly implicated in glyLDL-induced up-regulation of HSF1 and PAI-1 in EC. The present study also demonstrated increased expressions of HSF1 and PAI-1, in addition to NOX in STZ-induced diabetic mice, which provides an in vivo evidence for associations between NOX, HSF1, and PAI-1 under diabetic condition. Our findings support a recent report on the increased protein and mRNA of NOX in diabetic mice (47). Oxidative stress triggers Ras activation. Activated Ras may further stimulate ROS production via NOX activation (21). A positive feedback between Ras and NOX may play a critical role in glyLDL-induced up-regulation of HSF1 and PAI-1 in EC (Fig. 7).

Raf-1 is a common downstream target protein of Ras activation. Multiple studies demonstrated that the activation of Ras promotes the translocation of Raf-1 to membrane and increases Raf-1 phosphorylation (20, 48). Chen et al. (49) reported that the increased expression of PAI-1 was associated with an elevated phosphorylation of Raf-1 in vascular smooth muscle cells. Our previous study indicated that Raf-1 is required for ox-LDL-induced PAI-1 expression in EC (18). The present study demonstrated that the phosphorylation of Raf-1 was increased in EC incubated with glyLDL. Treatment with Raf-1 inhibitor blocked glyLDL-induced up-regulation of PAI-1 mRNA and Raf-1 phosphorylation in EC, which suggests that Raf-1 is implicated in glyLDL-induced PAI-1 production in EC. Raf-1 expression is increased in retinal EC under diabetic condition (50). A recent study demonstrated that the level of pRaf-1 was increased in retinal EC of diabetic rats (20). Our findings suggest a new functional role of Raf-1 in terms of glyLDL-induced PAI-1 production in vascular EC. The target for activated Raf-1 induced by glyLDL in EC, potentially other intracellular ROS generation systems, remains unclear.

![FIG. 6. Effect of insulin-deficient diabetes on signaling mediators and PAI-1 in diabetic mice. A, Plasma glucose levels in control (CTL) and STZ-diabetic mice (STZ-DM). B, The abundances of RAGE, H-Ras, NOX, HSF1, and PAI-1 proteins were determined in the hearts of control and STZ-DM using Western blot analysis and mouse-specific antibodies. C, PAI-1 mRNA levels were determined in hearts of control and STZ-DM mice using RT-PCR. Values in B and C are presented in percentage of control after normalization with β-actin protein or GAPDH mRNA (mean ± se, n = 5 animals/group). **, P < 0.01 vs. control.]

### TABLE 1. Effect of STZ-induced diabetes on glucose, PAI-1, HSF1, RAGE, NOX, and H-Ras in mice

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<tr>
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<th>Control</th>
<th>STZ-DM</th>
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<tr>
<td>pGlucose (mg/dl)</td>
<td>135.2 ± 10.4</td>
<td>549.8 ± 37.2</td>
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<tr>
<td>pPAI-1 (µg/ml)</td>
<td>3.1 ± 0.5</td>
<td>7.7 ± 0.6</td>
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<tr>
<td>hHSF-1 (fold)</td>
<td>0.9 ± 0.0</td>
<td>2.7 ± 0.6</td>
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<tr>
<td>hPAI-1 (fold)</td>
<td>0.9 ± 0.1</td>
<td>3.1 ± 0.5</td>
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<tr>
<td>hNOX4 (fold)</td>
<td>1.0 ± 0.0</td>
<td>2.9 ± 0.1</td>
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<tr>
<td>hH-Ras (fold)</td>
<td>1.0 ± 0.1</td>
<td>2.7 ± 0.5</td>
</tr>
<tr>
<td>hRAGE (fold)</td>
<td>1.0 ± 0.1</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>PAI-1 mRNA (fold)</td>
<td>1.0 ± 0.0</td>
<td>2.2 ± 0.2</td>
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Data are fold change compared with control except pGlucose and pPAI-1. Mean ± se (n = 5 animals/group). p, Plasma; h, heart; STZ-DM, streptozotocin-induced diabetes mellitus.

* P < 0.01.
The increase in PAI-1 mRNA in glyLDL-treated EC may be a consequence of increased transcription of PAI-1 gene, decreased degradation of PAI-1 mRNA, or both. Our previous data demonstrated that glyLDL, ox-LDL, or oxidized very LDL increased the transcription of PAI-1 gene (12, 46, 51). The increases of PAI-1 protein and mRNA in glyLDL-treated EC were consistent to that in hearts of STZ-diabetic mice. These findings do not exclude the possibility of the coexistence of decreased degradation of PAI-1 mRNA, which potentially contributes to elevated PAI-1 protein or mRNA as indicated by other groups (52).

Cells respond to environmental stresses, such as heat shock, ROS, and shearing force, via a variety of posttranslational modifications of proteins or stress responses. Stress responses in cells are mediated by Hsp. Increased expression of Hsp has been implicated in the pathogenesis of atherosclerosis (53). The transcription of Hsp genes is regulated by HSF. Interaction between HSF and heat shock responsive element in promoters of targeted proteins triggers the transcription of Hsp genes (54). The increased expression of HSF1 was detected in human atherosclerotic lesions (55), which suggest that stress response is enhanced under hypercholesterolemia. Our previous study demonstrated that glyLDL, ox-LDL, or oxidized very LDL enhanced the expression of HSF1 in vascular EC. HSF1 is required for the up-regulation of PAI-1 in vascular EC through the binding of HSF1 to PAI-1 promoter induced by the modified lipoproteins (12, 46, 51). The results of the present study further demonstrate that transmembrane signaling mediators, RAGE, H-Ras, Raf-1, and NOX are involved in glyLDL-induced up-regulation of HSF1 and PAI-1 in EC, which provides additional insight for diabetes-associated metabolic stress-induced up-regulation of stress-response mediator and fibrinolytic regulator in vasculature.

The results of the present study suggest that glyLDL increased H-Ras translocation as early as 5 min. The increased abundance of H-Ras reached a peak around 15 min after the start of incubation with glyLDL. Phosphorylation of Raf-1 was increased in EC within 5–10 min of exposure to glyLDL. Activated H-Ras may stimulate ROS production by activating NOX (20, 21). A recent study demonstrated that AGEs-LDL increased NOX activity in EC via RAGE (56). The abundance of NOX2 in EC was increased by glyLDL and reached a peak around 1 h. The activation of NOX increases the generation of ROS (21, 28). Our previous studies demonstrated that glyLDL significantly increased superoxide and H2O2 production in EC within 30 min and reached a peak at around 2 h (16). Elevated ROS may trigger the activation of H-Ras (20, 21). Oxidative stress activates HSF1 (57). Treatment with glyLDL increased HSF1 expression in EC as early as 2 h, which was peaked at 6 h. HSF1 further regulates the transcription of PAI-1 in EC induced by glyLDL as previously described (12). Taken together, glyLDL-induced PAI-1 expression in EC may be a consequence of the serial activation of RAGE, H-Ras/Raf-1, NOX, and HSF1 (Fig. 7).
We recently reported that mitochondria may be another source of ROS in glyLDL-treated EC (58). ROS may impair mitochondrial respiration chain activity and further increase ROS production (59, 60). The possible relationship between H-Ras/Raf-1 and mitochondria-derived ROS remains to be investigated.

In summary, the results of the present study indicate that NOX, H-Ras, and Raf-1 are implicated in the up-regulation of HSF1 or PAI-1 in EC induced by glyLDL via RAGE, which may contribute to oxidative stress, hypofibrinolysis, and thrombotic events under diabetic condition. The identification of signaling mediators involved in glyLDL-induced PAI-1 production provides potential pharmacological targets for the prevention of diabetes-associated metabolic stress-induced thrombotic events.

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