Original Article

Apoptosis Signal-Regulating Kinase 1 Mediates Cellular Senescence Induced by High Glucose in Endothelial Cells

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Vascular ageing is accelerated in patients with diabetes. However, the underlying mechanism remains unclear. Here, we show that high glucose induces activation of apoptosis signal-regulating kinase 1 (ASK1), an apoptosis-inducing signal that mediates endothelial cell senescence induced by hyperglycemia. High glucose induced a time-dependent increase in the levels of ASK1 expression and its activity in human umbilical vein endothelial cells (HUVECs). Incubation of endothelial cells with high glucose increased the proportion of cells expressing senescence-associated β-galactosidase (SA-β-gal) activity. However, transfection with an adenoviral construct including a dominant negative form of *ASK1* gene significantly inhibited SA-β-gal activity induced by high glucose. In addition, infection with an adenoviral construct expressing the constitutively active *ASK1* gene directly induced an increase in the levels of SA-β-gal activity. Activation of the ASK1 signal also enhanced plasminogen activator inhibitor-1 (PAI-1) expression in HUVECs. Induction of senescent endothelial cells in aortas and elevation of plasma PAI-1 levels were observed in streptozotocin (STZ) diabetic mice, whereas these changes induced by STZ were attenuated in ASK1-knockout mice. Our results suggest that hyperglycemia accelerates endothelial cell senescence and upregulation of PAI-1 expression through activation of the ASK1 signal. Thus, ASK1 may be a new therapeutic target to prevent vascular ageing and thrombosis in diabetic patients. *Diabetes* 55:1660–1665, 2006

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ASK1, apoptosis signal-regulating kinase 1; CA-ASK1, constitutively active form of *ASK1*; DN-ASK1, dominant negative form of *ASK1*; HUVEC, human umbilical vein endothelial cell; MAP, mitogen-activated protein; PAI-1, plasminogen activator inhibitor-1; SA-β-gal, senescence-associated β-galactosidase; STZ, streptozotocin.

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Ageing is an independent risk factor for atherosclerosis, and the incidence of cardiovascular events increases with age. Previous studies have shown that endothelial cells in atherosclerotic lesions show features of cellular senescence including senescence-associated β-galactosidase (SA-β-gal) staining and telomere shortening (1,2). Since repeated injuries of the endothelium and subsequent turnover of medial cells have been implicated in the genesis of atherosclerosis, the rate of telomere shortening appears to be higher in vascular cells susceptible to atherogenesis (3). Importantly, senescent vascular cell behaviors are consistent with the changes seen in atherosclerosis. For example, expression of inflammatory cytokines and adhesion molecules is upregulated in senescent vascular cells, and nitric oxide (NO) production is dramatically reduced in senescent endothelial cells (4–6). Conversely, NO is able to activate telomerase and delay endothelial cell senescence (7). In addition, introduction of telomerase catalytic component into endothelial cells extends life span and restores NO production in senescent endothelial cells (8). These findings suggest that vascular senescence may participate in the genesis of atherosclerosis.

Senescence was originally defined by the observation that primary cells limit their proliferative potential in cell culture (9). It has been proposed that some aspect of excessive telomere shortening induces cell-cycle arrest and characteristic features of senescence (10). Cellular senescence, however, can be induced prematurely in early passage cells by oxidative stress that causes DNA damage (11,12) or by strong mitogenic signals (13,14). A number of in vitro and in vivo studies have shown that oxidative stress is increased in diabetic patients (15,16). Thus, premature senescence might be accelerated in diabetic patients. Actually, it is reported that hyperglycemia induces premature senescence in human skin fibroblast (17), and glycated collagen I induces premature senescence-like phenotypic changes in association with reduced NO production in endothelial cells (18). However, the molecular mechanism by which hyperglycemia induces endothelial premature senescence remains unclear. Apoptosis signal-regulating kinase 1 (ASK1) is a member of the mitogen-activated protein (MAP) kinase kinase kinase group that activates the c-Jun NH_2-terminal kinase and p38 MAP kinase signal pathways (19). Recent studies have shown that mitochondria contain ASK1, which releases cytochrome C by an unknown mechanism that does not activate MAP kinases (20). Since mitochondria play a pivotal role in the genesis of both diabetes and cellular

senescence (21,22), we asked if ASK1 might be involved in the mechanism underlying the development of endothelial cell senescence induced by hyperglycemia. We found that ASK1 signaling is activated by hyperglycemia, and ASK1 activation may mediate cellular senescence induced by hyperglycemia in endothelial cells.

RESEARCH DESIGN AND METHODS

Cell culture and reagents. Human umbilical vein endothelial cells (HUVECs) were purchased from Sanko Junyaku (Tokyo, Japan) and cultured in endothelial basal medium-2 (Sanko Junyaku) supplemented with 2% FCS and antibiotics. Antibodies to ASK1, PAI-1, CD31, and α-tubulin were from Santa-Cruz Biotech and Carbiochem.

Adenoviral constructs. Replication-defective adenovirus vectors expressing a constitutively active form of *ASK1* gene (CA-ASK1) and a dominant negative form of *ASK1* gene (DN-ASK1) (mutant Lys 709 to Met), tagged hemagglutinin in the site of NH_2-terminal, have been described previously (19,23).

Western blot analysis. Protein extract (20 μg) was fractionated on SDS-PAGE gels and transferred to a polyvinylidine difluoride membrane (Immobilon-P; Millipore). The membrane was blocked with T-phosphate–buffered saline (1× PBS, 0.3% Tween 20) containing 3% dry milk and incubated with primary antibody (anti-ASK1, anti–α-tubulin, and anti–phospho-ASK1) overnight at 4°C. The immune complexes were detected by chemiluminescence methods (ECL; Amersham International).

Animal experiments. ASK1-knockout mice (24) were made diabetic mice by a single intraperitoneal injection of streptozotocin (STZ) (60 mg/kg; Sigma). Age-matched control groups were also created. Tail blood glucose was assayed 3 days after injection using glucose test strips (BM-Accutest; Roche Diagnostics, Basel, Switzerland) to confirm diabetes. All diabetic animals had blood glucose values >30 mmol/l. Mice were maintained under a constant temperature (23 ± 1°C) with a 12-h light and 12-h dark cycle for 1 week with free access to water and food. All animal experiments were performed in accordance with the Ethics Committee on Animal Experiments and Care of the Osaka University.

Measurement of PAI-1 levels by enzyme-linked immunosorbent assay. Total secreted PAI-1 and urokinase type plasminogen activator antigen levels (free plus complex forms) were measured (in duplicate) in conditioned culture media and plasma by using a commercial PAI-1 activity assay kit (CHEMICON). Absorption was measured at 405 nm with a Dynatech plate reader and converted to each respective antigen concentration by the use of appropriate standards and BioLinx software (Dynatech). Total secreted PAI-1 and urokinase type plasminogen activator antigen levels (24 h) were calculated in nanograms per milliliter per well (100 ml media per well).

Senescence-associated β-galactosidase activity. Senescence-associated β-galactosidase (SA-β-gal) activity was detected as described previously (25). Counting cells at four random fields per dish and assessing the percentage of SA-β-gal–positive cells from at least 1,000 cells per field obtained quantification of SA-β-gal–positive cells.

Statistical analysis. Results are expressed as means ± SE. Comparison among groups was performed by one-way ANOVA, followed by Duncan multiple-range test for differences between two groups; a Student's t test was used when appropriate. A value of $P < 0.05$ was considered significant.

RESULTS

High glucose induces upregulation of the ASK1 signaling in endothelial cells. We first examined whether high glucose induces upregulation of the ASK1 signaling in endothelial cells. As shown in Fig. 1*A*, incubation of HUVECs with high glucose (33 mmol/l) resulted in a time-dependent increase in the levels of ASK1 protein expression, whereas incubation for 24 h with an osmotic control mannitol had no significant effects (Fig. 1*B*). We also examined the effect of high glucose on the catalytic activity of ASK1 by using an anti–phospho-ASK1 antibody that monitors activating phosphorylation of ASK1 (26). High glucose induced a transient increase in the levels of endogenous ASK1 activity in endothelial cells (Fig. 1*B*), indicating that high glucose induces activation of the ASK1 signaling.

High glucose induces endothelial cell senescence through activation of ASK1. Next, we examined whether high glucose induces cellular senescence in endothelial cells. As shown in Fig. 2, the percentage of SA-β-gal–positive cells was increased in HUVECs after exposure to high glucose (33 mmol/l). However, an osmotic control mannitol had no significant effects (Fig. 2*B*). To investigate the relation between the ASK1 signaling and endothelial cell senescence, we next examined the effect of infection of HUVECs with an adenoviral construct expressing CA-ASK1 and DN-ASK1 on endothelial cell senescence. Upregulation of ASK1 activity with CA-ASK1 directly increased proportion of cells expressing SA-β-gal activity. Conversely, downregulation of ASK1 activity with DN-ASK1 inhibited SA-β-gal activity induced by high glucose, indicating that high glucose may induce endothelial cell senescence through activation of ASK1.

Senescent endothelial cells are not observed in aortas of STZ-diabetic ASK1-knockout mice. To investigate a direct link between ASK1 and endothelial cell senescence in vivo, we next examined whether senescent endothelial cells can be detected in aortas of STZ-diabetic ASK1-knockout mice. Western blot analysis confirmed that ASK1 was not expressed in aortas of ASK1-knockout mice (Fig. 3*A*). STZ-treated mice had an elevation of plasma glucose associated with decreased plasma insulin levels compared with control mice (Fig. 3*B* and *C*). SA-β-gal activity was observed in the aorta from STZ-diabetic mice (Fig. 3*D*). SA-β-gal–positive endothelial cells were detected on the luminal surface of these aortas. In contrast, SA-β-gal–positive endothelial cells were not detected in the aorta from STZ-diabetic ASK1-knockout mice (Fig. 3*D–F*). These results indicate that ASK1 may be essential for the induction of endothelial cell senescence in aortas of STZ-diabetic mice.

High glucose induces upregulation of endothelial PAI-1 expression and its release through activation of ASK1. Since the expression of PAI-1, a principal factor for thrombosis, is upregulated in the elderly, we next examined whether ASK1 mediates PAI-1 expression in HUVECs. Upregulation of ASK1 with CA-ASK1 directly enhanced endothelial PAI-1 expression and release into the medium from HUVECs. However, an osmotic control mannitol did not significantly enhance PAI-1 release from these cells (Fig. 4*B*). In addition, suppression of ASK1 with DN-ASK1 suppressed the levels of PAI-1 expression and release induced by high glucose (Fig. 4*A* and *B*). These results suggest that ASK1 mediates endothelial PAI-1 expression as well as endothelial cell senescence.

STZ treatment does not elevate plasma PAI-1 concentrations in ASK1-knockout mice. To investigate a link between ASK1 and PAI-1 release in vivo, we examined whether STZ treatment induces elevation of plasma PAI-1 levels in ASK1-knockout mice. As shown in Fig. 5, elevation of plasma PAI-1 levels was observed in STZ-diabetic mice. In contrast, this increase by STZ treatment was attenuated in ASK1-knockout mice. These results suggest that ASK1 may play an essential role in the elevation of plasma PAI-1 concentrations in diabetic mice.

DISCUSSION

In the present study, we demonstrated that high glucose induces activation of the ASK1 signal and cellular senescence in endothelial cells and that downregulation of the ASK1 signal by DN-ASK1 suppresses endothelial cell

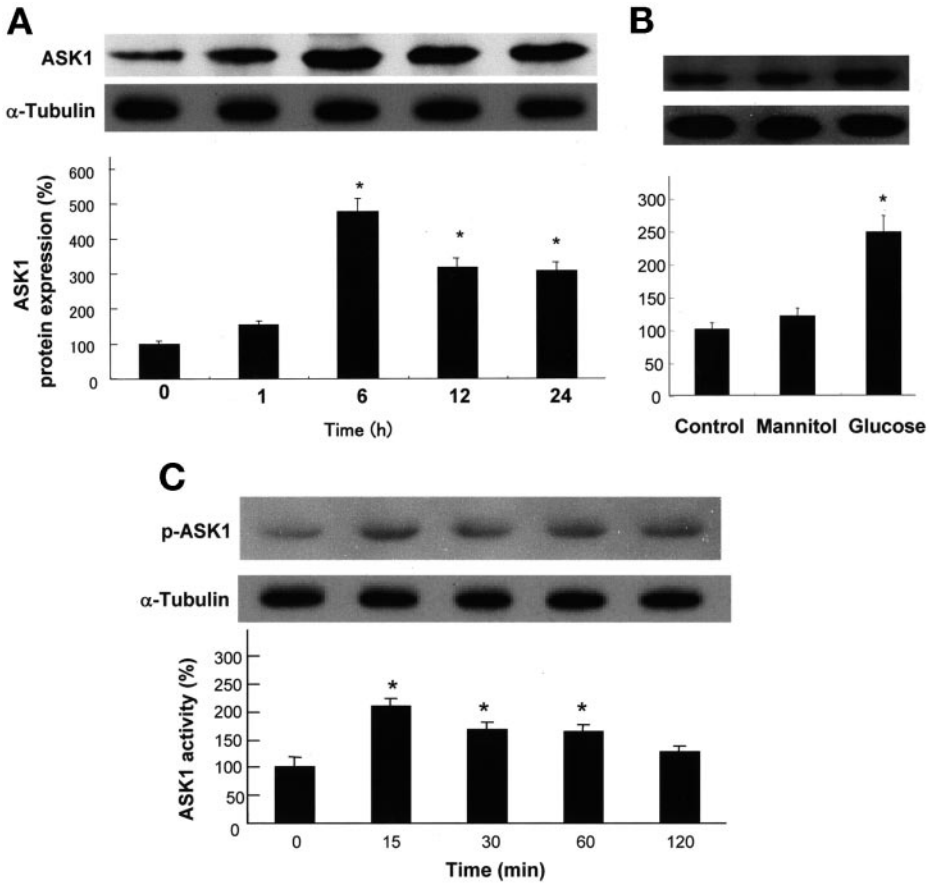

FIG. 1. High glucose induces upregulation of the ASK1 signal in endothelial cells. HUVECs were incubated with high glucose (33 mmol/l) for indicated times or mannitol (33 mmol/l) for 24 h. Western blot analyses of ASK1 expression (*A* and *B*) and phospho-ASK1 levels (ASK1 activity) (*C*) were performed with 20 μg of cell lysate. ASK1 expression and activities were quantified by densitometric analysis (data are normalized against α-tubulin and expressed as means ± SE). Data are presented as means ± SE in four experiments. *$P < 0.05$, significantly different from control HUVECs cultured in the absence of high glucose.

senescence induced by high glucose. In addition, activation of the ASK1 signal by CA-ASK1 directly induced premature senescence and upregulation of PAI-1 expression in endothelial cells. Furthermore, senescent endothelial cells are detected in aortas and plasma PAI-1 levels are elevated in STZ-diabetic wild-type mice, whereas these are not observed in STZ-diabetic ASK1-knockout mice.

Cellular senescence appears to play an important role in the ageing process. It is believed that accumulation of senescent cells in the vasculature results in failure of vascular homeostasis and function. Importantly, senescence is more accelerated in patients with diabetes compared with normal individuals, and these patients showed signs of premature senescence. However, the mechanism underlying the acceleration of senescence in these patients has not been defined. There are several stimuli that induce cellular senescence. These include telomere shortening, DNA damage, oxidative stress, sustained mitogen stimulation, and other cellular stresses (27–29). Senescence induced by telomere shortening is termed "replicative senescence," whereas senescence induced independent of telomere length is termed "premature

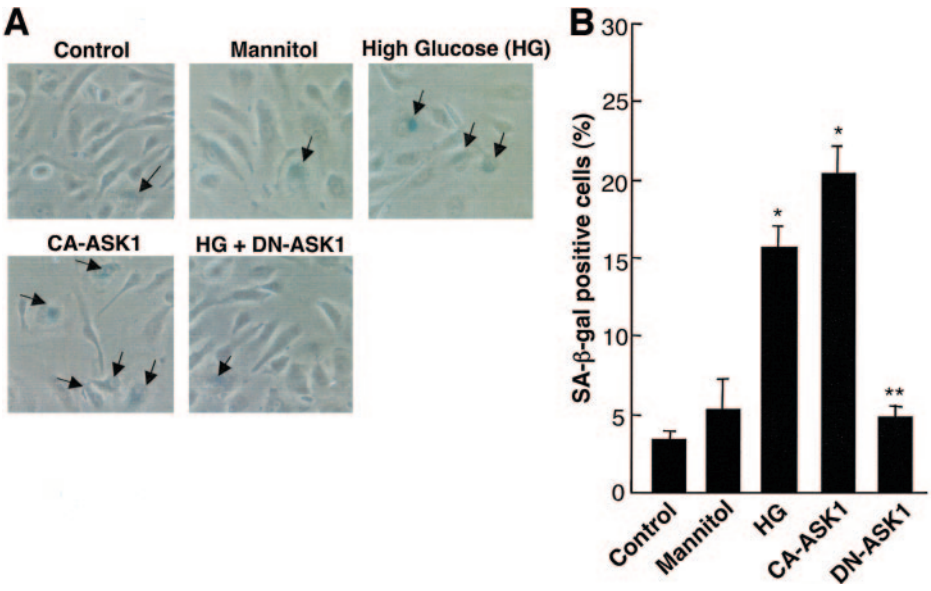

FIG. 2. ASK1 mediates endothelial cell senescence induced by high glucose. HUVECs were infected with CA-ASK1 or DN-ASK1 at a multiplicity of infection of 10. HUVECs were then treated with high glucose (33 mmol/l) or mannitol (33 mmol/l) for 24 h. *A*: Cells were stained for SA-β-gal as described in the text. *B*: Levels of SA-β-gal–positive cells were counted after the treatment as described in the text. Data are presented as means ± SE in four experiments. *$P < 0.05$, significantly different from control HUVECs. **$P < 0.05$, significantly different from HUVECs treated with high glucose.

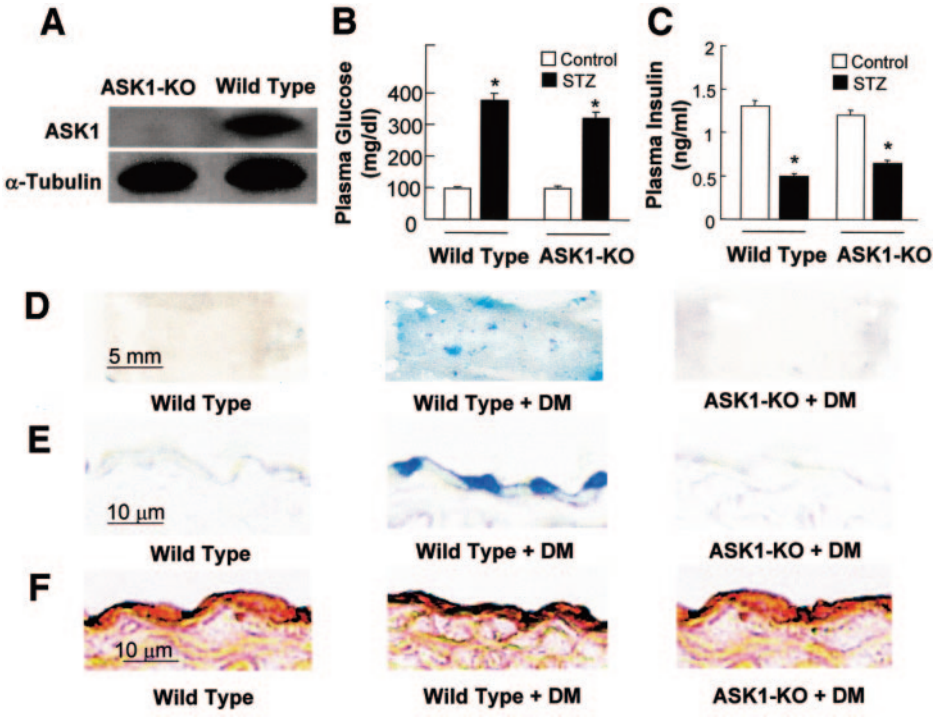

FIG. 3. Senescent endothelial cells are not detected in aortas of STZ-diabetic ASK1-knockout (KO) mice. *A*: Western blot analyses show ASK1 expression in mouse aortas. *B* and *C*: Plasma glucose and insulin levels were measured before and after treatment with STZ (60 mg/kg of body wt). *D*: Photographs show SA-β-gal activity (blue) in mouse aortas. *E*: Arrows indicate SA-β-gal–positive cells on the luminal surface in the cross-section of the mouse aorta. *F*: Section of aortas was also stained with CD31, an endothelial cell marker. Arrows indicate positive staining in the endothelium. STZ-diabetic (DM) mice were obtained as described in the text ($n = 3$).

senescence." It has been reported that proatherogenic factors impair telomerase activity and thereby may promote replicative senescence in endothelial cells, whereas NO, an anti-atherogenic factor, prevents age-related downregulation of telomerase activity and delays this senescence (7,30). Chen et al. (18) reported that glycated collagen I induces a premature senescence-like phenotype in endothelial cells. Although the molecular mechanism underlying induction of premature senescence is less well understood, several possible mechanisms have been reported previously. For example, oncogenic Ras promotes premature senescence in association with accumulation of p53 as well as p16 in vascular cells, which is mediated through activation of the MAP kinase cascade (3,30). Interestingly, modestly overactive p53 is reported to result in shortened life span and appearance of premature senescence, despite conferring extraordinary protection against cancer (31). Most recently, Wang et al. (32) reported that the stress-responsive Jun–NH_2-terminal kinase, an apoptosis-inducing signal (33), extends life span in *Drosophila* through activation of the transcription factor Foxo. These results suggest that apoptosis-related factors might participate in the mechanism underlying induction of premature senescence. Notably, ASK1 induces apoptosis through the mitochondria-dependent mechanism (20). Additional studies, however, are necessary to clarify this point.

The endothelium is a primary protective barrier that possesses strong anti-coagulatory properties. Importantly, senescence exaggerates vascular inflammation and thrombosis in the vessels, promoting the development of cardiovascular events (34). Senescent endothelial cells may contribute to the development of thrombosis through alteration of gene expressions in these cells. One of the important key genes for ageing-associated thrombosis is PAI-1, a major inhibitor of fibrinolysis. Accumulating evidence has shown that elevation of plasma PAI-1 levels is an independent risk factor for cardiovascular diseases as well as a clinical marker of impaired fibrinolysis. For example, the acute release of PAI-1 is a strong indepen-

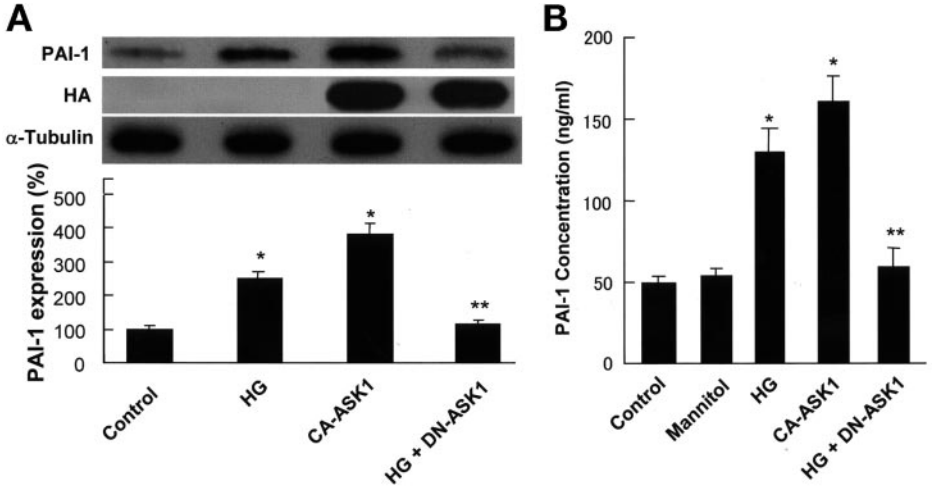

FIG. 4. Activation of the ASK1 signal induces upregulation of PAI-1 expression in endothelial cells. *A*: HUVECs were preinfected with an adenovirus vector expressing the CA-ASK1 or DN-ASK1 at a multiplicity of infection of 10. HUVECs were then treated with or without high glucose (33 mmol/l) or mannitol (33 mmol/l) for 24 h. Western blot analyses were performed as described in the text. *B*: Mutant ASK1 expression was determined by using antibody to the hemagglutinin (HG) tag. HUVECs were preinfected with an adenovirus vector as described in the legend of Fig. 4*A*. The medium was then collected after treatment with high glucose. PAI-1 concentrations in the medium were determined as described in the text. *$P < 0.05$, significantly different from control HUVECs cultured in the absence of high glucose. Data are presented as means ± SE in four experiments. **$P < 0.05$, significantly different from HUVECs treated with high glucose.

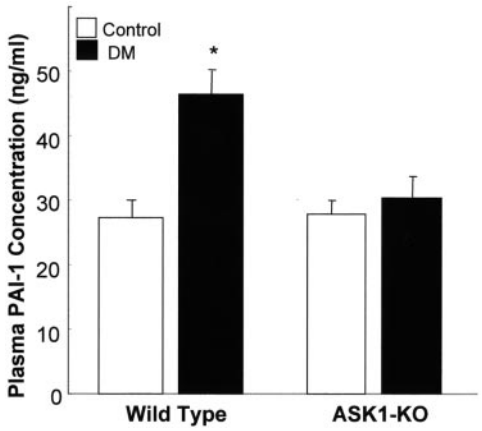

FIG. 5. Plasma PAI-1 concentrations were elevated in diabetic wild-type mice but not in diabetic ASK1-knockout (KO) mice. Diabetes (DM) was induced by STZ in both wild-type and ASK1-KO mice. Plasma PAI-1 concentrations were determined as described in the text. Data are presented as means ± SE in four experiments. *$P < 0.05$, significantly different from control.

dent predictor of death in ST-segment elevation myocardial infarction (35). High plasma PAI-1 concentrations precede a first acute myocardial infarction in both men and women (36). Interestingly, direct effects of PAI-1 on cardiovascular remodeling and metabolic profiles have been also demonstrated. PAI-1 enhances ventricular remodeling after myocardial infarction (37) and neointima formation after oxidative vascular injury in atherosclerotic-prone mice (38). In addition, disruption of the PAI-1 gene reduces the adiposity and improves the metabolic profile of genetically obese and diabetic *ob/ob* mice (39). These results suggest that PAI-1 plays a critical role in the pathogenesis of atherosclerosis as well as thrombosis. Importantly, plasma PAI-1 concentrations and PAI-1 expression in the arterial wall are elevated in patients with diabetes (40,41). In the present study, we demonstrated that suppression of the ASK1 signal inhibits elevation of plasma PAI-1 levels in diabetic mice. Our preliminary experiments also showed that ASK1 induced upregulation of p53 activity in endothelial cells (data not shown). Notably, previous studies suggest that p53 is implicated in the mechanism of cellular senescence and ageing (3,32) and that p53 phosphorylation at serine 15 is required for transcriptional induction of the PAI-1 gene (42), suggesting that ASK1-induced cellular senescence and PAI-1 upregulation may be mediated through the p53-dependent signaling pathways. Thus, the ASK1 signaling may be a new therapeutic target to prevent acceleration of the vascular senescence and thrombosis in diabetic patients.

In conclusion, we demonstrated for the first time to our knowledge that high glucose induces activation of the ASK1 signal that mediates the premature senescence in endothelial cells. Activation of ASK1 also induces upregulation of endothelial PAI-1 expression. These findings may provide a new insight into the molecular mechanism underlying the development of vascular complications in patients with diabetes.

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