FOXO1 impairs whereas statin protects endothelial function in diabetes through reciprocal regulation of Krüppel-like factor 2

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Aims
Krüppel-like factor 2 (KLF2) is implicated as a key molecule maintaining endothelial function. This study was designed to evaluate the reciprocal regulation of KLF2 by the forkhead transcription factor FOXO1, and the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor atorvastatin, in hyperglycaemic conditions.

Methods and results
Exposure of human umbilical vein endothelial cells to 30 mM glucose activated FOXO1 and suppressed KLF2. These effects were reversed by FOXO1 small interfering RNA. Adenoviral transfection of constitutively active FOXO1 suppressed KLF2 expression. Interestingly, atorvastatin inhibited FOXO1 by increasing phosphorylation and also by inhibiting nuclear localization and replenished KLF2 in high-glucose conditions. This effect of atorvastatin was attenuated by mevalonate. Chromatin immunoprecipitation analysis demonstrated that glucose increased whereas atorvastatin decreased FOXO1 binding to the promoter region of the KLF2 gene. In the vessels of Otsuka Long-Evans Tokushima Fatty rats, animal models of type 2 diabetes, FOXO1 was activated and KLF2 was suppressed, and this was reversed by atorvastatin treatment. The arteries from Otsuka Long-Evans Tokushima Fatty rats showed impairment of endothelium-dependent vasodilatation, and both atorvastatin and KLF2 gene therapies restored it.

Conclusions
Suppression of KLF2 by FOXO1 may be a plausible mechanism of diabetic endothelial dysfunction. High-glucose-induced, FOXO1-mediated KLF2 suppression was reversed by atorvastatin, suggesting that intensive statin treatment could be a therapeutic option in diabetic vascular dysfunction.

Keywords
FOXO1 • Statin • Krüppel-like factor 2 • Diabetes

1. Introduction
Endothelial dysfunction is an initial critical factor in the pathogenesis of diabetic vascular complications. In diabetes mellitus, endothelial dysfunction is characterized by impaired availability of endothelium-derived vasodilating factors, mainly nitric oxide.¹–⁴ Impaired endothelium-dependent vasodilatation has been demonstrated in patients with type 1 and type 2 diabetes,⁵,⁶ as well as in various animal models of diabetes.² A deregulated, chronic forkhead transcription factor FOXO1 activation with prolonged hyperglycaemia has been reported to induce pancreatic ß-cell failure, hypertriglyceridaemia, and hyperglycaemia in type 2 diabetes.⁷,⁸ However, the implications of FOXO1 activation in endothelial cells under diabetic conditions has not been investigated yet.

Krüppel-like factor 2 (KLF2), a main Krüppel-like factor expressed in endothelial cells (ECs), has been reported to play an important role in the maintenance of endothelial function. KLF2 affects the expression of various factors, including endothelial nitric oxide synthase (eNOS), which confers anti-inflammatory, anti-thrombotic, and anti-proliferative effects.⁹ Our preliminary experiment found that KLF2 expression was suppressed by a high concentration of glucose.¹⁰ We also observed that KLF2 contains forkhead factor-binding motifs in its promoter region. The presence of the forkhead factor-binding motifs in the promoter region and its reciprocal expression in ECs may indicate that KLF2 is suppressed in hyperglycaemic conditions by FOXO1 activation.

The present study investigated the hypothesis that FOXO1 suppresses KLF2, leading to endothelial dysfunction in under diabetic
conditions. Additionally, we evaluated the protective role of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), because an endothelium-protecting effect of statins in high-glucose conditions was previously reported.11 For these experiments, we used human umbilical vein endothelial cells (HUVECs) in high-glucose conditions with or without atorvastatin and evaluated the expression of FOXO1, KLF2, and eNOS. We also used the Otsuka Long-Evans Tokushima Fatty (OLETF) rats, as animal models of type 2 diabetes, to confirm the results in vivo.

2. Methods

2.1 Endothelial cell culture

HUVECs (Lonza) at passage four to six were plated onto 2% gelatin-coated dishes (Sigma) and maintained in EGM kit (Lonza). HUVECs were cultured with 20 mM of glucose for the high-glucose experiments. We exchanged the media for the indicated concentration of glucose at time 0. Atorvastatin was provided by Pfizer and was added at time 0.

2.2 Adenoviral vectors, lentiviral vectors and small interfering RNA

An adenovector expressing wild-type FOXO1 (Ad-FOXO1-WT) and constitutively active, triple mutant, FOXO1 (Ad-FOXO1-TM) were constructed as previously described.9 Briefly, the FOXO1-TM complimentary DNA (cDNA) for the indicated genes was subcloned into a shuttle vector, pAdTrack-CMV, which contains green fluorescent protein (GFP) under the control of a separate cytomegalovirus promoter. It is not possible to phosphorylate the FOXO1-AAA (TM-FOXO1) triple mutants because three phosphorylation sites are replaced by alanine residues. FOXO1-WT cDNA was subcloned into a shuttle vector, pAdTrack, which is only expressed with a single gene through the cytomegalovirus promoter. The shuttle vector containing the indicated cDNA was linearized and co-transformed into Escherichia coli BJS183 with the adenoviral plasmid pAdEasy-1 for homologue recombination. An adenoviral expressing green fluorescence protein (Ad-GFP) was used as a control. KLF2 adenoviral vector was constructed using human wild-type KLF2 plasmid (Origene). For gene transduction, HUVECs were infected with Ad-GFP horseradish peroxidase (SantaCruz Biotechnology, Inc.) or anti-rabbit IgG horseradish peroxidase (SantaCruz Biotechnology, Inc.) was used at a dilution of 1:2500. All the parameters in one set of figures were studied by loading the same amount of protein. We used one membrane for evaluating two molecules by stripping/reprobing once.

The proportion of intranuclear FOXO1 was analysed using an immunofluorescence microscope with Image pro Plus 4.5. For immunofluorescence staining, HUVECs were fixed and incubated with 1% bovine serum albumin in phosphate-buffered saline for 30 min at room temperature. Cells were then reacted with antibody to FOXO1 (1:50 dilution) overnight at 4 °C. Cells were incubated for 1 h with Alexa 488-conjugated donkey anti-rabbit IgG antibody (Molecular Probes) at a dilution of 1:1000.

For immunohistochemistry, carotid arteries were fixed with 10% neutral formaldehyde during overnight and embedded in paraffin blocks. Anti-PECAM1 and anti-FOXO1 were used to stain at a dilution of 1:50. Immunohistochemistry was performed with the R.T.U. VECTASTAIN kit (Vector). Each signal was developed with DAB (Vector) and counter-stained with haematoxylin (Sigma).

2.4 Nitrite assay

Nitrite concentrations in the conditioned media were determined as a surrogate marker of eNOS activity. Nitrite concentrations in culture media were measured using a colorimetric assay with the Griess reagent.15 A standard curve was established using sodium nitrite solutions of concentrations varying from 0 to 8 μM. The reaction was established in a 96-well microtiter plate, into which 100 μL aliquots of standards or samples were pipetted in triplicate, followed by the addition of 50 μL of 1% sulphanilamide (Sigma) in 2.5% H3PO4 and 50 μL of 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride (Sigma) in 2.5% H3PO4. Absorbance at 540 nm was measured in a microtiter plate reader.

2.5 Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was performed with a commercially available kit (Upstate Biotechnology).16 The antibody for FOXO1 was used for immunoprecipitation of the complex of FOXO1 protein and DNA fragments. The presence of DNA fragments from the KLF2 promoter region in the protein–DNA complex was analysed by PCR with primers for three different binding sites of FOXO1 on the KLF2 promoter at −609 bp (forward primer, 5′-GTG TGC CCC AGG CTG GTC-3′ and reverse primer, 5′-AGA GAG GCC TGC AGA ACC CT-3′), −754 bp (forward primer, 5′-GCC TTA TTG AGG TTG GGG AG-3′ and reverse primer, 5′-GAC CAG GCC CAA CAC CAC-3′), and −1082 bp (forward primer, 5′-GTA ACA GGA GTG ACA GTC CCT G-3′ and reverse primer, 5′-CTC CCC AAC CTC AAT AAG CC-3′).

2.6 Animal preparation

The experimental protocol was designed in accordance with the Guide for Experimental Animal Research issued by the Laboratory for Experimental Animal Research, Clinical Research Institute, Seoul National University Hospital. The OLETF strain of rat is a model of obese type 2...
diabetes. Their lean littermates are Long-Evans Tokushima Otsuka (LETO) rats. Male OLETF rats and LETO rats were gifts from Otsuka Pharmaceuticals (Tokushima, Japan). Atorvastatin was added to drinking water at 5 mg/kg/day for >4 weeks. Doses of 10–80 mg/kg atorvastatin in humans are equivalent to doses of 5–8 mg/kg in rats. For tissue harvesting, rats were first anaesthetized with an intraperitoneal injection of ketamine hydrochloride (50 mg/kg; Yuhan Corp. Bayer Korea) and then killed by cervical dislocation.

2.7 Organ chamber analysis for vascular reactivity
Abdominal aortic rings (4 mm long) from the indicated rats were used to assess vascular reactivity. Overexpression of KLF2 in the rat aortic ring was achieved by adenoviral transfection of 100 MOI in Dulbecco’s modified Eagle’s medium with 5% bovine serum albumin overnight. Rings were connected to isometric force displacement transducers (Grass Instruments) and suspended in organ chambers filled with 25 mL of gassed (95% O2 and 5% CO2) modified Krebs–Ringer bicarbonate solution as previously described. Using acetylcholine and sodium nitroprusside, endothelium-dependent and endothelium-independent vasodilatation, respectively, were evaluated using standard methods.

2.8 Statistical analysis
We expressed data as means ± SEM. Student’s unpaired two-tailed t-test between two groups and ANOVA for multiple comparisons followed by Tukey’s post hoc test were used to compare continuous variables. All calculations were performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). A value of P < 0.05 was considered statistically significant.

3. Results

3.1 Exposure to high glucose concentrations activated FOXO1 and suppressed KLF2 in endothelial cells
HUVECs were cultured in high glucose concentrations ranging from 12.5 to 100 mM. We observed no changes of cell viability in 50 mM glucose until day 4 (see Supplementary material online, Figure S1); therefore, we used 30 mM glucose as a physiologically high concentration of glucose for further experiments. We evaluated the expression of FOXO1 in physiologically high-glucose conditions. FOXO1 became unphosphorylated, which indicates that it is activated, with 30 mM glucose (Figure 1A). Expression of KLF2 was significantly decreased in high-glucose conditions, whereas there was no change in a control solution of mannitol of the same osmolarity (Figure 1B).

3.2 FOXO1 inhibited KLF2 and eNOS expression in endothelial cells
In the promoter region of KLF2, we found several putative inverse alignments of forkhead factor-binding motifs [WAARYAAAYW (W = A or T, R = A or G, and Y = C or T)] at −1082, −754, and −609 bp (Figure 2A). We investigated the role of FOXO1 in KLF2 expression by adenoviral transfection of constitutively active FOXO1 (Ad-TM-FOXO1), a main forkhead transcription factor expressed in HUVECs (validation of transfection efficacy is presented in see Supplementary material online, Figure S2). Ad-TM-FOXO1 transfection
completely inhibited the expression of KLF2 and eNOS (Figure 2B). Conversely, when FOXO1 was inhibited by small interfering RNA (siRNA), KLF2 expression, which was significantly suppressed by 75% with high glucose, was restored near to the control level. Expression of eNOS, which was also significantly suppressed by 63%, was also restored by FOXO1 siRNA, and correlated with KLF2 expression patterns. We measured nitrite concentration in the culture medium secreted according to eNOS activity, which showed the same pattern as eNOS expression (Figure 2C).

We then evaluated the role of KLF2 and FOXO1 in eNOS regulation, following two separate previous reports which observed eNOS regulation by FOXO1 or by KLF2. Augmented expression of KLF2 by transfection of KLF2 adenoviral vector (Ad-KLF2) induced eNOS expression by 10-fold (Figure 2D). However, Ad-TM-FOXO1 co-transfection significantly reduced eNOS expression in a dose-dependent manner, although KLF2 was kept highly expressed by Ad-KLF2 transfection. This showed that FOXO1 also inhibited eNOS directly though transcriptional suppression, besides the indirect regulation through KLF2 inhibition (Figure 2D).

### 3.3 Atorvastatin inhibited FOXO1, replenishing KLF-2 transcription in high-glucose conditions

To evaluate the hypothesized protective effect of statins against FOXO1, we treated HUVECs in high glucose with 0.01–1 μM of atorvastatin.
Atorvastatin. Atorvastatin phosphorylated FOXO1 in a dose-dependent manner, thus inactivating it (Figure 3A). In a dose-dependent manner, atorvastatin restored the KLF2 expression that was suppressed in high-glucose conditions (30 mM). p-FOXO1 means FOXO1 phosphorylated on serine 256. (B) Representative images and quantification by densitometry of the results of immunoblot analysis evaluating expression of p-FOXO1, FOXO1, KLF2, and eNOS following co-incubation with mevalonate (1 μM) for 24 h. The nitrite concentration in the same volume of culture media was compared by a colorimetric assay with Griess reagent.

Figure 3  Atorvastatin phosphorylates FOXO1, thereby inactivating it, and leads to derepression of KLF2 and eNOS. (A) Representative images and quantification by densitometry of the results of immunoblot analysis evaluating expression of p-FOXO1, FOXO1, KLF2, and eNOS after 30 h treatment with atorvastatin in high-glucose conditions (30 mM). p-FOXO1 means FOXO1 phosphorylated on serine 256. (B) Representative images and quantification by densitometry of the results of immunoblot analysis evaluating the contributing role of KLF2 in atorvastatin-induced eNOS expression. KLF2 was knocked down by lentiviral transfection of small hairpin KLF2 (Lenti-shKLF2). Lenti-shCon means negative control lentivirus. (C) Representative images and quantification by densitometry of the results of immunoblot analysis evaluating expression of p-FOXO1, FOXO1, KLF2, and eNOS following co-incubation of mevalonate (1 μM) for 24 h. The nitrite concentration in the same volume of culture media was compared by a colorimetric assay with Griess reagent.

3.4 Atorvastatin sequestered FOXO1 out of the nucleus, inhibiting its binding to the KLF2 promoter

The intracellular localization of FOXO1 following atorvastatin treatment was evaluated (Figure 4A). In the resting state, FOXO1 was located both in the cytoplasm and in the nucleus, whereas in high-glucose conditions, FOXO1 was located primarily in nucleus. However, with atorvastatin treatment, FOXO1 was translocalized out of the nucleus, even in high-glucose conditions, which suggests that FOXO1 was inactivated. Nuclear FOXO1 was significantly increased by 170% in high-glucose conditions (P < 0.001), but significantly decreased by 50% with atorvastatin treatment (P < 0.001).

Next, we evaluated whether the effect of atorvastatin on KLF2 induction was mediated through FOXO1 suppression, because atorvastatin is known to induce KLF2, mediated by MEF2. When FOXO1 was kept activated by Ad-TM-FOXO1, the induction of KLF2 by...
atorvastatin was completely inhibited (Figure 4B). The ChIP analysis showed that high-glucose conditions increased the binding of FOXO1 to the promoter of KLF2 at -609, -754, and -1082 bp, whereas atorvastatin significantly reversed it, suggesting that KLF2 expression is regulated by glucose or atorvastatin at the transcription level through modulation of FOXO1 binding to the KLF2 promoter regions (Figure 4C).

Finally, we knocked down FOXO1 using siRNA in order to confirm the role of FOXO1 in the regulation of KLF2 and eNOS by atorvastatin. Atorvastatin increased KLF2, which was potentiated by FOXO1 knockdown (Figure 4D). FOXO1 knockdown alone increased KLF2, showing that FOXO1 has an inhibitory action on KLF2. Both eNOS and its final oxidation product, nitrite, were also increased by atorvastatin, which was potentiated by FOXO1 siRNA. Interestingly, atorvastatin and FOXO1 siRNA showed additive effect on eNOS activation.

3.5 Endothelial dysfunction in the vessels of diabetic rats was normalized by atorvastatin

In order to investigate the clinical relevance of the in vitro findings, we performed animal experiments using OLETF rats as animal models of insulin-resistant, type 2 diabetes. The serum glucose level of OLETF rats was 2-fold higher than that of the control LETO rats. Body weight was also significantly greater for the OLETF rats than for the LETO rats. However, there were no significant differences in either serum glucose levels or body weight among the OLETF rats with or without atorvastatin treatment. The lipid levels were significantly reduced by atorvastatin in OLETF rats (See Supplementary material online, Table S1).
Consistent with in vitro findings, expression of KLF2 and eNOS was significantly reduced in the tissues of the OLETF rats compared with those of the control LETO rats. However, atorvastatin remarkably improved KLF2 and eNOS expression in the OLETF rats (Figure 5A).

We then examined the intracellular distribution of FOXO1 protein in order to evaluate its activity (Figure 5B). In the vessels from the OLETF rats, FOXO1 in the endothelial cells was densely stained in the nucleus (arrows in Figure 5B), which suggested that most of the FOXO1 was activated. However, in the vessels harvested from the LETO rats and the atorvastatin-treated OLETF rats, FOXO1 was stained in the cytoplasm (arrowheads in Figure 5B), suggesting that FOXO1 was inhibited by atorvastatin treatment.

In order to evaluate the functional significance of KLF2 replenishment by atorvastatin, we harvested vascular rings from the abdominal aorta and placed them into an organ chamber to assess both endothelium-dependent and endothelium-independent vasodilatation. Endothelium-independent vasodilatation was preserved in the OLETF rats at the same rate as that in the LETO rats (Figure 6A). However, endothelium-dependent vasodilatation in response to acetylcholine was significantly impaired in the OLETF rats compared with that in the LETO rats (Figure 6B). Atorvastatin treatment significantly improved endothelium-dependent vasodilatation, as did KLF2 replenishment by transfection of Ad-KLF2 (validation data presented in see Supplementary material online, Figure S4).

4. Discussion

The important finding of our study is that exposure of ECs to high-glucose concentrations activated FOXO1. FOXO1 suppressed KLF2 expression, resulting in endothelial dysfunction. We showed that atorvastatin restored KLF2 expression that was suppressed by FOXO1. This study has several novel findings. First, we showed that FOXO1 transcriptionally inhibited KLF2 expression, which has not been reported previously. Second, FOXO1 in ECs was activated in high-glucose conditions, which was inhibited by atorvastatin. Third, this regulation of FOXO1 activity by glucose or atorvastatin resulted in modulation of KLF2 and its downstream molecule, eNOS expression. Fourth, we confirmed that these in vitro findings were valid in vivo by using the OLETF rat as an animal model of insulin-resistant, type 2 diabetes. Finally, we demonstrated that the high-glucose/FOXO1/KLF2

**Figure 5** FOXO1 was activated whereas KLF2 was suppressed in the diabetic, OLETF rats, and this situation was normalized by statin treatment. (A) Immunoblot of KLF2 and eNOS in the tissues of the OLETF rats with or without atorvastatin treatment for 4 weeks. Tissues harvested from LETO rats were evaluated as the control. (B) Representative immunohistochemical images of carotid arteries stained with FOXO1. Arrows indicate nuclear FOXO1; arrowheads point to cytoplasmic FOXO1. An IgG stain used as the negative control. Magnification ×100; scale bars represent 50 μm.

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pathway was, at least partly, responsible for endothelial dysfunction observed in diabetes. Moreover, we suggest that statin treatment could reverse this pathological phenomenon. The results of our study are summarized in Figure 7.

4.1 FOXO1 activation in endothelial cells in high-glucose conditions

FOXO1 has been reported to activate the process of glucose production, i.e. gluconeogenesis in hepatocytes, which is required for survival during prolonged fasting or starvation. However, dysregulated, chronic FOXO1 activation results in fasting hyperglycaemia through an increase in hepatic gluconeogenesis, which is a prominent finding in diabetes.

In the present study, we showed that FOXO1 was activated by high glucose concentrations in HUVECs. This finding was consistent with a previous report in retinal microvascular endothelial cells. However, we demonstrated for the first time that FOXO1 activation impairs endothelial function by suppressing the gene expression of endothelial protective molecules, such as KLF2, in addition to a direct pro-apoptotic action, which had been regarded as the main action of forkhead transcription factor in ECs.

4.2 Dysfunction of the FOXO1/KLF2/eNOS axis in diabetic vascular failure

KLF2 is widely regarded as the most potent inducer of eNOS, especially mediating shear stress-induced eNOS expression. Although there have been two separate reports that observed eNOS regulation by FOXO1 or by KLF2, we found that FOXO1 is a negative transcriptional regulator of KLF2, leading to suppression of eNOS and vascular dysfunction in a diabetic animal model. We evaluated the functional implication of KLF2 suppression by FOXO1 only in terms of eNOS suppression in this study. However, the FOXO1/KLF2 pathway might exert more profound effects in vascular pathophysiology considering that more than 15–50% of flow-regulated genes are dependent on KLF2.

eNOS is suppressed in high-glucose conditions, but it is replenished by atorvastatin through activation of KLF2. Interestingly, FOXO1 knock-down alone increased eNOS activation and showed an additive effect with KLF2 induction by atorvastatin. This suggests that FOXO1 inhibited eNOS directly though transcriptional suppression, besides the indirect regulation through KLF2 inhibition.

Both FOXO1 and FOXO3a are expressed in mature ECs. However, the expression level of FOXO1 far exceeds that of...
KLF2 suppression by FOXO1 in diabetes

4.3 Inhibition of FOXO1 and improvement of endothelial function by statin

The effects of statins on cardiovascular diseases, including diabetes, are mainly attributed to their cholesterol-lowering properties. However, mounting evidence suggests pleiotropic effects of statins beyond lipid lowering. In this study, we confirmed that statins induced KLF2 expression (data with simvastatin not shown). In previous studies, the MEF2-binding site of the KLF2 promoter was suggested to be necessary for statin-mediated KLF2 induction. However, we observed that high glucose regulates the transcription of KLF2 by modulating FOXO1 binding in the more distant site than the proximal MEF2-binding site in its promoter region. Moreover, we confirmed FOXO1 primarily inhibited KLF2 expression. Therefore, we suggest that the up-regulated KLF2 expression with atorvastatin is primarily effected by the suppressed FOXO1 rather than by the activated MEF2.

4.4 Restoration of KLF2 for reversing vascular dysfunction in diabetes mellitus

In this study, we evaluated the functional implication of KLF2 modulation in vivo by measuring endothelium-dependent vasodilatation, which has been widely used as an index of endothelial function. In diabetic animal models, KLF2 expression was significantly decreased. Following treatment with atorvastatin, KLF2 and eNOS expression was restored. We confirmed that such a change in KLF2 and eNOS expression was well correlated with the endothelial-dependend vasodilatation of this vessel. Restoration of the endothelium-dependent vasodilatory response following statin treatment through eNOS activation was observed clinically in postmenopausal women12 and experimentally in thrombin-induced endothelial dysfunction.22 We also observed that statin treatment improved the endothelial function to the same extent as KLF2 replenishment by Ad-KLF2 transfection. We have not presented data from a specific knock-out experiment of KLF2 in the presence of statins, because we do not yet have effective KLF2 suppressors in rat models, such conditioned knock-out animals, which is a limitation of this study. However, these findings may be sufficient to suggest that statin treatment could reverse endothelial dysfunction, at least partly, by replenishing KLF2 expression in diabetic conditions.

In conclusion, suppression of KLF2 by FOXO1 in ECs might be a plausible mechanism of diabetic endothelial dysfunction, which can be reversed by statins. The findings of this study suggest that intensive statin treatment could be a potential therapeutic option in treatment of diabetic vascular dysfunction.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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