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Foxo1 Links Hyperglycemia to LDL Oxidation and Endothelial Nitric Oxide Synthase Dysfunction in Vascular Endothelial Cells

Jun Tanaka,¹ Li Qiang,¹ Alexander S. Banks,¹ Carrie L. Welch,¹ Michihiro Matsumoto,¹ Tadahiro Kitamura,² Yukari Ido-Kitamura,² Ronald A. DePinho,³ and Domenico Accili¹

OBJECTIVE—Atherosclerotic cardiovascular disease is the leading cause of death among people with diabetes. Generation of oxidized LDLs and reduced nitric oxide (NO) availability because of endothelial NO synthase (eNOS) dysfunction are critical events in atherosclerotic plaque formation. Biochemical mechanism leading from hyperglycemia to oxLDL formation and eNOS dysfunction is unknown.

RESEARCH DESIGN AND METHODS—We show that glucose, acting through oxidative stress, activates the transcription factor Foxo1 in vascular endothelial cells.

RESULTS—Foxo1 promotes inducible NOS (iNOS)-dependent NO-peroxynitrite generation, which leads in turn to LDL oxidation and eNOS dysfunction. We demonstrate that Foxo1 gain-of-function mimics the effects of hyperglycemia on this process, whereas conditional Foxo1 knockout in vascular endothelial cells prevents it.

CONCLUSIONS—The findings reveal a hitherto unsuspected role of the endothelial iNOS-NO-peroxynitrite pathway in lipid peroxidation and eNOS dysfunction and suggest that Foxo1 activation in response to hyperglycemia brings about proatherogenic changes in vascular endothelial cell function. *Diabetes* 58: 2344–2354, 2009

Cardiovascular disease (CVD) is the leading cause of death of diabetic patients. Type 2 diabetes increases CVD-related morbidity and mortality by two- to fourfold (1). Unlike microvascular diabetes complications, the benefit of tight glycemic control on the prevention of macrovascular complications remains unclear (2), owing possibly to the contribution of insulin resistance as an independent risk factor (3,4).

A growing consensus indicates that the adverse effects of hyperglycemia on diabetes complications are exerted through a shared pathway of oxidative stress, leading to

oxidative modification of lipid, protein, and DNA; activation of proinflammatory pathways; DNA damage; and cellular apoptosis (5). In contrast, the effects of “insulin resistance” are heterogeneous, primarily because the constellation of events commonly subsumed under this moniker is indeed an admixture of insulin resistance and excessive insulin sensitivity, at the cellular and organ level (6).

It is widely held that alterations of endothelial cell function are early events in atherosclerosis development. These perturbations include the modification of lipoproteins, loss of endothelium-dependent vasodilation (endothelial dysfunction, synonymous to endothelial nitric oxide synthase [eNOS] dysfunction), and increased expression of cellular adhesion molecules (7). These lead to the formation of fatty streaks, consisting of cholesterol-laden macrophages beneath the endothelium of large arteries. Several lines of evidence underscore the importance of oxidative modifications of native LDL and eNOS function in fatty streak formation (8,9). For example, macrophages become cholesterol-laden foam cells when cultured in the presence of oxidized, but not of native, LDL (10,11). Hyperglycemia has been linked to the generation of peroxynitrite, a highly potent oxidant that impairs eNOS activity, and glucose-induced eNOS dysfunction can be restored by antioxidants (12). However, the mechanisms by which hyperglycemia and oxidative stress increase oxLDL and cause eNOS dysfunction remain unclear.

In this study, we sought to identify a pathway linking diabetes to oxLDL formation and eNOS dysfunction. We show that the forkhead protein Foxo1 is activated by glucose and oxidative stress in endothelial cells to promote inducible NOS (iNOS)-dependent NO/peroxynitrite generation. The latter increases lipid peroxidation and causes eNOS dysfunction by disrupting eNOS dimerization. We demonstrate that Foxo1 gain-of-function mimics the effects of hyperglycemia on this process, while conditional Foxo1 knockout in vascular endothelial cells prevents it. The data reveal a seemingly novel mechanism, whereby iNOS-dependent NO/peroxynitrite generation by vascular endothelial cells promotes the early changes associated with the pathogenesis of atherosclerosis in diabetes.

RESEARCH DESIGN AND METHODS

Measurement of NO and reactive oxygen species/peroxynitrite production. We cultured primary human aortic endothelial cells (HAECs) (Lonza) in EGM-2 (endothelial growth medium-2; Lonzo) and used them between passages 3 and 5. We determined NO production using the NO-specific fluorescent dye 4,5-diaminofluorescein diacetate (DAF-2 DA; EMD Biosciences) as described elsewhere (13) and reactive oxygen species (ROS)/peroxynitrite production using 5-(and6)-carboxy 2,7-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA; Invitrogen). HAECs were grown to confluence and

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stimulated with high glucose or H₂O₂ in chamber slides (Lab-Tek), then serum-deprived for 2 h in EBM-2 (endothelial basal medium-2) supplemented with 100 μmol/l L-arginine, loaded with DAF-2 DA (3 μmol/l) or carboxy-H₂DCFDA (10 μmol/l) for 30 min at 37°C and washed three times with EBM-2. After fixation in 2% paraformaldehyde for 5 min at 4°C, we visualized NO and ROS/peroxynitrite production under microscope. In some assays, we transduced HAECs with Foxo1-ADA and DBD-Foxo1-ADA adenoviruses (14) 24 h before the experiment and cultured them with the iNOS inhibitor 1400W (10 μmol/l) for 3 h, or with the eNOS inhibitor L-NAME (100 μmol/l) (Calbiochem) for 30 min before adding DAF-2DA. We determined the total amount of NOx (nitrate and nitrite) in phenol-red free DMEM using a Nitric Oxide Quantitation Kit (Active Motif) after a 24-h culture in the presence or absence of NOS inhibitors. Insulin (1 μmol/l; Sigma-Aldrich) and A23187 (3 μmol/l; EMD Biosciences) were used to activate eNOS.

RNA isolation and expression studies. We extracted RNA using RNeasy Mini Kit and RNase-Free DNase Set (Qiagen). For real-time PCR analysis, we reverse transcribed total RNA using SuperScript II First-Strand Synthesis System (Invitrogen). Oligonucleotide primer sequences were as follows:

human (h)iNOS, 5'-tcgtggagacgggaagaagtc-3' and 5'-aagctcatctg gggggtaggc-3';

heNOS, 5'-gaccctcaccgctacaacatcc-3' and 5'-tccacgatgtgactgttgctc-3';
h36B4, 5'-tgctcaacatctccccctctc-3' and 5'-agacaaggccaggactctgttg-3';
hFoxo1, 5'-tgaattcaccagcccaaac-3' and 5'-gctaccaccaggatcaactgttg-3';
hFoxo3, 5'-tgactgatatggcagccacat-3' and 5'-cctgtctgttaaggagctgg-3';
hFoxo4, 5'-tgggctcaatctcactctccc-3' and 5'-agaagcaccctctctctgctga-3';
mouse (m)iNOS, 5'-ctcagcccaacaatacaagatgacc-3' and 5'-tgggtggaagat gctcatgcaaaat-3';

m36B4, 5'-tgctgaacatctccccctctc-3' and 5'-tctccacagacaatgccaggac-3';
mFoxo1, 5'-gggtcctgggccaataatgta-3' and 5'-gggtcctggcagatgtgtgagg-3';
mFoxo3, 5'-tgaactcctgctcagtcacc-3' and 5'-cgtgtcctgagcagatcaa-3';
mFoxo4, 5'-accactcctgctgagctcctc-3' and 5'-atcacagaaaggtggggaccac-3'

We performed PCR reactions in triplicate using a DNA Engine Opticon 3 System (MJ Research). Relative mRNA levels were calculated using a standard curve, with the PCR product for each primer set normalized to 36B4 mRNA level.

Western blotting. Cells were lysed and aortas were homogenized by Polytron immediately after dissection in RIPA buffer (150 mmol/l NaCl, 15 NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mmol/l Tris-HCl: pH8.0) containing protease inhibitor cocktail (Roche). The mixture was then sonicated and cellular debris were removed by centrifugation. SDS-PAGE and Western blotting were performed using standard techniques and ECL detection reagents (Amersham Bioscience) with anti-HA (12CA5; Boehringer Mannheim), anti-FLAG (M2; Sigma-Aldrich), anti-Foxo1 (H128; Santa Cruz Biotechnology), anti-eNOS (sc-653), anti-insulin receptor (InsR, C-19; all from Santa Cruz Biotechnology), anti-Akt(pS473), anti-Akt, anti-Foxo3, anti-Foxo1(pS253), anti-tyrosine Foxo (all from Cell Signaling), anti-InsR(pY1158/pY1162/pY1163) (Abcam), anti-iNOS (clone 6; BD Transduction Laboratories), and anti-actin (JLA20; Calbiochem) antibodies. SDS-resistant eNOS dimer formation was determined using low-temperature SDS-PAGE under reducing condition with anti-eNOS antibody (N-20; Santa Cruz Biotechnology) as described previously (12).

Transient transfection and luciferase assays. Expression plasmids encoding Foxo1-luciferase reporter gene (3xIgf1p-RE/pGL2 basic) have been described (15). Localization of Foxo1-green fluorescent protein (GFP) fusion protein was determined 48 h after Foxo1-GFP adenovirus infection. Human iNOS promoter (-3,997 to 89 from the transcription start site) was PCR-amplified with the following primers: 5'-caagacattcccgtgctgattg-3' and 5'-catcaaaagtgccgagagatt-3', and cloned into pGL3 basic luciferase reporter plasmid (iNOS/pGL3 basic). We cultured human umbilical vein endothelial cells (HUVECs) to confluence and transiently transfected them with 3xIgf1p-RE/pGL2 basic, iNOS/pGL3 basic, or pGL3 empty vector using FuGENE HD (Roche). We transduced cells with Foxo1-ADA or DBD-Foxo1-ADA adenoviruses 6 h after transfection. We carried out reporter assays with a Dual Luciferase Reporter Assay System (Promega) using Monolight 3010 Luminometer (BD Bioscience). We used plasmid pRL-CMV Renilla luciferase (Promega) to control for transfection efficiency.

Chromatin immunoprecipitation assays. We performed chromatin immunoprecipitation (ChIP) as described previously (15). To amplify the Foxo consensus binding site of the human iNOS promoter we used the following primers: 5'-agaagtgcagtcagcagatgct-3' and 5'-gcactagtaagcttgggtggaatg-3'.

siRNA-mediated Foxo knockdown. We transfected confluent HAECs with Foxo1, Foxo3, and Foxo4 siRNAs, singly or in combination (ON-TARGETplus SMARTpool, DARMACON), using TransIT-TKO transfection reagent (Mirus Bio). We determined transfection efficiency with siGLO Red (DARMACON).

LDL oxidation. We transduced confluent HAECs with Foxo1ADA or DBD-Foxo1ADA adenoviruses and cultured them at 37°C for 16 h. Thereafter, we incubated HAECs in phenol red-free DMEM supplemented with 0.1% BSA, 100

μmol/l L-arginine, and 100 μg/ml native LDL (Biomedical Technologies), in the absence or presence of iNOS inhibitor. After 24 h, we collected the medium and removed cells by low-speed centrifugation. We measured LDL oxidation products in the supernatant as thiobarbituric acid reactive substances (TBARS) using a TBARS assay kit (Cayman chemical) and SIN-1 (Sigma-Aldrich) as positive control. SIN-1 releases NO and superoxide to generate peroxynitrite.

Viral expression studies. Adenoviruses encoding HA-tagged Foxo1-ADA (constitutively nuclear), FLAG-tagged DNA binding-deficient (DBD)-Foxo1-ADA, and GFP-fused Foxo1 have been described previously (14,25). We transduced HAECs with Foxo1-ADA and DBD-Foxo1-ADA adenoviruses 24 h before the experiment.

Animal generation and analysis. The Columbia University Animal Care and Utilization Committee approved all animal experiments (protocol A444669). Tie2-cre (16) and *Foxo1^{fllox}* mice have been described (17). L1 mice are InsR transgenic/knockouts that express InsR exclusively in hepatocytes, brain, and pancreas β-cells (18). Wild-type, Foxo1-null, and floxed alleles were amplified by PCR with primers 5'-gcttagacagagatgttctcact-3', 5'-ccagagctttgtatcag gcaataa-3', and 5'-caagtcattattcagcacttga-3'. To induce diabetes, we administered a single intraperitoneal injection of streptozocin (STZ; Sigma-Aldrich) (200 mg/kg) in saline, and 2 weeks later, blood glucose values were measured with OneTouch Ultra system (LifeScan) to determine hyperglycemia. After the mice were killed, we dissected aortas, extracted mRNA, and collected serum for lipid peroxides measurements.

iNOS immunohistochemistry. Paraffin-embedded serial sections were prepared from the proximal aorta. iNOS was detected using a rabbit polyclonal antibody (ab15323, 1:100 dilution) (Abcam, Cambridge, MA) after antigen retrieval in 10 mmol/l citrate buffer with boiling for 10 min. Endothelial cells were identified with a goat polyclonal antibody against the endothelial marker PECAM-1/CD31/M-20 (SC-1506, 1:100 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA) after antigen retrieval in 1 mmol/l EDTA, pH8.0, and steaming for 20 min. Antigen detection was carried out using the ABCComplex/HRP kit (DAKO) followed by diaminobenzidine staining (DAB, Vector Laboratories). Sections were counterstained with hematoxylin.

Endothelial cells isolation. Liver and lung were digested in 2 mg/ml collagenase A (lung) and D (liver) solution (Roche diagnostics) for 45 min, followed by DNase I treatment (Sigma) for 5 min. Lysates were filtered through a cell strainer (100 mm and 40 mm) to remove debris. After washing twice, endothelial cells were enriched by taking the interphase of a 30% Histodenz (Sigma) and RPMI suspension of cells overlaid after spinning at 1500 × g for 20 min. Cells were further affinity isolated by mouse CD146 (LSEC) MicroBeads (Miltenyi Biotec) for liver and biotin-conjugated anti-mouse CD31 (BD Pharmingen) and streptavidin-MicroBeads (Miltenyi Biotec) for lung using MACS cell separation system (Miltenyi Biotec).

Statistical analyses. Values are expressed as means ± SE. Analyses of statistical significance were carried out using two-tailed Student's *t* test.

RESULTS

Glucose and oxidative stress promote iNOS-dependent NO and ROS/peroxynitrite generation in HAECs. NO has a dual effect on endothelial function (19,20). In physiologic conditions, NO is a potent vasodilator and protects endothelial cells from oxidative damage (21). In contrast, with oxidative stress, iNOS-derived NO can react with superoxide to yield peroxynitrite, a highly reactive oxidant (22), which in turn promotes LDL oxidation (8) and inhibits eNOS-dependent NO production (12). Incubation of HAECs in medium containing 25 mmol/l glucose for 48 h increased NO production four- to fivefold (Fig. 1A), with a concomitant increase of ROS/peroxynitrite (Fig. 1B). Incubation with H₂O₂, an inducer of oxidative stress, mimicked these effects (Fig. 1C). Measurements of gene expression indicated that both glucose and H₂O₂ increased iNOS, but not eNOS mRNA and protein, whereas nNOS mRNA was undetectable (Fig. 1D and E). Furthermore, the iNOS inhibitor 1400W, but not the eNOS inhibitor L-NAME, prevented H₂O₂- and glucose-induced NO production (Fig. 1F). These results indicate that exposure of HAECs to high glucose or oxidative stress promotes iNOS-dependent peroxynitrite generation through NO production, a hitherto unrecognized process.

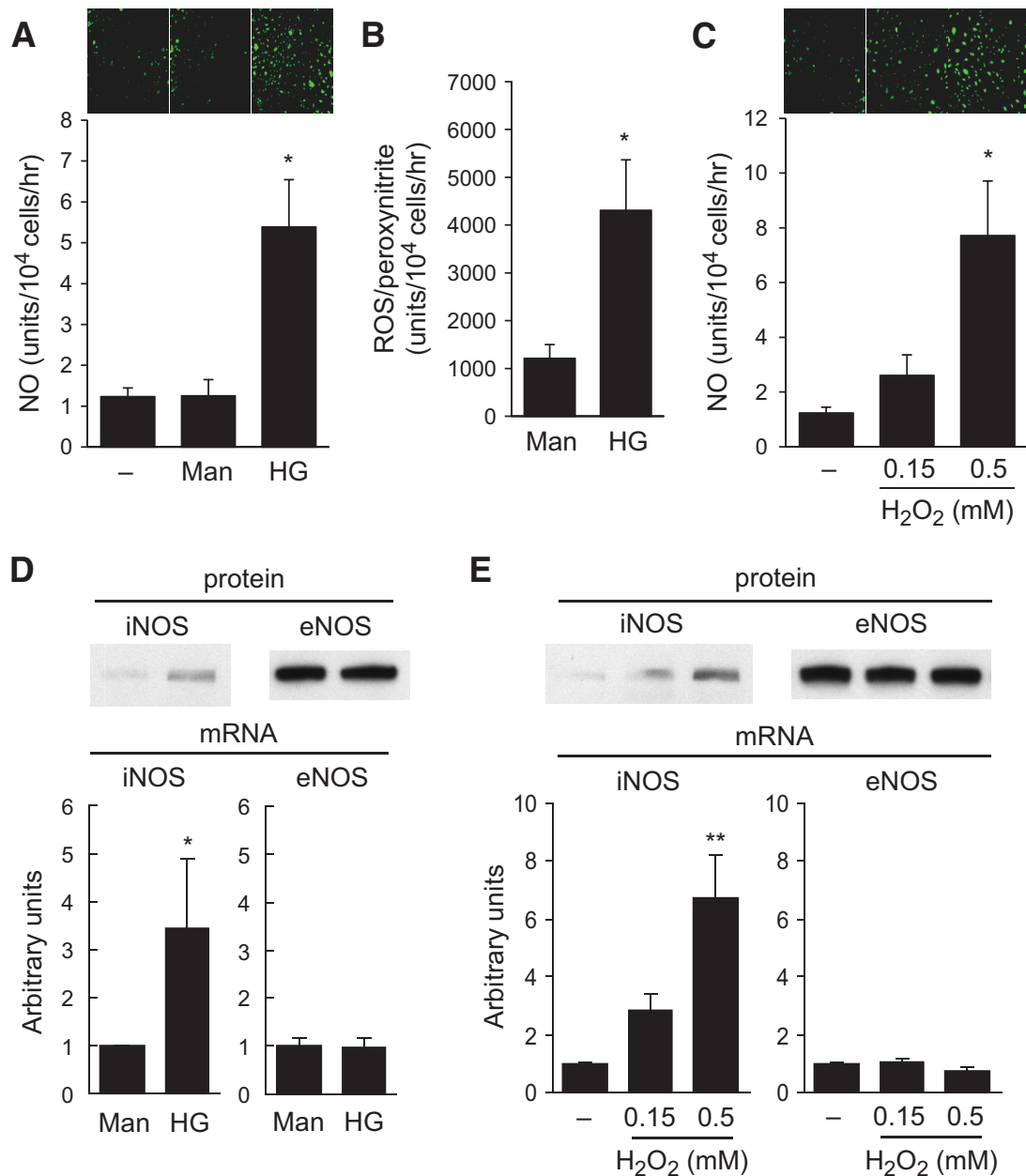


FIG. 1. iNOS-dependent NO and ROS/peroxynitrite generation in response to high glucose or oxidative stress in HAECs and in mice. *A–F:* HAECs were incubated in medium containing 5.5 mmol/l glucose (–), 25 mmol/l glucose (HG) or 25 mmol/l mannitol (Man) for 48 h, or H₂O₂ (0.15 or 0.5 mmol/l) for 12 h with (*E* and *F*) or without (*A–D*) either iNOS (1400W) or eNOS inhibitors (L-NAME). *A, C,* and *F:* Representative images (*upper panels*) and calculated relative intensities (*lower panels*) of NO production using DAF2-DA. *B:* ROS/peroxynitrite production using carboxy-H₂DCFDA. *D* and *E:* iNOS and eNOS protein (*upper panel*) and mRNA (*lower panel*) expression. *G:* Blood glucose, (*H*) aortic iNOS mRNA expression, (*I*) iNOS immunohistochemistry in aortic sections from C57BL/6J mice (first and second panel from the left) and from Tie2-cre/*Foxo1*^{fllox/fllox} and *Foxo1*^{fllox/fllox} mice (third and fourth panel from the left), and (*J*) plasma lipid peroxide levels (TBARS) in STZ-induced diabetic and saline-treated control mice ($n = 6$ for each group). The data were obtained two weeks after STZ injection. * $P < 0.05$; ** $P < 0.01$ by Student's *t* test. (A high-quality digital representation of this figure is available in the online issue.)

Increased iNOS expression and lipid peroxidation in diabetic mice. We investigated whether hyperglycemia, presumably resulting in oxidative stress, affects endothelial iNOS mRNA expression in vivo. When mice were rendered hyperglycemic by administration of STZ, aortic iNOS mRNA levels rose threefold (Fig. 1*G* and *H*). Immunohistochemistry on aortas isolated from STZ-induced diabetic mice indicated a selective increase in iNOS immunoreactivity in endothelial cells (Fig. 1*I*, first two panels from the left). We expected that the rise in NO levels in hyperglycemic conditions would result in peroxynitrite production through ROS, leading to increased lipid peroxidation (22,23). Accordingly, we ob-

served a twofold increase of lipid peroxides levels (measured as TBARS) in the plasma of STZ diabetic mice compared with normoglycemic controls (Fig. 1*J*).

iNOS activation by hyperglycemia in HAECs and in mice, with the attendant increase in oxLDL formation, represents an attractive mechanism to explain the early stages in the progression of atherosclerosis. Therefore, we sought to identify the biochemical mechanism underlying glucose regulation of iNOS in HAECs and in mice.

High glucose and insulin/growth factor withdrawal induces Foxo1 nuclear translocation. The forkhead protein Foxo1 integrates hormonal and nutrient cues with

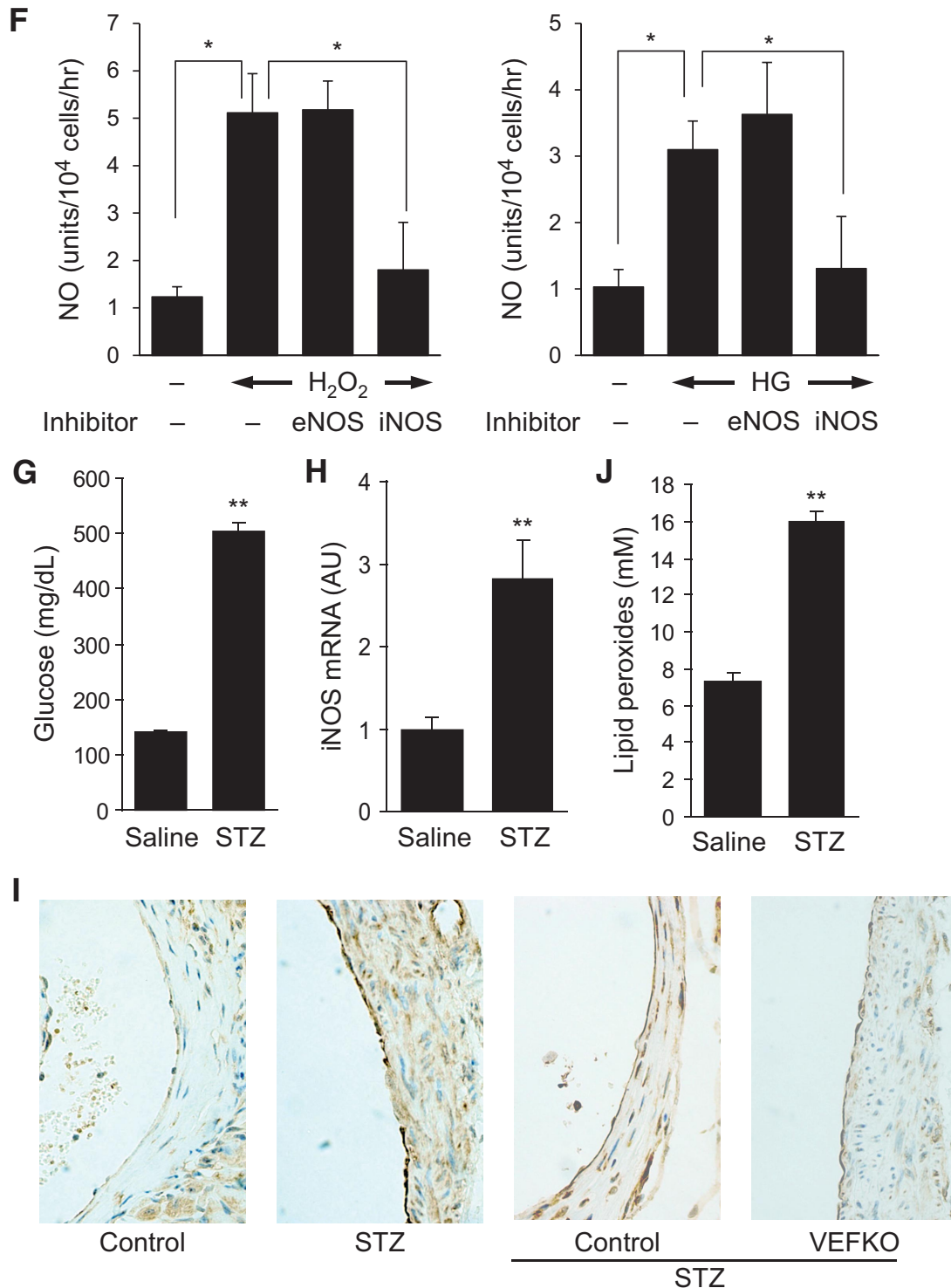


FIG. 1. Continued.

gene transcription (24). We asked whether glucose-induced oxidative stress and insulin/growth factor signaling affect the subcellular localization of a Foxo1-GFP fusion protein in HAECs. Under basal conditions (5.5 mmol/l glucose), we detected Foxo1-GFP in the cytoplasm; after incubation with increasing glucose concentrations or H₂O₂, Foxo1-GFP translocated to the nucleus, as did in response to insulin/growth factor withdrawal (Fig. 2A). To study the mechanism of glucose-induced translocation, we determined Foxo1 phosphorylation and acetylation in

cells grown in different glucose concentrations. We found that glucose decreased Foxo1 phosphorylation and acetylation in a dose-dependent manner (Fig. 2B). These data are consistent with our prior observations that glucose causes nuclear translocation of Foxo1 by promoting its deacetylation (15). Moreover, expression of a Foxo1-responsive reporter gene increased up to twofold in cells treated with high glucose or with H₂O₂ (but not with mannitol) in a time-dependent manner (Fig. 2C). These results indicate Foxo1 is activated by elevated glucose

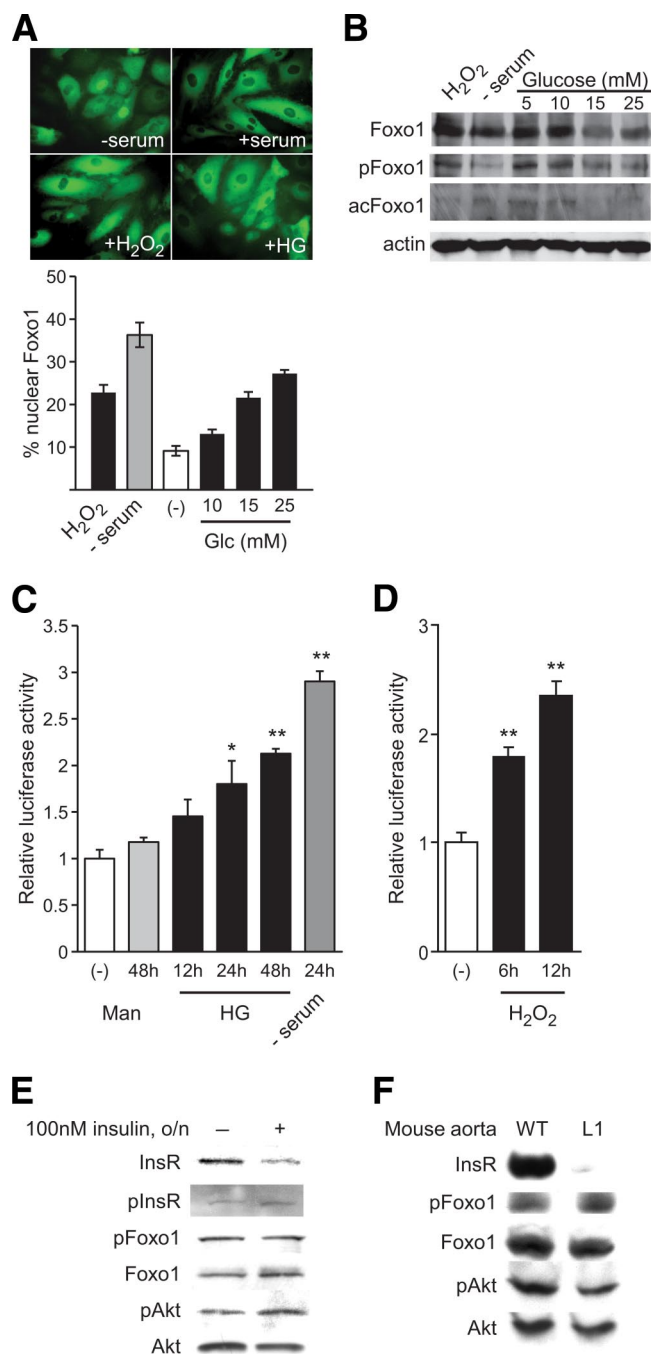


FIG. 2. Foxo1 activation and insulin receptor phosphorylation in HAECs and aortas. **A:** Foxo1-GFP localization in HAECs cultured with 2% FBS (+serum), 25 mmol/l glucose (HG), 0.5 mmol/l H₂O₂, or serum withdrawal (-serum). Data are quantified in the lower panel, which includes a dose-response curve for glucose-induced Foxo1 translocation. **B:** Foxo1 phosphorylation and acetylation determined by Western blotting with antiphospho-Foxo1 (Ser²⁵³) and antiacetyl-Foxo1 antibodies in HAECs treated with different glucose concentrations or H₂O₂ for 24 h. **C and D:** Foxo1 reporter assays in HUVECs transfected with 3xlgfBP-RE/Luc plasmid and treated with 25 mmol/l glucose (HG), mannitol (Man), or H₂O₂ (0.5 mmol/l) for the indicated lengths of time. **E and F:** Western blotting analysis of InsR, Foxo1, and Akt expression, InsR^{Y1158/Y1162/Y1163}, Foxo1^{S253}, and Akt^{S473} phosphorylation in HAECs incubated with 100 nmol/l insulin for 16 h to induce InsR downregulation (**E**), or in aortas dissected from mice lacking InsR in all cell types of the arterial wall (L1) (18) (**F**). **P* < 0.05; ***P* < 0.01 by Student's *t* test. (A high-quality digital representation of this figure is available in the online issue.)

levels and oxidative stress, as well as by insulin/growth factor withdrawal.

Insulin resistance fails to alter Foxo1 phosphorylation in HAECs and aorta. Although oxidative stress associated with hyperglycemia is thought to play a pathogenic role in atherosclerosis, outcome studies have failed to convincingly demonstrate that tight glycemic control prevents the macrovascular complications of diabetes (2). In addition to hyperglycemia, insulin resistance is also recognized as an independent risk factor for atherosclerosis (3,4). Theoretically, one would predict that insulin resistance in HAECs could also eventuate in increased Foxo1 activity, by way of reduced Akt-dependent Foxo1 phosphorylation, with increased nuclear retention (25). We examined this point in cultured cells and in mice.

First, we incubated HAECs in 100 nmol/l insulin overnight, to induce ligand-mediated receptor downregulation (26). The expectation was that a commensurate decrease in Akt and Foxo1 phosphorylation would occur, leading to increased nuclear Foxo1. The treatment caused a 50% decrease in InsR content but failed to affect phospho-InsR, phospho-Akt, and phospho-Foxo1 levels (Fig. 2E). Next, we investigated Foxo1 phosphorylation in aortas isolated from mice lacking InsR in all vascular endothelial compartments (referred to as L1) (18). Consistent with the result in insulin-treated HAECs, we detected near-normal Akt and Foxo1 phosphorylation in L1 mouse aortas (Fig. 2F). These findings can be explained by the presence of additional growth factor receptors (such as Igf1R) in HAECs (27), acting to preserve Foxo1 phosphorylation when insulin signaling is decreased. These data support a model in which hyperglycemia trumps insulin resistance as a metabolic cue regulating Foxo1 activity in endothelial cells.

Foxo1 activation mimics the effects of glucose and oxidative stress on NO and ROS production. To determine whether Foxo1 mediates iNOS-dependent NO and ROS production, we performed gain-of-function experiments in HAECs. Expression of constitutively nuclear Foxo1 (Foxo1-ADA) dose-dependently increased NO and ROS production (Fig. 3A-C) and was associated with increased iNOS but not eNOS expression (Fig. 3D). iNOS induction by Foxo1ADA was observed only in HAECs and not in mouse β TC-3 or in human THP-1 cells, suggesting that the Foxo1 effect on iNOS is specific for endothelial cells (data not shown). Addition of an iNOS, but not of an eNOS inhibitor, prevented the effect of Foxo1ADA (Fig. 3E). Finally, Foxo1 overexpression increased the levels of stable NO intermediates (NO_x) in the culture medium, an increase that was reversed by iNOS inhibition (Fig. 3F and G).

Activation of Foxo1 promotes oxidized LDL formation and impairs eNOS function by disrupting eNOS dimerization. Peroxynitrite has been shown to increase oxLDL levels and impair eNOS function, leading to blunted NO production (8) (12). We examined whether Foxo1 activation affects peroxynitrite production, LDL oxidation, and eNOS dimer formation in HAECs. We observed a dose-dependent increase in oxLDL levels in medium of HAECs transduced with Foxo1ADA (Fig. 4A), which was preempted by the addition of an iNOS inhibitor (Fig. 4B). Moreover, Foxo1 impaired eNOS dimer formation, an effect that was also reversed by the iNOS inhibitor. Glucose also decreased eNOS dimer formation in a dose-dependent manner, consistent with our hypothesis that hyperglycemia activates Foxo1 (Fig. 4C). Accordingly,

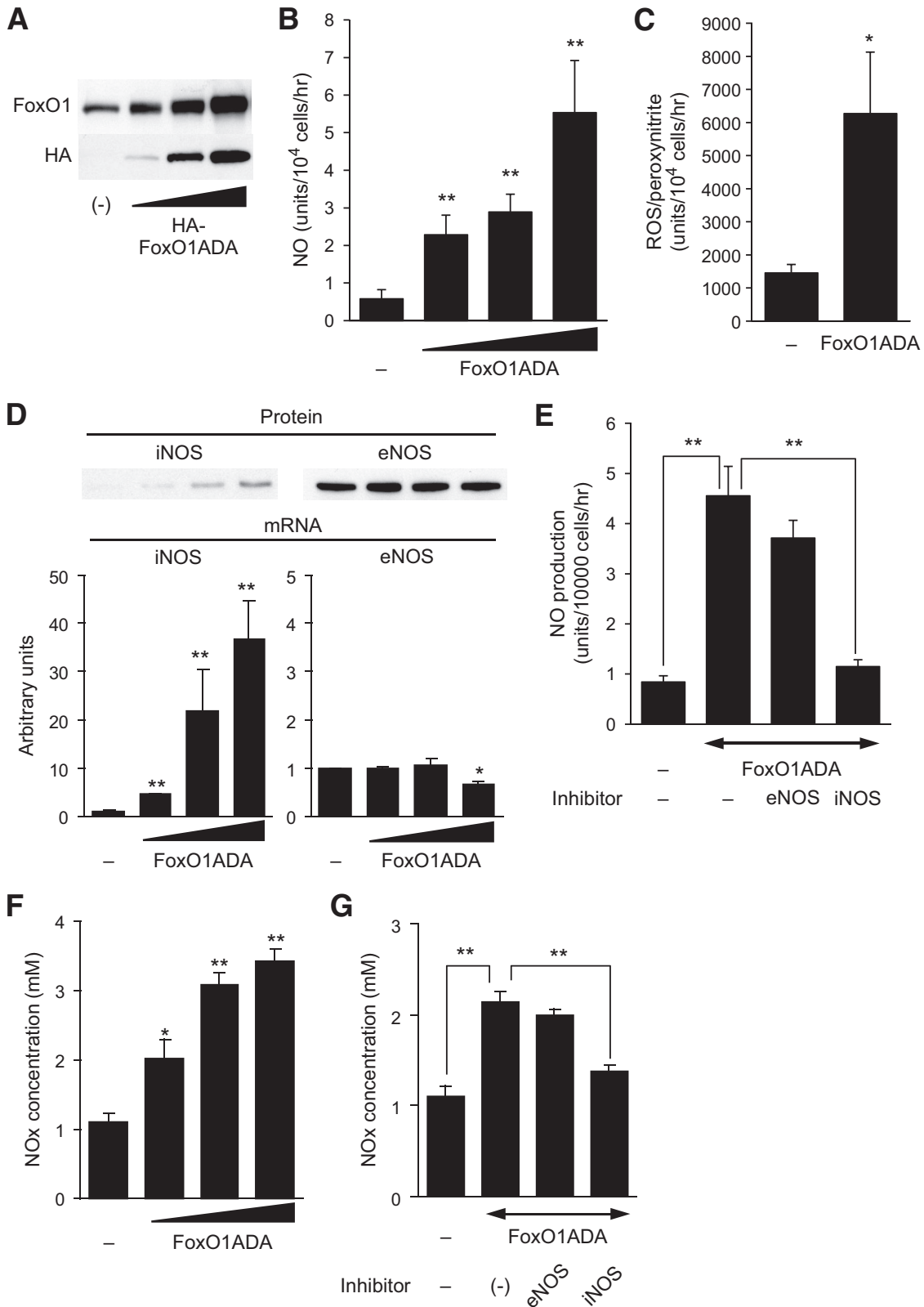


FIG. 3. Foxo1ADA increases iNOS mRNA, NO, and ROS/peroxynitrite generation. HAECs were transduced with increasing concentrations of HA-Foxo1ADA for 24 h (A–D and F) with or without pretreatment of the eNOS inhibitor, L-NAME, or iNOS inhibitor, 1400W (E and G). A: Endogenous and exogenous Foxo1 Western blotting using anti-Foxo1 and anti-HA antibodies. B and E: NO production using DAF-2DA. C: ROS/peroxynitrite production using carboxy-H₂DCFDA. D: iNOS and eNOS proteins (upper panel) and mRNA (lower panel) expression levels. F and G: Total amount of NOx concentration in the medium. *P < 0.05; **P < 0.01.

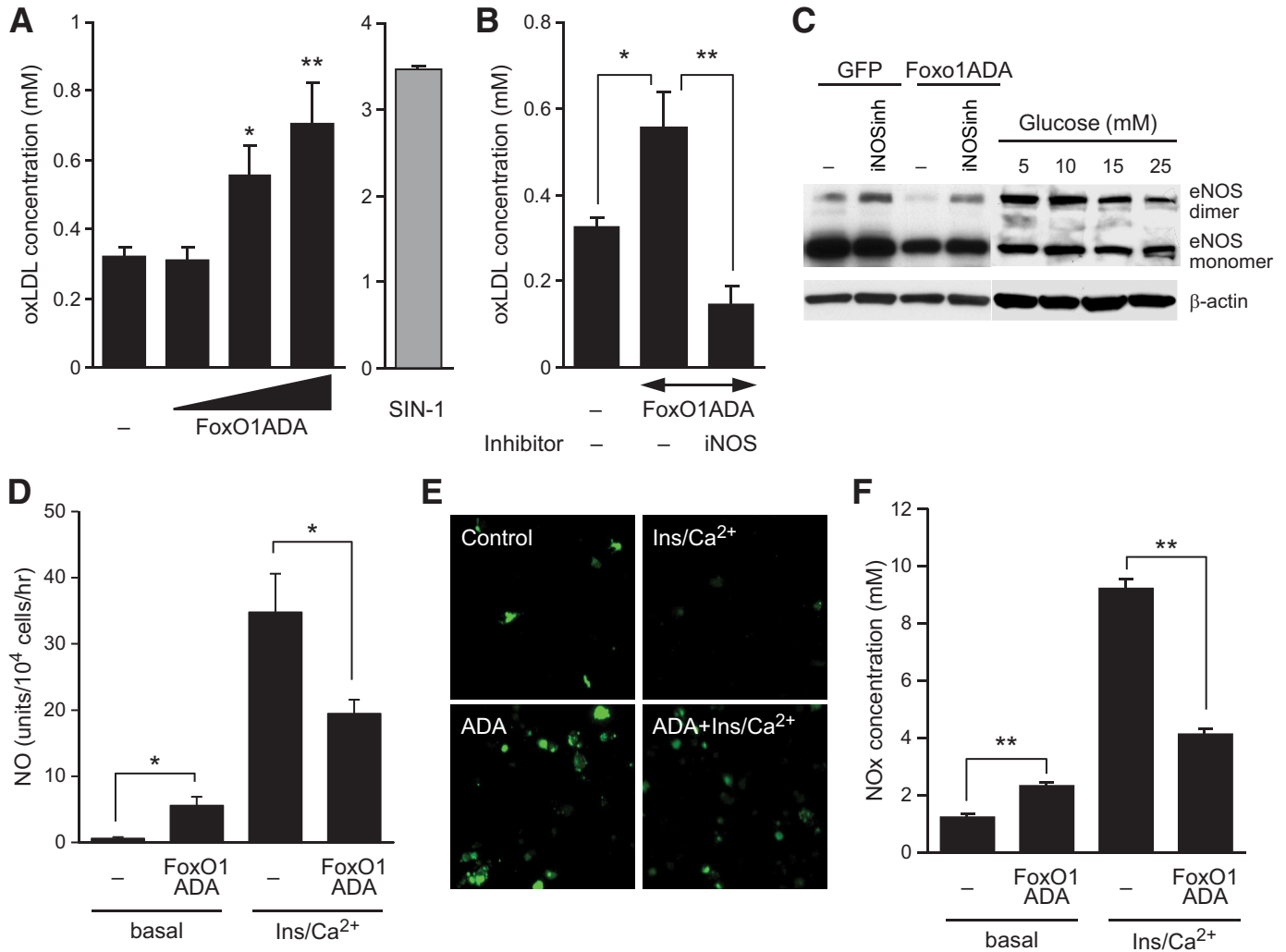


FIG. 4. LDL oxidation and eNOS dimerization in response to Foxo1ADA. **A:** LDL oxidation (measured as TBARS) in HAECs expressing Foxo1ADA and incubated with native LDL. Sin-1, a NO and superoxide donor, was used as a positive control to generate peroxynitrite. **B:** LDL oxidation in HAECs expressing Foxo1ADA and treated with the iNOS inhibitor, 1400W. **C:** Low-temperature western blotting to detect eNOS dimers in HAECs transfected with Foxo1ADA or treated with different glucose concentrations for 48 h. **D:** Basal and insulin/calcium ionophore-stimulated NO production using DAF2-DA. **E:** ROS production and **(F)** NOx concentration in the medium of HAECs transduced with Foxo1ADA and treated with insulin/calcium ionophore. * $P < 0.05$; ** $P < 0.01$ by Student's *t* test. (A high-quality digital representation of this figure is available in the online issue.)

eNOS-dependent NO production was decreased, whereas eNOS-independent basal NO production was increased (Fig. 4D), in cells transduced with Foxo1ADA. This effect was reflected in increased ROS production by Foxo1ADA, but not by eNOS activation (Fig. 4E). Measurements of stable NO intermediates (NOx) in the culture medium corroborated this result (Fig. 4F). These data indicate that Foxo1 activation in endothelial cells increases LDL peroxidation and decrease eNOS function through the iNOS-NO-peroxynitrite pathway.

Foxo1 binding to iNOS promoter is required for NO induction and LDL oxidation. Foxo1 can regulate gene expression in a DNA binding-independent manner (14). To determine whether Foxo1 binding to target DNA is required for iNOS induction and lipid peroxidation, we compared the effects of constitutively nuclear, DNA binding-competent (Foxo1ADA) and -defective (DBD-Foxo1ADA) Foxo1. DBD-Foxo1ADA failed to affect NO production and LDL peroxidation (Fig. 5A–C), demonstrating that DNA binding is required for iNOS induction. This conclusion is supported by gene reporter assays, indicat-

ing that Foxo1ADA, but not DBD-Foxo1ADA, increased expression of a reporter gene under the control of human iNOS promoter (Fig. 5D). Furthermore, ChIP assays show that Foxo1 binds to a forkhead site of the human iNOS promoter in intact chromatin isolated from HAECs (Fig. 5E).

Loss of Foxo1 function blocks iNOS induction in HAECs and in aortas of diabetic mice. We next asked whether Foxo1 loss-of-function prevented iNOS induction. To this end, we transfected HAECs with siRNA constructs targeting Foxo1, Foxo3, or Foxo4, singly or in combination. After transfection of phycoerythrin-labeled control siRNA, we observed red fluorescence in virtually all HAEC nuclei (Fig. 6A, blue), indicating high efficiency of siRNA delivery (Fig. 6A). Transfection of Foxo1, Foxo3, or Foxo4 siRNAs led to 92, 87, and 81% decreases in target mRNA expression, respectively. Cotransfection of the pooled siRNAs decreased Foxo1, Foxo3, and Foxo4 mRNA by 87, 77, and 82%, respectively (Fig. 6B). In untransfected HAECs, iNOS expression increased 2.8- and 2.5-fold after H_2O_2 and high glucose treatment, respectively. Foxo1

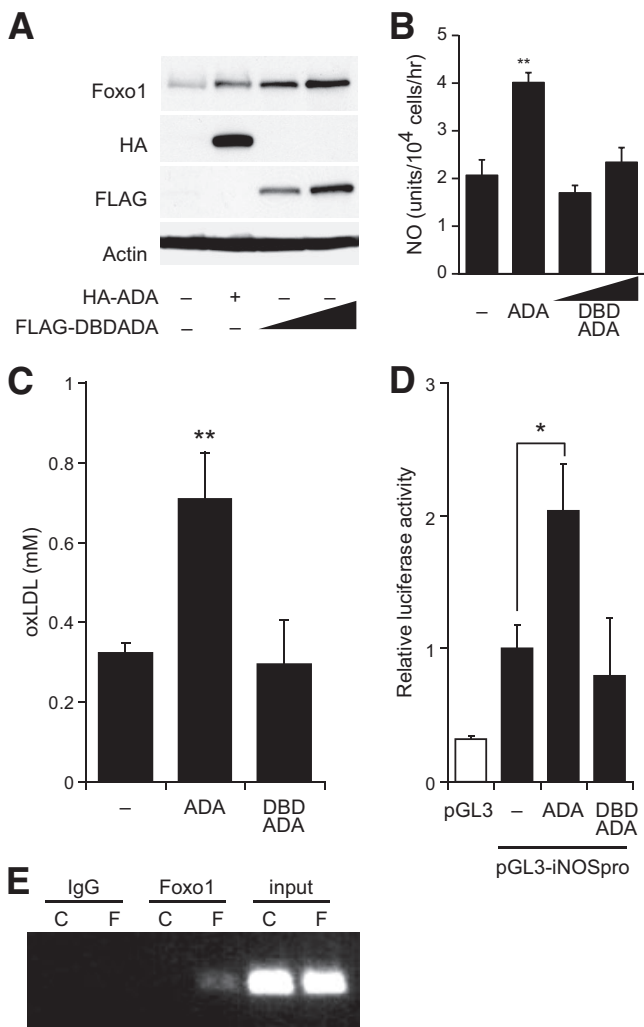


FIG. 5. Foxo1 binding to the iNOS promoter is required for NO production and LDL oxidation. **A:** Western blotting with anti-Foxo1, anti-HA, anti-FLAG, and antiactin antibodies in HAECs expressing HA-Foxo1ADA or FLAG-DBD-Foxo1ADA adenoviruses (14). **B:** NO production was estimated with DAF-2DA. **C:** LDL oxidation in HAECs cultured in the presence of native LDL. **D:** iNOS promoter reporter assays in transiently transfected HUVECs after transduction with HA-Foxo1ADA or FLAG-DBD-Foxo1ADA adenoviruses. **E:** ChIP assays of intact chromatin isolated from HAECs transduced with control ("C") or HA-Foxo1ADA ("F") adenoviruses, using rabbit control IgG and anti-Foxo1 antibodies. Total input DNA is shown on the right. * $P < 0.05$; ** $P < 0.01$.

knockdown blunted the rise of iNOS mRNA and protein in response to oxidative stress, whereas knockdown of either Foxo3 or Foxo4 had no effect (Fig. 6C). These data indicate that Foxo1 is the main Foxo isoform required for iNOS activation by oxidative stress.

To provide in vivo evidence for a role of Foxo1 in mediating the effects of hyperglycemia on iNOS and lipid peroxidation, we generated vascular endothelial cell-specific Foxo1 knockout mice (Tie2-cre/*Foxo1*^{fllox/fllox}) (Fig. 7A and B). Mice were born at term in Mendelian ratios and showed no gross or metabolic abnormalities (data not shown). We rendered them diabetic with STZ and measured aortic iNOS mRNA and serum lipid peroxides levels. We detected a 49% decrease of iNOS mRNA and a 32% decrease of serum lipid peroxide levels (Fig. 7C) in hyperglycemic Tie2-cre/*Foxo1*^{fllox/fllox} mice, compared with *Foxo1*^{fllox/fllox} controls. Immunohistochemical analyses indicated that the STZ-induced increase of iNOS protein was

blunted in Tie2-cre/*Foxo1*^{fllox/fllox} mice (Fig. 1I, third and fourth panel from the left). These results suggest that Foxo1 activation in diabetic endothelial cells is required for oxLDL generation.

DISCUSSION

The cardiovascular complications of diabetes represent a major threat to public health (28,29). Not only does excess morbidity and mortality for CVD already account for most of the financial burden of diabetes (28), but this complication appears to be uniquely resistant to tight glucose control (2,30). In addition, progress in reducing mortality from ischemic and nonischemic heart disease with lipid-lowering drugs is being offset by the soaring rates of diabetes-related CVD (31). Finally, it is becoming increasingly clear that effects of antidiabetic medications should be viewed in the context of CVD outcome studies and not simply of glucose control (32).

The clinical complexity of diabetes-related CVD stems from the heterogeneity of molecular mechanisms underlying atherosclerotic plaque development, progression, and eventual rupture. The present work focused on early events occurring in vascular endothelial cells and more precisely on the mechanisms by which hyperglycemia and insulin resistance affect oxLDL generation and endothelial dysfunction. Our data provide a mechanistic foundation for recent work, showing that ablation of Akt1, the predominant Akt isoform in endothelial cells, smooth muscle cells, and monocytes, leads to severe atherosclerosis in apolipoprotein E knockout mice, through a mechanism dependent on endothelial dysfunction (33). We propose that the next step in this process is activation of Foxo1 (through impaired Akt-dependent phosphorylation), followed by iNOS induction, generation of ROS/peroxynitrite, oxLDL production, impairment of eNOS activity, and endothelial dysfunction.

Two aspects of the identification of Foxo1 as an effector of oxidative stress damage in response to hyperglycemia have noteworthy pathophysiologic implications. First, it is apparent that Foxo1 is more readily activated in response to hyperglycemia than to insulin resistance in endothelial cells. This finding suggests that although insulin-resistant subjects are predisposed to atherosclerosis and suffer disproportionately from its consequences independently of diabetes (3,4), this may occur primarily through oxidative stress, rather than impaired insulin signaling per se. Indeed, the recent demonstration that increased free fatty acid levels can bring about oxidative stress in euglycemic conditions strengthens this conclusion (34). The failure of insulin resistance to activate Foxo1 (via de-phosphorylation) could be because of compensatory signals through IGF-1 receptors, which outnumber insulin receptors on vascular endothelial cells (27). Moreover, additional surface receptors for growth factors and fluid shear stress (35) enable vascular endothelial cells to maintain near-normal Akt phosphorylation through different pathways, when InsR signaling has been dampened or genetically ablated, as exemplified in Fig. 2F.

As a second consideration, it is interesting to compare and contrast the present findings with Foxo1 activation in response to oxidative stress in pancreatic β -cells (15). In β -cells, Foxo1 nuclear translocation protects against glucose toxicity, at least in the short term (15); whereas in vascular endothelial cells, Foxo1 activation boosts their oxidative capacity through ROS/peroxynitrite generation,

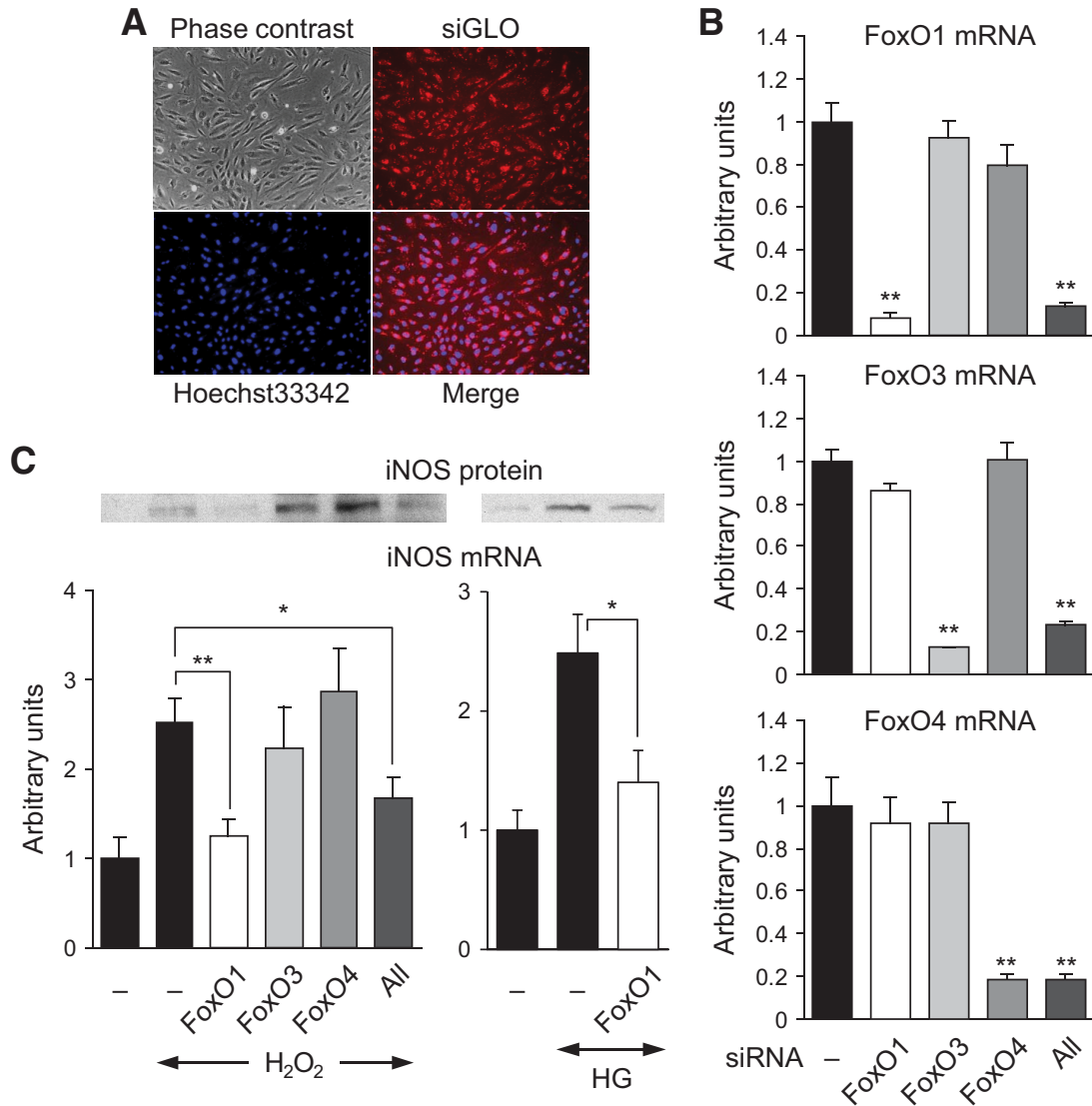


FIG. 6. siRNA-mediated Foxo1 knockdown prevents iNOS mRNA induction. *A*: Phase contrast image of transfected HAECs (*upper left*). siRNA transfection efficiency, determined using siGLO Red transfection indicator (red, *upper right*). Nuclei are visualized with Hoechst 33342 (blue, *lower left*). Merged images are shown in the lower right panel. *B*: Foxo isoform and (*C*) iNOS expression in HAECs transfected with Foxo1, Foxo3, and Foxo4 siRNA, singly or in combination. * $P < 0.05$; ** $P < 0.01$ by Student's *t* test. (A high-quality digital representation of this figure is available in the online issue.)

with attendant lipid peroxidation and eNOS dysfunction. This observation is consistent with the fact that Foxo1 increases iNOS transcription in endothelial cells but not in pancreatic β -cells or monocytes (data not shown). The present data dovetail with scattered evidence in the literature for a role of iNOS in the adverse metabolic consequences of hyperglycemia in skeletal muscle, adipose, and liver (36,37). In addition, although in advanced atherosclerotic lesions the role of macrophage-derived iNOS is quantitatively predominant, histopathology of fetal human aortic samples shows that LDL and oxLDL are frequently found in the absence of monocyte/macrophages, but the opposite is rare (38). Thus, our results suggest that iNOS activation by Foxo1 drives the generation of plasma lipid peroxides and endothelial dysfunction often seen in diabetes (36,39,40). Furthermore, iNOS has been shown in most (41–44), but not all studies (45), to promote atherosclerosis development. For example, administration of an iNOS inhibitor prevented atherogenic lesion progression in atherosclerosis-prone mice (46,47), and was associated

with lower plasma lipid peroxides levels (41,43,44). These results are consistent with our *in vivo* data, showing lower plasma lipid peroxides and aortic iNOS in STZ-induced diabetic mice lacking Foxo1 in vascular endothelial cells.

In conclusion, our data provide a model biochemical pathway through Foxo1, linking increased plasma glucose levels with the early pathophysiologic manifestations of atherosclerosis in diabetes. When viewed in the context of prior work, demonstrating that Foxo1 activation underlies many consequences of insulin resistance in liver and brain (48–50), this model can be subsumed under a unifying theory of the independent effects of impaired insulin action and hyperglycemia on the progression of diabetes and its complications.

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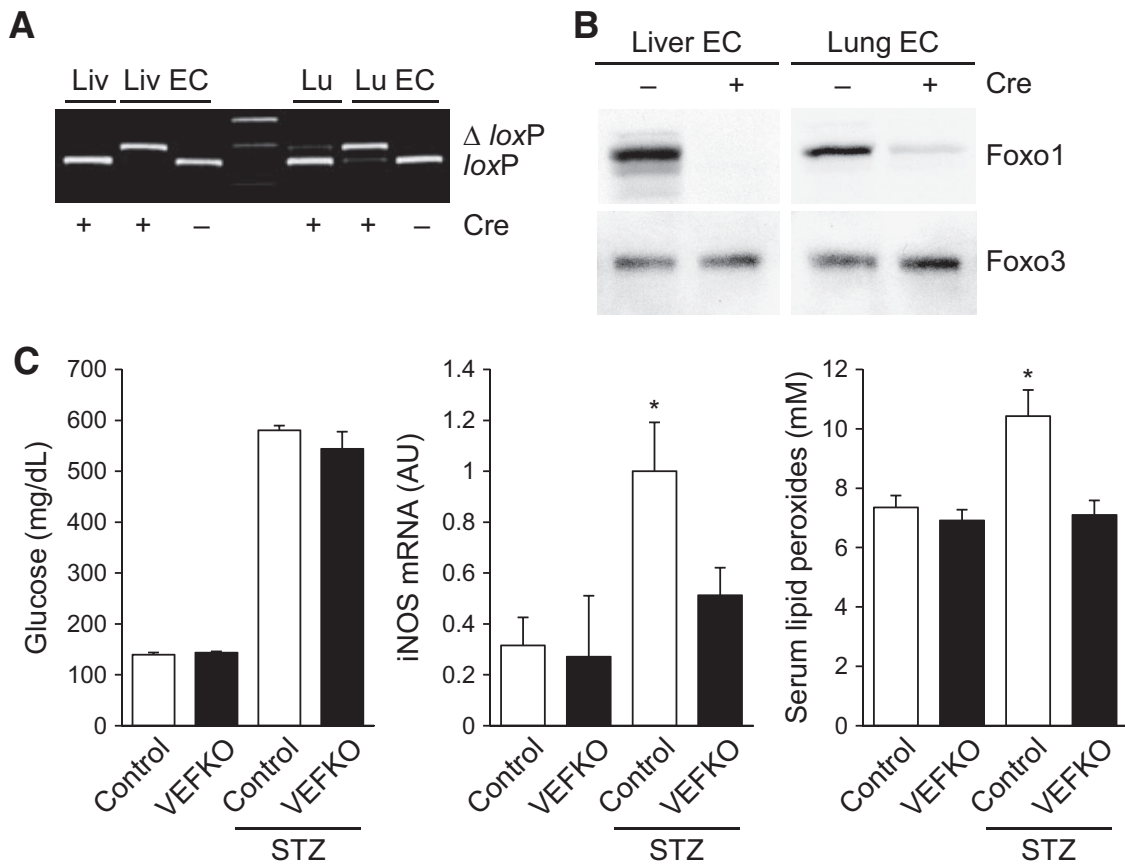


FIG. 7. Conditional ablation of Foxo1 in vascular endothelial cells (EC) impairs iNOS induction and lowers lipid peroxides levels in diabetic mice. A: PCR genotyping of whole tissue or affinity-purified liver and lung endothelial cells from *Tie2-cre/Foxo1^{fllox/fllox}* and *Foxo1^{fllox/fllox}* mice (17). **B:** Foxo1 and Foxo3 Western blot in endothelial cells isolated from *Tie2-cre/Foxo1^{fllox/fllox}* and *Foxo1^{fllox/fllox}* mice. **C:** Blood glucose, aortic iNOS mRNA, and serum lipid peroxides levels in *Tie2-cre/Foxo1^{fllox/fllox}* and *Foxo1^{fllox/fllox}* mice, examined 2 weeks after diabetes induction by STZ ($n = 12$ for each genotype). * $P < 0.05$ by Student's t test. VEFKO, vascular endothelial cell Foxo1 knockout.

No potential conflicts of interest relevant to this article were reported.

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