



Xuanchun Wang,^{1,2} Samuel M. Lockhart,^{1,3} Thomas Rathjen,^{1,4} Hassan Albadawi,⁵
 Ditte Sørensen,^{1,6,7} Brian T. O'Neill,¹ Nishant Dwivedi,¹ Simone R. Preil,⁸
 Hans Christian Beck,⁸ Sally L. Dunwoodie,^{9,10,11} Michael T. Watkins,⁵
 Lars Melholt Rasmussen,⁸ and Christian Rask-Madsen¹



Insulin Downregulates the Transcriptional Coregulator CITED2, an Inhibitor of Proangiogenic Function in Endothelial Cells

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In patients with atherosclerotic complications of diabetes, impaired neovascularization of ischemic tissue in the myocardium and lower limb limits the ability of these tissues to compensate for poor perfusion. We identified 10 novel insulin-regulated genes, among them *Adm*, *Cited2*, and *Ctgf*, which were downregulated in endothelial cells by insulin through FoxO1. CBP/p300-interacting transactivator with ED-rich tail 2 (CITED2), which was downregulated by insulin by up to 54%, is an important negative regulator of hypoxia-inducible factor (HIF) and impaired HIF signaling is a key mechanism underlying the impairment of angiogenesis in diabetes. Consistent with impairment of vascular insulin action, CITED2 was increased in cardiac endothelial cells from mice with diet-induced obesity and from *db/db* mice and was 3.8-fold higher in arterial tissue from patients with type 2 diabetes than control subjects without diabetes. CITED2 knockdown promoted endothelial tube formation and endothelial cell proliferation, whereas CITED2 overexpression impaired HIF activity *in vitro*. After femoral artery ligation, induction of an endothelial-specific HIF target gene in hind limb muscle was markedly upregulated in mice with endothelial cell deletion of CITED2, suggesting that CITED2 can

limit HIF activity *in vivo*. We conclude that vascular insulin resistance in type 2 diabetes contributes to the upregulation of CITED2, which impairs HIF signaling and endothelial proangiogenic function.

Coronary artery and peripheral vascular disease are major complications of diabetes. In these atherothrombotic diseases, angiogenesis is an important component in the complex response to acute and chronic ischemia. Diabetes is characterized by an impaired ability of hypoxic tissues to improve perfusion through angiogenesis (1). This impairment is caused by reduced activation of hypoxia-inducible factor (HIF), changes in growth factor signaling, abnormalities in inflammation accompanying the response to ischemia, and changes in mobilization and function of bone marrow-derived proangiogenic cells (1). There is obvious potential benefit from improving perfusion without, or in addition to, surgical intervention. However, despite efficacy of several strategies to improve angiogenesis in animal models of diabetes (1), there is no

¹Joslin Diabetes Center and Harvard Medical School, Boston, MA

²Huashan Hospital, Fudan University, Shanghai, People's Republic of China

³Queen's University Belfast, Belfast, U.K.

⁴Novo Nordisk A/S, Måløv, Denmark

⁵Department of Surgery, Massachusetts General Hospital, Boston, MA

⁶University of Southern Denmark, Odense, Denmark

⁷Danish Diabetes Academy, Odense, Denmark

⁸Center for Individualized Medicine of Arterial Diseases (CIMA), Odense University Hospital, Odense, Denmark

⁹Victor Chang Cardiac Research Institute, Sydney, New South Wales, Australia

¹⁰St Vincent's Clinical School, Faculty of Medicine, University of New South Wales, Sydney, New South Wales, Australia

¹¹School of Molecular Bioscience, University of Sydney, Sydney, New South Wales, Australia

Corresponding author: Christian Rask-Madsen, christian.rask-madsen@joslin.harvard.edu.

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X.W. and S.M.L. contributed equally to this work.

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established clinical pharmacological approach to improve angiogenesis in diabetes.

Insulin resistance is a central feature of type 2 diabetes (2). Insulin can promote angiogenesis by upregulating expression of vascular endothelial growth factor (VEGF) (3,4). In addition, insulin can act directly on vascular endothelial cells to promote angiogenesis. Knockout of the insulin receptor in endothelial cells in a mouse model of oxygen-induced retinopathy caused a pronounced reduction of retinal neovascularization, with the number of preretinal cells reduced by 57% (5). This reduction of retinal angiogenesis is similar in magnitude to the effect of anti-VEGF therapy in the same mouse model (6). Therefore, insulin resistance in endothelial cells may contribute to the impaired neovascular response to ischemia in obesity and type 2 diabetes.

Knowledge about the downstream effectors of the proangiogenic action of insulin on vascular endothelium is mostly limited to regulation of nitric oxide (NO) (7). Increased synthesis of NO (8–11) is one of the most established actions of insulin in endothelial cells, and NO is a critical mediator of angiogenesis (12). Insulin increases endothelial-derived NO through phosphorylation of endothelial NO synthase (eNOS) by Akt and by increasing expression of eNOS (2,9). Consistent with this mechanism, the bioavailability of endothelial-derived NO is decreased in patients with diabetes (13,14).

Insulin also acts as a potent inhibitor of FoxO transcription factors in many cell types, and endothelial cell FoxO has an angiostatic role in postnatal vascular homeostasis and angiogenesis (15,16). We therefore decided to investigate the ability of insulin to regulate 21 direct FoxO targets previously implicated in angiogenesis. These candidates were identified in adult mice harboring an inducible Cre transgene and “floxed” genes for the three major FoxO isoforms, FoxO1, 3, and 4 (16). The triple knockout mice developed hemangiomas in the uterus, liver, and skeletal muscle. The authors of this study hypothesized that genes that were differentially expressed in endothelial cells from hemangioma-prone and hemangioma-resistant vascular beds were driving proangiogenic function (16). Using gene array analysis, gene expression was measured in endothelial cells cultured from hemangioma-prone liver and compared with gene expression in endothelial cells from lung, which was not susceptible to the development of hemangioma. The promoter regions of genes differentially regulated in liver compared with lung endothelial cells were then searched for FoxO target sequences. In the majority of genes selected this way, chromatin immunoprecipitation confirmed that FoxO bound to their promoter region (16). These results prompt the question of how these direct FoxO target genes are regulated by physiological stimuli and disease.

We screened these candidates to determine if they were regulated by insulin in endothelial cells in a FoxO1-dependent manner. With this approach, we identified 10 novel insulin-regulated genes that may modulate angiogenesis in the context of insulin resistance. To confirm the validity of this concept, we further studied one of these genes, CBP/p300-interacting transactivator with ED-rich tail 2 (CITED2), a known inhibitor of HIF-1 α transactivation (17). In the

following sections, we describe how CITED2 expression in endothelial cells is downregulated by insulin and increased in insulin resistance and type 2 diabetes in animals and humans. We demonstrate that CITED2 inhibits endothelial cell HIF transactivation and propose that inhibition of CITED2 may improve angiogenesis in type 2 diabetes. In addition, the novel insulin-regulated gene program described here holds potential for further exploration of treatment targets in vascular complications of diabetes.

RESEARCH DESIGN AND METHODS

Additional methods are described in the Supplementary Data.

Animals

Mice with a floxed mutation in the *Foxo1* gene (16) were provided by Dr. Domenico Accili (Columbia University). Conditional knockout mice with gene deletion in vascular endothelial cells were generated by breeding mice with a Cre transgene under control of the VE-cadherin (*Cdh5*) promoter (stock number 006137; The Jackson Laboratory) (18) with *Insr*^{lox/lox} mice (19) or *Cited2*^{lox/lox} mice (20). *db/db* mice and age-matched *db/+* mice from the same colony were purchased from The Jackson Laboratory (stock number 000697).

Cell Culture

Primary mouse lung, liver, or myocardial endothelial cells were isolated and cultured as described previously (21). MS1 endothelial cells were purchased from the American Type Culture Collection and cultured in DMEM containing 5% FBS. Human umbilical vein endothelial cells (HUVECs) (Lonza) were cultured in EGM-2 medium (Lonza). HUVECs were used between passages two and five.

Human Mammary Artery Biopsies

Human mammary artery tissue was harvested from patients with type 2 diabetes or control subjects without diabetes undergoing coronary artery bypass grafting. Tissue left over from preparing the arterial graft was collected in the operating room and periadventitial tissue quickly removed. Parts of the cleaned vessel were either fixed in formalin or flash frozen. Immunohistochemistry of human arteries was performed on sections of paraffin-embedded tissue using a rabbit polyclonal antibody against CITED2 (catalog no. 7067; ProSci) after antigen retrieval by boiling in citrate buffer.

Adenovirus

Adenoviral constructs expressing mouse wild-type FoxO1 or a constitutively active T24A/S253D/S316A FoxO1 triple mutant were provided by Dr. Domenico Accili (22).

Real-time PCR and Western Blotting

Real-time PCR and Western blotting were performed as previously described (21). Primer sequences are shown in Supplementary Table 1. CITED2 Western blotting of mouse and human cells and tissue was performed using a polyclonal rat CITED2 antibody (catalog no. MAB5005; R&D Systems). In experiments where samples were prepared in several batches, the control conditions were set at 100% in individual experiments and therefore have no error estimates.

Transfection of HUVECs With Plasmids and Small Interfering RNA

To overexpress CITED2, HUVECs were transfected with a plasmid containing the full-length cDNA of the human CITED2 gene, a gift from Dr. Shoumo Bhattacharya (17) (plasmid no. 48184; Addgene). The backbone vector pcDNA 3.1 (Invitrogen) was used as a control. A plasmid expressing hypoxia-regulated element (HRE)-luciferase was a gift from Navdeep Chandel (23) (plasmid no. 26731; Addgene). For knockdown of CITED2, a small interfering (siRNA) with the sense sequence 5'-UGC AGA AGC UCA ACA ACC AdTdT-3' was used. An siRNA targeting a nonmammalian gene (eGFP) was used as a control.

Flow Cytometry and FACS

Flow cytometry of endothelial cells in culture was done after trypsin treatment to produce single-cell suspensions, stained with antibodies, and analyzed on an LSRII instrument (BD Biosciences). For FACS, the ventricles of the heart or both gastrocnemius muscles were minced with scalpels and then dissociated for 1 h in DMEM containing 2 mg/mL (560 units/mL) collagenase I (Worthington), 3 mg/mL Dispase (Roche), and 0.2 mg/mL (400 Kunitz units/mL) DNase I (Millipore). Filtered cells were stained with antibodies and sorted on a Moflo Legacy (Cytomation, currently Beckman Coulter) or BD FACSAria II SORP (BD Biosciences). Additional detail is described in the Supplementary Data.

Tube Formation Assay

HUVECs were transfected with CITED2 siRNA or plasmid as described above. After 48 h, cells were trypsin treated and 5,000 cells were plated on Matrigel. Tube formation was photographed through a phase-contrast microscope 4 h later. Images from randomly chosen microscopic fields were selected for analysis and analyzed by Angiogenesis Analyzer for ImageJ (<http://image.bio.methods.free.fr/>) by an observer unaware of culture conditions. The software automatically analyzes components of endothelial cell tubular networks (Supplementary Fig. 5).

Mouse Hind Limb Surgery

Femoral artery ligation was performed as described previously (24) with modifications as explained in the Supplementary Data.

Ethical Considerations

All protocols for animal use and euthanasia were reviewed and approved by the Animal Care Committee of the Joslin Diabetes Center and were in accordance with National Institutes of Health guidelines. The study of discarded arterial tissue from patients undergoing coronary artery bypass surgery was approved by the regional ethical committee in Denmark (Region Syddanmark, ID S-20100044) and a protocol was submitted to the Danish Data Protection Agency (Datatilsynet) as required by Danish law.

Statistical Analysis

Comparisons were made using paired Student *t* test for the cell culture studies and unpaired Student *t* test or Wilcoxon

rank-sum test for the clinical data and animal studies, with $P < 0.05$ considered statistically significant. In text and graphs, data are presented as the mean \pm SEM.

RESULTS

Identification of Insulin-Regulated FoxO1 Target Genes

Our aim was to discover novel insulin-regulated genes in endothelial cells with a role in angiogenesis. In the study by Paik et al. (16) described above, results were derived from analysis of endothelial cells from FoxO1/3/4 knockout mice. However, the described proangiogenic phenotype was prominent in FoxO1 knockout mice, whereas knockout of FoxO3, FoxO4, or both in combination did not result in susceptibility to hemangioma formation. Therefore, we reasoned that genes altered by FoxO1 knockout alone were more likely to have potent angiogenic function.

Endothelial cells were isolated from livers of *Foxo1^{lox/lox}* mice by two rounds of immunomagnetic isolation. The cultures were then infected with an adenovirus expressing

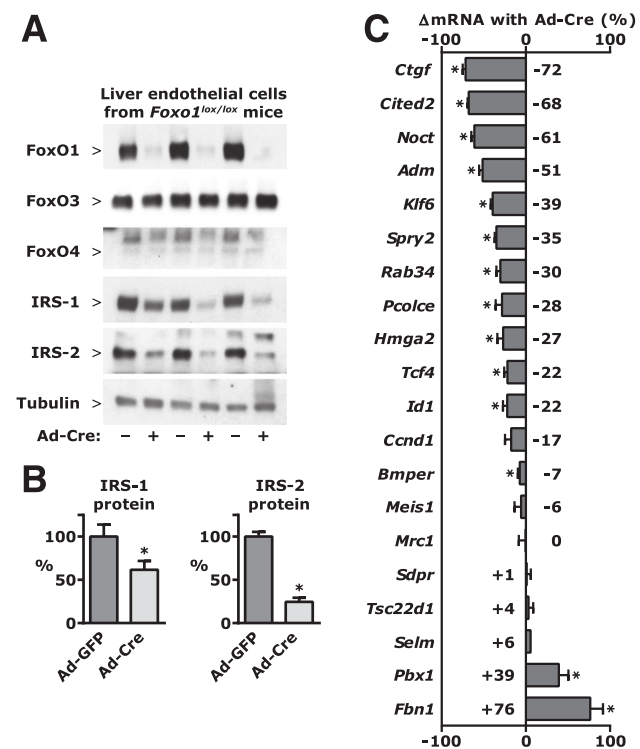


Figure 1—Gene expression in primary mouse endothelial cells with FoxO1 deletion. Primary mouse liver endothelial cells were isolated by immunomagnetic selection from mice with a “floxed” mutation of the *Foxo1* gene. Cultures were infected with adenovirus expressing Cre recombinase (Ad-Cre) or with control adenovirus expressing GFP only (Ad-GFP). **A**: Protein expression was measured in cell lysate by Western blotting. **B**: Quantitative analysis of IRS-1 and IRS-2 protein based on densitometry of Western blot. **C**: RNA was isolated from cell lysate and analyzed by real-time PCR. The changes in mRNA expression in Ad-Cre conditions relative to Ad-GFP conditions are depicted. Mean results from four independent experiments are shown. * $P < 0.05$.

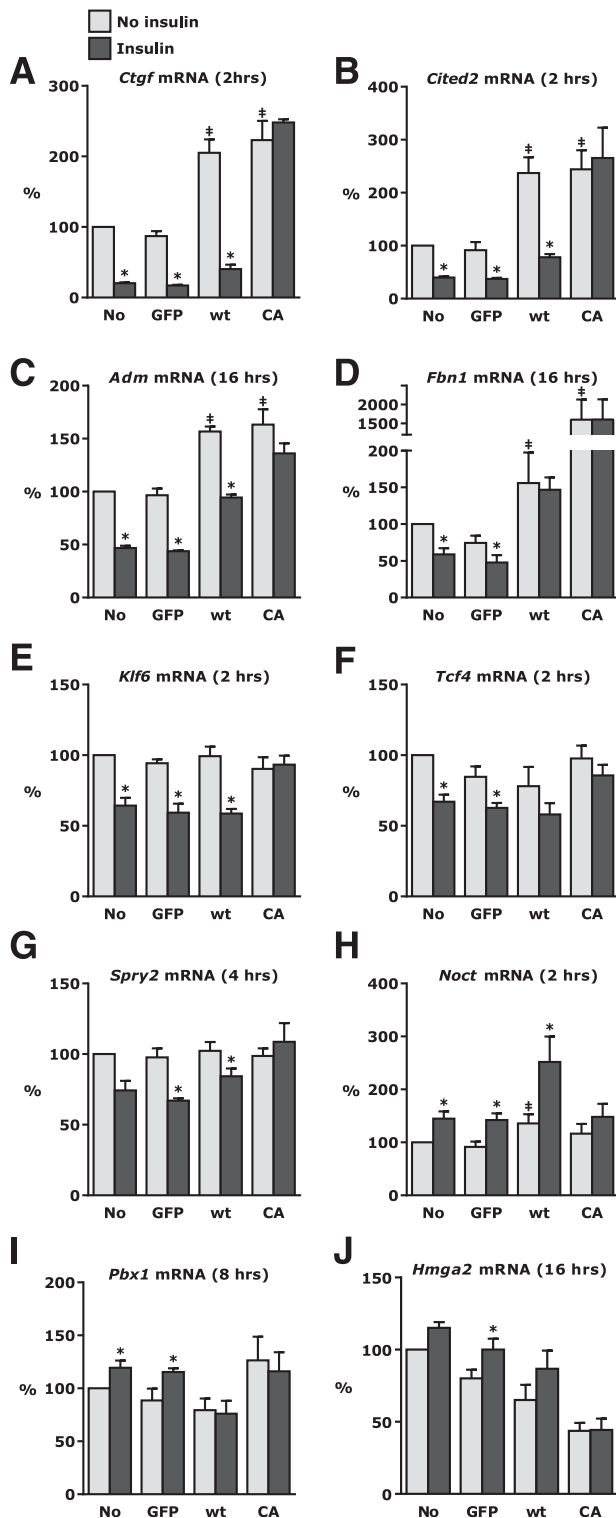


Figure 2—Gene regulation by FoxO1 and insulin in endothelial cells. A–J: MS1 endothelial cells were mock infected (No) or infected with adenovirus vectors expressing GFP (GFP), wild-type FoxO1 (wt), or a FoxO1 mutant made constitutively active by Ser to Ala mutations at three residues phosphorylated by Akt (CA). After 48 h, including serum starvation overnight, cells were stimulated with insulin (10 nmol/L, dark gray bars) or left untreated (light gray bars) for 2, 4, 8, or 16 h. RNA was isolated from cell lysate and analyzed by real-time PCR. For each gene, results from the time point with the largest insulin response are shown. All values are expressed relative to nontransfected controls. Mean values from

Cre recombinase or a control adenovirus. The proportion of CD31⁺ cells was 97.0 ± 0.4 and $98.2 \pm 0.3\%$, respectively, in recombined and control cultures (Supplementary Fig. 1A). This demonstrated a high purity of endothelial cells given that CD31⁺ cells costained for other endothelial markers, including VE-cadherin and VEGF receptor 2 (KDR) (Supplementary Fig. 1B and C). Cre-mediated recombination resulted in complete deletion of FoxO1 at the protein level without changes in FoxO3 or FoxO4 protein expression (Fig. 1A). FoxO1 knockout significantly changed expression of *Adm*, *Cited2*, *Ctgf*, *Fbn1*, *Hmga2*, *Klf6*, *Noct* (also known as *Ccrn4l*), *Pbx1*, *Spry2*, and *Tcf4* (Fig. 1C). These candidates were further studied as described below. Among the remaining genes, *Cnd1*, *Meis1*, *Mrc1*, *Sdpr*, *Selm*, and *Tsc22d1* were not regulated (Fig. 1C), whereas *Bmper*, *Id1*, *Pcolce*, and *Rab34* were regulated in the opposite direction of the change previously reported in cells cultured from Foxo1/3/4 knockout mice (16). *Nrep* (known as D0h4s114 or P311) was not detected.

In FoxO1-knockout cells, insulin receptor substrate-1 (IRS-1) and IRS-2 mRNA decreased by 73 ± 7 and $57 \pm 2\%$ (data not shown), and IRS-1 and IRS-2 protein decreased by 38 ± 12 and $75 \pm 6\%$ (Fig. 1B). These results are similar to previously published results describing that IRS-1 and IRS-2 are decreased in aortic endothelial cells from mice with endothelial-specific deletion of FoxO1/3/4 (25). Consequently, a knockout approach would not be useful to prove involvement of FoxO1 in insulin-stimulated signaling. We therefore used adenoviral vectors to express wild-type FoxO1, a constitutively active FoxO1 mutant or a control virus. It seemed important to study a wide time range of exposure to insulin as we expected the effect to be transient and the expression kinetics to differ for each gene. Because it was not feasible to expand primary mouse endothelial cells to include all control conditions at several time points, we used MS1 endothelial cells, which we have previously shown are sensitive to insulin (21). Cultures were serum starved overnight and treated with 10 nmol/L insulin for 2, 4, 8, or 16 h. mRNA expression results are shown in Fig. 2 for the time point with the largest change in the nontransfected condition.

Among the 10 genes selected, all were regulated by insulin (Fig. 2). We considered typical FoxO-dependent regulation to exist if insulin and FoxO caused reciprocal gene regulation and if insulin counteracted a change induced by wild-type FoxO1 overexpression but not one induced by overexpression of the constitutively active FoxO1 mutant. The expression of *Adm*, *Ctgf*, and *Cited2* was downregulated by insulin in a typical FoxO-dependent manner (Fig. 2A–C). *Klf6*, *Spry2*, and *Tcf4* were

three independent experiments are shown. * $P < 0.05$, compared with no insulin in the same condition with respect to adenovirus infection; ‡ $P < 0.05$, compared with insulin-untreated GFP condition.

downregulated by insulin but were not changed by FoxO overexpression (Fig. 2E–G). *Pbx1* was upregulated by insulin but was not changed by FoxO1 overexpression (Fig. 2I). Insulin also increased *Noct*, but this regulation may not be mediated through FoxO1 because *Noct* was increased by both FoxO1 expression and insulin treatment and the mutant FoxO1 did not prevent insulin regulation (Fig. 2H). *Fbn1* and *Hmga2* were regulated by insulin and ectopic FoxO1 expression in opposite directions, as expected (Fig. 2D and J), but these changes were not concordant with regulation after FoxO1 knockout in liver endothelial cells (Fig. 1C). Selection of genes for further study based on the criteria above is outlined in Supplementary Fig. 1.

Among the genes with typical regulation, *Ctgf* was downregulated by $80 \pm 1\%$ after 2 h, *Cited2* by $60 \pm 1\%$

after 2 h, and *Adm* by $54 \pm 2\%$ after 16 h (Fig. 2A–C). *Ctgf* encodes connective tissue growth factor (CTGF), a secreted protein that is a key effector of transforming growth factor- β signaling (26,27). *Cited2* encodes a transcriptional coregulator discussed extensively below, and *Adm* encodes adrenomedullin, a circulating vasodilator peptide hormone that is highly expressed in endothelial cells and vascular smooth muscle cells (28,29).

Ctgf, *Cited2*, and *Adm* were all expressed in endothelial cells freshly isolated from gastrocnemius muscle from C57BL/6 mice, although endothelial cell levels of *Ctgf* were relatively low compared with nonendothelial cells (Supplementary Fig. 2). CTGF and adrenomedullin are secreted by a number of cell types, and the importance of endothelial-derived CTGF and adrenomedullin relative to nonendothelial sources is uncertain. In contrast, the

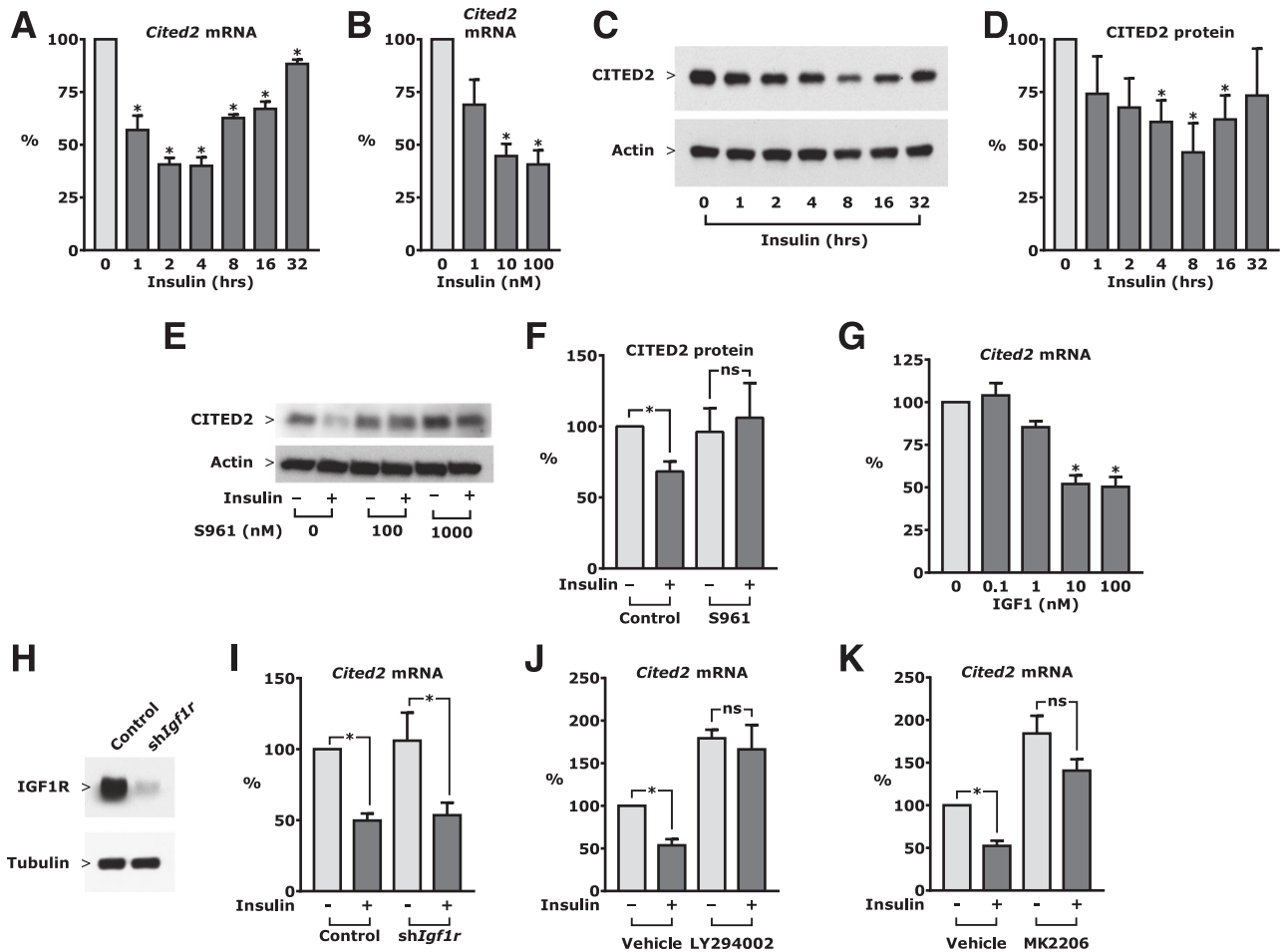


Figure 3—Features of insulin-dependent regulation of CITED2 in endothelial cells. A–K: MS1 endothelial cells were serum starved overnight and then treated with IGF1 (G) or insulin (all other panels) at a final concentration of 10 nmol/L unless otherwise indicated. RNA was isolated from cell lysate and analyzed by real-time PCR. Protein expression was measured in cell lysate by Western blotting. Mean values from three or four experiments are shown. * $P < 0.05$. A–D: Cells were treated for 2 h (B) or the duration listed. E and F: Cultures were treated with the insulin receptor antagonist S961 for 1 h followed by 10 nmol/L insulin for 8 h. G: Cultures were treated with IGF1 for 2 h. H and I: Cells were stably transfected with a lentiviral construct expressing a short hairpin RNA targeting the IGF1 receptor gene (sh*Igf1r*) and treated with insulin for 2 h (I). J and K: MS1 endothelial cells were serum starved overnight and then treated with the PI3K or Akt inhibitor LY294002 or MK2206, respectively, for 30 min, followed by treatment with 10 nmol/L insulin for 2 h, as indicated.

function of CITED2 as a transcriptional coregulator is cell autonomous. In addition, HIF is a key regulator of endothelial biology (30–33) and CITED2 has been shown to regulate HIF in nonvascular cells (17). As such, insulin-regulation of CITED2 in endothelial cells is likely important for endothelial function. Therefore, we proceeded by studying CITED2 to test our paradigm that genes regulated by insulin through FoxO are likely to be dysregulated in type 2 diabetes where vascular insulin signaling is impaired.

CITED2 Is Regulated by Insulin Through the Insulin Receptor, PI3K, and Akt

Insulin downregulated CITED2 mRNA with a nadir at 2 h in time-course experiments (Fig. 3A). The effect of insulin was potent and concentration dependent, with reductions in CITED2 mRNA of 31 ± 12 , 55 ± 6 , and $59 \pm 7\%$ by 1, 10, and 100 nmol/L insulin for 2 h (Fig. 3B). At the protein level, the maximal effect of insulin occurred after 8 h, where CITED2 was downregulated by $54 \pm 13\%$ (Fig. 3C and D).

The insulin receptor antagonist S961 efficiently blocked insulin-induced phosphorylation of Akt (Supplementary Fig. 3A) and prevented reduction of CITED2 protein after insulin treatment (Fig. 3E and F). Insulin-like growth factor-1 (IGF1) at 10 nmol/L reduced CITED2 mRNA by $48 \pm 5\%$, with no additional effect at 100 nmol/L (Fig. 3G). After RNAi-mediated downregulation of IGF1 receptor (IGF1R) protein to $91 \pm 4\%$ of control levels (Fig. 3H), insulin reduced CITED2 mRNA as efficiently as in control cells (Fig. 3I). These results demonstrate that although both insulin and IGF1 can reduce CITED2, the effect of insulin is mediated by the insulin receptor as opposed to the IGF1R. Treating endothelial cells with the PI3K inhibitor LY294002 or the Akt inhibitor MK2206 blocked insulin-stimulated downregulation of CITED2 mRNA (Fig. 3J–K). Therefore, insulin-stimulated downregulation of CITED2 is dependent on the insulin receptor, PI3K, Akt, and FoxO1. Exposure to high glucose concentrations for 78 h did not change the magnitude of insulin-induced downregulation of CITED2 compared with an osmotic control or low glucose concentrations (Supplementary Fig. 3B and C).

Next, we examined how CITED2 was regulated in endothelial cells in a vascular bed relevant to the vascular complications of diabetes. We isolated mouse myocardial endothelial cells by immunomagnetic isolation. In myocardial endothelial cells from control mice, insulin decreased CITED2 protein by $48 \pm 9\%$, but in mice with endothelial-specific knockout of the insulin receptor, insulin had no effect (Fig. 4A and B).

Endothelial cell insulin signaling through the PI3K/Akt pathway is impaired in animal models of obesity-associated insulin resistance (34,35). We therefore measured the expression of CITED2 mRNA in freshly isolated endothelial cells from mice with diet-induced obesity and from *db/db* mice. Cardiac ventricles were enzymatically dissociated and single-cell suspensions separated by FACS. This sorting

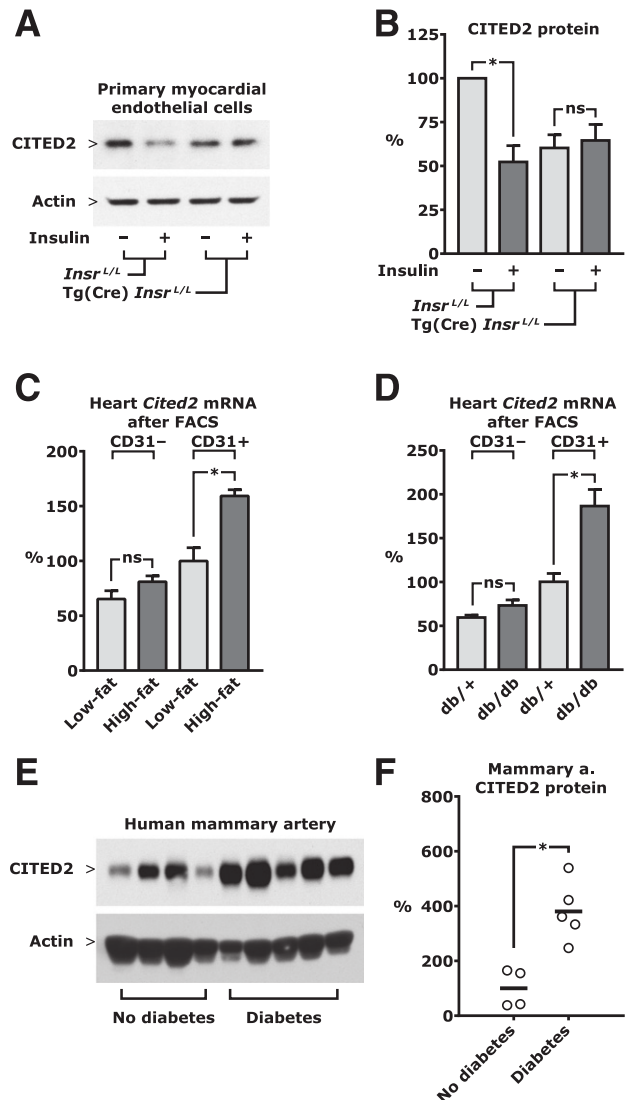


Figure 4—CITED2 expression in mouse myocardial endothelial cell culture, freshly isolated mouse cardiac endothelial cells, and human mammary arteries. **A** and **B**: Myocardial endothelial cells were isolated from mice with deletion of the insulin receptor gene (*Insr*) targeted to endothelial cells [Tg(Cre) *Insr*^{L/L}] or their controls (*Insr*^{L/L}). Cultures were serum starved overnight and then treated with 10 nmol/L insulin for 4 h. Protein expression was measured in cell lysate by Western blotting. **A**: Representative immunoblots. **B**: Quantitative analysis based on densitometry from three independent experiments. **C** and **D**: Mouse heart ventricles were enzymatically digested and sorted by FACS. Sequential gating excluded debris, doublets and aggregates, non-viable cells, and CD45⁺ cells and then selected for CD31⁺ and CD31⁻ cells. **C**: Real-time PCR was performed on samples from four mice on a regular low-fat diet and four mice on a high-fat diet. **D**: Real-time PCR was performed on samples from four *db/+* mice and three *db/db* mice. **E** and **F**: Human mammary artery tissue was obtained during coronary artery bypass surgery from five male obese patients with type 2 diabetes and four male nonobese control subjects without diabetes. Clinical characteristics are listed in Table 1. Artery left over after trimming the graft was immediately isolated from perivascular tissue and fixed in formalin (for histology) or frozen (for protein lysate). **E**: Protein expression was measured in tissue lysate by Western blotting. **F**: Quantitative analysis based on densitometry. **P* < 0.05.

resulted in efficient isolation of endothelial cell markers; in CD45⁻ CD31⁺ cells from the heart of *db/+* control mice, the endothelial cell markers *Pecam1* and *Kdr* were 35.5 ± 1.3 -fold and 50.8 ± 0.6 -fold higher, respectively, than in CD45⁻ CD31⁻ cells (Supplementary Fig. 4A and B). In CD45⁻ CD31⁺ cardiac cells isolated from mice on a high-fat diet, CITED2 mRNA was increased to $159 \pm 6\%$ of the level in mice on a low-fat diet (Fig. 4C). Similarly, in CD45⁻ CD31⁺ cardiac cells from *db/db* mice, CITED2 mRNA was increased to $187 \pm 19\%$ of the level in *db/+* mice (Fig. 4D). There was no difference in CITED2 mRNA in CD45⁻ CD31⁻ cells in mice on a low-fat versus a high-fat diet or in *db/db* versus *db/+* mice (Fig. 4C and D).

We next measured expression of CITED2 in vascular tissue from obese patients with type 2 diabetes compared with age-matched patients without obesity or diabetes. Insulin signaling through the PI3K/Akt pathway is impaired in patients with metabolic syndrome with insufficient compensation by hyperinsulinemia (2). We therefore expected increased expression of CITED2 in these patients. We collected mammary arteries from five obese patients with diabetes and four patients without obesity or diabetes undergoing coronary artery bypass surgery (Table 1). Patients in both groups had an average age of 61 years (Table 1). BMI was 35.9 ± 3.4 kg/m² in the group of patients with diabetes and 26.2 ± 0.9 kg/m² in control subjects. Lipid profiles were similar between both groups, consistent with the fact that all patients in both groups were treated with statins. There was a non-significant trend toward higher systolic and diastolic blood pressure in the group without diabetes despite the fact that all patients were treated with antihypertensive therapy. Immunohistochemistry of CITED2 revealed weak staining throughout the vascular wall but intense staining in nuclei of endothelial cells (Supplementary Fig. 4C). In tissue lysate, CITED2 was 3.8-fold higher in obese patients with diabetes than in the group without diabetes ($P = 0.003$ by Student *t* test; $P = 0.04$ by Wilcoxon rank-sum test) (Fig. 4E and F). These data, in a limited sample of patients, suggest that CITED2 is upregulated in vascular tissue in patients with obesity and type 2 diabetes and is consistent with the previous demonstration

that patients with type 2 diabetes exhibit endothelial cell insulin resistance (13,14). Importantly, this difference is maintained despite near optimal control of hemoglobin A_{1c} (HbA_{1c}), blood pressure, and plasma lipids in patients with diabetes and suggests a potential therapeutic benefit of CITED2 inhibition.

CITED2 Regulates Endothelial Proangiogenic Function and HIF Transactivation

Having demonstrated that CITED2 is upregulated in endothelial cells in diabetes, we next sought to examine the significance of these changes by characterizing the effects of CITED2 in endothelial cells. We used HUVECs for these experiments because human endothelial cells perform better in tube formation assays than primary rodent endothelial cells (36). As in MS1 endothelial cells (Fig. 3) and primary mouse myocardial endothelial cells (Fig. 4A and B), insulin decreased CITED2 protein in HUVECs (Fig. 5A). After 8 h of insulin treatment, CITED2 was 44% of control levels (Fig. 5B). HUVECs were transfected with siRNA targeting CITED2, which produced efficient knockdown with 95% reduction of CITED2 protein at 50 nmol/L siRNA (Fig. 5C). Compared with HUVECs transfected with a control siRNA, HUVECs with CITED2 knockdown increased several measures of tube formation on Matrigel (Fig. 5D and E and Supplementary Fig. 5). We then labeled HUVECs with EdU in order to determine the rate of DNA synthesis, a measure of cell proliferation (Fig. 5F and G). Knockdown of CITED2 increased EdU incorporation by 2.1 ± 0.3 -fold (Fig. 5G). We therefore conclude that CITED2 negatively affects tube formation and suppresses proliferation of endothelial cells.

CITED2 has been shown to regulate HIF transactivation in a number of cell types (17), but this effect has not been described in any vascular cells, including endothelial cells. Demonstration of this specific phenomenon is important because CITED2 can both activate and inhibit transcription factors by restricting the availability of CBP/p300 or by acting as a molecular scaffold recruiting these coactivators to the transcription factor complex. This effect must be measured by HIF transactivation rather than DNA binding. We therefore used a luciferase

Table 1—Patient characteristics

	No diabetes	Type 2 diabetes	<i>P</i>
	Mean \pm SEM	Mean \pm SEM	
Age (years)	61.7 \pm 3.7	61.6 \pm 4.5	0.99
BMI (kg/m ²)	26.2 \pm 0.9	35.9 \pm 3.4	0.02
HbA _{1c} (%)	5.6 \pm 0.0	7.0 \pm 0.0	0.03
Systolic blood pressure (mmHg)	147.0 \pm 6.2	134.4 \pm 6.1	0.15
Diastolic blood pressure (mmHg)	81.4 \pm 3.9	72.0 \pm 3.9	0.10
Total cholesterol (mmol/L)	4.1 \pm 0.4	4.3 \pm 0.5	0.82
LDL cholesterol (mmol/L)	2.2 \pm 0.3	2.0 \pm 0.3	0.53
HDL cholesterol (mmol/L)	1.2 \pm 0.2	1.0 \pm 0.1	0.38

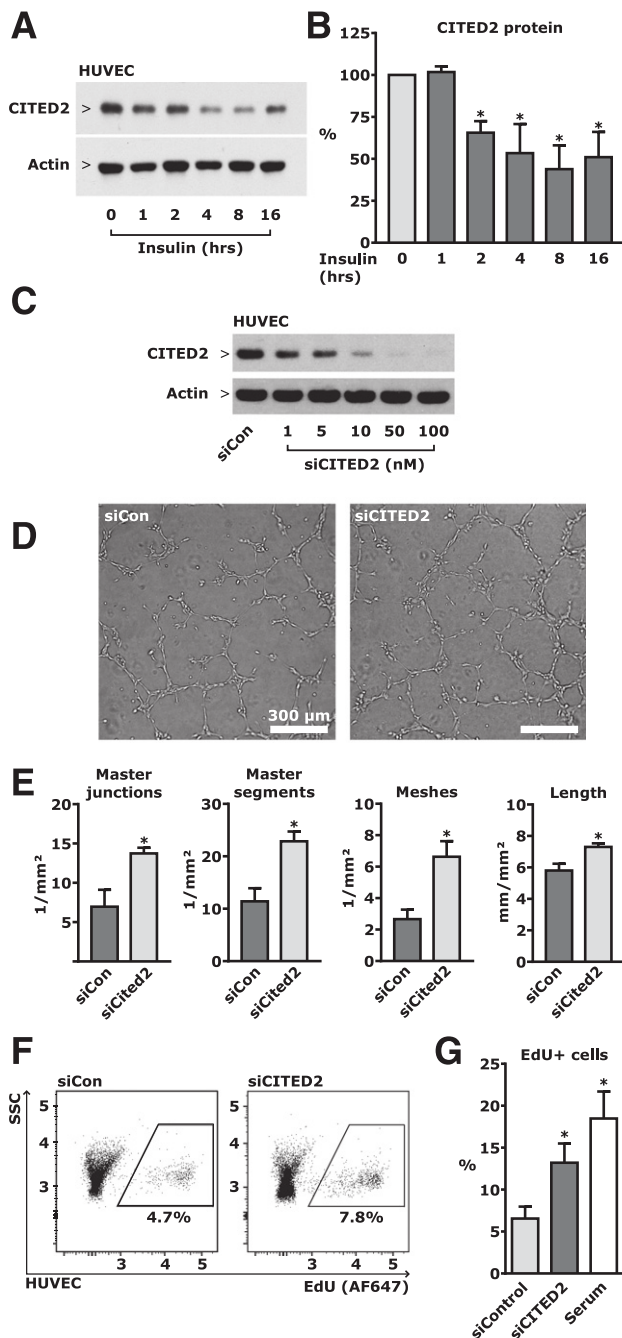


Figure 5—Proangiogenic endothelial cell function after reduced CITED2 expression. *A* and *B*: HUVECs were serum starved overnight and treated with insulin at 10 nmol/L for the times indicated. *A*: Western blot of HUVEC lysate. *B*: Quantitative analysis from three independent experiments. *C–G*: HUVECs were transfected with siRNA targeting CITED2 (siCITED2) or control siRNA (siCon). *C*: After transfection with siRNA, protein expression was measured in cell lysate by Western blotting. *D*: After siRNA transfection, HUVECs were plated on Matrigel and allowed to form tubes. Representative photos of phase-contrast microscopy are grayscale versions with adjusted contrast of the microphotos shown in Supplementary Fig. 5. *E*: Quantitative analysis of tube formation from three independent experiments. For explanation of the terms master junctions, master segments, meshes, and length, see Supplementary Fig. 5. *F* and *G*: HUVECs were transfected with siRNA 48 h before start of insulin treatment and serum starved 16 h before start of insulin treatment. Cultures were treated with 10 nmol/L insulin for 16 h and then labeled with EdU for an additional 4 h. EdU-labeled

reporter with a promoter containing hypoxia-regulated elements (HREs). Expression of this reporter in HUVECs showed that overexpression of CITED2 (Fig. 6A) decreased HRE-related activity by $59 \pm 4\%$ (Fig. 6B). Therefore, CITED2 impairs HIF transactivation in endothelial cells and inhibits endothelial cell proangiogenic function.

To establish that CITED2 regulates HIF in endothelial cells in vivo, we generated mice with cre-mediated recombination of the *Cited2* gene (20) after cross-breeding with *Cdh5-Cre* transgenic mice. In primary lung endothelial cells cultured from Tg(Cre) *Cited2*^{lox/lox} mice, CITED2 protein was reduced by $70 \pm 7\%$ compared with endothelial cells cultured from *Cited2*^{lox/lox} control mice (Fig. 6B and C). We studied these animals during hind limb ischemia after ligation of the femoral artery on one side.

We measured endothelin-1 (*Edn1*) mRNA in muscle lysate. Endothelin-1 is a HIF target gene (37) and is involved in angiogenesis (38). It is also expressed preferentially in endothelial cells, which we validated by measuring expression in nonischemic gastrocnemius muscle enzymatically dissociated and sorted by FACS. *Edn1* was 24.2 ± 3.2 -fold higher in CD31⁺ cells than in CD31⁻ cells (Fig. 6D). Therefore *Edn1* serves as a model HIF target gene specifically expressed in endothelial cells that can be used to assay changes in endothelial HIF activity in whole tissue lysate. HIF-1 α is among the most short-lived proteins with a half-life of a few minutes after restoration of normoxia after hypoxia (39), and HIF target genes are similarly short-lived with *Edn1* mRNA half-life being less than half an hour (33). We therefore measured *Edn1* in lysate from flash-frozen muscle. *Edn1* was increased in ischemic hind limb gastrocnemius muscle from C57BL/6 mice 24 h after femoral artery ligation (Supplementary Fig. 6). Expression subsequently decreased at 2, 3, and 7 days after surgery (Supplementary Fig. 6). In *Cited2*^{lox/lox} control mice, *Edn1* increased by 2.7 ± 0.4 -fold in the ischemic compared with the nonischemic leg (Fig. 6E). However, in Tg(Cre) *Cited2*^{lox/lox} mice, there was a 4.1 ± 0.4 -fold induction in *Edn1* ($P = 0.02$, compared with induction in *Cited2*^{lox/lox} mice) (Fig. 6E). This result indicates that CITED2 suppresses endothelial cell HIF transactivation in hypoxia and supports the concept that induction of CITED2 may contribute to dysfunction of HIF in type 2 diabetes.

DISCUSSION

Diabetes remains a risk factor for cardiovascular disease (40) despite control of plasma glucose, lowering of LDL cholesterol, and treatment of hypertension (41). Therefore, there is a need to reduce cardiovascular risk by targeting other factors that are dysregulated in diabetes.

cells were stained with Alexa Fluor 647 (AF647) using Molecular Probes Click-iT procedure and analyzed by flow cytometry. *F*: Representative scatter plots of side scatter (SSC) versus AF647 fluorescence from flow cytometry. *G*: Quantitative analysis of EdU incorporation based on three independent experiments. * $P < 0.05$.

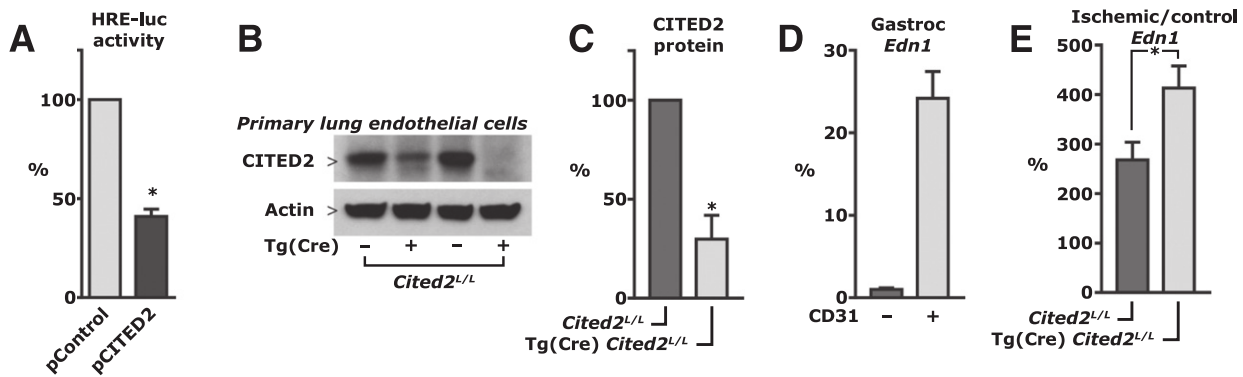


Figure 6—CITED2 regulates HIF transactivation in endothelial cells. **A:** HUVECs were cotransfected with a luciferase reporter gene driven by a promoter containing HREs, which bind HIF, and a Renilla control plasmid to allow normalization for transfection efficiency and cell number. The cultures were cotransfected with a plasmid expressing CITED2 (pCITED2) or a control plasmid (pControl) and luciferase activity was measured in cell lysate. **B and C:** Lung endothelial cells were isolated from mice with deletion of the *Cited2* gene targeted to endothelial cells [Tg(Cre) *Cited2*^{L/L}] or their controls (*Cited2*^{L/L}). **B:** Western blot prepared from cell lysate. **C:** Quantitation based on densitometry in cultures from three pairs of animals. **D:** Expression of *Edn1* mRNA in CD31⁻ and CD31⁺ cells in enzymatically dissociated gastrocnemius muscle. Cells were sorted by FACS after gating for single, viable, CD45⁻ cells. Tissue from three animals in each group was analyzed. **E:** *Edn1* expression in gastrocnemius muscle 24 h after femoral artery ligation. mRNA in the ischemic muscle relative to the contralateral, nonischemic gastrocnemius muscle is shown. Tissue from 12 animals in each group was analyzed. **P* < 0.05.

Insulin resistance in vascular endothelial cells is one such target. For example, we have previously shown that loss of insulin signaling in endothelial cells increases atherosclerosis in apolipoprotein E-null mice by up to threefold without changes in glucose tolerance, plasma lipids, or blood pressure (21). Insulin resistance in endothelial cells may also impair angiogenesis (5) and contribute to cardiovascular risk independent of atherosclerosis. However, little is known of the proangiogenic effector mechanisms that are activated by insulin in endothelial cells. Genes with angiogenic or angiostatic action that are regulated by insulin in endothelial cells are likely dysregulated in conditions with insulin resistance and may contribute to the impaired response to hypoxia. The identification of such genes can provide novel targets for improvement of angiogenesis in obesity and type 2 diabetes.

With the results presented here, we have identified novel insulin-regulated genes in endothelial cells. They were selected by a combined genetic and functional filter. Importantly, they were differentially regulated in endothelial cells by deletion of FoxO in tissues prone to hemangioma formation in FoxO-knockout animals (16). Therefore, they have a high likelihood of being mechanistically involved in endothelial cell homeostasis and angiogenesis. We validated this notion by demonstrating that one of these genes, CITED2, is downregulated by insulin in primary mouse and human endothelial cells and upregulated in cardiac endothelial cells isolated from obese animals and in vascular tissue from patients with obesity and type 2 diabetes. Furthermore, in keeping with predicted antiangiogenic function, CITED2 limits HIF transactivation in endothelial cells and negatively regulates endothelial cell tube formation and proliferation.

Although we did not study the other nine insulin-regulated genes further, we have provided a valuable resource for further study of the mechanisms leading from insulin resistance in endothelial cells to dysfunctional angiogenesis. These genes were not previously reported to be regulated by insulin, although one of them, the transcription factor *Klf6*, was shown to be upregulated by IGF1 (42). Several of the genes have previously been associated with angiogenic function, including *Ctgf* (26,27), *Adm* (28,29), *Pbx1* (43), *Spry2* (44), and *Tcf4* (45). However, a direct role for *Fbn1*, *Hmga2*, *Klf6*, and *Noct* in angiogenesis has not been demonstrated.

Our rationale for further exploring the role of CITED2 was that one of the best-described functions of CITED2 is negative regulation of HIF-1 α (17). Expression of CITED2 is induced by HIF-1 α . In turn, CITED2 inhibits HIF-1 α transactivation by competing with the transactivation domain of HIF-1 α for both p300 and CREB binding protein (CBP) (46). Activation of endothelial cell HIF is critical for angiogenesis. Deletion of HIF-1 β (*Arnt*) (30) or targeting both HIF- α isoforms (31) specifically in endothelial cells results in failure of vascular development and embryonic lethality. HIF- α isoforms have overlapping specificity but distinct function, and both HIF-1 α (32) and HIF-2 α (33) are necessary for normal angiogenesis in adulthood. Insulin may function as a proangiogenic factor through negative regulation of CITED2, thereby releasing a regulatory brake on HIF-dependent transcription.

Consistent with the ability of insulin to suppress CITED2 expression, this gene is upregulated in animal models and humans with insulin resistance. Expression of CITED2 is increased in liver of mice with diet-induced obesity or with the *db/db* mutation (47). More importantly, in gene array analysis

of skeletal muscle biopsies, CITED2 was one of the most downregulated genes during a euglycemic-hyperinsulinemic clamp in healthy volunteers (48). This was confirmed in an independent, unbiased screen of insulin-stimulated gene expression in skeletal muscle (49). In contrast, CITED2 was much less reduced during clamp in patients with type 2 diabetes than in healthy control subjects (48). Downregulation of hepatic CITED2 in mice limits gluconeogenesis and improves glucose tolerance (47). It is therefore possible that inhibition of CITED2 may improve both insulin sensitivity and angiogenesis. Given recent assertions that therapies for type 2 diabetes should be tested for efficacy in modifying cardiovascular morbidity or mortality alongside their gluoregulatory actions (50), CITED2 is an attractive therapeutic target.

With our study, we have identified a group of endothelial genes that are controlled by insulin through FoxO1 and are likely dysregulated in insulin resistance. One of these genes, CITED2, is upregulated in vascular tissue in patients with obesity and type 2 diabetes and suppresses HIF transactivation and proangiogenic function in endothelial cells. These results advance our understanding of insulin action on endothelial cells and points to new therapeutic approaches to improve angiogenesis in patients with obesity and type 2 diabetes.

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