

Mechanisms of Signal Transduction:

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11,12-Epoxyeicosatrienoic Acid-induced Inhibition of FOXO Factors Promotes Endothelial Proliferation by Down-Regulating $p27^{Kip1}$*

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Cytochrome P450-derived epoxyeicosatrienoic acids (EETs) stimulate endothelial cell proliferation and angiogenesis. In this study, we investigated the involvement of the forkhead box, class O (FOXO) family of transcription factors and their downstream target $p27^{Kip1}$ in EET-induced endothelial cell proliferation. Incubation of human umbilical vein endothelial cells with 11,12-EET induced a time- and dose-dependent decrease in $p27^{Kip1}$ protein expression, whereas $p21^{Cip1}$ was not significantly affected. This effect on $p27^{Kip1}$ protein was associated with decreased mRNA levels as well as $p27^{Kip1}$ promoter activity. 11,12-EET also stimulated the time-dependent phosphorylation of Akt and of the forkhead factors FOXO1 and FOXO3a, effects prevented by the phosphatidylinositol 3-kinase inhibitor LY 294002. Transfection of endothelial cells with either a dominant-negative or an "Akt-resistant"/constitutively active FOXO3a mutant reversed the 11,12-EET-induced down-regulation of $p27^{Kip1}$, whereas transfection of a constitutive active Akt decreased $p27^{Kip1}$ expression independently of the presence or absence of 11,12-EET. To determine whether these effects are involved in EET-induced proliferation, endothelial cells were transfected with the 11,12-EET-generating epoxygenase CYP2C9. Transfection of CYP2C9 elicited endothelial cell proliferation and this effect was inhibited in cells co-transfected with CYP2C9 and either a dominant-negative Akt or constitutively active FOXO3a. Reducing FOXO expression using RNA interference, on the other hand, attenuated $p27^{Kip1}$ expression and stimulated endothelial cell proliferation. These results indicate that EET-induced endothelial cell proliferation is associated with the phosphatidylinositol 3-kinase/Akt-dependent phosphorylation and inactivation of FOXO factors and the subsequent decrease in expression of the cyclin-dependent kinase inhibitor $p27^{Kip1}$.

The epoxyeicosatrienoic acids (EETs)[1] are biologically active eicosanoids generated by cytochrome P450 epoxygenases (1, 2). Epoxygenases of the CYP2B, -2C, and -2J subfamilies are expressed in vascular endothelial cells and metabolize arachidonic acid into a series of regio- and stereo-selective EETs (5,6-, 8,9-, 11,12-, and 14,15-EET) that are potent vasodilators and have been identified as endothelium-derived hyperpolarizing factors (3–5).

Recent reports have shown that EETs exert multiple effects on the vascular wall that are not directly related to changes in membrane potential or vascular tone. Indeed, EETs seem to act as second messengers in numerous signaling pathways (6, 7) and are involved in the regulation of inflammation, migration, apoptosis, hypoxia-reoxygenation injury, and platelet aggregation (8–13). Moreover, we and others have recently been able to demonstrate that EETs stimulate endothelial cell proliferation and elicit an angiogenic response (14–16).

Progression through the mammalian cell cycle requires the activation of cyclin-dependent kinases (CDKs) through association with regulatory subunits (cyclins) that phosphorylate the retinoblastoma gene product and the related pocket proteins from mid-G_1 to mitosis (17, 18). Cip/Kip cell cycle regulatory proteins are endogenous inhibitors of the cyclin/CDK complexes and play a crucial role in proliferation, migration and angiogenesis. The aim of the present study, therefore, was to determine whether the Cip/Kip family of cell cycle regulatory proteins is involved in EET-induced endothelial cell proliferation.

EXPERIMENTAL PROCEDURES

Materials—11,12-EET was purchased from Cayman Chemical (Massy, France). The cell proliferation enzyme-linked immunosorbent assay was purchased from Roche Molecular Biochemicals, protein A-Sepharose was from Amersham Biosciences, and protein G-Sepharose was from Zymed Laboratories Inc. (Berlin, Germany). α-dCTP was from Hartmann Analytic (Braunschweig, Germany). LY 294002 and all other chemicals were from Sigma (Heidelberg, Germany).

Cell Culture—Human umbilical vein endothelial cells (HUVEC) were purchased from Cell Systems/Clonetics (Solingen, Germany) and cultured in endothelial basal medium (Cell Systems/Clonetics) supplemented with hydrocortisone (1 μg/ml), bovine brain extract (3 μg/ml), penicillin (50 μg/ml), gentamycin (50 μg/ml), epidermal growth factor (10 μg/ml), and 8% fetal calf serum (Invitrogen). Second passage endothelial cells were used throughout the present study.

Plasmids and Transfection—HUVEC were grown to 60–70% confluence and then transfected with 2–2.5 μg of plasmids. Overexpression plasmids contained the cDNAs encoding wild-type Akt, dominant negative Akt (K179M or T308A, S473A), constitutive active Akt (T308D, S473D), wild-type FOXO3a, "Akt-insensitive"-FOXO3a A3, wild-type FOXO1, "Akt-insensitive" FOXO1 A3, $p27^{Kip1}$, and CYP2C9. Transfection was performed using the Superfect reagent (Qiagen, Hilden, Germany). Cells were incubated with the DNA-Superfect complexes at 37 °C for 4 h, followed by recovery in the presence of 4% fetal calf serum. Transfection efficiency was ~40% as determined using green fluorescent protein, and maximal levels of protein expression were observed between 24 and 48 h. The Akt constructs were kindly provided by Stefanie Dimmeler (Frankfurt am Main, Germany), the $p27^{Kip1}$ construct was from Ludger Hengst (Martinsried, Germany). The FOXO

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[1] The abbreviations used are: EET, epoxyeicosatrienoic acid; CDK, cyclin-dependent kinase; LY 294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; HUVEC, human umbilical vein endothelial cells; FOXO, forkhead box, class O; siRNA, small interfering RNA; BrdUrd, bromodeoxyuridine; FKHR, forkhead homolog 1; PI 3-K, phosphatidylinositol 3-kinase.

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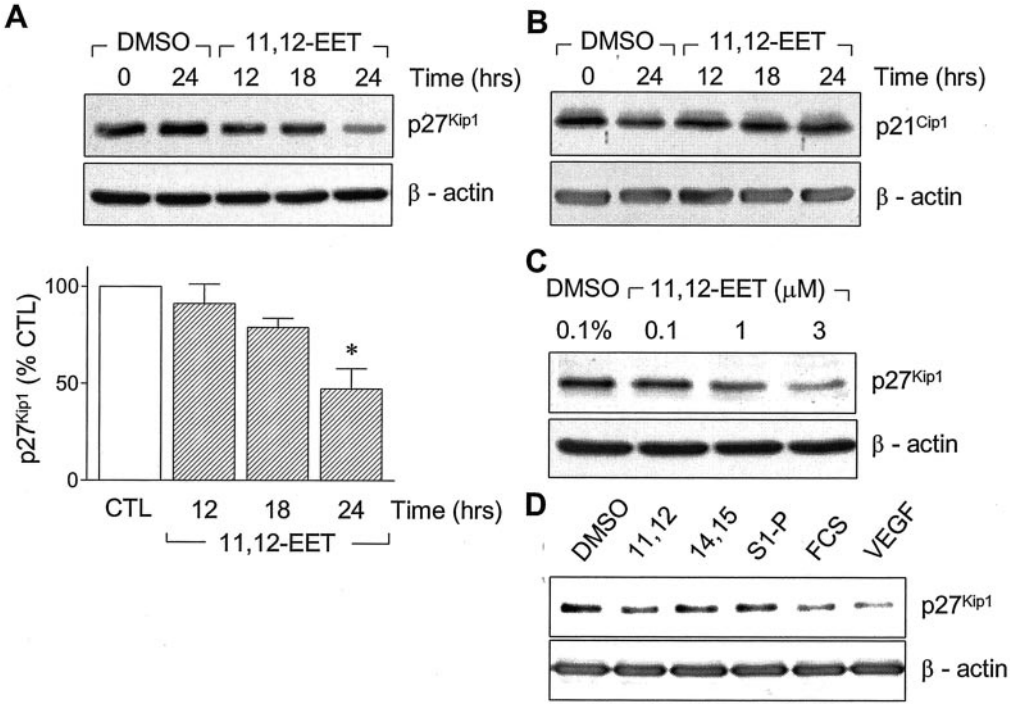

FIG. 1. **11,12-EET attenuates p27^{Kip1} protein expression.** *A*, Western blot and statistical analysis showing the effect of 11,12-EET on p27^{Kip1} protein expression. HUVEC were treated with solvent (*CTL*, 0.1% Me_2SO) or 11,12-EET (3 μM) for the times indicated. *B*, Western blot showing the time-dependent effect of 11,12-EET on p21^{Cip1} protein expression. *C*, Western blot showing the concentration-dependent effect of 11,12-EET on p27^{Kip1} expression after 24 h. *D*, Western blot showing the effect of different Akt-activating stimuli (500 nM sphingosine-1-phosphate (*S1-P*), 10% fetal calf serum (*FCS*), and 100 ng/ml vascular endothelial cell growth factor (*VEGF*)) and the EET-regioisomer 14,15-EET (3 μM) on p27^{Kip1} expression in endothelial cells 24 h after stimulation. Triton X-100-soluble cell fractions were prepared and subjected to SDS-PAGE. Western blotting was performed using p27^{Kip1} and β-actin antibodies. The β-actin signal demonstrates that equal amounts of proteins were loaded in each lane. The results represent data obtained in three independent experiments; *, $p < 0.05$ *versus* control.

plasmids as well as the pGL2-p27^{Kip1} luciferase promoter construct were from Boudewijn M. T. Burgering (Utrecht, Netherlands).

Small Interfering RNA—To silence FOXO gene expression, transfection of a siRNA duplex was performed using TransGene II transfection reagent (Mo Bi Tec, Göttingen, Germany) according to the manufacturer's protocol. The FOXO siRNA was synthesized by Eurogentec and corresponded to nucleotides 961 to 979 of the human FOXO1a coding region (GAGCGTGCCCTACTTCAAG). The siRNA also targeted the sequence for FOXO 3a. A non-related control siRNA that targeted the green fluorescent protein DNA sequence CCACTACCTGAGCACCCAG was used as a control. Incorporation of the siRNA duplex was monitored by confocal microscopy using fluorescin-conjugated siRNA.

Cell Proliferation Assays—To assay proliferation by bromodeoxyuridine (BrdUrd) incorporation, quiescent HUVEC were transfected with the plasmids indicated; after 24 h, the proliferative status of the endothelial cells was determined. Briefly, cells were incubated with BrdUrd (10 μM) for 2 h, and incorporated BrdUrd was detected by an enzyme-linked immunosorbent assay according to the manufacturer's instructions (Roche Molecular Biochemicals).

Immunoblotting—For Western blot analysis, cells were lysed in Triton X-100 lysis buffer (20 mM Tris-HCl, pH 8.0, containing 1% Triton X-100, 137 mM NaCl, 25 mM β−glycerophosphate, 1 mM sodium orthovanadate, 2 mM $Na_2H_2P_2O_7$, 2 mM EDTA, pH 8.0,10% glycerol, and protease inhibitors (100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin)), left on ice for 10 min, and centrifuged at 10,000 × *g* for a further 10 min. Proteins in the resulting supernatant or in the Triton-insoluble pellet were heated with Laemmli sample buffer and separated by SDS-PAGE as described previously (19). Proteins were detected with antibodies recognizing Akt, phospho-Ser^{473} Akt, FKHR, phospho-FKHR/FKHR-L1 (Cell Signaling Technology, Beverly, MA), p27^{Kip1} (BD Transduction Laboratories), p21^{Cip1} (BD Pharmingen), c-Myc, hemagglutinin (Santa Cruz Biotechnology Inc., Heidelberg, Germany) and β-actin (Sigma). Proteins were visualized by enhanced chemiluminescence using a commercially available kit (Amersham Biosciences).

Northern Blotting—HUVEC were starved of serum for 24 h before stimulation with 11,12-EET (3 μM) for the times indicated. Total RNA was isolated by guanidinium isothiocyanate and phenol extraction, and 20 μg of total RNA was used for Northern blotting. RNA was separated by electrophoresis on a 1.2% formaldehyde-denatured agarose gel, visualized with ethidium bromide, transferred to nylon membranes, and hybridized with the full-length p27^{Kip1} cDNA. Membranes underwent filmless autoradiographic analysis (BAS-1500 Bioimaging Analyzer; Fujifilm, Kyoto, Japan) before mRNA quantification. The autoradiographs were analyzed by scanning densitometry, and p27^{Kip1} mRNA levels were normalized to the respective 18S ribosomal RNA levels and expressed as the -fold increase in signal obtained compared with that in untreated cells.

Luciferase Assays—HUVEC were transiently co-transfected with a p27^{Kip1} promoter construct together with a LacZ construct; after 24 h, the cells were lysed, and luciferase activity was assayed according to the manufacturers protocol (Promega, Mannheim, Germany; Tropix, Bedford, MA). Values were corrected for transfection efficiency by measuring β-galactosidase activity.

Statistical Analysis—Data are expressed as mean ± S.E., and statistical evaluation was performed using Student's *t* test for unpaired data, one-way analysis of variance followed by a Bonferroni *t* test, or one-way analysis of variance for repeated measures, where appropriate. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Effect of 11,12-EET on the Expression of p27^{Kip1}—To determine whether or not EETs affect the expression of Cip/Kip, HUVEC were incubated with 11,12-EET and p21^{Cip1} and p27^{Kip1} levels were determined by Western blot analysis. Stimulation of endothelial cells with 11,12-EET induced a time-dependent decrease in p27^{Kip1} protein expression. p27^{Kip1} protein levels were reduced by 60% after 24 h of treatment with 3 μM 11,12-EET (Fig. 1*A*). The expression of the p27^{Kip1}-related protein p21^{Cip1}, however, was not significantly affected by EET stimulation (Fig. 1*B*). The EET-induced down-regulation of p27^{Kip1} was also concentration-dependent (Fig. 1*C*) and was comparable with the reduction of p27^{Kip1} seen after treatment with either vascular endothelial cell growth factor or fetal calf serum, endothelial mitogens known to decrease p27^{Kip1} expres-

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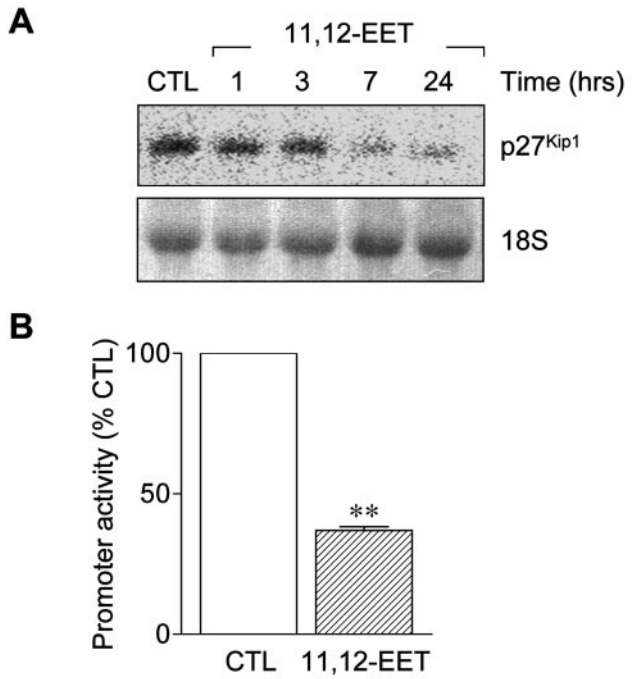

FIG. 2. **11,12-EET attenuates the transcription of p27^{Kip1}.** *A*, representative Northern blot showing the effect of 11,12-EET on p27^{Kip1} mRNA levels. HUVEC were treated with solvent (*CTL*, 0.1% Me_2SO) or 11,12-EET (3 μM) for the time indicated. The 18S signal is shown to demonstrate that equal amounts of RNA were loaded in each lane. *B*, HUVEC were transfected with a luciferase gene construct driven by the p27^{Kip1} promoter together with LacZ under the control of the cytomegalovirus promoter. After treatment with either solvent (*CTL*, 0.1% Me_2SO) or 11,12-EET (3 μM) for 24 h, luciferase activity relative to galactosidase activity was assessed. The results shown represent data obtained in four independent experiments; **, $p < 0.01$ *versus* control.

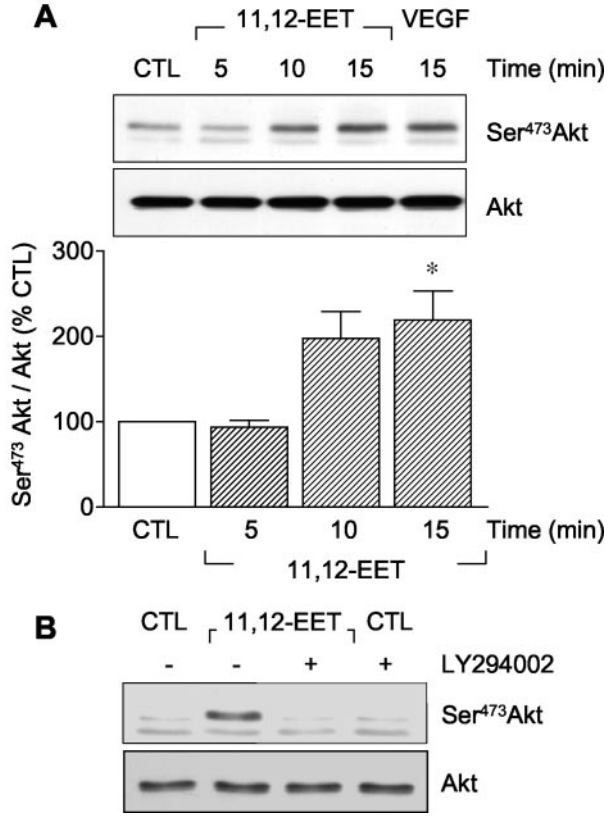

FIG. 3. **11,12-EET stimulates the PI 3-K-dependent phosphorylation of Akt.** *A*, Western blot and statistical summary showing the effect of 11,12-EET (3 μM) on the phosphorylation of Akt. After lysis, Triton X-100-soluble cell fractions were prepared and subjected to SDS-PAGE. Western blotting was performed using a phospho-specific Akt antibody (Ser^{473}). To ensure equal loading of protein in each lane, membranes were reprobed with antibody recognizing total Akt. In each experiment, a positive control (100 ng/ml vascular endothelial cell growth factor (*VEGF*), 15 min) for the activation of Akt was included. *B*, in some experiments, endothelial cells were treated with the PI 3-K inhibitor LY 294002 (10 μM) for 30 min before the addition of 11,12-EET (3 μM, 15 min); *, $p < 0.05$ *versus* control (pcDNA and solvent).

sion (20). In contrast, neither 14,15-EET nor sphingosine-1-phosphate significantly effected p27^{Kip1} expression.

Because the expression of p27^{Kip1} is determined by transcriptional as well as post-transcriptional mechanisms, we assessed whether the 11,12-EET-mediated down-regulation of p27^{Kip1} is caused by an effect on transcription. p27^{Kip1} mRNA levels were time-dependently decreased after the application of 11,12-EET (Fig. 2*A*). The decrease in mRNA levels was accompanied by a ~60% decrease in the activity of the p27^{Kip1} promoter (Fig. 2*B*).

Role for Akt in EET-induced Down-regulation of p27^{Kip1}—Protein kinase B/Akt plays an important role in regulating the transcription and subcellular distribution of p27^{Kip1}. We therefore determined whether or not Akt is implicated in the EET-induced signaling cascade. Treatment of endothelial cells with 11,12-EET led to a concentration- (data not shown) and time-dependent increase in the phosphorylation of Akt on Ser^{473} (Fig. 3*A*) that was prevented by pre-incubation with the phosphatidylinositol 3-kinase (PI 3-K) inhibitor LY 294002 (Fig. 3*B*).

LY 294002 also attenuated the 11,12-EET-induced decrease in p27^{Kip1} levels (Fig. 4*A*). Moreover, transfection of HUVEC with a dominant-negative Akt almost completely prevented the EET-induced down-regulation of p27^{Kip1} (Fig. 4*B*). Transfection of HUVEC with a constitutively active Akt, on the other hand, decreased p27^{Kip1} expression independently of the presence or absence of 11,12-EET, highlighting the importance of Akt in regulating p27^{Kip1} expression in endothelial cells.

Effect of 11,12-EET on the Activity Forkhead Transcription Factors—As a possible molecular mechanism by which the EET-induced activation of Akt could regulate p27^{Kip1} expression, we focused on the forkhead transcription factors, specifically the FOXO subfamily, which is involved in the Akt-dependent regulation of cell proliferation and survival (21). HUVEC express FOXO1 and FOXO3a (Fig. 5), whereas FOXO4 could not be detected (data not shown). 11,12-EET elicited the time-dependent phosphorylation of FOXO1 and FOXO3a (Fig. 5*A*), an effect that was abolished by pretreatment of HUVEC with LY 294002 (Fig. 5*B*).

Because 11,12-EET reduced p27^{Kip1} promoter activity and p27^{Kip1} is a direct target of the forkhead transcription factors, we determined the involvement of FOXO3a in the EET-induced down-regulation of p27^{Kip1}. Co-transfection of HUVEC with the p27^{Kip1} promoter construct and either a wild-type FOXO3a (FOXO3a WT) or an "Akt-insensitive" and thus constitutively active FOXO3a mutant (FOXO3a A3) prevented the EET-induced decrease in promoter activity (Fig. 6*A*). In cells transfected with FOXO3a A3, p27^{Kip1} promoter activity was, as expected, higher than that measured in cells transfected with wild-type FOXO3a. This increase in activity could not be inhibited by 11,12-EET, supporting the notion that the EET-induced inhibition of FOXO activity depends on Akt-mediated phosphorylation of FOXO proteins. Moreover, the EET-induced down-regulation of p27^{Kip1} protein expression was prevented by co-transfection with either the wild-type or constitutively active FOXO3a (Fig. 6*B*). Similar results were obtained when FOXO1 was overexpressed in endothelial cells (data not shown).

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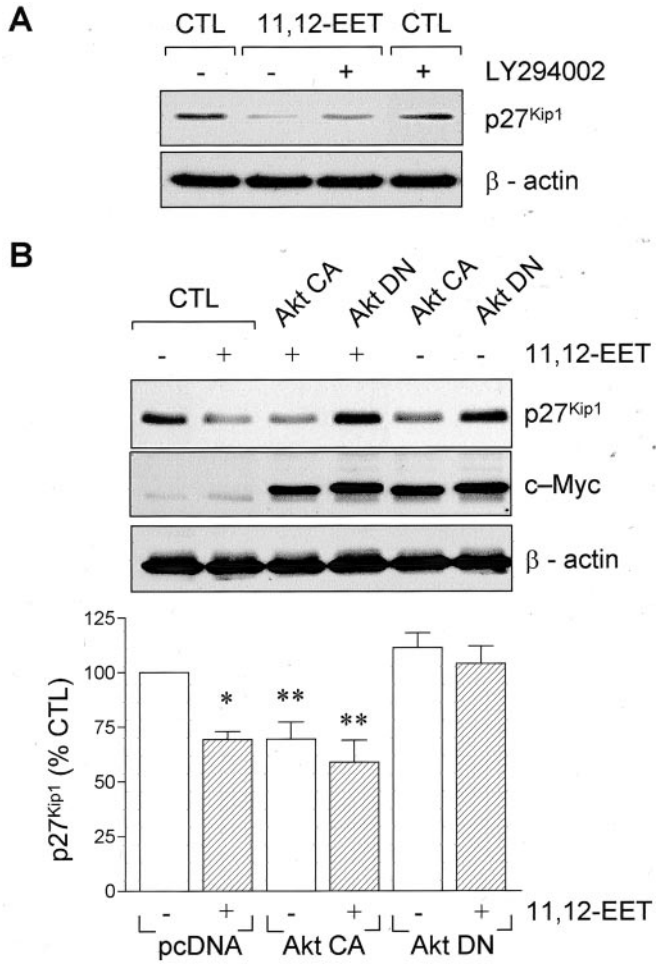

FIG. 4. **The PI 3-K/Akt pathway is involved in the EET-mediated down-regulation of p27^{Kip1}.** *A*, representative Western blots showing the effect of LY 294002 (10 μM) on the EET (3 μM, 24 h)-induced down-regulation of p27^{Kip1}. *B*, HUVEC were transfected with pcDNA3.1 (*CTL*), or the c-Myc-tagged T308D S473D Akt (*Akt CA*), T308A S473A Akt (*Akt DN*), or wild-type Akt constructs. After 24 h, cells were stimulated with 11,12-EET (3 μM, 24 h), and p27^{Kip1} expression was assessed by immunoblotting. The c-Myc signal demonstrates equal transfection of the Akt mutants and the β-actin signal demonstrates that equal amounts of proteins were loaded in each lane. The bar graph summarizes the results obtained in three additional experiments. *, $p < 0.05$; **, $p < 0.01$ *versus* control.

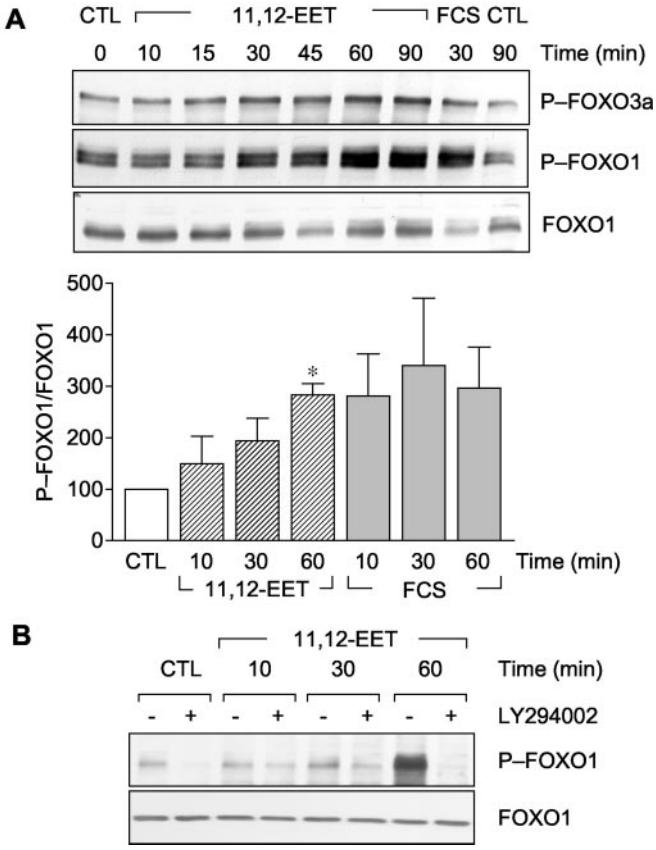

FIG. 5. **11,12-EET stimulates the phosphorylation of the forkhead factors FOXO3a and FOXO1.** *A*, HUVEC were incubated with either 11,12-EET (3 μM) or solvent (*CTL*, 0.1% Me_2SO) for the times indicated, and forkhead factor phosphorylation was analyzed by Western blotting using a specific antibody. In these experiments, 10% fetal calf serum (*FCS*) served as a positive control for FOXO phosphorylation. Membranes were reprobed with antibodies recognizing total FOXO1 to demonstrate that equal amounts of proteins were loaded in each lane. Representative Western blots together with a summary of the data obtained in four independent experiments are shown; *, $p < 0.05$ *versus* control. *B*, representative Western blots showing the effect of the PI 3-K inhibitor LY 294002 (10 μM, added 30 min before EET stimulation) on the 11,12-EET (3 μM)-induced phosphorylation of FOXO1. Identical results were obtained in three additional experiments.

The Mitogenic Effect of Cytochrome P450-derived EETs Depends on Akt and Forkhead Activity—To determine whether the effects observed are functionally involved in CYP2C9-induced proliferation, BrdUrd incorporation was assessed in CYP2C9-overexpressing HUVEC. Similar to the effect of the CYP2C9 product 11,12-EET, CYP2C9 overexpression resulted in a decrease in p27^{Kip1} expression (data not shown) and a 2-fold increase in BrdUrd incorporation compared with endothelial cells, which were transfected with an empty vector (Fig. 7). Overexpression of wild-type Akt in CYP2C9-expressing endothelial cells did not influence BrdUrd incorporation, but CYP2C9 overexpression failed to elicit proliferation in endothelial cells that were co-transfected with a dominant-negative Akt (Fig. 7*A*). Moreover, the co-transfection of HUVEC with CYP2C9 and either the wild-type or constitutively active FOXO3a completely prevented the cytochrome P450-induced proliferation of endothelial cells (Fig. 7*B*).

Effect of FOXO siRNA on Endothelial Cell Proliferation—To investigate whether endogenous FOXO factors are required for CYP2C9-induced endothelial proliferation, we attenuated FOXO gene expression using RNA interference. Transfection of endothelial cells with FOXO-specific siRNA significantly decreased the expression of FOXO protein compared with endothelial cells transfected with a control siRNA (expression was reduced by 66 ± 1.5%, $p < 0.001$, $n = 4$; Fig. 8*A*). To evaluate the consequences of decreased FOXO expression on CYP2C9-induced endothelial cell proliferation, we co-transfected endothelial cells with pcDNA3.1 or CYP2C9 and either a FOXO siRNA or a control siRNA.

The siRNA-induced reduction in FOXO expression was associated with a decrease in p27^{Kip1} protein levels (Fig. 8*B*), a finding that highlights the crucial role played by FOXO factors in the regulation of p27^{Kip1}. In cells treated with the FOXO siRNA, the expression of p27^{Kip1} was not influenced by the presence of 11,12-EET. In accordance with its effect on p27^{Kip1}, decreasing FOXO expression in cells transfected with pcDNA was in itself a significant stimulus of endothelial cell proliferation, compared with cells transfected with the control siRNA (Fig. 8*C*). The FOXO siRNA-induced proliferation of endothelial cells was such that we were unable to detect an additive effect of FOXO siRNA and CYP2C9 overexpression.

DISCUSSION

The results of the present investigation demonstrate that CYP2C9-derived 11,12-EET promotes endothelial proliferation

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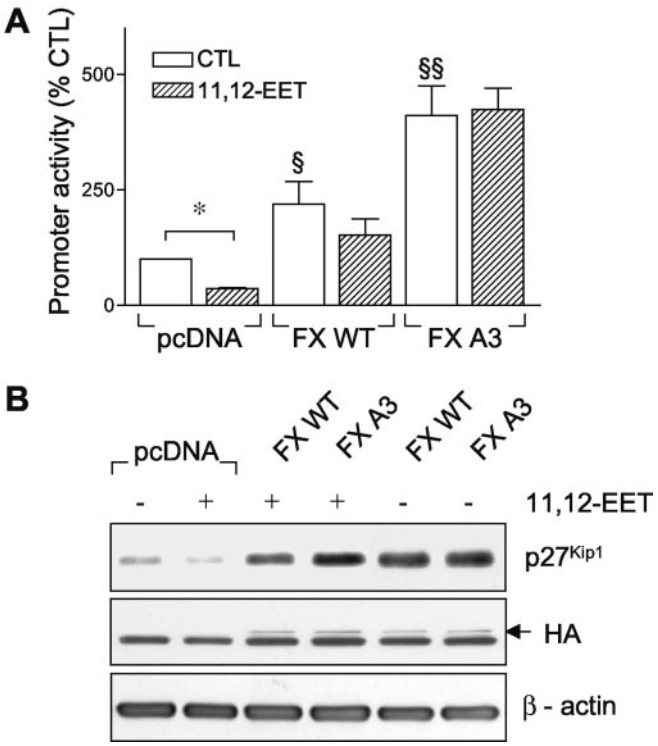

FIG. 6. **Overexpression of FOXO3a prevents the 11,12-EET-induced down-regulation of p27^{Kip1}.** *A*, HUVEC were co-transfected with a luciferase-coupled p27^{Kip1} promoter construct and LacZ together with either the FOXO3a (*FX WT*), FOXO3a A3 (*FX A3*), or pcDNA3.1 (*pcDNA*) plasmids and stimulated with either solvent (*CTL*, 0.1% Me_2SO) or 11,12-EET (3 μM) for 24 h. Luciferase activity relative to galactosidase activity was assessed and the results shown represent data obtained in three independent experiments; *, $p < 0.05$ *versus* the appropriate control; §, $p < 0.05$, §§, $p < 0.01$ *versus* pcDNA3.1 with solvent. *B*, HUVEC were transfected with either pcDNA3.1, FOXO3a, or the constitutively active FOXO3a A3. After an additional 24 h, cells were stimulated with either solvent or 11,12-EET (3 μM) for 24 h and p27^{Kip1} expression was monitored by immunoblotting. The β-actin signal demonstrates that equal amounts of proteins were loaded in each lane; the hemagglutinin (*HA*) signal demonstrates that similar amounts of the hemagglutinin-tagged FOXO constructs were expressed. Similar results were obtained in three independent experiments.

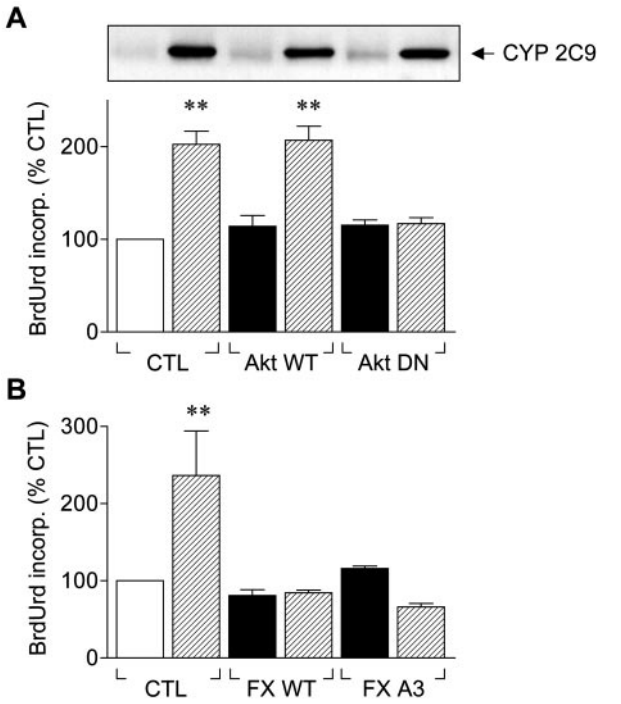

FIG. 7. **Modulation of the CYP2C9-induced proliferation of endothelial cells by Akt and FOXO3a.** *A*, HUVEC were transfected with either pcDNA3.1 (*open bars*), wild-type Akt (*Akt WT*), or a dominant-negative K179M Akt (*Akt DN*; *closed bars*), alone or in combination with CYP2C9 (*hatched bars*). After 24 h in culture, cells were incubated with bromodeoxyuridine (BrdUrd, 10 μM) for 2 h, and incorporated BrdUrd was detected by an enzyme-linked immunosorbent assay. The Western blot shows that CYP2C9 was equally expressed in these cells. *B*, HUVEC were transfected with either pcDNA3.1 (*open bars*), FOXO3a, or the constitutively active FOXO3a A3 (*FX A3*; *closed bars*), alone or in combination with CYP2C9 (*hatched bars*). After 24 h in culture, cells were incubated with BrdUrd (10 μM) for 2 h, and incorporated BrdUrd was detected by an enzyme-linked immunosorbent assay. The results represent data obtained in four independent experiments each performed in triplicate; **, $p < 0.01$ *versus* the appropriate control.

by down-regulating the expression of the cyclin-dependent kinase inhibitor p27^{Kip1}. The signal transduction cascade activated by 11,12-EET, which results in the down-regulation of p27^{Kip1}, involves activation of the PI 3-K/Akt signaling pathway and the subsequent phosphorylation and inhibition of the FOXO subfamily of forkhead transcription factors.

EETs are potent vasodilators (22) that, by acting as second messenger molecules, also play an important role in the regulation of vascular homeostasis (6, 7). The molecular mechanisms and signal transduction cascades that mediate the effects of EETs are not well defined, although the spectrum of kinases and phosphatases that can be stimulated by 11,12- and 14,15-EET is continuously expanding (6). EETs are reported to modulate endothelial cell gene expression (8, 10, 16) as well as the proliferation and migration of vascular cells and angiogenesis (9, 14–16). However, although the cytochrome P450 epoxygenases that generate EETs are expressed in native endothelial cells (5, 8), the expression of the enzyme is rapidly down-regulated (within 24 h) once the cells are isolated, making it difficult to assess the biological effects of EETs in cultured cells. However, we were recently able to show that overexpression of CYP2C9 in endothelial cells up-regulates the expression of cyclin D1 and thus stimulates endothelial cell proliferation (16). Because cell proliferation is governed by a complex interaction of CDKs and CDK inhibitors, we investigated whether additional regulators of cell cycle progression are targeted by cytochrome P450-derived EETs. Moreover, because a second non-catalytic function of cyclin D-CDK4 complexes is the sequestration of CDK inhibitors, including p21^{Cip1} and p27^{Kip1} (23, 24), we concentrated on the cyclin-dependent kinase inhibitor proteins. Our results show that 11,12-EET does not affect the expression of p21^{Cip1} but down-regulates the expression of p27^{Kip1}. Because the level of unbound Cip/Kip proteins may set an inhibitory threshold for the activation of cyclin E and A-CDK2, the down-regulation of these proteins is a requirement for mitogen-induced cell proliferation. Nuclear levels of CDK inhibitor proteins are decreased by sequestration into the cytosol; once the level has decreased sufficiently, cyclin E-CDK2 can facilitate its own activation by phosphorylating p27^{Kip1}, thus initiating its degradation (25–30). Indeed, the expression of p27^{Kip1} is cell cycle-dependent and highest in quiescent cells and decreases upon re-entry into the cell cycle (31).

Although phosphorylation was initially thought to determine cellular p27^{Kip1} levels, the FOXO subfamily of forkhead transcription factors is now known to directly regulate the transcription of the p27^{Kip1} gene (32, 33), which means that a proliferative stimulus that results in a decrease in p27^{Kip1} levels may also be associated with the inhibition of FOXO-induced transcription. Our data indicate that the proliferation of endothelial cells elicited by 11,12-EET is associated with a decrease in the activity of the p27^{Kip1} promoter and

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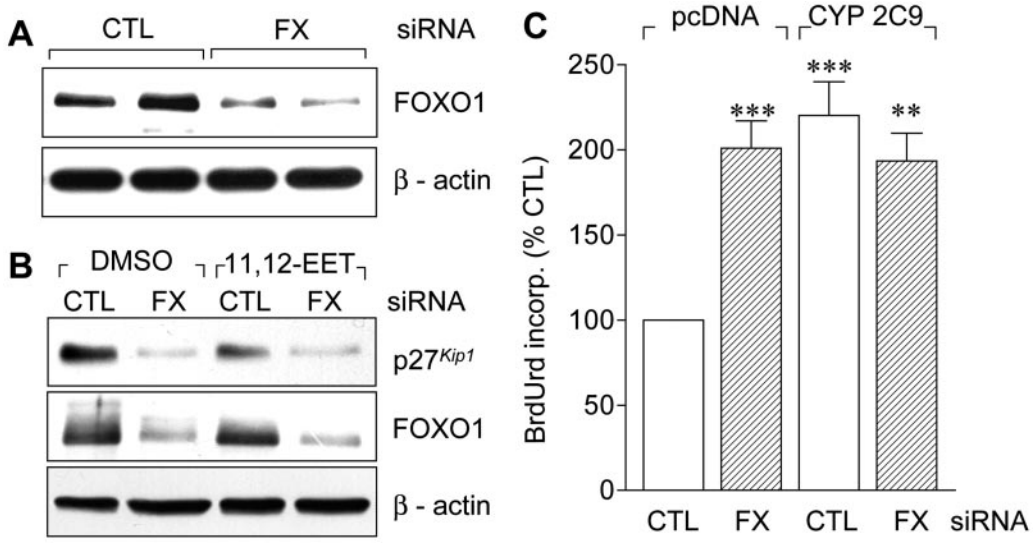

FIG. 8. **Silencing FOXO gene expression stimulates endothelial cell proliferation.** HUVEC (60% confluent) were transfected with either the FOXO siRNA (*FX*) or a control siRNA (*CTL*). *A*, representative Western blot showing the effect of FOXO siRNA on FOXO protein expression. To control for the equal loading of protein, the same blot was stripped and reprobed with an anti-β-actin antibody. *B*, Western blots showing the effect of FOXO siRNA (*FX*) on $p27^{Kip1}$ levels. Endothelial cells were transiently transfected with the indicated siRNA and cultured for a further 18 h before stimulation with 11,12-EET (3 μM, 24 h). *C*, HUVEC were serum-starved for 24 h and then co-transfected with pcDNA3.1, CYP2C9. and either FOXO siRNA or control siRNA. Twenty-four hours after transfection, cells were incubated with BrdUrd (10 μM) for 2 h, and incorporated BrdUrd was detected by an enzyme-linked immunosorbent assay. The results represent data obtained in three independent experiments each performed in triplicate; **, $p < 0.01$, ***, $p < 0.001$ *versus* control.

subsequently with a decrease in $p27^{Kip1}$ mRNA levels. These effects can be attributed to the phosphorylation and inhibition of FOXO factors inasmuch as cell stimulation with 11,12-EET time-dependently increased the phosphorylation of FOXO1 (formerly known as FKHR) and FOXO3a (formerly known as FKHR-L1). Moreover, overexpression of a constitutively active FOXO3a mutant largely prevented the 11,12-EET-induced down-regulation of $p27^{Kip1}$ expression and inhibited the CYP2C9-induced increase in endothelial cell proliferation.

Relatively little is known about the expression and biological functions of forkhead factors in endothelial cells. However, from work in numerous other cell types, it is evident that the FOXO family of forkhead transcription factors is regulated by the PI 3-K/Akt pathway (34–38), and the phosphorylation of FOXO proteins by Akt results in their cytoplasmic retention, inactivation, and proteosomal degradation (21, 39). The results of the present investigation indicate that the upstream signaling pathways leading to the 11,12-EET-induced phosphorylation of FOXO factors and down-regulation of $p27^{Kip1}$ in endothelial cells also involves activation of the PI 3-K/Akt pathway. Indeed, 11,12-EET induced the PI 3-K-dependent phosphorylation of Akt, and a selective PI 3-K inhibitor abolished the 11,12-EET-induced phosphorylation of FOXO proteins. Moreover, introducing a dominant-negative Akt into endothelial cells prevented both the 11,12-EET-induced down-regulation of $p27^{Kip1}$- and CYP2C9-induced endothelial cell proliferation. The important role of FOXO factors in endothelial cell proliferation was highlighted by the results of experiments in which FOXO expression was down-regulated using an siRNA approach. Indeed, the reduction in FOXO expression proved to be a strong stimulus for endothelial cell proliferation, and the effects observed were equivalent to those of CYP2C9 overexpression. The FOXO siRNA-stimulated increase in cell proliferation was associated with an attenuated expression of the forkhead target gene $p27^{Kip1}$, indicating that the FOXO-dependent regulation of $p27^{Kip1}$ expression plays a significant role in the regulation of endothelial cell growth.

We have previously shown that the induction of mitogen-activated protein kinase phosphatase 1 and the inhibition of c-Jun N-terminal kinase activity also play a crucial role in the regulation of CYP2C9-induced endothelial cell proliferation (16), and it remains to be demonstrated how the pathway outlined previously can be linked to the activation of the PI 3-K/Akt/FOXO cascade described here. There is, however, a precedent for the negative regulation of c-Jun N-terminal kinase after the activation of Akt, because Akt has been reported to phosphorylate and inactivate the kinase SEK1 and thus inactivate its substrate, c-Jun N-terminal kinase, in insulin-stimulated human embryonic kidney cells (40).

Exactly how 11,12-EET can elicit the activation of Akt remains unclear; although an extracellular EET receptor has been proposed on guinea pig monocytes (41) and rat aortic smooth muscle cells (42), there has been no report of such a receptor in endothelial cells. Certainly, part of the biological response to the exogenously applied EETs, as well as the overexpression of CYP2C9 in endothelial cells, can be accounted for by the transactivation of the EGF receptor, either by the release of epidermal growth factor/heparin-binding epidermal growth factor from the cell surface or the intracellular activation of the receptor by Src kinases (43–46).

Taken together, our findings demonstrate a novel signaling pathway by which cytochrome P450-derived 11,12-EET promotes endothelial cell proliferation and identify $p27^{Kip1}$ as a downstream target of EET signaling. The EET-induced proliferation involves the down-regulation of $p27^{Kip1}$ through PI3-K/Akt-regulated inactivation of the FOXO subfamily of forkhead transcription factors. Therefore, regulation of $p27^{Kip1}$ expression might be an important downstream target in EET-signaling, given that $p27^{Kip1}$ is a likely candidate to couple cell cycle progression with migration and angiogenesis (47–49).

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