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 p27Kip1 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 p27Kip1 protein expression, whereas p21Cip1 was not significantly affected. This effect on p27Kip1 protein was associated with decreased mRNA levels as well as p27Kip1 promoter activity. 11,12-EET also stimulated the time-dependent phosphorylation of Akt and of the forkhead factors FOXO1 and FOXO3α, 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 FOXO3α mutant reversed the 11,12-EET-induced downregulation of p27Kip1, whereas transfection of a constitutive active Akt decreased p27Kip1 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 FOXO3α. Reducing FOXO expression using RNA interference, on the other hand, attenuated p27Kip1 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 p27Kip1.

The epoxyeicosatrienoic acids (EETs) 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-G1 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 immunoassortment 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-CDT 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 (60 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 FOXO3α, “Akt-insensitive” FOXO3α A3, wild-type FOXO1, “Akt-insensitive” FOXO1 A3, p27Kip1, 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 p27Kip1 construct was from Ludger Hengst (Martinsried, Germany). The FOXO
plasmids as well as the pGL2-p27Kip1 luciferase promoter construct were from Boudevijn 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 correponded to nucleotides 961 to 979 of the human FOXO1a coding region (GAGCGTGGCCTACTTCGAG). 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 Na2HPO4, 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-Ser473 Akt, FKHR, phospho-FKHR/FKHR-L1 (Cell Signaling Technology, Beverly, MA), p27Kip1 (BD Transduction Laboratories), p21Cip1 (BD Pharmingen), e-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 p27Kip1 cDNA. Membranes underwent filmless autoradiographic analysis (LAS-1500 Bioimaging Analyzer; Fujifilm, Kyoto, Japan) before mRNA quantification. The autoradiographs were analyzed by scanning densitometry, and p27Kip1 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 p27Kip1 promoter construct together with a β-galactosidase 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 p27Kip1-To determine whether or not EETs affect the expression of Cip/Kip, HUVEC were incubated with 11,12-EET and p21Cip1 and p27Kip1 levels were determined by Western blot analysis. Stimulation of endothelial cells with 11,12-EET induced a time-dependent decrease in p27Kip1 protein expression. p27Kip1 protein levels were reduced by 60% after 24 h of treatment with 3 μM 11,12-EET (Fig. 1A). The expression of the p27Kip1-related protein p21Cip1, however, was not significantly affected by EET stimulation (Fig. 1B). The EET-induced down-regulation of p27Kip1 was also concentration-dependent (Fig. 1C) and was comparable with the reduction of p27Kip1 seen after treatment with either vascular endothelial cell growth factor or fetal calf serum, endothelial mitogens known to decrease p27Kip1 expres-
representative Northern blot showing the effect of 11,12-EET on p27
B expression or absence of 11,12-EET, highlighting the importance of
EET-induced activation of Akt could regulate p27
B as a possible molecular mechanism by which the
Factors that was prevented by pre-incubation with the phos-
Because 11,12-EET reduced p27
Kip1 expression, we focused on the forkhead transcription factors, specifically the FOXO subfamily, which is involved in the Akt-de-
primary Northern blot showing the effect of 11,12-EET on p27
Kip1 mRNA levels. HUVEC were treated with solvent (CTL, 0.1% Me2SO) 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 cytomeg-
avirus promoter. After treatment with either solvent (CTL, 0.1% Me2SO) 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.
Because the expression of p27
Kip1 is determined by tran-
scription as well as post-transcriptional mechanisms, we as-
essed 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. 2A). The decrease in mRNA levels was accom-
panied by a ~60% decrease in the activity of the p27
Kip1 promoter (Fig. 2B).
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 there-
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 Ser473 (Fig. 3A) that was prevented by pre-incubation with the phos-
phatidylinositol 3-kinase (PI 3-K) inhibitor LY 294002 (10
M, 15 min); *, p < 0.05 versus control (pcDNA and solvent).
LY 294002 also attenuated the 11,12-EET-induced decrease in p27
Kip1 levels (Fig. 4A). Moreover, transfection of HUVEC with a dominant-negative Akt almost completely prevented the EET-induced down-regulation of p27
Kip1 (Fig. 4B). 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 expres-
sion, we focused on the forkhead transcription factors, specifically the FOXO subfamily, which is involved in the Akt-de-
pendent 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. 5A), an effect that was abolished by pretreatment of HUVEC with LY 294002 (Fig. 5B).
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-in-
duced decrease in promoter activity (Fig. 6A). In cells transfec-
ted 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. 6B). Similar results were obtained when FOXO1 was overexpressed in endothelial cells (data not shown).
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 p27Kip1 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. 7A). 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. 7B).

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 ± 15%, p < 0.001, n = 4; Fig. 8A). 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 p27Kip1 protein levels (Fig. 8B), a finding that highlights the crucial role played by FOXO factors in the regulation of p27Kip1. In cells treated with the FOXO siRNA, the expression of p27Kip1 was not influenced by the presence of 11,12-EET. In accordance with its effect on p27Kip1, 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. 8C). 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...
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 cause a second non-catalytic function of cyclin D-CDK4 complex—perhaps the sequestration of CDK 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 elicted by 11,12-EET is associated with a decrease in the activity of the p27\(^{kip1}\) promoter and...
the PI 3-K/Akt pathway (34). The FOXO family of forkhead transcription factors is regulated by the PI 3-K/Akt pathway in numerous other cell types, it is evident that the FOXO proteins play a role in the regulation of endothelial cell growth. However, the functions of forkhead factors in endothelial cells. Certainly, part of the biological role in the regulation of endothelial cell growth. 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), and the phosphorylation of FOXO proteins by Akt results in their cytoplasmic retention, inactivation, and proteasomal 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 p27Kip1 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.

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. B, Western blots showing the effect of FOXO siRNA (FX) on p27Kip1 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.

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 over-expression 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 p27Kip1 as a downstream target of EET signaling. The EET-induced proliferation involves the down-regulation of p27Kip1 through PI3-K/Akt-regulated inactivation of the FOXO subfamily of forkhead transcription factors. Therefore, regulation of p27Kip1 expression might be an important downstream target in EET-signaling, given that p27Kip1 is a likely candidate to couple cell cycle progression with migration and angiogenesis (47–49).

Acknowledgments—We are indebted to Isabel Winter and Tanja Maria Mareczek for expert technical assistance.

REFERENCES
Cytochrome P450 2C9 and Endothelial Proliferation