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Cytochrome P450 2C9-induced Endothelial Cell Proliferation Involves Induction of Mitogen-activated Protein (MAP) Kinase Phosphatase-1, Inhibition of the c-Jun N-terminal Kinase, and Up-regulation of Cyclin D1*

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Cytochrome P450 (CYP)-derived epoxyeicosatrienoic acids (EETs) are important modulators of endothelial cell homeostasis. We investigated the signaling pathway linking the activation of CYP 2C9 to enhanced endothelial cell proliferation. Overexpression of CYP 2C9 in cultured human endothelial cells markedly increased proliferation. This effect was paralleled by an up-regulation of the G_1 phase regulatory protein, cyclin D1. The specific CYP 2C9 inhibitor, sulfaphenazole, prevented both the enhanced cell proliferation and up-regulation of cyclin D1. CYP 2C9 overexpression also decreased the activity of the c-Jun N-terminal kinase (JNK). Coexpression of wild type JNK with CYP 2C9 attenuated the CYP 2C9-induced increase in cyclin D1 expression and abolished the CYP 2C9-induced proliferation response. In contrast, cotransfecting dominant negative JNK with CYP 2C9 restored the CYP 2C9-mediated up-regulation of cyclin D1 and proliferation. The inactivation of JNK is linked to its dephosphorylation by dual specificity mitogen-activated protein (MAP) kinase phosphatases (MKPs). Overexpression of CYP 2C9 significantly increased the expression of MKP-1, as did incubation with 11,12-EET. These data demonstrate that the mitogenic effect of CYP 2C9 is due to the generation of EETs, which promote the MKP-1-mediated dephosphorylation and inactivation of JNK, effects ultimately culminating in the expression of cyclin D1 and endothelial cell proliferation.

Cytochrome P450 (CYP)[1] epoxygenases catalyze the epoxidation of arachidonic acid into a series of regio- and stereospecific epoxyeicosatrienoic acids (5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET) and play a crucial role in the regulation of vascular homeostasis (for a review, see Ref. 1). Initially, CYP-derived EETs were described to hyperpolarize and relax vascular smooth muscle cells as a consequence of the activation of large conductance Ca^{2+}-dependent K^+ channels (BK^+_{Ca}) (2–4). However, several more recently reported cellular actions of EETs cannot be attributed to the activation of BK^+_{Ca}, suggesting that EETs are more than just vasodilators. Indeed, EETs are reported to stimulate the ADP-ribosylation of G-proteins (5) as well as activate intracellular protein kinases including tyrosine kinases, the extracellular signal-regulated kinase 1 and 2 (ERK1/2), the p38 mitogen-activated protein (MAP) kinase, and protein kinase B (protein kinase B/Akt) (6–8). 11,12-EET has also been shown to exert anti-inflammatory effects by inhibiting the activation of the transcription factor NF-κB and decreasing the cytokine-induced expression of vascular cell adhesion molecule-1 (9).

CYP epoxygenases of the 2B, 2J, and 2C families are reported to be expressed in cultured and native endothelial cells (9–12); however, whereas expression of CYP 2J appears to be constitutive, stimuli such as pulsatile stretch or Ca^{2+} antagonists markedly enhance the expression of CYP 2C (11–13). The resulting CYP 2C-induced changes in vascular function/homeostasis cannot only be attributed to the synthesis of EETs because this enzyme also generates biologically relevant amounts of reactive oxygen species (ROS) (14).

We have reported previously that EETs activate MAP kinases and enhance endothelial cell growth (7). Therefore, the aim of the present investigation was to determine the cellular events linking CYP 2C with endothelial cell proliferation. We report here that the mitogenic effect of CYP 2C9 can be attributed to the generation of EETs, induction of MAP kinase phosphatase-1 (MKP-1), and inhibition of c-Jun N-terminal kinase (JNK) activity, effects ultimately culminating in the increased expression of cyclin D1 and endothelial cell proliferation.

EXPERIMENTAL PROCEDURES

Materials—11,12-EET was purchased from Biomol (Hamburg, Germany) or 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). Sulfaphenazole and all other chemicals were from Sigma.

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 exclusively in this study.

Plasmids and Transfection—HUVEC (50–60% confluent) were serum-starved for 24 h prior to transfection with 2–2.5 μg of plasmids. Plasmids included CYP 2C9 (pcDNA3 CYP 2C9), wild type JNK1 (pcDNA3Flag-JNK1), dominant negative JNK1 (pcDNA3.1Flag-JNK1

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[1] The abbreviations used are: CYP, cytochrome P450; EET, epoxyeicosatrienoic acid; MAP, mitogen-activated protein; MKP, MAP kinase phosphatase; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; BrdUrd, bromodeoxyuridine; ROS, reactive oxygen species; HUVEC, human umbilical vein endothelial cells; GST, glutathione *S*-transferase; CDK, cyclin-dependent kinase.

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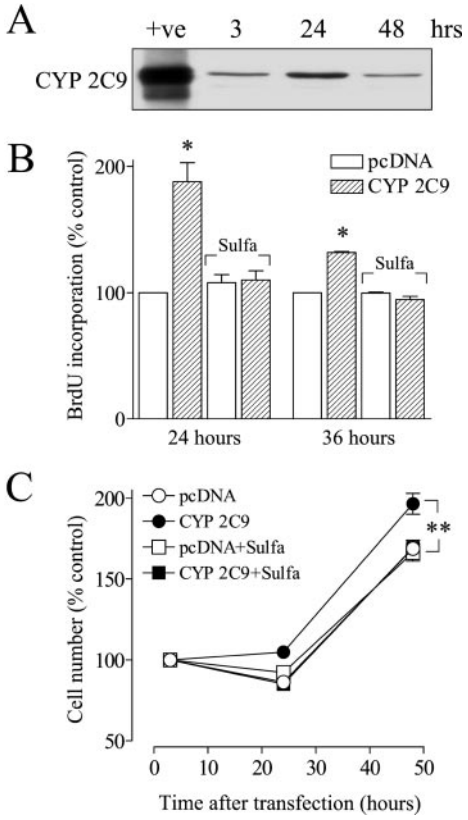

FIG. 1. **CYP 2C9 stimulates endothelial cell proliferation.** HUVEC were transfected with either pcDNA3.1 or CYP 2C9 and cultured for 3, 24, 36, or 48 h in the presence of 4% fetal calf serum and in the absence and presence of sulfaphenazole (10 μM). *A*, Western blot showing the expression of CYP 2C9 after transfection. *+ve*, positive control for CYP 2C9. *B*, at the relevant time point after transfection, cells were incubated with BrdUrd (*BrdU*, 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. *C*, at 3, 24, and 48 h after transfection, cells were harvested and counted. The results represent data obtained in four independent experiments each performed in triplicate; *, $p < 0.05$; **, $p < 0.01$ *versus* control.

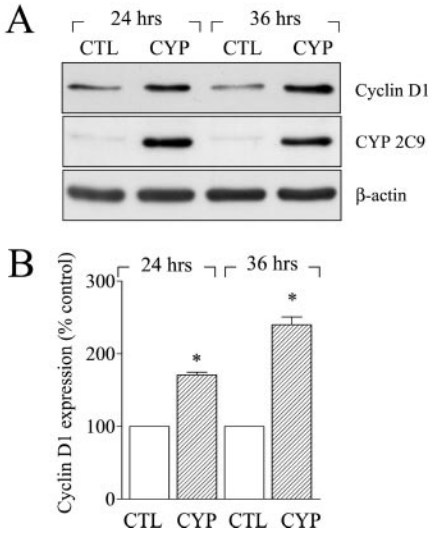

FIG. 2. **Overexpression of CYP 2C9 enhances the expression of cyclin D1 in endothelial cells.** Representative Western blot (*A*) and statistical analysis (*B*) showing the effect of CYP 2C9 (*CYP*) overexpression on cyclin D1 expression as compared with control vector- (*CTL*) transfected cells. At the times indicated, Triton X-100-soluble cell fractions were prepared and subjected to SDS-PAGE. Western blotting was performed using an anti-cyclin D1 antibody. Membranes were then reprobed with antibodies against CYP 2C9. The β-actin signal is shown to demonstrate that equal amounts of proteins were loaded in each lane; *, $p < 0.05$ *versus* control.

APF), wild type p38β (pcDNA3.1Flag-p38β), dominant negative p38β (pcDNA3.1Flag p38β AF), and the control plasmid (pcDNA3.1). 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 about 40% as determined using green fluorescent protein, and maximal levels of protein expression were observed between 24 and 48 h. The wild type JNK1 and dominant negative JNK1 expression plasmids were kindly provided by Dr. Roger J. Davis (Worcester, MA), and the wild type and dominant negative p38 constructs were a gift from Dr. Gang Pei (Shanghai, China).

Cell Proliferation Assays—To assay proliferation by bromodeoxyuridine (BrdUrd) incorporation, quiescent HUVEC were transfected with pcDNA3.1 CYP 2C9, wild type JNK1, a dominant negative JNK1, or combinations thereof. At 24 and 36 h after transfection, 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).

In separate series of experiments, endothelial cells were transfected as described and harvested after 3, 24, and 36 h. Cells were then counted using a cell counter (CASY1, Schärfe System), and protein was extracted for Western blot analysis.

JNK Assay—To assess JNK activity, endothelial cells were stimulated as described under "Results," rinsed twice with ice-cold phosphate-buffered saline, and then lysed in Triton 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). The protein extracts were incubated with 4 μl of rabbit anti-JNK1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at 4 °C and then with 20 μl of protein A/G-Sepharose. After 1 h at 4 °C, the immunoprecipitates were washed twice with Triton lysis buffer and kinase buffer (25 mM HEPES, pH 7.6, 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 2 mM dithiothreitol). The kinase assays were performed at 30 °C for 30 min using 2 μg of GST-c-Jun (Cell Signaling, Beverly, MA) as a substrate, 20 μM ATP, and 5 μCi of [γ-^{32}P]dATP (Hartmann Analytik, Braunschweig, Germany) in 30 μl of kinase buffer. The reactions were stopped by the addition of Laemmli sample buffer, and the products were resolved by SDS-PAGE (12%). The incorporation of [^{32}P]phosphate was visualized by autoradiography and quantified by scanning densitometry.

Immunoblotting—For Western blot analysis, cells were lysed in Triton lysis buffer, left on ice for 10 min, and centrifuged at 10,000 × *g* for 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 (15). Proteins were detected with antibodies recognizing cyclin D1, JNK1, MKP-1 (Santa Cruz Biotechnology), phospho-p38, and p38 MAP kinase (Cell Signaling). The CYP 2C9 antibody used was purified by Eurogentec (Seraing, Belgium) from the serum of rabbits immunized with a CYP 2C9 peptide (RRRKLPPGPTPLPIC). Proteins were visualized by enhanced chemiluminescence using a commercially available kit (Amersham Biosciences).

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

RESULTS

Effect of CYP 2C9 on Endothelial Cell Proliferation—To characterize the role of CYP 2C9 as a putative modulator of endothelial proliferation, BrdUrd incorporation was determined in human umbilical vein endothelial cells 24 and 36 h after transfection with either pcDNA3.1 or CYP 2C9. At 24 h after transfection, CYP 2C9 protein expression was maximal (Fig. 1*A*), and BrdUrd incorporation into endothelial cells was approximately twice that detected in control cells, *i.e.* cells transfected with pcDNA3.1 and cultured in the presence of 4% fetal calf serum (Fig. 1*B*). To ensure that the increase in BrdUrd incorporation was indeed mediated by CYP 2C9, endothelial cells were pretreated with the specific CYP 2C9 inhibitor

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sulfaphenazole (16). The CYP inhibitor significantly reduced BrdUrd incorporation into CYP 2C9-overexpressing cells so that values no longer differed from the control (Fig. 1*B*). Treatment of control cells with sulfaphenazole did not influence either BrdUrd incorporation or the expression of CYP 2C9 protein (data not shown). Under the same experimental conditions, basic fibroblast growth factor (100 ng/ml) and vascular endothelial growth factor (100 ng/ml) enhanced BrdUrd incorporation by 2.5 ± 0.1- and 2.8 ± 0.2-fold (n = 4), respectively. Overexpression of CYP 2C9 was also associated with a significant increase in the number of endothelial cells, an effect that was abolished by sulfaphenazole (Fig. 1*C*).

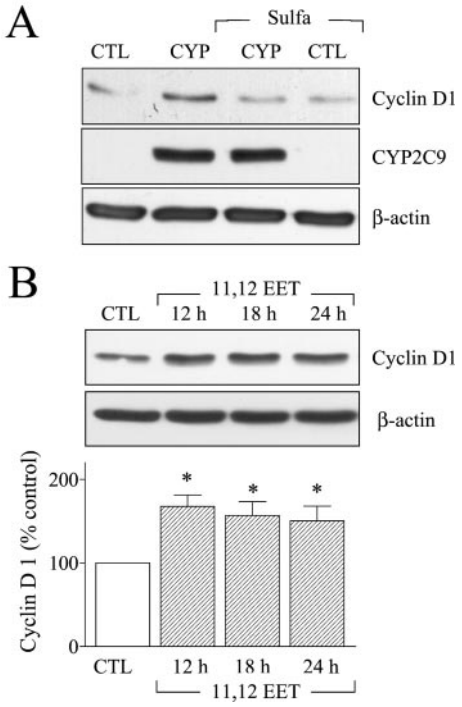

FIG. 3. **Sulfaphenazole prevents the CYP 2C9-induced up-regulation of cyclin D1, whereas 11,12-EET mimics the effects of CYP 2C9 overexpression.** *A*, representative Western blot showing the effect of CYP 2C9 overexpression on cyclin D1 expression in the absence and presence of sulfaphenazole (10 μM) 24 h after transfection. *CTL*, control. *B*, Western blot and densitometric analysis demonstrating the time-dependent effect of 11,12-EET (1 μM) on cyclin D1 expression. The Western blots shown are representative of data obtained in four independent experiments. The β-actin signal is shown to demonstrate that equal amounts of proteins were loaded in each lane; *, $p < 0.05$ *versus* control.

Effect of CYP 2C9 on the Expression of Cyclins—Extracellular mitogens induce cell proliferation via receptor-mediated signaling targeting the cell cycle machinery. Cyclins, especially the D-type cyclins, function as critical sensors of these mitogenic signals, which accumulate and assemble with cyclin-dependent kinases (CDKs) during G_1 phase to promote cell cycle progression. To investigate whether or not CYP 2C9 influences cyclin expression to induce cell proliferation, we determined the expression of cyclin D1 in endothelial cells transfected with either a control vector or CYP 2C9. CYP 2C9 significantly increased the expression of cyclin D1 as compared with control vector-transfected cells (Fig. 2). The increase in cyclin D1 expression was ~2-fold above control levels after 24 h and ~2.5-fold above control levels after 36 h. Incubation of endothelial cells with sulfaphenazole (30 μM) prevented the CYP 2C9-induced up-regulation of cyclin D1, whereas basal cyclin D1 expression in control vector-transfected cells was not affected by the CYP inhibitor (Fig. 3*A*). 11,12-EET, which is a product of the metabolism of arachidonic acid by CYP 2C9 (17), also enhanced the expression of cyclin D1 in cultured endothelial cells (Fig. 3*B*).

Effects of CYP 2C9 on the Activation/Inactivation of MAP Kinases—To further investigate the molecular mechanisms involved in the CYP 2C9-mediated increase in cyclin D1 expression, we assessed the effects of CYP 2C9 overexpression on the activation of the MAP kinases: ERK1/2, p38 MAP kinase, and JNK. The activation of the ERK1/2 and p38 MAP kinases was determined by Western blot analysis using phospho-specific antibodies. Although no change in the phosphorylation of

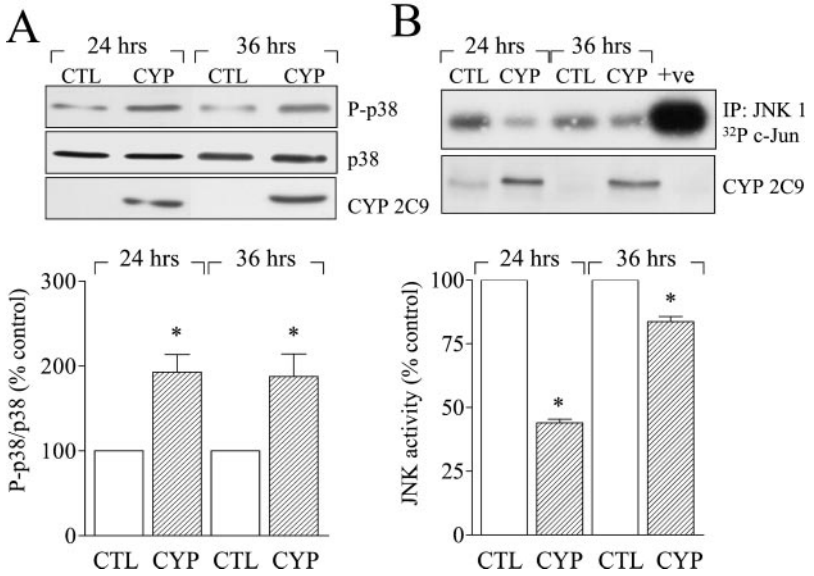

FIG. 4. **Overexpression of CYP 2C9 enhances the phosphorylation of p38 MAP kinase and decreases the activity of JNK1.** Quiescent endothelial cells were transiently transfected with either CYP 2C9 or control vector (*CTL*) plasmid. At the times indicated, the phosphorylation of the p38 MAP kinase (*A*) and the activity of JNK (*B*) were determined. *A*, representative Western blot demonstrating the effects of CYP 2C9 overexpression on p38 MAP kinase phosphorylation as compared with control vector-transfected cells. Membranes were probed with a phospho-specific antibody for p38 MAP kinase. To ensure equal loading of protein in each lane, membranes were reprobed with an antibody recognizing total p38 protein. The Western blot presented is representative of results obtained in three independent experiments. *B*, immunocomplex kinase assays were performed using a GST-Jun fusion protein as a substrate for JNK. A representative autoradiograph and the densitometric analysis from five independent experiments are shown; *, $p < 0.05$ *versus* control.

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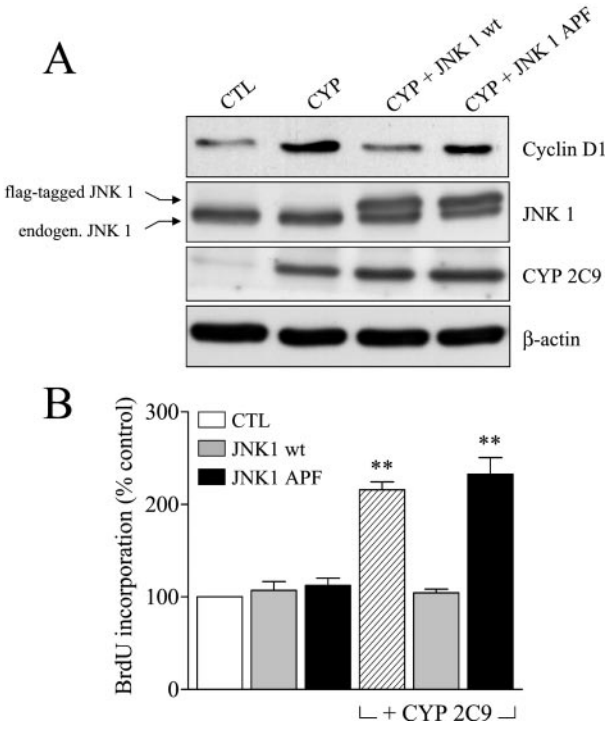

FIG. 5. **Overexpression of wild type JNK1 attenuates the CYP 2C9-induced up-regulation of cyclin D1 and endothelial cell proliferation.** *A*, representative Western blots showing the effects on cyclin D1 expression of coexpressing wild type JNK1 (*JNK1 wt*) or dominant negative JNK1 (*JNK1 APF*) with CYP 2C9 (24 h). To ensure equal expression of the transfected plasmids, membranes were reprobed with antibodies against JNK1 and CYP 2C9. The β-actin signal is shown to demonstrate that equal amounts of proteins were loaded in each lane. The Western blots shown are representative of results obtained in three independent experiments. *CTL*, control. *B*, HUVEC were transfected with pcDNA3.1, CYP 2C9, wild type JNK (*JNK1 wt*), a dominant negative JNK (*JNK1 APF*), or a combination of CYP 2C9 and either JNK1 wt or JNK1 APF. After 24 h in culture, cells were incubated with BrdUrd (*BrdU*, 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; **, $p < 0.01$ *versus* control.

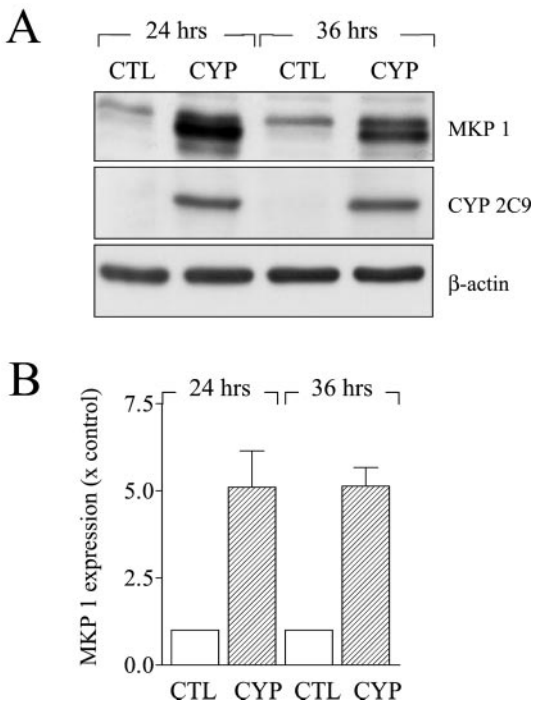

FIG. 6. **CYP 2C9 induces the expression of MAP kinase phosphatase MKP-1.** Representative Western blot (*A*) and statistical analysis (*B*) showing the effect of CYP 2C9 overexpression on the expression of MKP-1. At the times indicated, Triton X-100-soluble cell fractions were prepared and subjected to SDS-PAGE. Western blotting was performed using an MKP-1 antibody. Membranes were then reprobed with antibodies against CYP 2C9. The β-actin signal is shown to demonstrate that equal amounts of proteins were loaded in each lane. The results represent data obtained in four independent experiments; **, $p < 0.01$ *versus* control (*CTL*).

ERK1/2 phosphorylation could be detected (data not shown), the p38 MAP kinase was phosphorylated and thus activated in CYP 2C9-overexpressing endothelial cells (Fig. 4*A*). On the other hand, overexpression of endothelial cells with CYP 2C9 decreased the basal activity of JNK, assessed in an *in vitro* kinase assay using GST-Jun as a substrate (Fig. 4*B*). In these experiments, JNK activity was reduced by ~60 and 20% at 24 and 36 h after transfection, respectively.

Role of the p38 MAP Kinase and JNK in the CYP 2C9-induced Up-regulation of Cyclin D1—To delineate the role of the differential activation of JNK and p38 in the CYP 2C9 signaling cascade leading to enhanced cyclin D1 expression, endothelial cells were cotransfected with plasmids encoding for CYP 2C9 and either wild type or dominant negative forms of the p38 MAP kinase or JNK. Cotransfection of dominant negative as well as the wild type p38 MAP kinase with CYP 2C9 did not affect cyclin D1 expression (data not shown), excluding a role for p38 MAP kinase in the CYP 2C9-induced cyclin D1 up-regulation. The CYP 2C9-mediated up-regulation of cyclin D1 was, however, almost completely reversed by coexpression of wild type JNK1 with CYP 2C9. In contrast, cotransfecting dominant negative JNK1 with CYP 2C9 restored the CYP 2C9-induced increase in cyclin D1 expression (Fig. 5*A*). These alterations in cyclin D1 expression were paralleled by changes in BrdUrd incorporation. When endothelial cells were transfected with wild type or dominant negative JNK1, no changes in cyclin D1 expression were detected (data not shown), and BrdUrd incorporation was similar to that detected in cells transfected with pcDNA3.1 (Fig. 5*B*). As shown above, overexpression of CYP 2C9 increased BrdUrd incorporation. This effect was unaffected by cotransfection of CYP 2C9 and a dominant negative JNK1 but was completely abolished in cells cotransfected with CYP 2C9 and wild type JNK1 (Fig. 5*B*).

Effect of CYP 2C9 on the Expression of MKP-1—Inactivation or attenuation of JNK signaling is linked to the dephosphorylation of JNKs on their regulatory threonine and tyrosine residues by a family of dual specificity phosphatases. One phosphatase, which inhibits JNK *in vivo* and *in vitro*, is MKP-1 (18). We therefore examined the effects of CYP 2C9 on MKP-1 expression. Transfection of endothelial cells with CYP 2C9 increased the expression of MKP-1 protein by ~5-fold (Fig. 6). Moreover, the time course of MKP-1 induction paralleled the CYP 2C9-mediated decrease in JNK activity, suggesting a role for MKP-1 as a mediator of the inhibitory effect of CYP 2C9 on JNK.

Effects of 11,12-EET and H_2O_2 on Basal JNK Activity and MKP-1 Expression—Since CYP 2C9 generates EETs as well as ROS, both of which can affect the activation of MAP kinases as well as cell proliferation (7, 19, 20), we compared the effects of 11,12-EET and H_2O_2 on JNK activity and MKP-1 expression. Stimulation of endothelial cells with 11,12-EET (1 μM) elicited a marked and time-dependent decrease in basal JNK activity, which was pronounced 1 h after stimulation (Fig. 7*A*). Although 11,12-EET was applied only once, JNK activity remained attenuated over 12 h. Incubation of endothelial cells with H_2O_2 (50 μM), on the other hand, increased the activity of JNK1 (Fig. 7*B*).

In accordance with the effects of CYP 2C9 overexpression on JNK activity, MKP-1 protein levels were time dependently increased in response to stimulation with 11,12-EET (Fig. 8).

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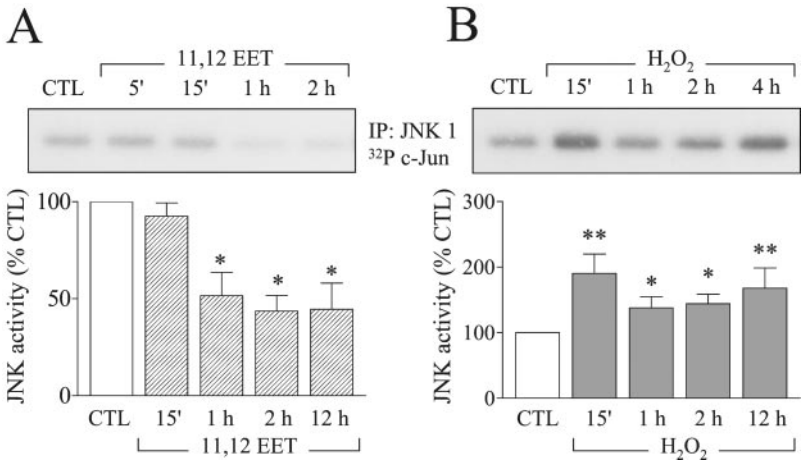

FIG. 7. **Effect of 11,12-EET and H_2O_2 on the activity of JNK1.** *A*, endothelial cells stimulated with 11,12-EET (1 μM) for the indicated time period. Immunocomplex kinase assays were then performed using GST-c-Jun as substrate for JNK. A representative autoradiograph and densitometric analysis from four independent experiments are shown. *CTL*, control. *B*, endothelial cells stimulated with H_2O_2 (50 μM) for the times indicated. Thereafter, immunocomplex kinase assays for JNK were performed. A representative autoradiograph and densitometric analysis from three independent experiments are shown. *IP*, immunoprecipitate.

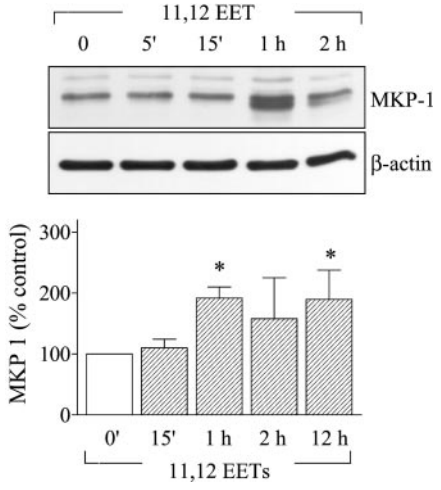

FIG. 8. **11,12-EETs induce the expression of MAP kinase phosphatase MKP-1.** Western blot and densitometric analysis demonstrating the effects of 11,12-EETs on MKP-1 expression. Endothelial cells were treated with 11,12-EET (1 μM) for the indicated time periods. Thereafter, incubations were stopped, and Triton X-100-soluble cell fractions were prepared. Western blotting was performed using an antibody recognizing MKP-1. The results shown represent the data obtained in four independent experiments; *, $p < 0.05$; **, $p < 0.01$ *versus* control.

The increase in MKP-1 expression paralleled the time course of JNK inactivation, suggesting that the up-regulation of MKP-1 was responsible for the EET-induced inhibition of JNK activity. Incubation of endothelial cells with H_2O_2 had no effect on MKP-1 expression (data not shown). CYP 2C9-derived EETs rather than ROS are therefore the most likely mediators of the enhanced cyclin D1 expression and endothelial cell proliferation.

DISCUSSION

We have demonstrated previously that the CYP 2C9 product, 11,12-EET, activates multiple signal transduction pathways in native and cultured endothelial cells and that overexpression of a CYP 2C epoxygenase enhances endothelial cell numbers (7). Therefore, we set out to elucidate the intracellular signal transduction cascade linking enhanced CYP expression and EET production with proliferation. The results of the present investigation demonstrate that the overexpression of CYP 2C9 in human endothelial cells stimulates cell proliferation by targeting the expression of the cell cycle regulatory protein, cyclin D1. The overexpression of CYP 2C9 increased the generation of EETs, which enhanced the expression of the immediate early gene product MKP-1. An MKP-1-mediated reduction in JNK activity resulted in an enhanced expression of cyclin D1, which mediated the proliferative effect of CYP 2C9 overexpression in endothelial cells.

Progression through the mammalian cell cycle requires the activation of CDKs by their association with the cyclin proteins that are regulatory subunits. Different CDK-cyclin holoenzymes are activated at specific phases of the cell cycle, and active CDK-cyclin complexes phosphorylate the retinoblastoma gene product (pRb) and the related pocket proteins p107 and p130 from mid-G_1 to mitosis. As a consequence of Rb hyperphosphorylation, the association between Rb and the transcription factor E2F is disrupted, facilitating the transcription of a bank of genes essential for DNA synthesis and S phase progression (21). We concentrated on the CYP 2C9-induced alterations in cyclin D1 expression as this relatively labile cyclin provides a link between mitogenic cues and the cell cycle machinery.

Numerous pathways have been reported to regulate the expression of cyclin D1, but the MAP kinase family is generally assumed to play a crucial role in this process (22). Depending on the cell type investigated, proliferative stimuli have been linked to the activation as well as the inactivation of ERK1/2, JNK, and the p38 MAP kinase (23). In the present investigation, we observed that the phosphorylation and thus presumably the activation of the p38 MAP kinase was enhanced in CYP 2C9-overexpressing endothelial cells, whereas the activity of JNK was attenuated, and the phosphorylation of ERK1/2 was unaffected. Of the MAP kinases affected by CYP 2C9, a link between kinase activity and cyclin D1 expression could only be made for JNK, and a decrease in JNK activity was associated with an increase in cyclin D1 expression. Moreover, coexpression of CYP 2C9 and JNK prevented the CYP 2C9-induced induction of cyclin D1 expression as well as the CYP 2C9-induced increase in endothelial cell proliferation. Cyclin D1 expression and proliferation were, however, enhanced in endothelial cells, which overexpressed both CYP 2C9 and a dominant negative JNK mutant. Overexpressing CYP 2C9 together with either a wild type or dominant negative p38 MAP kinase did not affect cyclin D1 levels.

The JNK/stress-activated protein kinase pathway is activated by numerous cellular stresses, and although it has been implicated in mediating apoptosis and growth factor signaling, its role in endothelial cell growth is unclear. The limited number of studies that specifically address the role of JNK in endothelial cell proliferation has, however, indicated that in vascular endothelial growth factor-treated and integrin-activated endothelial cells, the activation rather than the inactivation of JNK is associated with an increase in cyclin D1 expression, as well as endothelial cell proliferation and migration (24–26). It is, however, more than likely that JNK activity

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is differentially regulated by different proliferative stimuli as, for example, insulin-stimulated cell proliferation and survival is reported to involve the phosphatidylinositol 3-kinase-mediated inhibition of JNK activity (27). Although the precise molecular steps between CYP-dependent JNK inactivation and cyclin D1 up-regulation remain to be identified, our findings indicate a novel role for JNK in the control of cyclin D1 expression in endothelial cells.

To determine how CYP 2C9 attenuates the activity of JNK, we assessed the effects of CYP overexpression on the expression of MKP-1, a dual specificity protein phosphatase that is reported to exhibit a certain specificity/preference for JNK-1 (18, 28). CYP 2C9 overexpression resulted in the induction of MKP-1 with a time course that paralleled the decrease in JNK activity and the increase in cyclin D1 expression.

CYP 2C9 does not only generate EETs in endothelial cells, and this enzyme, like other CYP epoxygenases, generates ROS in amounts sufficient to affect intracellular signaling (14). Because ROS, such as superoxide anions and hydrogen peroxide (H_2O_2), have also been reported to affect MKP expression, MAP kinase activity, and cell proliferation (20, 29–31), we determined whether or not the effects of CYP 2C9 overexpression could be mimicked by incubating endothelial cells with 11,12-EET or H_2O_2. We found that 11,12-EET and H_2O_2 exert contrasting effects on JNK activity in endothelial cells in that EET stimulation decreased the JNK-mediated phosphorylation of c-Jun, whereas H_2O_2 increased kinase activity. Similarly, 11,12-EET, but not H_2O_2, induced the expression of MKP-1.

Although our results suggest that EET-mediated alterations in the MKP-1 expression and JNK activity regulate the expression of cyclin D1, we cannot rule out the possibility that additional parallel signaling pathways are also involved. For example, recent reports have suggested that EETs are capable of activating phosphatidylinositol 3-kinase (32), which results in the activation of the protein kinase B/Akt (8). The activation of protein kinase B/Akt could contribute to CYP 2C9-induced cell proliferation by inhibiting the glycogen synthase kinase-3β-dependent phosphorylation and degradation of cyclin D1 (33) as well as the phosphorylation and degradation of $p21^{Cip1}$ (34). Indeed, $p21^{Cip1}$ expression is enhanced in endothelial cells overexpressing CYP 2C9,[2] and $p21^{Cip1}$ has been shown to be a positive regulator of cyclin D-dependent kinases and to promote cell cycle progression by acting as an essential assembly factor for cyclin D-CDK complexes (35–37). A further possibility is that EET signaling involves cross-talk with the epidermal growth factor receptor (32) and that this receptor may function directly as a transcription factor to enhance cyclin D1 expression (38). Thus, it is certainly feasible that at least part of the proliferative effects of CYP 2C9 overexpression in endothelial cells can also be attributed to the parallel activation of signaling cascades not directly affecting JNK.

Epoxygenase-derived EETs are now recognized as important modulators of intracellular signal transduction cascades, but it is not clear exactly how EETs can initiate their effects on cell signaling. Two modes of cell activation by EETs have been proposed. The first involves the activation of a putative extra- or intracellular EET receptor (39), and the second involves the incorporation of EETs into the plasma membrane where they may associate with effector molecules such as ADP-ribosyltransferases (40), the small GTP-binding protein Ras (41), or protein kinase A (42). Although the initial steps in the signal cascade remain to be elucidated, the results of the present study clearly demonstrate that CYP epoxygenase-derived EETs induce the expression of the immediate early gene product MKP-1, which dephosphorylates and inactivates JNK, thus enhancing cyclin D1 levels and promoting endothelial cell proliferation.

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[2] M. Potente, unpublished observations.

REFERENCES

1. Fleming, I. (2001) *Circ. Res.* **89,** 753–762
2. Campbell, W. B., Gebremedhin, D., Pratt, P. F., and Harder, D. R. (1996) *Circ. Res.* **78,** 415–423
3. Li, P. L., Zou, A. P., and Campbell, W. B. (1997) *Hypertension* **29,** 262–267
4. Campbell, W. B., Falck, J. R., and Gauthier, K. (2001) *Med. Sci. Monit.* **7,** 578–584
5. Li, P. L., and Campbell, W. B. (1997) *Circ. Res.* **80,** 877–884
6. Hoebel, B. G., and Graier, W. F. (1998) *Eur. J. Pharmacol.* **346,** 115–117
7. Fleming, I., Fisslthaler, B., Michaelis, U. R., Kiss, L., Popp, R., and Busse, R. (2001) *Pfluegers Arch. Eur. J. Physiol.* **442,** 511–518
8. Chen, J. K., Capdevila, J., and Harris, R. C. (2001) *Mol. Cell. Biol.* **21,** 6322–6331
9. Node, K., Huo, Y., Ruan, X., Yang, B., Spiecker, M., Ley, K., Zeldin, D. C., and Liao, J. K. (1999) *Science* **285,** 1276–1279
10. Lin, J. H. C., Kobari, Y., Zhu, Y., Stemerman, M. B., and Pritchard, K. A., Jr. (1996) *Endothelium* **4,** 219–229
11. Fisslthaler, B., Popp, R., Kiss, L., Potente, M., Harder, D. R., Fleming, I., and Busse, R. (1999) *Nature* **401,** 493–497
12. Fisslthaler, B., Hinsch, N., Chataigneau, T., Popp, R., Kiss, L., Busse, R., and Fleming, I. (2000) *Hypertension* **36,** 270–275
13. Fisslthaler, B., Popp, R., Michaelis, U. R., Kiss, L., Fleming, I., and Busse, R. (2001) *Hypertension* **38,** 1427–1432
14. Fleming, I., Michaelis, U. R., Bredenkotter, D., Fisslthaler, B., Dehghani, F., Brandes, R. P., and Busse, R. (2001) *Circ. Res.* **88,** 44–51
15. Fleming, I., Fisslthaler, B., and Busse, R. (1996) *J. Biol. Chem.* **271,** 11009–11015
16. Mancy, A., Dijols, S., Poli, S., Guengerich, P., and Mansuy, D. (1996) *Biochemistry* **35,** 16205–16212
17. Daikh, B. E., Lasker, J. M., Raucy, J. L., and Koop, D. R. (1994) *J. Pharmacol. Exp. Ther.* **271,** 1427–1433
18. Hirsch, D. D., and Stork, P. J. S. (1997) *J. Biol. Chem.* **272,** 4568–4575
19. Chen, J.-K., Falck, J. R., Reddy, K. M., Capdevila, J., and Harris, R. C. (1998) *J. Biol. Chem.* **273,** 29254–29261
20. Ruiz-Gines, J. A., Lopez-Ongil, S., Gonzalez-Rubio, M., Gonzalez-Santiago, L., Rodriguez-Puyol, M., and Rodriguez-Puyol, D. (2000) *J. Cardiovasc. Pharmacol.* **35,** 109–113
21. Ekholm, S. V., and Reed, S. I. (2000) *Curr. Opin. Cell Biol.* **12,** 676–684
22. Cook, S. J., Balmanno, K., Garner, A., Millar, T., Taverner, C., and Todd, D. (2000) *Biochem. Soc. Trans.* **28,** 233–240
23. Wilkinson, M. G., and Millar, J. B. (2000) *FASEB J.* **14,** 2147–2157
24. Pedram, A., Razandi, M., and Levin, E. R. (1998) *J. Biol. Chem.* **273,** 26722–26728
25. Oktay, M., Wary, K. K., Dans, M., Birge, R. B., and Giancotti, F. G. (1999) *J. Cell Biol.* **145,** 1461–1469
26. Pedram, A., Razandi, M., and Levin, E. R. (2001) *Endocrinology* **142,** 1578–1586
27. Desbois-Mouthon, C., Cadoret, A., Blivet-Van Eggelpoel, M. J., Bertrand, F., Caron, M., Atfi, A., Cherqui, G., and Capeau, J. (2000) *Endocrinology* **141,** 922–931
28. Hofken, T., Keller, N., Fleischer, F., Goke, B., and Wagner, A. C. (2000) *Biochem. Biophys. Res. Commun.* **276,** 680–685
29. Tournier, C., Thomas, G., Pierre, J., Jacquemin, C., Pierre, M., and Saunier, B. (1997) *Eur. J. Biochem.* **244,** 587–595
30. Baas, A. S., and Berk, B. C. (1995) *Circ. Res.* **77,** 29–36
31. Irani, K. (2000) *Circ. Res.* **87,** 179–183
32. Chen, J.-K., Wang, D.-W., Falck, J. R., Capdevila, J., and Harris, R. C. (1999) *J. Biol. Chem.* **274,** 4764–4769
33. Diehl, J. A., Cheng, M., Roussel, M. F., and Sherr, C. J. (1998) *Genes Dev.* **12,** 3499–3511
34. Rössig, L., Badorff, C., Holzmann, Y., Zeiher, A. M., and Dimmeler, S. (2002) *J. Biol. Chem.* **277,** 9684–9689
35. LaBaer, J., Garrett, M. D., Stevenson, L. F., Slingerland, J. M., Sandhu, C., Chou, H. S., Fattaey, A., and Harlow, E. (1997) *Genes Dev.* **11,** 847–862
36. Cheng, M., Olivier, P., Diehl, J. A., Fero, M., Roussel, M. F., Roberts, J. M., and Sherr, C. J. (1999) *EMBO J.* **18,** 1571–1583
37. Sherr, C. J., and Roberts, J. M. (1999) *Genes Dev.* **13,** 1501–1512
38. Lin, S. Y., Makino, K., Xia, W., Matin, A., Wen, Y., Kwong, K. Y., Bourguignon, L., and Hung, M. C. (2001) *Nat. Cell. Biol.* **3,** 802–808
39. Wong, P. Y., Lai, P. S., and Falck, J. R. (2000) *Prostaglandins Other Lipid Mediat.* **62,** 321–333
40. Li, P.-L., Chen, C.-L., Bortell, R., and Campbell, W. B. (1999) *Circ. Res.* **85,** 349–356
41. Muthalif, M. M., Uddin, M. R., Fatima, S., Parmentier, J., Khandekar, Z., and Malik, K. U. (2001) *Prostaglandins* **65,** 33–43
42. Imig, J. D., Inscho, E. W., Deichamnn, P. C., Reddy, K. M., and Falck, J. R. (1999) *Hypertension* **33,** 408–413

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