0026-895X/04/6504-934–943$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 65:934–943, 2004

Vol. 65, No. 4
2882/1136432
Printed in U.S.A.

Aryl Hydrocarbon Receptor Activation and Cytochrome P450 1A Induction by the Mitogen-Activated Protein Kinase Inhibitor U0126 in Hepatocytes

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Received August 5, 2003; accepted December 22, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

The aryl hydrocarbon receptor (AhR) is involved in various processes such as cytochrome P450 (P450) 1A induction after xenobiotic exposure. It is also considered to play a major role in cell proliferation and differentiation. Recent evidences have suggested a cross-talk between AhR functions and the mitogen-activated protein kinase (MAPK) cascade. We now report that 1,4-diamino-2,3-dicyano-1,4-*bis*[2-aminophenylthio]butadiene (U0126), a specific inhibitor of MAPK kinase (MEK) MEK1/2, elicits a marked increase in CYP1A1 expression at both mRNA and protein levels associated with a significant increase of enzyme activity in primary rat hepatocytes and a human hepatoma cell line. This induction occurred independently of MEK/extracellular signal-regulated kinase (ERK) activation and in the absence of ERK1 and ERK2 expression. The effect of U0126 was mediated by its ability to transactivate xenobiotic responsive element (XRE)-driven genes, as demonstrated by transfection assays with an XRE-driven luciferase construct in the human B16A2 hepatoma cell line. CYP1A1 modulation was abolished by a cotreatment with resveratrol, an established AhR antagonist, arguing for AhR activation by U0126. Such an effect was demonstrated by direct in vitro ligand binding competition assays using rabbit liver cytosol, showing that this compound binds AhR with an $EC_{50} = 25 \times 10^{-6}$ M. Moreover, we demonstrated that U0126 is a substrate for several P450s including human CYP1A2, -1A1, and -1B1. We conclude that the widely used specific inhibitor of MEK/ERK, U0126, also acts as a potent AhR activator and an inducer of related genes. Such effects on the AhR may have an impact on biological functions attributed previously to MAPK inhibition.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and polycyclic aromatic hydrocarbons (PAH) are potent inducers of several genes, including some encoding "Phase I" and "Phase II" xenobiotic-metabolizing enzymes. These enzymes include cytochrome P450 (P450), glutathione transferases, NADPH: quinone reductases, and UDP-glucuronosyl transferases. TCDD and PAH effects are mediated by activation of the aryl hydrocarbon receptor (AhR), a cytosolic protein that forms complexes with two 90-kDa heat shock proteins and some other proteins. After ligand binding, the AhR is translocated and localized in the nucleus followed by an heterodimerization with the AhR nuclear translocator (Arnt) protein and acts as a transcriptional factor. Several reports have also shown constitutive activation of the AhR in the absence of exogenous ligand under certain conditions (Singh et al., 1996; Crawford et al., 1997; Chang and Puga, 1998; Adachi et al.,

This work was supported by the Institut National de la Santé et de la Recherche Médicale, the Ligue Nationale contre le Cancer, comité d'Ille et Vilaine, and United States Public Health Service grants R01-CA90426 and P30-ES00267.

ABBREVIATIONS: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AhR, aryl hydrocarbon receptor; OPZ, oltipraz; 3MC, 3-methylcholanthrene; BaP, benzo[*a*]pyrene; P450, cytochrome P450; MAPK, mitogen-activated protein kinase; PAH, polycyclic aromatic hydrocarbon; EROD, ethoxyresorufin *O*-deethylation; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase; EGF, epidermal growth factor; XRE, xenobiotic-responsive element; PD98059, 2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one; U0126, 1,4-diamino-2,3-dicyano-1,4-*bis*[2-aminophenylthio]butadiene; βNF, β-naphthoflavone; TBS, Tris-buffered saline; αNF, α-naphthoflavone; Q-PCR, quantitative reverse transcription-polymerase chain reaction; Arnt, aryl hydrocarbon receptor nuclear translocator; HPLC, high-performance liquid chromatography; DMSO, dimethyl sulfoxide; siRNA, small interfering RNA; GFP, green fluorescent protein; SSC, standard saline citrate; APCI, atmospheric pressure chemical ionization.

2001); however a physiological ligand for the AhR has yet to be identified.

AhR knockout mice exhibit decreased liver size, hepatic portal fibrosis (Gonzalez and Fernandez-Salguero, 1998; Lahvis et al., 2000), and decreased constitutive expression of certain xenobiotic-metabolizing enzymes. The AhR is also proposed to play an important role in cell and tissue homeostasis in vivo by participating with other signaling mechanisms in controlling cell proliferation and differentiation without a requirement of exogenous ligands, as shown in mouse hepatoma cells (Ma and Whitlock, 1996). Evidence has been presented that AhR function might be affected by processes involving the mitogen-activated protein kinase (MAPK) signaling cascade (Reiners et al., 1997). The serine/threonine extracellular signal-regulated kinases (ERK) ERK1/2 are critically implicated in many intracellular signaling events such as proliferation and differentiation (Talarmin et al., 1999). Indeed, two peaks of mitogen-activated protein kinase kinase (MEK) activation have been demonstrated in hepatocytes in G_1 phase, leading to transactivation of genes involved in the S phase. The MEK/ERK pathway has been shown to be crucial in controlling hepatocyte morphology (Rescan et al., 2001). Both endogeneous and exogeneous compounds can modulate both AhR function and MAPK activity. Thus, the flavonoid PD98059 has been reported to be both a specific MEK1/2 inhibitor and an AhR antagonist, being a suppressor of induction of CYP1A1 and -1B1 and NADPH quinone oxidoreductase 1, three enzymes induced by PAHs and TCDD (Reiners et al., 1998). Another compound, U0126, initially developed as an anti-inflammatory drug, has been demonstrated recently to be a very specific potent inhibitor of MEK1/2 (Favata et al., 1998), raising the question of whether it might also act as an AhR antagonist.

We now report that U0126 has AhR ligand properties and behaves as a potent CYP1A inducer in both rat primary hepatocytes and human hepatoma cells in contrast to the antagonistic effects of PD98059. These effects occur at the transcriptional level, are mediated by xenobiotic-responsive element (XRE) sequences, and are independent of U0126 inhibition of MEK/ERK phosphorylation. We also demonstrate that P450s are able to catalyze the NADPH-dependent metabolism of U0126.

Materials and Methods

Chemicals. U0126 and EGF were obtained from Promega (Madison, WI), and 3MC, DMSO, resveratrol, and α-naphthoflavone (αNF) were from Sigma-Aldrich (St Louis, MO). PD98059 was supplied from Calbiochem (Darmstadt, Germany). Oltipraz (OPZ) was kindly provided by Dr. F. Ballet (Aventis, Antony, France). TCDD was purchased from Wellington Laboratories (Guelph, ON, Canada), and [1,6-^{3}H]TCDD (specific activity, 28 Ci/mmol) was from Terrachem (Lenexa, KS). TCDD stock and radiolabeled TCDD were dissolved in nonane/ethanol (50:50, v/v) and toluene/ethanol (14:86, v/v) mixtures, respectively.

Microsomal Preparations. Microsomes were prepared from rat and human liver samples. Male Sprague-Dawley rats (125–150 g each) were purchased from Harlan (Indianapolis, IN) and treated with either corn oil (0.5 ml/animal/day) or 40 mg β-naphthoflavone (7,8-benzoflavone; βNF) in corn oil (once/day, 3 days). Rats were killed using a CO_2 atmosphere, and liver microsomes were prepared using differential centrifugation (Guengerich, 2001). Human liver microsomes were prepared from two samples, denoted HL 111 and HL 114. Some of the properties of these samples have been described in earlier work (Guengerich, 1988).

Liver Cell Cultures and Treatments. Hepatocytes were isolated from either Sprague-Dawley male rats (Janvier, Les-Genest-St-Isle, France) (150–200g) or MF1 wild or ERK1 knockout mouse livers by a two-step perfusion procedure using 0.025% collagenase (Boehringer Ingelheim GmbH, Gagny, France) buffered with 0.1 M HEPES, pH 7.4, as described previously (Guguen et al., 1975). The cells were plated at a density of 10^3/cm^2 in 60-mm diameter dishes in 2 ml of Williams' E medium (Laboratories Eurobio, Les Ulis, France) containing 2.5 mM glutamine, 1 mg/ml bovine serum albumin, 5 μg/ml insulin, and 100 IU/ml penicillin/100 μg/ml streptomycin and supplemented with 10% fetal calf serum (v/v). Four hours after plating, the medium was replaced with fresh medium without calf serum but with 5×10^{-5} M hydrocortisone hemisuccinate (Aventis). Primary hepatocytes were treated either 24 h after seeding or after synchronization at the restriction point, i.e., 48 h after seeding (Rescan et al., 2001). For siRNA interference studies, ERK2-specific sequences were synthesized as synthetic oligonucleotides (unpublished sequence; patent pending) and annealed to form a short double-stranded RNA with 3′-dithymidine overhang. Control siRNA was directed against a nonmammalian gene green fluorescent protein (GFP). ERK2 siRNAs were introduced into ERK1−/− mouse hepatocytes by lipid-mediated transfection using LipofectAMINE2000 (Invitrogen, Carlsbad, CA). The lipid complex was added dropwise and incubated for 4 h. CYP1A activity and inactivation of ERK2 were analyzed 48 h later.

Human hepatoma B16A2 cells were used after 3 weeks at confluence in Williams' E medium as described previously (Glaise et al., 1998). All stock solutions of chemicals used in this study were dissolved in DMSO and used at the final percentage of 0.7% in culture media.

RNA Isolation, Northern Blotting, and Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction. Total RNA from each sample was isolated using a SV RNA Kit (Promega), and CYP1A1 mRNA levels were analyzed using standard procedures. A 10-μg sample of total RNA was loaded on a denaturing 1% (w/v) agarose-6% (v/v) formaldehyde gel for electrophoresis and transferred with 20× standard saline citrate (SSC) (3 M trisodium citrate and 0.3 M NaCl, pH 7.0) onto a nylon filter (N^+ hybond; Amersham Biosciences Inc., Arlington Heights, IL). The integrity and relative amounts of RNA were assessed by methylene blue staining. Prehybridization and hybridization were performed according to the method described by Church and Gilbert (1984). cDNA probes were ^{32}P-labeled by random priming using a Rediprime labeling kit and [α-^{32}P]dCTP (Amersham Biosciences). Membranes were washed with 3× SSC/0.1% SDS (v/v) for 30 min and then twice with 1× SSC/0.1% SDS (v/v) for 10 min at 65°C. The two probes used were the human CYP1A1-specific cDNA probe, a gift from I. de Waziers (INSERM U490, Paris, France), and the P450 C6 cDNA probe complementary to rat CYP1A1 mRNA (Affolter et al., 1986). After hybridization, filters were stripped of the previous probes and rehybridized with nick-translated genomic 18S probe used as control. Relative mRNA amounts were determined by densitometry (Densilab, Microvision Instruments, Evry, France). Real-time quantitative reverse transcription-polymerase chain reaction (Q-PCR) for rat CYP1A1 mRNA was performed using an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) using Sybergreen as fluorescence probe (Eurogentec, Seraing, Belgium). The sequences of CYP1A1 primers were the following: forward primer, 5′-AAAGATCCAGGAGGAGGAGTTAGACACA-3′; reverse primer, 5′-GAGGTCTGTCAGAAAGCCGG-3′. One microgram of extracted mRNA from treated rat hepatocytes was reverse-transcribed by incubation with 0.5 mM deoxynucleoside-5′-triphosphate, 500 ng of random primers (Promega), and 0.01 M dithiothreitol in 1× first-strand buffer in the presence of 200 IU of SuperScript (Invitrogen). The reaction mixture for Q-PCR was performed with 3% of reverse-transcribed product in the presence of 1× master mix reagents (3.5

mM $MgCl_2$, 300 μM deoxynucleoside-5′-triphosphate, 0.25 IU hot goldStart enzyme, and SYBR Green) (Eurogentec, Seraing, Belgium). The Q-PCR assay was performed for 40 cycles simultaneously with 18S rRNA to normalize RNA loading.

Total Cell Extract Preparation. Cells were scraped, after washing in phosphate-buffered saline, in homogenization buffer [60 mM β-glycerophosphate, 15 mM *p*-nitrophenylphosphate, 25 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.2, 15 mM EGTA, 15 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM vanadate, 1 mM NaF, 1 mM phenylphosphate, 100 μM benzamidine, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml soybean trypsin inhibitor] supplemented with 100 μM phenylmethanesulfonyl fluoride. This step was performed at 4°C, and extracts were immediately stored at −20°C in blue loading buffer, whereas an aliquot was kept apart for protein estimation by the method described by Bradford (1976).

Immunoblotting Analysis. Cell lysates were resolved by denaturating SDS-polyacrylamide gel electrophoresis (10% gel, w/v). Proteins were transferred to nitrocellulose membranes by using a transblot apparatus (Millipore Corporation, St Quentin-Yvelines, France) overnight at 35 V in transfer buffer [0.5 mM Tris, 5 mM glycine, 10% ethanol (v/v), and 0.1% SDS(w/v)]. Transfer was checked by Ponceau red dying. Subsequently, filters were rinsed in Tris-buffered saline (TBS), pH 7.4, blocked with TBS-3% (w/v) bovine serum albumin for 2 h at room temperature, rinsed twice in washing buffer (TBS + 0.1% Tween 20, v/v), and hybridized for 2 h with diluted CYP1A1/2 antibody in TBS-3% bovine serum albumin at room temperature or overnight at 4°C with diluted anti-phosphoERK antibody (Cell Signaling Technology Inc., Beverly, MA) that detects phosphorylated (and therefore activated) ERK1/2. This antibody was a mouse monoclonal antiserum directed against a synthetic phosphothreonine/tyrosine peptide corresponding to residues 202 to 204 of human ERK1/2 MAPK (Cell Signaling Technology). The same blots were subsequently stripped and reprobed with a polyclonal antibody that recognizes ERK1 and ERK2 to verify equal amounts of the protein in the various samples (Cell Signaling Technology) or mouse myosin light chain (Sigma, St. Louis, MO) for the siRNA experiments. After three washes in the washing buffer and once in TBS, membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After three washes in wash buffer and twice in TBS, proteins were detected by the enhanced chemiluminescence kit procedure (Amersham Biosciences), and band intensity was measured by densitometry.

Determination of CYP1A1/2 Activity in Cultured Cells. Ethoxyresorufin *O*-deethylation (EROD) (catalyzed mainly by CYP1A1/2) was measured according to the procedure described by Burke and Mayer (1983). Briefly, liver cells plated in 96 wells were washed twice with phosphate-buffered saline at 37°C, and then 1.5 mM salicylamide was added to block phase II-conjugating enzymes. 7-Ethoxyresorufin (3 μM) was added one min later, and fluorescence of resorufin generated from the conversion of ethoxyresorufin to resorufin was measured every 2 min for 30 min at 37°C.

Transfection Assays. Confluent human hepatoma B16A2 cells were transfected with the jetPEI-Gal kit (Polyplus, Illkirch, France). The different plasmids used encoded the luciferase reporter gene and differed by the XRE constructs of the promoter as described previously (Le Ferrec et al., 2002). The p1A1-FL construct containing the −1566, +73 5′ region of the human CYP1A1 gene upstream of the firefly luciferase reporter gene was a gift from Professor R. Barouki (INSERM U490, Paris, France) and has been described previously (Morel and Barouki, 1998). Successive deletions of the 5′-flanking region were performed by PCR. The PCR products were subcloned into the luciferase reporter gene plasmid pGL3-basic (Promega) to yield the p1A1-FL(−1460) and p1A1-FL(−800) plasmids. A double-stranded oligonucleotide (CGACCTCAGGCTACGTGAGAATAGTG-CACTCAGGCTAGCGTGAGAAAGTGCACTCAGGCTAGCGTAGA-ATTGAGCT) containing three XRE sequences (underlined) was inserted between the KpnI and SacI sites of the pGL3-promoter to give pGL3-XRE3. B16A2 cells were cotransfected with the pRL-SV40 vector that codes for *Renilla* luciferase (Promega) plus the p1A1-FL constructs. Similar experiments were performed with a basic control consisting of the promoterless pGL3-luciferase construct (pGL3-basic) and a pGL3-promoter plasmid containing the SV40 promoter upstream of the luciferase gene. One hour after treatment, 500 μl of transfection medium (serum-free Williams E medium) containing 1 μg of luciferase reporter plasmid were added to confluent B16A2 cells along with 0.1 μg of the pRL-SV40 DNA and 4 μl of jetPEI-Gal. The cells were incubated for 24 h. Dual luciferase assays (firefly and *Renilla*) were performed with a Promega kit as recommended by the manufacturer.

In Vitro AhR Binding Competition Assay. The AhR binding competition assay was performed with rabbit liver cytosol as receptor source after the procedure used by Casper et al. (1999). Briefly, 1 ml of 0.8 mg cytosol prepared in 20 mM HEPES, pH 7.6, containing 1.5 mM EDTA, 10% glycerol (v/v), 20 mM KCl, and 5 mM $CaCl_2$ was incubated with various concentrations of U0126, 3MC, or BaP (used as positive control) dissolved in DMSO in the presence of 2 nM [1,6-^{3}H]TCDD for 4 h at 4°C. Unbound ligand was then removed by incubating the samples with 2% activated charcoal (w/v), suspending in cytosol buffer for 90 min at 4°C, and centrifuging at 15,000*g* for 10 min. Radioactivity was estimated by scintillation counting. Measurements were done in duplicate, and binding competition assays were repeated four times for U0126.

U0126 Metabolism Assay. Incubations of U0126 (20 μM, freshly dissolved in DMSO to 2 mM) were done in liver microsomes or *Escherichia coli* membranes in which human CYP1A1, -1A2, or -1B1 was coexpressed with human NAPDH-P450 reductase (Parikh et al., 1997). Reactions were fortified with an NADPH-generating system (Guengerich, 2001). After varying times, aliquots (0.30 ml) were mixed with CH_2Cl_2 (1.0 ml) using a vortex device. The layers were separated by centrifugation (3000*g* for 10 min), and 0.8 ml of the organic layer was transferred to a glass Reacti-vial (Pierce, Rockford, IL) and evaporated to dryness under an N_2 stream. The residues were stored at −20°C and dissolved (immediately before HPLC analysis) in 100 μl of CH_3CN. A 50-μl aliquot was injected onto a 6.2 × 80 mm Zorbax octadecylsilane HPLC column (3 μm; Mac-Mod, Chadds Ford, PA), which was eluted with a 16 to 68% gradient (v/v) of increasing CH_3OH in H_2O over 15 min (flow rate, 2.0 ml/min). The effluent passed through a ThermoSeparations UV3000R rapid-scanning detector (ThermoSeparations, Sunnyvale, CA) using the wavelength range of 220 to 400 nm for data collection. The 310-nm traces were used to estimate the amount of U0126 remaining.

The HPLC traces showed two peaks for U0126, eluting at 11 and 13 min. When dry U0126 (stored sealed from the manufacturer at −20°C) was dissolved in DMSO and injected into the HPLC column within 60 s, the two peaks were still observed. The two peaks had almost identical UV and mass spectra (MH^+ 381) and therefore seem to be isomers, as discussed later. Both peaks decreased during incubations, and the sum of the areas (310 nm absorbance) of the two peaks was used to estimate concentrations of U0126.

Mass Spectrometry. HPLC/atmospheric pressure chemical ionization (APCI) mass spectrometry studies on U0126 enzymatic products were done using a Finnigan TSQ 7000 triple quadrupole mass spectrometer (ThermoSeparations) operating in the positive ion mode using an APCI source set at a corona current of 5 μA and vaporizer temperature of 400°C. N_2 was used as the sheath gas (80 psi) to assist nebulization and as the auxiliary gas (10 psi) to assist with desolvation. The stainless steel capillary was heated to 210°C, and the APCI source and mass spectrometer parameters were optimized to obtain maximum sensitivity. The tube lens and the heated capillary were operated at 87 and 20 V, respectively, and the electron multiplier was set at 2000 V. HPLC conditions (Econosphere C18 octadecylsilane, 5 μm, 2.1 × 150 mm column; Alltech Associates, State College, PA) were the following: flow rate, 1.25 ml min^{-1} using a linear gradient of 20 to 75% CH_3OH (v/v) over a time of 15 min.

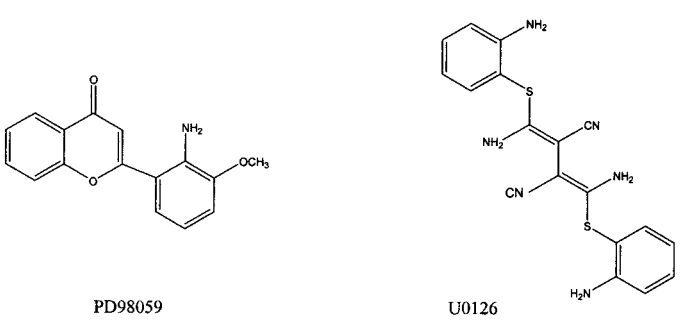
Statistical Analysis. Differences between group mean values were determined by a one-factor analysis of variance followed by

Dunnett's test for post hoc analysis for pairwise comparison of mean values.

Results

Effect of U0126 on EROD Activity and Implication of MEK/ERK Signaling Pathway. To analyze the effect of U0126 on CYP1A, we exposed primary rat hepatocytes to 50 μM U0126 for 24 h and then measured EROD activity. The effects were compared with those obtained with 75 μM PD98059, another MAPK inhibitor recognized as an AhR antagonist (Reiners et al., 1998) (Scheme 1). No cytotoxicity was detected by either light microscopy examination or the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide toxicity assay (results not shown). U0126 induced EROD activity as effectively as 3MC, a prototypical CYP1A inducer, whereas PD98059 had no significant effect (Fig. 1A). Interestingly, PD98059 suppressed CYP1A induction not only by 3MC as already reported but also by U0126 (Fig. 1A). Cotreatment of cells with U0126 and 3MC showed that U0126 was without such an antagonist effect. The different effects exerted by these two MEK inhibitors on CYP1A activity suggested an absence of correlation between ERK activity and CYP1A induction. To address this hypothesis, we analyzed the effect of EGF, a well-known MAPK activator. EGF treatment did not modulate EROD activity by itself, but it slightly decreased U0126-mediated induction (Fig. 1A). EGF stimulation of ERK phosphorylation was strongly decreased after 1, 3, 7, and 24 h of cotreatment with either U0126 or PD98059, confirming their inhibitory effects in our culture conditions (Fig. 1B) and leading to the conclusion that U0126 and PD98059 have similar effects on ERK inhibition, although they exert opposite effects on P450 activity. Similarly, 3MC did not influence ERK1 and ERK2 phosphorylation (Fig. 1B). Taken together, these results tend to prove an absence of correlation between ERK phosphorylation and induction of P450 activity. In addition, no changes in p38 MAPK were observed whatever the treatment except for a slight increase in the presence of EGF (Fig. 1B), indicating that this pathway is not implicated in CYP1A induction by U0126.

To demonstrate an absence of correlation between CYP1A induction by U0126 and the MEK/ERK pathway, we measured CYP1A activity in primary hepatocytes isolated from ERK1−/− knockout mice. In this model, we confirmed that U0126 strongly induced EROD activity at levels similar to those in wild-type mice and with 3MC (data not shown). We also inactivated ERK2 in the same cells by transfection with specific RNA interference. Transfection efficiency was first optimized using siRNA-GFP coupled to rhodamine. In optimal experiments, more than 90% of the cells seemed positive for rhodamine after 3 h of LipofectAMINE transfection. As shown in Fig. 1C, siERK2 RNA transfection causes ~90% disappearance of the corresponding protein compared with a control siGFP RNA duplex (Fig. 1C). In ERK1 and -2−/− hepatocytes, CYP1A induction by U0126 was still effective (induction by a factor 11), providing direct evidence that in the absence of a functional MEK/ERK pathway, U0126 was still able to induce P450 activity.



Scheme 1. Chemical structures of MAPK inhibitors used in this study.

Effects of Dose and Time on P450 Modulation by U0126. U0126 strongly induced CYP1A activity in rat hepatocytes in a dose-dependent manner after 24 and 48 h of treatment (Fig. 2A). An induction was detectable with a concentration as low as 3 μM (data not shown) and reached a maximum at 50 μM. No synergy was observed with a cotreatment of U0126 and 3MC. However, a 3-h treatment with 50 μM U0126 led to a transient inhibition of the activity (to 63%); this inhibition was reversed after 7 h (data not shown). Such an early inhibition was confirmed by measuring CYP1A activity in rat liver microsomes in the presence of U0126 (data not illustrated). An inhibition of EROD activity was obtained, being concentration-dependent and reaching 70% with a 50 μM concentration, indicating that U0126 caused direct inhibition of CYP1A activity (probably as a competitive substrate, vide infra). A similar effect of U0126 was obtained by using *E. coli* recombinant human CYP1A2 (data not shown). U0126 had no effect on pentoxyresorufin *O*-deethylation catalyzed by CYP2B nor on 6β-hydroxylation of testosterone catalyzed by CYP3A in rat hepatocytes, whatever the concentration used and the duration of exposure.

Induction of CYP1A activity by U0126 was correlated with an increase at both mRNA and protein level (Fig. 2, B and C). A marked increase (10-fold) was observed at mRNA levels as early as 3 h after the beginning of treatment (Fig. 2B). It reached a maximum after 12 h and then decreased during the following 12 h, representing approximately 4-fold the control value after 24 and 48 h of treatment. After 3 h of treatment by U0126, no variation in the protein level was observed; it became significant only after 24 h and reached the same maximum level as obtained with 3MC after 48 h (Fig. 2C). 3MC and U0126 induced both transcripts, protein and activity, at similar levels.

Mechanism of Induction of CYP1A by U0126. Because CYP1A induction by PAHs is mediated through a well-known transcriptional mechanism and U0126 led to results similar to those obtained with 3MC, we hypothesized that U0126 acts through the same mechanism. The effects of actinomycin D, an inhibitor of transcriptional activation, and cycloheximide, a protein-synthesis inhibitor, were investigated on CYP1A1 mRNA induction mediated by U0126. As shown in Fig. 3A, a 12-h treatment with actinomycin D (3 μg/ml) fully blocked the concentration-dependent increase of CYP1A1 mRNA, arguing for a transcription-mediated CYP1A induction effect of U0126. Requirement of protein synthesis in the induction process was analyzed by treating the cells with cycloheximide. A 12-h treatment with cycloheximide (10 μg/ml) had no effect on basal CYP1A1 mRNA expression measured by Q-PCR but led to a superinduction after U0126 cotreatment (Fig. 3B). Superinduction of CYP1A1 mRNA was dose-dependent and also occurred with 3MC, as reported previously (Ma et al., 2000).

The involvement of the XRE sequence, recognized by the

ligand-AhR-Arnt complex in CYP1A1 transcriptional induction of CYP1A, was then investigated. For this purpose, we transfected various human CYP1A promoter constructions, in front of a luciferase reporter gene, containing one or several XRE sequences into the differentiated human hepatoma cell line B16A2. In those cells, 50 μM U0126 significantly induced both CYP1A1 mRNA levels and EROD activity (data not shown). Transfection of B16A2 cells with plasmid constructs containing the 1460 fragment of the CYP1A promoter led to a significant induction after treatment with 3MC, U0126, or a combination of U0126 and 3MC (Fig. 4). However, a single XRE element was not sufficient to obtain a transcriptional induction with either 3MC or U0126, whereas a three-XRE sequence led to the same transcriptional induction effectiveness as with the entire promotor sequence.

To assess whether U0126-induced transcription is mediated through the AhR, we used the known antagonist resveratrol (Casper et al., 1999; Ciolino and Yeh, 1999). This compound has been shown to bind AhR and prevents AhR-Arnt transactivation of XRE-driven genes. Resveratrol prevented the luciferase activity induction mediated by U0126 with the 1460 plasmid construct (Fig. 5A). Implication of the AhR was further confirmed by treating rat hepatocytes with 100 μM resveratrol for 12 h, which resulted in a complete blockade of CYP1A mRNA induction by U0126 (as well as by 3MC and OPZ, used as positive controls) (Fig. 5B). Resveratrol inhibition was concentration-dependent and was effec-

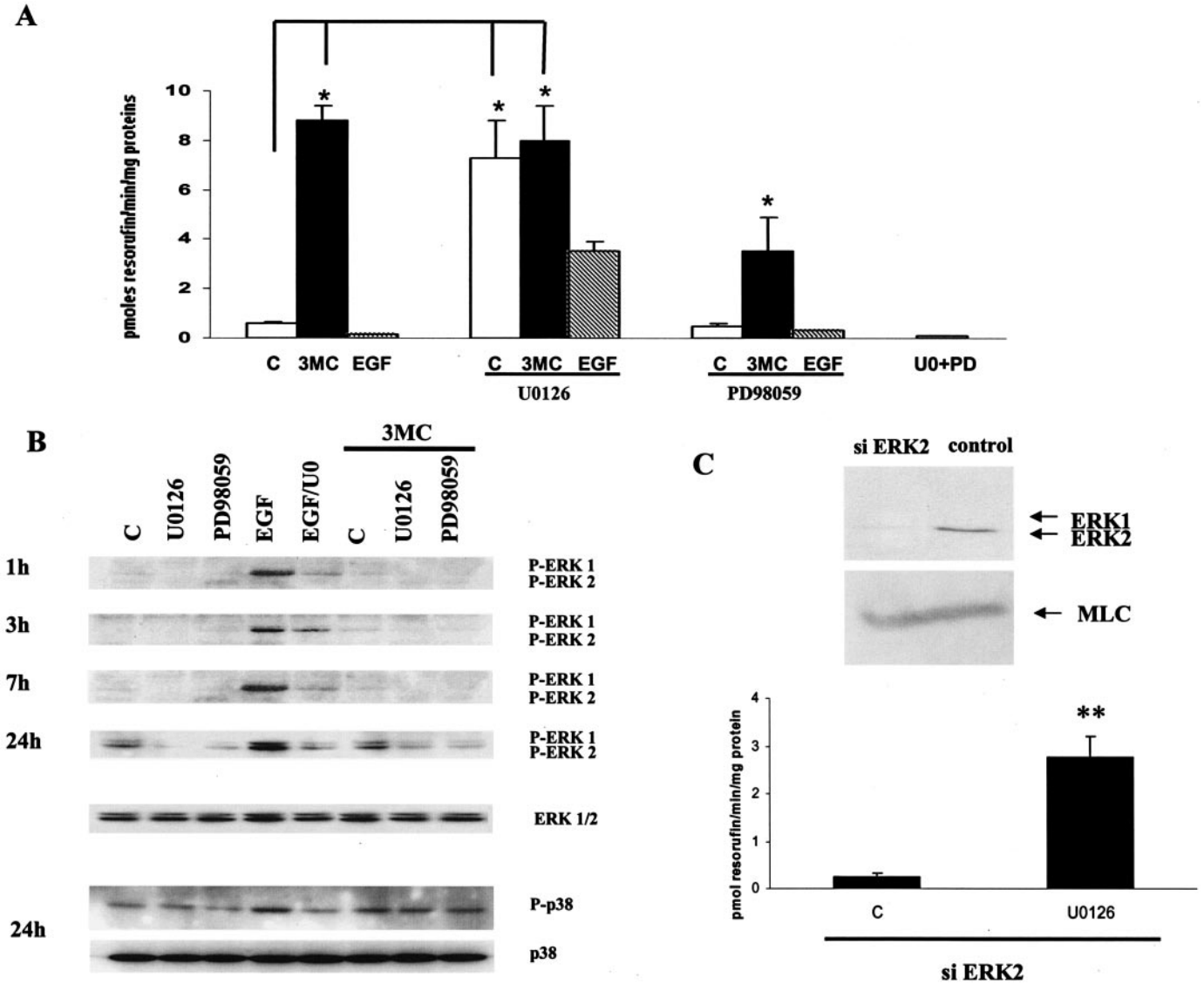


Fig. 1. Effects of U0126 on EROD activity and MEK/ERK signaling pathway. A, effect of MAPK inhibitors on EROD activity in the presence or absence of 3MC. EROD activity was measured in rat hepatocytes treated with 75 μM PD98059 or 50 μM U0126 in the absence (□) or presence (■) of either 5 μM 3MC or 50 ng/ml EGF (▨). Statistical analysis (Dunnett's test) was performed in this representative experiment reproduced three times by comparing U0126-, 3MC-, or U0126/3MC-treated and control cells (⋆, $p < 0.05$). B, ERK and p38 immunoblots of rat hepatocytes treated with 50 μM U0126, 75 μM PD98059, 50 ng/ml EGF, or EGF plus 50 μM U0126 (EGF/U0) in the presence or absence of 3MC at the indicated times of treatment. Total ERK1/2 was analyzed for protein quantitation. C, CYP1A activity in ERK1−/− mouse hepatocytes transfected with siRNA ERK2 (siERK2) or siRNA GFP (control) and the corresponding immunoblot showing the inactivation of ERK2 in the siERK2-transfected cells with myosin light chain hybridization for protein quantitation. Statistical analysis (Dunnett's test) was performed in a representative experiment by comparing U0126-treated with control cells (⋆⋆, $p < 0.01$).

tive at a concentration as low as 25 μM (data not shown). In vitro AhR ligand binding competition assays were performed with U0126 using rabbit liver cytosol as a source of AhR. The AhR ligands 3MC and BaP were used as controls. Our results showed that U0126 displaced [1,6-^{3}H]TCDD with an EC_{50} of $25 \pm 9 \times 10^{-6}$ M, whereas 3MC was as potent as nonradioactive TCDD in inhibiting binding to the AhR with an EC_{50} $= 1 \times 10^{-9}$ M. Competition efficiency of BaP was higher, with its EC_{50} of 1×10^{-8} M in our model.

P450-Dependent Metabolism of U0126. Preliminary experiments with rat and human liver microsomes and recombinant CYP1A2 indicated that U0126 was a P450 substrate and disappeared in the presence of NADPH (results not presented). Subsequent studies showed that U0126 disappeared in liver microsomes prepared from either control rats or the two human samples (Fig. 6A). Treatment of rats with βNF (a CYP1 inducer) or exposure of microsomes to αNF (an inhibitor of the CYP1 family) (Shimada et al., 1998) did not modify U0126 disappearance rates in rat liver, indicating that other P450s are involved in U0126 metabolism. However, recombinant human CYP1A1, -1A2, and -1B1 were all found to catalyze the disappearance of U0126 (Fig. 6B). Relatively little has been published about the chemistry of U0126 (Middleton et al., 1957), but it seems to be rather complex, with facile interconversion of the *E,E*, the *E,Z*, and the *Z,Z* isomers and slower rearrangement to the heterocyclic ring systems (Duncia et al., 1998). Freshly dissolved commercial U0126 yielded two HPLC peaks, which seemed to be isomers (t_R = 11.8, 13.8 min). We could not determine whether the apparent isomerization was so rapid that it occurred within minutes or whether the commercial (dry) product was already a mixture. The two peaks were collected, evaporated to dryness, dissolved in DMSO, and tested independently in the XRE-driven reporter. No differences were observed between the two peaks and the parent compound (data not shown). The only new product detected in HPLC analysis was an apparently more polar compound (t_R = 10.0 min). This compound was not completely characterized; it showed a loss of the typical UV spectrum in the region of 300 nm (the two substrate peaks had λ_{max} = 310 nm), and mass spectrometry showed an apparent MH^+ of 379 (M_r = 378) or loss of 2 atomic mass units from the substrate. This loss of 2 atomic mass units corresponds to a formal dehydrogenation, although the site of oxidation (and which isomer it came from) could not be established from the available data. HPLC/UV-visible analysis showed a band at 480 nm, suggesting an increased conjugation to produce this change in the chromophore. The same compound seemed to be formed in the different microsomal and re-

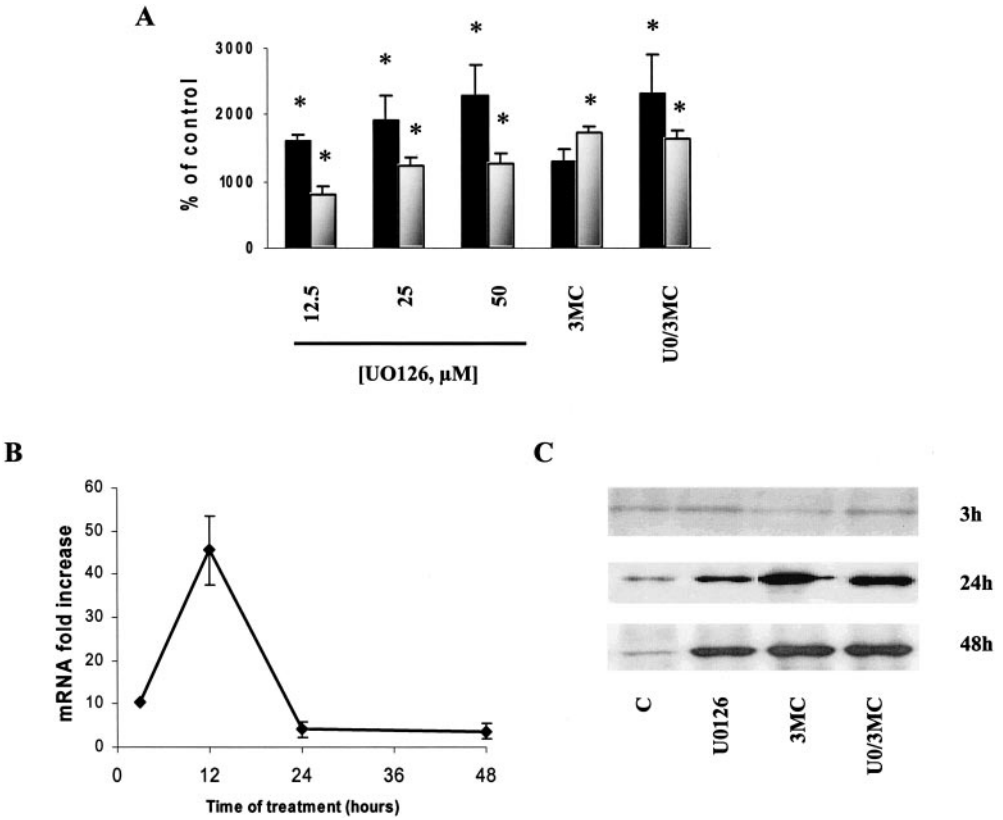


Fig. 2. Time-dependent effect of U0126 on CYP1A activity, protein, and mRNA levels. A, primary rat hepatocytes were treated with U0126 at various concentrations for 24 (■) or 48 h (■) and compared with the effect of 5 μM 3MC or cotreatment with 5 μM 3MC and 50 μM U0126 (U0/3MC). CYP1A activity was estimated by the EROD assay performed on intact cells, as described under *Materials and Methods*. The values are expressed as means ± S.D. of four measurements and as a percentage of the control, which are 0.13 and 0.22 pmol/min/mg protein for 24- and 48-h treated cells, respectively. Statistical analysis (Dunnett's test) was performed by comparing U0126-treated with control cells (⋆, $p < 0.05$). mRNA (B) and protein (C) levels were analyzed in primary rat hepatocytes treated with 50 μM U0126, 5 μM 3MC alone, or a combination of 50 μM U0126 and 5 μM 3MC (U0/3MC) for either 3, 12, 24, or 48 h. B, total RNA was isolated and subjected to Northern blot analysis. Hybridization of the same filters was performed successively with CYP1A1-specific cDNA and an 18S oligonucleotide probe used as control. The results obtained with this semiquantitative technique were expressed as percentages of U0126-treated versus control cells and represented the mean ± S.D. of three independent experiments. C, 10 μg of rat microsomes were analyzed by Western blot using a specific CYP1A1 antibody. The results are representative of an experiment reproduced three times.

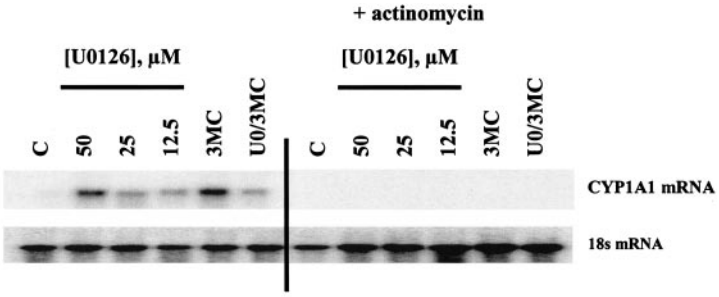
combinant P450 systems, although the data might not discern possible differences.

Discussion

Besides its function as a ligand-activated transcriptional factor required for the induction of some phase 1 and phase 2 xenobiotic-metabolizing enzymes, the AhR plays a role in cell differentiation and homeostasis (Schmidt et al., 1996; Gonzalez and Fernandez-Salguero, 1998). During recent years, several inhibitors of the major MAPK pathways have been described. Thus, the flavonoid compound PD98509 was reported to be both a specific inhibitor of the MAPK kinases MEK1/MEK2 and an AhR antagonist (Reiners et al., 1998). The present report shows that unlike PD98509, U0126—another specific MEK1/MEK2 inhibitor widely used to analyze MAPK implications in various biological functions—is a potent CYP1A inducer and is also a CYP1 substrate in hepatocytes. Moreover, our results clearly demonstrate that U0126 effects on MAPK and CYP1A modulation are not related; they are caused by its ability to bind the AhR and activate its transactivating function.

A variety of chemicals such as halogenated aromatic compounds (TCDD) and PAHs (3MC and BaP) are potent AhR agonists and inducers of several detoxifying enzymes. Our results support the conclusion that U0126 is an AhR ligand and an inducer of related genes in hepatocytes. First, in vitro binding assays clearly showed that U0126 competed in vitro with radiolabeled TCDD, although with a relatively high EC_{50} (25×10^{-6} M). Second, the induction pattern was quite similar to that elicited by 3MC, a prototypical PAH (Poland and Glover, 1974), occurring at the same time and reaching similar levels. Third, when added simultaneously, the two compounds had no synergistic effects. Fourth, the mechanism of P450 induction was transcriptional and involved the XRE sequences. Fifth, the addition of the AhR antagonist

A



B

	[U0126], μM					
	3	6	12.5	25	50	3MC
control	2	4	6	14	22	37
+ cycloheximide	134	526	980	1556	1885	4882

Fig. 3. Effects of actinomycin D and cycloheximide on the induction of CYP1A mRNA expression by U0126. Cultures (24-h-old) cultures were maintained for 12 h in the absence (C) or presence of 50, 25, or 12.5 μM U0126, 50 μM U0126 plus 5 μM 3MC (U0/3MC), or 5 μM 3MC, both in the absence or presence of 3 μg/ml actinomycin D (A) or 10 μg/ml cycloheximide (B). RNA was analyzed by either Northern blot hybridization (A) or Q-PCR (B) and compared with 18S rRNA used as loading and amplification control, respectively. Q-PCR results are expressed as a percentage of the corresponding control (no effect on basal CYP1A1 mRNA expression was observed). The experiment has been performed in triplicate with two independent rat hepatocyte cultures.

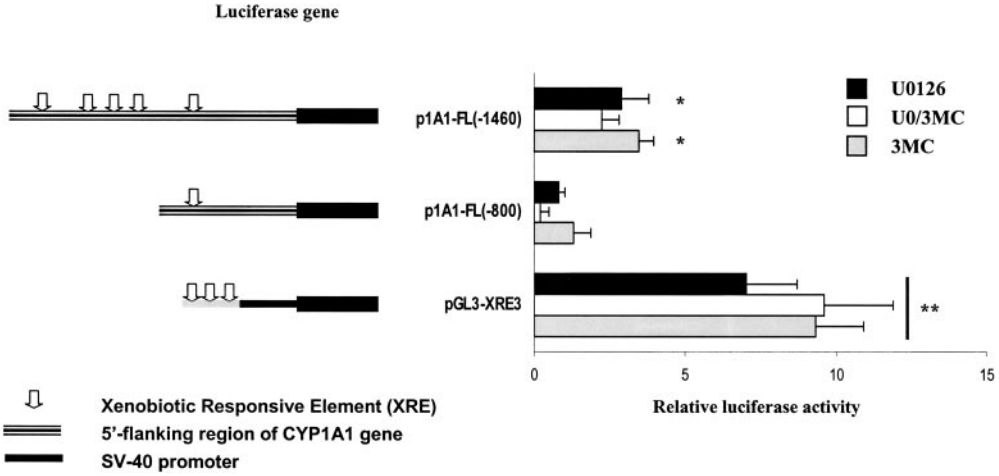


Fig. 4. Activation of the AhR-XRE pathway by U0126 in human hepatoma cells. Transfection assay with a luciferase reporter plasmid bearing different promoter constructs containing one or several XRE sequences (arrows) was performed in the absence (control) or presence of 50 μM U0126 (U0126), 50 μM U0126 plus 5 μM 3MC (U0/3MC), or 5 μM 3MC (3MC). A representative set of experiments performed in triplicate is shown. The values are expressed as means ± S.D. of three measurements. Statistical analysis (Dunnett's test) was performed by comparing differentially treated cells for each plasmid construct. U0126, 3MC, or U0126/3MC treatment significantly induced luciferase activity (⋆, $p < 0.05$; ⋆⋆, $p < 0.01$). The control values expressed as arbitrary luciferase activity were 11.82 ± 3.00, 7.15 ± 1.71, and 0.52 + 0.02 for p1A1-FL(−1460), p1A1-FL(−800), and pGL3-XRE3 constructs, respectively.

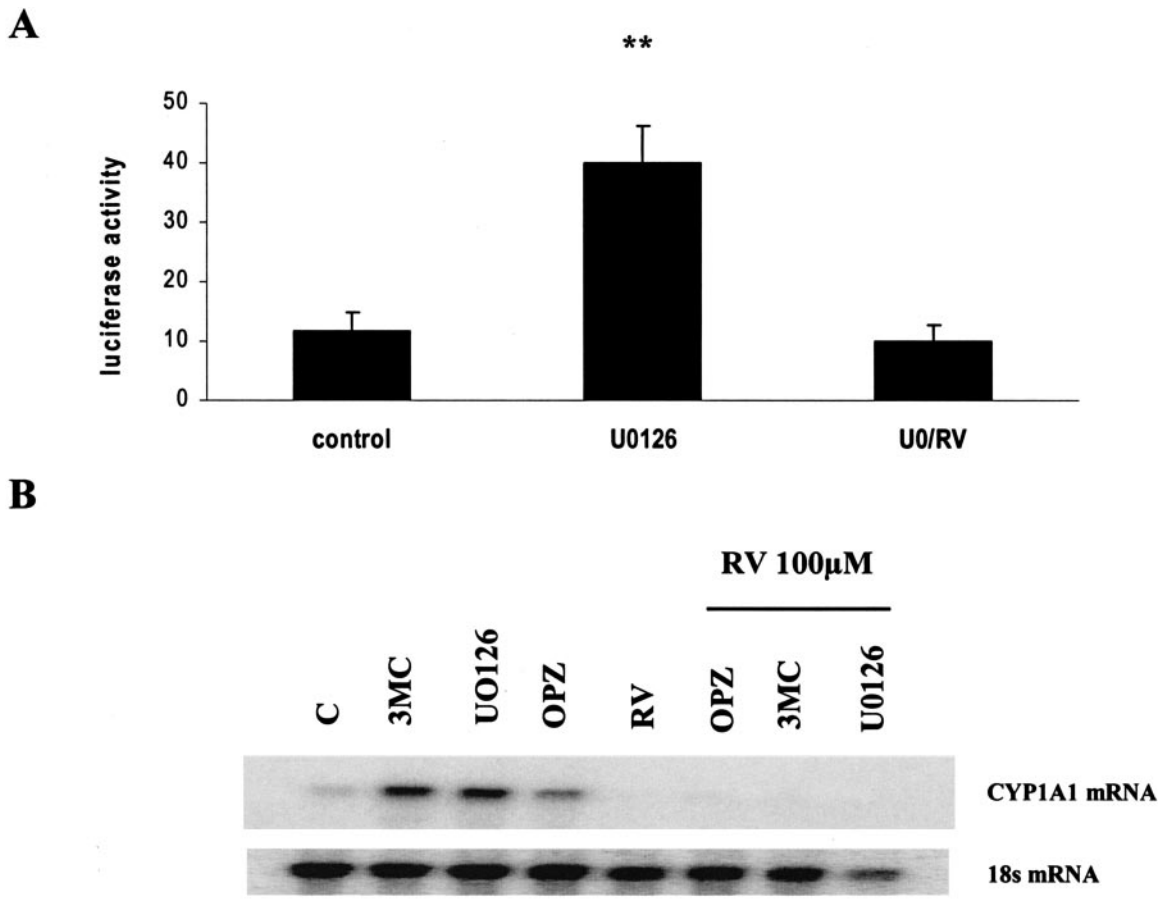


Fig. 5. Effect of resveratrol on CYP1A1 induction by U0126. A, effect of resveratrol on luciferase activity using the responsive p1A1-FL(−1460) construct transfected in human hepatoma cells treated with U0126 or 3MC in the presence or absence of 100 μM resveratrol (RV). The values are expressed as means ± S.D. of three measurements. U0126 significantly increased luciferase activity, which was totally blocked by resveratrol (⋆⋆, $p < 0.01$). B, effect of resveratrol on primary rat hepatocytes. Cells were maintained under control conditions or treated with 5 μM 3MC, 50 μM U0126, or 50 μM OPZ in the presence or absence of 100 μM RV during 12 h. Total RNA was isolated from control and treated cells and subjected to Northern blot analysis. Hybridization of the same filters was performed successively with a CYP1A1-specific cDNA probe and an 18S rRNA oligonucleotide probe used as a control.

resveratrol completely blocked P450 induction by U0126. Several other nonhalogenated aromatic compounds and PAHs, including flavonoids, dithiolethiones, thiazolium compounds, retinoids, carotenoids, and benzimidazoles, are also known to be AhR agonists, indicating that various chemicals

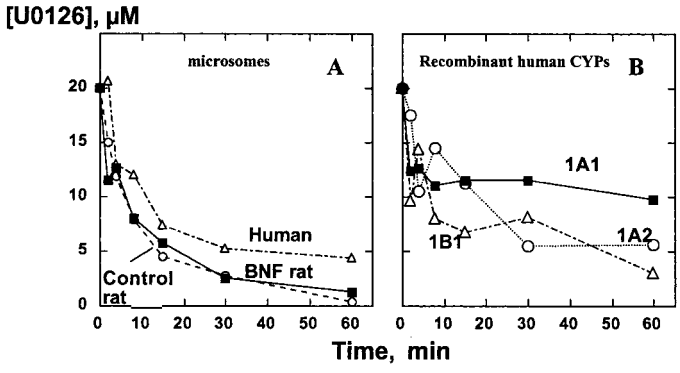


Fig. 6. NADPH-dependent metabolism of U0126. U0126 (20 μM) was incubated with either rat (A) (control, ○; βNF, an inhibitor of the CYP1A family, ■) or human (△) liver microsomes (1.3 μM total CYP) or *E. coli* membranes (B) in which NADPH-P450 reductase and either human CYP1A1 (■), CYP1A2 (○), or CYP1B1 (△) were coexpressed. The P450 concentrations in B were 0.60, 1.3, and 2.0 μM for CYP1A1, CYP1A2, and CYP1B1, respectively.

with quite different chemical structures can modulate the expression of AhR-dependent genes. To our best knowledge, no compound with a chemical structure very similar to that of U0126 has already been shown to be an AhR agonist. U0126 has to be classified as a weak AhR ligand, in the same range as indole-3-carbinol (60×10^{-6} M), resveratrol (6×10^{-6} M), and 7-ketocholesterol (7×10^{-6} M), previously studied using the same in vitro approach (Casper et al., 1999; Savouret et al., 2001). Similar binding assays performed with the last two compounds have shown that they exhibit a higher affinity for AhR in living cultured cells compared with cytosols, with $EC_{50} = 10^{-7}$ and 5×10^{-7} M, respectively, in living cells (Casper et al., 1999; Savouret et al., 2001). This suggests that cytosol preparation is detrimental to the AhR. Whether U0126 would also display a stronger affinity for AhR in such conditions deserves further investigation.

Several studies have demonstrated that AhR plays a role in biological processes in the absence of exogenous ligands by using null AhR mice or cells deficient in the AhR (Sadek and Allen-Hoffmann, 1994a; Schmidt et al., 1996). The AhR has been demonstrated to be implicated in cell differentiation and homeostasis whether concomitantly activated by exogenous ligands or not (Sadek and Allen-Hoffmann, 1994b; Singh et al., 1996). Because cell differentiation also involves

the MEK/ERK pathway, it could be postulated that a link exists between AhR activation in the absence of exogeneous ligands and the MEK/ERK pathway. In the present study, we demonstrated that P450 induction mediated by U0126 is unrelated to MEK/ERK activation. Such a conclusion is taken from our results showing that 1) although being both ERK inhibitors, U0126 and PD98059 had different effects on CYP1-catalyzed EROD activity in the absence of any detectable ERK phosphorylation; 2) unlike U0126, 3MC did not modulate MEK/ERK activities; 3) EGF strongly induced ERK phosphorylation without affecting basal P450 activity; and 4) U0126 still induced CYP1A activity in mouse hepatocytes defective for ERK1 and ERK2.

U0126 was also found to exert a direct transient inhibition of EROD activity. This effect on rat liver microsomes, similarly observed with 3MC, was not AhR-dependent and could be interpreted as a competition between ethoxyresorufin and U0126 at the active site of the enzyme because U0126 was shown to be a P450 substrate. The dual effects of U0126, i.e., inhibition and induction of P450s, seem to be different from those reported with several flavonoids that act as AhR antagonists and agonists at low and high concentrations, respectively. They are rather comparable with those previously obtained with OPZ, a synthetic chemoprotective compound derived from dithiolethiones (Langouët et al., 1997). Interestingly, preliminary experiments indicated that, as reported with OPZ (Le Ferrec et al., 2002), an increased intracellular calcium content occurred before P450 induction by U0126, as shown by the complete blockage of induction by the addition of BAPTA-AM, a chelator of intracellular calcium.

The strongest inhibition of MAPK in rat hepatocytes was found to occur with a concentration of 50 μM U0126 (Coutant et al., 2002). Compared with the data obtained with other cell systems, such a concentration seems to be quite high. Indeed, Satoh and coworkers (2000) showed that in the mouse hippocampal cell line HT22, 10 μM U0126 was required to inhibit ERK phosphorylation, whereas 0.1, 0.5, and 1 μM U0126 were sufficient to obtain similar effects on ERK in Cos7 (Favata et al., 1998), Hepa1 (Tan et al., 2002), and cortical neuron cells (Satoh et al., 2000), respectively. The high concentration of U0126 required to obtain MEK/ERK inhibition in rat and human hepatocytes could be related to its metabolism in these cells. Indeed, by using rat and human microsomes fortified with NADPH and recombinant human CYP1A1, -1A2, and -1B1, we demonstrated that U0126 is indeed a P450 substrate. The competition between ethoxyresorufin and U0126 for CYP1-catalyzed activity provides an additional argument that U0126 is a substrate of the CYP1A family. However, the use of rat microsomes treated with βNF or αNF, an inducer and inhibitor of CYP1 family, respectively, did not markedly modify the rates of disappearance of U0126, suggesting that other uncharacterized P450s are responsible for most of the oxidative metabolism and consequently for the production of the main metabolite detected by HPLC. Such a biotransformation lowers the concentration of the parent compound in the cells. U0126 has been shown to directly act on ERK activation (Favata et al., 1998), giving an explanation for why higher concentrations are required to inhibit MEK/ERK in hepatocytes. Another explanation could be chemical degradation of U0126, because the AP1-suppressive activity, mediated through the MEK/ERK pathway, has been shown to decrease depending on the prolonged storage time of U0126 in DMSO. Such an effect has been attributed to a cyclization of U0126, leading to a less active derivative (Duncia et al., 1998). However, the marked differences between the effective U0126 concentrations in hepatocytes and in other in vitro models do not support such a hypothesis. In contrast, either U0126 or a metabolite could be considered responsible for CYP1A induction. Interestingly, a marked induction was observed with a concentration as low as 3 μM, providing further support that U0126 acts as a P450 inducer independent of its MEK/ERK inhibitory effects.

MAPK cascades trigger various intracellular signals to intracellular targets. MEK/ERK represents one of the three major MAPK signaling cascades and is inhibited by PD98059 and U0126. Our results show that at their most effective inhibitory concentrations in rat hepatocytes, these compounds differently modulated the AhR and related genes, leading to hypothesize that the effects on AhR and related genes might influence some biological processes. Furthermore, it is well established that in some models, the induction of CYP1 family results in an increase in the formation of electrophiles and reactive oxygen species that may have deleterious consequences. Because U0126 is both a substrate and inducer of some P450s, differences in its effects should be expected according the cell type and species. This important point deserves further investigation. U0126 has been reported to block adipocyte (Shimba et al., 2001) and thymocyte (Tsukumo et al., 2002) differentiation inhibited by AhR ligands on the basis of its ability to inhibit the MEK/ERK pathway. However, in the light of our observations, it may be postulated that U0126 effects on cell differentiation could also at least in part result from AhR activation.

In summary, our results provide the first demonstration that U0126, a specific inhibitor of MEK/ERK, behaves as an AhR agonist, induces related genes, and is also a P450 substrate. We postulate that such properties could interfere with some MAPK-related and/or nonrelated biological processes.

Acknowledgments

We thank Dr. Gilles Pages (Centre National de la Recherche Scientifique-UMR 6543, Nice, France) for giving us the ERK1−/− mice, Professor Robert Barouki (INSERM U490, Paris, France) for the p1A1-FL construct, and Dr. E. LeFerrec (INSERM U620, Rennes, France) for helpful discussion.

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