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Okadaic Acid Potentiates 3-Methylcholanthrene-Induced *CYP2A8* Gene Expression in Primary Cultures of Syrian Hamster Hepatocytes: Possible Involvement of Activator Protein-1

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SUMMARY

In Syrian hamster liver, treatment with 3-methylcholanthrene (3-MC) markedly induces an isozyme of cytochrome P450 (CYP), CYP2A8. To elucidate the mechanism of this induction, we studied the effect of okadaic acid (OA), an inhibitor of serine threonine protein phosphatases 1 and 2A, on 3-MC-induced CYP2A8 expression in primary cultures of Syrian hamster hepatocytes. The addition of OA to the cultured hepatocytes at a concentration of 1 nM potentiated 3-MC- (0.1 and 1 μM) induced expression of mRNA and protein of CYP2A8 and its associated coumarin 7-hydroxylase activity. In addition, OA not only induced c-*fos* and *jun*-D mRNA, components of transcription factor activator protein-1 (AP-1), with an increase in AP-1 binding activity in the nucleus, but also activated AP-1-dependent gene transcription in the hepatocytes. The dose-dependent effect of OA on 3-MC-induced CYP2A8 expression corresponded to that of OA on c-*fos* and *jun*-D mRNA induction and on the activation of AP-1-dependent gene transcription. The expression of c-*fos* and *jun*-D mRNA induced by OA preceded the expression of CYP2A8 mRNA potentiated by cotreatment with 3-MC and OA. Treatment with anisomycin and cycloheximide also potentiated 0.1 μM 3-MC-induced coumarin 7-hydroxylase activity, induced c-*fos* and *jun*-D mRNA expression, and activated AP-1-dependent gene transcription in the hepatocytes. Furthermore, 3-MC-induced CYP2A8 expression was potentiated in the hepatocytes transfected with c-Jun expression plasmid. These results suggest that AP-1, inducible by serine threonine protein kinase, may be one of the components of the signal transduction system from 3-MC to CYP2A8 gene expression.

CYP represents a multigene family of hemoproteins that catalyze the oxidative biotransformation of structurally and chemically diverse compounds of both an endogenous and an exogenous nature (1). Of the 12 gene families characterized in mammals (2), several subfamily proteins are inducible by specific drugs and chemicals (1, 2). A number of studies have been devoted to elucidation of the mechanisms involved in the regulation of various constitutive and inducible *CYP* genes. Of the genes studied, *CYP1A1* has been relatively well characterized regarding the induction mechanism by PAHs, such as 3-MC, and HAHs, such as TCDD, in mice and rats. The mechanism by which the *CYP1A1* gene is transcriptionally activated involves the binding of ligand to AhR (3). Then, the ligand-bound AhR translocates to the nucleus (4), where it associates with enhancer elements in the 5′-flanking region of the *CYP1A1* gene, which are referred to as dioxin-responsive element or xenobiotic-responsive element (5). Although both *CYP1A1* and *CYP1A2* are transcriptionally activated by PAHs and HAHs concomitantly in mice and rats, they are controlled by different modes of regulation because the 5′-flanking sequence of the *CYP1A1* gene is quite different from that of *CYP1A2* gene (6), and 3-MC induces only CYP1A1 mRNA, not CYP1A2 mRNA, in several cell types (2). Recent studies by Quattrochi *et al.* (6, 7) have shown that an xenobiotic-responsive element-like sequence, AP-1 binding sites, and a conserved TATA box, all of which are located in the 5′-flanking region of the *CYP1A2* gene, and the ligand-bound AhR were important for the overall expression of the *CYP1A2* gene by 3-MC.

In addition to these mechanisms of transcriptional activation by PAH and HAH, recent studies identified a critical role

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ABBREVIATIONS: CYP, cytochrome P450; AhR, aryl hydrocarbon receptor; AP-1, activator protein-1; HAH, halogenated aromatic hydrocarbon; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; 3-MC, 3-methylcholanthrene; NF, naphthoflavone; OA, okadaic acid; PAH, polycyclic aromatic hydrocarbon; PBS, phosphate-buffered saline; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

for specific signal transduction pathways in the control of 3-MC- and TCDD-induced *CYP* gene expression. Notably, protein phosphorylation seems to be a determinant not only in the regulation of TCDD-induced *CYP* gene expression (8, 9) but also in the final expression of many overt toxicities and lethalities by TCDD (10). Furthermore, cAMP-dependent protein kinase and growth hormone receptor-associated protein kinase were reported to affect the expression of the *CYP2B* and *CYP2C* gene families (11, 12).

We previously demonstrated that 3-MC induced the *CYP2A8* and *CYP1A2* genes, but not the *CYP1A1* gene, in Syrian hamster liver (13, 14), whereas Sagami *et al.* (15) reported that the *CYP1A1* gene was induced by 3-MC in extrahepatic tissues, such as the lung, in Syrian hamsters. In the livers of mice and rats, 3-MC induces the *CYP1A1* and *CYP1A2* but not the *CYP2A* gene family (1). This suggests that the molecular mechanism by which the expression of the *CYP2A8* gene is controlled in hamster liver is quite different from that of *CYP1A1* and *CYP2A* gene expression in other species. However, no studies have been done on the mechanism by which 3-MC induces *CYP2A8* gene expression in Syrian hamster liver. Because protein kinases have been reported to be involved in PAH- and HAH-induced gene activation, as mentioned above (8–10), protein phosphorylation may play an important role in the signal transduction system from 3-MC to *CYP2A8* gene expression.

OA, a polyether fatty acid isolated from marine sponges, has been shown to be a potent tumor promoter (16). Instead of activating protein kinase C like phorbol ester tumor promoters, OA specifically inhibits serine threonine phosphoprotein phosphatases 1 and 2A, leading to an increase in the phosphorylation state of many cellular proteins (16). Interestingly, OA treatment mimicked the effects of several growth hormones and cytokines whose intracellular signaling systems involved the protein kinase-mediated cascade (17).

In the current study, we sought to elucidate the mechanism by which 3-MC induced CYP2A8. We first established a primary culture system of Syrian hamster hepatocytes that faithfully reproduced 3-MC-induced CYP2A8 expression as observed *in vivo*. Then, we examined the effect of OA on the induction of the *CYP2A8* gene by 3-MC using this culture system to determine whether any factors related to protein phosphorylation are involved in the induction mechanism. We found for the first time that OA was capable of potentiating 3-MC-induced *CYP2A8* gene expression in the hepatocytes, and we obtained results suggesting that the transcription factor AP-1 may be involved in the mechanism of 3-MC-induced *CYP2A8* gene expression.

Experimental Procedures

Animals and materials. Female Std:Syrian hamsters (8–10 weeks old; Nippon SLC, Hamamatsu, Japan) were used for the preparation of primary cultures of hepatocytes. Waymouth's MB 752/1 media, OPTI-MEM I reduced serum media, Lipofectin reagent, and TRIZOL reagent were obtained from GIBCO BRL (Gaithersburg, MD), and transferrin, selenium, and insulin were from Boehringer-Mannheim Biochemica (Mannheim, Germany). Collagen-coated plastic dishes were obtained from Toyobo Engineering (Osaka, Japan). Dexamethasone, 3-MC, OA, forskolin, and collagenase were purchased from Wako Pure Chemicals (Osaka, Japan). Anisomycin was purchased from Sigma Chemical (St. Louis, MO). *CYP2A8* cDNA probe was prepared as described previously (18). Oligonucleotide probes of mouse and rat c-*fos*, and murine *jun*-D were purchased from Oncogene Science (Uniondale, NY). [α-^{32}P]dCTP (110 TBq/mmol) and [γ-^{32}P]ATP (185 TBq/mmol) were purchased from Amersham International (Buckinghamshire, UK). Poly(dI-dC)/poly(dI-dC) was purchased from Pharmacia (Uppsala, Sweden). AP-1 consensus sequence synthetic oligonucleotide (5′-CGCTTGATGAGTCAGCCGGAA-3′) and mutant AP-1 consensus sequence synthetic oligonucleotide (5′-CGCTTGAGTCAGACGCCGGAA-3′) were purchased from Trevigen (Gaithersburg, MD) and Greiner Japan (Tokyo, Japan), respectively (19). Rabbit polyclonal anti-hamster CYP2A8 antibody was prepared as described previously (20). Renaissance Western Blot Chemiluminescence Reagent was purchased from DuPont-New England Nuclear (Boston, MA). AP-1-dependent reporter gene, -73 Col-LUC, was kindly provided by Dr. M. Karin and Dr. F.-X. Claret (University of California, San Diego), and c-Jun expression plasmid, pRSV-c-Jun, was kindly provided by Dr. H. Iba (University of Tokyo, Tokyo, Japan) and Dr. T. Curran (St. Jude Children's Research Hospital, Memphis, TN) (21, 22). pRSV-2 vector was prepared from pRSV-c-Jun (21). pSV-β-galactosidase control plasmid was purchased from Promega (Madison, WI).

Isolation and culture of hepatocytes. Syrian hamster hepatocytes were isolated according to the method described for rat hepatocyte preparation using EGTA and collagenase (23). The cells were dispersed in Waymouth's MB752/1 medium containing bovine serum albumin (2%), insulin (0.5 mg/liter), transferrin (0.5 mg/liter), selenium (0.5 μg/liter), and dexamethasone (1 nM) (24) and seeded onto collagen-coated plastic dishes at a density of 2×10^6 cells/4 ml/60-mm dish. The cultures were maintained at 37° in a CO_2-humidified incubator, and the medium was renewed every day. Cells were treated with 3-MC and OA, dissolved in dimethylsulfoxide, 72 hr after the onset of the culture. The final concentration of dimethylsulfoxide in the culture medium was 0.1%. Control plates received dimethylsulfoxide alone at the same concentration.

Measurement of coumarin 7-hydroxylase activity. At 24 hr after treatment with the chemicals, cells were washed with ice-cold PBS, scraped, and homogenized by ultrasonication in 50 mM Tris·HCl, pH 7.4, 0.154 M KCl, and 1 mM EDTA. Microsomal fractions from cell homogenates were prepared as described previously (25); suspended in 50 mM Tris·HCl, pH 7.4, 0.154 M KCl, 1 mM EDTA, and 20% glycerol; and stored at −75° until use. Coumarin 7-hydroxylase activity in microsomal fractions was determined as described by Lu *et al.* (26). Protein concentrations were measured using the Pierce protein assay kit (Pierce Chemical, Rockford, IL) with bovine serum albumin as a standard.

Western blotting. Microsomal proteins of hepatocytes (10 μg of protein) were electrophoresed on 10% polyacrylamide gels in the presence of sodium dodecyl sulfate according to the method of Laemmli (27), and resolved proteins were transferred onto polyvinylidene difluoride membranes. After blocking of the nonspecific binding sites, the membrane was incubated with anti-CYP2A8 antibody in PBS with 0.05% Tween-20/bovine serum albumin (1%) for 1 hr. After washing, the membrane was incubated with peroxidase-labeled secondary antibody for 1 hr in PBS with 0.05% Tween-20/bovine serum albumin (1%). After additional washing, antibody binding proteins were visualized using Renaissance Western Blot Chemiluminescence Reagent.

Northern hybridization. Extraction of total RNA from the whole cells was done using TRIZOL reagent according to the manufacturer's instruction. For the preparation of cytosolic RNA, cells were washed with PBS, scrapped, and collected. Then, cells were suspended in 0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris·HCl, pH 8.6, 0.5% Nonidet P-40, 1 mM dithiothreitol, and 1000 units/ml RNase inhibitor and remained on ice for 5 min to extract the cytosolic RNA. After centrifugation, the supernatant was treated with proteinase K (50 μg/ml) for 30 min at 37°. The proteins in the solution were extracted with phenol/chloroform, and the RNA in the aqueous phase

was precipitated with isopropanol. The total RNA (10 μg) was denatured by heating at 65° for 5 min in 20 mM 3-*N*-morpholino propanesulfonic acid, pH 7.0, containing 2.2 M formaldehyde and 50% (v/v) formamide. The RNA was electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde. Resolved RNA was transferred onto a nylon membrane in 20× standard saline citrate (1× = 0.15 M sodium chloride with 15 mM sodium citrate). After UV cross-linking, the membrane was subjected to hybridization under the conditions described by Church and Gilbert (28). The hybridization was done at 60° for the *CYP2A8* cDNA probe and at 50° for the c-*fos* and *jun*-D oligonucleotide probes. The membrane was then washed with 2× standard saline citrate containing 0.1% sodium dodecyl sulfate at 65° (*CYP2A8*) or at 55° (c-*fos* and *jun*-D) for 30 min. The membrane was exposed to X-ray film for autoradiography for 1–2 days.

Transfection and reporter gene assays. Transient DNA transfections were performed according to the lipofection method. First, we examined the optimal conditions for transfection in the hepatocytes using pRSV-Luc, luciferase expression plasmid. We found that transfection efficiency depended on the concentration of Lipofectin reagent in the medium. The best transfection efficiency was obtained when the Lipofectin/DNA complex was prepared with 10 μl of Lipofectin and 50 μg of DNA in 2 ml of medium (4-fold and 3-fold increase compared with the use of 2 μl and 20 μl of Lipofectin, respectively). Therefore, we prepared the Lipofectin/DNA complex with 10 μl of Lipofectin reagent. The hepatocytes were cultured for 48 hr and washed with PBS three times before changing of the medium to 2 ml of OPTI-MEM I medium containing Lipofectin/DNA complex. Lipofectin/DNA complexes were prepared according to the manufacturer's instruction. Each dish received 2 μg of the AP-1-dependent reporter gene, -73 Col-LUC, which contained the AP-1 consensus sequence (29, 30), and 2 μg of pSV-β-galactosidase control plasmid and/or 1 to 50 μg of c-Jun expression plasmid, pRSV-c-Jun (21, 22). pRSV-2, which does not contain c-*jun*, was transfected as a negative control for the c-Jun expression experiments. The hepatocytes were exposed to the Lipofectin/DNA complex for 16 hr, and then the medium was changed to Waymouth's MB752/1 medium containing various concentrations of OA or other chemicals. After an additional cultivation, reporter enzyme activities in the hepatocytes were assayed or RNAs were extracted from the hepatocytes. The luciferase (31) and β-galactosidase (32) assays were carried out as described previously. All plasmids were prepared by equilibrium sedimentation in CsCl gradients. To correct for differences in transfection efficiencies between dishes within given experiments, the luciferase activities in the cell extracts were normalized to the β-galactosidase activity.

Nuclear extract preparation and gel mobility shift assay. Nuclear extracts from primary cultures of the hepatocytes treated by OA were prepared according to the procedure of Kadonaga and Tjian (33). The extracts were dialyzed against 25 mM HEPES-KOH, pH 7.8, containing 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10% glycerol (v/v), 50 mM KCl, and 0.5 mM EDTA. After precipitates were removed by centrifugation, aliquots of the extracts were stored at −75° until use. AP-1 consensus sequence synthetic oligonucleotide was end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase and purified using a Sephadex G-25 column. The labeled oligonucleotide probes (20,000–30,000 cpm, 0.125 pmol) were mixed with 2 μg of poly(dI-dC)/poly(dI-dC) and 1 or 4 μg of nuclear extract proteins in a final volume of 20 μl of solution containing 25 mM HEPES-KOH, pH 7.8, 5.0 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10% glycerol (v/v), 50 mM KCl, and 0.5 mM EDTA. The samples were incubated at room temperature for 30 min (19) and electrophoresed on 4% nondenaturing polyacrylamide gels using 50 mM Tris·HCl, pH 8.5, 0.38 M glycine, and 2 mM EDTA as a running buffer. Gels were dried under vacuum and were exposed to X-ray film for autoradiography. Normal and mutant AP-1 consensus sequence synthetic oligonucleotides (25 pmol) were used as cold competitors.

Results

Potentiation of 3-MC-induced coumarin 7-hydroxylase activity by OA in primary cultures of hepatocytes. We first studied the effects of 3-MC on the induction of CYP2A8 in primary cultures of Syrian hamster hepatocytes grown in serum-free hormonally defined medium. The hepatocytes, which had been precultured for 3 days, were treated with 3-MC at various concentrations and incubated for an additional 24 hr. Microsomes were then prepared from the hepatocytes, and the activity of coumarin 7-hydroxylase, specific for CYP2A, was measured. As shown in Fig. 1, 3-MC induced the coumarin 7-hydroxylase activity in a dose-dependent manner at concentrations ranging from 0.1 nM to 0.1 μM.

Second, we examined the effects of various chemicals on 3-MC-induced coumarin 7-hydroxylase. Cotreatment of 0.1 μM and 1 μM 3-MC with 1 nM OA markedly increased coumarin 7-hydroxylase activity compared with the treatment with 3-MC alone (Fig. 1), whereas OA alone did not induce the activity, even at concentrations of 10 nM to 1 μM (data not shown). Anisomycin (0.2 μM) and cycloheximide (0.1 μM), which are known to activate intracellular kinases (34), also potentiated the induction of coumarin 7-hydroxylase activity in cotreatment with 0.1 μM 3-MC (Fig. 1). On the other hand, dexamethasone, which was reported to be an essential factor for 3-MC-induced *CYP1A2* gene expression in rat hepatocytes (35), and forskolin, which was shown to potentiate phenobarbital-induced coumarin 7-hydroxylase activity in mouse hepatocytes (36), failed to potentiate the coumarin 7-hydroxylase induction by 3-MC (Fig. 1).

Fig. 2 illustrates the dose-dependent effects of OA (0.1–30 nM) on the coumarin 7-hydroxylase activity of the hepatocytes in the presence of 0.1 μM 3-MC. OA was effective over a narrow range of concentrations, with maximal activation of the enzyme activity at 3 nM (Fig. 2), when measured at 24 hr

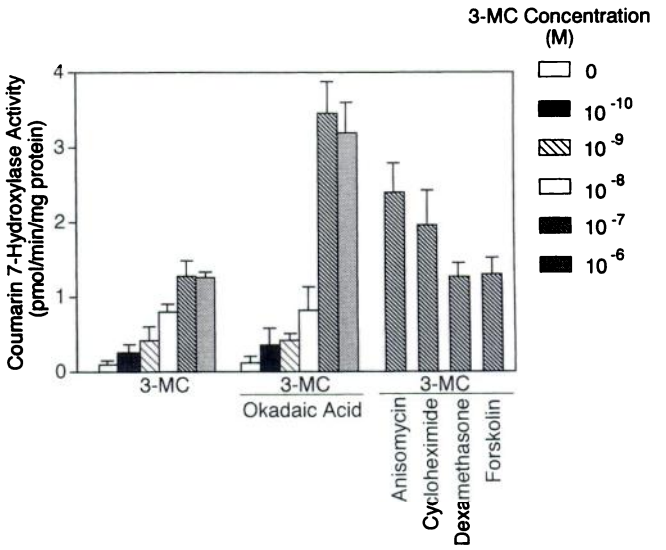

Fig. 1. Effects of various chemicals on 3-MC-induced coumarin 7-hydroxylase activity in primary cultures of Syrian hamster hepatocytes. Hepatocytes that had been precultured for 3 days were treated with chemicals in the presence of different concentrations of 3-MC. Chemicals were added at the following concentrations: OA, 1 nM; anisomycin, 0.2 μM; cycloheximide, 0.1 μM; dexamethasone, 1 μM; and forskolin, 0.1 mM. Microsomes were prepared from hepatocytes 24 hr after treatment and subjected to the enzyme assay. Each value represents the mean ± standard error of three determinations.

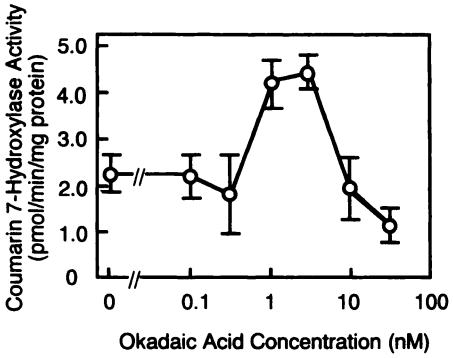

Fig. 2. Dose-dependent effects of OA on 3-MC-induced coumarin 7-hydroxylase activity in primary cultures of Syrian hamster hepatocytes. Hepatocytes that had been precultured for 3 days were treated with OA at different concentrations in the presence of 0.1 μM 3-MC. Microsomes were prepared from hepatocytes 24 hr after treatment and subjected to the enzyme assay. Each value represents the mean ± standard error of three determinations.

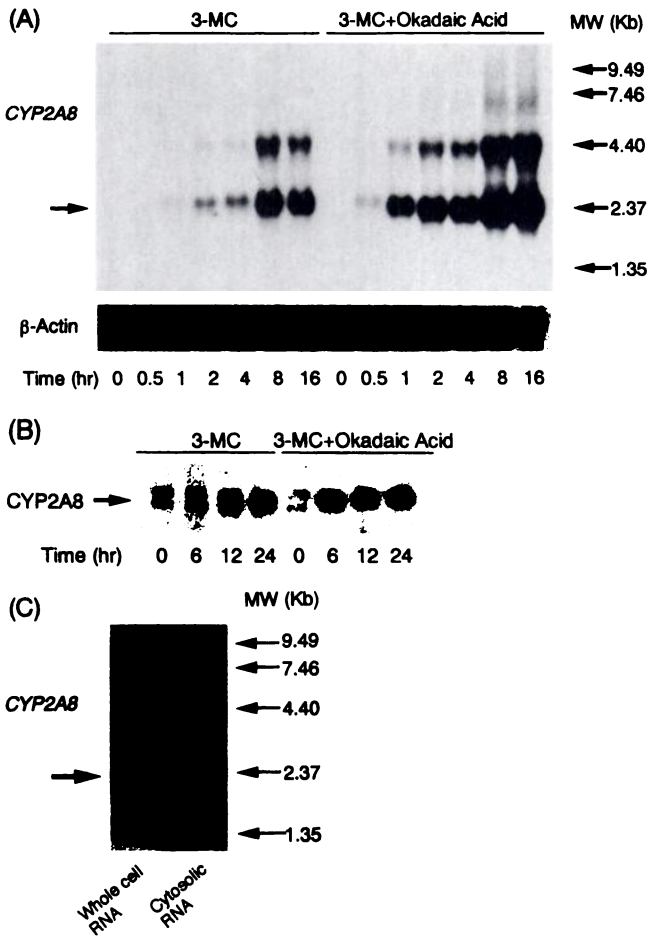

Fig. 3. Time course analysis of the effects of OA on 3-MC-induced CYP2A8 mRNA expression (A) and CYP2A8 protein expression (B) in primary cultures of Syrian hamster hepatocytes. Hepatocytes that had been precultured for 3 days were treated with 0.1 μM 3-MC alone or cotreated with 1 nM OA and 0.1 μM 3-MC. A, Total RNAs were prepared from hepatocytes at indicated times, and CYP2A8 mRNA expression was analyzed by Northern blot. Total RNA (10 μg) was applied to each lane. *Arrow*, CYP2A8 mRNA signal. Uniform RNA loading is demonstrated by β-actin probe. B, Microsomes were prepared from hepatocytes at indicated times, and CYP2A8 protein expression was analyzed by Western blot using anti-CYP2A8 antibody. Microsomal protein (10 μg) was applied to each lane. *Arrow*, CYP2A8 protein signal. C, Northern blot analysis of whole-cell RNA and cytosolic RNA. RNAs were prepared from whole-cell homogenate or cytosolic fraction, which were treated with 0.1 μM 3-MC for 8 hr. Hybridization probe was the same as A.

after the additions of 3-MC and OA. OA treatment, at concentrations of >30 nM, effected morphological changes and detachment of cells from the culture dish surface, which might be attributed to the toxicity of OA.

Potentiation of 3-MC-induced CYP2A8 mRNA and protein expression by OA in primary cultures of hepatocytes. Fig. 3, A and B, shows the time-dependent effects of 3-MC alone (0.1 μM) and with OA (1 nM) on CYP2A8 mRNA and protein levels in the hepatocytes that had been precultured for 3 days. As shown in Fig. 3A, a strongly hybridizing band was detected with an size of ~2.37 kb, which corresponded to CYP2A8 mRNA. A band at 4.40 kb was also detected with lesser intensity. We used the 5′ region of *CYP2A8* cDNA with 606 base pairs, which is specific for the detection of CYP2A8 mRNA, as a hybridization probe and 3-MC-induced 4.40-kb band over the same time course as the band at 2.37 kb (Fig. 3A). Furthermore, we detected only 2.37-kb band, not a 4.40-kb band, in the cytosolic RNA of 3-MC-treated hepatocytes (Fig. 3C). Therefore, we considered the 4.40-kb band to be a nuclear precursor mRNA for CYP2A8. Treatment with 3-MC alone induced CYP2A8 mRNA expression at 2 hr after the addition, and the level of the mRNA attained its maximum at 8 hr, which was maintained for ≤16 hr. On the other hand, cotreatment of 3-MC with OA induced CYP2A8 mRNA at 0.5 hr after the treatment, and the maximal level of CYP2A8 mRNA, observed at 8 hr, was 7.8-fold higher than that obtained by the treatment with 3-MC alone, as quantified with a densitometer (Fig. 3A).

We examined the time course changes in CYP2A8 protein expression in the hepatocytes using Western blotting analysis. As shown in Fig. 3B, a significant band of CYP2A8 protein was observed at 12 hr after 3-MC treatment, and the intensity of the band increased up to 24 hr. However, cotreatment with OA and 3-MC effected a significant increase in the CYP2A8 protein level at 6 hr after treatment and the maximal amount induced by OA and 3-MC was higher than that obtained by treatment with 3-MC alone (Fig. 3B). The time course changes in the protein expression paralleled those in the mRNA expression (Fig. 3, A and B).

Effects of α- and β-NF on 3-MC-induced CYP2A8 in primary cultures of hepatocytes. α-NF is known to inhibit the induction of CYP1A1, functioning as an AhR antagonist, whereas β-NF is an inducer of CYP1A1 and CYP1A2, functioning through the AhR (10). To study whether the AhR is involved in 3-MC-induced CYP2A8 in hamster hepatocytes, we examined the effects of α- and β-NF on 3-MC-induced CYP2A8 protein expression in hamster hepatocytes. Although the addition of 10 μM α-NF alone did not cause an increase in CYP2A8 in the hepatocytes, the addition of 10 μM β-NF alone increased CYP2A8, and the amount of CYP2A8 induced by β-NF was comparable to that was induced by 0.1 μM 3-MC (Fig. 4A). Pretreatment with 10 μM α-NF inhibited the induction of CYP2A8 by 3-MC, but the pretreatment of β-NF did not affect 3-MC-induced CYP2A8 expression (Fig. 4A). Because the treatment of β-NF alone could induce CYP2A8, we next examined the effect of OA on β-NF-induced CYP2A8 expression. As a result, 1 nM OA potentiated β-NF-

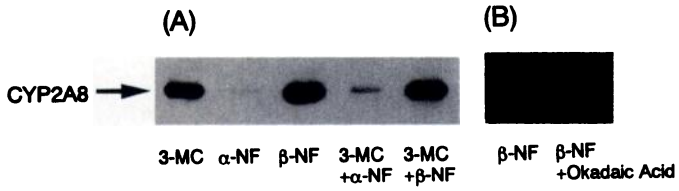

Fig. 4. Effects of α- and β-NF on 3-MC-induced CYP2A8 in primary cultures of Syrian hamster hepatocytes. A, Hepatocytes that had been precultured for 3 days were treated with 10 μM α- or β-NF. After cultivation for 3 hr, 0.1 μM 3-MC was added. Microsomes were prepared 24 hr after the final addition, and CYP2A8 protein expression was analyzed by Western blot. B, Hepatocytes were treated with 10 μM β-NF alone or cotreated with 10 μM β-NF and 1 nM OA. After cultivation for 24 hr, microsomes were prepared, and CYP2A8 protein expression was analyzed by Western blot.

induced CYP2A8 expression and affected 3-MC-induced CYP2A8 (Fig. 4B) in a similar manner. These data are consistent with the idea that 3-MC-induced CYP2A8 was mediated through the AhR and suggested that OA interacted with AhR-mediated induction.

Induction of c-*fos* and *jun*-D mRNA by OA in primary cultures of hepatocytes. Because OA has been reported to stimulate gene transcription mediated by AP-1 (37, 38), we investigated the possible involvement of the Fos and Jun families, the components of AP-1, in the potentiation by OA of 3-MC-induced CYP2A8 gene expression in the hepatocytes. We detected c-*fos* and *jun*-D mRNAs using rat and mouse c-*fos* DNA probes and murine *jun*-D cDNA probe in the hepatocytes. Fig. 5A shows time course changes in c-*fos* and *jun*-D mRNA expression in the hepatocytes treated with 1 nM OA. The levels of c-*fos* and *jun*-D mRNAs increased significantly 0.5 and 1 hr after the OA treatment, respectively, and declined immediately thereafter, which preceded the OA-potentiated CYP2A8 mRNA expression (Fig. 3A). We also studied dose-dependent effects of OA on c-*fos* and *jun*-D mRNA induction in the hepatocytes (Fig. 5B). Marked increases in c-*fos* and *jun*-D mRNA levels were observed with 1 and 3 nM OA at 1 hr after the treatment, and this dose-dependent effect corresponded to that of OA on 3-MC-induced coumarin 7-hydroxylase activity (Fig. 2). However, we could not detect the expression of c-*fos* or *jun*-D mRNA with 3-MC treatment alone at the concentrations used in this experiment (data not shown).

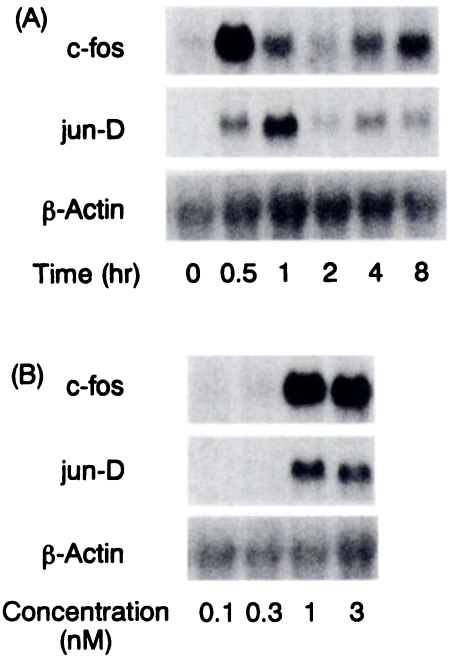

Fig. 5. Effects of OA on the induction of c-*fos* and *jun*-D mRNAs in primary cultures of Syrian hamster hepatocytes. A, Hepatocytes precultured for 3 days were treated with 1 nM OA. Total RNAs were prepared from hepatocytes at indicated times, and c-*fos* and *jun*-D mRNA expression was analyzed by Northern blot. Total RNA (20 μg) was applied to each lane. B, Hepatocytes were treated with different concentrations of OA for 1 hr. Total RNAs were prepared from hepatocytes, and c-*fos* and *jun*-D mRNA expression was analyzed by Northern blot. Total RNA (20 μg) was applied to each lane. Uniform RNA loading is demonstrated by β-actin probe.

Induction of c-*fos* and *jun*-D mRNA by anisomycin and cycloheximide in primary cultures of hepatocytes. Because both anisomycin and cycloheximide potentiated 3-MC-induced coumarin 7-hydroxylase activity (Fig. 1), we studied the effects of anisomycin and cycloheximide on c-*fos* and *jun*-D mRNA expression in the hepatocytes. Anisomycin at 0.2 μM markedly induced c-*fos* and *jun*-D mRNA expression 1 hr after treatment (Fig. 6). Cycloheximide (0.1 μM) slightly increased c-*fos* and *jun*-D mRNA levels (Fig. 6). The dose-dependency of these chemicals on c-*fos* and *jun*-D mRNA induction was almost the same as that observed in the potentiation of 3-MC-induced coumarin 7-hydroxylase (data not shown). We also examined the effects of dexamethasone and forskolin on c-*fos* and *jun*-D mRNA levels 1 hr after treatment but could not observe the induction of the mRNA at any of the concentrations examined (Fig. 6).

Induction of AP-1 binding activity by OA in nuclear extracts of hepatocytes. To investigate whether the induction of c-*fos* and *jun*-D mRNAs results in increased AP-1

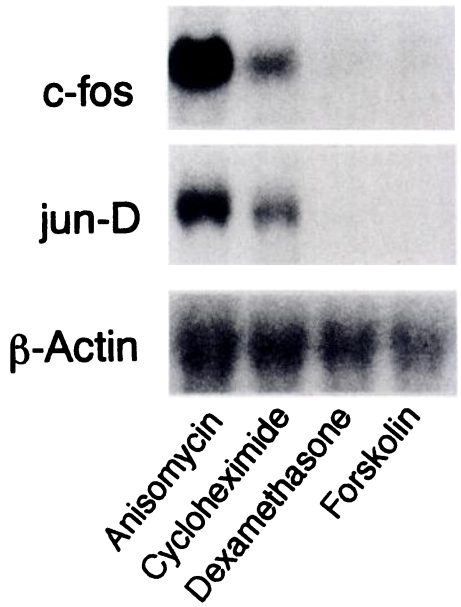

Fig. 6. Effects of various chemicals on the induction of c-*fos* and *jun*-D mRNAs in primary cultures of Syrian hamster hepatocytes. Hepatocytes precultured for 3 days were treated with anisomycin (0.2 μM), cycloheximide (0.1 μM), dexamethasone (1 μM), or forskolin (0. 1 mM) for 1 hr. Total RNAs were prepared from hepatocytes, and c-*fos* and *jun*-D mRNA expression was analyzed by Northern blot. Total RNA (20 μg) was applied to each lane. Uniform RNA loading is demonstrated by β-actin probe.

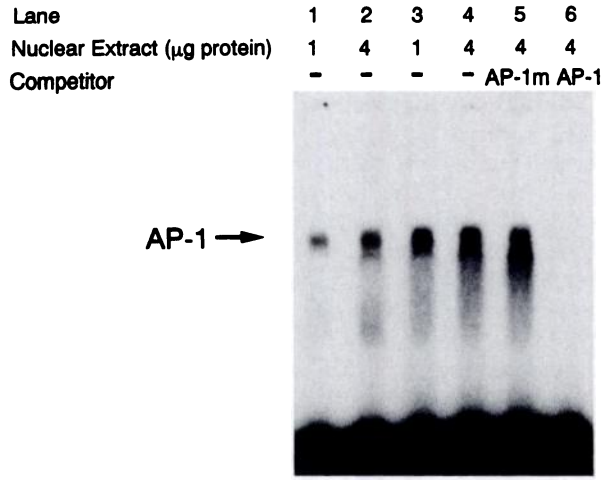

Fig. 7. Induction of AP-1 binding activity by OA in Syrian hamster hepatocytes analyzed by gel mobility shift assay. Nuclear extracts were prepared from untreated or OA-treated (1 nM for 1 hr) hepatocytes. Nuclear extracts (1 and 4 μg of protein) were incubated with ^{32}P-labeled AP-1 consensus sequence. The nuclear extracts prepared from OA-treated hepatocytes were incubated with labeled AP-1 in the presence of a 200-fold molar excess of unlabeled AP-1 mutant (*lane 5*) and in the presence of a 200-fold molar excess of unlabeled AP-1 (*lane 6*). *Arrow*, major AP-1 binding activity.

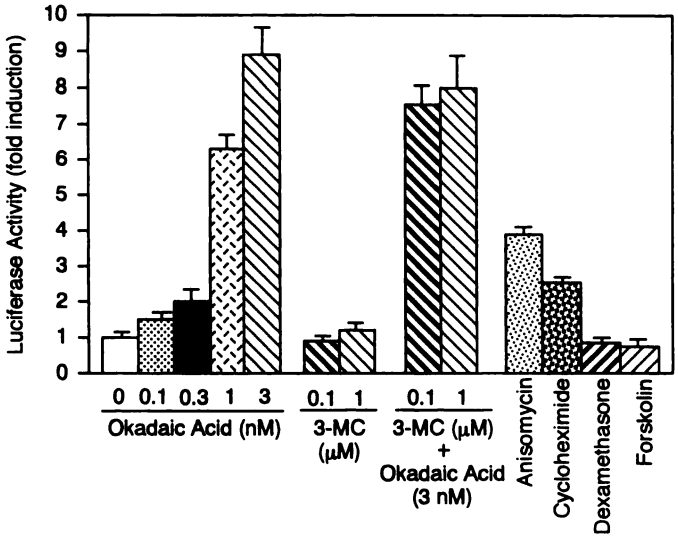

Fig. 8. Effects of various chemicals on AP-1-dependent transcriptional activity in primary cultures of Syrian hamster hepatocytes. Hepatocytes precultured for 2 days were transfected with the AP-1-dependent reporter gene, -73 Col-LUC reporter, and β-galactosidase expression plasmid for 16 hr. Cells were treated with OA (0–3 nM), 3-MC (0.1 and 1 μM), 3-MC (0.1 and 1 μM) with OA (3 nM), anisomycin (0.2 μM), cycloheximide (0.1 μM), dexamethasone (1 μM), or forskolin (0.1 mM) for 6 hr, and then luciferase and β-galactosidase activities were determined. Luciferase activities normalized by β-galactosidase activities were calculated relative to the level of luciferase activity in nontreated cells, which was given an arbitrary value of 1.0. Each value represents the mean ± standard error of three determinations.

binding activity, gel mobility shift assays were performed with a radiolabeled DNA probe containing the AP-1 site using nuclear extracts from untreated or OA-treated hepatocytes. Marked inductions of AP-1 complexes were obtained by the treatment of hepatocytes with 1 nM OA for 1 hr (Fig. 7, *lanes 3 and 4*), whereas a barely detectable complex was present with nuclear extracts from untreated hepatocytes (Fig. 7, *lanes 1 and 2*). The specificity of AP-1 complex was examined in competition experiments by adding a 200-fold excess of an unlabeled probe to the mixture (Fig. 7, *lane 6*). Furthermore, the mutant AP-1 probe, which contains mutations that interfere with the binding of AP-1, failed to bind to the AP-1 complex (Fig. 7, *lane 5*).

Induction of AP-1-dependent transcriptional activity by OA in primary cultures of hepatocytes. Karin (39) reported that changes in AP-1 protein levels did not mirror the transcriptional activity of AP-1. Therefore, in addition to the Northern blotting analysis for c-*fos* and *jun*-D mRNA and gel mobility shift assay for AP-1, we measured the transcriptional activity of an AP-1-dependent reporter gene in the hepatocytes treated with OA (Fig. 8). The addition of OA dramatically increased the transcriptional activity of an AP-1-dependent reporter gene, and the dose-dependency of the transcriptional activation was parallel to that of OA in potentiation of the induction of the enzyme activity of CYP2A8 and mRNA level of c-*fos* and *jun*-D (Figs. 2, 5B, and 8). We also observed that anisomycin and cycloheximide, but not dexamethasone and forskolin, activated the transcription of the AP-1-dependent reporter gene in the hepatocytes (Fig. 8). We could observe neither the transcriptional activation of the AP-1-dependent reporter gene by 3-MC alone nor the synergistic effect of 3-MC and OA on the transcriptional activity in the hepatocytes (Fig. 8).

Involvement of AP-1 in the potentiation of 3-MC-induced CYP2A8 expression in primary cultures of hepatocytes. To confirm the possibility that AP-1 can potentiate 3-MC-induced CYP2A8 expression in hamster hepatocytes, we examined the effect of c-Jun expression on 3-MC-induced CYP2A8 mRNA expression. The expression of c-Jun by pRSV-c-Jun, c-Jun expression plasmid, not only activated AP-1-dependent gene transcription but also potentiated 0.1 μM 3-MC-induced CYP2A8 expression in hamster hepatocytes (Fig. 9, A and B). These effects of c-Jun expression plasmid were dependent on the amount of expression plasmid (Fig. 9, A and B). The transfection with pRSV-2 that does not contain c-*jun* did not affect AP-1-dependent gene transcription or 3-MC-induced CYP2A8 expression (Fig. 9, A and B). The activation of AP-1-dependent reporter gene and potentiation of 3-MC-induced CYP2A8 expression in the hepatocytes transfected with 50 μg of pRSV-c-Jun were comparable to those with 1 nM OA (Figs. 3A, 8, and 9). However, c-Jun expression did not affect low concentrations (0.1 and 1 nM) of 3-MC-induced CYP2A8 mRNA expressions (Fig. 9C). These results were consistent with the result of OA-potentiated CYP2A8 expression and strongly suggested that c-Jun, a potent component of AP-1, might be involved in the potentiation of 3-MC-induced CYP2A8 expression in the hamster hepatocytes.

Discussion

Except for the induction of the *CYP1A1* gene by PAHs or HAHs, the detailed molecular mechanism underlying the induction of *CYP* genes is obscure. This might be in part due to the absence of stable cell culture model systems in which inductions of *CYP* genes can be demonstrated. In the current study, we developed a primary culture system of Syrian hamster hepatocytes that responded to 3-MC with CYP2A8 gene induction in a manner comparable to that reported in the liver *in vivo* (13, 14) (Fig. 1). Using this culture system,

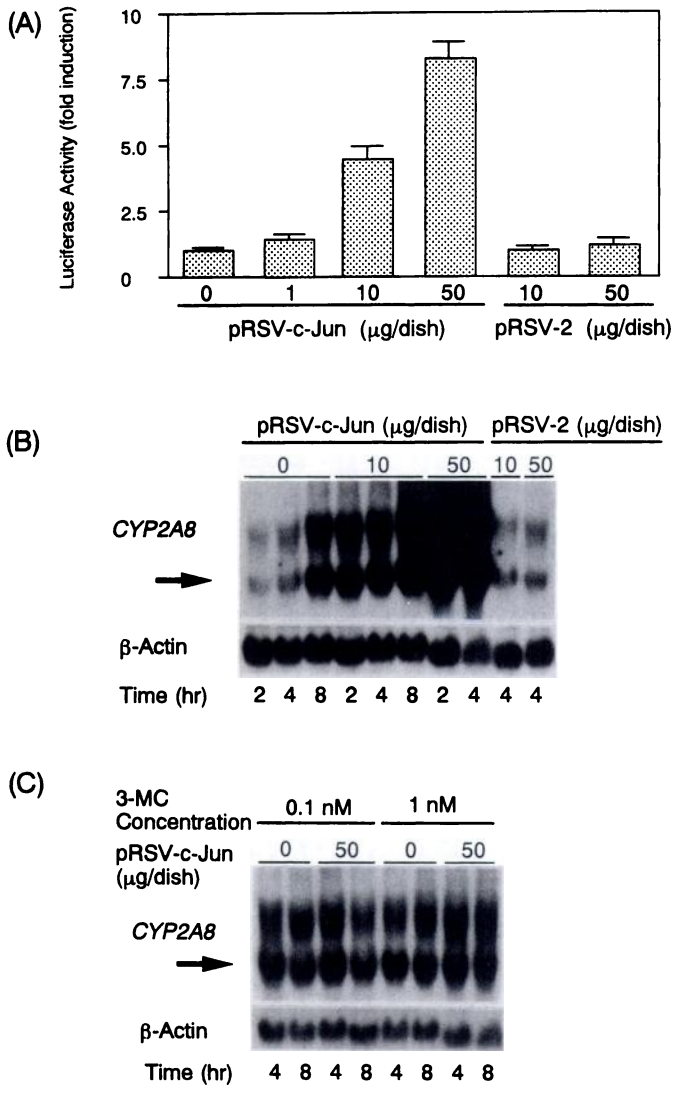

Fig. 9. Effect of c-Jun expression on 3-MC-induced CYP2A8 expression in primary cultures of Syrian hamster hepatocytes. Hepatocytes precultured for 2 days were transfected with c-Jun expression plasmid, pRSV-c-Jun, at the indicated concentrations shown in the figure; AP-1-dependent reporter gene; and β-galactosidase expression plasmid for 16 hr. A, The media were changed, and the cells were cultivated for an additional 8 hr. After harvest, luciferase and β-galactosidase activities were determined. Luciferase activities normalized by β-galactosidase activities were calculated relative to the level of luciferase activity in the cell without pRSV-c-Jun. pRSV-2 was transfected as a negative control. Each value represents the mean ± standard error of three determinations. B, The media were changed, and cells were treated with 0.1 μM 3-MC. Total RNAs were prepared at indicated times, and CYP2A8 mRNA expressions were analyzed by Northern blot. pRSV-2 was transfected as a negative control. Uniform RNA loading is demonstrated by β-actin probe. C, The cells were treated with 1 or 0.1 nM 3-MC. Total RNAs were prepared at indicated times, and CYP2A8 mRNA expressions were analyzed by Northern blot. Uniform RNA loading is demonstrated by β-actin probe.

we investigated the components of the signal transduction pathway and the nature of the transcription factors mediating 3-MC-induced CYP2A8 gene expression. The results of the current work clearly demonstrate that OA potentiated 3-MC-induced CYP2A8 gene expression (Figs. 1–3) and suggest that the induction of CYP2A8 was mediated by AhR in primary cultures of Syrian hamster hepatocytes (Fig. 4). Although it has been reported that dexamethasone and phorbol ester affected the expression of CYP1A2 by 3-MC (35) and of CYP1A1 by TCDD (9), respectively, this is the first report that shows that OA potentiated the induction of *CYP* genes by PAHs and HAHs.

We then studied the mechanism by which OA potentiated 3-MC-induced CYP2A8 expression. Because OA increased the intracellular phosphorylation state of various proteins (16), the results suggested that the protein phosphorylation process was involved in the potentiation of the 3-MC-induced CYP2A8 gene expression. The identification of the proteins that were responsible for the effects of OA is fairy difficult because of the diversity of the substrates of serine threonine protein phosphatases 1 and 2A (16). On the other hand, OA has been reported to increase the levels of Fos and Jun via a protein phosphorylation process in human T cells (40) and rat phenochromocytoma PC12 cells (41). OA was also shown to regulate the activity of AP-1 not only at the transcriptional level but also at the post-translational level (39). For example, protein kinase A and a novel family of kinases that are structurally related to mitogen-activated protein kinase phosphorylate c-Fos and activate its transcriptional activity (39). Jun kinase and stress activating protein kinase, which have been shown to be activated by OA, also phosphorylate c-Jun and stimulate its ability to activate transcription (39). In fact, Park *et al.* (38) reported that OA regulated the expression of MyoD1 negatively and inhibited myogenesis by the activation and induction of AP-1. These reports led us to examine the effects of OA on AP-1 expression in hamster hepatocytes. As a result, we observed not only the expression of c-*fos* and *jun*-D mRNAs with an increase in nuclear AP-1 but also the activation of AP-1-dependent gene transcription by OA in the hepatocytes (Figs. 5, 7, and 8). More detailed experiments have given evidence of the involvement of AP-1 in OA potentiation of CYP2A8 gene expression by 3-MC. First, the concentrations of OA that potentiated the 3-MC-induced coumarin 7-hydroxylase activity were identical to those of OA that induced c-*fos* and *jun*-D mRNAs and activated the AP-1-dependent gene transcription (Figs. 2, 5B, and 8). Second, low concentrations of anisomycin and cycloheximide, which were capable of activating the Jun kinase/stress activating protein kinase (34), potentiated the induction of CYP2A8 mRNA by 3-MC, induced c-*fos* and *jun*-D mRNAs, and activated AP-1-dependent gene transcription like OA did in the hepatocytes (Figs. 1, 6, and 8). However, dexamethasone and forskolin, which had been reported to affect the induction of CYP1A2 and Cyp2a-5, respectively (35, 36), did not affect the CYP2A8 mRNA induction by 3-MC, the expression of c-*fos* and *jun*-D mRNA, or the activation of AP-1-dependent gene transcription in the hepatocytes (Figs. 1, 6, and 8). Third, the time course changes in c-*fos* and *jun*-D mRNA expression induced by OA preceded the changes in OA-potentiated CYP2A8 mRNA expression (Figs. 3A and 5A). Fourth, 3-MC-induced CYP2A8 mRNA expression was potentiated in the hepatocytes transfected with c-Jun expression plasmid (Fig. 9). There is a possibility that another transcription factor could be involved in the OA action because OA can phosphorylate many proteins, including other transcription factors. However, all of these results strongly suggest that AP-1 may be involved in this response as one of the factors that are affected by OA.

Three members of the Jun family, c-Jun, Jun-B, and

Jun-D, have been identified (42), whereas the Fos family includes c-Fos, Fos-B, Fos-B2, Fra-1, and Fra-2 (21). These proteins seem to be expressed differently in various cell types, can form heterodimers with each other, and bind to the same DNA consensus sequence. They seem to have different effects on cell functions, and the complex of c-Fos and c-Jun has the most potent transcriptional activity (43). However, we detected only c-*fos* and *jun*-D mRNAs in Syrian hamster hepatocytes using mouse and rat probes. We could not detect any other *fos* and *jun* families using mouse, rat, or human probes, probably because these probes were not suitable for the detection of hamster *fos* and *jun*. Nevertheless, we believe that OA might induce c-Jun as well as Jun-D in the hepatocytes because OA stimulated AP-1 binding activity in the hepatocyte nuclear extracts (Fig. 7).

We can speculate on various possible pathways in which AP-1 could be involved in the mechanism of the OA-potentiated induction of CYP2A8 by 3-MC. It is possible that AP-1 could bind directly to the transcriptional regulatory region of the CYP2A8 gene. Puga *et al*. (44) found that HAH activated protein kinase C, followed by the induction of c-Fos and Jun-D in cultured murine hepatoma cells independent of AhR, and predicted that the induction of AP-1 might lead to the elucidation of the molecular basis of HAH-induced tumor promotion. Therefore, there is a possibility that AP-1, which may be induced by 3-MC and OA, directly induced CYP2A8 expression independent of AhR. For this reason, we measured the transcriptional activity of the AP-1-dependent reporter gene when 3-MC alone was added to the hepatocytes. Our results indicated that 3-MC could not affect the AP-1-dependent transcriptional activity in the hamster hepatocytes, which differed from HAH in murine hepatoma cells (Fig. 8). Furthermore, OA alone could not induce CYP2A8 in the hepatocytes (Fig. 1). This result showed that the AP-1 alone could not induce the CYP2A8 gene. Another possibility is that AP-1 may increase 3-MC-dependent transcription factors, such as AhR. OA seems to interact with AhR-mediated induction because the inductions by 3-MC as well as by β-NF, another AhR agonist, were potentiated by OA (Fig. 4). In fact, Moore *et al*. (9) reported that phorbor ester potentiated the TCDD-induced *CYP1A1* gene expression associated with the increase of nuclear AhR levels and the binding of nuclear extracts to dioxin-responsive element. According to this hypothesis, OA would potentiate CYP2A8 expression at any concentration of 3-MC. However, this possibility could be ignored because OA potentiated the expression of CYP2A8 only when 3-MC was present at high concentrations (Fig. 1). We then speculated that AP-1 and 3-MC-dependent transcription factor, both of which might bind to the transcriptional regulatory region of the CYP2A8 gene, cooperated to induce the CYP2A8 gene. According to this hypothesis, we can understand why OA or expression of c-Jun potentiates CYP2A8 expression only when 3-MC is present at the two highest levels, 0.1 and 1 μM (Figs. 1 and 9). A small amount of AP-1 is present in the untreated (OA not added) hepatocytes (Fig. 7, *lanes 1 and 2*). We believe that the resting level of AP-1 in the untreated (OA not added) hepatocytes is sufficient to induce the small amount of CYP2A8 observed after the addition of low concentrations of 3-MC (<10 nM). Even if OA induced high levels of AP-1 in the hepatocytes, this excess AP-1 is not required to induce small amounts of CYP2A8. Furthermore, we suppose that the resting levels of AP-1 in the untreated hepatocytes are not sufficient to induce large amounts of CYP2A8 corresponding to high concentrations of 3-MC. High levels of AP-1 induced by OA are required for the induction of large amounts of CYP2A8 in the hepatocytes treated with high concentrations of 3-MC (0.1 and 1 μM). Therefore, we believe that AP-1 is necessary to *trans*-activate the CYP2A8 gene mediated by a 3-MC-dependent transcription factor, such as AhR.

In summary, we have shown that OA potentiated the 3-MC-induced CYP2A8 gene expression associated with the induction of AP-1 in Syrian hamster hepatocyte cultures. We also showed that AP-1 might be one of the components of the signal transduction system from 3-MC to CYP2A8 gene expression. Further studies, especially involving the determination of transcriptional regulatory regions of the CYP2A8 gene, are required to understand completely the mechanism of signal transduction by which 3-MC activates transcription of the CYP2A8 gene.

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References

1. Conney, A. H. Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons: G. H. A. Clowes Memorial Lecture. *Cancer Res.* **42**:4875-4917 (1982).
2. Gonzalez, F. J. Molecular genetics of the P-450 superfamily. *Pharmacol. Ther.* **45**:1-38 (1990).
3. Whitlock, J. P., Jr. The regulation of cytochrome P-450 gene expression. *Annu. Rev. Pharmacol. Toxicol.* **26**:333-369 (1986).
4. Tukey, R. H., R. R. Hannah, M. Negishi, D. W. Nebert, and H. J. Eisen. The Ah locus: correlation of intranuclear appearance of inducer-receptor complex with induction of cytochrome P1–450 mRNA. *Cell* **31**:275-284 (1982).
5. Fujisawa-Sehara, A., K. Sogawa, M. Yamane, and Y. Fujii-Kuriyama. Characterization of xenobiotic responsive elements upstream from the drug-metabolizing cytochrome P-450c gene: a similarity to glucocorticoid regulatory elements. *Nucleic Acids Res.* **15**:4179-4191 (1987).
6. Quattrochi, L. C., and R. H. Tukey. The human cytochrome Cyp1A2 gene contains regulatory elements responsive to 3-methylcholanthrene. *Mol. Pharmacol.* **36**:66-71 (1989).
7. Quattrochi, L. C., T. Vu, and R. H. Tukey. The human CYP1A2 gene and induction by 3-methylcholanthrene: a region of DNA that supports AH-receptor binding and promoter-specific induction. *J. Biol. Chem.* **269**:6949-6954 (1994).
8. Reiners, J.-J., Jr., A. Scholler, P. Bischer, A. R. Cantu, and A. Pavone. Suppression of cytochrome P450 Cyp1a-1 induction in murine hepatoma 1c1c7 cells by 12-O-tetradecanoylphorbol-13-acetate and inhibitors of protein kinase C. *Arch. Biochem. Biophys.* **301**:449-454 (1993).
9. Moore, M., T. R. Narasimhan, M. A. Steinberg, X. Wang, and S. Safe. Potentiation of CYP1A1 gene expression in MCF-7 human breast cancer cells cotreated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and 12-O-tetradecanoylphorbol-13-acetate. *Arch. Biochem. Biophys.* **305**:483-488 (1993).
10. Enan, E., and F. Matsumura. Evidence for a second pathway in the action mechanism of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD): significance of Ah-receptor mediated activation of protein kinase under cell-free conditions. *Biochem. Pharmacol.* **49**:249-261 (1995).
11. Sidhu, J. S., and C. J. Omiecinski. cAMP-associated inhibition of phenobarbital-inducible cytochrome P450 gene expression in primary rat hepatocyte cultures. *J. Biol. Chem.* **270**:12762-12733 (1995).
12. Tollet, P., M. Hamberg, J.-A. Gustafsson, and A. Mode. Growth hormone signaling leading to *CYP2C12* gene expression in rat hepatocytes involves phospholipase A2. *J. Biol. Chem.* **270**:12569-12577 (1995).
13. Sunouchi, M., M. Fukuhara, Y. Ohno, and A. Takanaka. Effects of various inducers on the activities of cytochrome P450-mixed function oxidases and aflatoxin B1 activation in microsomes of hamster livers. *J. Toxicol. Sci. (Tokyo)* **13**:193-204 (1988).
14. Fukuhara, M., T. Nohmi, K. Mizokami, M. Sunouchi, M. Ishidate, Jr., and A. Takanaka. Characterization of three forms of cytochrome P450 induc-

ible by 3-methylcholanthrene in golden hamster livers with special reference to aflatoxin B1 activation. *J. Biochem. (Tokyo)* **106:**253-258 (1989).
15. Sagami, I., T. Ohmachi, H. Fujii, and M. Watanabe. Pulmonary microsomal cytochrome P-450 from 3-methylcholanthrene-treated hamster: purification, characterization, and metabolism of benzo[*a*]pyrene. *J. Biochem. (Tokyo)* **100:**449-457 (1986).
16. Cohen, P., C. F. B. Holmes, and Y. Tsukitani. Okadaic acid: a new probe for the study of cellular regulation. *Trends Biochem. Sci.* **15:**98-102 (1990).
17. Guy, G. R., X. Cao, S. P. Chuna, and Y. H. Tan. Okadaic acid mimics multiple changes in early protein phosphorylation and gene expression induced by tumor necrosis factor or interleukin-1. *J. Biol. Chem.* **267:**1846-1852 (1992).
18. Fukuhara, M., K. Nagata, K. Mizokami, Y. Yamazoe, A. Takanaka, and R. Kato. Complete cDNA sequence of major 3-methylcholanthrene-inducible cytochrome P-450 isozyme (P-450-AFB) of Syrian hamster with high activity toward aflatoxin B1. *Biochem. Biophys. Res. Commun.* **162:**265-272 (1989).
19. Xie, T., M. Belinsky, Y. Xu, and A. K. Jaiswal. ARE- and TRE-mediated regulation of gene expression: response to xenobiotics and antioxidants. *J. Biol. Chem.* **270:**6894-6900 (1995).
20. Sunouchi, M., Y. Ishimi, K. Mizokami, M. Fukuhara, A. Takanaka, F. Hanaoka, and M. Yamada. Preparation and characterization of monoclonal antibodies against a form of hamster cytochrome P-450 high specific to aflatoxin B1. *Biochem. Biophys. Res. Commun.* **153:**441-447 (1988).
21. Suzuki, T., H. Okuno, T. Yoshida, T. Endo, H. Nishina, and H. Iba. Difference in transcriptional regulatory function between c-Fos and Fra-2. *Nucleic Acids Res.* **19:**5537-5542 (1991).
22. Sonnenberg, J. L., F. J. Rauscher III, J. I. Morgan, and T. Curran. Regulation of proenkephalin by Fos and Jun. *Science (Washington D. C.)* **246:**1622-1625 (1989).
23. Tohkin, M., T. Yagami, and T. Matsubara. Mastoparan, a peptide toxin from wasp venom, stimulates glycogenolysis mediated by an increase of the cytosolic free Ca^{2+} concentration but not by an increase of cAMP in rat hepatocytes. *FEBS Lett.* **260:**179-182 (1990).
24. Nemoto, N., and J. Sakurai. Activation of *Cyp*1a1 and *Cyp*1a2 genes in adult mouse hepatocytes in primary culture. *Jpn. J. Cancer Res.* **84:**272-278 (1993).
25. Fukuhara, M., K. Mizokami, M. Sakaguchi, Y. Niimura, K. Kato, S. Inouye, and A. Takanaka. Aflatoxin B1-specific cytochrome P-450 isozyme (P450-AFR) inducible by 3-methylcholanthrene in golden hamsters. *Biochem. Pharmacol.* **39:**463-469 (1990).
26. Lu, A. Y., R. Kuntzman, S. West, M. Jacobson, and A. H. Conney. Reconstituted liver microsomal enzyme system that hydroxylates drugs, other foreign compounds, and endogenous substrates. II. Role of the cytochrome P-450 and P-448 fractions in drug and steroid hydroxylations. *J. Biol. Chem.* **247:**1727-1733 (1972).
27. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* **227:**680-685 (1970).
28. Church, G. M., and W. Gilbert. Genomic sequencing. *Proc. Natl. Acad. Sci. USA* **81:**1991-1995 (1984).
29. Angel, P., M. Imagawa, R. Chiu, B. Stein, R. J. Imbra, H. J. Rahmsdorf, C. Jonat, P. Herrlich, and M. Karin. Phorbol ester-inducible genes contain a common *cis* element recognized by a TPA-modulated *trans*-acting factor. *Cell* **49:**729-739 (1987).
30. Deng, T., and M. Karin. JunB differs from c-Jun in its DNA-binding and dimerization domains, and represses c-Jun by formation of inactive heterodimers. *Genes Dev.* **7:**479-490 (1993).
31. Brasier, A. R., J. E. Tate, and J. F. Habener. Optimized use of the firefly luciferase assay as a reporter gene in mammalian cell lines. *Biotechniques* **7:**1116-1122 (1989).
32. Norton, P. A., and J. M. Coffin. Bacterial β-galactosidase as a marker of Rous sarcoma virus gene expression and replication. *Mol. Cell. Biol.* **5:**281-280 (1985).
33. Kadonaga, J. T., and R. Tjian. Affinity purification of sequence-specific DNA binding proteins. *Proc. Natl. Acad. Sci. USA* **83:**5889-5893 (1986).
34. Cano, E., and L. C. Mahaderan. Parallel signal processing among mammalian MAPKs. *Trends Biochem. Sci.* **20:**117-122 (1995).
35. Silver, G., L. M. Reid, and K. S. Krauter. Dexamethasone-mediated regulation of 3-methylcholanthrene-induced cytochrome P-450*d* mRNA accumulation in primary rat hepatocyte cultures. *J. Biol. Chem.* **265:**3134-3138 (1990).
36. Salonpaa, P., O. Pelkonen, A. Kojo, M. Pasanen, M. Negishi, and H. Raunio. Cytochrome P4502A5 expression and inducibility by phenobarbital is modulated by cAMP in mouse primary hepatocytes. *Biochem. Biophys. Res. Commun.* **205:**631-637 (1994).
37. Pshenichkin, S. P., and B. C. Wise. Okadaic acid increases nerve growth factor secretion, mRNA stability, and gene transcription in primary cultures of cortical astrocytes. *J. Biol. Chem.* **270:**5994-5999 (1995).
38. Park, K., M. Chung, and S.-J. Kim. Inhibition of myogenesis by okadaic acid, an inhibitor of protein phosphatases 1 and 2A, correlates with the induction of AP1. *J. Biol. Chem.* **267:**10810-10815 (1992).
39. Karin, M. The regulation of AP-1 activity by mitogen-activated protein kinases. *J. Biol. Chem.* **270:**16483-16486 (1995).
40. Thevenin, C., S.-J. Kim, and J. H. Kehr. Inhibition of protein phosphatases by okadaic acid induces AP1 in human T cells. *J. Biol. Chem.* **266:**9363-9366 (1991).
41. Haby, C., D. Aunis, and J. Zwiller. Okadaic acid induces activator protein 1 activity and immediate early gene transcription in rat pheochromocytoma cells: mechanism of action. *Biochem. Pharmacol.* **48:**819-825 (1994).
42. Ryder, K., and D. Nathans. Induction of protooncogene c-jun by serum growth factors. *Proc. Natl. Acad. Sci. USA* **85:**8464-8467 (1988).
43. Castellazzi, M., G. Spyrou, N. La-Vista, J. Dangy, F. Piu, M. Yaniv, and G. Brun. Overexpression of c-jun, junB, or junD affects cell growth differently. *Proc. Natl. Acad. Sci. USA* **88:**8890-8894 (1991).
44. Puga, A., D. W. Nebert, and F. Carrier. Dioxin induces expression of *c-fos* and *c-jun* proto-oncogenes and a large increase in transcription factor AP-1. *DNA Cell Biol.* **11:**269-281 (1992).

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