# 行政院國家科學委員會專題研究計畫 成果報告

## DHA 調控 phenobarbital 誘發初代肝細胞 cytochrome P450 2B1 表現之機制探討(第 2 年)
## 研究成果報告(完整版)

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# DHA down-regulates phenobarbital-induced cytochrome P450 2B1 gene expression in rat primary hepatocytes by attenuating CAR translocation

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**Abstract**

The constitutive androstane receptor (CAR) plays an important role in regulating the expression of detoxifying enzymes, including cytochrome P450 2B (CYP 2B). Phenobarbital (PB) induction of human CYP 2B6 and mouse CYP 2b10 has been shown to be mediated by CAR. Our previous study showed that PB-induced CYP 2B1 expression in rat primary hepatocytes is down-regulated by both n-6 and n-3 polyunsaturated fatty acids (PUFAs), especially docosahexaenoic acid (DHA); however, the mechanism for this down-regulation by DHA was previously unknown. The objective of the present study was to determine whether change in CAR translocation is involved in the down-regulation by n-6 and n-3 PUFAs of PB-induced CYP 2B1 expression in rat primary hepatocytes. We used 100 μM arachidonic acid, linoleic acid, eicosapentaenoic acid, and DHA to test this hypothesis. PB triggered the translocation of CAR from the cytosol into the nucleus in a dose-dependent and time-dependent manner in our hepatocyte system, and the CAR distribution in rat primary hepatocytes was significantly affected by DHA. DHA treatment decreased PB-inducible accumulation of CAR in the nuclear fraction and increased it in the cytosolic fraction in a dose-dependent manner. The down-regulation of CYP 2B1 expression by DHA occurred in a dose-dependent manner, and a similar pattern was found for the nuclear accumulation of CAR. The results of immunoprecipitation showed a CAR/RXR heterodimer bound to nuclear receptor binding site 1 (NR-1) of the PB-responsive enhancer module (PBREM) of the CYP 2B1gene. The EMSA results showed that PB-induced CAR binding to NR-1 was attenuated by DHA. Taken together, these results suggest that attenuation of CAR translocation and decreased subsequent binding to NR-1 are involved in DHA's down-regulation of PB-induced CYP 2B1 expression.


*Keywords:* Constitutive androstane receptor; CAR; CYP 2B1; PUFAs; Docosahexaenoic acid; DHA; Hepatocytes

## Introduction

Hepatic cytochrome P450s (CYPs) display diverse functions in the metabolism of biological compounds, including endogenous molecules such as steroid hormones (You, 2004) and xenochemicals such as drugs and environmental pollutants (Iwase et al., 2006; Arlt et al., 2006). Phenobarbital (PB) has been identified as a potent inducer of the CYP 2B subfamily in both human and rodent primary hepatocytes (Wang et al., 2004; Li et al., 2006). Increased CYP 2B activity induced by xenochemicals may result in the accelerated metabolism of co-administered drugs and lead to decreased therapeutic efficacy and increased toxicity (Lin, 2006). PB-responsive enhancer module (PBREM), which responds to numerous PB-type inducers, has been identified in the promoter of human and rat CYP 2B genes (Sueyoshi et al., 1999). PBREM contains a nuclear factor-1 (NF-1) binding site flanked by two DR-4 nuclear receptor-binding motifs, NR-1 and NR-2 (Honkakoski and Negishi, 1998). Specific mutation of these NR motifs results in a complete loss of responsiveness to PB induction (Honkakoski et al., 1998).

The constitutive androstane receptor (CAR), which is present mostly in the cytosol of noninduced mammalian liver and hepatocytes, has been recognized as a transcription factor. It plays an important role not only in the regulation of hepatic fatty acid oxidation, gluconeogenesis (Miao et al., 2006), and the metabolism of steroid hormones and bilirubin (Yamamoto et

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al., 2003), but also in the modulation of xenochemical-metabolizing gene expression, including that of the phase I enzymes, such as CYPs 2B, 2C, and 3A, and the phase II enzymes, such as glutathione S-transferase alpha 1 (GSTA1) and UDP-glucuronosyltransferase 1A1 (UGT1A1) (Pascussi et al., 2003a; Reschly and Krasowski, 2006). Upon activation by PB-type inducers, CAR translocates from the cytosol to the nucleus and forms a heterodimer with retinoid X receptor (RXR). Subsequently, the heterodimer binds to NR-1 and trans-activates the target genes (Kawamoto et al., 1999; Kim et al., 2001).

N-6 and n-3 polyunsaturated fatty acids (PUFAs) have been identified to play an important role in energy metabolism and in endogenous hormone synthesis (Spector and Yorek, 1985). N-6 and n-3 PUFAs have also been shown to regulate the expression of many genes, such as acetyl-CoA carboxylase (Suchankova et al., 2005), leptin (Reseland et al., 2001), and fatty acid synthetase (Kim et al., 2004). PUFAs have also been studied for their role in the regulation of gene expression involved in the prevention of cardiovascular diseases (Le Jossic-Corcos et al., 2005), tumor development (Trombetta et al., 2007), and neurodegenerative disorders (Kim and Takahashi, 2006). However, studies of the roles of PUFAs in the expression of detoxifying genes are relatively scarce. Our previous study showed that PB-induced CYP 2B1 expression in rat primary hepatocytes is down-regulated by both n-6 and n-3 PUFAs, especially docosahexaenoic acid (DHA) (Li et al., 2006). However, the mechanism for this down-regulation by DHA was not fully understood. In the present study, we used the rat primary hepatocyte culture system to study whether CAR plays a crucial role in the down-regulation by n-6 and n-3 PUFAs of PB-induced CYP 2B1 expression.

## Materials and methods

*Materials.* Cell culture medium (RPMI-1640) was purchased from Gibco-BRL (Gaithersburg, MD); collagenase type I was from Worthington Biochemical (Lakewood, NJ); Matrigel and ITS$^+$ (insulin, transferrin, selenium, bovine serum albumin, and linoleic acid) were from Collaborative Biomedical Products (Bedford, MA); and arachidonic acid (AA), linoleic acid (LA), eicosapentaenoic acid (EPA), and DHA were from Cayman Chemical (Ann Arbor, MI). Anti-CAR antibody (sc-13065) and anti-RXRα antibody (sc-553) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-CYP 2B1 antibody (H72520M) was purchased from Meridian Life Science, Inc. (Saco, ME). Anti-hsp90 antibody was purchased from Stressgen Bioreagents (Ann Arbor, MI). Anti-C23 antibody was purchased from BD Transduction Laboratories (San Diego, CA). Phenobarbital, butylated hydroxytoluene, α-tocopheryl succinate, sodium chloride, dexamethasone, potassium chloride, potassium phosphate, magnesium sulfate, calcium chloride, and sodium bicarbonate were from Sigma Chemical (St. Louis, MO).

*Hepatocyte isolation and culture.* Male Sprague–Dawley rats (weighing 250–300 g) were purchased from the National Animal Breeding and Research Center (Taipei, Taiwan). Hepatocytes were isolated by a modification of the two-step collagenase perfusion method described previously (Seglen, 1976). After isolation, hepatocytes ($3 \times 10^6$ cells/dish) were plated on collagen-coated 60-mm plastic tissue dishes in RPMI-1640 medium (pH 7.38) supplemented with 10 mM HEPES, 1% ITS$^+$, 1 μM dexamethasone, 100 IU penicillin/ml, and 100 μg streptomycin/ml. Cells were incubated at 37 °C in a 5% $CO_2$ humidified incubator. After 4 h, cells were washed with phosphate-buffered saline (PBS) to remove any unattached or dead cells, and the same medium supplemented with Matrigel (233 mg/L) and 0.1 μM dexamethasone was added. Thereafter, the medium was changed daily. The protocol for each experiment is described in the corresponding figure legend.

*Fatty acid preparation.* PUFA samples were prepared and complexed with fatty acid–free bovine serum albumin at a 6:1 molar ratio before addition to the culture medium. At the same time, 20 μM α-tocopheryl succinate and 0.1% butylated hydroxytoluene were added to the culture medium to prevent lipid peroxidation.

*Cytosolic and nuclear extracts and whole cell lysates preparation.* After each experiment, hepatocytes were washed twice with cold PBS and were then scraped from the dishes with PBS. Cell homogenates were centrifuged at 2000×*g* for 5 min. The supernatant was discarded, and the cell pellet was allowed to swell on ice for 15 min after the addition of 350 μl of hypotonic extraction buffer containing 10 mM HEPES, 1 mM $MgCl_2$, 1 mM EDTA, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.5% Nonidet P-40 (NP-40), 4 μg/ml leupeptin, 20 μg/ml aprotinin, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). After centrifuged at 6000×*g* for 15 min, the resulting supernatant was used as cytosolic fraction for Western blot analysis and the pellet containing nuclei were extracted by gentle mixing with 50 μl of hypertonic extraction buffer containing 10 mM HEPES, 0.4 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 4 μg/ml leupeptin, 20 μg/ml aprotinin, 0.2 mM PMSF, and 10% glycerol at 4 °C for 30 min. The samples were then centrifuged at 10000×*g* for 15 min. The supernatant containing the nuclear proteins was collected and stored at −80 °C until the electrophoretic mobility shift assay (EMSA) and Western blot analysis were performed. For whole cell lysates preparation, hepatocytes were washed twice with cold PBS and were then incubated in 200 μl of RIPA lysis buffer containing 140 mM NaCl, 1 mM $NaH_2PO_4$, 9 mM $Na_2HPO_4$, 0.1% SDS, 0.5% sodium deoxy-cholate (NaDOC), and 1% NP-40 at 4 °C for 30 min. The cells were then scraped and centrifuged at 10000×*g* for 15 min. The supernatant was used as whole cell lysates protein for Western blot analysis.

*EMSA.* EMSA was carried out as described previously (Pustylnyak et al., 2005a). Three micrograms of nuclear extract, poly (dI-dC), and biotinylated double-stranded NR-1 consensus oligonucleotides (5′-GATCTGTACTTTTCC-TGACCTGATC-3′) (Pustylnyak et al., 2005a) were mixed with the binding buffer (LightShift Chemiluminescent EMSA Kit; Pierce Chemical Co., Rockford, IL) to a final volume of 20 μl, and the mixture was incubated at 27 °C for 30 min. An unlabeled double-stranded NR-1 oligonucleotide and a double-stranded NF-κB oligonucleotide (5′-AGTTGAGGCGACTTTCCCAGGC-3′) were used to confirm competitive binding and specific binding, respectively. The nuclear protein–DNA complex was separated by 8% Tris/boric acid/EDTA–polyacrylamide gel electrophoresis and was then transferred to Hybond N$^+$ membranes. The membranes were treated with streptavidin-horseradish peroxidase, and the nuclear protein–DNA bands were developed with a SuperSignal West Pico kit (Pierce Chemical Co.).

*Immunoprecipitation.* Nuclear extracts were diluted to 1 μg/μl with IP buffer (40 mM Tris–HCl [pH 7.5], 1% NP-40, 150 mM NaCl, 5 mM EGTA, 1 mM DTT, 1 mM PMSF, 20 mM NaF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM sodium vanadate). The diluted nuclear extracts (80 μg) were then incubated with 0.8 μg anti-CAR antibody for 6 h at 4 °C, mixed with 4 μl protein A–Sepharose suspension, and incubated for an additional 1 h. Immunoprecipitates and the supernatants were collected by centrifugation at 16000×*g* for 1 min. The pellet was washed three times with 300 μl IP buffer and was then subjected to Western blotting.

*Western blotting for CAR, RXRα, and CYP 2B1.* Cytosolic and nuclear extracts and whole cell lysates proteins were prepared from rat primary hepatocytes as described in the previous paragraph. For CAR, RXRα, hsp90, and C23 protein expression, 15 μg of cytosolic protein and 20 μg of nuclear protein and whole cell lysates protein were separated on a 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel, and the separated proteins were transferred to polyvinylidene difluoride membranes. After blocking, the membrane was incubated with anti-CAR antibody (1:500), anti-RXRα antibody (1:500), and anti-hsp90 (1:500) for 1 h at room temperature, or with anti-C23 antibody (1:500) for 2 h at room temperature. Thereafter, the membranes were incubated with the secondary anti-rabbit or anti-mouse IgG-horseradish peroxidase conjugate for 1 h at room temperature, and the protein bands were visualized by enhanced chemiluminescence plus Western blotting detection reagent (Amersham Biosciences, Boston, MA). For CYP 2B1 protein expression, immunoblotting was carried out as described previously (Li et al., 2006).

*Northern blotting for CYP 2B1.* Total RNA was extracted from rat primary hepatocytes with 0.5 mL TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. For Northern blotting, 20 μg of each RNA sample was electrophoresed on a 1% agarose gel containing 6% formaldehyde and then transferred to a Hybond-$N^+$ nylon membrane (Amersham, Little Chalfont, United Kingdom) as previously described (Yang and Krauss, 1997). For hybridization with cDNA, the membrane was prehybridized at 42 °C for 1 h in a solution containing 10× Denhardt's reagent (0.2% Ficoll, 0.2% polyvinylpyrrolidone, and 0.2% bovine serum albumin), 5× saline-sodium phosphate-EDTA buffer (750 mM NaCl, 50 mM $NaH_2PO_4$, 5 mM EDTA), 2% SDS, 50% formamide, and 100 mg/L of single-stranded sheared salmon sperm DNA. The membrane was then hybridized in the same solution with $^{32}$P-labeled CYP 2B1 cDNA probe at 42 °C overnight. The hybridized membrane was washed once or twice in 2× SSC buffer (SSC/0.05% SDS) at room temperature and then at 52 °C for 10 min in 0.1× SSC/0.1% SDS. Autoradiography was performed by exposing the membrane to Kodak SuperRx X-ray film (Pierce, Rockford, IL) at −80 °C with an intensifying screen.

*cDNA preparation and RT-PCR analysis.* Forty hours after attachment, hepatocytes were treated with (5, 25, and 100 μM) or without DHA for 24 h. Total RNA was extracted according to manufacturer's instructions. Total RNA was isolated and subjected to RT-PCR with specific CAR and GAPDH primers. Amounts of 0.1 μg total RNA were reversely transcribed with superscript II reverse transcriptase (Stratagene, Heidelberg, Germany) in a 20-μl final volume of the reaction buffer, which consisted of 5 mM $MgCl_2$, 1 mM of each deoxyribonucleotide triphosphate, 2.5 U RNase inhibitor, and 2.5 mM oligo(dT). The conditions of PCR amplification and CAR primers design were carried out as described previously (Pustylnyak et al., 2005a). For the synthesis of complementary DNA, the reaction mixtures were incubated at 45 °C for 15 min; the reaction was stopped by denaturing the reverse transcriptase by heating the mixture to 99 °C for 5 min. To the cDNA sample, a PCR mixture containing 4 mM $MgCl_2$, 200 μM of each deoxyribonucleotide triphosphate, a dilution of the cDNA preparation, 5 μl of 10× buffer, 0.2 U Taq polymerase, and 0.6 pmol CAR primer was added to a total volume of 50 μl. The sequences of the RT-PCR CAR primers were as follows: 5′-ACCAGATCTCCCTTCTCAAG-3′ (forward) and 5′-CTCGTACTGGAACCCTA-3′ (reverse) which amplify a 146-bp fragment (bp 799–944) of the CAR (GenBank Accession No. NM_022941). The sequences of the RT-PCR GAPDH primers were as follows: 5′-CCATCACCATCTTCCAGGAG-3′ (forward) and 5′-CCTGCTTCACCACCTTCTTG-3′ (reverse), which amplify a 576-bp fragment (bp 253–828) of the GAPDH (GenBank Accession No. AB017801). For CAR and GAPDH PCR amplification, the samples were heated to 95 °C for 5 min and then immediately cycled 30 times through a 1-min denaturing step at 95 °C, a 0.5-min annealing step at 59 °C, and a 1-min elongation step at 72 °C. The amplified PCR products of CAR and GAPDH mRNA were separated on 1.5% agarose gels alongside markers.

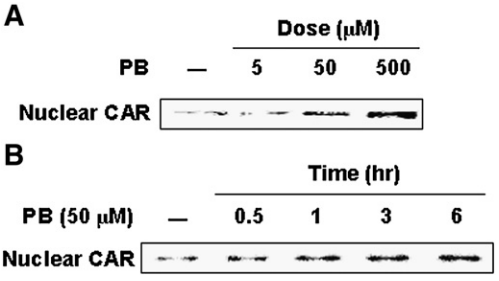


Fig. 1. Nuclear accumulation of CAR in rat primary hepatocytes after PB treatment. Hepatocytes were cultured as described in Materials and methods. (A) Forty hours after attachment, hepatocytes were treated with or without various concentrations of PB for 1 h, and aliquots of total hepatocyte nuclear extracts (20 μg) were used for Western blot analysis. (B) Hepatocytes were treated with or without 50 μM PB for various time periods, and aliquots of total hepatocyte nuclear extracts (20 μg) were used for Western blot analysis. One representative experiment out of three independent experiments is shown.

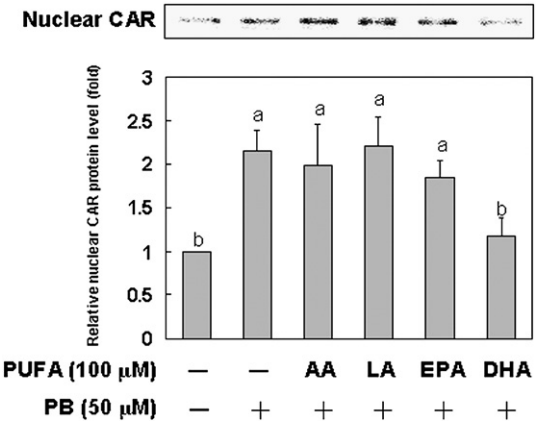


Fig. 2. Effect of n-6 and n-3 PUFAs on PB-induced nuclear accumulation of CAR. After attachment, hepatocytes were pretreated with or without 100 μM AA, LA, EPA, and DHA for 24 h before the addition of PB. One hour after PB addition, cells were harvested and aliquots of total hepatocyte nuclear extracts (20 μg) were used for Western blot analysis. Values are means ± SD, $n = 3$. Bars with different letters are significantly different ($P < 0.05$).

*Statistical analysis.* Data were analyzed by using analysis of variance (SAS Institute, Cary, NC). The significance of the difference between mean values was determined by Duncan's test; $P$ values $< 0.05$ were taken to be statistically significant.

## Results

### *Effect of PB on the nuclear accumulation of CAR in rat primary hepatocytes*

Transactivation of the nuclear orphan receptor CAR was identified as a crucial event in PB-inducible CYP 2B gene expression in mouse primary hepatocytes (Kawamoto et al., 1999). To determine the effect of PB on the nuclear accumulation of CAR in rat primary hepatocytes, cells were treated with increasing concentrations of PB (5–500 μM) for 1 h. As shown in Fig. 1A, the nuclear accumulation of CAR was increased by PB in a dose-dependent manner. Also, the nuclear accumulation of CAR with 50 μM PB was time dependent (Fig. 1B). In the following experiments, 50 μM PB and a 1-h exposure were used to examine CAR translocation.

### *Effect of n-6 and n-3 PUFAs on PB-inducible nuclear accumulation of CAR*

Our previous study (Li et al., 2006) showed that n-6 and n-3 PUFAs down-regulate PB-inducible CYP 2B1 gene expression in rat primary hepatocytes. To study whether CAR translocation is involved in this down-regulation, we pretreated cells with 100 μM AA, LA, EPA, and DHA. As shown in Fig. 2, DHA significantly attenuated the nuclear accumulation of CAR; this effect was not found for the other n-6 and n-3 PUFAs.

### *Dose effect of DHA on the nuclear translocation of CAR and CYP 2B1 gene expression induced by PB*

To further demonstrate whether the down-regulation of CYP 2B1 gene expression by DHA was through attenuation of CAR

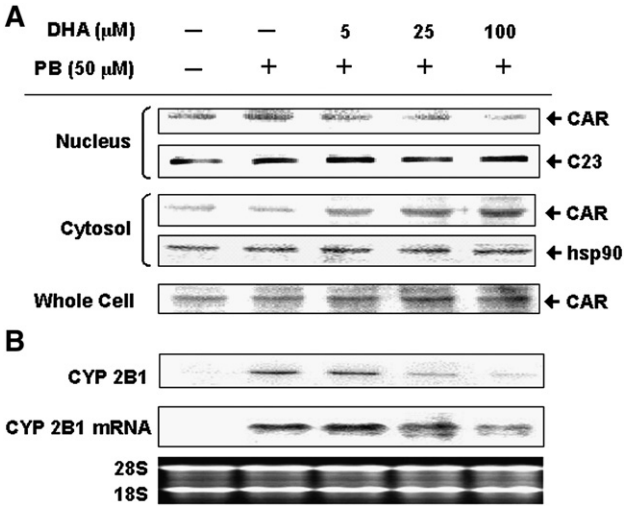


Fig. 3. Effect of various concentrations of DHA on CAR nuclear translocation and CYP 2B1 expression by PB. (A) After attachment, hepatocytes were treated with or without various concentrations of DHA for 24 h before the addition of PB, and the cells were then incubated with PB for another 1 h. Western blot analysis was performed for hepatocyte nuclear and cytosolic extracts and whole cell lysates proteins (20 μg, 15 μg, and 20 μg, respectively), after electrophoresis on 10% SDS–PAGE gels using primary antibodies, anti-CAR, anti-hsp90, and anti-C23. Hsp90 and C23 were used as the cytosolic and nucleus protein loading control, respectively. (B) After attachment, hepatocytes were incubated with or without DHA for 24 h before the addition of PB, and the cells were then incubated with PB for another 20 h. Microsomal proteins (7.5 μg) were used for Western blot analysis and total RNA extracts (20 μg) were used for Northern blot analysis. Uniform RNA loading was demonstrated by the ethidium bromide staining of the 18S and 28S rRNA bands, which were used as the internal standards. One representative experiment out of three independent experiments is shown.

nuclear translocation, we performed Northern and Western blotting to detect CYP 2B1 gene expression and Western blotting to examine the distribution of CAR in rat primary hepatocytes. As shown in Fig. 3A, DHA treatment decreased PB-inducible accumulation of CAR in the nuclear fraction and increased it in the cytosolic fraction in a dose-dependent manner. A similar pattern was found for CYP 2B1 protein and mRNA expression after DHA treatment (Fig. 3B). CYP 2B1 expression was related to CAR nuclear translocation in rat primary hepatocytes.

*Time effect of PB on nuclear NR-1 complex formation*

In the present study, the nuclear protein CAR was found to accumulate after 0.5 h of PB exposure, which indicates that the binding of CAR to NR-1 may occur in our rat primary hepatocytes. EMSA was used to examine the effect of PB on NR-1 complex formation. NR-1 complex formation began to appear at 3 h and was still present at 20 h (Fig. 4).

*Effect of n-6 and n-3 PUFAs on PB-inducible nuclear NR-1 complex formation*

EMSA was performed to further examine whether the down-regulation of PB-inducible CYP 2B1 gene expression by n-6 and n-3 PUFAs was mediated by attenuating the formation of the

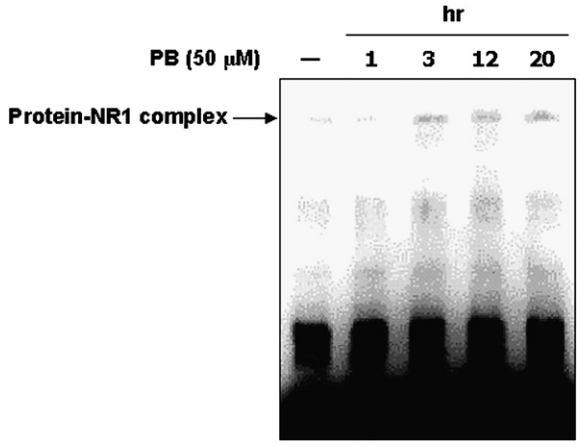


Fig. 4. Time effect of PB on protein–NR-1 complex formation. After attachment, hepatocytes were incubated with or without 50 μM PB for various time periods. Aliquots of total hepatocyte nuclear extracts (6 μg) were used for EMSA. One representative experiment out of three independent experiments is shown.

NR-1 complex. As shown in Fig. 5, NR-1 complex formation was significantly attenuated by 100 μM DHA but not by the other n-6 and n-3 PUFAs.

*Identification of the component in the NR-1 complex*

In the present study, the nuclear accumulation of CAR was significantly decreased by DHA (Fig. 2), and subsequent NR-1 complex formation was attenuated by DHA as well (Fig. 5). The CAR-RXR heterodimer is formed after CAR translocates from the cytosol into the nucleus, and the heterodimer is required for CAR binding to NR-1 and activation of the target gene. To further identify whether the component in the NR-1 complex was CAR, we performed immunoprecipitation experiments to

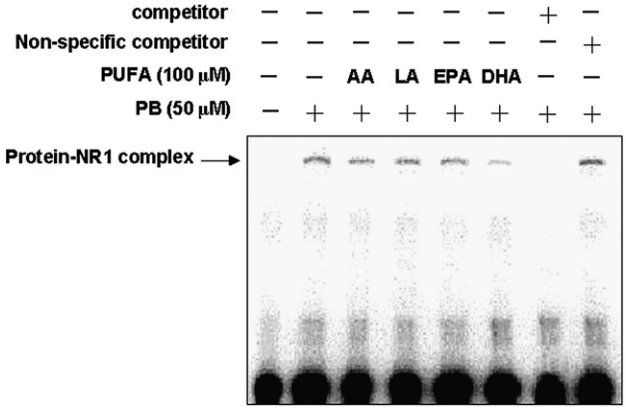


Fig. 5. Effect of n-6 and n-3 PUFAs on PB-inducible nuclear NR-1 complex formation. After attachment, hepatocytes were pretreated with or without 100 μM AA, LA, EPA, and DHA for 24 h before the addition of PB, and the cells were then incubated with PB for another 20 h. Aliquots of total hepatocyte nuclear extracts (6 μg) were used for EMSA. To confirm the specificity of the nucleotide, 100-fold cold probe (biotin-unlabeled NR-1) and NF-κB probe were included in the EMSA. One representative experiment out of three independent experiments is shown.

provide direct evidence for CAR-RXR heterodimer formation and adduct formation between CAR and NR-1. As shown in Fig. 6A, the pattern of CAR accumulation after immunoprecipitation with anti-CAR antibody was consistent with the result of DHA attenuation of PB-induced nuclear CAR accumulation (Fig. 2). Moreover, the CAR-RXRα heterodimer was formed in the nucleus of our rat primary hepatocyte culture system (Fig. 6B). The NR-1 complex was increased after PB exposure and was attenuated by DHA; this indicated that CAR may play an important role in protein–DNA binding. As shown in Fig. 6C, CAR was removed from nuclear extracts by immunoprecipitation before EMSA; the results showed that CAR immunoprecipitation leads to no NR-1 complex formation in the PB-treated group. This indicated that the attenuation of NR-1 complex formation by DHA may be through the inhibition of nuclear CAR translocation.

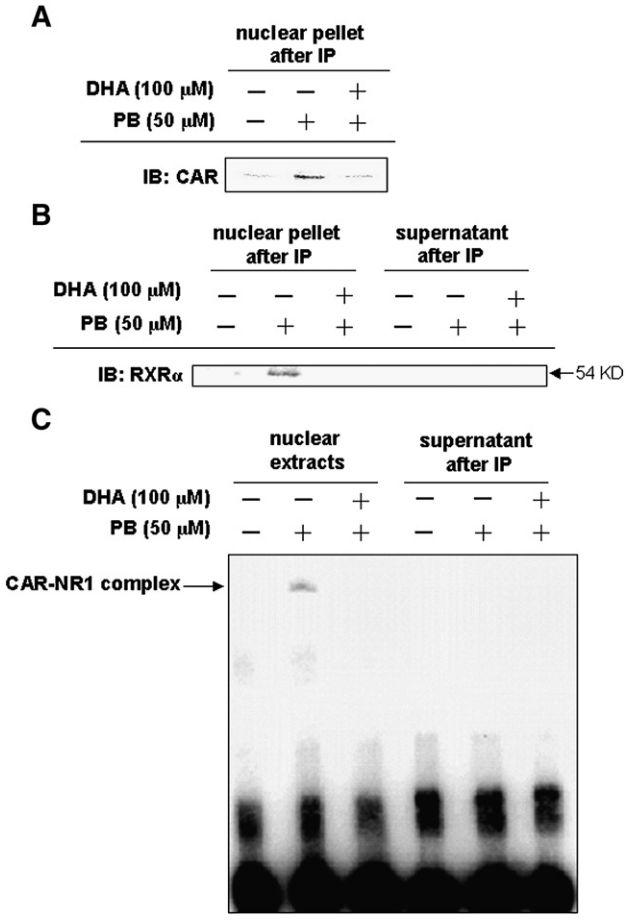


Fig. 6. Identification of the component in the NR-1 complex by immunoprecipitation (IP). After attachment, hepatocytes were treated with or without DHA for 24 h, and the cells were then incubated with PB for another 1 h. Nuclear extracts were then subjected to IP with anti-CAR antibody. (A) Aliquots of pellet after IP (20 μg) were used for Western blot analysis with anti-CAR antibody. (B) Aliquots of pellet and supernatant after IP (20 μg) were used for Western blot analysis with anti-RXRα antibody. (C) Hepatocytes were treated with or without DHA for 24 h, and the cells were then incubated with PB for another 20 h. Aliquots of nuclear extracts and the supernatant after IP with anti-CAR antibody (6 μg) were used for EMSA. One representative experiment out of three independent experiments is shown.

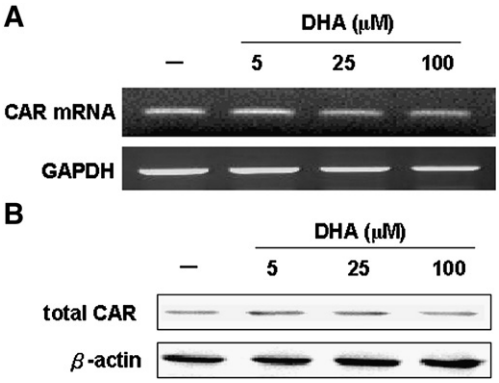


Fig. 7. Effect of various concentrations of DHA on CAR gene expression. After attachment, hepatocytes were treated with or without various concentrations of DHA for 24 h. (A) Total RNA was isolated from hepatocytes and was subjected to RT-PCR with specific CAR and GAPDH primers as described in Materials and methods. (B) Aliquots of total protein (20 μg) were used for Western blot analysis. GAPDH and β-actin were used as internal controls. One representative experiment out of three independent experiments is shown.

### *Effect of DHA on CAR gene expression*

A recent study showed that CAR gene expression was not affected by colchicine in primary human hepatocytes (Dvorak et al., 2007); however, cocaine was shown to down-regulate CYP 2C gene expression through decreased CAR gene expression in human U373 MG astrocytoma cells (Malaplate-Armand et al., 2005). To determine the role of CAR gene expression in down-regulation of CYP 2B1 gene expression by DHA in rat primary hepatocytes, CAR mRNA and protein levels were assayed. As shown in Figs. 7A and B, CAR mRNA and protein levels were not significantly affected by treatment with different concentrations of DHA.

## Discussion

The nuclear orphan receptor CAR activates the transcription of detoxifying genes, such as CYP 2B (Faucette et al., 2006). CAR accumulates in the nucleus in response to PB and PB-type inducers in rat liver (Pustylnyak et al., 2005a) and mouse primary hepatocytes (Hosseinpour et al., 2006). To demonstrate whether PB triggers the nuclear accumulation of CAR in our rat primary hepatocyte culture system, we subjected the cell nuclear extracts to Western blotting. We showed that a basal level of nuclear CAR is present in untreated hepatocytes (Fig. 1), which agrees with the results of a previous study (Hosseinpour et al., 2006). Furthermore, the nuclear accumulation of CAR was increased by PB in a dose-dependent manner and was dramatically increased within 30 min after treatment with 50 μM PB (Fig. 1). These results support the notion that the nuclear accumulation of CAR is rapid after PB treatment in mouse primary hepatocytes (Kawamoto et al., 1999).

In our previous study (Li et al., 2006), PB-induced CYP 2B1 expression in rat primary hepatocytes was down-regulated by both n-6 and n-3 PUFAs, especially AA and DHA. AA down-regulates CYP 2B1 expression through $PGE_2$ and the cAMP-dependent PKA pathway; however, the mechanism by which

DHA down-regulates CYP 2B1 expression was unclear. Recent studies suggested that DHA may affect the localization of transcription factors and regulate their target gene expression (Calviello et al., 2007). PUFAs inhibit the translocation of carbohydrate-responsive element–binding protein (ChREBP) from the cytosol into the nucleus, and this results in the suppression of hepatic glycolysis and lipogenesis (Dentin et al., 2005). We were thus interested in investigating the role of CAR in the down-regulation of PB-induced CYP 2B1 expression by n-6 and n-3 PUFAs. The dosage selection of PUFAs was performed in our previous study (Li et al., 2006). Maximal concentration used caused no cell damage was 100 μM. Our data showed that the nuclear accumulation of CAR induced by PB was significantly attenuated by DHA but not other PUFAs. DHA treatment decreased PB-inducible CAR accumulation in the nuclear fraction and increased it in the cytosol in a dose-dependent manner. Nuclear CAR accumulation was consistent with CYP 2B1 protein and mRNA expression. These results suggest that CAR is involved in the regulation of CYP 2B1 transcription and translation and DHA plays a role in CAR nuclear translocation.

PBREM was found to have sequence homology in different species, such as in the mouse cyp 2b10, human CYP 2B6, and rat CYP 2B1/2 genes (Sueyoshi et al., 1999). The NR-1 site in PBREM is the most conserved sequence in CYP 2B gene families, and many studies have suggested that NR-1 is the most important motif within the PBREM for mediating the transactivation of CYP 2B genes by PB (Sueyoshi et al., 1999; Wang and Negishi, 2003). After exposure to PB and PB-type inducers, the translocated CAR forms a heterodimer with RXR within the nucleus and subsequently binds to PBREM and enhances its activity (Honkakoski et al., 1998). A response element for the CAR/RXR heterodimer has been described as a DR-4 element for modulating CYP 2B expression (Honkakoski et al., 1998; Frank et al., 2003); however, a CAR/RXR heterodimer was shown to bind to the ER-6 (everted repeat with a 6-bp spacer) element of the CYP 3A4 gene (Sueyoshi et al., 1999). Moreover, many other CAR functional response elements were identified, including DR-2, -3, and -5 and ER-8 (Handschin and Meyer, 2003). These observations suggest that CAR-mediated gene expression may be regulated through the action of different CAR functional response elements.

In the present study, we found that CAR began to accumulate in the nucleus within 30 min after PB treatment (Fig. 1B) and that the NR-1 complex was formed after 3 h of PB exposure (Fig. 4). The EMSA results suggested that the nuclear CAR may form a heterodimer with RXR and bind to NR-1 within 3 h after PB exposure in rat primary hepatocytes. Attenuation of nuclear NR-1 complex formation was found only in the DHA-treated group (Fig. 5). This result agrees with our previous study showing that the mechanism by which n-3 PUFAs down-regulate CYP 2B1 expression may be different from that for n-6 PUFAs (Li et al., 2006). Another previous study showed that the nuclear NR-1 complex is formed after xenochemical exposure in rat liver (Pustylnyak et al., 2005a); however, the component of the NR-1 complex was not shown. In the present study, we used the same NR-1 oligonucleotide for EMSA and carried out immunoprecipitation to further identify the component in the NR-1 complex. We found that the CAR-RXRα heterodimer was formed in the nucleus of our rat primary hepatocyte culture system after PB exposure (Fig. 6). After CAR removal from the nuclear extracts by immunoprecipitation before EMSA, no NR-1 complex formation was found in the PB-treated group. These results suggest that CAR translocates into the nucleus and forms a CAR/RXRα heterodimer after PB exposure in rat primary hepatocytes.

A previous study showed that phosphorylation/dephosphorylation events might be crucial for PB-induced CYP 2B and 3A expression in rat primary hepatocytes (Joannard et al., 2000). Serine/threonine protein phosphatase inhibitor (okadaic acid) and $Ca^{2+}$/calmodulin-dependent protein kinase II inhibitor (KN-62) both have been shown to affect murine PB-induced CYP 2B10 enzyme activity (Posti et al., 1999) and CAR mRNA level (Pustylnyak et al., 2005b). Hosseinpour et al. (2006) showed that the dephosphorylation of serine-202 is a required step in the regulation of nuclear CAR translocation. Furthermore, it was recently reported that AMP-activated protein kinase activation by inducers is involved in PB-induced CYP 2B expression and in the modulation of nuclear CAR translocation and transcription (Shindo et al., 2007). A recent study showed that PB-induced CAR-mediated CYP 2B1 transcription was repressed by epidermal growth factor and growth hormone in rat primary hepatocytes (Bauer et al., 2004). Furthermore, hepatocytes growth factor (HGF) treatment was effective to repress the induction of endogenous CYP 2b gene by PB and TCPOBOP in mouse primary hepatocytes (Koike et al., 2007). The inhibitory effect of HGF on CYP 2b10 expression was attributed to its increase of extracellular signal-regulated kinase (ERK)1/2 phosphorylation in the cytosol. Giving 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene (U0126), an inhibitor of HGF downstream kinase mitogen-activated protein kinase kinase (MEK), induced the CYP 2b10 gene and up-regulated the CAR-mediated promoter activity in the absence of TCPOBOP. The results suggest that phosphorylation of ERK1/2 plays an important role in sequestering CAR in the cytosol of mouse primary hepatocytes. DHA was reported to be involved in ERK1/2 activation in malignant peripheral nerve sheath tumor cells (Mashour et al., 2005) and in rat vascular smooth muscle cells (Machida et al., 2005). PB-induced gene expression has been shown to be regulated by several serine/threonine protein phosphatases (Kawamura et al., 1999; Pustylnyak et al., 2005b) and kinases (Marc et al., 2000). The relationship between DHA and phosphorylation/dephosphorylation events such as ERK1/2 activation is our future study.

PUFAs inhibit hepatic fatty acid synthesis by suppressing SREBP-1c nuclear abundance. This suppression may be through several mechanisms, including attenuation of SREBP-1c translocation, suppression of SREBP-1c gene transcription, and enhancement of SREBP-1c mRNA decay and proteasomal degradation (Jump et al., 2005). In the present study, n-6 and n-3 PUFAs were used to pretreat hepatocytes for 24 h before PB treatment. The amount of nuclear CAR was lower in the DHA-treated group than in the PB-treated group, which suggests that DHA may down-regulate CAR transcription. However, results

of Figs. 7A and B did not support this notion. CAR mRNA and protein levels were not significantly affected by treatment with different concentrations of DHA. CAR governs not only CYP 2B1 but also other xenochemical-metabolizing enzymes and transporters, such as CYP 2C and 3A, UGT1A1, GSTA1, and MRP-2 (multidrug-resistance protein 2) and MRP-3 (Huang et al., 2003; Pascussi et al., 2003b; Shelby and Klaassen, 2006). The results of the present study support an important role for the nutritional factor DHA in detoxifying gene expression and provide a possible molecular mechanism for DHA's action in regulating nuclear CAR translocation and CAR-NR-1 binding activity.

In summary, the results of the present study indicate that PB-induced CYP 2B1 expression in rat primary hepatocytes is down-regulated by DHA. Attenuation of CAR nuclear translocation and decreased nuclear CAR-NR-1 complex formation contribute to this down-regulation of CYP 2B1 gene expression by DHA.

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Research Article

# Docosahexaenoic acid downregulates phenobarbital-induced cytochrome P450 2B1 gene expression in rat primary hepatocytes *via* the c-Jun NH2-terminal kinase mitogen-activated protein kinase pathway

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Mitogen-activated protein kinase (MAPK) pathways play central roles in the transduction of extracellular stimuli into cells and the regulation of expression of numerous genes. Docosahexaenoic acid (DHA) was shown to be involved in the regulation of expression of drug metabolizing enzymes (DMEs) in rat primary hepatocytes in response to xenobiotics. Cytochrome P450 2B1 (CYP 2B1) is a DME that is dramatically induced by phenobarbital-type inducers. The constitutive androstane receptor (CAR) plays a critical role in regulating the expression of DMEs, and the phosphorylation/dephosphorylation of CAR is an important event in CYP 2B1 expression. In the present study, we determined the effect of DHA on MAPK transactivation and its role in CYP 2B1 expression induced by phenobarbital. c-Jun NH2-terminal kinase (JNK) JNK1/2 and ERK1/2 were activated by phenobarbital in a dose-dependent manner. DHA (100 μM) inhibited JNK1/2 and ERK2 activation induced by phenobarbital in a time-dependent manner. Both SP600125 (a JNK inhibitor) and SB203580 (a p38 MAPK inhibitor) inhibited CYP 2B1 protein and mRNA expression induced by phenobarbital. SB203580 significantly increased the intracellular 3′-5′-cyclic adenosine monophosphate (cAMP) concentration compared with a control group ($p < 0.05$). Our results suggest that inhibition of JNK activation by DHA is at least part of the mechanisms of DHA's downregulation of CYP 2B1 expression induced by phenobarbital.

**Keywords:** Affiliations / CYP 2B1 / DHA / Hepatocytes / Mitogen-activated protein kinases / PUFA

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## 1 Introduction

Fish oils are rich in the *n* - 3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). In humans, EPA (20:5, *n* - 3) and DHA (22:6, *n* - 3) are derived from the essential fatty acid α-linolenic acid (ALA) (18:3, *n* - 3) and play an important role in energy metabolism and endogenous hormone synthesis [1]. Many studies have shown that DHA is more effective than EPA in regulating cell proliferation, apoptosis, and inflammatory cytokine production [2–4].

DHA is a predominant structural fatty acid in the central nervous system, and its availability is crucial for central nervous system development [5]. Epidemiologic studies and clinical trials have shown that DHA exerts anti-atherosclerotic [6], anti-inflammatory [7, 8], and anti-aging [9] effects. Furthermore, DHA was recognized as a potent inhibitor of the growth of various tumor cells, including human breast cancer cells [3], pancreatic cancer cells [10], human colon adenocarcinoma cells [11], and prostate cancer cells [12]. The antitumor effect of DHA was indicated by increased caspase activity, increased DNA fragments,

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**Abbreviations: cAMP,** 3′-5′-cyclic adenosine monophosphate; **CYP 2B1,** cytochrome P450 2B1; **EPA,** eicosapentaenoic acid; **DHA,** docosahexaenoic acid; **DME,** drug metabolizing enzyme; **ERK1/2,** extracellular signal-regulated kinases 1/2; **JNK,** c-Jun NH2-terminal kinase; **MAPK,** mitogen-activated protein kinase; **PB,** phenobarbital

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and a loss of mitochondrial membrane potentials. The mechanism of action of DHA was thought to be through the regulation of a signal transduction pathway such as inhibition of the Akt/NFκB cell survival pathway [3]. The early atherosclerotic lesions caused by inflammatory processes were inhibited by DHA through a reduction in mitogen-activated protein kinase (MAPK) p42/p44 activity [13]. These results support a role of DHA in the signal transduction pathway.

Exposure to xenobiotics and drugs activates drug metabolizing enzymes (DMEs), and this event affords protection to animals. DMEs consist of phase I enzymes, phase II metabolizing enzymes, and phase III transporters, which are abundant either at the basal unstimulated level or are elevated after exposure to xenobiotics [14]. Cytochrome P450 2B1 (CYP 2B1) is a DME induced by phenobarbital (PB) in both human and rodent primary hepatocytes [15, 16]. A variety of dietary nutrients such as vitamin E and fatty acids, have been shown to influence the CYP 2B1 gene expression induced by PB in rat primary hepatocytes [15, 17]. Prostaglandin production and transcription factor activation are the mechanisms underlying the modulation of CYP 2B1 expression by dietary nutrients.

MAPKs belong to the serine/threonine protein kinase family, and they include c-Jun NH2-terminal kinase (JNK), extracellular signal-regulated kinases 1/2 (ERK1/2), p38 MAPK, and big MAPK (BMK) [18]. Many studies have shown that the MAPKs play a critical role in gene expression. p38-type MAPK was reported to be involved in the repression by abscisic acid, a plant growth inhibitor, of *Arabidopsis thaliana Ku* (*AtKu*) gene expression in *A. thaliana* [19]. MAPK inhibitor was shown to block the 17ss-estradiol-induced estrogen-related receptor α expression in both estrogen receptor-positive MCF-7 and estrogen receptor-negative SKBR3 breast cancer cells [20]. The expression of cannabinoid-induced tissue inhibitors of MMPs and subsequent suppression of Hela cell invasion was prevented by pretreatment of cells with inhibitors of MAPKs [21]. The MAPK cascade is considered to be a major signaling system by which extracellular stimuli are transduced into the cells [22, 23]. Treatment of primary cultures of rat hepatocytes with PB induced CYP 2B expression, and the ERK MAPK pathway was reported to be involved in the transcriptional regulation of rat CYP 2B gene expression [24]. In the present study, we used the rat primary hepatocyte culture system to study the effect of DHA on MAPK transactivation induced by PB and the link between the MAPK pathway and CYP 2B1 gene expression induced by PB.

## 2 Materials and methods

### 2.1 Chemicals

Cell culture medium (RPMI-1640) and penicillin–streptomycin solution were from GIBCO-BRL (Gaithersburg, MD); Matrigel and ITS$^+$ (insulin, transferrin, selenium, BSA, and linoleic acid) were from Collaborative Biomedical Products (Bedford, MA); collagenase type I was from Worthington Biochemical (Lakewood, NJ); TRIzol reagent was from Invitrogen (Carlsbad, CA); dexamethasone, HEPES, sodium bicarbonate, butylated hydroxytoluene, α-tocopheryl succinate, calcium chloride, magnesium chloride, PB, albumin, and bovine serum essentially fatty acid free were from Sigma Chemical (St. Louis, MO); DHA and cyclic AMP EIA kit were from Cayman Chemical (Ann Arbor, MI); SP600125 (JNK inhibitor) and PD98059 (MAPK/ERK kinase (MEK) inhibitor) were from TOCRIS (Ellisville, MO); SB203580 (p38 MAPK inhibitor) was from Biosource (Camarillo, CA); antibody against CYP 2B1 (H72520M) was from Meridian Life Science (Saco, ME); antibodies against JNK and phospho-JNK were from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies against ERK, phospho-ERK (Thr202/Tyr204), p38, and phospho-p38 (Thr180/Tyr 182) were from Cell Signaling Technology (Beverly, MA).

### 2.2 Hepatocyte isolation and culture

Male Sprague-Dawley rats (weighing 250–300 g) were purchased from the National Animal Breeding and Research Center (Taipei, Taiwan). Hepatocytes were isolated by a modification of the two-step collagenase perfusion method described previously [25]. After isolation, hepatocytes ($3 \times 10^6$ cells *per* dish) were plated on collagen-coated 60-mm plastic tissue dishes in RPMI-1640 medium (pH 7.38) supplemented with 10 mM HEPES, 1% ITS$^+$, 1 μM dexamethasone, 100 IU penicillin/mL, and 100 μg streptomycin/mL. Cells were incubated at 37°C in a 5% $CO_2$ humidified incubator. After a 4 h attachment period, cells were washed with PBS to remove any unattached or dead cells, and the same medium supplemented with Matrigel (233 mg/L) and 0.1 μM dexamethasone was added. Thereafter, the medium was changed daily. The protocol for each experiment is described in the corresponding figure legend. The rats were treated in compliance with the *Guide for the Care and Use of Laboratory Animals*.

### 2.3 Fatty acid preparation

DHA samples were prepared and complexed with fatty acid-free BSA at a 6:1 molar ratio before addition to the culture medium. At the same time, 0.1% butylated hydroxytoluene and 20 μM α-tocopheryl succinate were added to the culture medium to prevent lipid peroxidation.

### 2.4 Northern blotting for CYP 2B1

RNA was extracted from primary rat hepatocytes with 0.5 mL TRIzol reagent (Invitrogen, Carlsbad, CA). The extract was allowed to react at room temperature for 5 min,

 

0.1 mL chloroform was added, and the sample was incubated for an additional 3 min. The samples were centrifuged at 12 000 × *g* for 15 min at 4°C. The aqueous phase was transferred to a fresh tube, and the RNA was precipitated by the addition of 0.5 mL isopropyl alcohol. The RNA samples were allowed to sit at room temperature for 10 min and were then centrifuged at 12 000 × *g* for 20 min at 4°C. The resulting RNA pellets were washed twice with 70% ice-cold ethanol. For Northern blot analysis, 20 μg of each RNA sample was electrophoresed on a 1%-agarose gel containing 6% formaldehyde and was transferred to a Hybond-$N^+$ nylon membrane (Amersham, Little Chalfont, UK) as previously described [26]. For hybridization with cDNA, the membrane was prehybridized at 42°C for 1 h in a solution containing 10 × Denhardt's reagent (0.2% Ficoll, 0.2% polyvinylpyrrolidone, and 0.2% BSA), 5 × saline-sodium phosphate-EDTA (750 mM NaCl, 50 mM $NaH_2PO_4$, 5 mM EDTA), 2% SDS, 50% formamide, and 100 mg/L of single-stranded sheared salmon sperm DNA. The membrane was then hybridized in the same solution with $^{32}$P-labeled CYP 2B1 cDNA probe at 42°C overnight. The hybridized membrane was washed once or twice in 2 × SSC buffer (SSC/0.05% SDS) at room temperature and then at 55°C for 10 min in 0.1 × SSC/0.1% SDS. Autoradiography was performed by exposing the membrane to Kodak SuperRx X-ray film (Pierce, Rockford, IL) at −80°C with an intensifying screen.

### 2.5 Western blotting for CYP 2B1 and MAPKs

Cells were washed twice with cold PBS and were harvested in 500 μL of 20 mM potassium phosphate buffer (pH 7.0). Cell homogenates were centrifuged at 9 000 × *g* for 30 min at 4°C. The resultant supernatant portion was then ultracentrifuged at 105 000 × *g* for 1 h at 4°C. The protein content of the microsomal fraction was measured by using the Coomassie Plus Protein Assay Reagent Kit (Pierce Chemical Company, Rockford, IL). SDS polyacrylamide gels made with 7.5% polyacrylamide were prepared as described by Laemmli [27]. For CYP 2B1, 7.5 μg of microsomal protein was applied to each gel. After electrophoresis, the separated proteins were transferred to polyvinylidine difluoride membranes. The nonspecific binding sites in the membranes were blocked with 5% nonfat dry milk in 15 mM Tris-150 mM NaCl buffer (pH 7.4) at 4°C overnight. After blocking, the membrane was incubated with anti-CYP 2B1 antibody at 37°C for 1 h. Thereafter, the membrane was incubated with the secondary peroxidase-conjugated anti-rabbit IgG at 37°C for 1 h, and the immunoreactive bands were developed by adding hydrogen peroxide and 3,3′-diaminobenzidine tetrachloride as the substrates for peroxidase. For the detection of MAPKs, the membranes were incubated overnight at 4°C with anti-JNK, anti-ERK1/2, and anti-p38 MAPK or anti-phospho-activated JNK1/2, ERK1/2, and p38 MAPK antibodies. The bands were detected by using an enhanced chemiluminescence plus Western blotting detection reagent (Amersham Biosciences, Boston, MA).

### 2.6 Measurement of intracellular cAMP concentrations

Intracellular 3′-5′-cyclic adenosine monophosphate (cAMP) concentrations were measured by using the cAMP EIA kit (Cayman Chemical). Forty hours after attachment, hepatocytes were treated with 20 μM of SP600125, PD98059, or SB203580 for 20 h. Cell extracts were prepared as described by Beck and Omiecinski [28].

### 2.7 Statistical analysis

Data were analyzed by using one-way analysis of variance (SAS Institute, Cary, NC). The significance of the difference among mean values was determined by Duncan's test; $p$ values <0.05 were taken to be statistically significant.

## 3 Results

### 3.1 Effect of phenobarbital on early activation of MAPKs in rat primary hepatocytes

It was reported previously that the MAPK pathway is involved in the PB induction of CYP 2B in primary cultures of rat hepatocytes [24]. To demonstrate the importance of the MAPK pathway in the PB induction of CYP 2B in our culture system, hepatocytes were treated with increasing concentrations of PB (0.05 – 5 mM) for 15 min. As shown in Fig. 1, both JNK1/2 and ERK1/2 were activated by PB in

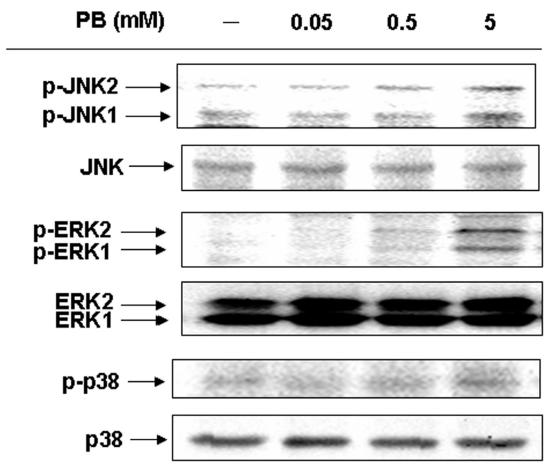


**Figure 1.** Effect of PB on the early activation of MAPKs in rat primary hepatocytes. Forty hours after attachment, hepatocytes were incubated with or without various concentrations of PB for 15 min, and aliquots of total hepatocyte extracts (30 μg) were used for Western blot analysis. One representative experiment out of three independent experiments is shown.

 

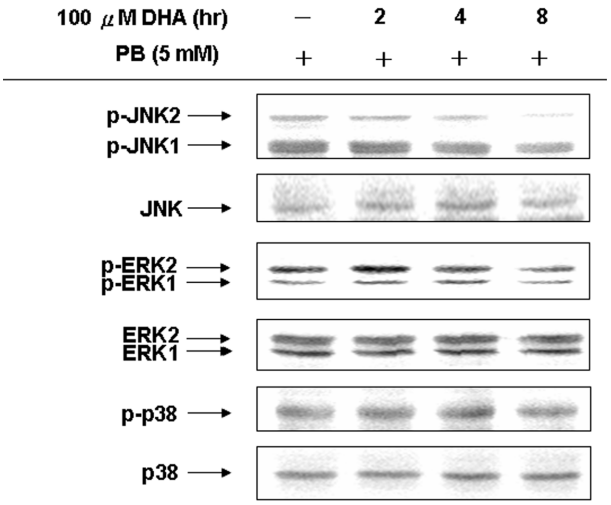


**Figure 2.** Effect of DHA on PB-induced MAPK activation. Forty hours after attachment, hepatocytes were pretreated with or without 100 μM DHA for 2, 4, and 8 h before the addition of PB. Fifteen minutes after PB addition, cells were harvested and aliquots of total hepatocyte extracts (30 μg) were used for Western blot analysis. One representative experiment out of three independent experiments is shown.

a dose-dependent manner; p38 activity, however, was not affected by PB treatment.

### 3.2 Effect of DHA on phenobarbital-induced MAPK activation

DHA (100 μM) was shown to downregulate PB-induced CYP 2B1 expression in rat primary hepatocytes [15, 29]. Also, DHA was shown to modulate the phosphorylation of MAPKs in response to mitogens in human T cells [30] and human umbilical vein endothelial cells [31]. The effect of DHA (100 μM) on PB-induced MAPK phosphorylation was assessed in the present study. As shown in Fig. 2, pretreatment with 100 μM DHA caused a time-dependent inhibition of JNK1/2 and ERK2 phosphorylation but not p38 in the presence of PB. Maximal inhibition was observed in hepatocytes pretreated with DHA for 8 h.

### 3.3 Effect of specific MAPK inhibitors on CYP 2B1 protein and mRNA expression

To assess the individual role of the MAPK pathways in PB-induced CYP 2B1 expression, hepatocytes were pretreated with 20 μM of SP600125 (a JNK inhibitor), PD98059 (an MEK inhibitor), or SB203580 (a p38 MAPK inhibitor) for 20 h and then incubated with PB for another 20 h. As shown in Fig. 3, both SP600125 and SB203580 inhibited PB-induced CYP 2B1 protein and mRNA expression, but PD98059 showed no effect.

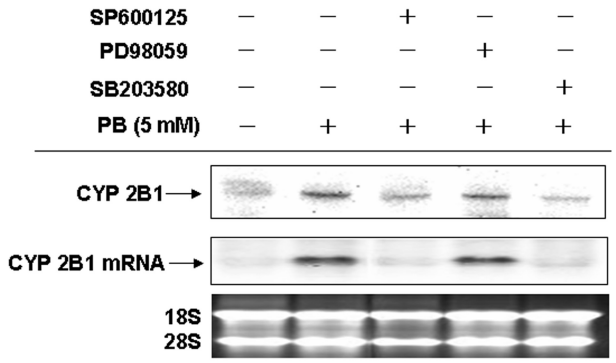


**Figure 3.** Effect of specific MAPK inhibitors on CYP 2B1 protein and mRNA expression. Forty hours after attachment, hepatocytes were pretreated with or without 20 μM specific MAPK inhibitors for 20 h before the addition of PB. After PB addition, the cells were incubated for another 20 h. (A) Western blot analysis. (B) Northern blot analysis. Uniform RNA loading was demonstrated by the ethidium bromide staining of the 18S and 28S rRNA bands, which were used as the internal standards. One representative experiment out of three independent experiments is shown.

### 3.4 Effect of specific MAPK inhibitors on intracellular cAMP concentrations

An increase in the intracellular cAMP level was reported to inhibit PB-induced CYP 2B1 mRNA expression [32]. SB203580 was shown to inhibit PB-induced CYP 2B1/2 mRNA expression in rat primary hepatocytes [24]; however, this effect was considered to be p38-independent. It was postulated that the inhibitory effect of SB203580 was due to its stimulation of cAMP production. To demonstrate the hypothesis, we assayed the intracellular cAMP concentrations of hepatocytes treated with 20 μM of specific MAPK inhibitors for 20 h. As shown in Fig. 4, the intracellular cAMP concentration was significantly increased by SB203580 ($p < 0.05$) but not by SP600125 or PD98059.

## 4 Discussion

In our previous study, we found that $n-6$ and $n-3$ PUFAs downregulate PB-induced CYP 2B1 gene expression through different pathways in rat primary hepatocytes. Arachidonic acid increased intracellular prostaglandin $E_2$ synthesis, and prostaglandin $E_2$ subsequently activated the cAMP-dependent PKA pathway to downregulate CYP 2B1 gene expression [15]. DHA, by contrast, attenuated the translocation of constitutive androstane receptor [29]. However, other possible mechanisms involved in the modulation of PB-induced CYP 2B1 gene expression by $n-3$ PUFAs were not fully understood.

Previous studies showed that the MAPKs are important signal enzymes involved in many facets of cellular regulation. The MAPK cascade is a major signaling system by

 

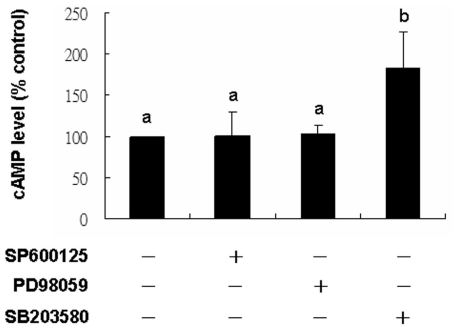


**Figure 4.** Effect of specific MAPK inhibitors on intracellular cAMP concentrations. Forty hours after attachment, hepatocytes were treated with or without 20 μM specific MAPK inhibitors for 20 h. The cells were then washed twice with cold PBS and were lysed and scraped into 0.8 mL ice-cold 70% ethanol. Cell debris was pelleted at 2000 × *g*, and the resulting supernatant fluid was lyophilized and stored at −20°C until analyzed. cAMP concentrations in cells without any specific MAPK inhibitor treatment are expressed as 100% (control), and the concentrations in the other groups were calculated in comparison with the control. Values are the mean ± SD of three independent experiments. Values not sharing the same letter are significantly different ($p < 0.05$).

which an extracellular stimulus is transduced into cells [22, 23]. MAPKs have been reported to be involved in the regulation of the induction of phase II DMEs by several xenobiotics [33, 34]; however, less is known about such a role in the control of phase I enzymes. A recent study indicated that PB induced the gene expression of the phase I enzyme CYP 2B1/2 by activating MAPK phosphorylation [24]. In the present study, we found that 5 mM PB significantly evoked phosphorylation of JNK1/2 and ERK1/2 but not p38 (Fig. 1). Our result agrees with that of Joannard *et al*. [24], who showed that phosphorylation of JNK1/2 and ERK1/2 was rapidly increased by 5 mM PB within 15 min. However, phosphorylation of p38 was found in their study but not in ours. In the present study, 5 mM PB significantly increased ERK1/2 phosphorylation (Fig. 1), which is consistent with a finding of Hodges *et al*. [35] who showed that treatment of hepatocytes for 12 h with 1.0 mM PB resulted in a statistically significant activation of p42/44 MAPK. These results suggest that MAPK phosphorylation may be involved in the PB-mediated changes in biological response in rat primary hepatocytes.

In order to preserve the PB induction response within the CYP 2B subfamily, we used a Matrigel overlay procedure in our culture system. This method differs from that of others [24, 35]. Our preliminary study found that MAPK phosphorylation was less significant in the presence of Matrigel than in the absence of Matrigel (data not shown). A Matrigel overlay during cell culture is important for PB-induced CYP 2B expression; however, it may wrap up cells and affect MAPK phosphorylation in response to PB.

DHA has been shown to affect gene expression. DHA-inhibited LPS-induced cyclooxygenase 2 (COX-2) expression in RAW 264.7 cells [36], and the effect was through the modulation of the TLR-mediated signaling pathway. In another study, dietary supplementation with fish oil showed a beneficial effect on patients with immune-related renal diseases [37]. Mice given deoxynivalenol had IgA hyperelevation and intramesangial IgA deposition that mimicked the early stages of human IgA nephropathy [38]. *In vivo* study showed that deoxynivalenol-induced phosphorylation of ERK1/2 and JNK1/2 was significantly suppressed in spleens of mice fed with fish oil. Two primary *n* - 3 PUFAs in fish oil, EPA, and DHA were used to confirm the *in vivo* findings. Both EPA and DHA significantly suppressed IL-6 superinduction by deoxynivalenol in RAW 264.7 cells, as well as impaired deoxynivalenol-induced ERK1/2 and JNK1/2 phosphorylation. Human umbilical vein endothelial cells stimulated with TNF-α resulted in enhanced MAPK expression (ERK1/2, JNK, and p38) and MAPK activation (JNK and p38) [31]. Pretreatment of human umbilical vein endothelial cells with DHA significantly decreased MAPK expression (ERK1/2, JNK, and p38) and MAPK activation (JNK and p38) stimulated by TNF-α. In contrast with the downregulation of gene expression by DHA, some studies showed an upregulatory effect of DHA on gene expression. DHA slightly enhanced both IL-1β and phorbol 12-myristate 13-acetate-induced COX-2 expression in rat vascular smooth muscle cells [39]. Also, IL-1β and phorbol 12-myristate 13-acetate induced both rapid and prolonged activation of p44/42 MAPK, but not p38 MAPK, was stimulated by DHA. Thus, on the basis of evidence that DHA regulates gene expression through different MAPK pathways, the objective of the present study was to investigate the role of the MAPK pathways in DHA's downregulation of CYP 2B1 expression induced by PB.

In the present study, pretreatment of rat primary hepatocytes with DHA significantly reduced PB-induced JNK1/2 and ERK2 phosphorylation, but not p38 phosphorylation (Fig. 2). A previous study showed that monocytes treated with okadaic acid (a serine/threonine phosphatase 2A inhibitor) before LPS stimulation resulted in a dose-dependent and significant increase in JNK activity compared with LPS-stimulated cells; however, monocytes treated with 5 μM 1,2-dioleoyl-sn-glycerol-3-phosphate (a PP2A activator) before LPS stimulation showed a substantial decrease in JNK activity [40]. In another study, clinical inhibition of PP2A activity in L199P transgenic mice caused the activation of ERK and JNK [41]. These results suggest that PP2A plays a negative role in the regulation of the JNK signaling pathway. An *in vitro* study showed that DHA causes increased ceramide formation, and this could result from DHA-induced activation of sphingomyelinase in the plasma membranes [42]. Jurkat leukaemic cells incubated with 10 μM DHA resulted in a four-fold increase in ceramide formation as early as 3 h after treatment. Long-

 

chain ceramide has been shown to activate PP1 and PP2A *in vitro*, and this activation is stereospecific [43]. The stimulatory effect of DHA on PP2A was possibly through a connected pathway for DHA action, and by this pathway, DHA increased ceramide levels and led to stimulation of PP2A [42]. In the present study, PB-induced JNK1/2 and ERK1/2 phosphorylation; however, SP600125 and SB203580 inhibited the CYP 2B1 protein and mRNA expression induced by PB (Figs. 1 and 3). This implicates the JNK pathway in the induction of CYP 2B1 expression by PB. Also, DHA preincubation for 8 h significantly decreased JNK1/2 phosphorylation induced by PB in rat primary hepatocytes. This effect might be partially attributed to the stimulation of ceramide generation by DHA and resulted in enhancement of PP2A activity. Activation of PP2A has been validated to result in a substantial decrease in JNK activity [40].

Cell uptake of long-chain fatty acid has been demonstrated to occur expeditiously in a lipid raft-dependent manner. 3T3-L1 fibroblasts and adipocytes incubated with 173 μM oleate resulted in uptake of oleate into cells *via* lipid rafts within 10 min. The uptake was nearly linear over the course of 10 min in 3T3-L1 fibroblasts, and was linear over the course of 40 s in 3T3-L1 adipocytes [44]. Although no evidence describes the uptake of DHA into hepatocytes, the presence of lipid rafts in rat primary hepatocytes [45] supports the possibility that DHA uptake into hepatocytes occurs *via* the same mechanism. Stimulatory effect of DHA on ceramide production occurred as early as 3 h after treatment [42]. Based on the evidence mentioned above, it is logical to deduce that DHA downregulates PB-induced CYP 2B1 expression in rat primary hepatocytes involves stimulation of ceramide production, activation of PP2A, and inhibition of JNK signaling pathway.

In our previous studies, 100 μM DHA was shown to downregulate PB-induced CYP 2B1 expression in rat primary hepatocytes [15, 29]. A DHA concentration of 100 μM was used to evaluate its effect on MDA-MB-231 breast cancer cell growth [46] and Jurkat T cell proliferation [47]. A clinical study showed that in cystic fibrosis patients supplemented with 70 mg of DHA/kg of body weight/d for 6 wks, the plasma DHA level increased from 20 μg/mL (60 μM) to 80 μg/mL (243.5 μM) [48]. This suggests that 100 μM DHA is an achievable level in human plasma.

In the present study, we used inhibitors of the MAPK pathways to validate the relation between MAPK activation and PB-induced CYP 2B1 gene expression. Our results showed that phosphorylation of JNK1/2 and ERK1/2 was significantly increased by PB (Fig. 1); however, PB-induced CYP 2B1 gene expression was inhibited by SP600125 (JNK inhibitor) and SB203580 (p38 MAPK inhibitor) (Fig. 3). Because PB did not activate the p38 pathway in our culture system (Fig. 1), and SB203580 was shown to significantly increase cAMP production in hepatocytes (Fig. 4), our results suggest that PB-induced CYP 2B1 expression was p38-independent. Meanwhile, PD98059 (ERK inhibitor) showed no effect on PB-induced CYP 2B1 expression (Fig. 3), which suggests that the downregulation of PB-induced CYP 2B1 expression by DHA found in previous studies may be *via* the JNK pathway.

Both SB203580 and SB202474 (an inactive analog of SB-203580) were shown to potentiate the cAMP accumulation in rat pinealocytes [49]. In rat primary hepatocytes, an increase in the intracellular cAMP level led to inhibition of CYP 2B1 expression induced by PB [32]. Although SB203580 pretreatment inhibited both CYP 2B1 protein and mRNA expression induced by PB in rat primary hepatocytes in the present study (Fig. 3), this effect was p38-independent. The p38 pathway was not activated by PB (Fig. 1), and DHA showed no inhibitory effect on p38 activation. To ascertain whether the inhibitory effect of SB203580 was through increases in the intracellular cAMP level, we assayed the intracellular cAMP concentration. SB-203580 significantly increased the intracellular cAMP concentration compared with the control, but SP600125 showed no effect (Fig. 4). This result supports the notion that the p38 pathway is not involved in the CYP 2B1 expression induced by PB. The same result was substantiated by Joannard *et al*. [24].

In summary, the results of the present study indicate that DHA downregulates PB-induced CYP 2B1 gene expression in rat primary hepatocytes *via* the JNK MAPK pathway. Future study is warranted to clarify the relationship between DHA and ceramide production, ceramide and PP2A activation, and PP2A and JNK activity in rat primary hepatocytes.

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