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n-6 and n-3 Polyunsaturated fatty acids down-regulate cytochrome P-450 2B1 gene expression induced by phenobarbital in primary rat hepatocytes☆

Chien-Chun Li[a], Chong-Kuei Lii[a], Kai-Li Liu[a], Jaw-Ji Yang[b], Haw-Wen Chen[a,*]

[a]*Department of Nutrition, Chung Shan Medical University, Taichung 402, Taiwan*
[b]*School of Dentistry, Chung Shan Medical University, Taichung 402, Taiwan*

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Abstract

In mammals, polyunsaturated fatty acids (PUFAs) act not only as an important energy source, but also as substrates for cellular membrane and hormone formation. They also play key roles in cellular metabolism and gene regulation. The objective of the present study was to determine whether individual n-6 and n-3 PUFAs affect cytochrome P-450 2B1 (CYP 2B1) expression induced by phenobarbital (PB) in primary rat hepatocytes. We used 100-μM arachidonic acid (AA), linoleic acid, eicosapentaenoic acid and docosahexaenoic acid (DHA) to test this hypothesis. Phenobarbital-induced CYP 2B1 expression was down-regulated by n-6 and n-3 PUFAs, especially AA and DHA. Prostaglandin (PG) E_2 but not PGE_3 was found to down-regulate PB-induced CYP 2B1 expression. The cyclooxygenase inhibitor indomethacin (20 μM) attenuated the down-regulation of CYP 2B1 gene expression by n-6 and n-3 PUFAs induced by PB, and maximal attenuation was found in the AA-treated group. We also studied the PGE_2 downstream cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) pathway to determine its role in the down-regulation of CYP 2B1 expression by AA with the use of 0.4 mM of the adenylate cyclase inhibitor 9-(tetrahydro-2′-furyl)adenine] (SQ22536) and 7.5 μM of the PKA inhibitor H-89. Both inhibitors attenuated the down-regulation of CYP 2B1 expression by AA. These results suggest that PB-induced CYP 2B1 expression is down-regulated by n-6 and n-3 PUFAs through different pathways. Prostaglandin E_2 and the cAMP-dependent PKA pathway were involved in AA down-regulation of CYP 2B1 expression, whereas the down-regulation by n-3 PUFAs is not fully understood yet and the glucocorticoid receptor/constitutive androstane receptor/retinoid X receptor signal transduction cascade can be involved.

Keywords: PUFAs; Arachidonic acid; Docosahexaenoic acid; Phenobarbital; CYP 2B1; Hepatocytes

1. Introduction

Linoleic acid (LA) and linolenic acid are essential fatty acids that cannot be synthesized by mammals and that must be obtained through food intake. Both fatty acids act as an important energy source and as substrates for membrane formation and hormone biosynthesis [1]. The n-6 and n-3 PUFAs have been recognized to play crucial roles in cellular physiologic events, such as nutrient metabolism [2] and gene expression [3]. Many hepatic genes involved in lipid and carbohydrate metabolism, such as fatty acid synthetase [4], acetyl-CoA carboxylase [5] and stearyl-CoA desaturase-1 [6], have been shown to be down-regulated by n-6 and n-3 PUFAs. In contrast, mRNA and protein levels of the glucose transporter 4 in adipose tissue of male Sprague–Dawley rats were increased by n-3 PUFAs [7]. n-6 and n-3 PUFAs have also been shown to modulate the expression of nonlipogenic genes, such as sterol regulatory element binding protein 1c [8], CD 36 [9], and cyclin D1 and early growth response factor-1, the expression of which was regulated by the mitogen-activated protein kinase/extracellular signal-regulated kinase signal transduction pathway [10]. Recent studies have shown that n-3 PUFAs act in contrast with n-6 PUFAs in the modulation of cellular proliferation, differentiation and signal transduction in tumor progression [11,12], and in the pathogenesis of atherosclerosis [10].

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* Corresponding author. Tel.: +886 4 2473 0022x11748; fax: +886 4 2324 8192.
E-mail address: hawwen@csmu.edu.tw (H.-W. Chen).

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Arachidonic acid (AA) is derived from LA through the desaturase and elongase system. Arachidonic acid is the principal precursor of the two-series eicosanoids. n-3 PUFAs, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are found primarily in fish oil and can also be synthesized from α-linolenic acid [13]. Both AA and EPA are substrates for cyclooxygenase (COX), but AA generates two-series eicosanoids and EPA generates three-series eicosanoids. Previous studies have shown that prostaglandin E_2 (PGE_2) and prostaglandin E_3 (PGE_3) derived from n-6 and n-3 PUFAs play important roles in the modulation of immune response, chronic inflammation and cell division [14,15], and both eicosanoids show differential effects on COX-2 expression [16], T-cell proliferation and cytokine production [14].

The cytochrome (CYP) P-450 enzymes are a superfamily of proteins that play an important role in the metabolism of many foreign chemicals and endogenous compounds [17,18]. So far, at least 17 distinct CYP gene families have been recognized in mammals [19], and they are known to be modulated by many chemicals [17]. In addition to chemicals, CYP gene families were found to be regulated by nutritional factors, such as dietary lipids and garlic juice [20,21]. Previous studies have shown that CYP gene expression is modulated by dietary lipids [20,22]. Cytochrome P-450 2B1 (CYP 2B1), one of the hepatic CYP superfamilies, is known to be highly inducible by phenobarbital (PB) [23]. The induction of CYP 2B1 expression by PB has been studied extensively in biology and toxicology [24]. Anticancer drugs such as cyclophosphamide and ifosfamide are substrates for CYP 2B1 and have been widely used for cancer therapy [17,25]. These anticancer CYP prodrugs are converted to cytotoxic metabolites not only to tumors but also to host cells [26,27]. For this reason, we chose CYP 2B1 as a target gene to investigate whether the CYP 2B1 gene expression was modulated by specific PUFAs that are present in our dietary lipids. In our previous study, it showed that CYP 2B1 gene expression was down-regulated by PGE_2 through the cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) pathway [23]. However, the individual effects of n-6 and n-3 PUFAs on CYP 2B1 gene expression have not been investigated. In the present study, we used the primary rat hepatocyte culture system to study the effects of n-6 and n-3 PUFAs on CYP 2B1 expression induced by PB and the possible mechanisms involved in this gene regulation.

2. Materials and methods

2.1. Chemicals

Cell culture medium (RPMI-1640) was obtained from GIBCO-BRL (Gaithersburg, MD); Matrigel and ITS^+ (insulin, transferrin, selenium, bovine serum albumin and linoleic acid) were from Collaborative Biomedical Products (Bedford, MA); collagenase type É was from Worthington Biochemical (Lakewood, NJ); *N*-(2-[*p*-bromocinnamylamino] ethyl)-5-isoquinolinesulfonamide dihydrochloride (H-89), indomethacin, PB, butylated hydroxytoluene, α-tocopheryl succinate, β-nicotinamide adenine dinucleotide, sodium pyruvate, sodium chloride, potassium chloride, potassium phosphate, magnesium sulfate, calcium chloride and sodium bicarbonate were from Sigma Chemical (St. Louis, MO); 9-(tetrahydro-2ℓ-furyl)adenine] (SQ22536) was from Calbiochem (San Diego, CA); and AA, LA, EPA, DHA, PGE_3, PGE_2 and the PGE_2-monoclonal EIA kit were from Cayman Chemical (Ann Arbor, MI).

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 [28]. After isolation, hepatocytes (3×10^6 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 4-h attachment, cells were washed with phosphate-buffered saline 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* [29].

2.3. Lactate dehydrogenase leakage assay

The effect of n-6 and n-3 PUFAs on cell damage was evaluated by use of the lactate dehydrogenase (LDH) leakage assay. According to the method of Moldeus et al. [30], LDH activity was monitored in a portion of cell-free medium and was compared with the total activity achieved after cell lysis. After the cell-free medium was removed, the cells were lysed with 20 mM potassium phosphate buffer containing 0.5% Triton X-100, removed with a cell scraper, and then centrifuged at $10{,}000\times g$ for 30 min at 4°C. The supernatant portion was used for the analysis. NADH (0.2 mM final concentration), pyruvate (1.36 mM final concentration), and the sample were mixed in 1 ml of Krebs–Henseleit buffer. The rate of change in absorbance at 340 nm due to NADH oxidation was recorded.

2.4. Fatty acid preparation

Polyunsaturated fatty acid samples in concentrations ranging from 1 to 100 μM 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, 0.1% butylated hydroxytoluene and 20 μM α-tocopheryl succinate were added to the culture medium to prevent lipid peroxidation.

2.5. Determination of hepatocyte PGE2 level

Forty hours after attachment, hepatocytes were treated with 100 μM AA, LA, EPA or DHA for 4 h. Cells were incubated with PB for another 20 h. In another experiment, hepatocytes were pretreated with or without 20 μM indomethacin for 1 h and were then treated with 100 μM AA for 4 h, and finally incubated with PB for another 20 h. Hepatocytes in the 0-, 1- and 10-μM AA groups were not pretreated with indomethacin but were further incubated with PB for another 20 h. Fifty-microliter aliquots of culture medium were taken and analyzed for PGE_2 by using the PGE_2-monoclonal EIA kit according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI).

2.6. 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 75% 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 [31]. For hybridization with cDNA, the membrane was prehybridized at 42°C for 2 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 (750 mM NaCl, 50 mM NaH_2PO_4, 5 mM EDTA), 2% sodium dodecyl sulfate (SDS), 50% formamide and 100 mg/L of single-strand 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.

2.7. Western blotting for CYP 2B1

Sodium dodecyl sulfate polyacrylamide gels made with 7.5% acrylamide were prepared as described by Laemmli [32]. For CYP 2B1, 7.5 μg of microsomal protein was applied to each lane. After electrophoresis, the separated proteins were transferred to polyvinylidene difluoride membranes. The nonspecific binding sites on the membranes were blocked with 5% nonfat dry milk in 15 mM Tris–150 mM NaCl buffer (pH 7.4) at 4°C overnight. Polyclonal antibody against CYP 2B1 was obtained from Chemicon International (Temecula, CA). A goat peroxidase-conjugated antirabbit IgG was used to detect the immunoreactive bands. Incubation with primary and secondary antibodies was performed at 37°C for 30 min. For color development, hydrogen peroxide and 3,3′-diaminobenzidine tetrachloride were used as the substrates for peroxidase.

2.8. 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 one-way analysis of variance and Duncan's test; P values $<.05$ were taken to be statistically significant.

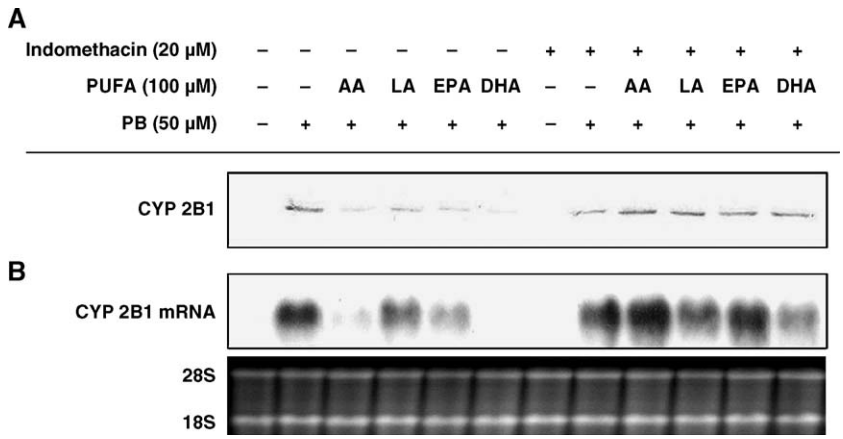

Fig. 1. Effect of indomethacin (a cyclooxygenase inhibitor) on the modulation of CYP 2B1 expression by n-6 and n-3 PUFAs in the presence of PB. Hepatocytes were cultured as described in Materials and Methods. Forty hours after attachment, hepatocytes were pretreated with or without 20 μM indomethacin for 1 h, and the cells were then incubated with 100 μM AA, LA, EPA or DHA for 4 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. Results

3.1. Lactate dehydrogenase leakage

Lactate dehydrogenase leakage was increased by 1.07±0.37% and 6.48±3.33% in hepatocytes treated with 200 μM EPA and DHA, respectively, compared with that in cells not treated with PUFAs (0.2±0.03%). However, the LDH leakage of hepatocytes treated with 200 μM AA and LA was increased by only 0.49±0.39% and 0.47±0.14%, respectively. The LDH leakage of hepatocytes treated with 100 μM PUFAs was not significantly different from that of untreated cells. We chose to use 100 μM PUFAs because the LDH leakage results showed that concentrations greater than 100 μM caused cell damage. In our study, the effects of n-6 and n-3 PUFAs observed were not due to cell viability.

3.2. Effect of n-6 and n-3 PUFAs on CYP 2B1 expression induced by PB in the presence or absence of indomethacin

As shown in Fig. 1A, CYP 2B1 protein expression was down-regulated by 100 μM AA, LA, EPA and DHA in the presence of PB. The effect was more significant in the AA- and DHA-treated groups. The CYP 2B1 mRNA expression pattern was similar to the protein expression pattern (Fig. 1B).

We showed previously that PB induction of CYP 2B1 gene expression in primary rat hepatocytes is down-regulated by PGE_2. Prostaglandin E_2 is derived from AA through the action of COX. Thus, to further determine whether the end products of the COX metabolic pathway are involved in the down-regulation of CYP 2B1 gene expression by n-6 and n-3 PUFAs, we used 20 μM of the COX inhibitor indomethacin. As shown in Fig. 1, indomethacin attenuated the down-regulation of CYP 2B1 gene expression by the n-6 and n-3 PUFAs in the presence of PB, and the maximal attenuation effect was found in the AA-treated group.

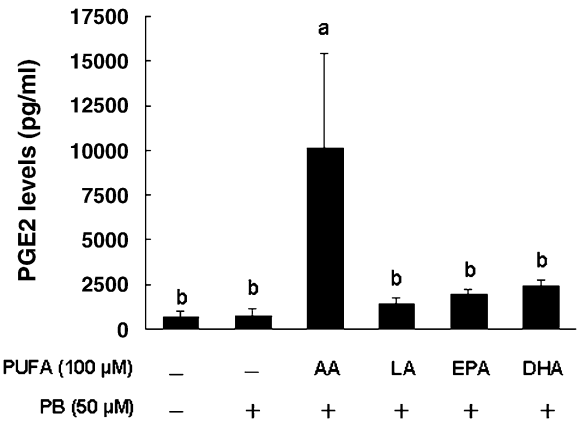

Fig. 2. Effect of n-6 and n-3 PUFAs on prostaglandin E_2 (PGE_2) production. Hepatocytes were treated with 100 μM AA, LA, EPA or DHA for 4 h and were then incubated with PB for another 20 h. Fifty-microliter aliquots of the media were removed, and PGE_2 concentrations were measured by ELISA. Values are means±SD ($n=3$). Bars with different letters are significantly different ($P<.05$).

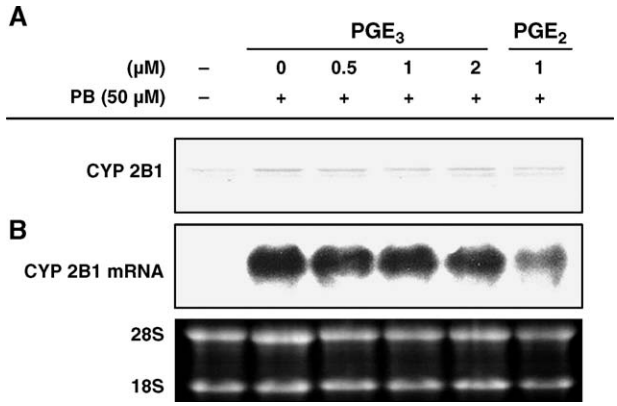

Fig. 3. Effect of various concentrations of PGE_3 on CYP 2B1 expression in the presence of PB. Forty hours after attachment, hepatocytes were incubated with PGE_3 or PGE_2 for 15 min before the addition of PB; the cells were then incubated with PB for another 20 h. (A) Western blot analysis. (B) Northern 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.

3.3. Effect of n-6 and n-3 PUFAs on PGE2 production

As shown in Fig. 2, the PGE_2 concentration was significantly higher in the AA-treated group than in the other groups ($P<.05$). The PGE_2 concentration did not differ significantly among the groups treated with PB alone, LA, EPA or DHA.

3.4. Effect of PGE3 on CYP 2B1 expression

To investigate whether the regulatory effect of n-3 PUFAs on CYP 2B1 expression in the presence of PB is mediated by PGE_3, we assayed the effect of 0, 0.5, 1 and

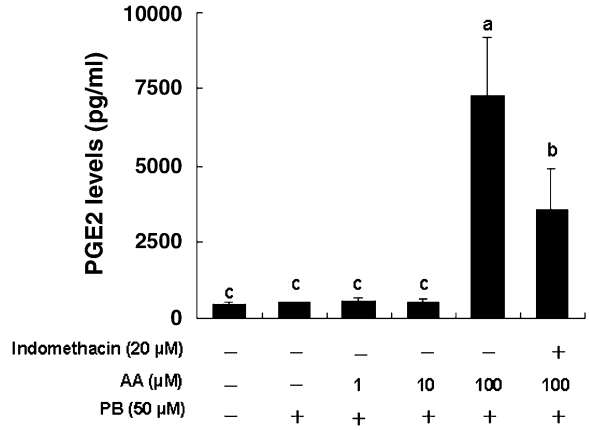

Fig. 4. Effect of AA dose on prostaglandin E_2 (PGE_2) production in the presence or absence of indomethacin. Forty hours after attachment, hepatocytes were pretreated with or without 20 μM indomethacin for 1 h and were then treated with 100 μM AA for 4 h. Hepatocytes in the 0-, 1- and 10-μM AA groups were not pretreated with 20 μM indomethacin. Cells were further incubated with PB for another 20 h, and 50-μL aliquots of the medium were removed. Prostaglandin E_2 was measured by ELISA. Values are means±SD ($n=3$). Bars with different letters are significantly different ($P<.05$).

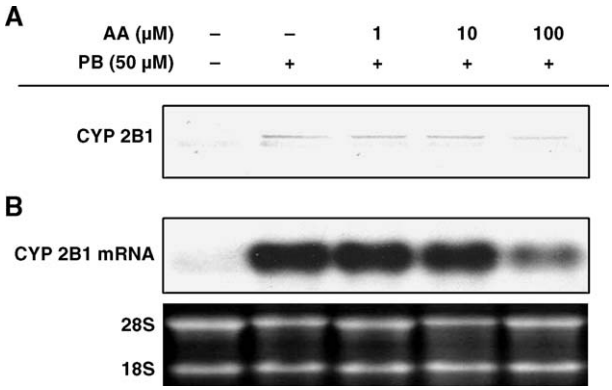

Fig. 5. Effect of AA dose on CYP 2B1 expression in the presence of PB. Forty hours after attachment, hepatocytes were pretreated with 0, 1, 10 and 100 μM AA for 4 h before the addition of PB. After PB addition, the cells were incubated for another 20 h. (A) Western blot analysis. (B) Northern blot analysis. 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.

2 μM PGE_3 on CYP 2B1 expression. As shown in Fig. 3, CYP 2B1 mRNA expression was not down-regulated by PGE_3, but was down-regulated by 1 μM PGE_2.

3.5. Effect of AA dose on PGE2 production in the presence or absence of indomethacin

To investigate whether there was a dose-response relation in the effect of AA on PGE_2, we studied the effect of 0, 1, 10 and 100 μM AA on the PGE_2 level in supernatant fluid from hepatocyte culture medium. As shown in Fig. 4, the PGE_2 level was significantly higher in the group treated with 100 μM AA than in the groups not treated with AA. This PGE_2-increasing effect was not seen with 1 or 10 μM AA. To confirm whether the PGE_2 production was decreased by COX inhibitor in our system, we added 20 μM indomethacin. Indomethacin significantly decreased PGE_2 production in the group treated with 100 μM AA in the presence of PB (Fig. 4).

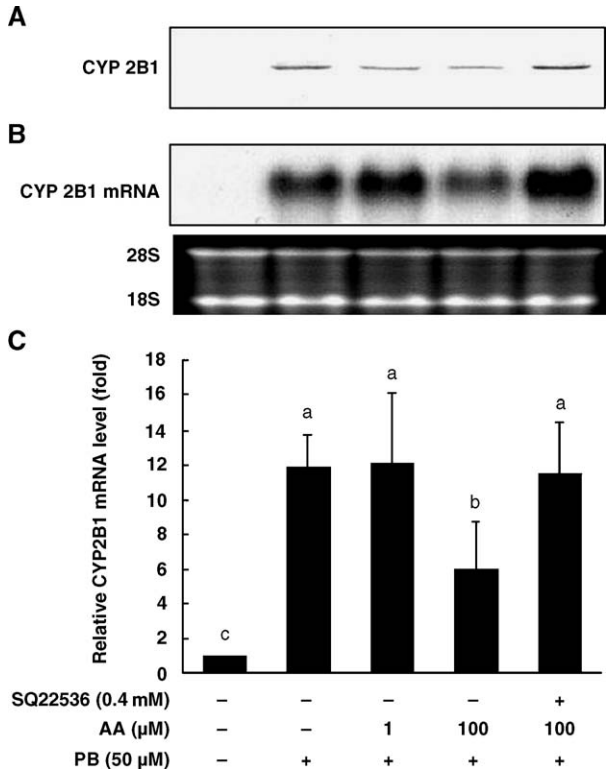

Fig. 6. Effect of SQ22536 (an adenylate cyclase inhibitor) on the down-regulation of CYP 2B1 expression by AA in the presence of PB. Forty hours after attachment, hepatocytes were pretreated with or without 0.4 mM SQ22536 for 1 h and were then treated with 100 μM AA. Hepatocytes in the 1-μM AA group were not pretreated with SQ22536. After 4 h, PB was added, and 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 four independent experiments is shown. (C) CYP2B1 mRNA levels were normalized to 18S rRNA and are expressed relative to the CYP2B1 mRNA level in untreated cells. Values are means±SD ($n=4$). Bars with different letters are significantly different ($P<.05$).

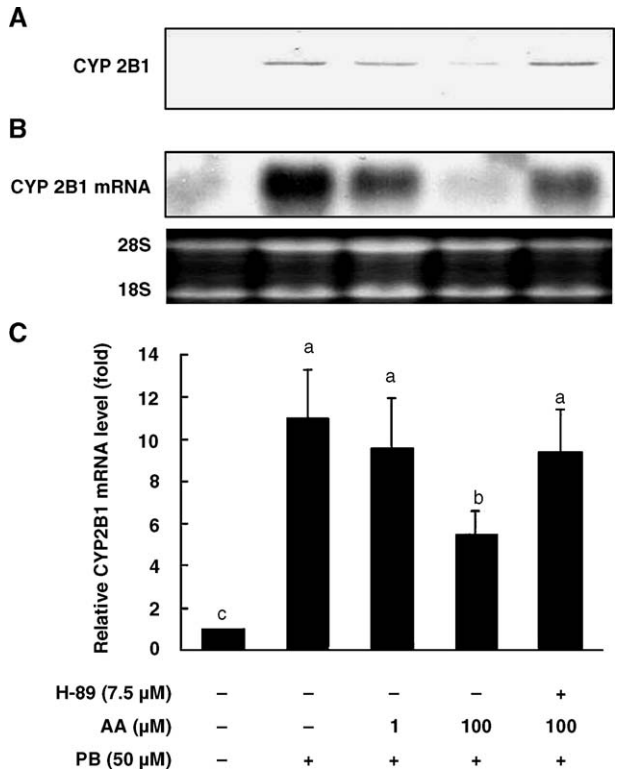

Fig. 7. Effect of H-89 (a PKA inhibitor) on the down-regulation of CYP 2B1 expression by AA in the presence of PB. Forty hours after attachment, hepatocytes were pretreated with or without 7.5 μM H-89 for 1 h and were then treated with 100 μM AA. Hepatocytes in the 1-μM AA group were not pretreated with H-89. After 4 h, PB was added, and 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 four independent experiments is shown. (C) CYP2B1 mRNA levels were normalized to 18S rRNA and are expressed relative to the CYP2B1 mRNA level in untreated cells. Values are means±SD ($n=4$). Bars with different letters are significantly different ($P<.05$).

3.6. Effect of AA dose on CYP 2B1 expression

To study whether the down-regulation of CYP 2B1 expression by AA was consistent with AA-mediated PGE_2 production, we used 0, 1, 10 and 100 μM AA. CYP 2B1 protein and mRNA expression were down-regulated by AA at a concentration of 100 μM; such a regulatory effect was not seen with 0, 1 or 10 μM AA (Fig. 5).

3.7. Effect of adenylate cyclase inhibitor on AA down-regulation of CYP 2B1 expression

As shown in Fig. 6A, 0.4 mM of the adenylate cyclase inhibitor SQ22536 attenuated the down-regulation of CYP 2B1 protein expression by 100 μM AA. A similar pattern was found for mRNA expression (Fig. 6B).

3.8. Effect of PKA inhibitor on AA down-regulation of CYP 2B1 expression

As shown in Fig. 7, 7.5 μM of the PKA inhibitor H-89 attenuated the down-regulation of CYP 2B1 gene expression by 100 μM AA in the presence of PB.

4. Discussion

The n-6 and n-3 PUFAs are known to have multiple effects on cellular metabolism and gene expression [3,33]. Their effects on gene expression may be due to either the direct action of the n-6 and n-3 PUFAs themselves or to the action of some of their metabolites [34]. Previous studies have shown that CYP gene expression is modulated by dietary lipids [20,22]. The objective of this study was to investigate the effects of n-6 and n-3 PUFAs on CYP 2B1 gene expression and possible mechanisms involved. In the present study, we showed that 100 μM of n-6 and n-3 PUFAs can down-regulate the expression of CYP 2B1 induced by PB in primary rat hepatocytes, especially in the AA- and DHA-treated groups, and this effect is significantly attenuated by the COX inhibitor indomethacin (Fig. 1). These results suggest that metabolites of the COX pathway play an important role in the down-regulation of CYP 2B1 expression by n-6 and n-3 PUFAs in the presence of PB in primary rat hepatocytes.

The incorporation of PUFAs into T cells was demonstrated by Denys et al. [35]. The concentration of PUFAs used was 20 μM, and it was lower than that used in our system. They showed that PUFAs are incorporated into phospholipids. Their results supported the incorporation of PUFAs into primary rat hepatocytes in our system.

Cyclooxygenase, which is also known as PG synthase, is the key enzyme for PG synthesis. Prostaglandin E_2, one of the end products of the COX metabolic pathway, can down-regulate PB-induced CYP 2B1 expression in primary rat hepatocytes [36], and this down-regulation is through the EP_2 receptor [23]. In the present study, PGE_2 levels were significantly greater in the AA-treated group than in the other groups (Fig. 2). It has been demonstrated that the phospholipase A_2 (PLA_2), which catalyzes the hydrolysis of the *sn*-2 fatty acyl bond of membrane phospholipids to liberate free fatty acids such as AA, was activated by n-6 and n-3 PUFAs [37]. In addition to PLA_2, COX is required for PG synthesis. Cyclooxygenase 1 has been demonstrated to be present in our culture system [23]. Dietary n-3 PUFAs effectively reduced PGE_2 release compared with n-6 PUFA in bone cells [38] and brain-metastatic melanoma [39]. These observations are consistent with our results. In the present study, PGE_2 levels were not significantly different among EPA- or DHA-treated and untreated hepatocytes, and this can be due to the 43% PGE_3 specificity of PGE_2 EIA kit. In addition to eicosanoid precursors, PB was reported to increase PGE_2 production in rat liver [40]; however, this effect was not found in the present study (Figs. 2 and 4). Our results are consistent with those of Lee and Edwards [41] who showed that PB did not increase PGE_2 formation in hepatocytes. The metabolism of AA by COX gives rise to the two-series eicosanoids (PGs) and thromboxanes; however, n-3 PUFAs act as substrates for COX to produce three-series PGs and thromboxanes. Our data showed that PGE_2 was not significantly increased in the EPA- and DHA-treated groups (Fig. 2), but that PB-induced CYP 2B1 expression was down-regulated by both n-3 PUFAs (Fig. 1). This suggests that a PG-independent pathway may be involved in the down-regulation of CYP 2B1 expression by n-3 PUFAs.

The intracellular cAMP level is rapidly increased by PGE_3 treatment in human pulmonary microvascular endothelial cells [42], and a previous study showed that increased intracellular cAMP levels suppress the induction of CYP 2B1 by PB in primary rat hepatocytes [43]. We found that PGE_3, in contrast with PGE_2, could not down-regulate CYP 2B1 expression induced by PB, even at higher concentrations than found to be effective for PGE_2 (Fig. 3). To date, many studies have reported that the biological activity of PGE_3 is much less than that of PGE_2 [16,44]. This suggests that PGE_3 is not involved in the down-regulation of CYP 2B1 expression by n-3 PUFAs.

Desaturation and elongation reactions are required for the conversion of the essential fatty acids LA and α-linolenic acid to AA and DHA. At least two fatty acid Ä6 desaturase [45] and elongase isozymes exist in primary rat hepatocytes [46]. In the present study, the down-regulation of CYP 2B1 expression by LA was not as potent as that by AA (Fig. 1). This result suggests that LA may need time to be converted to AA, unlike AA, which can be directly metabolized to PGE_2 in the culture system. Although n-3 PUFAs are not metabolized to PGE_2, indomethacin can still block PGE_2 synthesis from endogenous precursors in the n-3 PUFA-treated groups. This partially explains why indomethacin attenuated the down-regulation in the n-3 PUFA groups.

Two COX isoforms, COX-1 and COX-2, have been identified and characterized [47]. A previous study showed both basal COX-2 and basal COX-1 mRNA in primary rat hepatocytes [48]. However, only the constitutive COX-1 mRNA and not the inducible COX-2 mRNA was detected in

primary rat hepatocytes in our previous study [23]. In the present study, the COX inhibitor indomethacin was found to attenuate the down-regulation of CYP 2B1 gene expression by n-6 and n-3 PUFAs (Fig. 1) and to significantly decrease the PGE_2 level in the 100-μM AA-treated group in the presence of PB (Fig. 4). These results suggest that the intrinsic expression of functionally active COX-1 is required for AA's down-regulation of CYP 2B1 expression induced by PB in primary rat hepatocytes.

In several studies, PGE_2 concentrations in the nanomolar range were reported to be required for its physiologic action [49]; however, concentrations in the micromolar range were required to stimulate hepatocyte DNA synthesis [41]. In our previous study, 0.01–1 μM PGE_2 was found to down-regulate CYP 2B1 expression induced by PB in primary rat hepatocytes [23], and the most potent effect was at 1 μM. In the present study, addition of 100 μM AA, which yielded significantly greater PGE_2 than did 0, 1 or 10 μM AA (Fig. 4), had a down-regulatory effect on CYP 2B1 expression induced by PB that was similar to the effect of PGE_2. This result suggests that AA metabolite PGE_2 is involved in the down-regulation of PB-induced CYP 2B1 expression by AA.

Prostaglandin E_2-mediated cAMP production is involved in the regulation of various cellular physiologic responses [50] and signal transduction pathways [51]. Sidhu and Omiecinski [43] showed that activation of a cAMP-dependent PKA pathway results in suppression of PB-inducible CYP gene expression. In our previous study, PGE_2 down-regulated CYP 2B1 expression induced by PB through a cAMP-dependent PKA pathway in primary rat hepatocytes [23]. To study whether AA-mediated PGE_2 production and the downstream cAMP-dependent PKA pathway play a critical role in AA's down-regulation of CYP 2B1 expression induced by PB, we used the adenylate cyclase inhibitor SQ22536 and the PKA inhibitor H-89. Both SQ22536 and H-89 attenuated the effect of AA on CYP 2B1 expression induced by PB (Figs. 6 and 7). These results suggest that the cAMP-dependent PKA pathway is involved in the down-regulation of CYP 2B1 expression by AA.

The induction of CYP 2B gene expression by PB is regulated by intracellular constitutive androstane receptor (CAR) translocation [52]. Constitutive androstane receptor has been shown to bind to the PB response element of CYP 2B gene promoter as a heterodimer with the retinoid X receptor (RXR) [53]. Phenobarbital not only triggers the translocation of CAR from the cytoplasm into the nucleus, but also stimulates CAR activity for the induction of CYP 2B gene expression, and these steps are dependent on phosphorylation/dephosphorylation status of cells [54]. Previous studies showed that the expression of CAR and RXR is under the regulation of glucocorticoid receptor (GR) [55,56]. In GR knockout mice, the expression of CYP 2B was significantly inhibited [57]. Expression of several CYP gene families including CYP 2B was down-regulated by tubulin network-disrupting agent colchicines. Its action was on the GR-[CAR/RXR] signal transduction cascade [58]. Decreased GR activity by PUFAs, especially AA and DHA, was found in a previous study [59]. It is possible that GR/CAR/RXR signal transduction cascade was involved in the down-regulation of CYP 2B1 expression by n-6 and n-3 PUFAs.

Future works are warranted to clarify the relationship between PKA and CAR translocation and the GR/CAR/RXR signal transduction cascade in the down-regulation of CYP 2B1 expression by n-3 PUFAs.

In summary, the results of the present study indicate that PB-induced CYP 2B1 expression in rat hepatocytes is down-regulated by both n-6 and n-3 PUFAs, especially AA and DHA. Prostaglandin E_2 formation and the cAMP-dependent PKA pathway are at least part of the mechanisms for AA's down-regulation of CYP 2B1 gene expression induced by PB, whereas the down-regulation by n-3 PUFAs is not fully understood yet and the GR/CAR/RXR signal transduction cascade can be involved.

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