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 (3×10^6 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

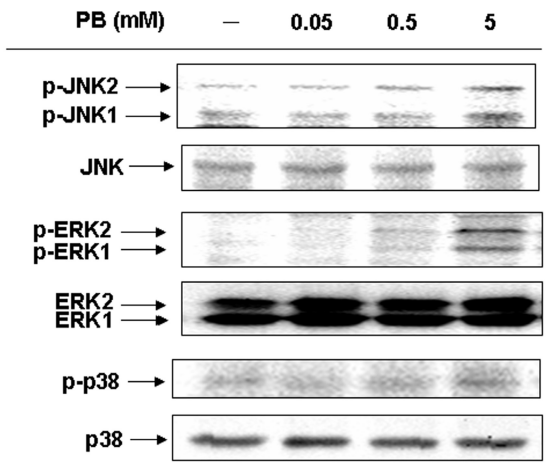

Figure 1. Effect of PB on the early activation of MAPKs in rat primary hepatocytes. Forty hours after attachment, hepatocytes were incubated with or without various concentrations of PB for 15 min, and aliquots of total hepatocyte extracts (30 μg) were used for Western blot analysis. One representative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

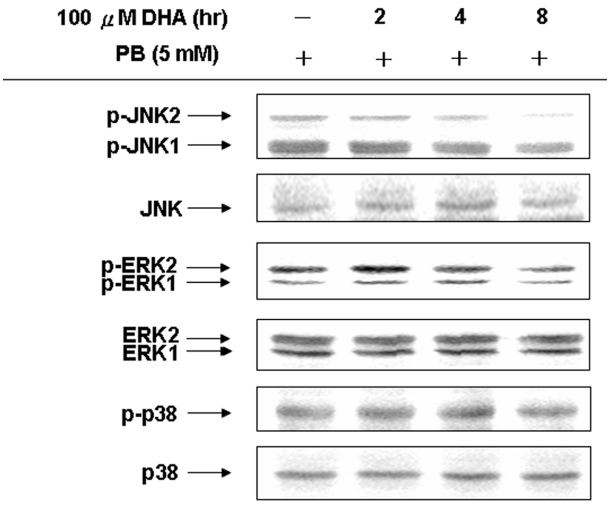

Figure 2. Effect of DHA on PB-induced MAPK activation. Forty hours after attachment, hepatocytes were pretreated with or without 100 μM DHA for 2, 4, and 8 h before the addition of PB. Fifteen minutes after PB addition, cells were harvested and aliquots of total hepatocyte extracts (30 μg) were used 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.

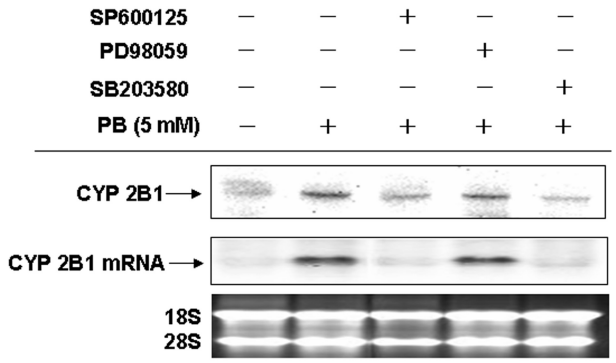

Figure 3. Effect of specific MAPK inhibitors on CYP 2B1 protein and mRNA expression. Forty hours after attachment, hepatocytes were pretreated with or without 20 μM specific MAPK inhibitors for 20 h before the addition of PB. After PB addition, the cells were incubated for another 20 h. (A) Western blot 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

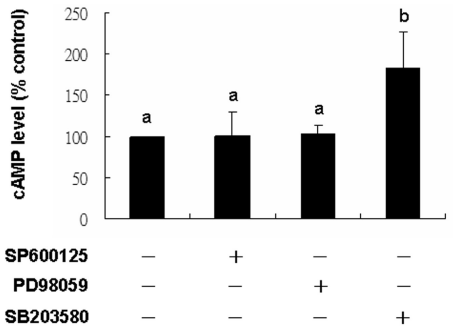

Figure 4. Effect of specific MAPK inhibitors on intracellular cAMP concentrations. Forty hours after attachment, hepatocytes were treated with or without 20 μM specific MAPK inhibitors for 20 h. The cells were then washed twice with cold PBS and were lysed and scraped into 0.8 mL ice-cold 70% ethanol. 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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The authors have declared no conflict of interest.

5 References

[1] Spector, A.-A., Yorek, M.-A., Membrane lipid composition and cellular function, *J. Lipid Res*. 1985, *26*, 1015 – 1035.

[2] Merendino, N., Loppi, B., D'Aquino, M., Molinari, R., *et al*., Docosahexaenoic acid induces apoptosis in the human PaCa-44 pancreatic cancer cell line by active reduced glutathione extrusion and lipid peroxidation, *Nutr. Cancer* 2005, *52*, 225 – 233.

[3] Schley, P.-D., Jijon, H.-B., Robinson, L.-E., Field, C.-J., Mechanisms of omega-3 fatty acid-induced growth inhibition in MDA-MB-231 human breast cancer cells, *Breast Cancer Res. Treat.* 2005, *92*, 187 – 195.

[4] Weldon, S.-M., Mullen, A.-C., Loscher, C.-E., Hurley, L. A., *et al*., Docosahexaenoic acid induces an anti-inflammatory profile in lipopolysaccharide-stimulated human THP-1 macrophages more effectively than eicosapentaenoic acid. *J. Nutr. Biochem.* 2007, *18*, 250 – 258.

[5] Singh, M., Essential fatty acids, DHA and human brain, *Indian J. Pediatr.* 2005, *72*, 239 – 242.

[6] von Schacky, C., N-3 PUFA in CVD: Influence of cytokine polymorphism, *Proc. Nutr. Soc.* 2007, *66*, 166 – 170.

[7] Mori, T. A., Beilin, L. J., Omega-3 fatty acids and inflammation, *Curr. Atheroscler. Rep.* 2004, *6*, 461 – 467.

[8] Simopoulos, A.-P., Omega-3 fatty acids in inflammation and autoimmune diseases, *J. Am. Coll. Nutr.* 2002, *21*, 495 – 505.

[9] Cole, G.-M., Lim, G.-P., Yang, F., Teter, B., *et al*., Prevention of Alzheimer's disease: Omega-3 fatty acid and phenolic anti-oxidant interventions, *Neurobiol. Aging* 2005, *26*, 133 – 136.

[10] Zhang, W., Long, Y., Zhang, J., Wang, C., Modulatory effects of EPA and DHA on proliferation and apoptosis of pancreatic cancer cells, *J. Huazhong Univ. Sci. Technol. Med. Sci*. 2007, *27*, 547 – 550.

[11] Danbara, N., Yuri, T., Tsujita-Kyutoku, M., Sato, M., *et al*., Conjugated docosahexaenoic acid is a potent inducer of cell cycle arrest and apoptosis and inhibits growth of colo 201 human colon cancer cells, *Nutr. Cancer* 2004, *50*, 71 – 79.

[12] Narayanan, N.-K., Narayanan, B.-A., Reddy, B. S., A combination of docosahexaenoic acid and celecoxib prevents prostate cancer cell growth *in vitro* and is associated with modulation of nuclear factor-kappaB, and steroid hormone receptors, *Int. J. Oncol.* 2005, *26*, 785 – 792.

[13] Bousserouel, S., Brouillet, A., Béréziat, G., Raymondjean, M., *et al*., Different effects of n-6 and n-3 polyunsaturated fatty acids on the activation of rat smooth muscle cells by interleukin-1 beta, *J. Lipid Res.* 2003, *44*, 601 – 611.

[14] Xu, C., Li, C.-Y., Kong, A.-N., Induction of phase I, II and III drug metabolism/transport by xenobiotics, *Arch. Pharm. Res.* 2005, *28*, 249 – 268.

[15] Li, C.-C., Lii, C.-K., Liu, K.-L., Yang, J.-J., *et al*., n-6 and n-3 polyunsaturated fatty acids down-regulate cytochrome P-450 2B1 gene expression induced by phenobarbital in primary rat hepatocytes, *J. Nutr. Biochem*. 2006, *17*, 707 – 715.

[16] Wang, Y., Wang, S., Liu, Y., Yan, L., *et al*., Characterization of metabolites and cytochrome P450 isoforms involved in the microsomal metabolism of aconitine, *J. Chromatogr. B. Anal. Technol. Biomed. Life Sci.* 2006, *844*, 292 – 300.

[17] Tsai, C.-F., Lii, C.-K., Yang, J.-J., Liu, K.-L., *et al*., Prostaglandin E2 is involved in the increase of cytochrome P-450 2B1 expression by alpha-tocopheryl succinate in primary rat hepatocytes in the presence of phenobarbital, *Nutr. Cancer*. 2001, *41*, 188 – 195.

[18] Naor, Z., Benard, O., Seger, R., Activation of MAPK cascades by G-protein-coupled receptors: The case of gonadotropin-releasing hormone receptor, *Trends Endocrinol. Metab*. 2000, *11*, 91 – 99.

[19] Liu, P.-F., Chang, W.-C., Wang, Y.-K., Munisamy, S.-B., *et al*., Differential regulation of Ku gene expression in etiolated mung bean hypocotyls by auxins, *Biochim. Biophys. Acta* 2007, *1769*, 443 – 454.

[20] Hu, P., Kinyamu, K.-H., Wang, L., Martin, J., *et al*., Estrogen induces ERRalpha gene expression and chromatin structural, *J. Biol. Chem*. 2008, *283*, 6752 – 6763.

[21] Ramer, R., Hinz, B., Inhibition of cancer cell invasion by cannabinoids via increased expression of tissue inhibitor of matrix metalloproteinases-1, *J. Natl. Cancer Inst.* 2008, *100*, 59 – 69.

[22] Chang, L., Karin, M., Mammalian MAP kinase signalling cascades, *Nature* 2001, *410*, 37 – 40.

[23] Cowan, K.-J., Storey, K.-B., Mitogen-activated protein kinases: New signaling pathways functioning in cellular responses to environmental stress, *J. Exp. Biol*. 2003, *206*, 1107 – 1115.

[24] Joannard, F., Rissel, M., Gilot, D., Anderson, A., *et al*., Role for mitogen-activated protein kinases in phenobarbital-induced expression of cytochrome P450 2B in primary cultures of rat hepatocytes, *Toxicol. Lett*. 2006, *161*, 61 – 72.

[25] Seglen, P.-O., Preparation of isolated rat liver cells, *Methods Cell Biol.* 1976, *13*, 29 – 83.

[26] Yang, J.-J., Krauss, R.-S., Extracellular ATP induces anchorage-independent expression of cyclin A and rescues the transformed phenotype of a ras-resistant mutant cell line, *J. Biol. Chem.* 1997, *272*, 3103 – 3108.

[27] Laemmli, U.-K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* 1970, *227*, 680 – 685.

[28] Beck, N.-B., Omiecinski, C.-J., Lack of modulation by phenobarbital of cyclic AMP levels or protein kinase A activity in rat primary hepatocytes, *Biochem. Pharmacol.* 1999, *58*, 1109 – 1114.

[29] Li, C.-C., Lii, C.-K., Liu, K.-L., Yang, J.-J., *et al*., DHA down-regulates phenobarbital-induced cytochrome P450 2B1 gene expression in rat primary hepatocytes by attenuating CAR translocation, *Toxicol. Appl. Pharmacol.* 2007, *225*, 329 – 336.

[30] Denys, A., Hichami, A., Khan, N.-A., Eicosapentaenoic acid and docosahexaenoic acid modulate MAP kinase (ERK1/ERK2) signaling in human T cells, *J. Lipid Res*. 2001, *42*, 2015 – 2020.

[31] Xue, H., Wan, M., Song, D., Li, Y., *et al*., Eicosapentaenoic acid and docosahexaenoic acid modulate mitogen-activated protein kinase activity in endothelium, *Vascul. Pharmacol*. 2006, *44*, 434 – 439.

[32] Sidhu, J.-S., Omiecinski, C.-J., cAMP-associated inhibition of phenobarbital-inducible cytochrome P450 gene expression in primary rat hepatocyte cultures, *J. Biol. Chem.* 1995, *270*, 12762 – 12773.

[33] Yu, R., Lei, W., Mandlekar, S., Weber, M.-J., *et al*., Role of a mitogen-activated protein kinase pathway in the induction of phase II detoxifying enzymes by chemicals, *J. Biol. Chem*. 1999, *274*, 27545 – 27552.

[34] Yu, R., Mandlekar, S., Lei, W., Fahl, W. E., *et al*., p38 mitogen-activated protein kinase negatively regulates the induction of phase II drug-metabolizing enzymes that detoxify carcinogens, *J. Biol. Chem*. 2000, *275*, 2322 – 2327.

[35] Hodges, N.-J., Orton, T.-C., Strain, A.-J., Chipman, J.-K., Potentiation of epidermal growth factor-induced DNA synthesis in rat hepatocytes by phenobarbitone: possible involvement of oxidative stress and kinase activation, *Carcinogenesis* 2000, *21*, 2041 – 2047.

[36] Lee, J.-Y., Plakidas, A., Lee, W.-H., Heikkinen, A., *et al*., Differential modulation of Toll-like receptors by fatty acids: preferential inhibition by n-3 polyunsaturated fatty acids, *J. Lipid Res.* 2003, *44*, 479 – 486.

[37] Donadio, J.-V., Omega-3 polyunsaturated fatty acids: a potential new treatment of immune renal disease, *Mayo Clin. Proc.* 1991, *66*, 1018 – 1028.

[38] Moon, Y., Pestka, J.-J., Deoxynivalenol-induced mitogen-activated protein kinase phosphorylation and IL-6 expression in mice suppressed by fish oil, *J. Nutr. Biochem.* 2003, *14*, 717 – 726.

[39] Machida, T., Hiramatsu, M., Hamaue, N., Minami, M., *et al*., Docosahexaenoic acid enhances cyclooxygenase-2 induction by facilitating p44/42, but not p38, mitogen-activated protein kinase activation in rat vascular smooth muscle cells, *J. Pharmacol. Sci.* 2005, *99*, 113 – 116.

[40] Shanley, T.-P., Vasi, N., Denenberg, A., Wong, H.-R., The serine/threonine phosphatase, PP2A: Endogenous regulator of inflammatory cell signaling, *J. Immunol.* 2001, *166*, 966 – 972.

[41] Kins, S., Kurosinski, P., Nitsch, R.-M., Götz, J., Activation of the ERK and JNK signaling pathways caused by neuron-specific inhibition of PP2A in transgenic mice, *Am. J. Pathol.* 2003, *163*, 833 – 843.

[42] Siddiqui, R.-A., Jenski, L.-J., Harvey, K.-A., Wiesehan, J.-D., *et al*., Cell-cycle arrest in Jurkat leukaemic cells: A possible role for docosahexaenoic acid, *Biochem. J.* 2003, *371*, 621 – 629.

[43] Chalfant, C.-E., Kishikawa, K., Mumby, M.-C., Kamibayashi, C., *et al*., Long chain ceramides activate protein phosphatase-1 and protein phosphatase-2A. Activation is stereospecific and regulated by phosphatidic acid, *J. Biol. Chem.* 1999, *274*, 20313 – 20317.

[44] Pohl, J., Ring, A., Ehehalt, R., Schulze-Bergkamen, H., *et al*., Long chain fatty acid uptake into adipocytes depends on lipid raft function, *Biochemistry* 2004, *43*, 4179 – 4187.

[45] Nourissat, P., Travert, M., Chevanne, M., Tekpil, X., *et al*., Ethanol induces oxidative stress in primary rat hepatocytes through the early involvement of lipid raft clustering, *Hepatology* 2008, *47*, 59 – 70.

[46] Wu, M., Harvey, K.-A., Ruzmetov, N., Welch, Z.-R., *et al*., Omega-3 polyunsaturated fatty acids attenuate breast cancer growth through activation of a neutral sphingomyelinase-mediated pathway, *Int. J. Cancer* 2005, *117*, 340 – 348.

[47] Verlengia, R., Gorjao, R., Kanunfre, C.-C., Bordin, S., *et al*., Comparative effects of eicosapentaenoic acid and docosahexaenoic acid on proliferation, cytokine production, and pleiotropic gene expression in Jurkat cells, *J. Nutr. Biochem*. 2004, *15*, 657 – 665.

[48] Jumpsen, J.-A., Brown, N.-E., Thomson, A.-B.-R., Paul Man, S.-F., *et al*., Fatty acids in blood and intestine following docosahexaenoic acid supplementation in adults with cystic fibrosis, *J. Cyst. Fibros*. 2006, *5*, 77 – 84.

[49] Ho, A.-K., Price, L., Mackova, M., Chik, C.-L., Potentiation of cyclic AMP and cyclic GMP accumulation by p38 mitogen-activated protein kinase (p38MAPK) inhibitors in rat pinealocytes, *Biochem. Pharmacol.* 2001, *62*, 1605 – 1611.

