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Docosahexaenoic acid down-regulates phenobarbital-induced cytochrome P450 2B1 gene expression in rat primary hepatocytes via the sphingomyelinase/ceramide pathway☆

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Abstract

Docosahexaenoic acid (DHA) regulates the expression of cytochrome P450 2B1 (CYP 2B1) in rat primary hepatocytes in response to xenobiotics. Ceramide, a lipid signaling molecule, is involved in various physiological processes and can be generated by the hydrolysis of sphingomyelin via sphingomyelinase (SMase). DHA activates SMase and increases ceramide formation *in vitro*. Ceramides differentially enhance adenylyl cyclase activity *in vitro* depending on the chain length of their fatty acids. In addition, the cAMP-dependent PKA pathway down-regulates CYP 2B1 expression induced by phenobarbital (PB). In the present study, we determined the effect of DHA on SMase transactivation and the downstream pathway in CYP 2B1 expression induced by PB. SMase was activated by DHA 2 h after treatment, and D609 (an SMase inhibitor) attenuated the inhibition of PB-induced CYP 2B1 expression by DHA. Ceramide formation reached a maximum 3 h after DHA administration. C2-ceramide dose-dependently inhibited PB-induced CYP 2B1 expression and increased intracellular cAMP concentrations. SQ22536 (an adenylyl cyclase inhibitor) and H89 (a PKA-specific inhibitor) partially reversed the inhibition of PB-induced CYP 2B1 expression by C2-ceramide. These results suggest that stimulation of SMase, generation of ceramide and activation of the cAMP-dependent PKA pathway are involved in the inhibition exerted by DHA.

Keywords: Ceramide; CYP 2B1; DHA; Phenobarbital; Sphingomyelinase

1. Introduction

Ceramide, a lipid second messenger, is generated from sphingomyelin by the action of sphingomyelinase (SMase) or by *de novo* synthesis through ceramide synthase [1]. Sphingomyelin belongs to a class of sphingolipids that are ubiquitous in eukaryotic cells; sphingolipids are especially plentiful in plasma membranes and related cell membranes, such as Golgi apparatus and lysosomes [2]. Ceramide is involved in diverse physiological processes, including cell growth, differentiation and programmed cell death [3].

Recently, ceramide or ceramide analogues were shown to be antiproliferative and pro-apoptotic against various cancer cell lines, including the androgen receptor-negative prostate cancer cell line PC3 [4], breast cancer cell lines [5], OVCAR-3 ovarian carcinoma cells and HCT116 human colon carcinoma cells [6], and CNE2 nasopharyngeal carcinoma cells [7]. Sphingomyelin, the precursor of ceramide, inhibits 1,2-dimethylhydrazine-induced colon cancer in CF1 mice [8]. SMase activity is significantly decreased in human colorectal carcinomas [9]. Because of the involvement of sphingolipid breakdown products in anti-proliferative pathways of cells, products such as ceramide are recognized as tumor-suppressor lipids [10].

Docosahexaenoic acid (DHA), a unique polyunsaturated fatty acid abundant in fish oils, plays a pivotal role in anti-atherosclerosis [11], anti-inflammation [12,13] and anti-aging [14]. In addition, DHA inhibits the growth of certain tumor cells, including human breast cancer cells [15], pancreatic cancer cells [16] and human colon adenocarcinoma cells [17]. A variety of mechanisms have been proposed for the antitumor activity of DHA. DHA was reported to increase ceramide formation in Jurkat leukemic cells [18]. As mentioned already, an increase in ceramide exerts antiproliferative and pro-apoptotic effects. Thus, it is possible that the role of DHA in signal transduction is through ceramide production.

Drug metabolizing enzymes (DMEs) are induced by xenobiotics and drugs. DMEs are composed of Phase I enzymes, Phase II metabolizing enzymes and Phase III transporters, which are either abundant at the basal unstimulated level or elevated after exposure

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to xenobiotics [19]. Cytochrome P450 2B1 (CYP 2B1) is a Phase I enzyme induced by phenobarbital (PB)-type inducers [20,21]. Cyclophosphamide (CPA) is a chemotherapeutic agent frequently used in the treatment of human malignancies including pancreatic cancer [22] and head and neck cancer [23]. The oxazaphosphorine anticancer prodrug CPA is mainly activated by a liver P450 2B-catalyzed 4-hydroxylation that yields active and cytotoxic metabolites [24]. Dietary nutrients such as vitamin E and DHA have been shown to influence PB-induced CYP 2B1 gene expression in rat primary hepatocytes [21,25]. The underlying mechanisms of this influence are changes in prostaglandin E_2 (PGE_2) production and alteration of transcription factor translocation, respectively. The physiologic effect of PGE_2 is mediated through prostaglandin E receptors, which belong to the family of G-protein-coupled receptors [26,27]. Binding of PGE_2 to prostaglandin E receptor 2 causes increased intracellular cAMP formation [28], which leads to PKA activation. cAMP-associated inhibition of PB-induced CYP 2B1 expression was reported by Sidhu and Omiecinski [20]. The constitutive androstane receptor (CAR), a transcription factor, plays an important role in the modulation of the gene expression of DMEs, including CYP 2B, 2C and 3A [29]. Upon activation by PB-type inducers, CAR translocates from the cytosol to the nucleus and forms a heterodimer with retinoid X receptor. Subsequently, the heterodimer binds to NR-1 and transactivates the target genes [30,31]. DHA was shown to down-regulate PB-induced CYP 2B1 gene expression in rat primary hepatocytes by attenuating CAR translocation [21]. Because of its effect on intracellular ceramide formation, DHA may regulate PB-induced CYP 2B1 expression through a novel pathway besides CAR.

In the present study, we used rat primary hepatocytes to study the effect of DHA on SMase transactivation and the link between the SMase product ceramide 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, USA); Matrigel and ITS^+ (insulin, transferrin, selenium, bovine serum albumin and linoleic acid) were from Collaborative Biomedical Products (Bedford, MA, USA); collagenase type I was from Worthington Biochemical (Lakewood, NJ, USA); TRIzol reagent was from Invitrogen (Carlsbad, CA, USA); dexamethasone, *N*-[2-hydroxythyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES), sodium bicarbonate, butylated hydroxytoluene, α-tocopheryl succinate, calcium chloride, *N*-acetyl-D-sphingosine (C2-ceramide), 9-(Tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22536), *N*-(2-[*p*-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide dihydrochloride (H89), *O*-Tricyclo[5.2.1.$0^{2,6}$]dec-9-yl dithiocarbonate potassium salt (D609), magnesium chloride, phenobarbital, albumin and bovine serum essentially fatty acid free were from Sigma (St. Louis, MO, USA); DHA and cyclic AMP EIA kit were from Cayman Chemical (Ann Arbor, MI, USA); and porcine brain ceramides were from Avanti Polar Lipids (Albaster, AL, USA).

2.2. Hepatocyte isolation and culture

Male Sprague-Dawley rats (weighing 250–300 g) were purchased from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan). Hepatocytes were isolated by a modification of the two-step collagenase perfusion method described previously [32]. 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 phosphate-buffered saline (PBS) to remove any unattached or dead cells, and the same medium was 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. Lactate dehydrogenase leakage assay

The effect of C2-ceramide on cell damage was evaluated by use of the lactate dehydrogenase (LDH) leakage assay. According to the method of Moldéus et al. [33], 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 0.01 mM potassium phosphate buffer, removed with a cell scraper and then centrifuged at 7000×*g* for 10 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 (118.07 mM NaCl, 4.83 mM KCl, 0.96 mM KH_2PO_4, 2.45 mM $MgSO_4\cdot7H_2O$, 2.19 mM $CaCl_2\cdot7H_2O$ and 23.81 mM $NaHCO_3$, pH 7.4). The rate of change in absorbance at 340 nm due to NADH oxidation was recorded.

2.4. Fatty acid preparation

Docosahexaenoic acid 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, 0.1% butylated hydroxytoluene and 20 μM α-tocopheryl succinate were added to the culture medium to prevent lipid peroxidation.

2.5. Northern blotting for CYP 2B1

RNA was extracted from rat primary hepatocytes with 0.5 ml TRIzol reagent. 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 [34]. 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 (750 mM NaCl, 50 mM NaH_2PO_4, 5 mM EDTA), 2% sodium dodecyl sulfate (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, USA) at −80°C with an intensifying screen.

2.6. Western blotting for CYP 2B1

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 9000×*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). SDS polyacrylamide gels made with 7.5% polyacrylamide were prepared as described by Laemmli [35]. 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.

2.7. cDNA Preparation and RT-PCR analysis

Total RNA was extracted as for Northern blotting. 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 CYP 2B1 primer design were carried out as described previously [36]. 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 CYP 2B1 primer was added to a total volume of 50 μl. The sequences of the RT-PCR CYP 2B1 primers were as follows: 5′-GCTCAAGTACCCC-CATGTCG-3′ (forward) and 5′-ATCAGTGTATGGCATTTTACTGCGG-3′ (reverse), which amplify a 109-bp fragment. 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 CYP 2B1 and GAPDH PCR amplification, the samples were heated to 95°C for 5 min and then immediately cycled 23 times through a 1-min denaturing step at 95°C, a 1-min annealing step at 54°C and a 1-min elongation step at

72°C. The amplified PCR products of CYP 2B1 and GAPDH mRNA were separated on 2.0% agarose gels alongside markers.

2.8. Sphingomyelinase activity analysis

Forty hours after attachment, hepatocytes were treated with or without 100 μM DHA for various time periods. After treatment, cells were harvested with 500 μl of 1× reaction buffer and sonicated, then centrifuged at 9000×*g* for 30 min at 4°C. The supernatant fraction was used for assay. Intracellular SMase activity was measured by using the Amplex Red Sphingomyelinase Assay Kit, A-12220, according to the manufacturer's instructions (Molecular Probes, UK). The fluorescence was determined by Series BioAssay Reader (PerkinElmer Instrument).

2.9. Measurement of intracellular cAMP concentrations

Forty hours after attachment, hepatocytes were treated with various concentrations of C2-ceramide for 24 h. Cell extracts were prepared as described by Beck and Omiecinski [37]. Intracellular cAMP concentrations were measured by using the cAMP EIA kit according to the manufacturer's instructions (Cayman Chemical).

2.10. Determination of cellular ceramides

Rat primary hepatocytes (1×10^7) were seeded into 10-cm dishes (Falcon, BD Falcon, San Jose, CA, USA) and grown in RPMI-1640 for 40 h before treatment with 100 μM DHA for 3, 6 and 12 h. After treatment, cells were harvested into 2 ml of ice-cold buffered saline solution (135 mM NaCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, 10 mM HEPES, pH 7.2) and centrifuged at 400×*g* for 5 min at 4°C. The cell pellet was resuspended in 350 μl of buffered saline solution and sonicated twice for 10 s. Unbroken cells were pelleted by centrifugation at 400×*g* for 5 min at 4°C and discarded [38]. The protein concentration of the supernatant was determined by the method of Lowry et al. [39].

Aliquots of protein (0.4 mg) were used to extract lipids according to the method described by Bligh and Dyer [40] with 1 ml of 100:100:1 (vol/vol/vol) $CHCl_3$/MeOH/HCl at 4°C for 20 min. Additionally, 8 μl of 25 mM C2-ceramide was added to the protein as an internal standard. The organic phase was dried under nitrogen. Dried lipids were digested with 500 μl of 100 mM KOH (dissolved in methanol) at 37°C for 1 h, followed by 500 μl of chloroform, 270 μl of buffered saline solution and 30 μl of 100 mM EDTA at 25°C for 20 min to extract lipids. Extracted lipids were dried under nitrogen and resuspended in 1 ml of 100:100:1 (vol/vol/vol) $CHCl_3$/MeOH/HCl. Lipids were resolved on a Silica-60 TLC plate (Merck, Darmstadt, Germany) with 100:5:2 (vol/vol/vol) CH_3Cl/MeOH/CH_3COOH as a mobile phase by a modification of the method described previously [41]. Individual lipid classes were visualized by dipping each plate into $CuSO_4$ (3%)–H_3PO_4 (8%) for 30 s, drying the thin-layer chromatography (TLC) plate and charring the plate on a hot plate. Densitometric scanning of the bands was performed by a scanner (Paperfection 1250, EPSON), and bands were quantitatively evaluated by Image Reader LAS-1000 (Fuji Film, Tokyo, Japan) with Image Gauge software (v. 3.46, Fuji Film, Tokyo, Japan).

2.11. Statistical analysis

Data were analyzed by using analysis of variance (SAS Institute, Cary, NC, USA). The significance of the difference between mean values was determined by one-way analysis of variance followed by Tukey's test or by a two-tailed Student's *t* test; *P* values $<.05$ were taken to be statistically significant.

3. Results

3.1. Effect of DHA incubation time on SMase activity in rat primary hepatocytes

DHA has been reported to enhance neutral SMase activity in breast tumor tissues and MDA-MB-231 breast cancer cells [42]. In the present study, significant activation of SMase by 100 μM DHA was observed after a 2-h incubation ($P<.0001$); the activity then decreased to near basal levels at 3 h (Fig. 1).

3.2. Effect of D609 (an SMase inhibitor) on CYP 2B1 expression inhibited by DHA in the presence of PB

CYP 2B1 expression was significantly induced by 50 μM PB and was dose-dependently inhibited by up to 100 μM DHA. Pretreatment with D609 (10 μg/ml; an SMase inhibitor) significantly reversed the inhibition of PB-induced CYP 2B1 expression exerted by 100 μM DHA (Fig. 2).

3.3. Effect of DHA on ceramide formation

Ceramide plays a critical role in apoptosis [43,44], and many studies have demonstrated the role of SMase in the hydrolysis of cellular sphingomyelin and the generation of ceramide [45–47]. We treated hepatocytes with 100 μM DHA for 0, 3, 6 and 12 h, and determined ceramide formation. A representative scan of ceramide on a TLC plate is shown in Fig. 3A. Ceramide formation reached a maximum after 3 h of incubation and decreased thereafter (Fig. 3B). A significant effect of DHA was found at 3 and 6 h ($P<.0001$).

3.4. Effect of C2-ceramide on cell viability

The effect of C2-ceramide on cell viability was determined by the LDH leakage assay (Fig. 4). LDH leakage of cells increased with increasing concentrations of C2-ceramide (5–40 μM) compared with that in untreated cells (1.10±0.22%); however, the effect was not significant. When the concentration of C2-ceramide was increased to 50 μM, the LDH leakage of cells increased by 15.96±2.62%, and this value was significantly greater than in the other groups. We chose to use 40 μM C2-ceramide because the LDH leakage results showed that concentrations greater than 40 μM caused cell damage. In our study, the effects of C2-ceramide observed were not due to cell viability. Meanwhile, 100 μM DHA was chosen because concentrations greater than this were found to cause cell damage, as reported in our previous study [48].

3.5. Effect of C2-ceramide on CYP 2B1 expression in the presence of PB

CYP 2B1 expression was significantly induced by 50 μM PB, and the expression was dose-dependently down-regulated by C2-ceramide (Fig. 5). The results of Western blot and Northern blot experiments were consistent.

3.6. Effect of C2-ceramide on the intracellular cAMP concentration

Intracellular cAMP concentrations were significantly increased in cells treated with 40 μM C2-ceramide for 24 h (Table 1, $P<.05$).

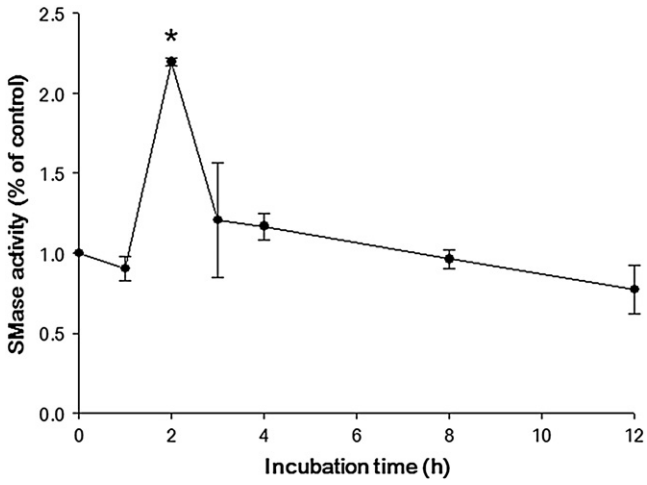

Fig. 1. Effect of DHA incubation time on SMase activity in rat primary hepatocytes. After attachment, hepatocytes were treated with 100 μM DHA for various time periods. Aliquots of total protein (12.5 μg) were used to determine SMase activity by using an Amplex Red Sphingomyelinase Assay Kit (Molecular Probes, UK). Each point represents the mean±S.D. of three independent experiments. Values were analyzed by one-way ANOVA followed by Tukey's *post hoc* test to compare SMase activity in DHA-treated cells to controls. *$P<.0001$.

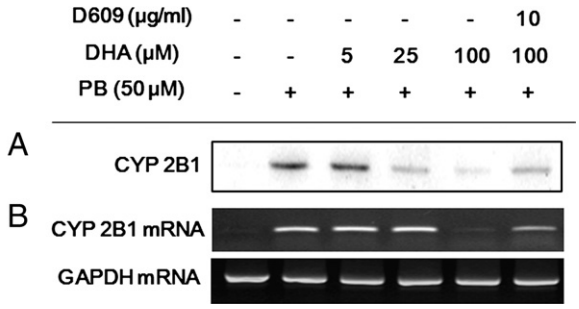

Fig. 2. Effect of D609 (an SMase inhibitor) on CYP 2B1 expression inhibited by DHA in the presence of PB. Forty hours after attachment, hepatocytes were pretreated with or without D609 for 1 h before the addition of DHA. After various concentrations of DHA were added for 4 h, PB was added and the cells were incubated for another 20 h. (A) Aliquots of microsomal protein (20 μg) were used for Western blot analysis. (B) Total RNA was isolated from hepatocytes and was subjected to RT-PCR with specific CYP 2B1 and GAPDH primers as described in Materials and Methods.

3.7. Effect of SQ22536 and H89 on CYP 2B1 expression inhibited by C2-ceramide in the presence of PB

The cAMP-dependent PKA pathway was reported to be involved in the down-regulation of CYP 2B1 expression induced by PB in rat primary hepatocytes [20,48]. Because the intracellular cAMP concentration was significantly increased by 40 μM C2-ceramide (Table 1), we used SQ22536 (an adenylyl cyclase inhibitor) and H89 (a PKA-specific inhibitor) to determine events upstream and downstream of cAMP on CYP 2B1 expression inhibited by C2-ceramide in the presence of PB. Both SQ22536 and H89 reversed the CYP 2B1 expression inhibited by C2-ceramide, although the effect was mild (Fig. 6).

4. Discussion

Hepatic CYP 2B1, a Phase I DME, plays an important role in the biotransformation system. Several chemotherapeutic agents, such as CPA and ifosfamide, require bioactivation by hepatic CYP 2B1 for their antitumor activity [49]. Our previous study showed that PB-induced CYP 2B1 gene expression in rat primary hepatocytes is down-regulated by DHA, and our results indicated that the attenuation of CAR translocation and decreased nuclear CAR-NR-1 complex formation contribute to DHA's down-regulatory action [21]. Thereafter, mitogen-activated protein kinase was found to play a critical role in the suppression of PB-induced CYP 2B1 expression by DHA in rat primary hepatocytes as well [50]. In addition, c-Jun NH2-terminal kinase is activated by PB in a dose-dependent manner, and this activation is inhibited by DHA. However, other possible mechanisms for this down-regulation by DHA were not fully understood.

Ceramides are recognized to be an important second messenger in diverse signaling pathways within cells, especially for apoptosis. Ceramides are also reported to be involved in the expression of DMEs. Previous studies showed that the expression of genes such as CYP 2C11 [51] and CYP 3A4 [52] is suppressed by ceramides. To our knowledge, the present study is the first to determine the effect of ceramide on PB-induced CYP 2B1 expression in rat primary hepatocytes.

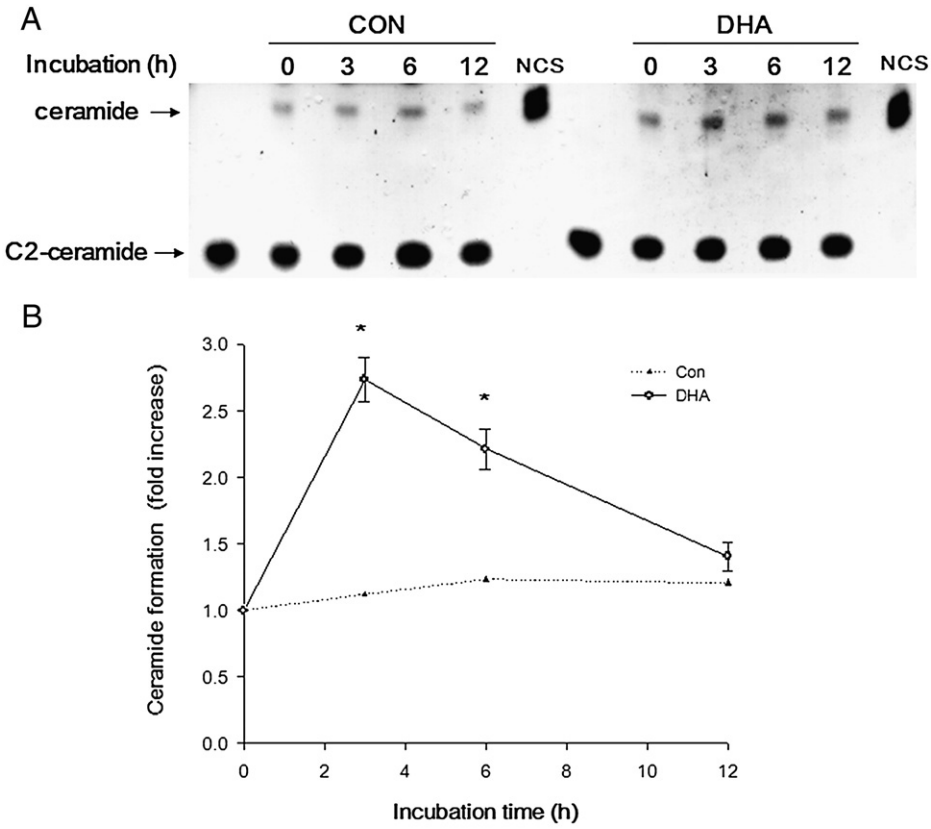

Fig. 3. Effect of DHA on ceramide formation in rat primary hepatocytes. After attachment, hepatocytes were treated with or without 100 μM DHA for various time periods. Aliquots of total protein (0.4 mg) were used to extract lipids, and ceramides were resolved by TLC. (A) Representative scan of ceramide on a TLC plate. C2-ceramide was added as an internal standard. A natural ceramide standard (NCS; porcine brain ceramides) was run simultaneously to identify the location of ceramides. (B) Ceramides were quantified by densitometry analysis. Each point represents the mean±S.D. of three independent experiments. The levels in control cells were set to 1. Values were analyzed by Student's *t* test, relative to control at each time point. *$P<.0001$.

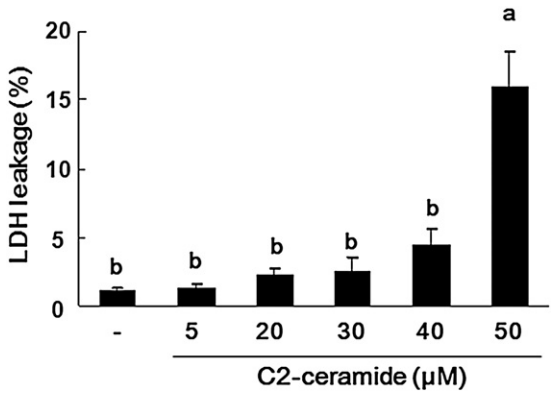

Fig. 4. Effect of C2-ceramide on cell viability in rat primary hepatocytes. Rat primary hepatocytes were treated with various concentrations of C2-ceramide for 24 h, and cell viability was measured by using the LDH leakage assay. Values are the mean±S.D. of three independent experiments. Values not sharing the same letter are significantly different ($P<.05$).

A previous study showed that treatment of cells with exogenous bacterial SMase for 25 min results in cleavage of 90–95% of total sphingomyelin and the concomitant generation of ceramide in human lung fibroblasts (WI38) [53]. In another study, treatment of rat primary hepatocytes with interleukin-1β (IL-1β) caused a rapid turnover of sphingomyelin and an increase in intracellular ceramide. The increased ceramide was found to down-regulate CYP 2C11 gene expression [51]. Chun et al. [52] found that cells treated with bacterial SMase and tumor necrosis factor-α (TNF-α) had increased intracellular ceramide and this significantly suppressed CYP 3A4 gene expression in human colon carcinoma HT-29 cells. Neutral SMase activity of human aortic smooth muscle cells treated with apoC-I, apoC-I-enriched HDL and TNF-α (positive control) increased two- to threefold after 10 min and then decreased over 60 min [54]. SMase, especially the neutral type, is activated by PUFAs, including arachidonic acid (AA; 20:4 n-6), octadecenoic acid (18:1 n-9), eicosapentaenoic acid (EPA; 20:5 n-3) and DHA (22:6 n-3) in human neutrophils [45]. The stimulatory effect of PUFAs on SMase activity suggests that the physiologic effect of PUFAs is through SMase activation and ceramide generation. Thus, we were interested in investigating the role of SMase and ceramide in the down-regulation of PB-induced CYP 2B1 expression by DHA.

Barsacchi et al. [55] showed that activation of both acidic SMase and neutral SMase by TNF-α was rapid in onset in U937 cells: SMase activity increased after 2 min of cytokine treatment, peaked at 5 min

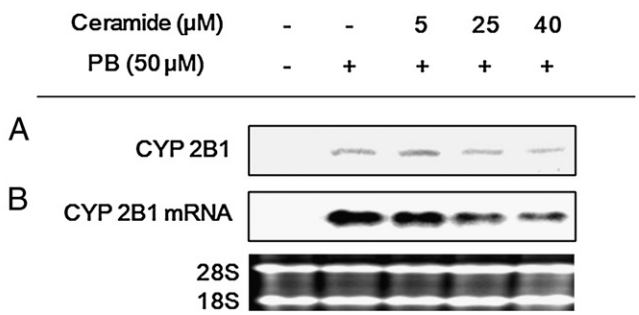

Fig. 5. CYP 2B1 expression was inhibited by C2-ceramide in rat primary hepatocytes after PB treatment. After attachment, hepatocytes were pretreated with or without various concentrations of C2-ceramide for 4 h before the addition of PB, and the cells were then incubated with PB for another 20 h. (A) Microsomal proteins (7.5 μg) were used for Western blot analysis. (B) 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.

Table 1
Effect of C2-ceramide on the intracellular cAMP concentration

Treatment	cAMP concentration (pg/mg protein)
Control	94.9±14.2[b]
C2-ceramide (μM)	
10	111.7±8.9[b]
20	113.0±7.4[b]
40	133.4±9.9[a]

After attachment, hepatocytes were treated with various concentrations of C2-ceramide for 24 h.
Values are mean±S.D., $n=3$. Values were analyzed by one-way ANOVA followed by Tukey's *post hoc* test. Values not sharing the same letter are significantly different ($P<.05$).

and then diminished, and returned to basal levels after 30 min. No further increase in acidic SMase activity was noted during the 4-h period. However, a second prolonged phase of activation of neutral SMase commenced 90 min after TNF-α administration and continued for the rest of the observation period. In the present study, SMase activity peaked at 2 h of treatment with 100 μM DHA and then returned to basal levels in rat primary hepatocytes (Fig. 1). Our results were not consistent with those of Barsacchi et al. [55]. Inconsistency in the kinetics of ceramide generation in response to stimuli in different cell types has been addressed by Jaffrézou et al. [56].

Tricyclodecan-9-yl xanthate (D609) is an acidic SMase inhibitor [57], and D609 at 25 μg/ml was found to selectively inhibit TNF-α-activated acidic SMase activity in U937 cells [55]. In the present study, pretreatment with D609 (10 μg/ml) reversed the down-regulation of PB-induced CYP 2B1 expression by DHA (Fig. 2). These results suggest that the SMase-dependent ceramide pathway may play an important role in DHA's down-regulation of PB-induced CYP 2B1 expression in rat primary hepatocytes.

In the present study, the intracellular ceramide level was increased approximately two- to threefold 3 h after DHA administration (Fig. 3). These results support a role for DHA in activating SMase, hydrolyzing sphingomyelin and generating ceramide in rat primary hepatocytes. Its action is similar to that of Fas ligands, TNF-α and heat shock protein [58–60]. SMase activity reached a peak 2 h after DHA administration, and ceramide production reached a maximum 3 h after DHA treatment in our culture system. The timetable is logical, because SMase activation occurs first, followed by ceramide generation.

The role of ceramides in apoptosis has been proposed in many previous studies. NB16 cells treated with 20 μM C2-ceramide for 20 h lost approximately 75% of cell viability, but only 25% of cells were

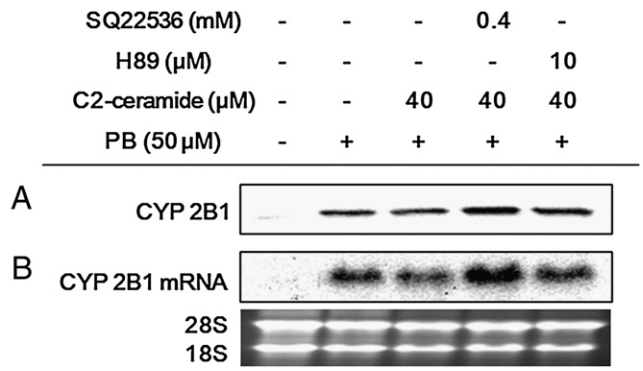

Fig. 6. Effect of SQ22536 (an adenylyl cyclase inhibitor) and H89 (a PKA-specific inhibitor) on CYP 2B1 expression inhibited by C2-ceramide in the presence of PB. After attachment, hepatocytes were pretreated with or without SQ22536 or H89 for 1 h before the addition of C2-ceramide. After C2-ceramide addition for 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 three independent experiments is shown.

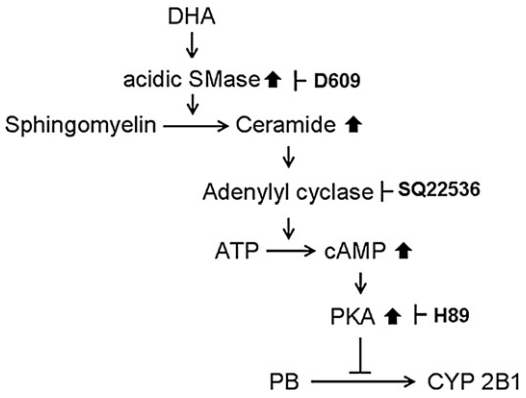

Fig. 7. Model showing pathways that mediate DHA down-regulate PB-induced CYP 2B1 expression in rat primary hepatocytes.

scored apoptotic [61]. The apoptotic effect of ceramides is differential in different cell types. Mu et al. [62] showed that 100 μM C2-ceramide induced apoptosis in CEM leukemic cells but not in granulosa cells. In addition to ceramide itself, other factors can cause apoptosis via stimulation of ceramide generation. Fatty acids, such as 0.4 mM palmitate, induce apoptosis by increasing ceramide generation in cultured bovine retinal pericytes [63]. In the present study, we chose to use both 100 μM DHA and 40 μM C2-ceramide because the LDH leakage results showed concentrations greater than those that caused cell damage. For DHA, in addition to its cytotoxic effect, 100 μM DHA is an achievable level in human plasma [64]. On the basis of the effect of 100 μM DHA on SMase activity and ceramide production, we propose that ceramide is one of the pathways responsible for regulation of PB-induced CYP 2B1 by DHA in rat primary hepatocytes.

Expression of CYP 2C11 in rat hepatocytes and CYP 3A4 in human colon carcinoma HT-29 cells is suppressed by C2-ceramide and C6-ceramide, respectively [51,52]. In the present study, PB-induced CYP 2B1 protein and mRNA expression was down-regulated by C2-ceramide in a dose-dependent manner (Fig. 5). In our previous study, we reported that the cAMP-dependent PKA pathway is involved in AA's down-regulation of PB-induced CYP 2B1 expression [48]. The long-chain ceramides, C18- and C24-ceramides, were found to stimulate adenylyl cyclase (AC) II in HEK 293 cells as potent as phorbolester TPA. However, short-chain C2-ceramide showed a relatively weak stimulatory effect [65]. In the present study, ceramide played a role in down-regulating PB-induced CYP 2B1 expression by DHA, and C2-ceramide dose-dependently increased the intracellular cAMP level (Table 1). These results indicate that the short-chain C2-ceramide (40 μM) has a significant stimulatory effect on intracellular cAMP concentrations in rat primary hepatocytes. Although C2-ceramide was not as potent as C18/C24 ceramides in stimulating AC II activity, C2-ceramide at a concentration of 20 μM enhanced AC II activity threefold in HEK 293 cells [65]. These results suggest that the physiologic effect of ceramides may depend on their chain length.

In addition to the stimulation of AC by ceramides, the ceramide derivative ceramide-1-phosphate (C-1-P) was found to enhance phospholipase A_2 (PLA_2) activity in a recent study [66]. PLA_2 is responsible for the release of AA from cell membrane phospholipids in response to stimuli [67]. Released AA is converted to physiologically active metabolites such as prostaglandins, leukotrienes and other bioactive products via oxygenases [cyclooxygenase (COX), lipoxygenase and P450] [68]. Constitutive COX-1 mRNA is present in rat primary hepatocytes [28]; this indicates that the metabolites of the COX pathway may be involved in ceramide signaling transduction.

As shown in Table 1, 40 μM C2-ceramide significantly increased the intracellular cAMP concentration. We studied events upstream and downstream of cAMP in the C2-ceramide inhibition of PB-induced CYP 2B1 expression. As shown in Fig. 6, both SQ22536 and H89 partially reversed the PB-induced CYP 2B1 expression suppressed by C2-ceramide. These results suggest that the cAMP-dependent PKA pathway may be involved in the down-regulation of PB-induced CYP 2B1 expression by C2-ceramide.

The findings of the present study are schematically presented in Fig. 7. This figure shows that stimulation of acidic SMase and production of ceramide, subsequent enhancement of adenylyl cyclase activity, generation of cAMP and activation of cAMP-dependent PKA pathway were involved in inhibition of PB-induced CYP 2B1 expression by DHA.

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