Prostaglandin E₂ Is Involved in the Increase of Cytochrome P-450 2B1 Expression by α-Tocopheryl Succinate in Primary Rat Hepatocytes in the Presence of Phenobarbital

Ching-Feng Tsai, Chong-Kuei Lii, Jaw-Ji Yang, Kaili Liu, Wea-Lung Lin, and Haw-Wen Chen

Abstract: The modulation of cytochrome P-450 2B1 expression by α-tocopheryl succinate and whether prostaglandin E₂ is involved in this modulation in primary rat hepatocytes in the presence of phenobarbital were investigated. A primary rat hepatocyte culture model that faithfully reproduces the phenobarbital response observed in vivo was used. Intracellular α-tocopherol content was dose dependently increased by α-tocopheryl succinate incubation. Hepatocytes were demonstrated to have prostaglandin E₂-synthesizing capability. α-Tocopheryl succinate inhibited prostaglandin E₂ synthesis by hepatocytes and increased cytochrome P-450 2B1 expression in the presence of phenobarbital; however, it had little effect on intracellular cAMP level. To mimic the exogenous source of prostaglandin E₂ from non-parenchymal cells, various concentrations of prostaglandin E₂ were added to the cell culture. High doses of exogenous prostaglandin E₂ (100 and 1,000 nM) inhibited the cytochrome P-450 2B1 expression in the presence of phenobarbital compared with low doses (1 and 10 nM); however, the presence of high doses of prostaglandin E₂ had no effect on intracellular cAMP level. Forskolin significantly increased intracellular cAMP level and inhibited cytochrome P-450 2B1 expression in the presence of phenobarbital. The results of this study indicate that α-tocopherol succinate increases cytochrome P-450 2B1 expression via its inhibition of prostaglandin E₂ synthesis in the presence of phenobarbital; however, changes in intracellular cAMP level are not related to cytochrome P-450 2B1 expression.

Introduction

The liver is the major organ involved in bioactivation, biotransformation, and detoxification of numerous compounds of endogenous and exogenous origin (1). The enzyme systems present in the liver include mixed-function oxidases and conjugation enzymes. The cytochrome P-450s belong to the mixed-function oxidases (2). To date, 14 gene families of cytochrome P-450s have been identified and characterized in mammals (3), and certain cytochrome P-450s are inducible by distinct classes of chemical agents (4,5). Phenobarbital is well known for its sedative/antiseize properties in the central nervous system (6,7) and has a number of pleiotropic effects in the liver, including gene induction (8), tumor promotion in rodents (9), and disruption of gap-junctional intercellular communication (10). In rat liver, cytochrome P-450 2B and 3A gene families have been shown to be induced by phenobarbital and phenobarbital agonist via transcriptional activation (8,11). It has been recognized that most liver processes are controlled by a variety of receptors and intracellular second messengers that transduce extracellular stimuli (12,13).

Hepatic cytochrome P-450 2B1 activity was found to be affected by chemicals as well as dietary factors such as dietary lipid and vitamin E in rodents (14–16). In our previous animal studies, dietary α-tocopherol acetate was found to increase hepatic cytochrome P-450 2B1 activity in the presence or absence of phenobarbital induction, although the mechanism was not determined (15,16). Previous studies (12,13) have found that liver processes are related to receptors on cells and changes in the intracellular second-messenger level. We suspected that dietary α-tocopherol acetate affects the synthesis of endogenous compounds that regulate cytochrome P-450 2B1 activity and protein level in the presence or absence of phenobarbital. Dietary vitamin E has been found to decrease prostaglandin E₂ production in macrophages (17). Prostaglandin E₂ was found to affect the physiological and pathological response via influence of intracellular cAMP level (18,19). Sidhu and Omiecinski (13) demonstrated a striking inhibition of phenobarbital-mediated cytochrome P-450 gene induction by cAMP and protein kinase A activators and suggested a negative regulatory role for the cAMP signal transduction pathway in phenobarbital gene induction. In this study, a primary rat hepatocyte culture model was used to study the mechanism involved in α-tocopheryl succinate modulation of cytochrome P-450 2B1 activity.
protein and mRNA expression in the presence of phenobarbital. This primary rat hepatocyte culture system can faithfully reproduce the phenobarbital induction response observed in vivo (20–22).

Materials and Methods

Materials

Cell culture medium was obtained from GIBCO-BRL (Gaithersburg, MD); Matrigel and ITS+ (insulin, transferrin, selenium, bovine serum albumin, and linoleic acid) from Collaborative Biomedical Products (Bedford, MA); forskolin, collagenase type IV, dexamethasone, phenobarbital, and α-tocopheryl succinate from Sigma; and prostaglandin E₂ from Cayman Chemical (Ann Arbor, MI).

Isolation and Culture of Hepatocytes

Eight-week-old male Sprague-Dawley rats, with an average body weight of 250–300 g, were purchased from the National Animal Breeding and Research Center (Taipei, Taiwan). Rat hepatocytes were isolated by a modification of the two-step collagenase perfusion method described by Seglen (23) and cultured with a protocol described by Sidhu and Omiecinski (13). To isolate healthy hepatocytes efficiently, an isodensity Percoll centrifugation method was applied as described by Kreamer et al. (24). This method not only increases cell viability but also minimizes the contamination of other nonparenchymal cells. Finally, Percoll was washed twice with ice-cold phosphate-buffered saline, 0.5 ml of hexane was added to each dish, and cells of three dishes were scraped and transferred to an Eppendorf tube; α-tocopheryl acetate (200 µl) was added as the internal standard. After 2 min of mixing, the hexane layer was transferred to another Eppendorf tube, and the remainder was extracted four times with hexane. The pooled hexane was then evaporated under nitrogen, the residue was redissolved in 350 µl of filtered high-performance liquid chromatography-grade methanol by mixing, and 50 µl of the mixture was injected into a high-performance liquid chromatography system for α-tocopherol analysis. Protein was measured by the method of Lowry et al. (28).

cDNA Probe

One pair of oligonucleotide primers was designed on the basis of the published sequences of 2B1: 5'-GGATGGGAAAAGAGGAGTGTGGA-3' (forward) and 5'-CTGGAGGTGGTGAGAGAG-3' (reverse). mRNA obtained from hepatocytes was used as the template for reverse transcription-polymerase chain reaction. The polymerase chain reaction conditions were set as follows: denaturing at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min for 35 cycles followed by a 7-min extension at 72°C. The band corresponding to the DNA fragment of cytochrome P-450 2B1 was labeled with [α-32P]dCTP and used as a probe for Northern blot analysis.

RNA Preparation and Northern Blot Analysis

RNA of cells was extracted with 1 ml of TRIzol reagent. The extract was allowed to react at room temperature for 5 min, 0.2 ml of 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 addition of 0.5 ml of isopropyl alcohol. The RNA samples were allowed to sit at room temperature for 10 min and then centrifuged at 12,000 g for 10 min at 4°C. The resultant RNA pellets were washed twice with 75% ice-cold ethanol. For Northern blot analysis, 20 µg of each RNA sample were electrophoretically separated by 1% agarose gel containing 6% formaldehyde and transferred to a HyBond membrane as previously described (29). For hybridization with cDNA, the membrane was prehybridized for 2 h at 42°C in a solution containing 10× Denhardt’s reagent (0.2% Ficoll, 0.2% poly-
vinylpyrolidone, and 0.2% bovine serum albumin), 5× SSPE (750 mM NaCl, 50 mM NaH₂PO₄, 5 mM EDTA), 2% sodium dodecyl sulfate (SDS), 50% formamide, and 100 µg/ml of single-strand sheared salmon sperm DNA. The membrane was then hybridized in the same solution with ³²P-labeled 2B1 cDNA probe at 42°C overnight. Autoradiography was performed by exposing the membrane to Kodak SuperRx X-ray film at −80°C with an intensifying screen.

SDS-Polyacrylamide Gel Electrophoresis and Immunodetection

SDS polyacrylamide gels made with 10% acrylamide were prepared as described by Laemmli (30). For cytochrome P-450 2B1, 12.5 µg of microsomal protein were applied to each gel. After electrophoresis, proteins separated on SDS-polyacrylamide gels were transferred to polyvinylidene difluoride membranes. The nonspecific binding sites on the polyvinylidene difluoride membranes were blocked with 5% nonfat dry milk in 15 mM Tris-150 mM NaCl buffer (pH 7.4) at 4°C overnight. Polyclonal antibodies against cytochrome P-450 2B1 were obtained from Chemicon International (Temecula, CA). A goat peroxidase-conjugated anti-rabbit IgG was used to detect the immunoreactive bands. Incubation with primary antibody and secondary antibody 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.

Enzyme-Linked Immunosorbent Assay Determination of Prostaglandin E₂ Synthesis of Hepatocytes

The capability of hepatocytes to synthesize prostaglandin E₂ was determined by measuring the prostaglandin E₂ released into the medium. The assay was performed by using the prostaglandin E₂ EIA kit-monoclonal (Cayman).

Enzyme-Linked Immunosorbent Assay Determination of Intracellular cAMP Level

Intracellular cAMP level was measured using the cAMP EIA kit (Cayman). Cells were stimulated for 20 h with various concentrations of α-tocopheryl succinate, prostaglandin E₂, and forskolin (0.1 mM). Cell extracts were prepared as described by Beck and Omiecinski (12).

Statistical Analysis

Data were analyzed by using analysis of variance (SAS Institute, Cary, NC). Tukey’s test was used to evaluate the significance of the difference between means; \( P < 0.05 \) was taken to be statistically significant.

Results

Dose Response and Time Course of Cytochrome P-450 2B1 Expression

First, the lowest dose of phenobarbital to induce cytochrome P-450 2B1 expression was determined to avoid the regulation of cytochrome P-450 2B1 expression by α-tocopheryl succinate to be overcome by phenobarbital overdose induction during experiments. Results of Western blotting showed that 0.1 mM phenobarbital can effectively induce cytochrome P-450 2B1 expression after 20 h of treatment, although no lower dose was tested (Fig. 1A). This dose was consistent with that used in cAMP-associated inhibition of phenobarbital-induced cytochrome P-450 2B1 gene expression in primary rat hepatocyte cultures (13). In other studies (12,31), 1 mM phenobarbital was used to induce cytochrome P-450 2B1 expression in primary rat hepatocytes. Second, the minimal period required for cytochrome P-450 2B1 expression induced by 0.1 mM phenobarbital was determined. Results of Western

![Figure 1](image-url) Western blot analyses of cytochrome P-450 2B1 expression. A: dose response of cytochrome P-450 2B1 expression to various concentrations of phenobarbital (PB). B: time course of phenobarbital-induced cytochrome P-450 2B1 expression.
blotting demonstrated that cytochrome P-450 2B1 expression was identified beginning at 15 h of culture and continued to increase at 20 h of culture; so 20 h of culture was selected for use in experiments (Fig. 1B). Unless otherwise stated, the cells were cultured with 0.1 mM phenobarbital for 20 h to induce the cytochrome P-450 2B1 expression.

**Effect of α-Tocopheryl Succinate on Cytochrome P-450 2B1 Expression**

In our previous studies (15,16), activity of cytochrome P-450 2B1 was increased by dietary α-tocopheryl acetate in the presence or absence of phenobarbital. To determine whether this effect is present in an in vitro model as well and to determine the mechanism responsible for this effect, a primary rat hepatocyte culture was used. At 48 h after plating, hepatocytes were incubated with various concentrations of α-tocopheryl succinate (0, 5, 10, 20, and 50 µM) for another 24 h. Intracellular α-tocopherol content was dose dependently increased by α-tocopheryl succinate incubation (Fig. 2). This result suggested that α-tocopheryl succinate can be efficiently incorporated into hepatocytes. Western and Northern blot analyses revealed that α-tocopheryl succinate enhanced cytochrome P-450 2B1 expression in the presence of phenobarbital (Fig. 3), and maximum enhancement was noted at 10 µM α-tocopheryl succinate. α-Tocopheryl succinate at >10 µM showed no further enhancement. The results indicate that α-tocopheryl succinate can increase cytochrome P-450 2B1 expression in the presence of phenobarbital in vitro.

**Effect of α-Tocopheryl Succinate on Prostaglandin E₂ Synthesis by Hepatocytes**

On the basis of previous evidence that prostaglandin E₂ synthesis is decreased by vitamin E (17), we hypothesized that the effect of α-tocopheryl succinate on cytochrome P-450 2B1 expression may occur through inhibition of prostaglandin E₂ synthesis. To test this hypothesis, the prostaglandin E₂-synthesizing capability of hepatocytes and the effect of α-tocopheryl succinate on hepatocyte prostaglandin E₂ synthesis were studied. In this study, hepatocytes were found to have the capability to synthesize prostaglandin E₂, and α-tocopheryl succinate significantly inhibited prostaglandin E₂ synthesis by hepatocytes (Fig. 4). There

![Figure 2](image2.png)

**Figure 2.** Dose response of α-tocopheryl succinate incorporation into hepatocytes. Values are means ± SD of 3 independent experiments, each measured in duplicate by high-performance liquid chromatography. Groups not sharing the same letter (a–d) are significantly different (*P* < 0.05).

![Figure 3](image3.png)

**Figure 3.** Effects of various concentrations of α-tocopheryl succinate (α-TS) on cytochrome P-450 2B1 expression in the presence of phenobarbital. A: Western blot analyses. B: Northern blot analyses; ribosomal 18S and 28S RNA hybridization levels were used as normalization standards.
was a 60–70% decrease in prostaglandin E₂ release of cells coincubated with α-tocopherol succinate and phenobarbital compared with those incubated with phenobarbital only.

**Effect of Exogenous Prostaglandin E₂ on Cytochrome P-450 2B1 Expression**

In addition to parenchymal hepatocytes, nonparenchymal cells are present in the liver. The nonparenchymal cell, such as sinusoidal endothelial cells or Kupffer cells, which are hepatic residential macrophages, are the main producers of prostanoids (32,33). To further examine the effect of prostaglandin E₂ on cytochrome P-450 2B1 expression of hepatocytes, exogenous prostaglandin E₂ was added to hepatocytes. Results of Western and Northern blot analysis consistently showed that exogenous prostaglandin E₂ at 100 and 1,000 nM dramatically inhibited cytochrome P-450 2B1 expression in the presence of phenobarbital (Fig. 5, A and B).

Also, 0.1 mM forskolin completely inhibited cytochrome P-450 2B1 expression in the presence of phenobarbital (Fig. 5C). These results suggest that the high doses of exogenous prostaglandin E₂ (100 and 1,000 nM) inhibited cytochrome P-450 2B1 expression and forskolin (0.1 mM) completely inhibited the expression in the presence of phenobarbital.

**Effects of α-Tocopheryl Succinate and Exogenous Prostaglandin E₂ on Intracellular cAMP Level**

Although α-tocopherol succinate suppressed the prostaglandin E₂-synthesizing capability of hepatocytes, intracellular cAMP level was not significantly different among hepatocytes treated with various concentrations of α-tocopherol succinate (Fig. 6). This result indicates that the effect of α-tocopherol succinate on cytochrome P-450 2B1 expression induced by phenobarbital was not related to the changes in intracellular cAMP level. Prostaglandin E₂ addition had no effect on intracellular cAMP level, and forskolin functioned as an adenylyl cyclase activator to significantly increase intracellular cAMP level compared with prostaglandin E₂ (Fig. 7). These results suggest that hepatocytes possess adenylyl cyclase activity, and the effect of prostaglandin E₂ on intracellular cAMP level was not observed.

**Discussion**

In our previous animal studies, we found that dietary α-tocopherol acetate plays a role in increasing cytochrome P-450 2B1 activity in the presence or absence of phenobarbital induction (15,16), although the mechanism was not identified. In this study, we used a primary rat hepatocyte culture that faithfully reproduces the phenobarbital induction response observed in vivo (20–22) to try to determine the mechanism involved in α-tocopherol succinate enhancement of cytochrome P-450 2B1 expression in the presence of phenobarbital. Because adult rat hepatocytes maintained in primary culture rapidly lose the ability to respond to phenobarbital (34), a Matrigel overlay, which maintains the hepatocyte’s response to phenobarbital induction of cytochrome P-450 2B1 expression, was successfully applied to determine the mechanism responsible for α-tocopherol succinate enhancement of cytochrome P-450 2B1 expression in the presence of phenobarbital. The extent of incorporation of α-tocopherol into the hepatocytes with various concentrations of α-tocopherol succinate incubation was first determined. As expected, results showed that α-tocopherol was efficiently and dose dependently incorporated into the cells after 24 h of incubation (Fig. 2). In this incorporation study, cytochrome P-450 2B1 expression in the presence of phenobarbital was greater when α-tocopherol succinate was added to hepatocytes than when no α-tocopherol succinate was added. Western and Northern blot analyses showed consistent results (Fig. 3).

Hepatocytes were demonstrated to have prostaglandin E₂-synthesizing capability. The prostaglandin E₂-synthesizing capability of hepatocytes was significantly inhibited by α-tocopherol succinate (Fig. 4). Isolated rat hepatocytes have been used to investigate the capability of hepatocytes to synthesize and/or degrade eicosanoids, and results showed that hepatocytes appear to be the site of degradation, instead of synthesis, of eicosanoids in the liver (32). The capability of hepatocytes to synthesize prostaglandin E₂ was <22 fmol/10⁶ cells/30 min. The inhibition of prostaglandin E₂ synthesis in macrophages by vitamin E was demonstrated in a previous study (17). Our result that α-tocopherol succinate inhibited prostaglandin E₂ synthesis of hepatocytes is consistent with this finding. In macrophages (17), vitamin E had decreased prostaglandin E₂-synthesizing capability. On the basis of aforementioned findings, the effect of α-tocopherol succinate on the α-tocopherol succinate-induced inhibition of cytochrome P-450 2B1 expression was examined.
copheryl succinate on the cytochrome P-450 2B1 expression in the presence of phenobarbital may be explained, at least in part, by its inhibition of prostaglandin E2 synthesis (Fig. 4). Thus α-tocopheryl succinate addition resulted in the greater cytochrome P-450 2B1 expression in the presence of phenobarbital than with no α-tocopheryl succinate addition. A previous study by Sidhu and Omiecinski (13) demonstrated that increased intracellular cAMP level inhibits phenobarbital-induced cytochrome P-450 2B1 gene expression and suggested a negative regulatory role for the cAMP signal transduction pathways in phenobarbital gene induction. Intracellular cAMP level was found to be regulated by prostaglandin E2 and this alteration was found to be responsible for the physiological and pathological end points of prostaglandin E2, (18,19). Although the prostaglandin E2-synthesizing capability of hepatocytes was significantly inhibited by α-tocopheryl succinate, the intracellular cAMP level was not significantly different among hepatocytes treated with various concentrations of α-tocopheryl succinate (Fig. 6). This result indicated that the effect of α-tocopheryl succinate on cytochrome P-450 2B1 expression in the presence of phenobarbital was not related to changes in intracellular cAMP level.

The liver is composed of parenchymal and nonparenchymal cells. In contrast to the low prostaglandin E2-synthesizing capability of hepatocytes, the nonparenchymal cells, such as sinusoidal endothelial cells or Kupffer cells, which are hepatic residential macrophages, are the main producers of prostanoids (32,33). The effect of extrahepatic prostaglandin E2 on intracellular cAMP level was studied using forskolin as the positive control. We found that intracellular cAMP level was not affected by prostaglandin E2; however, the levels of cytochrome P-450 2B1 protein and mRNA in the presence of phenobarbital were suppressed by 100 and 1,000 nM prostaglandin E2 (Fig. 5). In comparison, cAMP level was significantly increased by forskolin (Fig. 6), and cytochrome P-450 2B1 mRNA level in the presence of phenobarbital was suppressed completely (Fig. 5). In a previous study (35), prosta-

Figure 5. Effects of various concentrations of prostaglandin E2 and forskolin on cytochrome P-450 2B1 expression in the presence of phenobarbital. A: Western blot analyses. B: Northern blot analyses; ribosomal 18S and 28S RNA hybridization levels were used as normalization standards. C: Northern blot analyses for forskolin (0.1 mM). Similar results were observed in 3 independent experiments.
glandin $E_2$ increased the level of cAMP in hepatocytes slightly. However, phenobarbital exposure had no effect on intracellular cAMP level and protein kinase A activity of hepatocytes (12), and it was suggested that alterations in cAMP levels and associated protein kinase A activity were not involved in the cytochrome $P-450$ 2B1 expression induced by phenobarbital. The difference between our study and that of Beck and Omiecinski (12) was in the stimuli added to the cell cultures. In our study, prostaglandin $E_2$ and phenobarbital were added; however, only phenobarbital was added in the study of Beck and Omiecinski.

In summary, the results of this study indicate that $\alpha$-tocopheryl succinate increases cytochrome $P-450$ 2B1 expression in the presence of phenobarbital via its inhibition of prostaglandin $E_2$ synthesis, while changes in intracellular cAMP level are little implicated in this mechanism. Endogenous and exogenous prostaglandin $E_2$ seem to be involved in regulation of cytochrome $P-450$ 2B1 expression in the presence of phenobarbital. Further studies are needed to elucidate the mechanism involved in prostaglandin $E_2$ regulation of cytochrome $P-450$ 2B1 expression in the presence of phenobarbital.

Acknowledgments and Notes

This work was supported by the National Science Council of Taiwan, ROC, under Contract NSC-89-2320-B-040-055. Address correspondence to H.-W. Chen, Dept. of Nutrition, Chung Shan Medical University, Taichung 402, Taiwan.

Submitted 8 March 2001; accepted in final form 25 June 2001.

References
