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※ 維生素 E 對 in vivo 肝臟和 in vitro 肝細胞 cytochrome P450 2B1 表現之影響及機制探討

Effect of vitamin E on in vivo and in vitro cytochrome P450 IIB expression and the possible mechanisms involved ※

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α-Tocopherol Acetate Supplementation Enhances Rat Hepatic Cytochrome PROD Activity in the Presence of Phenobarbital Induction

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Abstract: *Hepatic cytochrome P-450 enzymes play important roles in bioactivation of chemical carcinogens, biotransformation of many endogenous compounds, and detoxification of numerous xenobiotics. These enzyme activities have been shown to be regulated by various dietary factors. In our previous study, hepatic cytochrome pentoxyresorufin O-dealkylase (PROD) activity was decreased in rats fed an α-tocopherol acetate-deficient diet compared with rats fed α-tocopherol acetate-adequate or -supplemented diets. The objective of the present study was to investigate whether the modulatory effect of dietary α-tocopherol acetate on hepatic cytochrome PROD activity is influenced by the presence of phenobarbital. Weanling male Sprague-Dawley rats were fed the AIN-76 diet for four days, fasted for two days, then fed semipurified diets that were α-tocopherol acetate deficient, adequate, or supplemented with 5 and 15 g/kg α-tocopherol acetate for four days. Liver and plasma α-tocopherol concentrations were dose dependently regulated by dietary α-tocopherol acetate level. Inhibition of lipid peroxidation by dietary α-tocopherol acetate was dose dependent. Hepatic total cytochrome P-450 content was significantly greater in rats fed diets supplemented with 5 and 15 g/kg α-tocopherol acetate than in rats fed an α-tocopherol-adequate diet ($p < 0.05$). Hepatic cytochrome PROD activity was significantly greater in rats fed diets supplemented with 5 and 15 g/kg α-tocopherol acetate than in rats fed α-tocopherol acetate-deficient and -adequate diets ($p < 0.05$). These results suggest that, in the presence of phenobarbital, dietary α-tocopherol acetate efficiently affects tissue α-tocopherol levels and inhibits lipid peroxidation and that diets supplemented with 5 or 15 g/kg α-tocopherol acetate enhance hepatic cytochrome PROD activity compared with α-tocopherol acetate-deficient or -adequate diets.*

Introduction

It is well established that vitamin E, which is composed of eight isomers, is an important lipid-soluble antioxidant. Among these isomers, α-tocopherol is known to have the highest biological activity (1) and is the most abundant hydrophobic antioxidant *in vivo* (2). Vitamin E scavenges lipid peroxyl radicals by donating the phenolic hydrogen to radicals, which leads to the formation of lipid hydroperoxides and reversibly oxidized vitamin E (3). In addition to its free radical-scavenging capability, vitamin E is also highly reactive toward singlet oxygen (4). It cooperates with other antioxidants (5,6) to protect different structures against oxidative damage and lipid peroxidation, often measured as the production of malondialdehyde (7). In previous studies, it has been shown that vitamin E supplementation efficiently inhibits liver lipid peroxidation (8,9). This supports the role of vitamin E as a lipophilic antioxidant.

Cytochrome *P*-450 enzymes play a multifunctional role in animals. They are involved in the bioactivation of chemical carcinogens (10,11), the biotransformation of many endogenous compounds, and the detoxification of numerous xenobiotics (10–13). Changes in cytochrome *P*-450 enzyme activity may influence the health of animals. Hepatic microsomal cytochrome *P*-450 enzymes are the predominant enzymes involved in the phase I metabolism of various endogenous and exogenous compounds. Glutathione *S*-transferases (GST), a family of cytosolic enzymes, are important in the phase II metabolism of these compounds via conjugation reaction. Cytochrome *P*-450 enzymes and NADPH cytochrome *P*-450 reductase constitute mixed-function oxidases (14). Cytochrome *P*-450 enzymes are present in multiple forms and have broad but distinct substrate specificities (15). Cytochrome 1, 2, and 3 families are thought to be responsible for most drug metabolism (16). Cytochrome *P*-450 enzymes are the predominant proteins present in the hepatic endoplasmic reticulum, representing about 5–25% of the total protein content. It is well established that cytochrome *P*-450 IIB1 activity can be induced by phenobarbital (17), which is also an effective hepatopromoter of hepatic foci and tumor development (8). Cytochrome *P*-450 IIB1 activity is induced not only by phenobarbital but also by barbiturates with sedative/anticonvulsant properties (18). Cytochrome *P*-450 enzymes have been found to be affected by various dietary factors, including vitamin E (19).

In addition to its pivotal antioxidant function, vitamin E has been shown to play an important role in drug oxidation

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via its influence on cytochrome *P*-450 enzymes (19). However, findings concerning the relation between dietary vitamin E and hepatic cytochrome *P*-450 enzyme activity have been inconsistent (20–23). Intratracheal administration of liposome-associated α-tocopherol in rats conserves the cytochrome *P*-450 concentration and confers protection against paraquat-induced lung damage compared with plain liposomes (20). Diets supplemented with 0.06% α-tocopherol caused significant increases in hepatic total cytochrome *P*-450 content and NADPH-cytochrome *P*-450 reductase activity in rats compared with α-tocopherol-adequate diets ($p < 0.05$) (21). Diets deficient in vitamin E depressed total cytochrome *P*-450 level by 15% compared with diets adequate in vitamin E (22). However, in another study, dietary α-tocopherol did not modify the hepatic total cytochrome *P*-450 content (0, 0.05, 0.5, 5 IU/g diet). However, the specific activities of cytochrome *P*-450 involved in aflatoxin B_1 metabolism have been found to be affected by dietary α-tocopherol. Microsomal cytochrome *P*-450 IIB1 activity was decreased in the deficient group (0 IU) to 65% of that in the control group (0.05 IU). Dietary supplementation of 0.5 IU of α-tocopherol increased cytochrome *P*-450 IIB1 activity to 128% of the activity of rats receiving dietary supplementation of 0.05 IU α-tocopherol, whereas dietary supplementation with 5 IU of α-tocopherol reduced the specific cytochrome *P*-450 activity of rats compared with dietary supplementation with 0.5 IU of α-tocopherol (23). These results suggest that the effect of dietary α-tocopherol on cytochrome *P*-450 IIB1 activity is not dose dependent (0–5 IU).

In our previous study (24), we found that dietary α-tocopherol acetate selectively affects hepatic cytochrome PROD activity but not hepatic cytochrome *N*-nitrosodimethylamine demethylase activity. Changes in activities of cytochrome *P*-450s play an important role in carcinogenesis. Phenobarbital is an effective hepatopromoter and inducer of hepatic cytochrome *P*-450s. In this study, we investigate whether the modulatory effect of dietary α-tocopherol acetate on hepatic cytochrome PROD activity is influenced by the presence of phenobarbital. A fasted-refed model was employed following the methods of Wade and co-workers (25), who reported that the effect of experimental diets on hepatic cytochrome *P*-450 activity can be observed within a short period.

Materials and Methods

Animal Treatment and Microsome Preparation

Weanling male Sprague-Dawley rats were purchased from the National Animal Breeding and Research Center (Taipei, Taiwan). The rats were housed in individual stainless steel cages in air-conditioned quarters (22°C) with a 12:12-hour light-dark cycle. The rats were fed the AIN-76A rodent diet for four days, then fasted for two days. Sixteen rats were then randomly assigned to four groups and received diets that were α-tocopherol acetate deficient, adequate, or supplemented with 5 and 15 g/kg α-tocopherol acetate for four days (Table 1). All rats were provided ad libitum access to feed and tap water for four days before they were sacrificed by an overdose of CO_2. All rats were injected with phenobarbital at a daily dose of 75 mg/kg body wt ip for three days before sacrifice. After sacrifice, liver cytosolic and microsomal fractions of rats were prepared by differential centrifugation (26).

Determination of Cytochrome *P*-450 Activities and Protein Concentration

Microsomal total cytochrome *P*-450 content and NADPH cytochrome *P*-450 reductase activity were determined by the methods of Omura and Sato (27) and Phillips and Langdon (28), respectively. The activity of PROD was assayed according to the method of Lubet and colleagues (29). A fluorescence spectrophotometer (model F4500, Hitachi, Tokyo, Japan) was used to measure resorufin at 515-nm excitation and 555-nm emission with the entrance and exit slits set at 10 nm. Cytosolic GST activity was determined by the method of Habig and associates (30). Sample and reference cuvettes were read for five minutes in a dual-beam spectro-

Table 1. Composition of Experimental Diets

	Diet			
			Supplemented	
Ingredient	Deficient	Adequate	5 g/kg	15 g/kg
Corn oil, tocopherol-stripped	17			
Corn oil		17	17	17
Casein	20	20	20	20
Sucrose	23	23	23	23
Cornstarch	15	15	15	15
Cellulose	5	5	5	5
AIN-76 mineral mixture	3.5	3.5	3.5	3.5
Vitamin mixture, tocopherol devoid	1			
AIN-76 vitamin mixture		1	1	1
DL-Methionine	0.3	0.3	0.3	0.3
α-Tocopherol acetate			0.425	1.275
Choline bitartrate	0.2	0.2	0.2	0.2

photometer set at 340 nm. Activity was expressed as nanomoles of 1-chloro-2,4-dinitrobenzene conjugate formed per milligram of protein per minute. Protein was measured by the method of Lowry and others (31).

Determination of Hepatic and Plasma α-Tocopherol Concentration and Hepatic Lipid Peroxidation

Hepatic and plasma α-tocopherol concentrations were determined using a modification of the procedure of Catignani and Bieri (32). Fifty microliters of internal standard (α-tocopheryl acetate in ethanol) and hepatic homogenate (100 µl taken from 0.1 g of liver and 1 ml of 50 mM potassium phosphate buffer, pH 7.0) or 100 µl of plasma were mixed by vortexing for one minute. To extract the lipid, 200 µl of high-performance liquid chromatography (HPLC)-grade hexane were added and mixed for an additional one minute. Phases were separated by centrifugation at 2,000 rpm for two minutes. The hexane layer was withdrawn and evaporated under nitrogen. The residue was redissolved in 50 µl of filtered HPLC-grade methanol by mixing, and 20 µl of the mixture were injected into an HPLC instrument. The HPLC instrument was purchased from Hitachi and consisted of a model L-6200A intelligent pump, a model L-4200 UV-VIS detector, a model D-6000 interface, and an LC organizer. The column was 3.9 mm × 30 cm stainless steel packed with micro Bondapak C_{18}. A 3 × 22 mm guard column (precolumn) packed with Bondapak C_{18} was attached to the primary column. The detector wavelength was 290 nm, with a sensitivity of 0.01 absorbance unit full scale. The solvent was 100% HPLC-grade methanol, and the flow rate was 1.2 ml/min. Peak-to-area ratios of samples were converted to micrograms of α-tocopherol using a standard curve prepared with samples containing a constant amount of α-tocopherol acetate combined with different amounts of α-tocopherol standard. Plasma total lipid was determined by the sulfo-phospho-vanillin reaction, as described by Frings and Dunn (33).

Liver lipid peroxidation was measured by assaying thiobarbituric acid-reactive substance (TBARS) using a modification of the procedure described by Fraga and co-workers (34). Liver samples were homogenized in 50 mM potassium phosphate buffer (pH 7.4). To the liver homogenate, 0.5 ml of 3% sodium dodecyl sulfate (SDS), 2 ml of 0.1 N HCl, 0.3 ml of 10% phosphotungstic acid, and 1 ml of 0.7% 2-thiobarbituric acid were added. The mixture was heated in boiling water for 30 minutes, then TBARS were extracted into 5 ml of 1-butanol. After centrifugation, the fluorescence of the butanol layer was measured at 515-nm excitation and 555-nm emission in a fluorescence spectrophotometer (model F4500, Hitachi). The values were expressed in nanomoles per gram of liver. A malondialdehyde standard curve was also prepared using 1,1,3,3-tetramethoxypropane.

Gel Electrophoresis

SDS polyacrylamide gels made with 10% acrylamide were prepared as described by Laemmli (35). For cytochrome *P*-450 IIB1, 1 µg of liver microsomal protein from 16 rats or 1 µg of pooled liver microsomal protein from four rats in each group was applied to each gel. After electrophoresis, proteins separated on SDS-polyacrylamide gels were transferred to polyvinylidene difluoride membranes. The nonspecific binding sites on the polyvinylidine difluoride membranes were blocked with normal goat serum in 15 mM Tris-150 mM NaCl buffer (pH 7.4) at 4°C overnight. Polyclonal antibodies against cytochrome *P*-450 IIB1 were obtained from Chemicon International (Temecula, CA). An avidin-peroxidase and biotinylated anti-mouse IgG kit were used to detect the immunoreactive bands. Each incubation with primary antibody, secondary biotinylated antibody, and avidin-peroxidase complex was performed at 37°C for 30 minutes. For color development, hydrogen peroxide and 3,3′-diaminobenzidine tetrachloride were used as the substrates for peroxidase. The density of each band was quantitated by AlphaImager 2000 (Alpha Innotech, San Leandro, CA).

Statistical Analysis

All analyses were conducted in duplicate for each sample. Data were analyzed by analysis of variance (SAS Institute, Cary, NC). Duncan's test was used to evaluate the significance between means; $p < 0.05$ was considered to be statistically significant.

Results

Hepatic and Plasma α-Tocopherol Concentrations and Hepatic Lipid Peroxidation

Dietary α-tocopherol acetate significantly and dose dependently affected hepatic and plasma α-tocopherol concentrations ($p < 0.05$). Rats fed diets supplemented with 5 and 15 g/kg α-tocopherol acetate had significantly greater hepatic α-tocopherol content than rats fed α-tocopherol acetate-deficient and -adequate diets ($p < 0.05$), and the α-tocopherol acetate supplementation of 15 g/kg resulted in significantly greater hepatic α-tocopherol content in rats than the α-tocopherol acetate supplementation of 5 g/kg ($p < 0.05$). Plasma α-tocopherol concentration was increased significantly by increasing dietary α-tocopherol acetate level ($p < 0.05$) (Table 2). Liver lipid peroxidation measured as TBARS formation was dose dependently inhibited by increasing dietary α-tocopherol acetate level. Rats fed a diet deficient in α-tocopherol acetate had significantly greater hepatic lipid peroxidation than rats fed an α-tocopherol acetate-adequate diet or diets supplemented with 5 or 15 g/kg α-tocopherol acetate ($p < 0.05$). Rats fed α-tocopherol acetate-adequate diets had significantly greater hepatic lipid peroxidation than rats fed diets supplemented with 15 g/kg α-tocopherol acetate ($p < 0.05$), but the difference between rats fed control and 5 g/kg α-tocopherol acetate-supplemented diets was not statistically significant (Table 2).

Table 2. Effect of Dietary α-Tocopherol Acetate Concentration on Hepatic and Plasma α-Tocopherol Concentrations and Lipid Peroxidation in the Presence of Phenobarbital[a,b]

Group	α-Tocopherol		TBARS,
	μg/g liver	mg/g total lipid	nmol/g liver
Deficient	ND[§]	1.01 ± 0.07[§]	467 ± 27*
Adequate	6.7 ± 2.0[‡]	2.86 ± 0.42[‡]	151 ± 35[†]
Supplemented			
5 g/kg	71.7 ± 14.6[†]	6.95 ± 1.13[†]	119 ± 14[†,‡]
15 g/kg	320.7 ± 69.7*	9.23 ± 0.91*	100 ± 10[‡]

a: Values are means ± SD of 4 rats in each group. ND, nondetectable; TBARS, thiobarbituric acid-reactive substances.

b: Groups not sharing a symbol (*,†,‡,§) are significantly different ($p < 0.05$).

Hepatic Cytochrome *P*-450 Enzymes and GST Activities

Rats fed diets supplemented with 5 g/kg α-tocopherol acetate had significantly greater hepatic total cytochrome *P*-450 content than rats fed α-tocopherol acetate-deficient or -adequate diets ($p < 0.05$); also rats fed diets deficient in α-tocopherol acetate had significantly greater hepatic total cytochrome *P*-450 content than rats fed α-tocopherol acetate-adequate diets ($p < 0.05$). The effect of dietary α-tocopherol acetate on hepatic total cytochrome *P*-450 content was not dose dependent (Table 3). Hepatic cytochrome *P*-450 reductase activity of rats fed diets supplemented with 5 g/kg α-tocopherol acetate was significantly greater than that of rats fed an α-tocopherol acetate-adequate diet or a diet supplemented with 15 g/kg α-tocopherol acetate ($p < 0.05$) but was not significantly different from that of rats fed α-tocopherol acetate-deficient diets (Table 3). Rats fed diets supplemented with 5 or 15 g/kg α-tocopherol acetate had significantly greater hepatic cytochrome PROD activity than rats fed α-tocopherol acetate-deficient or -adequate diets ($p < 0.05$), and the hepatic cytochrome PROD activity of rats fed diets deficient in α-tocopherol acetate was significantly greater than that of rats fed α-tocopherol acetate-adequate diets ($p < 0.05$) (Table 3). Hepatic cytosolic GST activity was significantly greater in rats fed α-tocopherol acetate-adequate diets than in rats fed α-tocopherol acetate-deficient diets or diets supplemented with 5 or 15 g/kg α-tocopherol acetate ($p < 0.05$) (Table 3).

Cytochrome *P*-450 IIB1 Protein Level

Western blotting analysis showed that rats fed diets supplemented with 5 g/kg α-tocopherol acetate had a significantly higher level of cytochrome *P*-450 IIB1 than rats fed α-tocopherol acetate-deficient or -adequate diets in the presence of phenobarbital (Table 4). Hepatic cytochrome *P*-450 *IIB1* protein level of pooled liver microsomes from four dietary groups showed a pattern consistent with the quantified protein level presented in Table 4 (Figure 1). These results were similar to the activity measurement.

Discussion

In the present study, a fasted-refed model was used as described by Wade and colleagues (25), whose experiment showed that the effect of experimental diets on hepatic cytochrome *P*-450 activity can be observed within a short period. The results of a previous study (17) and this study demonstrate that the experimental diets can efficiently affect tissue level by using this model. Plasma and liver α-tocopherol level reflected the dietary α-tocopherol acetate content. In contrast, liver lipid peroxidation, as determined by TBARS formation, was negatively correlated with dietary α-tocopherol acetate content. These results are in agreement with that of previous studies which reported that tissue α-tocopherol content can be efficiently affected by dietary α-tocopherol acetate level and that dietary α-tocopherol acetate is effective in the inhibition of tissue lipid peroxidation (8,9,36).

Rats fed diets supplemented with 5 or 15 g/kg α-tocopherol acetate had significantly greater hepatic total cytochrome *P*-450 content than rats fed control diets ($p < 0.05$). Although several previous studies have reported that α-tocopherol or α-tocopherol acetate can affect hepatic total cytochrome *P*-450 content (20–22), other studies found no effect (23,24). In this study, higher dosages of α-tocopherol acetate (supplementation of 5 and 15 g/kg) resulted in greater hepatic total cytochrome *P*-450 content than the lower dosages of α-tocopherol acetate (α-tocopherol acetate-deficient

Table 3. Effect of Dietary α-Tocopherol Acetate Concentration on Hepatic Total Cytochrome *P*-450 Content, Cytochrome NADPH *P*-450 Reductase, Cytochrome PROD, and GST Activities in the Presence of Phenobarbital[a,b]

Group	Total *P*-450 Content, nmol/mg protein	NADPH *P*-450 Reductase Activity, nmol·min^{-1}·mg protein^{-1}	PROD Activity, pmol·min^{-1}·mg protein^{-1}	GST Activity, nmol·min^{-1}·mg protein^{-1}
Deficient	1.1 ± 0.1[†]	574 ± 57*,[†]	572 ± 33[†]	2,289 ± 256[†]
Adequate	0.7 ± 0.1[‡]	533 ± 76[†]	445 ± 60[‡]	3,317 ± 395*
Supplemented				
5 g/kg	1.3 ± 0.1*	623 ± 36*	1,252 ± 131*	2,269 ± 116[†]
15 g/kg	1.2 ± 0.1*,[†]	514 ± 43[†]	1,177 ± 68*	2,565 ± 170[†]

a: Values are means ± SD of 4 rats in each group. PROD, pentoxyresorufin *O*-dealkylase; GST, glutathione *S*-transferase.

b: Groups not sharing a symbol (*,†,‡) are significantly different ($p < 0.05$).

Table 4. Effect of Dietary α-Tocopherol Acetate Concentration on Cytochrome *P*-450 IIB1 Protein Level in the Presence of Phenobarbital[a,b]

Group	Protein Level, IDV
Deficient	20,020 ± 1,333†
Adequate	14,960 ± 1,333‡
Supplemented	
5 g/kg	24,640 ± 2,806*
15 g/kg	22,440 ± 1,512*,†

a: Values are means ± SD of 4 rats in each group. IDV, integrated density value.

b: Groups not sharing a symbol (*,†,‡) are significantly different ($p < 0.05$).

and -adequate diets). However, the effect of dietary α-tocopherol acetate on hepatic total cytochrome *P*-450 content was not dose dependent. Our result was in agreement with that of Murray (21), but in contrast to that of Williams and co-workers (22). This may have been due to differences in experimental period and dosage of α-tocopherol acetate. Hepatic NADPH cytochrome *P*-450 reductase activity was significantly greater in rats fed diets supplemented with 5 g/kg α-tocopherol acetate than in rats fed an α-tocopherol acetate-adequate diet or a diet supplemented with 15 g/kg α-tocopherol acetate ($p < 0.05$). The hepatic NADPH cytochrome *P*-450 reductase activity was not increased with increasing dietary α-tocopherol acetate. Hepatic cytochrome PROD activity was significantly greater in rats fed diets supplemented with 5 or 15 g/kg α-tocopherol acetate than in rats fed α-tocopherol acetate-deficient or -adequate diets in the presence of phenobarbital induction ($p < 0.05$). In our previous study (24), we found that rats fed α-tocopherol acetate-adequate and -supplemented diets had significantly greater hepatic cytochrome PROD activity than rats fed α-tocopherol acetate-deficient diets ($p < 0.05$). These results suggest that α-tocopherol acetate may play an important role in maintaining hepatic cytochrome PROD activity in the presence or absence of cytochrome *P*-450 IIB1 inducer (e.g., phenobarbital). However, the level of α-tocopherol acetate required to maintain cytochrome PROD activity seems to be greater in the presence than in the absence of phenobarbital. The capability of α-tocopherol acetate to maintain hepatic cytochrome PROD activity seems not to be related to its inhibition of lipid peroxidation. Rats fed α-tocopherol acetate-deficient diets had significantly greater liver lipid peroxidation than rats fed α-tocopherol acetate-adequate diets; however, the hepatic cytochrome PROD activity was significantly greater in rats fed α-tocopherol acetate-deficient diets than in rats fed α-tocopherol acetate-adequate diets ($p < 0.05$). The ability of α-tocopherol acetate to maintain hepatic cytochrome PROD activity needs to be studied in future experiments. The lack of correlation between hepatic total cytochrome *P*-450 content and PROD activity is that the total cytochrome *P*-450 content measures total isoforms of cytochrome *P*-450 rather than a specific cytochrome *P*-450 isoform. Cytosolic GST activity was significantly greater in rats fed α-tocopherol acetate-adequate diets than in rats fed α-tocopherol acetate-deficient diets or diets supplemented with 5 or 15 g/kg α-tocopherol acetate ($p < 0.05$). In our previous study (24), we found that the dietary α-tocopherol acetate adequacy or supplementation can enhance cytosolic GST activity compared with α-tocopherol acetate deficiency. However, this relationship was not observed in the present study, and this may have been due to the interaction between α-tocopherol acetate and phenobarbital.

The results presented in Table 4 show that a diet supplemented with 5 g/kg α-tocopherol acetate significantly enhanced cytochrome *P*-450 IIB1 protein level compared with α-tocopherol acetate-deficient or -adequate diets ($p < 0.05$). The Western blotting results were similar to the results of activity measurement, although not completely consistent. The difference between these two assays is that cytochrome PROD activity includes cytochrome *P*-450 IIB1 and IIB2 activities, and the effect of cytochrome *P*-450 IIB2 cannot be excluded.

In summary, the results suggest that dietary α-tocopherol acetate can enhance cytochrome PROD activity in the presence or absence of phenobarbital, according to the data of this and a previous study (24). In the presence of phenobarbital, however, the amount of dietary α-tocopherol acetate needed to enhance cytochrome PROD activity is greater than that in the absence of phenobarbital. Additional studies are needed to determine the role of dietary α-tocopherol acetate in chemical detoxification that is specifically metabolized by cytochrome *P*-450 IIB1.

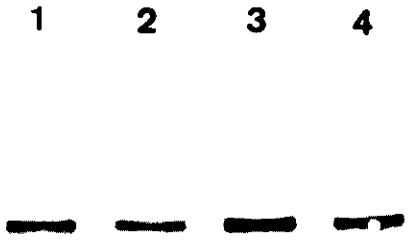

Figure 1. Western blot analysis of liver microsomal protein samples against anti-cytochrome *P*-450 IIB1. 1, Deficient; 2, adequate, 3, supplemented with 5 g/kg α-tocopherol acetate; 4, supplemented with 15 g/kg α-tocopherol acetate.

Acknowledgments and Notes

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References

1. Machlin, LJ: Vitamin E. In *Handbook of Vitamins*, LJ Machlin (ed). New York: Dekker, 1991, pp 99–144.
2. Burton, GW, Joyce, A, and Ingold, KU: Is vitamin E the only lipid-soluble, chain-breaking antioxidant in human blood plasma and erythrocyte membranes? *Arch Biochem Biophys* **221,** 281–290, 1983.
3. McCay, PB: Vitamin E: interactions with free radicals and ascorbate. *Annu Rev Nutr* **5,** 323–340, 1985.
4. Frankel, EN: The antioxidant and nutritional effects of tocopherols, ascorbic acid and β-carotene in relation to processing of edible oils. In *Nutritional Impact of Food Processing*, JC Somagyi and HR Muller (eds). Basel: Karger, 1989, pp 297–312.
5. Wefers, H, and Sies, H: Antioxidant defense: vitamin E and C and β-carotene. In *Oxy Radicals in Molecular Biology and Pathology*, PA Cerutti, I Fridovich, and JM McCord (eds). New York: Liss, 1988, pp 481–490.
6. Willson, RL: Free radical protection: why vitamin E, not vitamin C, β-carotene or glutathione? In *Biology of Vitamin E*, R Porter and J Whelan (eds). London: Pitman, 1983, pp 19–44.
7. Stam, H, Hulsmann, WC, Jongkind, JF, van der Kraaij, AMM, and Koster, JF: Endothelial lesions, dietary composition and lipid peroxidation. *Eicosanoids* **2,** 1–14, 1989.
8. Lii, CK, Ko, JJ, and Chen, HW: No inhibition of γ-glutamyl transpeptidase-positive foci by vitamin E with or without phenobarbital. *Nutr Cancer* **27,** 200–205, 1997.
9. Cho, S-H, and Choi, Y-S: Lipid peroxidation and antioxidant status is affected by different vitamin E levels when feeding fish oil. *Lipids* **29,** 47–52, 1994.
10. Guengerich, FP: Oxidation of toxic and carcinogenic chemicals by human cytochrome *P*-450 enzymes. *Chem Res Toxicol* **4,** 391–407, 1991.
11. Conney, AH: Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons: G. H. A. Clowes Memorial Lecture. *Cancer Res* **42,** 4875–4917, 1982.
12. Conney, AH: Pharmacological implications of microsomal enzyme induction. *Pharmacol Res* **19,** 317–366, 1967.
13. Guengerich, FP: Reactions and significance of cytochrome *P*-450 enzymes. *J Biol Chem* **266,** 10019–10022, 1991.
14. Bidlack, WR, Brown, RC, and Mohan, C: Nutritional parameters that alter hepatic drug metabolism, conjugation, and toxicity. *Fed Proc* **45,** 142–148, 1986.
15. Gonzalez, FJ: The molecular biology of cytochrome *P*450s. *Pharmacol Rev* **40,** 243–288, 1988.
16. Wrighton, SA, and Stevens, JC: The human hepatic cytochromes *P*450 involved in drug metabolism. *Crit Rev Toxicol* **22,** 1–22, 1992.
17. Chen, H-W, Lii, C-K, Wu, M-H, Ou, C-C, and Sheen, L-Y: Amount and type of dietary lipid modulate rat hepatic cytochrome *P*-450 activity. *Nutr Cancer* **29,** 174–180, 1997.
18. Rice, JM, Diwan, BA, Hu, H, Ward, JM, Nims, RW, et al.: Enhancement of hepatocarcinogenesis and induction of specific cytochrome *P*450-dependent monooxygenase activities by the barbiturates allobarbital, aprobarbital, pentobarbital, secobarbital and 5-phenyl- and 5-ethylbarbituric acids. *Carcinogenesis* **15,** 395–402, 1994.
19. Campbell, TC, and Hayes, JR: Role of nutrition in the drug-metabolizing enzyme system. *Pharmacol Rev* **26,** 171–197, 1974.
20. Suntres, ZE, Hepworth, SR, and Shek, PN: Protective effect of liposome-associated α-tocopherol against paraquat-induced acute lung toxicity. *Biochem Pharmacol* **44,** 1811–1818, 1992.
21. Murray, M: *In vitro* and *in vivo* studies of the effect of vitamin E on microsomal cytochrome *P*450 in rat liver. *Biochem Pharmacol* **42,** 2107–2114, 1991.
22. Williams, DE, Carpenter, HM, Buhler, DR, Kelly, JD, and Dutchuk, M: Alterations in lipid peroxidation, antioxidant enzymes, and carcinogen metabolism in liver microsomes of vitamin E-deficient trout and rat. *Toxicol Appl Pharmacol* **116,** 78–84, 1992.
23. Cassand, P, Decoudu, S, Leveque, F, Daubeze, M, and Narbonne, JF: Effect of vitamin E deficiency intake on *in vitro* activation of aflatoxin B_1. *Mutat Res* **319,** 309–316, 1993.
24. Chen, HW, Lii, CK, Sung, WC, and Ko, YJ: Effect of vitamin E on rat hepatic cytochrome *P*-450 activity. *Nutr Cancer* **31,** 178–183, 1998.
25. Wade, AE, White, RA, Walton, LC, and Bellows, JT: Dietary fat—a requirement for induction of mixed function oxidase activities in starved-refed rats. *Biochem Pharmacol* **34,** 3747–3754, 1985.
26. Huang, CJ, Cheung, NS, and Lu, VR: Effects of deteriorated frying oil and dietary protein levels on liver microsomal enzymes in rats. *J Am Oil Chem Soc* **65,** 1796–1803, 1988.
27. Omura, T, and Sato, R: The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J Biol Chem* **239,** 2370–2378, 1964.
28. Phillips, AH, and Langdon, RG: Hepatic triphosphopyridine nucleotide-cytochrome *c* reductase: isolation, characterization, and kinetic studies. *J Biol Chem* **237,** 2652–2660, 1962.
29. Lubet, RA, Mayer, RT, Cameron, JW, Nims, RW, Burke, MD, et al.: Dealkylation of pentoxyresorufin: a rapid and sensitive assay for measuring induction of cytochrome(s) *P*-450 by phenobarbital and other xenobiotics in the rat. *Arch Biochem Biophys* **238,** 43–48, 1985.
30. Habig, WH, Pabst, MJ, and Jakoby, WB: Glutathione *S*-transferase. The first enzymatic step in mercapturic acid formation. *J Biol Chem* **249,** 7130–7139, 1974.
31. Lowry, O, Rosebrough, N, Farr, N, and Randall, R: Protein measurement with the Folin phenol reagent. *J Biol Chem* **193,** 265–275, 1951.
32. Catignani, GL, and Bieri, JG: Simultaneous determination of retinol and α-tocopherol in serum or plasma by liquid chromatography. *Clin Chem* **29,** 708–712, 1983.
33. Frings, CS, and Dunn, RT: A colorimetric method for determination of total serum lipids based on the sulfo-phospho-vanillin reaction. *Am J Clin Pathol* **53,** 89–91, 1970.
34. Fraga, CG, Leibovitz, BE, and Tappel, AL: Lipid peroxidation measured as thiobarbituric acid-reactive substances in tissue slices: characterization and comparison with homogenates and microsomes. *Free Radical Biol Med* **4,** 155–161, 1988.
35. Laemmli, UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685, 1970.
36. Chen, HW, Lii, CD, Wang, ML, Ou, CC, and Wang, MF: Effects of vitamin E deficiency and dietary linoleate on serum thromboxane synthesis in male Sprague-Dawley rats. *Prostaglandins Leukot Essent Fatty Acids* **53,** 405–411, 1995.

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Prostaglandin E_2 Is Involved in the Increase of Cytochrome *P*-450 2B1 Expression by α-Tocopheryl Succinate in Primary Rat Hepatocytes in the Presence of Phenobarbital

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Abstract: The modulation of cytochrome P-*450 2B1 expression by α-tocopheryl succinate and whether prostaglandin E_2 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 E_2-synthesizing capability. α-Tocopheryl succinate inhibited prostaglandin E_2 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 E_2 from nonparenchymal cells, various concentrations of prostaglandin E_2 were added to the cell culture. High doses of exogenous prostaglandin E_2 (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 E_2 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-tocopheryl succinate increases cytochrome* P-*450 2B1 expression via its inhibition of prostaglandin E_2 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/antiseizure 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-tocopheryl 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-tocopheryl 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 E_2 production in macrophages (17). Prostaglandin E_2 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

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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 E_2 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 Williams' E medium using low-speed centrifugation. Cell viability was >90% as determined by trypan blue exclusion. Hepatocytes were plated at 0.6×10^6 cells/ml in 5 ml of complete Williams' E medium on 60-mm plastic tissue culture dishes coated with type III collagen. The initial concentration of dexamethasone was 1 μM; after the 3-h attachment period, the dexamethasone concentration was reduced to 0.1 μM. The reason for reduction in dexamethasone level was that dexamethasone can modulate the gene expression in vivo and in vitro (25,26). Medium was changed on a daily basis.

Matrigel Overlay

A stock solution of Matrigel (5 mg/ml) was prepared, and a dilute concentration (233 μg/ml final concentration) was added as an overlay at 4 h after plating.

Gene Induction Treatment

After 48 h of plating, cells were treated with various concentrations (0–50 μM) of α-tocopheryl succinate. After another 4 h, 0.1 mM phenobarbital was added to the cells to induce the cytochrome *P*-450 2B1 expression for another 20 h. To investigate the effect of exogenous prostaglandin E_2 on cytochrome *P*-450 2B1 expression, cells were pretreated with various concentrations of prostaglandin E_2 (0–1,000 nM) for 15 min before the addition of 0.1 mM phenobarbital.

Intracellular α-Tocopherol Determination

α-Tocopherol content of cells was determined using a modification of the procedure of Catignani and Bieri (27). Briefly, medium was aspirated, and cells were 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′-GGATGGGAAAGAGGAGTGTGGA-3′ (forward) and 5′-CTGGAGGATGGTGGTGAAGAAG-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-^{32}P]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 NaH_2PO_4, 5 mM EDTA), 2% sodium dodecyl sulfate (SDS), 50% foramide, and 100 μg/ml of single-strand sheared salmon sperm DNA. The membrane was then hybridized in the same solution with ^{32}P-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 polyvinylidine 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 E_2 Synthesis of Hepatocytes

The capability of hepatocytes to synthesize prostaglandin E_2 was determined by measuring the prostaglandin E_2 released into the medium. The assay was performed by using the prostaglandin E_2 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 E_2, 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

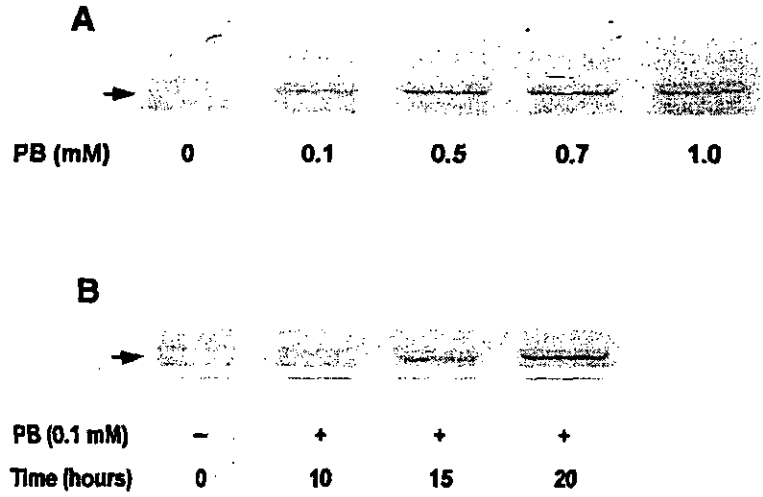

Figure 1. Western blot analyses of cytochrome *P*-450 2B1 expression. A: dose response of cytochrome *P*-450 2B1 expression to various concentrations of phenobarbital (PB). B: time course of phenobarbital-induced cytochrome *P*-450 2B1 expression.

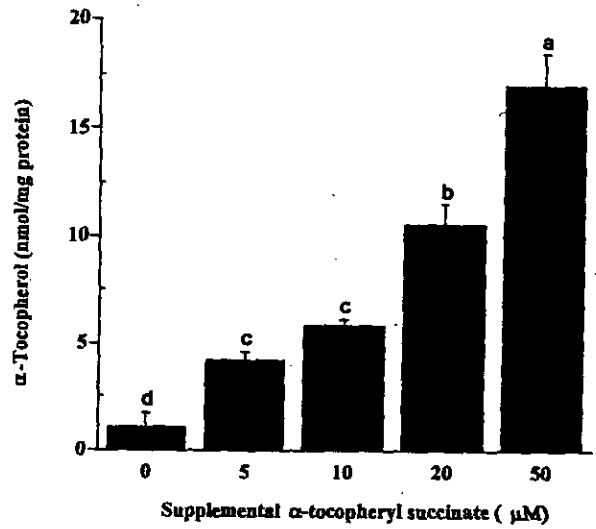

Figure 2. Dose response of α-tocopheryl succinate incorporation into hepatocytes. Values are means ± SD of 3 independent experiments, each measured in duplicate by high-performance liquid chromatography. Groups not sharing the same letter (a–d) are significantly different ($P < 0.05$).

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 E_2 Synthesis by Hepatocytes

On the basis of previous evidence that prostaglandin E_2 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 E_2 synthesis. To test this hypothesis, the prostaglandin E_2-synthesizing capability of hepatocytes and the effect of α-tocopheryl succinate on hepatocyte prostaglandin E_2 synthesis were studied. In this study, hepatocytes were found to have the capability to synthesize prostaglandin E_2, and α-tocopheryl succinate significantly inhibited prostaglandin E_2 synthesis by hepatocytes (Fig. 4). There

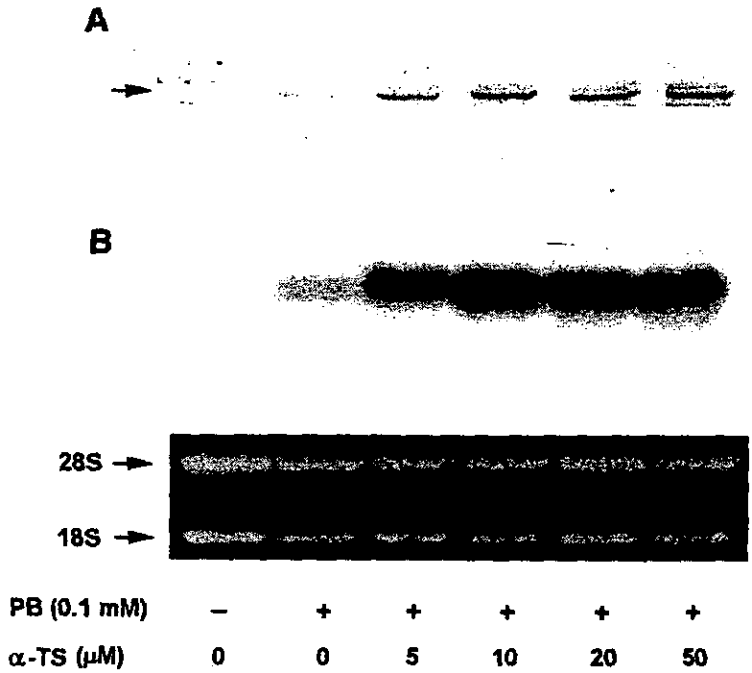

Figure 3. Effects of various concentrations of α-tocopheryl succinate (α-TS) on cytochrome *P*-450 2B1 expression in the presence of phenobarbital. A: Western blot analyses. B: Northern blot analyses; ribosomal 18S and 28S RNA hybridization levels were used as normalization standards.

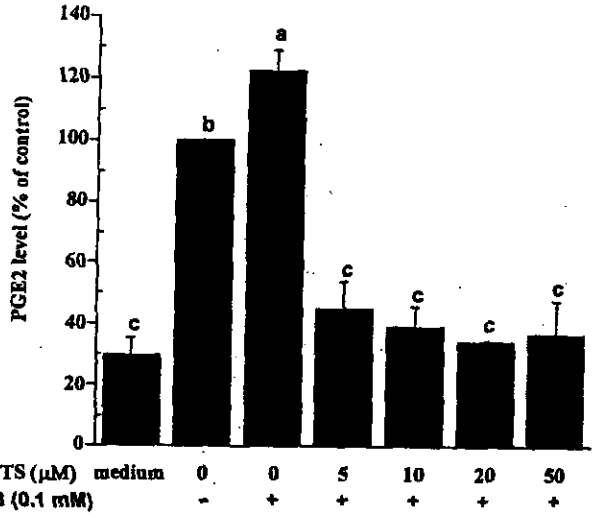

Figure 4. Effects of various concentrations of α-tocopheryl succinate on prostaglandin E_2 (PGE_2)-synthesizing capability of hepatocytes in the presence of phenobarbital. Values are means ± SD of 3 independent experiments. Prostaglandin E_2 level of no α-tocopheryl succinate and phenobarbital addition group was 104.8 ± 10.1 pg/ml, and it was regarded as 100%. Groups not sharing the same letter (a–c) are significantly different ($P < 0.05$).

was a 60–70% decrease in prostaglandin E_2 release of cells coincubated with α-tocopheryl succinate and phenobarbital compared with those incubated with phenobarbital only.

Effect of Exogenous Prostaglandin E_2 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 E_2 on cytochrome *P*-450 2B1 expression of hepatocytes, exogenous prostaglandin E_2 was added to hepatocytes. Results of Western and Northern blot analysis consistently showed that exogenous prostaglandin E_2 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 E_2 (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 E_2 on Intracellular cAMP Level

Although α-tocopheryl succinate suppressed the prostaglandin E_2-synthesizing capability of hepatoyctes, intracellular cAMP level was not significantly different among hepatocytes treated with various concentrations of α-tocopheryl succinate (Fig. 6). This result indicates that the effect of α-tocopheryl succinate on cytochrome *P*-450 2B1 expression induced by phenobarbital was not related to the changes in intracellular cAMP level. Prostaglandin E_2 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 E_2 (Fig. 7). These results suggest that hepatocytes possess adenylyl cyclase activity, and the effect of prostaglandin E_2 on intracellular cAMP level was not observed.

Discussion

In our previous animal studies, we found that dietary α-tocopheryl 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-tocopheryl 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-tocopheryl 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-tocopheryl 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-tocopheryl succinate was added to hepatocytes than when no α-tocopheryl succinate was added. Western and Northern blot analyses showed consistent results (Fig. 3).

Hepatocytes were demonstrated to have prostaglandin E_2-synthesizing capability. The prostaglandin E_2-synthesizing capability of hepatocytes was significantly inhibited by α-tocopheryl 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 E_2 was <22 fmol/10^6 cells/30 min. The inhibition of prostaglandin E_2 synthesis in macrophages by vitamin E was demonstrated in a previous study (17). Our result that α-tocopheryl succinate inhibited prostaglandin E_2 synthesis of hepatocytes is consistent with this finding. In macrophages (17), vitamin E had decreased prostaglandin E_2-synthesizing capability. On the basis of aforementioned findings, the effect of α-to-

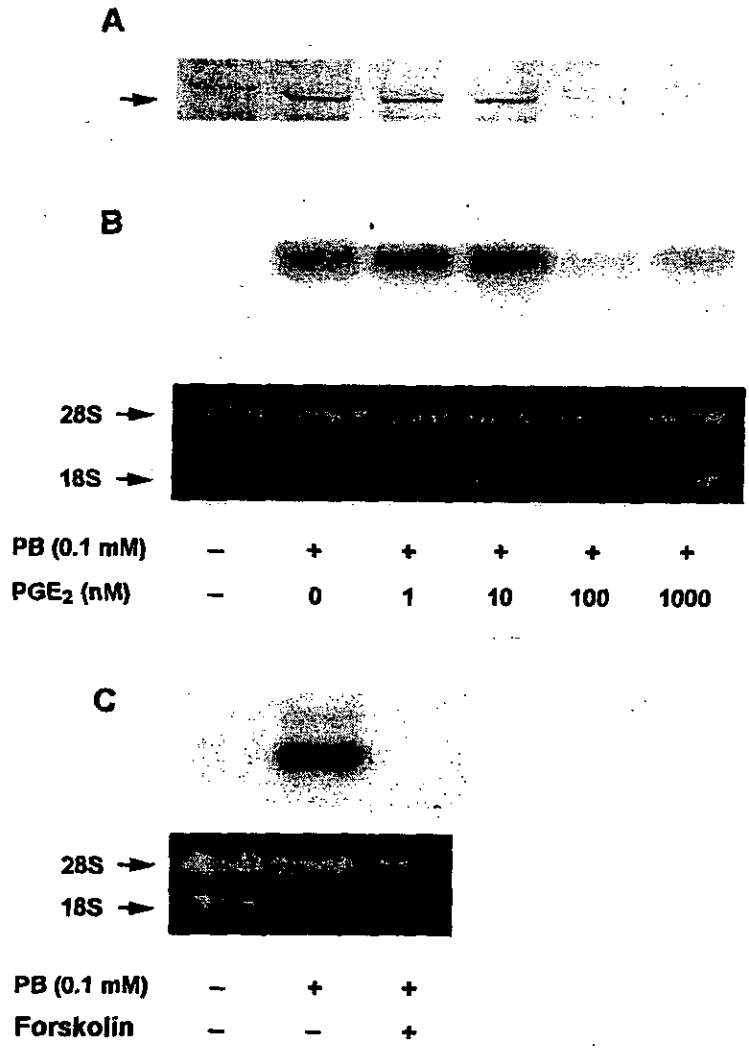

Figure 5. Effects of various concentrations of prostaglandin E_2 and forskolin on cytochrome *P*-450 2B1 expression in the presence of phenobarbital. A: Western blot analyses. B: Northern blot analyses; ribosomal 18S and 28S RNA hybridization levels were used as normalization standards. C: Northern blot analyses for forskolin (0.1 mM). Similar results were observed in 3 independent experiments.

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 E_2 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 E_2, and this alteration was found to be responsible for the physiological and pathological end points of prostaglandin E_2 (18,19). Although the prostaglandin E_2-synthesizing capability of hepatoyctes 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 E_2-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 E_2 on intracellular cAMP level was studied using forskolin as the positive control. We found that intracellular cAMP level was not affected by prostaglandin E_2; 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 E_2 (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-

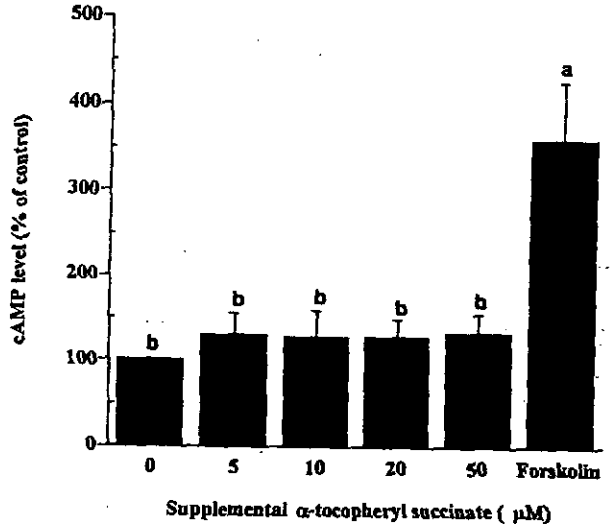

Figure 6. Effect of various concentrations of α-tocopheryl succinate on intracellular cAMP level; 0.1 mM forskolin was used as positive control in the presence of phenobarbital. Values are means ± SD of 3 independent experiments, each measured in triplicate by enzyme-linked immunosorbent assay. Intracellular cAMP level of no α-tocopheryl succinate and phenobarbital addition group was 79.3 ± 1.5 fmol/μg protein, and it was regarded as 100%. Groups not sharing the same letter (a, b) are significantly different ($P < 0.05$).

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

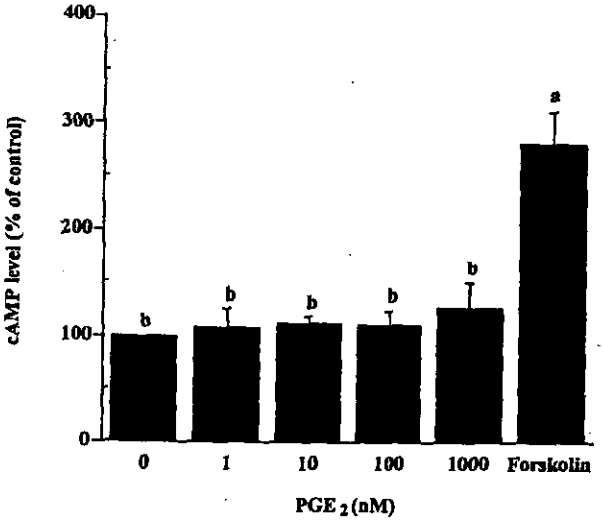

Figure 7. Effects of various concentrations of prostaglandin E_2 and forskolin (0.1 mM) on intracellular cAMP level in the presence of phenobarbital. Values are means ± SD of 3 independent experiments, each measured in triplicate by enzyme-linked immunosorbent assay. Intracellular cAMP level of no α-tocopheryl succinate and phenobarbital addition group was 83.1 ± 35.4 fmol/μg protein, and it was regarded as 100%. Groups not sharing the same letter (a, b) are significantly different ($P < 0.05$).

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 α-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

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References

1. Conney AH: Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons: G. H. A. Clowes Memorial Lecture. *Cancer Res* **42**, 4875–4917, 1982.
2. Bidlack WR, Brown RC, and Mohan C: Nutritional parameters that alter hepatic drug metabolism, conjugation, and toxicity. *Fed Proc* **45**, 142–148, 1986.
3. Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, et al.: P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* **6**, 1–42, 1996.
4. Okey AB: Enzyme induction in the cytochrome *P*-450 system. *Pharmacol Ther* **45**, 241–298, 1990.
5. Nelson DR, Kamataki T, Waxman DJ, Guengerich FP, Estabrook RW, et al.: The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA Cell Biol* **12**, 1–51, 1993.
6. Ishihara H, Martin BL, Brautigan DL, Karaki H, Ozaki H, et al.: Calyculin A and okadaic acid: inhibitors of protein phosphatase activity. *Biochem Biophys Res Commun* **159**, 871–877, 1989.
7. Ormandy GC and Hope RS: Pertussis toxin potentiates seizures induced by pilocarpine, kainic acid and *N*-methyl-D-aspartate. *Brain Res* **553**, 51–57, 1991.
8. Hardwick JP, Gonzalez FJ, and Kasper CB: Transcriptional regulation of rat liver epoxide hydratase, NADPH-cytochrome *P*-450 oxidoreductase, and cytochrome *P*-450b genes by phenobarbital. *J Biol Chem* **258**, 8081–8085, 1983.
9. Shinozuka H, Lombardi B, and Abanobi SE: A comparative study of the efficacy of four barbiturates as promoters of the development of γ-glutamyltranspeptidase-positive foci in the liver of carcinogen-treated rats. *Carcinogenesis* **3**, 1017–1020, 1982.
10. Ruch RJ and Klaunig JE: Effects of tumor promoters, genotoxic carcinogens and hepatocytotoxins on mouse hepatocyte intercellular communication. *Cell Biol Toxicol* **2**, 469–483, 1986.
11. Gonzalez FJ: Molecular genetics of the P-450 superfamily. *Pharmacol Ther* **45**, 1–38, 1990.

12. Beck NB and Omiecinski CJ: Lack of modulation by phenobarbital of cyclic AMP levels or protein kinase A activity in rat primary hepatocytes. *Biochem Pharmacol* **58**, 1109–1114, 1999.
13. Sidhu JS and Omiecinski CJ: cAMP-associated inhibition of phenobarbital-inducible cytochrome P450 gene expression in primary rat hepatocyte cultures. *J Biol Chem* **270**, 12762–12773, 1995.
14. Chen HW, Lii CK, Wu MH, Ou CC, and Sheen LY: Amount and type of dietary lipid modulate rat hepatic cytochrome *P*-450 activity. *Nutr Cancer* **29**, 174–180, 1997.
15. Chen HW, Lii CK, Sung WC, and Ko YJ: Effect of vitamin E on rat hepatic cytochrome *P*-450 activity. *Nutr Cancer* **31**, 178–183, 1998.
16. Lii CK, Sung WC, Ko YJ, and Chen HW: α-Tocopherol acetate supplementation enhances rat hepatic cytochrome PROD activity in the presence of phenobarbital induction. *Nutr Cancer* **32**, 37–42, 1998.
17. Beharka AA, Wu D, Han SN, and Meydani SN: Macrophage prostaglandin production contributes to the age-associated decrease in T cell function which is reversed by the dietary antioxidant vitamin E. *Mech Ageing Dev* **93**, 59–77, 1997.
18. Kuver R, Savard C, Oda D, and Lee SP: PGE generates intracellular cAMP and accelerates mucin secretion by cultured dog gallbladder epithelial cells. *Am J Physiol* **267**, G998–G1003, 1994.
19. Kawaguchi H, Pilbeam CC, Harrison JR, and Raisz LG: The role of prostaglandins in the regulation of bone metabolism. *Clin Orthop Related Res* **313**, 36–46, 1995.
20. Sidhu JS, Farin FM, and Omiecinski CJ: Influence of extracellular matrix overlay on phenobarbital-mediated induction of CYP2B1, 2B2, and 3A1 genes in primary adult rat hepatocyte culture. *Arch Biochem Biophys* **301**, 103–113, 1993.
21. Sidhu JS, Farin FM, Kavanagh TJ, and Omiecinski CJ: Effect of tissue-culture substratum and extracellular matrix overlay on liver-selective and xenobiotic inducible gene expression in primary rat hepatocytes. *In Vitro Toxicol* **7**, 225–242, 1994.
22. Sidhu JS and Omiecinski CJ: Modulation of xenobiotic-inducible cytochrome P450 gene expression by dexamethasone in primary rat hepatocytes. *Pharmacogenetics* **5**, 24–36, 1995.
23. Seglen PO: Preparation of isolated rat liver cells. *Methods Biol* **13**, 29–83, 1976.
24. Kreamer BL, Staecker JL, Sawada N, Sattler GL, Hsia MTS, et al.: Use of a low-speed, iso-density Percoll centrifugation method to increase the viability of isolated rat hepatocyte preparations. *In Vitro Cell Dev Biol* **22**, 201–211, 1986.
25. Arancibia S, Estupina C, and Tapia-Arancibia L: Rapid reduction in somatostatin mRNA expression by hypothalamic neurons induced by dexamethasone. *Neuroreport* **11**, 3163–3167, 2000.
26. Chang MM-J, Juarez M, Hyde DM, and Wu R: Mechanism of dexamethasone-mediated interleukin-8 gene suppression in cultured airway epithelial cells. *Am J Physiol* **280**, L107–L115, 2001.
27. Catignani GL and Bieri JG: Simultaneous determination of retinol and α-tocopherol in serum or plasma by liquid chromatography. *Clin Chem* **29**, 708–712, 1983.
28. Lowry O, Rosebrough N, Farr N, and Randall R: Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**, 265–275, 1951.
29. Yang JJ and Krauss RS: Extracellular ATP induces anchorage-independent expression of cyclin A and rescues the transformed phenotype of a *ras*-resistant mutant cell line. *J Biol Chem* **272**, 3103–3108, 1997.
30. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685, 1970.
31. Sidhu JS and Omiecinski CJ: An okadaic acid-sensitive pathway involved in the phenobarbital-mediated induction of CYP2B gene expression in primary rat hepatocyte cultures. *J Pharmacol Exp Ther* **282**, 1122–1129, 1997.
32. Bissell DM and Guzelian PS: Phenotypic stability of adult rat hepatocytes in primary monolayer culture. *Ann NY Acad Sci* **349**, 85–98, 1980.
33. Tran-Thi TA, Gyufko K, Henninger H, Busse R, and Decker K: Studies on synthesis and degradation of eicosanoids by rat hepatocytes in primary culture. *J Hepatol* **5**, 322–331, 1987.
34. Kroll B, Kunz S, Klein T, and Schwarz LR: Effect of lindane and phenobarbital on cyclooxygenase-2 expression and prostanoid synthesis by Kupffer cells. *Carcinogenesis* **20**, 1411–1416, 1999.
35. Hashimoto N, Watanabe T, Ikeda Y, Yamada H, Taniguchi S, et al.: Prostaglandins induce proliferation of rat hepatocytes through a prostaglandin E_2 receptor EP_3 subtype. *Am J Physiol* **272**, G597–G604, 1997.

計畫結果自評

本計畫已完成,結果已發表在國外 SCI 期刊,期刊附在本報告中.