RESEARCH ARTICLES

Effect of (N-hydroxymethyl)-2-propylpentamide on Rat Hepatic Cytochrome P450

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Abstract

The effect of (N-hydroxymethyl)-2-propylpentamide (HPP), a novel valproic acid (VPA) derivatives possessing anticonvulsant activity, on rat hepatic cytochrome P450 was studied in *ex vivo* and *in vitro* system. In *ex vivo* study, HPP at doses of 100 and 200 mg/kg/day or VPA at 250 mg/kg/day were given intraperitoneally to male Wistar rats once daily for 7 days. On the day after, rat liver microsomes were prepared and determined for total CYP contents as well as CYP activities (ethoxyresorufin O-dealkylation for CYP1A1, methoxyresorufin O-dealkylation for CYP1A2, benzyloxy- & pentoxyresorufin O-dealkylation for CYP2B1/2B2 and aniline 4-hydroxylation for CYP2E1). In *in vitro* study, inhibitory effects of HPP at final concentrations of 0.1, 1, 10, 100 and 1000 μM on β–napthoflavone-induced CYP1A1/1A2, phenobarbital-induced CYP2B1/2B2 and ethanol-induced CYP2E1 activities were studied. The results showed that VPA at the dose studied did not have any effect on total CYP contents and all CYP activities. However, HPP at 100 and 200 mg/kg/day significantly induced CYP1A1 and CYP2B1/2B2 activities. In addition, HPP at 100 and 1000 μM significantly inhibited CYP2B1/2B2 activities *in vitro* with IC_{50} of about 752 μM. These results suggested that the inhibitory effect of HPP on CYP2B1/2B2 activities may be, in part, responsible for the prolongation of barbiturate sleeping time after single dose administration of HPP. The induction effect of HPP, but not VPA, on CYP1A1 and CYP2B1/2B2 activities after being administered for 7 days may be resulted from the direct effect of HPP or its metabolites. Further studies are needed to clarify the metabolic pathways of HPP and the CYPs involved as well as the effect of HPP on human CYPs. *In vivo* studies to verify the potential of drug interaction and carcinogenic risk are also needed.

Key words : (N-hydroxymethyl)-2-propylpentamide, rat hepatic cytochrome P450

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ผลของ (เอน-ไฮดรอกซีเมทธิล)-2-โพรพิลเพนทามายด์ ต่อเอนไซม์ไซโตโครมพี 450 ในตับหนูขาว

นารีรัตน์ รักสุนทร[1], สมทรง ลาวัณย์ประเสริฐ[2,4], นวลศรี นิวัติศัยวงศ์[3], มยุรี ตันติสิระ[2], ชำนาญ ภัตรพานิช[3] และพรพิมล กิจสนาโยธิน[2]

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บทคัดย่อ

ศึกษาผลของ (เอน-ไฮดรอกซีเมทธิล)-2-โพรพิลเพนทามายด์ (เอชพีพี) อนุพันธ์ตัวใหม่ของวัลโปรอิคแอซิด (วีพีเอ) ซึ่งมีฤทธิ์ต้านชักต่อเอนไซม์ไซโตโครมพี 450 ในตับหนูขาว แบบ *ex vivo* และ *in vitro* โดยใน *ex vivo* ทำโดยฉีดเอชพีพีขนาด 100 และ 200 มิลลิกรัม/กิโลกรัม/วัน วีพีเอขนาด 250 มิลลิกรัม/กิโลกรัม/วัน แก่หนูขาวเพศผู้ทางหน้าท้องเป็นเวลา 7 วัน วันถัดมาเตรียมไมโครโซมจากตับหนู เปรียบเทียบปริมาณไซโตโครมพี 450 รวมและสมรรถนะของไซโตโครมพี 450 (โดยใช้เอทอกซีรีโซรูฟิน โอ-, เมทอกซีรีโซรูฟิน โอ-, เบนซิลอกซีรีโซ-รูฟิน โอ- และ เพนทอกซีรีโซรูฟิน โอ-ดีอัลคิเลชั่นและอนิลิน-4-ไฮดรอกซีเลชั่น สำหรับแสดงสมรรถนะของ CYP1A1, CYP1A2, CYP2B1/2B2 และ CYP2E1 ตามลำดับ) ส่วนใน *in vitro* จะศึกษาฤทธิ์ยับยั้งเอนไซม์ของเอชพีพีที่ความเข้มข้นสุดท้าย 0.1, 1, 10, 100 และ 1000 ไมโครโมลาร์ ต่อเอนไซม์ CYP1A1/1A2, CYP2B1/2B2 และ CYP2E1 ที่ถูกเหนี่ยวนำก่อนด้วย เบต้าแนพโทฟลาโวน พีโนบาบิทัล และเอทธานอล ตามลำดับ ผลการศึกษาพบว่า วีพีเอในขนาดที่ใช้ศึกษาไม่มีผลต่อปริมาณไซโตโครมพี 450 รวมและสมรรถนะของ CYP ที่ศึกษา แต่เอชพีพีขนาด 100 และ 200 มิลลิกรัม/กิโลกรัม/วัน มีผลเหนี่ยวนำสมรรถนะของ CYP1A1 และ CYP2B1/2B2 อย่างมีนัยสำคัญทางสถิติ นอกจากนี้ยังพบว่าเอชพีพีความเข้มข้น 100 และ 1000 ไมโครโมลาร์ มีผลยับยั้งสมรรถนะของ CYP2B1/2B2 ใน *in vitro* อย่างมีนัยสำคัญทางสถิติ โดยมีค่า IC_{50} ประมาณ 752 ไมโครโมลาร์ จึงมีผลต่อการเพิ่ม barbiturate sleeping time ในหนูถีบจักรเมื่อให้เอชพีพีเพียงครั้งเดียวตามที่มีรายงานมาก่อน ส่วนการที่เอชพีพีในขนาด 100 และ 200 มิลลิกรัม/กิโลกรัม/วัน เมื่อให้ติดต่อกัน 7 วัน มีฤทธิ์เหนี่ยวนำสมรรถนะของ CYP1A1 และ CYP2B1/2B2 ซึ่งไม่พบผลนี้เมื่อให้วีพีเอนั้นอาจเกิดจากผลของเอชพีพีเองหรือเมแทบอไลต์ของเอชพีพี อย่างไรก็ตามควรมีการศึกษาเพิ่มเติมเพื่อหาวิถีกระบวนการเมแทบอลิซึมของเอชพีพีโดยเฉพาะ CYP ที่เกี่ยวข้องและผลของเอชพีพีต่อ CYP isozymes ต่างๆ ที่พบในคน เพื่อประเมินศักยภาพในการก่อให้เกิดปฏิกิริยาระหว่างยาและการเพิ่มความเสี่ยงต่อสารก่อมะเร็งในคนได้ชัดเจนยิ่งขึ้น

คำสำคัญ: (เอน-ไฮดรอกซีเมทธิล)-2-โพรพิลเพนทามายด์, ไซโตโครมพี 450

Introduction

(N-hydroxymethyl)-2-propyl-pentamide (HPP) is one of valproic acid (VPA) derivatives[1]. Chemical structures of HPP and VPA are shown in figure 1 and 2, respectively. Study in mice has shown that HPP possessed a higher anti-convulsant activity and relative safety margin, comparing to its parent compound, VPA[2]. Furthermore, a single dose of HPP (75 mg/kg body weight) given intraperitoneally to mice significantly prolonged barbiturate sleeping time[2]. This probably resulted from the direct depressant effect on CNS or indirect inhibition effect on CYP2B which is responsible for barbiturate clearance in rodents. The serious adverse effects of VPA, hepatotoxicity and teratogenicity, might result from VPA itself or its CYP2B metabolite, 2-n-propyl-4-pentanoic acid (4-ene-VPA)[3,4]. Additionally, it has shown that VPA is a potent inducer of rat CYP2B1/2B2[5]. In line with these finding, n-(2-propylpentanoyl) urea (VPU), one of VPA derivatives has demonstrated an inhibitory effect on human CYP2C9 and CYP1A1/1A2 *in vitro*[6] and an induction effect on rat CYP2B1/2B2[7]. Since HPP is a derivative of VPA whose many significant adverse effects resulted from induction or inhibition effects on CYPs involving in its own metabolism, it is interesting to investigate the effect of HPP on rat CYPs, especially CYP2B which involves in VPA metabolism as well as other CYPs involving in bioactivation of procarcinogens and promutagens, including CYPs 1A1, 1A2, 2B1, 2B2 and 2E1[8].

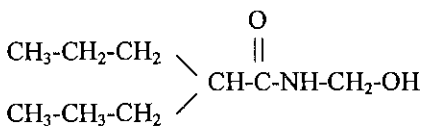

Figure 1 Chemical structure of HPP

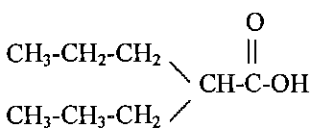

Figure 2 Chemical structure of VPA

Materials and Methods

Adult male Wistar rats (250-300 g) obtained from National Laboratory Animal Center, Nakornpathom were used. The animals were housed in animal care facility for about 1 week before the experimentation.

Experimental Chemical

4-Aminophenol, aniline hydrochloride, benzyloxyresorufin (BR), bovine serum albumin (BSA), dimethylsulfoxide (DMSO), ethoxyresorufin (ER), glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PD), methoxyresorufin (MR), β-naphthoflavone (β-NF), nicotinamide adenine dinucleotide phosphate (NADP), pentoxyresorufin (PR), resorufin, Trisma base, and VPA were purchased from Sigma, USA. Acetonitrile was purchased from J.T. Backer, USA. Ethanol absolute and glycerol were purchased from Carlo Erba, USA. Phenobarbital (PB) was purchased from May&Backer, England. Polyethyleneglycol 400 (PEG400) was purchased from T. Chemical Ltd. Partnership, Thailand. HPP was synthesized by the method of C. Patarapanich[1].

Experimental Methods

1. An *ex vivo* study

Rats were randomly assigned into 4 groups of 6 rats each. Control group, rats were given PEG400 (the diluent of VPA and HPP) intraperitoneally, once daily for 7 days. VPA group: rats were given VPA (250 mg/kg/d), HPP group 1: rats were given HPP (100 mg/kg/d) and HPP group 2: rats were given HPP (200 mg/kg/d) in the same manner. On the day after 7 days of compound administration, rats were sacrificed for preparation of liver microsomes by differential centrifugation and kept at –80 °C until assay. Hepatic

microsomal protein concentration was determined according to the method of Lowry et al[9].

Total CYP content determination

Total CYP contents in microsomal subfractions were determined spectrophotometrically by the method of Omura and Sato[10]. The quantity of CYP was calculated from the absorbance difference (450-490 nm) after reduced by sodium dithionite and bubbled with carbon monoxide. The extinction coefficient of 91 mM^{-1} cm^{-1} was used for a calculation.

Alkoxyresorufin O-dealkylation assay

The O-dealkylations of ethoxy-, methoxy-, benzyloxy- and pentoxy-resorufin by liver microsomes were determined according to the method of Burke and Mayer[11] and Lubet et al.[12] with slight modifications. Each 1 ml of reaction mixture contained 0.1 M Tris buffer, pH 7.4, alkoxyresorufin (5 μM), NADPH regenerating system [comprising NADP (1 mM), G6P (5mM), and magnesium chloride (3 mM)], and microsomal sample (containing 100 gM of protein). Three tubes of 1 ml of reaction mixture were prepared for each sample (1 tube for a blank and the remaining 2 tubes for a sample). The reaction was started by the addition of 10 μl of G6PD (100 units/ml) in 20 mM potassium phosphate buffer, pH 7.4 after a 2 minutes preincubation. Ten microlitre of 20 mM potassium phosphate buffer, pH 7.4 was used in placed of G6PD in the blank. After 5 minutes of incubation at 37 ° C, the reaction was terminated by adding 1 ml of methanol (HPLC grade).

The O-dealkylations of alkoxy-resorufins were determined by measuring the amount of resorufin formed by fluorescence spectrophotometer (excitation λ = 556 nm and emission λ = 589 nm) and expressed as a function of time and amount of protein.

Aniline 4-hydroxylation

The 4-hydroxylation of aniline by liver microsomes was determined according to the method of Schenkman et al.[14], utilizing aniline hydrochloride as a substrate. The reaction was determined by measuring the amount of a metabolite, 4-aminophenol, by spectrophotometer at 630 nm and expressed as a function of time and amount of protein.

2. An *in vitro* study

Rats were randomly assigned into 3 groups of 4 rats each. β-NF group, rats were given β-NF (80 mg/kg/d) intraperitoneally, once daily for 2 days. Four rats were given corn oil in the same manner. PB group, rats were given PB sodium (80 mg/kg/d) intraperitoneally, once daily for 3 days. Four rats were given sterile water in the same manner. Ethanol group, short-term heavy ethanol treatment was used according to the method of Hu, Ingelman-Sundberg and Lindros[15] with some modification. Four rats were given water in the same manner. The inhibition effects of HPP on CYP were investigated by performing co-incubation of HPP with marker substrate for each CYP isoform as the method described above. HPP solutions (0.1, 1, 10, 100 and 1000 μM final concentrations) were dissolved in each of 0.5 mM substrate solution (ER, MR or BR) for studying inhibition effect of HPP on CYP1A1, CYP1A2 and CYP2B1/2B2 activities. Acetonitrile (1% final concentration) was used for dissolving HPP (0.1, 1, 10, 100 and 1000 μM) to study inhibitory effect on CYP2E1.

Data Analysis

All numeric data were presented as mean±SD or % of control activity. The data were analyzed by one way analysis of variance (ANOVA) followed by Student-Newman-Keuls (S-N-K) test. The statistical significant level was $p<0.05$.

For estimation of IC_{50}, the % of inhibition was transformed to probit unit by using transformation table of Fisher and Yates. The linear regression method was used to fit a curve between probit unit and log dose by using Crikcet graph program (Macintosh® computer).

Results

An *Ex vivo* study

The results of this study showed that neither VPA nor HPP (100 and 200 mg/kg/d) had significant effect on total CYP contents. Whereas VPA had no significant effect on any CYP catalytic activities, HPP showed induction effect on some CYP activities. The strongest induction effects were seen on the CYP2B1/2B2 activities with both substrates (BR and PR) used, meanwhile the relative weaker effect was seen on CYP1A1 (or EROD activity). In contrast, there were no significant effects of HPP on CYP1A2 (or MROD activity) and CYP2E1 (or aniline 4-hydroxylase activity) (figure 3).

An *in vitro* study

As shown in figure 4, both β-NF and PB pretreatment significantly ($p < 0.05$) increased total CYP contents and the activities of CYP1A1/1A2 (in β-NF group) and CYP2B1/2B2 (in PB group). Ethanol pretreatment only significantly increased CYP2E1 activity, without increasing the total CYP contents.

Regarding the effect of solvents used for dissolving HPP on CYP activity, rate of aniline 4-hydroxylation by CYP2E1 was almost completely inhibited by DMSO at 1% (v/v) final concentration. Acetonitrile at 0.1 and 1% (v/v) final concentration did not significantly affect the same catalytic activity of CYP2E1[13]. Since the limitation of HPP solubility, 1% (v/v) of acetonitrile final concentration was used in the study of HPP on CYP2E1 (data not shown).

The results of inhibition study showed that HPP at high concentration exhibited selective inhibitory effect on CYP isoforms activities. While all the concentrations used showed no inhibitory effect on CYP1A1, CYP1A2 and CYP2E1 (figure 5), HPP at 100 and 1000 μM significantly decreased the rate of benzyloxyresorufin O-dealkylase (CYP 2B1/2B2 activities) with IC_{50} of about 752 μM. The inhibition effect seemed to be dose-dependent.

Discussion and conclusion

HPP at 100 and 200 mg/kg/day exhibited induction effect on rat hepatic microsomal enzymes, not only CYP2B1/2B2 but also CYP1A1 activities. The CYP2B was more highly induced than CYP1A1. Both CYP1A2 and CYP2E1 were not affected by HPP. Previously, it was found that intraperitoneal injection of PEG400 had no effect on rat liver microsomal total CYP contents as well as all CYP activities as compared to sterile water[7]. Therefore, effects of HPP on rat liver microsomal activities in this study were not contributed by PEG400. VPA at 250 mg/kg/day showed no effect on all CYP isoforms studied. This result was consistent with the finding of Kiatkosolkul[7]. Furthermore, an earlier study by Rogier et al.[5] also showed that intraperitoneal injections of 100 mg/kg/day of VPA to rats once daily for 10 days demonstrated no induction effect on CYP2B1/2B2 activities. However, VPA demonstrated a potent induction effect on CYP2B1/2B2 in the *in vitro* hepatic cell culture system as well as *in vivo*, when administered by continuous infusion for two weeks. It has been proposed that the absence of induction effect on CYP2B following intraperitoneal administration of VPA may be a consequence of the short half-life of VPA in rats (10-20 minutes)[16]. In line with this finding, it has also been found that other derivatives of VPA, N-(2-propylpentanoyl) urea (VPU) and valproyl morpholine (VPM), exhibited induction and inhibition effects on CYPs. VPU has been demonstrated to be an inducer of rat liver CYP2B1/2B2 *in vivo*[7] as well as an inhibitor of human liver CYP2C9 and CYP1A1/1A2 *in vitro*[6]. VPM was recently shown to be an inducer of rat liver CYP2B1/2B2 and CYP1A1 *in vivo* as well as an inhibitor of CYP2B1/2B2 *in vitro*[17].

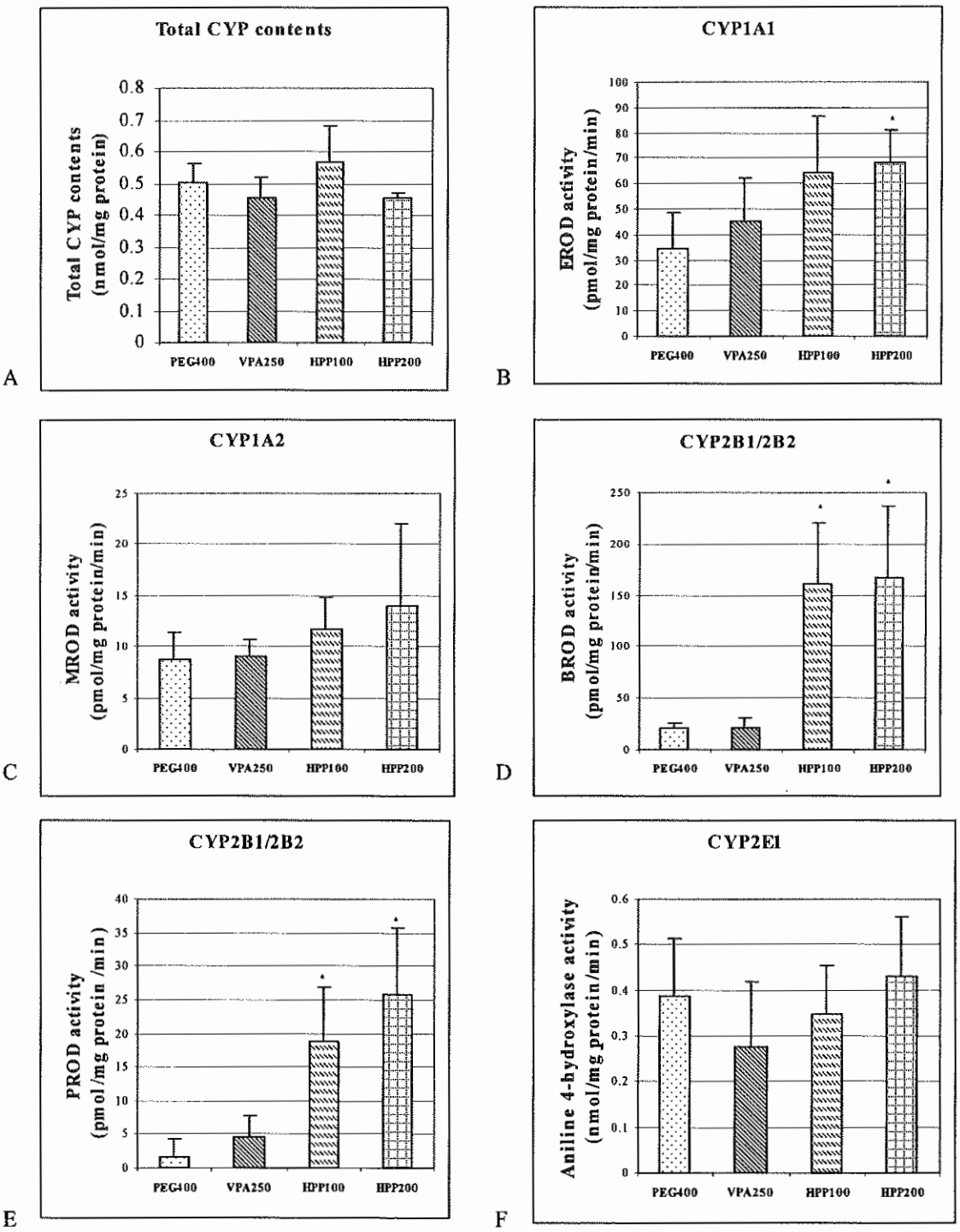

Figure 3 Effects of HPP and VPA on total CYP contents (A), CYP1A1 (B), CYP1A2 (C), CYP2B1/2B2 (D, E) and CYP2E1 (F) activities in *ex vivo* system. Rat were given PEG400 (control), VPA (250 mg/kg/d) and HPP (100 and 200 mg/kg/d) for 7 days. Liver microsomes were prepared and determined for the total CYP contents, ethoxy- (EROD), methoxy- (MROD), benzyloxy- (BROD), pentoxyresorufin O-dealkylase (PROD) and aniline 4-hydroxylase activities. Values are mean ± standard deviation (n=6). *Significantly different from control was determined by one-way ANOVA followed by S-N-K at $p < 0.05$.

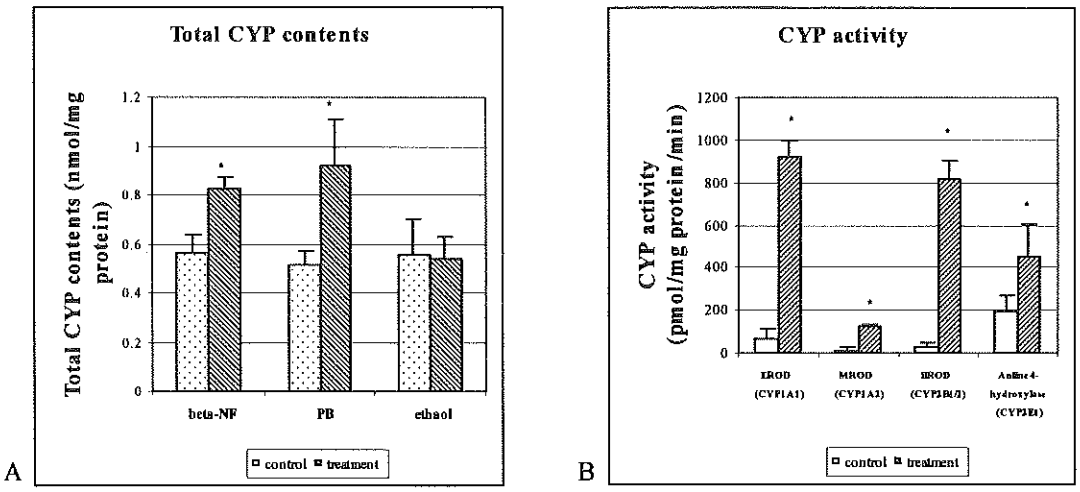

Figure 4 Effect of β–NF, phenobarbital (PB) and ethanol pretreatment on rat microsomal total CYP contents (A), CYP1A1, CYP1A2, CYP2B1/2B2 and CYP2E1 activities (B). Rats were given β–NF, PB or ethanol (as described in materials and methods). Controls of β–NF, PB and ethanol treatment groups were given corn oil, sterile water and water, respectively. Values are mean ± standard deviation (n=4). *Significantly different from control was determined by student's *t* test at $p < 0.05$.

Although CYP2B1/2B2 are not expressed in human, they play an important role in anticonvulsant metabolism including VPA and PB in rat[18]. Regarding VPA metabolism, CYP2B subfamily is responsible for the formation of 4-ene-VPA, a potent hepatotoxic and teratogenic metabolite of VPA[4,19]. Induction effect of VPA on CYP2B1 has also been suggested to contribute substantially to the hepatotoxic effect of VPA[5]. In parallel with VPA, all derivatives of VPA including VPU, VPM and HPP have shown an induction effect as well as inhibition effect on CYP2B. It is interesting to explore whether CYP2B involved in their metabolism or the formation of toxic metabolite, as VPA. CYP1A has been of particular interest due to their ability to activate procarcinogens and promutagens both in rat and human, namely polycyclic aromatic hydrocarbon and aflatoxin B1. Meanwhile CYP2B involves in bioactivation of aflatoxin B1[8,20,21], increased activities of CYP in this subfamily by HPP may increase animal susceptibility to the adverse effect of CYP mediated activation of toxins and carcinogens. Further study to verify its carcinogenicity should be conducted.

In contrast to β–NF and PB, ethanol pretreatment slightly increased total CYP but significantly increased CYP2E1 activity. The induction of CYP2E1 has been proposed to arise through multiple mechanisms. One of the possible mechanisms of ethanol induction appeared to occur via stabilization of the CYP2E1 mRNA. Transcriptional activation of CYP2E1 gene has also been reported[22]. Induction effects of PB and β–NF involve the particular gene transcriptional activation[23]. It is known that some organic solvents can affect the activities of several CYPs. A study of Busby et al.[24] suggested that induction or inhibition effects of solvent were substrate-dependent for a given CYP. In order to keep organic solvent minimal and constant in quantity, HPP were solubilized in the substrate solution. Whereas DMSO showed an inhibition effect on CYP2E1 activity, acetonitrile did not show any noticeable change on this enzyme activity at con-

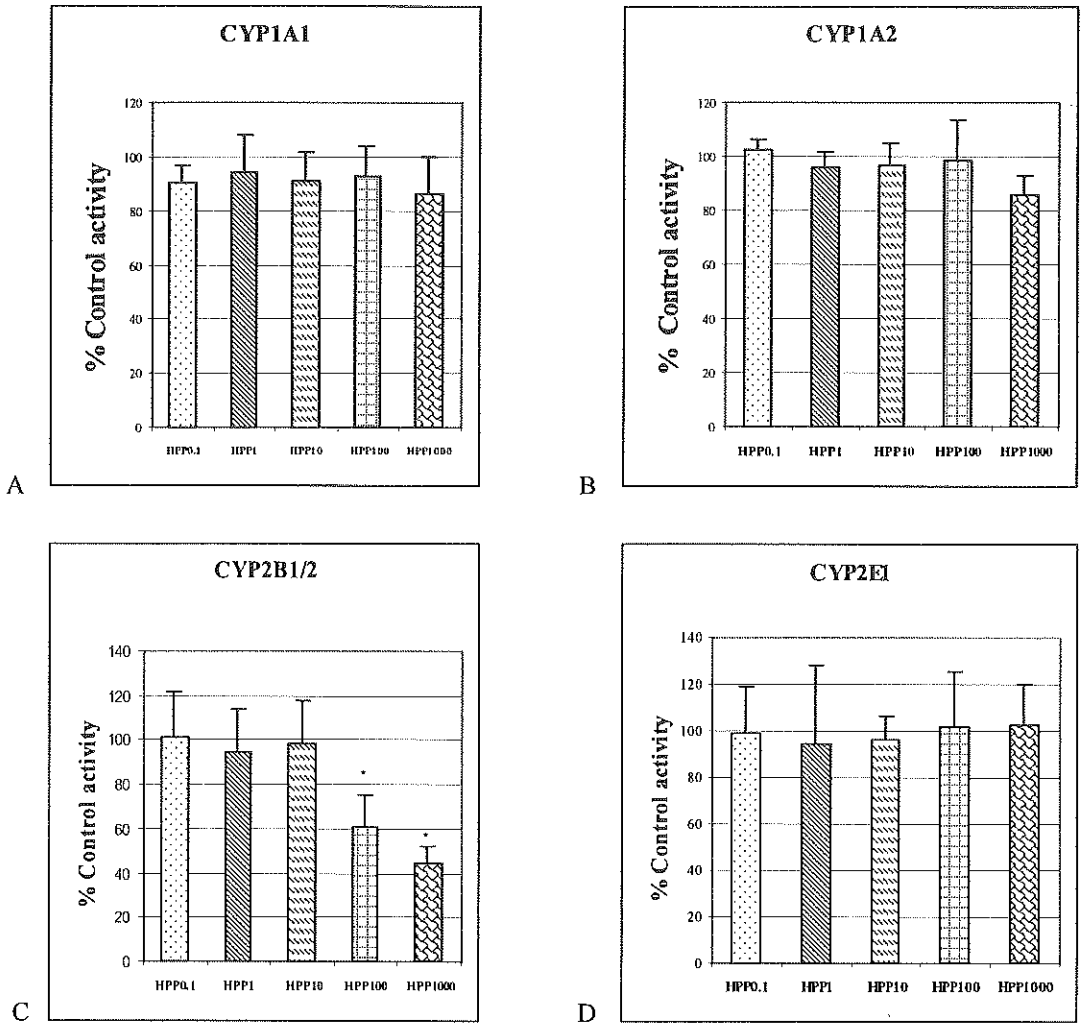

Figure 5 Effect of HPP on CYP1A1 (A), CYP1A2 (B), CYP2B1/2B2 (C) and CYP2E1 (D) activities in *in vitro* system. The effects of HPP at final concentrations of 0.1, 1, 10, 100 and 1000 μM on ethoxy- (EROD), methoxy- (MROD), benzyloxyresorufin O-dealkylase and aniline 4-hydroxylase of CYP1A1, CYP1A2, CYP2B1/2B2 and CYP2E1 activities, respectively were determined by co-incubation of each concentration of HPP with β–NF-induced (for CYP1A1/1A2), PB-induced (for CYP2B1/2B2) or ethanol-induced (for CYP2E1) rat liver microsomes. Values are % control activity (n=6). *Significantly different from control was determined by one-way ANOVA followed by S-N-K at $p < 0.05$.

centration ≤ 1% (data not shown). Due to the limit solubility of HPP, 1% acetonitrile was used as the solvent in the inhibition study on CYP2E1. HPP at final concentration of 100 and 1000 μM inhibited rat CYP2B1/2B2 activities with IC_{50} of about 752 μM. The decrease of CYP2B1/2B2 activity indicated that HPP might be a competitive reversible inhibitor. HPP may be a substrate for CYP2B1/2B2 similar to VPA[20] and may be, in part, responsible for prolongation of barbiturate sleeping time after single dose administration of HPP[2]. However, further *in vitro* study to investigate whether HPP could be a mechanism-based inhibitor, should be conducted.

In conclusion, seven-day administrations of HPP exhibited selective induction effect on rat hepatic microsomal

CYP. HPP 100 and 200 mg/kg/d demonstrated no effects on rat hepatic CYP contents, CYP1A2 and CYP2E1 activities. In contrast, HPP induced CYP1A1 and CYP2B1/2B2 activities. The induction effect on CYP2B was stronger than on CYP1A1. Furthermore, HPP at final concentration of 100 and 1000 μM inhibited CYP2B1/2B2 activities with IC_{50} of about 752 μM. This finding suggested that HPP may be, in part, responsible for prolongation of barbiturate sleeping time after single dose administration of HPP. Further studies are needed to clarify the metabolic pathway of HPP and the CYPs involved as well as the effect of HPP on human common CYPs. *In vivo* studied to verify the potential of drug interaction and carcinogenic risk are also needed.

Acknowledgements

This study was supported primarily by a grant from the Faculty of Pharmaceutical Sciences, and in part by the Graduate School, Chulalongkorn University.

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