Effects of huperzine A on liver cytochrome P-450 in rats

MA Xiao-Chao[1], WANG Hai-Xue[2], XIN Jian[1], ZHANG Ting[1], TU Zeng-Hong[1,3]

[1]*State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China*
[2]*Department of Pharmacology, School of Pharmacy, Fudan University, Shanghai 200032, China*

KEY WORDS Huperzine A; cytochrome P-450; cytochrome P-450 CYP1A2; liver; rat

ABSTRACT

AIM: To predict possible drug interaction and assure safety medication of huperzine A (HupA). **METHODS:** The effects of HupA on activities and expressions of cytochrome P-450 (CYP) were examined. Liver microsomes and total mRNA were prepared from rats treated orally with 0, 0.1 (pharmacological dose), 1, or 2 mg/kg huperzine A for 2 weeks. Phenobarbital, 3-methylcholanthrene (3-MC), ethanol, and dexamethasone were used as positive controls. Total CYP protein was assayed by carbon monoxide difference spectrum. Activity of isoenzyme was detected with specific probe. Expression of CYP protein and mRNA was analyzed with Western blot and RT-PCR. **RESULTS:** No changes of isoenzyme expression and catalytic activities were found in rats treated with 0.1 mg/kg huperzine A. Huperzine A 1, 2 mg/kg parallelly increased CYP1A2 activity, protein and mRNA, although they were minor when contrasted with 3-MC. Huperzine A 1, 2 mg/kg got no effects on CYP2C11, CYP2B1/2, 2E1 and 3A. **CONCLUSION:** Activity and expression of liver CYP isoenzymes were not affected in rats treated with pharmacological dose of HupA, but HupA at toxicological dose may elicit a slight inductive response of CYP1A2. The CYP1A2 induction produced by HupA is related to transcription enhancement.

INTRODUCTION

Acetylcholinesterase inhibitors (AChEI), such as physostigmine and tacrine, were identified to possess the action of ameliorating cognitive dysfunction of Alzheimer's disease (AD)[1]. However, short duration of action, low bioavailability, frequent side effects, and dose-dependent hepatotoxicity limited their clinical values[2,3]. New AChEI that has greater therapeutic window, longer duration of action, and fewer side effects is desirable. Huperzine A (HupA) is a purified compound derived from herb club moss (*Huperzia serrata*) found in China. It is a potent, reversible, and selective AChEI exhibiting memory-enhancing activities in animal and clinical trials. Compared with tacrine and physostigmine, HupA has a longer duration of action, higher therapeutic index and its peripheral cholinergic side effects are minimal at therapeutic doses[4,5].

Treatment for AD patients with HupA needs a long course and combined medications are often required[6]. Combining and successive medications are the common reasons for inhibition or induction of cytochrome P-450 (CYP) and adverse drug reaction may thus occur. It is necessary to identify the effects of HupA on CYP expression, which will be beneficial for understanding the consequences of pharmacology, toxicology, and therapeutics of HupA and other combined drugs[7], while limited data are available about effects of HupA on liver CYP up to now. Present study was attempted to ex-

[3] Correspondence to Prof TU Zeng-Hong.
Phn 86-21-5080-6600, ext 1107.
E-mail zhtu@mail.shcnc.ac.cn
Received 2002-09-27 Accepted 2003-01-10

amine the effects of HupA on liver CYP to indicate possible drug interactions and avoid possible adverse drug reactions.

MATERIALS AND METHODS

Chemicals and reagents HupA, purity 98 %, was supplied by the Department of Phytochemistry, Shanghai Institute of Materia Medica. Monoclonal antibodies (rabbit anti-rat) to CYP1A2, 2C11, 2E1, and 3A were obtained from Chemcon International, Inc (Temecula, CA, USA). The secondary antibody (goat anti-rabbit) was purchased from Sino-American Co (Luoyang, China). Deoxynucleotide triphophates (dNTP), guanidinium thiocyanate (Tri-reagent), *Taq* DNA polymerase and reverse transcriptase (M-MLV) were obtained from Gibco (Grand Island, NY, USA). NADPH, resorufin, erythromycin, 7-ethoxy/7-pentoxyresorufin (ER/PR), *p*-nitrophenol, 4-nitrocatechol, phenobarbital (PB), 3-methylcholanthrene (3-MC), and bovine serum albumin (BSA) were procured from Sigma Chemical Co (St Louis, MO, USA). All other reagents were of the highest quality available commercially.

Animals and treatment Sprague-Dawley rats (about 2-month old, 201 g±20 g) were supplied by Shanghai Experimental Animal Center, Chinese Academy of Sciences (Grade II, Certificate No 005). The rats were randomly divided into eight groups (A to H) and each group contained 6 male rats. Groups from A to D were treated with HupA 0.1, 1, and 2 mg/kg or placebo (distilled water, DW) for 2 weeks, respectively. Groups from E to H were treated with 3-MC (dissolved in vegetable oil, 20 mg/kg, ip), PB (dissolved in saline, 50 mg/kg, ip), ethanol (10 % in drinking water) or dexamethasone (dissolved in vegetable oil, 80 mg/kg, ip) for 3 d served as positive controls. Animal facilities were maintained under 12-h light/dark cycle (light on at 7:30 AM), (22±2) °C, and 50 %-70 % relative humidity with food and water available *ad libitum*. Twenty-four hours after the last dose, rats were weighed and killed. Each liver was removed, weighed, and stored at -80 °C.

Microsomal preparation Liver tissues were homogenized in 4 volumes of ice-cold potassium phosphate buffer 0.25 mol/L, pH 7.25, containing KCl 0.15 mol/L, edetic acid 0.01 mol/L, and DTT 0.1 mmol/L. Subcellular fractionation of the liver homogenate was centrifuged at 14 000×*g* for 20 min. The supernatant was centrifuged at 105 000×*g* for 60 min to separate the microsomes[8]. The microsomal pellet was washed and resuspended in microsome dilution buffer containing potassium phosphate buffer 0.1 mol/L, pH 7.25, containing edetic acid 0.01 mol/L, and DTT 0.1 mmol/L. Microsomal samples were aliquoted, frozen, and stored at -80 °C for further analysis. The protein content of each microsomal preparation was determined by the method[9]. Total CYP450 protein in liver microsomes was assayed by carbon monoxide difference spectrum after reduction with sodium dithionite[10].

Isoenzymes activities 7-Methoxyresorufin-*O*-demethylase (MROD, CYP1A2), 7-pentoxyresorufin-*O*-deethylase (PROD, CYP2B1/2), *p*-nitrophenol hydroxylase (PNPH, CYP2E1), and erythromycin *N*-demethylase (ERDM, CYP3A) activities were examined for selective activities of CYP isoforms. Sample for MROD activity was analyzed by fluorometry for the amount of resorufin produced[11]. PROD assay was performed as Rutten *et al*[12]. PNPH activity was determined by measuring the formation of 4-nitrocatechol according to the method of Reinke and Moyer *et al*[13]. ERDM activity was determined by measuring the formation of formaldehyde according to the method of Wrighton[14].

Western blot analysis CYP1A2, 2C11, 2E1, and 3A isoenzymes in liver microsomes were detected by Western blot analysis using monoclonal antibodies against CYP1A2, 2C11, 2E1, and 3A. Glutamate dehydrogenase (55.4 kDa) was used as standard for molecular weight determination. Microsomal samples were loaded on a 5 % polyacrylamide stacking gel with a 10 % resolving gel. After SDS-PAGE, the proteins were transferred electrophoretically to 0.45-μm nitrocellulose membranes. The membrane was blocked with a 2 % BSA solution, incubated with the primary antibodies (1:500 dilution) and horseradish peroxidase-conjugated secondary antibodies (1:1000 dilution) subsequently. Protein band was visualized by chemiluminescence with ECL detection kit (Amersham, UK). Band intensities were quantitated with scanning densitometry.

RT-PCR analysis Total hepatocyte RNA was extracted from liver tissue using Trizol Reagent. Synthesis of cDNA was carried out with reverse transcriptase (M-MLV, 0.1 U/μg RNA). Total RNA 2 μg was reverse transcribed into single-stranded cDNA using the buffer of Tris-HCl 50 mmol/L (pH 8.3), KCl 75 mmol/L, $MgCl_2$ 3 mmol/L, DTT 10 mmol/L, oligo-dT 0.5 μg, and dNTP 0.5 mmol/L incubated at 37 °C for 1 h. After inactivation at 95 °C for 5 min, 100 ng of cDNA products was added to a 50 μL PCR reaction mixture consisting 50 pmol primers, dNTP 0.2 mmol/L; Tris-

HCl 10 mmol/L (pH 8.3), KCl 50 mmol/L, $MgCl_2$ 1.5 mmol/L, and *Taq* DNA polymerase 0.5 μL. Primer sequences of *CYP1A2* were TCCCTCAGGAGAAGATTGT (sense) and ACCTGCCACTGGTTTATG (anti-sense), amplicon 337 bp. β-Actin was amplified as an internal control with primer sequences of CCTCTATGCCAACACAGTGC (sense) and GTACTCCTGCTTGCTGATCC (anti-sense), amplicon 211 bp. PCR was performed 30 cycles under conditions of denaturation at 94 °C for 50 s, annealing at 53 °C for 50 s, and extention at 72 °C for 1 min. PCR products were electrophoresed on 3 % agarose gels.

Statistics Data were expressed as mean±SD and the statistical significance of differences were calculated using one-way ANOVA. $P<0.05$ was considered statistically significant.

RESULTS

Microsomal protein and total CYP No mortality was found during 2-week treatment with HupA. HupA 1 and 2 mg/kg produced the onset of cholinergic hyperactivity such as hypersalivation and muscular fasciculation in the first week's administration. Body and liver weights were decreased in rats treated with HupA 2 mg/kg, while relative liver weight had a trend to increase. Microsomal protein was decreased by about 2.9 % in HupA 1 mg/kg and by 4.4 % in HupA 2 mg/kg groups, but there was no significant difference as compared with DW control. Consistent with the changes of microsomal protein, total CYP protein was decreased in rats treated with HupA 2 mg/kg (Tab 1).

Isoenzymes activity A significant increase of MROD activity was found in rats treated with HupA 2 mg/kg ($P<0.01$, Tab 2). In HupA 1 and 2 mg/kg-treated groups, MROD activity was increased by 20 % and 61 % respectively. But HupA-induced MROD activity increase was minor when contrasted with 3-MC, which increased MROD activity by 485 %. HupA did not produce significant changes in the microsomal activity of PROD, PNPH, or ERDM in rats at any doses.

Tab 1. Relative liver weight, microsomal protein, and total CYP protein of rats treated with HupA for 2 weeks. Distilled water (DW) and phenobarbital (PB) were employed as controls. *n*=6. Mean±SD. [a]*P*>0.05, [b]*P*<0.05 *vs* DW control.

Groups	Liver weight/ g·kg^{-1} (body weight)	Protein/ g·L^{-1}	Total CYP protein/mmol·g^{-1} (protein)
DW	35.2±1.8	12.5±1.0	0.98±0.03
PB	35.5±1.4	14.6±1.3	1.74±0.15
Hup A 0.1 mg/kg	35.1±1.0	12.4±1.1	0.97±0.03
Hup A 1 mg/kg	35.3±1.0	12.2±0.7	0.966±0.021[a]
Hup A 2 mg/kg	36.0±2.2	12.0±1.0	0.945±0.025[b]

Tab 2. Effects of HupA on MROD activity, CYP1A2 protein and mRNA in rat liver. Distilled water (DW) and 3-methylcholanthrene (3-MC) were employed as controls. *n*=6. Mean±SD. [b]*P*<0.05, [c]*P*<0.01 *vs* DW control.

Groups	MROD activity/ nmol·min^{-1}·g^{-1} (protein)	CYP1A2 protein	*CYP1A2* mRNA
DW	69±11	693±120	0.15±0.04
3-MC	402±63	6502±1086	1.9±0.3
Hup A 0.1 mg/kg	67±10	709±142	0.15±0.06
Hup A 1 mg/kg	83±13	991±168[c]	0.32±0.07[c]
Hup A 2 mg/kg	110±17[b]	1408±323[c]	0.44±0.09[c]

CYP1A2 protein expression was expressed by band density. CYP1A2 mRNA was expressed as relative desity to β-actin.

Western blot analysis CYP isoenzymes in liver microsomes were separated by SDS-polyacrylamide gel electrophoresis and probed with anti-rat CYP1A2, 2C11, 2E1, and 3A antibodies (Fig 1). Apparent changes of immunoblotting bands were noted in microsomal samples cross-reacted with anti-CYP1A2 antibody. Densitometric analysis revealed that CYP1A2 expression was increased by 43 % and 103 % in rats treated

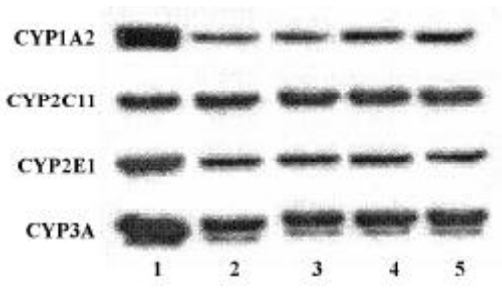

Fig 1. Western bolt analysis of liver microsomes from rats treated with HupA 0, 0.1, 1, or 2 mg/kg for 2 weeks. Lane 1: 3-MC (CYP1A2), DW control (CYP2C11), ethanol (CYP2E1) and dexamethasone (CYP3A); Lane 2: DW control; Lane 3: HupA 0.1 mg/kg; Lane 4: HupA 1 mg/kg; Lane 5: HupA 2 mg/kg.

with HupA 1 and 2 mg/kg, while 3-MC increased CYP1A2 expression by 838 % (Tab 2). HupA-related changes of CYP2C11, 2E1, and 3A proteins were not found after 2-week treatment.

RT-PCR analysis Apparent increase in band intensity of *CYP1A2* mRNA was noted in rats treated with HupA 1 and 2 mg/kg (Fig 2, Tab 2). *CYP1A2* mRNA band intensity obtained by scanning densitometry was divided by the intensity of β-actin band. Relative *CYP1A2* mRNA content was increased by 113 %, 194 %, and 1193 % in rats treated with HupA 1, 2 mg/kg, and 3-MC, respectively.

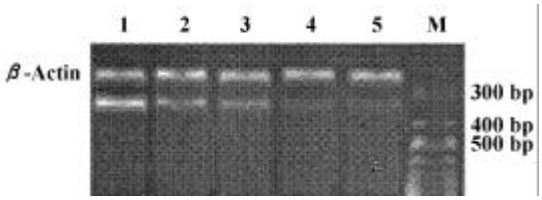

Fig 2. RT-PCR analysis of liver *CYP1A2* mRNA in rats treated with HupA 0, 0.1, 1, or 2 mg/kg for 2 weeks. Lane 1: 3-MC; Lane 2: HupA 2 mg/kg; Lane 3: HupA 1 mg/kg; Lane 4: HupA 0.1 mg/kg; Lane 5: DW control; Lane M: Mark.

DISCUSSION

In the present study, decreases of liver microsomal protein and total CYP were noted in rats treated with HupA 2 mg/kg. Liver microsomal protein and total CYP levels are always related to liver functional status, such as blood and oxygen supply, which can be interfered by AChEI. Over dose of AChEI increases ACh at the celiac ganglion and thus increases sympathetic activity via the hepatic nerve[15]. When ACh is released in the celiac ganglion and causes an action potential that propagates through the hepatic nerve, sinusoidal vascular space and tissue perfusion will be decreased[16]. For rats, HupA 0.1 mg/kg is a pharmacological dose[17], and 2 mg/kg is 20 times of pharmacological dose. It is suspected that overdoses of HupA affect liver functional status and decrease microsomal protein and total CYP.

Significant increases of CYP1A2 activity and protein were consistent with the increase of *CYP1A2* mRNA, which indicate that the induction produced by HupA is related to transcription enhancement, while the induction produced by HupA 2 mg/kg was minor in contrast with 3-MC, a generally accepted CYP1A2 inducer. Interestingly, HupA-induced CYP1A2 expression was not paralleled by total CYP increase. On adverse, a reduction in total CYP was found in rats treated with HupA 2 mg/kg, which suggests that only total CYP may not always be adequate to indicate enzyme induction, especially when the extent of induction is weak.

In light of our results, the therapeutic doses of HupA in human should not be high enough to elicit an induction response of CYP1A2. CYP1A2, about 13 % of total CYP isoenzymes in human liver, is an enzyme with both pharmacologic and toxicologic significance[18], which involves metabolic clearance of many drugs and has been implicated in the metabolic activation of certain procarcinogens and promutagens[19]. CYP1A2 activity can be induced by environmental factor like smoking and caffeinated drinks, and some drugs, such as insulin and modafinil. At the same time, CYP1A2 activity can be inhibited by other drugs, such as cimetidine, methoxsalen, quinolones, furafylline, citalopram, fluoxetine, fluvoxamine, and moclobemide[20]. So doctors of AD patients should pay attention to the factors interfering CYP1A2 to avoid possible drug interactions and assure safety of HupA medication.

In conclusion, activity and expression of liver CYP were not affected in rats treated with pharmacological dose of HupA, but at toxicological dose of HupA may elicit a slight inductive response of CYP1A2. The CYP1A2 induction produced by HupA is related to transcription enhancement. Because CYP1A2 is involved in the metabolism of numerous important drugs, further studies are required to assess whether HupA causes a clinically relevant interaction with any CYP1A2 substrates and/or inhibitors.

REFERENCES

1 Susan DI. Behavioral evaluation of cholinergic drugs. Life Sci 1997; 60: 1145-52.
2 Winblad B, Adem A, Backman L, Nordberg A, Elinder F, Arhem P. Cholinesterase inhibitors in Alzheimer's disease: evaluation of clinical studies. In: Becker R, Giacobini E, editors. Cholinergic basis for Alzheimer therapy. Boston: Birkh user; 1991. p 238-43.
3 Watkins PB, Zimmerman HJ, Knapp MJ, Gracon SI, Lewis KW. Hepatotoxic effects of tacrine administration in patients with Alzheimer's disease. JAMA 1994; 271: 992-8.
4 Tang XC, He XC, Bai DL. Huperzine A: a novel acetylcholinesterase inhibitor. Drugs Fut 1999; 24: 647-63.
5 Wang H, Tang XC. Anticholinesterase effects of huperzine A, E2020, and tacrine in rats. Acta Pharmacol Sin 1998; 19: 27-30.
6 Tang XC. Huperzine A (Shuangyiping): a promising drug for Alzheimer's disease. Acta Pharmacol Sin 1996; 17: 481-4.

7 Curtis JO, Rory PR, Vinayak PH. Concise review of the cytochrome P450 isoenzymes and their roles in toxicology. Toxicol Sci 1999; 48: 151-6.
8 Parmar D, Dhawan A, Dayal M, Seth PK. Immunochemical and biochemical evidences for expression of phenobarbital and 3-methycholanthrene inducible isoenzymes of cytochrome P450 in rat brain. Int J Toxicol 1998; 17: 1-8.
9 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folinphenol reagent. J Biol Chem 1951; 193: 265-75.
10 Omura T, Sato R. The carbon monoxide-binding protein of liver microsomes. I. Evidence for its hemoprotein nature. J Biol Chem 1964; 293: 2370-8.
11 Nerurkar PV, Park SS, Thomas PE, Nims RW, Lubet RA. Methoxyresorufin and benzyloxyresorufin: substrates preferentially metabolized by cytochromes P4501A2 and 2B, respectively, in the rat and mouse. Biochem Pharmacol 1993; 46: 933-43.
12 Rutten AA, Falke HE, Catsburg JF, Wortelboer HM, Blaauboer BJ, Doorn L, *et al*. Interlaboratory comparison of microsomal ethoxyresorufin and pentoxyresorufin *O*-dealkylation determinations: standardization of assay conditions. Arch Toxicol 1992; 66: 237-44.
13 Reinke LA, Moyer MJ. *p*-Nitrophenol hydroxylation: a microsomal oxidation which is highly inducible by ethanol. Drug Metab Dispos 1985; 13: 548-52.
14 Wrighton SA, Maurel P, Schuetz EG, Watkins PB, Young B, Guzelian PS. Identification of the cytochrome P-450 induced by macrolide antibiotics in rat liver as the glucocorticoid responsive cytochrome P-450p. Biochemistry 1985; 24: 2171-8.
15 Robert FS, Gavin EA, James AR, Raleigh DC, Ronald PM, Ronald GT. Development and characterization of a new model of tacrine-induced hepatotoxicity: role of the sympathetic nervous and hypoxia-reoxygenation. J Pharmacol Exp Ther 1997; 282: 1591-9.
16 Gardemann A, Puschel GP, Jungermann K. Nervous control of liver metabolism and hemodynamics. Eur J Biochem 1992; 207: 399-411.
17 Cheng DH, Ren H, Tang XC. Huperzine A, a novel promising acetylcholinesterase inhibitor. Neuroreport 1996; 8: 97-101.
18 Shimada T, Yamazaki H, Mimura M, Inui Y, Guengerich FP. Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. J Pharmacol Exp Ther 1994; 270: 414-23.
19 Guengerich FP, Shimada T. Oxidation of toxic and carcinogenic chemicals by cytochrome P450 enzymes. Chem Res Toxicol 1991; 4: 391-407.
20 Pelkonen O, Maenpaa J, Taavitsainen P, Rautio A, Raunio H. Inhibition and induction of human cytochrome P450 (CYP) enzymes. Xenobiotica 1998; 28: 1203-53.