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

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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 values²,³. 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 cholinergeric side effects are minimal at therapeutic doses⁴,⁵.

Treatment for AD patients with HupA needs a long course and combined medications are often required⁶. 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 drugs⁷, while limited data are available about effects of HupA on liver CYP up to now. Present study was attempted to ex-
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 triphosphates (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 placebo (distilled water, DW) for 2 weeks, respectively. 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 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), KC1 75 mmol/L, MgCl2 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, MgCl₂ 1.5 mmol/L, and Taq DNA polymerase 0.5 μL. Primer sequences of CYP1A2 were TCCCTCAGGAGAACATTG (sense) and ACCTGCACTGTTATATG (anti-sense), amplicon 337 bp. β-Actin was amplified as an internal control with primer sequences of CTTCAATGGCAGAGA CACGATG (sense) and GTACTCCTGCTTGCTGAT (anti-sense), amplicon 211 bp. PCR was performed 30 cycles under conditions of denaturation at 94 °C for 30 s, annealing at 53 °C for 50 s, and extension 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 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. *P*<0.05, *P*<0.01 vs DW control.

<table>
<thead>
<tr>
<th>Groups</th>
<th>MROD activity/ CYP1A2 protein</th>
<th>CYP1A2 mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol·min⁻¹·g⁻¹ (protein)</td>
<td></td>
</tr>
<tr>
<td>DW</td>
<td>69±11</td>
<td>693±120</td>
</tr>
<tr>
<td>3-MC</td>
<td>402±63</td>
<td>6502±1086</td>
</tr>
<tr>
<td>Hup A 0.1 mg/kg</td>
<td>67±10</td>
<td>709±142</td>
</tr>
<tr>
<td>Hup A 1 mg/kg</td>
<td>83±13</td>
<td>991±168</td>
</tr>
<tr>
<td>Hup A 2 mg/kg</td>
<td>110±17</td>
<td>1408±323</td>
</tr>
</tbody>
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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
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.

**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**