Potential inhibition of cytochrome P450 3A4 by propofol in human primary hepatocytes

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Abstract
AIM: Hepatic cytochrome P450 isoenzymes constitute a superfamily of hemoproteins that play a major role in the metabolism of endogenous compounds and in the detoxification of xenobiotic molecules. P450 3A4 is one of the most important forms in human being, and mediates the metabolism of around 70 % of therapeutic drugs and endogenous compounds. Propofol, a widely used intravenous anesthetic drug, is known to inhibit cytochrome P450 activities in isolated rat hepatocytes. The goal of this study was to evaluate the potential efficacy of propofol on P450 3A4 in a dose-dependent manner to understand its drug-drug interaction.

METHODS: Hepatocytes were isolated from liver specimens from hepatic angioma patients undergone hepatic surgery. Primary incubated hepatocytes were treated with 0, 0.01, 0.05, 0.1, 0.5, and 1.0 mM propofol for 24 hours. P450 3A4 activity was measured with Nash's colorimetry. The protein expression was assessed by Western blot analysis.

RESULTS: A dose-dependent inhibitory effect of propofol was observed in cytochrome P450 3A4 activity. A minimal dosage of propofol (0.01 mM) induced a significant inhibition of P450 3A4 activity, although its regular dosages (0.01-0.1 mM) showed no inhibitory effect on the cellular protein expression of P450 3A4.

CONCLUSION: Propofol may be a potential CYP3A4 inhibitor as this anesthetic can inhibit isoenzyme activity significantly and reduce the metabolic rate of CYP3A4 substrates. This inhibition occurs at post-expression level, and concentration of propofol used clinically does not affect CYP3A4 protein expression. propofol may thus induce drug interaction of cytochrome P450 3A4 activity at the dosage used clinically.

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INTRODUCTION
Cytochrome P450 enzymatic system is essential for the biotransformation of xenobiotics and drugs, and is a superfamily of haemoproteins[1]. One group of particular interest for those caring for the critically ill is CYP 3A4, which metabolizes many drugs used in the critical patients, including midazolam, lignocaine, alfentanil, erythromycin and cyclosporin[2,3]. Propofol (2, 6-diisopropylphenol) is widely used for anesthetic induction as well as for chronic sedation in ICU. Unlike midazolam, propofol does not undergo phase I metabolism, but is metabolized mostly through direct glucuronidation in the liver. Recent works have shown that several cytochrome P450 isoforms do participate in its metabolism, especially the CYP2B6 isofrom. Both in vitro and in vivo, the inhibitory effects of propofol on cytochrome P450 activities have been already described, thus, propofol may potentially alter the metabolism of many co-administered anesthetics such as alfentanil and midazolam. However, the mechanism of propofol’s inhibitory effect still remains unclear[4-6].

Since primary cultures of human hepatocytes represent a unique in vitro system to study the potential of drugs to induce phase I and phase II enzymatic reaction involved in drug metabolism. Many researchers have successfully used human hepatocyte cultures to investigate the effect of various drugs on cytochrome P-450 induction and drug metabolism[7,8]. The goal of this study was to evaluate the inhibitory effect of propofol on cytochrome P450 and its 3A4 isoform activities, as well as on protein expression of CYP3A4 isofrom during 24 hours culture.

MATERIALS AND METHODS
Materials
Propofol was kindly provided by Astra-Zeneca. Rabbit anti-human CYP3A4 polyclonal antibody was purchased from Chemicon (San Diego, CA). HRP tagged sheep anti-rabbit antibody was purchased from PharMingen (Mannheim, Germany). Collagenase IV glucose 6-phosphate, erythromycin, Lowry’s phenol reagent, glucose 6-phosphate transferase, acetate ammonium, acetyl-acetone, and NADP were purchased from Sigma Chemical (St. Louis, MO). Newborn calf serum was obtained from Gibco (Paisley, UK). RPMI 1640 medium was purchased from Seromed (Berlin, Germany). dexamethasone and insulin were obtained from BioWhittaker (Walkersville, MD). Penicillin G/streptomycin were obtained from Gibco Laboratories (Grand Island, NY). All other reagents used in this study were of AR or CP grade.

Isolation and culture of human hepatocytes
Surgical liver biopsies (30-50 g) were taken from hepatic angioma patients undergone hepatic surgery after informed consent was obtained. Patients had no known liver lesions, nor had they received P450s induced medication during the weeks before surgery. None of the patients was habitual consumers of alcohol or other drugs. A total of 8 liver biopsy specimens (from ten men and two women) were used. The patients aged from 18 to 54 years. Human hepatocytes were
isolated using modified collagenase digestive technique according to the method of Liddle[9]. Liver tissues were cut into small pieces about 1 mm³ and washed three times by 4 °C equilibrium liquid to remove the leftover blood, then the liver pieces were digested for 30 min by RPMI 1640 medium containing 0.05% collagenase. The dissociated hepatocytes were placed into cold Hank’s medium for 3 times and centrifugated at 600 rpm for 40 s, at 800 rpm for 50 s and at 1000 rpm for 60 s. Percoll-gradient centrifugation (1000–

1 200 r/min, 10 min) was required to get rid of remained blood thoroughly. Concentration and density of Percoll liquid were 35% (v/v) and 1.08-1.09 g/ml respectively. Viability at plating was greater than 90% after trypan blue elimination and hepatocytes were plated in RPMI 1640 medium supplemented with 10⁻² M dexamethasone, 10⁻² M insulin, 100 U/ml penicillin G, 100 µg/ml streptomycin, and 5% bovine calf serum. The hepatocytes (3x10⁶) were plated onto 60-mm culture plates previously coated with type I (rat-tail) collagen. The cells were allowed to attach for 4 h at 37 °C and 5% CO₂, during which the media were replaced with serum-free media with the supplements listed above and changed every 24 h thereafter.

Treatment of cultures
For CYP inhibition experiments, propofol was dissolved in dimethylsulfoxide and added to cultured human hepatocytes at a final concentration of 0.01, 0.05, 0.1, 0.5 and 1.0 mM, respectively (concentration of DMSO in culture medium was 0.1% v/v). Treatments were started after medium renewal, and stopped after 24 hrs of co-culture in fresh RPMI 1640 medium, the control cells were run parallely.

Cellular lysates protein and CYP3A4 activity assays
Total protein concentration of cellular lysates was determined by the method of Lowry et al[10]. The spectra were recorded using a Shimadzu UV-250 double-beam spectrophotometer. The final protein concentration of cellular lysates was adjusted to 0.2 mg/ml using Tris-HCl buffer (pH 7.4). CYP3A4-specific activity was determined by N-demethylation of erythromycin using the Nash method listed above[11].

Immunooquantitation of CYP3A4 isoform protein by Western blot analysis
Cellular lysates were obtained from cultured human hepatocytes, which were incubated with propofol for the indicated time points and resolved by SDS-PAGE with vertical mini-gel electrophoresis equipment. Samples of liver microsomal protein (10 µg/lane) were denatured in 10 µl loading buffer (4 µl distilled water, 1 ml 0.5M Tris-HCl, pH 6.8, 0.8 ml glycerol, 1.6 ml 10% w/v SDS, 0.4 ml-mercaptoethanol, 0.05 ml 0.05% w/v pyronin Y) and were separated on a 10% w/v resolving gel. Proteins were transferred from the polyacrylamide gel to the nitrocellulose sheets by electrophoresis, and probed with rabbit anti-human CYP3A4 polyclonal antibody (not cross-reactive with other rat P450s) according to the protocol. CYP3A4 protein was detected by conjugation to the primary antibody by a HRP-linked sheep anti-rabbit second antibody using diaminobenzidine as a substrate[12].

Statistical analysis
The data were analyzed using χ² test. P<0.05 was considered statistically significant.

RESULTS
Inhibition of CYP3A4 activity by propofol in hepatocytes primary culture
Information on the donor livers is presented in Table 1. We first determined the inhibition of propofol in primary cultures of human hepatocytes. The hepatocytes were treated with propofol at concentrations of 0.01, 0.05, 0.1, 0.5 and 1.0 mM respectively for 24 h, then we investigated the effect of propofol on CYP3A4 activity determined by N-demethylation of erythromycin in the medium, as shown in Figure 1. A significant decrease of CYP3A4 activity was detected in hepatocytes pretreated even with 0.01 and 0.05 mM propofol which were relatively lower, and the inhibitory effect showed a dose-dependent increase when the concentration of propofol was higher. Compared with the controls, the total cellular lysate protein in the cultured medium remained unchanged in all groups.

| Table 1 Inforrmations of donors and their drug history |
|-------------|-----------------|------------------|
| HH | Age (y) | Gender (F/M) | Drug history | Cell viability % |
| 1 | 42 | M | Fentanyl, ranitidine, dopamine | 88 |
| 2 | 38 | M | Vercuronium, fentanyl, ephedrine | 86 |
| 3 | 18 | M | Vasopressin, phenobarbital, rocuronium | 90 |
| 4 | 26 | F | Phenobarbital, fentanyl, vercuronium | 92 |
| 5 | 39 | F | Midazolam, fentanyl, lidocaine | 80 |
| 6 | 54 | M | Phenobarbital, rocuronium | 84 |
| 7 | 49 | M | Fentanyl, ephedrine | 87 |
| 8 | 44 | M | Fentanyl, ranitidine, dopamine | 85 |

Figure 1 Effect of propofol on CYP3A4 activity in primary cultured hepatocytes. Hepatocytes prepared from donors were treated for 24 h with 0, 0.01, 0.05, 0.1, 0.5, and 1.0 mM propofol. At the end of this time, the medium was changed and erythromycin at 0.4 mM was added to the cells. After incubated for 30 min, aliquots of the medium were removed, and N-demethylation of erythromycin activity was determined as described above. Each value represented the mean of triplicate treatments with SD indicated by the vertical bars.*P <0.01.

Figure 2 Effect of propofol on CYP3A4 protein expression. Hepatocytes prepared from donors were treated for 24 h with 0, 0.01, 0.05, 0.1, 0.5, and 1.0 mM propofol. CYP3A4 was analyzed in sonicates of whole cells as described in the paragraph of Materials and Methods. 20 micrograms of sonicated protein were applied per well.

Change of CYP3A4 isoform protein
Hepatocytes were treated with propofol at a concentration of 0.01, 0.05, 0.1, 0.5 and 1.0 mM, respectively for 24 hrs, and total protein synthesis was measured as described previously (Kostubsky et al., 1997). The data in Figure 2 show a high expression of CYP3A4 proteins in hepatocytes, but
concentrations of propofol used clinically did not decrease total protein synthesis, even very high concentration of propofol (0.5 and 1.0 mM) could slightly reduce the hepatic CYP3A4 protein expression after 24 hours of culture. That meant the regular dosages used clinically (0.01-0.1 mM) had no significantly inhibitory effect on the cellular protein expression of P450 3A4.

DISCUSSION

In vitro studies with hepatic cells, in particular the human cultured hepatocytes, have offered a defined system for studying the direct effects of individual xenobiotic molecules on the regulation of hepatic CYP expression and activity in man.[11] This approach does not require any prior knowledge of the metabolism or disposition of the test compound, but instead uses the ability of a drug to induce or inhibit the metabolism of isofom-specific substrates, such as the conversion of testosterone to 6-hydroxytestosterone or N-demethylation of erythromycin by CYP3A1.[12] This method by utilizing intact human hepatocytes for studying the metabolic activities, but not the micromolar suspensions, can provide quick and reproducible estimates of CYP3A4 metabolic capacity and protein levels. Primary cultures of human hepatocytes are responsive to induction of CYPs and can be used to assess the interactions resulted from the effect of CYPs during multiple drug therapy. The present study was conducted to examine the potential down-regulation by propofol on CYP expression. To this end, specific monooxygenase activities for CYP3A4 isozymes and de novo CYP protein synthesis were examined.[15,16]

In our studies, the activity of CYP3A4 isoform in 8 Chinese patients was obviously lower than that of others reported in Caucasians. Although CYP3A4 drug metabolising activities vary widely among individuals, it has a unimodal population distribution and does not appear to be subjected to genetic polymorphism as is seen with other CYP isoforms (2D6, 2C9 and 2C19).[17-20] The wide interrace variability is likely, in part, to be caused by ethnic or cultural differences, perhaps related to an interaction between habit and diet. Hence we could not draw any conclusion about the normal distribution characteristics of CYPs in Chinese, because of the limited sample number and experimental conditions. More detailed and complete studies should be performed for analysing the distribution of CYPs among Chinese in the near future.[21]

Propofol has been shown to interfere with the metabolism of alfentanil and sufentanil by inhibiting CYP2B1 and CYP1A1. Propofol inhibits CYP2E1 only to a limited extent as its molecules are too large to bind effectively to the active sites of the enzyme. Thus, propofol may potentially alter the metabolism or disposition of the test compound, but still remains unclear.[25,26] This is the first report on whether different concentrations of propofol can down-regulate the protein expression of CYP3A4 in hepatocytic primary culture. According to the present result, we could see the regular dosages used clinically (0.01-0.1 mM) had no inhibitory effect on the cellular protein expression of P450 3A4, although a minimum dosage of propofol (0.01 mM) induced a significant inhibition of P450 3A4 activity. The intrinsic inhibition mechanisms of propofol on P450s have been investigated by several groups but still remain unclear.[25,26] It has been demonstrated that propofol exhibits a concentration dependent inhibitory effect on CYP2B1 and 1A1 by binding to the haemoloyt of the enzymes. Some researchers have used homologic P450 models, they postulated plausible mechanisms for enzyme catalysis, inhibition, and activation, based on its 3D structure. The sites of effect that propofol binds to CYP3A4 hemeprotein maybe 214, 218 active site residues, then competitive inhibition of propofol and other CYP3A4 substrates may occur because active sites of P450s have been occupied.[27] This postulation may explain the potential inhibitory mechanism of propofol on CYP3A4.

In the present study, a dosage-dependent inhibitory effect of propofol was observed on cytochrome P450 3A4 activity, while the mean plasma concentration of propofol in clinical TCI anesthesia was 0.01-0.05 mM as described by others,[28-30] indicating that a minimum clinical dosage of propofol (0.01 mM) could induce a significant inhibition of P450 3A4 activity. Since CYP3A4 is a predominant isofrom of CYP3A in adult humans, change of hepatic CYP3A4 activity will change the metabolism of most therapeutic drugs. Firstly, a large number of intravenous anesthetics and sedative agents (including diazepam, midazolam, fentanyl, lidocaine, etc.) are substrates of CYP3A4 isoform, N-hydroxylation and N-dealkylation reactions of anesthetics can be reduced when propofol administration causes drug accumulation, oversedation and postoperative awake delay.[31,32] Secondly, amiodarone, quinidine, nifedipine, berberine and ciclosporin are also eliminated through CYP3A4, thus competitive inhibition should be noticed and avoided especially when more than one substrates are administrated by patients with impaired liver function. These findings are in accordance with pharmacokinetic studies that have shown reduced clearance of midozolam when combined with fentanyl in cirrhotics, but overdosage of anti-arrhythmic have a greater clinical significance than that of other drugs[33,34]. Thirdly, as CYP3A4 also plays an important role in biotransformation and detoxification of many endogenous substrates, reduction of CYP3A4 activity may result in inactivation of endogenous substances including cholesterol, bile acid, sex hormones and glucocorticoids, thus causing more extensive physiopathologic changes in patients with hepatic diseases. These changes, in turn, will affect the drug metabolic enzymes.[35,36]

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