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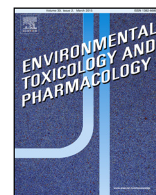

An improved *in vitro* method for screening toxin and medicine targeting CYP2E1

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ABSTRACT

The cytochrome P450 enzyme 2E1 (CYP2E1) presents in both microsome and mitochondrion, which influences the metabolism of many xenobiotics. The mice active liver homogenate was prepared for the medicinal incubation and mitochondrion was extracted for chemical screening targeting CYP2E1 enzyme. Representative CYP2E1 inducers (ethanol and pyrazole) and inhibitors (diallyldisulfide and kaempferol) were applied to evaluate the effectiveness of homogenate-mitochondrial system. In parallel, the *in-vitro* microsomal method targeting CYP2E1 was also operated for comparison. The results showed that in homogenate-mitochondrial method, the protein level and activity of CYP2E1 were increased by ethanol and pyrazole; reduced by diallyldisulfide and kaempferol, and this homogenate-mitochondrial method is convenient with good repeatability and reproducibility in screening chemicals targeting CYP2E1, especially for the inducers. Thus, the homogenate-mitochondrial method might be effective in screening both CYP2E1 inhibitor and inducer.

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1. Introduction

The *in-vitro* models for evaluating drug stability/bioconversion are of great value at the early stage of drug discovery and development since they provide a well-defined environment for specific studies in contrast to the complex *in-vivo* environment. The liver microsome has been applied to predict hepatic glucuronidation, drug-drug interaction and pharmacokinetic behavior based on the clearance concepts (Iwatsubo et al., 1997; Obach et al., 2006). The extracted microsome for predicting hepatic availability and medicinal screening have been primarily conducted using compounds metabolized by Cytochrome P450s, a group of crucial enzymes in drug metabolism (Obach, 2011). Especially, the Cytochrome P450 2E1 (CYP2E1) is a potential target in the treatment of liver diseases, which plays a crucial role in the biotransformation of numerous hepatotoxins and drugs. CYP2E1 is widely distributed in various cells, tissues and organs (Botto et al., 1994; Molina-Ortiz et al., 2014); in hepatocytes, the CYP2E1 located in both endoplasmic reticulum (ER) and mitochondrion (Lewis and Roberts, 2005; Robin et al., 2005). The microsomal method has been widely used as a tool to select CYP2E1 inhibitors, such as diallyldisulfide (DADS) and some flavonoids (Lu et al., 2011; Shih et al., 2013), by targeting on ER CYP2E1, asthe ER resident CYP2E1 mainly exists in the microsome (Shih et al., 2013). This evaluation system is composed of microsomal CYP450 enzyme mixture, NADPH, specific probe and sample substrate in phosphate buffer. The 4-nitrophenol (4-NP) is a convenient substrate as a probe for the measurement of hepatic CYP2E1 activity *in-vitro* (Tassaneeyakul et al., 1993). The *in vitro* microsomal method is based on the interaction between CYP2E1 and its substrate in the enzyme reaction condition, which is directly related to the CYP2E1 spatial structure alteration. However, the application of this classical microsomal method in chemical screening is still having some limitations. Firstly, the microsomal CYP2E1 can be easily degraded by proteasome present on the ER (Kitam et al., 2012). Secondly, the microsomal CYP2E1 is difficult acquired at normal cell lines for its lost in subcultured hepatocytes (Wilkening and Bader, 2003). Thirdly, the microsomal method reflects the reaction between enzyme and compound, which could not always show the real effects of compound to enzyme in normal intracellular environment. Take the CYP2E1 inducer for instance, the bioactivity of CYP2E1 is increased by up-regulation its expression in some pathological conditions such as alcoholic liver disease and diabetes (Gonzalez, 2005; Wu and Cederbaum, 2005). Numerous foods and drugs are potential substrates to CYP2E1 and responsible for hamful side effects; therefore, the screening of CYP2E1 induc-

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ers and inhibitors is necessary to ensure the healthy medications for humans. In this study, we explored an improved method based on liver homogenate-mitochondrial CYP2E1 for the screening of medicines and toxic chemicals.

2. Materials and methods

2.1. Animals

A total of 30 Kun-Ming mice (4- weeks old, 25 ± 2 g of body weight) of different batches (Birth dates: 08/03, 2014; 11/01, 2014 and 04/03, 2015, respectively) were procured from the animal center of Fourth Military Medical University (Xi'an, China). All mice were maintained in an air- and humidity-controlled room with a 12 h light and dark cycle. Animals were free to access feed and water throughout the experiment. All animal experimental protocols were reviewed and approved by the university ethical committee for the care and use of laboratory animals.

After fasting for 10 h, mice were euthanized by intraperitoneal injection with 10% chloral hydrate (Sigma, Shanghai, China) and their heart and liver were exposed; the circulating blood was removed by Ringer's solution through left ventricular perfusion. The liver homogenates were prepared separately to extract microsome and mitochondrion.

2.2. Liver homogenate-mitochondrial method

The liver homogenate and mitochondrion were prepared as per the method described previously with minor modification (Pallotti and Lenaz, 2007). The liver was removed and chilled on ice, minced with scissors and washed thrice in solution I (0.25 mM sucrose, 50 mM Tris-HCl, 35 mM EDTA, pH 7.4 medium and 0.4% BSA). Subsequently the liver was homogenized in cold solution I at the ratio of 1 g: 10 mL by a two-step-based homogenization: a large clearance pestle (0.12 mm) used for the initial sample reduction, and a small clearance pestle (0.05 mm) used to form the final homogenate. A total of 10 mL homogenate was prepared from 1 g of mice liver and every 1 mL was subpackaged for further medicine incubation and mitochondrion extraction. In order to evaluate the effectiveness of homogenate-mitochondrial CYP2E1 method, ethanol (CAS: 64-17-5, Sigma-Aldrich) and pyrazole (PZ, CAS: 288-13-1, Sigma-Aldrich) were taken as indicators for the CYP2E1 induction (Lu et al., 2008), and (DADS, CAS: 2179-57-9, Sigma-Aldrich) and kaempferol (KM, CAS: 520-18-3, Sigma-Aldrich) as the indicators of CYP2E1 inhibition (Sapkota et al., 2014; Wang et al., 2015). These 4 chemicals (standards) were separately incubated in the homogenate at 37 °C for 30–60 min and the control sample was incubated with mitochondrial solution I without any exogenous chemicals. A volume of 1 mL homogenate was incubated with ethanol (2, 4, 8, 16, 32, 64, 128, 512 mM), PZ, DADS and KM (2, 4, 8,16, 32, 64, 128, 256, 512 μM), respectively.

The mitochondrion was extracted from homogenate after incubation with the chemicals. The mitochondrial extraction was obtained from the typical differential centrifugation procedure used for the mitochondrial preparations (Borralho et al., 2015). The homogenate was centrifuged at 2,200 *g* for 15 min at 4 °C. After decanting the supernatant, the pellet was resuspended in Solution I, and subjected to a second centrifugation step. The two supernatants were combined and centrifuged at 11,600 *g* for 30 min at 4 °C. The pellet was resuspended in Solution II (0.21 mM mannitol, 0.07 mM sucrose, 5 mM Tris-HCl, pH 7.4, 1 mM EDTA) at a ratio of 1 mL Solution II per gram of starting material.

The protein level and activity of homogenate-mitochondrial CYP2E1 could be affected by some factors, such as homogenate solvent (mitochondrial solution I or TMS buffer), dilution ratio (1:20, 1:10, 1:5 and 1:1), temperature (0 °C, 25 °C and 37 °C), and incubation duration (5, 10, 15, 30 and 60 min). In this study, we optimized the chemical incubation condition. In order to assess the repeatability of our method, every concentration of each chemical had 6 technical repeats in each batch. Furthermore, the reproducibility of the homogenate-mitochondrial method was evaluated through comparing the experimental data obtained by three different laboratory technicians.

2.3. Liver homogenate preparation and microsome extraction

For the microsome preparation, the liver was removed and chilled on ice, minced with scissors and washed thrice in microsomal TMS buffer (20 mM Tris-HCl, 5 mM $MgCl_2$, 0.25 M Sucrose, pH 7.5). Subsequently the liver was homogenized in cold TMS buffer at the ratio of 1 g: 4 mL. For the microsomal extraction procedure, a total of 1 g liver without chemical incubation was homogenized in microsomal TMS buffer by 1:4 (w/v) on the ice (Lewis and Roberts, 2005).

The microsomal preparation was followed by Zhang et al. with some modification (Zhang et al., 2008). The liver homogenate was centrifuged at 9,000 *g* for 20 min without prior incubation with any chemicals. Then, the supernatant was fractionated (1 mL each) into 2 mL tubes contain 200 μL of $CaCl_2$ (88 mM) and kept in shaker for 5 min with ice. Finally, the mixture was centrifuged at 27,,000 *g* for 20 min and pellet was resuspended in 50 mM Tris-HCl mixed with 20% glycerol.

2.4. CYP2E1 protein level determination

Liver microsomal and mitochondrial proteins were harvested and their concentrations were analyzed using bicinchoninic acid (BCA) protein assay kit according to the manufacturer's protocol. Sample proteins were separated using SDS-PAGE with a 12% separating gel by loading equal amounts of protein per lane, subsequently, transferred to a PVDF membrane under 200 V for 1.5 h. The membrane was blocked with 5% fat-free milk in TBST buffer (25 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 5 mL Tween 20) for 2 h prior to incubation over night with anti CYP2E1 primary antibodies (Boster, Wuhan, China; 1:500) at 4 °C. Following the overnight incubations, the membrane was washed three times in TBST buffer and then incubated with an anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Sungene Biotech, Tianjin, China 1:3000) for 2 h at 37 °C. Bands of immunoreactive proteins were detected using a ECL Western blot kit (Vazyme Biotech, Nanjing, China) for the HRP tagged secondary antibody. The bands were scanned and the intensity of each protein band was analyzed using a Gel-pro Analyzer 4.5 (Media Cybernetics, USA). In order to determine the contamination in the mitochondrion and microsome faction of each, the mitochondrial indicator CoxIV and microsomal indicator cytochrome P450 reductase (CPR) were determined by Western blot and these two antibody were purchased from Abcam (Shanghai, China). There was no contamination in the mitochondrial fraction using CPR and CoxIV for microsome and mitochondria by western blot (data not shown).

2.5. CYP2E1 activity assay

CYP2E1 activity was measured as described by Tassaneeyakul et al. (1993). The relative activity of CYP2E1 was expressed as the ratio of the chemical-interfered CYP2E1 activity to control CYP2E1 without ethanol intervention. The mitochondrial CYP2E1 enzyme pre-incubated with chemicals was incubated with 4-NP, NADPH regenerating system in phosphate buffered solution (PBS, 0.05 mol/L, pH 7.4). Part of the extracted CYP2E1 protein was boiled

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