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Inter-individual and inter-organ variability in the bioactivation of paracetamol by human liver and kidney tissues

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ABSTRACT

Paracetamol (PAR) overdose is associated with massive hepatic injury; it may induce kidney toxicity as well. It is essential to measure organ-specific activities of related CYPs for evaluating the overdose cases. Available HPLC-based methods require high amounts of tissue samples. In order to develop liquid chromatography mass spectrometry (LC–MS)-based methods to process small amounts of human tissues, liver and kidney samples were obtained. Individual microsomes were prepared and incubated with PAR (for quantifying bioactivation), with nifedipine (for measuring CYP3A4 activity) and with *p*-nitrophenol (for measuring CYP2E1 activity). The small amount of tissue microsomes was sufficient to measure both the formation of NAPQI and the activities of CYP enzymes. Although the sample size in group was relatively low, both NAPQI formation and activity of CYP2E1 were significantly higher in males compared to females in kidney. Considerable variations in the metabolic capacity of individuals were observed for both organs.

1. Introduction

Paracetamol (PAR) is one of the most commonly used analgesic and antipyretic drugs in the world. Although it is considered to be safe, serious liver toxicity has been reported when taken above the therapeutic doses (Proudfoot, Wright, 1970; Curry et al., 1982). The mechanism of toxicity has been postulated as protein covalent binding, and other mechanistic hypotheses have been suggested (Xie et al., 2015). The two-electron oxidized electrophilic intermediate, *N*-acetyl-*p*-benzoquinone imine (NAPQI), accounts for liver necrosis. NAPQI forms adducts with sulphydryl moieties on cellular proteins in the centrilobular region of the liver, and the resultant adduct evokes a partial immunologic response (Forfar et al., 1980; Williams et al., 2010). It has recently been reported that mitochondrial damage and nuclear DNA fragmentation are critical to PAR hepatotoxicity in humans (McGill et al., 2012). CYP2E1 and CYP3 A4 have been reported as the major enzymes that catalyse PAR oxidation to NAPQI (Chen et al., 1998). In addition to liver toxicity, although less frequent, renal insufficiency occurs in approximately 1–2% of patients who have had a PAR overdose (Prescott, 1983). On the other hand, kidney medulla tissue that has been damaged has relatively lower levels of CYP isoforms compared to the liver; it is instead rich in prostaglandin endoperoxide synthase (PTGS). This enzyme also catalyses two-electron oxidation of PAR to NAPQI, which involves the formation of a one-electron oxidation product, called *N*-acetyl-*p*-benzosemiquinone imine free radical. This radical likely contributes to the nephrotoxicity of the drug (Testa and Kramer, 2010).

When hepatotoxicity cases are observed, PAR-induced nephrotoxicity is also seen (Larson et al., 2005). However, PAR- associated nephrotoxicity can also occur in the absence of liver toxicity. A wide inter-individual variability has been reported in PAR-associated liver toxicity in humans. One of the main causes is thought to be the variability in the activities of major cytochrome P450 isozymes that are responsible for phase I-mediated bioactivation of PAR to NAPQI.

Genetic polymorphism of phase I and phase II enzymes involved in PAR biotransformation and phenotypic properties may explain inter-individual differences. Cytochrome P450 s (especially CYP2E1, CYP3A4, CYP1A1 and CYP2D6), phase-II enzymes, (UDP-

Abbreviations: Acn, acetonitrile; GSH, glutathione; GST, glutathione S-transferase; HKM, human kidney microsomes; HLM, human liver microsomes; HPLC, high-performance liquid chromatography; I.S., internal standard; LC–MS, liquid chromatography mass spectrometry; NAPQI, *N*-acetyl-*p*-benzoquinone imine; NAPQI-CYS, cysteine conjugate of NAPQI; NAPQI-SG, NAPQI-glutathione conjugate; [$^2D_3$]-NAPQI-SG, glutathione conjugate of deuterium- labelled NAPQI; NRS, NADPH-regenerating system; PAR, paracetamol; PTGS, prostaglandin endoperoxide synthase; SE, standard error; SRM, selected reaction monitoring; UV, ultraviolet

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glucuronosyltransferase, sulfotransferase, glutathione S-transferases (GSTs)) and *N*-deacetylase are essential for PAR-induced hepatotoxicity and nephrotoxicity (Bessems and Vermeulen, 2001; Zhao and Pickering, 2011). CYP2E1 has been reported to be the major form responsible for the bioactivation of PAR in humans (Chen et al., 1998). This finding has been confirmed by animal experiments; CYP2E1 knockout mice have been shown to be resistant against hepatotoxicity (Lee et al., 1996; Zaher et al., 1998; Gonzaloes, 2001). CYP2E1 is known to be inducible by endogenous testosterone and by small chain alcohols and alkanes that are frequently present in environmental pollutants.

CYP3A4 is known to be one of the most important P450 isoforms because it is responsible for the metabolism of almost 50% of marketed drugs, and it has been suggested as a risk factor for drug-induced liver injury (Li and Schlicht, 2014). In addition, it is also highly inducible; medications, food constituents and unintentional exposure to environmental pollutants may drastically increase the expression of the enzyme in liver and other tissues.

In the studies that investigated PAR-associated liver or kidney injuries, an accurate and precise measurement of enzyme activity is a necessity. Although several successful methods have been reported to measure the activities of CYP450s in tissue samples, a scaled-down method is required, especially for human biological samples that are not routinely accessible and that are obtained with very limited amount when they are accessible. Therefore, we aimed to develop relevant methods for measuring the activity of CYP3 A4 and CYP2E1 and for quantifying PAR bioactivation in a limited amount of human tissue samples. Once developed, we applied the methods to the liver and kidney samples obtained from surgical patients. Although liver injury following PAR overdose and its associated mechanisms has been investigated extensively, extrahepatic pathways have not described comprehensively in literature. In the current study, we aimed to provide renal data in terms of CYP450-mediated bioactivation of PAR and individual variability with regard to metabolism. The capacity of each tissue to generate NAPQI and the specific activities of CYP2E1 and CYP3 A4 were determined by an isotope-dilution LC tandem mass spectrometry method that was operated in selected reaction monitoring (SRM) or full scan mode. Furthermore, the ability of microsomal fractions from different individuals to activate PAR, reactive NAPQI and individual CYP2E1 and CYP3A4 activities in both liver and kidney tissues were evaluated in this study.

2. Materials and methods

2.1. Chemicals and reagents

PAR was a gift from Abdi İbrahim Pharmaceutical Company (Turkey). *p*-Nitrophenol was obtained from Fluka. Nifedipine, mass spectrometry-grade solvents and all other chemicals were obtained from Sigma-Aldrich. Glutathione conjugate of deuterium-labelled NAPQI ([$^2D_3$]-NAPQI-SG) was synthesized in house from *N*-(4- hydroxyphenyl-2,3,5,6-^{2}H)acetamide (purchased from MSD Isotopes, Montreal, Quebec, Canada), according to the method described by Thatcher and Murray (Thatcher and Murray, 2001). The product was purified by successive preparative high- performance liquid chromatography (HPLC). The chemical structure and purity of the synthesized [$^2D_3$]- NAPQI-SG was determined by mass spectrometry (liquid chromatography [LC] tandem mass spectrometry [MS]), as described in Section 2.3.

2.2. Preparation of microsomal fractions

Microsomal fractions were isolated from human liver and kidney tissues obtained from patients who were admitted to Ege University Hospitals for an operation. The whole procedure was approved by the Ethical Committee of Ege University (doc. number 10-5.1/1). Microsomal fractions from nine human livers (HLM) and eight human kidneys (HKM) were prepared freshly by differential centrifugation, as described elsewhere (Guengerich and Bartleson, 2007). The protein content of the microsomes was determined using the method described by Lowry and modified by Miller (Miller, 1959).

2.3. Quantification of NAPQI formation

Individual microsomes (1 mg/mL protein) were incubated with PAR (250 μM) in the presence of NADPH (500μM), NADPH-regenerating system (NRS; 500 micM glucose-6-phosphate and 1 U/mL glucose-6-phosphate dehydrogenase) and glutathione (GSH; 5 mM) in potassium phosphate buffer at a pH of 7.40. The reaction was started by adding NADPH. Incubations were carried out in a shaking waterbath for 1 h at 37 °C. The reaction was terminated by adding 50 μ L of phosphoric acid (10%). [$^2D_3$]-NAPQI-SG was added as an I.S. to each tube at a final concentration of 5 μM. After the centrifugation, 25 μL of the sample was injected onto the Phenomenex inertsil ODS-2 HPLC column (150 mm × 4.6 mm × 5 μm), which was equipped with a guard column that used an HPLC system that consisted of a Thermo Accela system controller, an LC-gradient pump, a column oven, an injector and an ultraviolet (UV) detector (254 nm). An acetonitrile (Acn) gradient was formed in water (containing 0.2% formic acid) starting with 1% Acn and increasing to 99% Can over 20 min. The gradient returned to 10% after 2 min and back to 1% after 3 min. The flow rate was 0.5 mL/min. The HPLC was coupled to a triple quadrupole mass spectrometer (Thermo QuantumAccess Max) that was equipped with an electrospray ionisation source. The mass spectrometer was operated in the SRM mode with positive ionisation for the $[M+H]^+$ ion of NAPQI-SG to neutral loss of pyroglutamic acid moiety and glycine moiety of the conjugate, respectively (*m/z* 457 to *m/z* 328 and to *m/z* 382), and for the corresponding transitions of the stable isotope-labelled I.S. [$^2D_3$]-NAPQI (*m/z* 460 to *m/z* 331 and to *m/z* 385). Nitrogen was used as the nebulizer, auxiliary and sheath gas. Vaporizer and capillary temperatures were 350°C and 370°C, respectively. Collision energy was 20 V for both the authentic and the labelled compound.

2.4. NADPH dependency of NAPQI-SG formation

In order to explore whether NAPQI-SG formation is dependent on a continuous electron flow for the catalytic oxidation reaction, incubations with microsomes both from livers and kidneys were performed in the absence of NRS. Several HLM and HKMs were used for this purpose. These samples were processed similarly to the samples harvested from the incubations that contained NRS.

2.5. Measurement of microsomal CYP2E1 activity

CYP2E1 activity was measured by *p*-nitrophenol hydroxylation to 4-nitrocatechol catalysed by the enzyme (Elbarbry et al., 2006) with modifications; the reported HPLC method was transferred to LC–MS in the current study. The precursor and product ions of *p*-nitrophenol were determined by direct infusion of 1 ppm of the pure compound into the MS. Precursor and product ions in negative mode of ESI for *p*-nitrophenol and its metabolite, 4-nitrocatechol, were shown in Fig. 1A. HLMs and HKMs were incubated with *p*-nitrophenol in the presence of NADPH and NRS in 50 mM of phosphate buffer (pH: 6.80, with 2 mM MgCl and 1 mM ascorbic acid) in a final volume of 0.5 mL. To obtain the calibration line, 200, 100, 5 and 2.5 μM of *p*-nitrophenol solutions were prepared in mobile phase. The reaction was started by adding NRS and maintaining the solution for 30 min at 37 °C. The reaction was stopped by adding ice-cold phosphoric acid (10%). The incubation media were stirred thoroughly and then centrifuged at 12,000 × *g* for 20 min. Next, 50 μ L of supernatants were injected onto a Symmetry Shield RP18 HPLC column (100 mm × 4.6 mm × 3.5 μ m) that was equipped with a guard column that used the same HPLC system as described in Section