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Drug Metabolism Enzyme Expression and Activity in Primary Cultures of Human Proximal Tubular Cells

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Abstract

We previously catalogued expression and activity of organic anion and cation, amino acid, and peptide transporters in primary cultures of human proximal tubular (hPT) cells to establish them as a cellular model to study drug transport in the human kidney [*Toxicology* 228, 200–218 (2006)]. Here, we extend our analysis to drug metabolism enzymes. Expression of 11 cytochrome P450 (CYP) enzymes was determined with specific antibodies. CYP1B1, CYP3A4, and CYP4A11 were the only CYP enzymes readily detected in total cell extracts. These same CYP enzymes, as well as CYP3A5 and possibly CYP2D6, were detected in microsomes from confluent hPT cells, although expression levels varied among kidney samples. In agreement with Western blot data, only activity of CYP3A4/5 was detected among the enzyme activities measured. Expression of all three glutathione S-transferases (GSTs) known to be found in hPT cells, GSTA, GSTP, and GSTT, was readily detected. Variable expression of three sulfotransferases (SULTs), SULT1A3, SULT1E, and SULT2A1, and three UDP-glucuronosyltransferases (UGTs), UGT1A1, UGT1A6, and UGT2B7, was also detected. When examined over the course of cell growth to confluence, expression of all enzymes was generally maintained at readily measurable levels, although they were often lower than in fresh tissue. These results indicate that primary cultures of hPT cells possess significant capacity to metabolize many classes of drugs, and can be used as an effective model to study drug metabolism.

Keywords

Human kidney; proximal tubular cells; primary cell culture; drug metabolism; cytochrome P450; glutathione S; transferase; UDP; glucuronosyltransferase; sulfotransferase

1. Introduction

In a recent study (Lash et al., 2006), we catalogued protein expression and activity of a large number of plasma membrane transporters, including many involved in the uptake or efflux of organic anions and cations, neutral amino acids, and small peptides. The study demonstrated that primary cultures of human proximal tubular (hPT)¹ cells express a wide array of carriers and generally maintain their expression throughout the time period in which cells grow to confluence (i.e., typically 5 days). Moreover, expression levels of several of the carriers were found to exhibit significant variability among human kidney samples, consistent with the

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existence of genetic polymorphisms. These results validated the hypothesis that hPT cells can be used to study drug transport and that findings can be applied to clinical situations and drug design and development.

For several years, we have used primary cultures of hPT cells as model systems to characterize metabolism and mechanisms of cytotoxicity of the environmental contaminant and putative human carcinogen trichloroethylene (Cummings and Lash, 2000; Cummings et al., 2000a; Lash et al., 2001, 2003, 2005). Although these cells have been shown to express many drug metabolism enzymes and respond to toxicants by undergoing a wide range of responses, including necrosis, apoptosis, cell proliferation, and induction of key signaling molecules involved in stress response, complete information is lacking about their ability to metabolize drugs by the classical Phase I and Phase II reaction pathways. The chief advantage of using these cells as a model system is their direct derivation from the *in vivo* human kidney. Results obtained in metabolism and toxicity studies with these cells can be directly applied, after appropriate *in vitro*-to-*in vivo* extrapolation, to humans without the need to account for the uncertainties inherent in extrapolating data from studies using various animal models, such as cells from rats or mice (Kedderis, 1997).

Unlike rat proximal tubules, which express several CYP enzymes, including CYP2E1, CYP2B1/2, CYP2C11, CYP3A1/2, and CYP4A2/3 (Cummings et al., 1999), hPT cells appear to express a much more limited number of CYP enzymes (Ahmet et al., 1997; Cummings et al., 2000a). Human kidney does not appear to express CYP2E1 or CYP2C enzymes. However, CYP3A4/5 and CYP4A11 enzymes, which are important in the metabolism of many P-glycoprotein substrates and arachidonic acid, respectively, are expressed at relatively high levels in the proximal tubules (Cummings et al., 2000a).

The other major Phase I drug metabolism enzyme system whose expression has been studied in human kidney is that of the flavin-containing monooxygenases (FMOs) (Cashman and Zhang, 2006; Krause et al., 2003). In contrast to human liver, which expresses primarily FMO3 as well as several other FMO enzymes at significant levels, human kidney expresses primarily FMO1, somewhat lower levels of protein for FMO5, and very low levels of protein for FMO3 (Krause et al., 2003). Nishimura and Naito (2006) determined mRNA expression profiles of several Phase I and Phase II drug metabolism enzymes in a broad range of human tissues. As with the protein expression studies in human kidneys, they found that for FMOs in human kidney, by far the major mRNA expressed was that of FMO1; mRNA for FMO2, FMO3, FMO4, and FMO5 were 4.0%, 0.09%, 24.7%, and 12.6%, respectively, of that for FMO1.

The major Phase II drug metabolism enzymes that we assess in the present paper and that have been studied in human kidney are the GSH *S*-transferases (GSTs), sulfotransferases (SULTs), and UDP-glucuronosyltransferases (UGTs). Because of our interest in GSH-dependent bioactivation of halogenated solvents such as trichloroethylene, we previously studied GSTs in human and rat kidney (Cummings et al., 2000a, 2000b); unlike rat PT cells, which only express GST α isozymes, human PT cells express GSTA, GSTP, and GSTT isozymes. Nishimura and Naito (2006) found that mRNA for GSTP was the most prominently expressed GST isoform, whereas mRNAs for GSTA, GSTM, and GSTT isoforms were expressed at variable levels up to 30% of that for GSTP. Whereas hPT cells express GSTA, GSTP, and GSTT, GSTM is only found in distal segments of the nephron (Campbell et al., 1991).

¹Abbreviations: AMMC, 3-(2-*N,N*-diethyl-*N*-methylaminoethyl)-7-methoxy-4-methylcoumarin; BOMC, butyloxymethylcoumarin; CYP, cytochrome P450; Dulbecco's Modified Eagle's Medium : Ham's F12 Medium, DMEM:F12; FMO, flavin-containing monooxygenase; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; GSH, glutathione; GST, GSH *S*-transferase; hPT, human proximal tubular; mc, monoclonal; ms, microsomes; PBS, phosphate-buffered saline; pc, polyclonal; SULT, sulfotransferase; UGT, UDP-glucuronosyltransferase.

Nishimura and Naito (2006) found mRNA expression for several isoforms of SULTs, including SULT1A1, SULT1A3, SULT1B1, SULT1E1, and SULT2B1. mRNA for SULT1A1 was the most highly expressed form. These investigators also determined mRNA expression for several isoforms of UGTs: mRNA for UGT1A6 and UGT1A9 were the most prominently expressed, with significantly lower levels found for UGT1A1, UGT2B10, UGT2B15, UGT2B17, and UGT8.

In the present work, we sought to characterize further the capabilities of primary cultures of hPT cells to handle drugs. We hypothesize that primary cultures of hPT cells express a tissue- and cell type-specific complement of Phase I and Phase II enzymes, that expression of these enzymes are maintained at measurable levels throughout the course of culture to confluence, and that these cells can be used as a model system to study the metabolism of diverse classes of drugs.

2. Materials and Methods

2.1. Materials

Antibodies used for Western blotting to determine expression of drug metabolism enzymes are summarized in Table 1, showing the source, catalogue number (where applicable), species reactivity, and notes on some key properties. All other chemicals, including CYP substrates, were of the highest grade available and were purchased from commercial vendors. Doubly-distilled, deionized water was used for all solutions and buffers.

2.2. Isolation and primary culture of hPT cells

hPT cells were derived from whole human kidneys procured by Life Legacy Foundation (formerly International Bioresearch Solutions; Tucson, AZ). All tissue was scored by a pathologist as normal (i.e., derived from non-cancerous, non-diseased tissue). Cell isolation procedures were based on those originally described by Todd et al. (1995) and modified (Cummings and Lash, 2000; Cummings et al., 2000a) with use of sterile conditions (i.e., all instruments and glassware were autoclaved and all buffers were filtered through a 0.2- μ m pore-size filter). Renal cortex and outer stripe were cut into slices, washed with sterile phosphate-buffered saline (PBS), minced, and the pieces were placed in a trypsinization flask filled with 300 ml of sterile, filtered Hanks' buffer, containing 25 mM NaHCO₃, 25 mM HEPES, pH 7.4, 0.5 mM EGTA, 0.2% (w/v) bovine serum albumin, 50 μ g/ml gentamicin, 1.3 mg/ml collagenase, and 0.59 mg/ml CaCl₂, which was filtered prior to use. Whole kidneys were perfused with Wisconsin medium and kept on ice until they arrived at the laboratory, which was usually within 24 hr of removal from the donor. Kidneys used for these studies came from 14 donors (age, mean $\pm$ SEM): 8 males (48.1 $\pm$ 4.5), 6 females (56.3 $\pm$ 2.5). Detailed demographics of donors are summarized in Table 2.

All buffers were continuously bubbled with 95% O₂/5% CO₂ and were maintained at 37°C. Minced cortical pieces from whole kidneys were subjected to collagenase digestion for 60 min, after which the supernatant was filtered through a 70- μ m mesh filter to remove tissue fragments, centrifuged at 150 $\times$ g for 7 min, and the pellet resuspended in Dulbecco's Modified Eagle's Medium : Ham's F12 Medium (DMEM:F12; 1:1). Approximately 5 to 7 $\times$ 10⁶ cells were obtained per 1 g of human kidney cortical tissue.

hPT cells were resuspended in 2 ml of DMEM:F12 and diluted to 500 ml with cell culture medium, which was serum-free and hormonally-defined. Basal medium was a 1:1 mixture of DMEM:F12. Standard supplementation included 15 mM HEPES, pH 7.4, 20 mM NaHCO₃, antibiotics for day 0 through day 3 only (192 IU penicillin G/ml + 200 μ g streptomycin sulfate/ml or 50 μ g gentamicin/ml) to inhibit bacterial growth, 2.5 μ g amphotericin B/ml to inhibit

fungal growth, 5 µg bovine insulin/ml (= 0.87 µM), 5 µg human transferrin/ml (= 66 nM), 30 nM sodium selenite, 100 ng hydrocortisone/ml (= 0.28 µM), 100 ng epidermal growth factor/ml (= 17 nM), and 7.5 pg 3,3',5-triiodo-DL-thyronine/ml (= 111 nM) (Lash et al., 1995a). Cells were seeded at densities of 50–100 µg protein per cm² ($0.5\text{--}1.0 \times 10^6$ cells/ml) on polystyrene T-75 tissue culture flasks. Cultures were grown at 37°C in a humidified incubator under an atmosphere of 95% air/5% CO₂ at pH 7.4. Cultures were grown to confluence (generally 5 days) prior to experiments, unless otherwise stated. Cells were harvested by either scraping the flasks with a Teflon scraper or by brief incubation with Cellstripper (Cellgro, Herndon, VA) (in Ca²⁺- and Mg²⁺-free Hanks' buffer).

2.3. Isolation of microsomes

hPT cells were grown to confluence on collagen-coated, T-75 flasks, were harvested by scraping the flasks with a Teflon scraper, and were homogenized in 11 ml of buffer (100 mM potassium phosphate, pH 7.4, 1 mM EDTA, 150 mM KCl) using a Dounce tissue homogenizer. Microsomes were isolated from hPT cell homogenates by initially centrifuging at 9,000 g for 5 min. The post-mitochondrial supernatant was then centrifuged for 60 min at 105,000 g. The resulting pellets were resuspended in buffer and centrifuged an additional 60 min at 105,000 g to produce the "washed" microsomal fraction. Microsomal pellets were resuspended in buffer containing 10% (v/v) glycerol and were stored at –80°C until used. Purity of the microsomal fraction was estimated by measurement of marker enzymes for several subcellular compartments as described previously (Lash et al., 1995b). Results indicated that cross-contamination of the microsomal fraction with cytoplasm was < 5%, and contamination with mitochondria, lysosomes, and plasma membranes was < 2%.

2.4. Western blots

Cell lysate or microsomal protein (50 µg) was loaded onto a 12% (w/v) SDS polyacrylamide gel. Following electrophoresis, protein was transferred to a nitrocellulose membrane. The membrane was blocked with 5% (w/v) milk powder solution in Tris-buffered saline, followed by incubation with the primary antibody for 4 hr. The membrane was then washed three times with Tween Tris-buffered saline and then incubated for 1 hr with the appropriate secondary antibody conjugated to alkaline phosphatase. The membrane was washed 3 to 6 times with Tween Tris-buffered saline and then band(s) were visualized by incubating the membrane in a BCIP/NBT solution (Promega). Alkaline phosphatase staining intensity was determined by scanning laser densitometry with a Fuji Science Imaging System connected to a Macintosh G4 computer. Molecular weight standards were run alongside most samples to confirm molecular weights of proteins detected by the antibodies. When available, purified proteins were also run alongside samples as positive controls. In Western blots of some total cell extracts, expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or actin was also determined as a loading control. The antibody for actin (Sigma) was a polyclonal rabbit antibody that detects the 42-kDa protein in all mammalian species tested, including humans. The antibody for GAPDH (Calbiochem) was a monoclonal mouse antibody that recognizes the monomeric 36-kDa protein in canine, chicken, fish, frog, human, mouse, porcine, rabbit, and rat.

2.5. CYP enzyme assays

The following enzyme assays were performed by incubating microsomes from confluent hPT cells (1 mg protein/ml) to determine activities of specific CYP enzymes: Ethoxyresorufin deethylation (CYP1A1), phenacetin hydroxylation (CYP1A2), pentoxyresorufin deethylation (CYP2B6), diclofenac hydroxylation (CYP2C9), mephenytoin hydroxylation (4-hydroxy metabolite) (CYP2C19), 3-(2-*N,N*-diethyl-*N*-methylamino)ethyl-7-methoxy-4-methylcoumarin (AMMC) hydroxylation (CYP2D6), and hydroxylation of butyloxymethylcoumarin (BOMC; Vivid Red) or felodipine (CYP3A4).

All assays were measured by incubating microsomes (0.5 or 1 mg protein per time point) from at least 3 different kidneys at 37°C for up to 60 min with 1 mM NADPH and substrate at the indicated concentration. All assays were performed in the absence of NADPH to determine NADPH-dependent rates of metabolism.

Ethoxyresorufin deethylase and pentoxyresorufin deethylase activities were measured in a fluorescent plate reader (excitation = 535 nm, emission = 580 nm) using 1.7 μ M and 2 μ M substrate concentrations, respectively (Lubet et al., 1985). A standard resorufin curve was constructed with 0, 1, 5, 10, 20, 50, and 100 pmol/ml.

Phenacetin hydroxylation was measured by incubating microsomes with NADPH and 30 μ M phenacetin. Incubation mixtures were extracted with acetonitrile, centrifuged, evaporated to dryness under nitrogen, and product measured by LC/MS, using a Supelco, Discovery Amide C16 column (100 $\times$ 2.1 mm, 5 μ m particle size). Mobile phase was 40:60 acetonitrile : 0.1% (v/v) formic acid, and detection was with the electrospray positive ion mode at 0.2 ml/min (isocratic). Analytes were 4-acetamidophenol (m/z 152.1 to 110.2) and D₄-paracetamol (m/z 156.1 to 114.1) and the assay range was 5 to 1000 ng 4-acetamidophenol/ml.

Diclofenac hydroxylation was measured by incubating microsomes with NADPH and 7.5 μ M diclofenac. Incubation mixtures were extracted with acetonitrile, centrifuged, evaporated to dryness under nitrogen, and product measured by LC/MS, using a Phenomenex, Synergi Max RP column (50 $\times$ 2.0 mm, 4 μ m particle size). Mobile phase was 50:50 acetonitrile : 0.1% (v/v) formic acid, and detection was with the electrospray positive ion mode at 0.27 ml/min (isocratic). Analytes were 4'-hydroxydiclofenac (m/z 312.0 to 231.1) and ¹³C₆-4'-hydroxydiclofenac (m/z 318.0 to 237.1) as internal standard. A standard curve was constructed with 0, 10, 25, 50, 100, 250, and 500 ng 4-hydroxydiclofenac/ml.

Mephenytoin hydroxylation was measured by incubating microsomes with NADPH and 50 μ M mephenytoin. Incubation mixtures were extracted with acetonitrile, centrifuged, evaporated to dryness under nitrogen, and product measured by LC/MS, using a Varian, Polaris C₁₈ column (50 $\times$ 2.0 mm, 5 μ m particle size). Mobile phase was 75:25 acetonitrile : 0.1% (v/v) formic acid, and detection was with the electrospray positive ion mode at 0.2 ml/min (isocratic). Analytes were 4'-hydroxymephenytoin (m/z 235.1 to 150.2) and 3-acetamidophenol (m/z 152.1 to 110.2) as internal standard. A standard curve was constructed with 0, 5, 10, 25, 50, 100, 250, 500, 750, 1000, 1500, and 2000 ng mephenytoin/ml.

AMMC hydroxylation was measured by incubating microsomes, NADPH, and 2 μ M AMMC and product was measured in a fluorescent plate reader (excitation = 390 nm, emission = 460 nm).

BOMC (Vivid Red) hydroxylation was measured by incubating microsomes, NADPH, and 3 μ M BOMC and product was measured in a fluorescent plate reader (excitation = 535 nm, emission = 580 nm).

Felodipine hydroxylation was measured by incubating microsomes with NADPH and 10 μ M felodipine. Incubation mixtures were extracted with acetonitrile, centrifuged, evaporated to dryness under nitrogen, and product measured by LC/MS, using a Varian, Polaris C₁₈ column (50 $\times$ 2.0 mm, 5 μ m particle size). Mobile phase was a gradient of 0.1% (v/v) formic acid (A) and acetonitrile (B) at 275 μ l/min for 0.1 min (90% A, 10% B), 220 μ l/min for 1.5 min (25% A, 75% B), 450 μ l/min for 3.0 min (90% A, 10% B), and 450 μ l/min for 3.5 min (90% A, 10% B). Detection was with electrospray positive ion mode. Analytes were dehydrofelodipine (m/z 382.1 to 354.2) and D₃-dehydrofelodipine (m/z 387.1 to 359.1) as internal standard. A standard curve was constructed with 0, 20, 40, 60, 80, and 100 ng dehydrofelodipine/ml.

2.6. Data analysis

Protein determinations were done using the bicinchoninic acid (BCA) protein determination kit from Sigma, with bovine serum albumin as the standard. Results are expressed as means $\pm$ SEM. For inhibition studies, significant differences for means were first assessed by a one-way analysis of variance. When significant *F* values were obtained with the analysis of variance, the Fisher's protected least-significance *t* test was performed to determine which means were significantly different from one another, with two-tail probabilities < 0.05 considered significant.

3. Results

3.1. Expression of CYP enzymes

To determine the capacity of hPT cells to catalyze Phase I oxidation reactions, protein expression of 11 CYP enzymes was determined with specific antibodies. Of the 11 enzymes, only five showed detectable expression. Protein expression was not detected for CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, or CYP2E1. Immunoblots using total cell extracts of three different cell cultures showed expression of CYP1B1, CYP3A4, and CYP4A11, with some maintenance of expression throughout the five days of culture to confluence (Figure 1). In each case, expression was clearly higher at Day 0, when cells were first isolated and plated. Expression levels were maintained from Day 3 to Day 5.

CYP2D6 protein expression was detected in most of the samples of microsomes of confluent (i.e., Day 5) hPT cells as faint bands at approximately 50 kDa (data not shown). Use of purified CYP2D6 as a positive control, however, suggested that the polyclonal antibody does not react very strongly with the protein, implying that the actual levels of expression may be higher than they appear from the Western blots. As described below, however, no activity with a CYP2D6-specific substrate was detected, suggesting that this CYP enzyme may indeed not be expressed in hPT cells.

Human kidney expresses at least two forms of CYP3A, CYP3A4 and CYP3A5. Although previous immunohistochemical studies suggested that CYP3A enzymes are expressed primarily in glomeruli (Campbell et al., 1991), we determined their expression in microsomes from confluent hPT cells (data not shown). CYP3A4 protein was expressed in 12 of 13 samples tested. Of the 12 samples showing expression, protein was detected at similar levels except for that in microsomes from one culture, which appeared to be significantly lower than those of the other cultures. In contrast, CYP3A5 was detected as relatively faint bands only in 7 of 13 samples.

CYP4A11 protein was readily detected in all 13 samples of microsomes from confluent hPT cells (data not shown). Expression levels appeared to vary modestly among the samples.

3.2. CYP enzyme activities

Activities of several CYP enzymes were measured with selective substrates (Table 3). Of the seven CYP enzyme activities determined, only assays for CYP3A4 detected measurable activity. Two different substrates were used to measure CYP3A4 in microsomes from confluent hPT cell cultures, BOMC and felodipine (Figure 2). In each case, variable levels of activity were detected among the 13 samples tested. Although the felodipine hydroxylation assay should be the more sensitive one, being an LC/MS as opposed to a fluorometric method, felodipine hydroxylation exhibited a significant fraction that was NADPH-independent. In contrast, very low BOMC hydroxylation occurred in the absence of NADPH. For both substrates, incubations in the absence of substrate exhibited very low signal, indicating little interference with other components of the assay system.

For BOMC hydroxylation (Figure 2A), samples that had the highest activity were ms4, 5, 8, and 11. The pattern was somewhat different for felodipine hydroxylation (Figure 2B), being highest for ms1, 4, 6, 8, and 9.

3.3. Expression of Phase II conjugation enzymes

Isozymes from each of the families of enzymes (GSTs, SULTs, and UGTs) were expressed but at variable levels. The SULTs were generally the most difficult of the three Phase II enzyme families to detect by Western blot analysis.

GSTA and GSTT were much more readily detected than GSTP (Figure 3). For GSTT (Figure 3C), two bands were detected, suggesting some lack of specificity in the antibody. Based on molecular weight, the band of interest is indicated in the figure. Examination of GST protein expression at Day 0, Day 3, and Day 5 suggests that whereas GSTP and GSTT expression levels appeared to remain fairly constant during time in culture, expression was significantly lower for GSTA at Day 3 and Day 5 as compared to that at Day 0 in all three cell cultures.

Expression of three SULT isozymes, SULT1A3, SULT1E, and SULT2A1, were determined in extracts of three cell cultures (Figure 4). For SULT1E (Figure 4B) and for SULT2A1 (Figure 4C), two bands were detected as for GSTT above, again suggesting some lack of specificity in the antibodies. Based on molecular weight, the appropriate bands of interest are indicated in the figure. For SULT1A3 and SULT1E, expression levels were maintained throughout the five days of primary culture. For SULT2A1, however, expression level was lower for Day-3 and Day-5 as compared to that for Day-0 in cultures 1 and 2, but were still readily detected. For SULT2A1, expression level was only slightly lower for Day-3 and Day-5 as compared to that for Day-0 cells.

Expression of three UGT isozymes, UGT1A1, UGT1A6, and UGT2B7, were determined in extracts of three cell cultures (Figure 5). Variable levels of expression were detected for the first two UGT isozymes over the course of growth to confluency. For example, for UGT1A1, expression level actually increased in culture 1 during the course of culture, was highest on Day-3 in culture 2, and was only detectable on Day-0 and Day-5 in culture 3. For UGT1A6, expression was only detected on Day-0 in culture 1, was detected at all three days in culture 2 but decreased from Day-0 to Day-5 (particularly at Day-5), and decreased only slightly over the course of growth in culture 3. In contrast, expression level of UGT2B7 was readily detected and was maintained at approximately constant level over the course of growth in all three cultures.

4. Discussion

The present study extends our previous work (Lash et al., 2006) in characterizing the handling of drugs in primary cultures of hPT cells. Previously, we elaborated the capacity of hPT cells to transport drugs and other xenobiotics across the basolateral and brush-border plasma membranes, quantifying expression and activity of several membrane carriers that catalyze either uptake or efflux of organic anions, organic cations, neutral amino acids, and small peptides. Once chemicals enter the PT cell, most are substrates for either Phase I (i.e., oxidative) or Phase II (i.e., conjugation) enzymes that either detoxify or bioactivate them. These metabolic transformations lead ultimately to either excretion or convert the chemicals to forms that are involved in other cellular reactions, which can be either therapeutic or cytotoxic.

A critical consideration in understanding and developing primary cultures of hPT cells as a model for study of drug metabolism is that unlike the liver, which is overwhelmingly comprised of relatively uniform hepatocytes, the kidney is a highly differentiated and heterogeneous organ, possessing numerous cell types, each of which has distinctive biochemistry, physiology,

and morphology. Drug metabolizing enzymes exhibit selective localizations along cell types of the nephron. Even within the proximal tubule itself, there is heterogeneity and while PT cells express many of the same Phase I and Phase II enzymes that are found in hepatocytes, levels of many of the proteins are only 5 to 20% of those in hepatocytes. Consequently, it is necessary to analyze highly concentrated samples and to allow blots to develop for prolonged periods of time to enhance detection. Because of these methodological adaptations, blots often result with relatively high backgrounds, making it difficult to distinguish between specific and nonspecific interactions between antibodies and proteins. An additional variable is the quality of each commercial antibody. In spite of these limitations and caveats, protein expression of several Phase I and Phase II drug metabolism enzymes was characterized, using purified proteins, when available, as positive controls.

Expression of 11 CYP enzymes (CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2D6, CYP2E1, CYP3A4, CYP3A5, and CYP4A11) were determined in either whole cell extracts or microsomes derived from confluent, primary cultures of hPT cells. Previous studies (Ahmet et al., 1997; Cummings et al., 2000a) showed that CYP2E1, which is involved in the hydroxylation of a diverse array of halogenated solvents, other organic solvents, and drugs such as acetaminophen (Boogaards et al., 1995), is not expressed in hPT cells. Of the 11 CYP enzymes whose expression was determined in this study, the only ones that exhibited consistently detectable expression were CYP1B1, CYP3A4/5, CYP4A11, and possibly CYP2D6. Fluorometric and LC/MS enzymatic assays confirmed the absence of activity of CYP1A1, CYP2B6, CYP2D6, CYP2C9, and CYP2C19. The absence of detected activity with the CYP2D6-specific substrate and the uncertain ability to detect CYP2D6 protein on the Western blot of hPT cell microsomes, suggests that CYP2D6 may not be expressed to a significant extent in hPT cells. Previous studies (Cummings et al., 2000a) demonstrated the activity of CYP4A11 with lauric acid as substrate. CYP3A4 activity was demonstrated with both BOMC and felodipine as substrates. In both cases, activity was determined in microsomes from 13 cell cultures; considerable interindividual variability was found, although the pattern differed slightly with each substrate. Attempts to correlate the relative level of activity with demographic characteristics of kidney donors, however, failed to show any patterns. Nonetheless, the high degree of interindividual variability is consistent with the long known existence of genetic polymorphisms for CYP3A4 and other CYP enzymes (Guengerich, 1991; Haener et al., 1996).

Expression of three classes of Phase II enzymes were also studied. Primary cultures of hPT cells were shown to express three isoforms of GST, GSTA, GSTP, and GSTT, which confirms previous studies of ours (Cummings et al., 2000a). While the GSTA antibody produced the strongest signal in Western blots, GSTA expression tended to decrease with time in culture, particularly in cultures 1 and 2 (cf. Figure 3, top panel). In contrast, GSTT expression was maintained throughout five days of culture. Although expression was clearly detected for three SULT isoforms, SULT1E appeared to be the most readily detectable SULT isoform expressed in hPT cells and the best maintained throughout culture to confluence. This contrasts with the findings of Nishimura and Naito (2006), who reported SULT1A1 mRNA to be the most highly expressed in human kidney. Finally, expression of three isoforms of UGT were determined. Again, whereas Nishimura and Naito (2006) reported expression of two mRNAs for UGT1A isoforms as being the major ones detected in human kidney, with much lower expression of several mRNAs for UGT2B isoforms, our results on protein expression suggested that UGT2B7 may be the major isoform found in hPT cells. A possible explanation for the differences between the present results and those of Nishimura and Naito is the ethnic makeup of the donor population. In the current study, for example, donors comprised only one Asian subject. Analysis of Phase II enzyme expression in larger and more diverse group of donors could result in different patterns of enzyme expression.

In summary, the present results validate the use of primary cultures of hPT cells as a model to study drug metabolism by most of the major Phase I and Phase II enzymes. Together with previous work on expression of FMO (Cashman and Zhang, 2006; Krause et al., 2003), these studies show that primary cultures of hPT cells possess high levels of CYP3A4/5, CYP4A11, FMO1, GSTA, GSTP, GSTT, SULT1E, UGT2B7, and lower levels of CYP1B1, possibly CYP2D6, SULT1A3, SULT2A1, UGT1A1, and UGT1A6. Taken together with our previous work on plasma membrane transporters relevant for the reabsorption and secretion of many classes of drugs (Lash et al., 2006), primary cultures of hPT cells can be effectively used to study the uptake, intracellular metabolism, and excretion of most major classes of therapeutic drugs and toxic chemicals. An important application of these findings for drug development is that the cellular model can be used to assess the potential for serious drug-drug interactions at the levels of both membrane transport and intracellular metabolism, which can in turn influence therapeutic efficacy or toxicity.

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Figure 1. Western blot analysis of CYP1B1, CYP3A4, and CYP4A11 expression in total cell extracts of hPT cells

Confluent hPT cell primary cultures (50 µg protein) at either Day 0, Day 3, or Day 5 were subjected to SDS-PAGE and then transferred to a nitrocellulose blot that was then incubated with the appropriate antibody. Blots were scanned by a Fuji Image Scanner.

Figure 2. CYP3A4 enzyme assays in microsomes from hPT cells

Microsomes were isolated from 13 confluent, primary cultures of hPT cells. *A. BOMC hydroxylation.* Incubations were performed in 24-well plates. Product was measured by the increase in fluorescence in a plate reader. *B. Felodipine hydroxylation.* Incubations were performed in 24-well plates. Product was measured by LC/MS. Results for both assays are means $\pm$ SE of measurements from 3 separate incubations with each preparation of microsomes. Incubations were also performed in the absence of NADPH or substrate as controls.

Figure 3. Western blot analysis of GST isozyme expression in hPT cells

hPT cells (50 μ g protein) at either Day 0, Day 3, or Day 5 were subjected to SDS-PAGE and then transferred to nitrocellulose blots that were then incubated with either a goat anti-rat GSTA pc antibody, a rabbit anti-human GSTP pc antibody, or a rabbit anti-human GSTT pc antibody. Blots were scanned by a Fuji Image Scanner. The arrow in panel C indicates the band of interest, based on molecular weight.

Figure 4. Western blot analysis of SULT isozyme expression in hPT cells
hPT cells (50 μ g protein) at either Day 0, Day 3, or Day 5 were subjected to SDS-PAGE and then transferred to nitrocellulose blots that were then incubated with either a rabbit anti-human SULT1A3 pc antibody, a rabbit anti-human SULT1E pc antibody, or a rabbit anti-human SULT2A1 pc antibody. Blots were scanned by a Fuji Image Scanner. The arrows in panels B and C indicate the band of interest, based on molecular weight.

Figure 5. Western blot analysis of UGT isozyme expression in hPT cells
hPT cells (50 µg protein) at either Day 0, Day 3, or Day 5 were subjected to SDS-PAGE and then transferred to nitrocellulose blots that were then incubated with either a rabbit anti-human UGT1A1 pc antibody, a rabbit anti-human UGT1A6 pc antibody, or a rabbit anti-human UGT2B7 pc antibody. Blots were scanned by a Fuji Image Scanner.

Table 1

Summary of antibodies used in studies of transporter expression.

Protein	Source	Catalogue No.	Species Reactivity	Notes
CYP1A1/2	NCI/H. Gelboin		Human	Mouse anti-Human mc
CYP1B1	Gentest	458211	Human	Rabbit anti-Human pc
CYP2A6	NCI/H. Gelboin		Human	Mouse anti-Human mc
CYP2B6	NCI/H. Gelboin		Human	Mouse anti-Human mc
CYP2C8	NCI/H. Gelboin		Human	Mouse anti-Human mc
CYP2C9	NCI/H. Gelboin		Human	Mouse anti-Human mc
CYP2C19	NCI/H. Gelboin		Human	Mouse anti-Human mc
CYP2D6	NCI/H. Gelboin		Human	Mouse anti-Human mc
CYP2E1#1	R. Novak		Rabbit, Rat, Human	Goat anti-Rabbit pc
CYP2E1#2	NCI/H. Gelboin		Human	Mouse anti-Human mc
CYP3A4/5	NCI/H. Gelboin		Human	Mouse anti-Human mc
CYP4A11	J.M. Lasker		Human	Rabbit anti-Human pc
GSTA	Oxford Biomedical	GS09	Rat, Human, Mouse	Goat anti-Rat pc
GSTM	Oxford Biomedical	GS23	Rat, Human, Mouse	Goat anti-Rat pc
GSTP	Oxford Biomedical	GS72	Human	Rabbit anti-Human pc
GSTT	Oxford Biomedical	GS56	Human	Rabbit anti-Human pc
UGT1A1	Gentest	458411	Human	Rabbit anti-Human pc
UGT1A6	Gentest	458416	Human	Rabbit anti-Human pc
UGT2B7	Gentest	458427	Human	Rabbit anti-Human pc
SULT1A3	PanVera	P2238	Human	Rabbit anti-Human pc
SULT1E	PanVera	P2237	Human	Rabbit anti-Human pc
SULT2A1	PanVera	P2236	Human	Rabbit anti-Human pc

Abbreviations: CYP, cytochrome P450; GST, glutathione *S*-transferase; mc, monoclonal; pc, polyclonal; SULT, Sulfotransferase; UGT, UDP-glucuronosyltransferase.

Table 2

Summary of human kidneys used in project.

Experiment #	Source Code	Date Cells Plated	Age	Sex	Race
1	IBS-3-011	5/2/2003	49	M	Caucasian
2	IBS-4-001	5/23/2003	40	M	Hispanic
3	IBS-4-002	5/30/2003	59	F	Caucasian
4	IBS-4-003	6/10/2003	56	F	Caucasian
5	IBS-4-004	6/26/2003	52	M	Caucasian
6	IBS-4-005	7/2/2003	55	F	Caucasian
7	IBS-4-006	8/9/2003	62	M	Asian
8	IBS-4-007	8/30/2003	48	M	Caucasian
9	IBS-4-008	9/16/2003	67	F	Caucasian
10	IBS-4-009	10/1/2003	50	F	Caucasian
11	IBS-4-010	10/24/2003	51	F	Caucasian
12	IBS-4-011	11/7/2003	64	M	Caucasian
13	IBS-4-012	12/17/2003	47	M	Caucasian
14	IBS-4-013	1/5/2004	23	M	African American
		Mean ± SEM:	48.1 ± 4.5	For 8 M	
			56.3 ± 2.5	For 6 F	
	11 Caucasian	1 African American	1 Hispanic	1 Asian	

Table 3

Qualitative summary of CYP enzyme assays.

Enzyme	Substrate	Result
CYP1A1	Ethoxyresorufin	< 1 pmol/ml
CYP1A2	Phenacetin	< 5 ng/ml
CYP2B6	Pentoxifyresorufin	< 1 pmol/ml
CYP2C9	Diclofenac	< 10 ng/ml
CYP2C19	Mephenytoin	< 5 ng/ml
CYP2D6	3-(2- <i>N,N</i> -diethyl- <i>N</i> -methylamino)ethyl)-7-methoxy-4-methylcoumarin (AMMC)	< 20 ng/ml
CYP3A4	Butyloxymethylcoumarin (BOMC; Vivid Red)	Variable: Highest in microsomes 4, 5, 8, 10, 11
CYP3A4	Felodipine	Variable: Highest in microsomes 4, 6, 8, 9