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Effect of Chronic Renal Insufficiency on Hepatic and Renal UDP- Glucuronyltransferases in Rats

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Running Title: Are Hepatic and Renal UDP-Glucuronyltransferases Affected in Rats by
Chronic Renal Insufficiency?

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Abbreviations: CRI, Chronic Renal Insufficiency; UGT, UDP-glucuronyltransferase;
E2-3G, β -estradiol 3-glucuronide; E2-17G, β -estradiol 17-glucuronide; 4-MU, 4-
methylumbelliferone; MUG, 4-methylumbelliferone glucuronide; M3G, morphine 3-
glucuronide; ER, endoplasmic reticulum; EDTA, ethylenediamine tetracetate; ANOVA,
one-way analysis of variance

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ABSTRACT

Significant evidence exists regarding altered CYP450 enzymes in chronic renal insufficiency (CRI), although none exists for the Phase II enzymes. The objective of this study was to investigate the effect of CRI on hepatic and renal UDP-glucuronyltransferase (UGT) enzymes. Three groups of rats were included: CRI induced by the 5/6th nephrectomy model, control and control pair-fed (CPF) rats. UGT activities were determined in liver and kidney microsomes by the 3- and 17-glucuronidation of β -estradiol (E2-3G and E2-17G), glucuronidation of 4-methylumbelliferone (4-MUG) and 3-glucuronidation of morphine (M3G). UGT isoforms responsible for these catalytic activities were screened using recombinant rat UGT1A1, UGT1A2, UGT1A3, UGT1A7, UGT2B2, UGT2B3, and UGT2B8. UGT protein levels were examined by Western blot analysis using polyclonal antibodies. There was no significant difference between CRI and CPF rats in hepatic and/or renal E2-3G (UGT1A1), E2-17G (UGT2B3), 4-MUG (UGT1A6), and M3G (UGT2B1) formation. Formation of E2-17G and 4-MUG in the liver, and E2-3G and 4-MUG in the kidney was significantly reduced ($p < 0.05$) in CPF and CRI rats, as compared to control rats. The downregulated glucuronidation activities were accompanied by corresponding reductions in protein content of specific UGT isoforms. These results suggest that CRI does not appear to influence the protein levels or catalytic activity of most of the major hepatic or renal UGT enzymes. The observed downregulation of hepatic and renal UGTs in CRI and CPF rats could be caused by restricted food intake in these groups of rats. (Key word: CRI, food restriction, drug metabolism, UGT)

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INTRODUCTION

UDP-glucuronyltransferase (UGT) enzymes are a major class of Phase II enzymes that catalyze the conjugation of glucuronic acid to many drugs and certain endogenous compounds [Jansen et al., 1992; Monteith et al., 1990; Miners and Machenzie, 1991; Tephly and Burchell, 1990]. The water-soluble glucuronides are readily eliminated from the body via biliary and renal excretion [Fisher et al., 2001]. UGT enzymes are a family of membrane-bound enzymes mainly located in the rough endoplasmic reticulum (ER) of mammalian liver and various extrahepatic tissues, including the gastrointestinal tract, the kidney and olfactory epithelium [Tukey et al., 2000]. Drug glucuronidation is influenced by many factors, including age, disease states, smoking, diet, drug therapy, etc [Miners and Machenzie, 1991].

Chronic renal insufficiency (CRI) is a condition in which there is a progressive loss of renal function. Many diseases can cause CRI, including diabetes, hypertension, polycystic kidney disease, rapidly progressive glomerulonephritis, renal disposition diseases, and renal vascular disease. Other risk factors include a family history of kidney diseases, increasing age, pregnancy, and medications. In patients with CRI, many types of renal function are affected, including glomerular blood flow and filtration, tubular secretion, reabsorption and renal parenchymal mass. The kidney is one of the major organs responsible for the elimination of drugs and their metabolites from the body. Therefore it is not surprising that in CRI the kinetics of drugs that are primarily eliminated by the kidney could be significantly modified. Hepatic function has often been assumed to be unchanged in patients with CRI as compared to patients with normal renal function. However, there is significant evidence that the disposition of highly

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metabolized drugs could also be altered in CRI [Touchette and Slaughter, 1991; Gibson, 1986]. For example, the beta-blocking agent bufuralol, is extensively metabolized by oxidation and conjugation in the body. After an oral dose, there was a five-fold increase in the AUC value of patients with severe renal failure, compared to healthy volunteers [Balant et al., 1980]. Another example is the anti-HIV drug zidovudine, which is primarily eliminated in the liver by glucuronidation. Singlas et al (1989) demonstrated that after a single oral dose of zidovudine, the mean AUC values were significantly higher in the renal failure group ($11.7 \mu\text{mol}\cdot\text{L}^{-1}\cdot\text{h}$) than in normal population ($5.2 \mu\text{mol}\cdot\text{L}^{-1}\cdot\text{h}$). Since the increase in AUC_{oral} cannot be fully explained by the decrease in Cl_{ren} , it is possible that hepatic glucuronidation of zidovudine may be decreased in renal failure. Since a number of extensively glucuronidated drugs have a narrow therapeutic window (e.g. zidovudine, morphine), altered glucuronidation is likely to have important clinical consequences [Miners and Mackenzie, 1991]. In rats induced with renal failure using the nephrectomy model, Ali M., et al (1979) showed that the rate of glucuronidation of phenolphthalein but not 4-nitrophenol by liver was significantly ($p<0.05$) reduced. However, the specific UGT isoforms affected by renal dysfunction and the exact mechanism underlying this phenomenon has not been systemically studied.

The UGTs are classified into two families, UGT1 and UGT2, in both rats and humans [Tukey et al., 2000]. In the rat UGT1 family, UGT1A1 and UGT1A6 are two important isoforms responsible for the glucuronidation of endogenous compounds (bilirubin, estradiol, etc) and xenobiotic small planar phenols (4-methylumbelliferone, 4-nitrophenol) respectively. Of the rat UGT2 family, UGT2B1 is an important isoform involved in the glucuronidation of steroids [Narayanan et al., 2000]. Most UGT isoforms

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are not only expressed in the liver but appear to have a distinct pattern of extrahepatic expression, particularly in the kidney, GI tract, lung, etc [Fisher et al., 2001]. Kidneys from different species have shown glucuronyltransferase activities towards various compounds [Peters and Jansent, 1988; Anders., 1980; Coughtrie et al., 1987; Guellet et al., 1995]. So kidney may not only be a major excretory organ but also might play an important role in the glucuronidation of drugs. Therefore, it is especially necessary to examine whether renal glucuronyltransferase activities are influenced in subjects with renal dysfunction.

The specific aims of this study were to determine the possible effect of chronic renal insufficiency (CRI) on hepatic and renal UGTs and to elucidate a possible mechanism to explain this phenomenon if observed. These aims were carried out by first characterizing glucuronidation activities of recombinant rat UGTs (UGT1A1, UGT1A2, UGT1A3, UGT1A7, UGT2B2, UGT2B3, and UGT2B8) towards estradiol, 4-methylumbelliferone (4-MU), and morphine. This effect was further investigated by determining hepatic and renal UGT catalytic activity and protein content in rats surgically induced with CRI as well as control and control pair-fed animals.

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MATERIALS AND METHODS

Materials

4-methylumbelliferone (4-MU), β -estradiol, morphine, their glucuronides and UDP-glucuronic acid (UDPGA) were purchased from Sigma Chemical (St Louis, MO). All reagents used for HPLC analysis were HPLC grade and purchased from Fisher Scientific Products (Pittsburgh, PA). Rat recombinant UGT enzymes and antibodies were prepared as previously described (Miles et al., 2005; Ritter et al., 1999).

Induction of Chronic Renal Insufficiency by the 5/6th Nephrectomy Method (Remnant Kidney Model)

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) (n=13), weighing 150-200g, were housed in the animal care facility in Virginia Commonwealth University (VCU), Richmond, VA and allowed to acclimatize for 7 days before surgical procedures. The surgical protocol was approved by the VCU Institutional Animal Care and Use Committee (IACUC).

CRI was induced in rats by the method published previously by our laboratory [Rege B., 2003]. Animals were randomly divided into three groups: control (Control) (n=4), control pair-fed (CPF) (n=5), and chronic renal insufficiency (CRI) (n=4). CRI was induced by the 5/6th nephrectomy method in which two-thirds of left kidney and the complete right kidney were excised. CPF animals were subjected to a sham operation in which animals underwent the same surgical procedures without the removal of kidney mass. They were fed the same amount of food as the weight-matched CRI animals based on the food intake of the CRI animals from the previous day. Rats in the control group were allowed free access to food and were not subjected to a surgical procedure. Five

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weeks after the surgeries, animals were sacrificed and the liver and kidney were removed and stored at -70°C until preparation of microsomes. Microsomes were prepared from liver and kidney tissues by the differential centrifugation method as described previously by Sarkar M.A., et al (1992). Microsomes were suspended in a buffer containing 10mM Tris Acetate (pH.7.4), 20% (w/v) glycerol, 1mM ethylenediamine tetracetate (EDTA) and stored at -70°C. Total microsomal protein concentrations were determined using the Bio-Rad Protein Assay kit (Bio-Rad, CA).

UGT Catalytic Activity

Incubation conditions

UGT1A1, UGT1A6 and UGT2B1 catalytic activities were evaluated in microsomal incubations using estradiol [King et al., 1996], 4-MU [Falany and Tephly, 1983], and morphine [Pritchard et al., 1993] as probe substrates, respectively. To investigate the specificity of these probe substrates, estradiol, 4-MU and morphine glucuronidation activity of recombinant rat UGT1A1, UGT1A2, UGT1A3, UGT1A7, UGT2B2, UGT2B3 and UGT2B8 enzymes expressed in HepG2 cells were also measured. The concentration of microsomal protein, UDPGA and incubation time were optimized in preliminary experiments (data not shown) to ensure glucuronidation experiments were carried out under initial rate conditions. The ER lumenal localization of the active site of UGT proteins results in the phenomenon of microsomal latency, which can be overcome by adding detergents, albumin, or lipids to disrupt the membrane [Fisher et al., 2001]. Triton X-100 was used in these experiments to remove the latency and observe optimal enzyme efficiency (data not shown). Substrate concentrations were determined based on the kinetic profiles of enzymatic reactions (data not shown).

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The enzyme activity assay mixture (250 μ l) contained 50mM Tris-HCl (pH7.4), MgCl₂ (10mM for the estradiol assay, 5mM for the 4-MU and the morphine assay), microsomal protein or cell lysate expressed with rat UGT isoform (1.0mg/ml for estradiol and morphine assay, 0.4mg/ml for 4-MU assay), 0.02% Triton X-100, 3mM UDPGA, and substrate (150 μ M estradiol, 1000 μ M 4-MU or 20mM morphine). After pre-incubation at 37°C for 3 minutes, the reaction was initiated by addition of UDPGA and was incubated at 37°C for 30 minutes (estradiol and morphine) or 15 minutes (4-MU). Then the reaction was terminated by addition of 5 μ l of 70% perchloric acid.

Analytical method

The proteins were removed by centrifugation at 14,000rpm for 5 minutes and a portion of the supernatant (100 μ l for estradiol assay, 20 μ l for 4-MU and morphine assay) was injected into a HPLC system for analysis.

Estradiol assay: Estradiol (E2) and its metabolites, estradiol 3- (E2-3G) and 17-glucuronide (E2-17G), were separated on a HPLC system previously described by Gentest (BD Bioscience company, MA) with slight modification. The HPLC system was equipped with a Waters Alliance 2690 separation module and column oven (45°C), a 5 μ m, 4.6mm \times 250mm ODS column (Beckman, CA) and a Brownlee RP-18 guard column (Perkin Elmer, CT). Mobile phase A was 10% methanol. Mobile phase B was 100% acetonitrile. Mobile phase C was 30% acetonitrile and 1mM perchloric acid. Initial mobile phase composition was 85% mobile phase A, 0% mobile phase B and 15% mobile phase C. Elution of E2, E2-3G and E2-17G was carried out by an increase in mobile phase B to 85% over 15 minutes, while mobile phase C remained constant during the run. The flow rate was 1.0ml/min. UV detection was at 280nm for estradiol and its

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metabolites.

4-Methylumbelliferone assay: The glucuronide metabolite of 4-MU was determined by dilution of the supernatant 5-fold before directly injecting into the HPLC column. 4-MU and 4-MU glucuronide (4-MUG) were analyzed according to the method published by Narayanan R., et al (2000) with slight modification. The separation was achieved on a 5 μ m, 250mm \times 3.0mm Luna-CN column (Phenomenex, CA) with a Brownlee CN guard column (Perkin Elmer, CT). The mobile phase consisted of acetonitrile (solvent A) and 20mM ammonium phosphate buffer (pH4.5) (solvent B). 4-MU and 4-MUG were resolved using the following gradient: t=0 min, %A=90; t=0.5, A%=70%; t=4.5, A%=90; total run time=10 min. The flow rate was held at 0.5ml/min. 4-MU and 4-MUG were detected by fluorescence detection with excitation wavelength at 290nm and emission wavelength at 400nm.

Morphine assay: Morphine (M) and morphine 3-glucuronide (M3G) were separated based on a previously published method [Innocenti et al., 2001], on a 5 μ m, 250mm \times 4.6mm ODS column and a guard column. The mobile phase consisted of 73.5% of 10mM sodium phosphate in 1mM sodium dodecyl sulfate (pH2.1) and 26.5% of acetonitrile. The flow rate was 1.0ml/min. Morphine and M3G were detected by fluorescence detection with the excitation wavelength at 210nm and the emission wavelength at 340nm.

Estradiol, 4-MU, and morphine metabolites were quantified from standard curves of glucuronides. All the analytical methods were validated according to FDA Guidelines for Bioanalytical Methods Validation and were well within the acceptance criteria (+15%). The limit of detection (LOD) and quantification (LOQ) were determined to be

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0.29 and 0.98 μ M for E2-3G, respectively. The LOD and LOQ were determined to be 0.13 and 0.45 μ M for E2-17G, respectively. The lowest calibration standards of 4-MUG and M3G were determined to be the experimental LOQ, which was 5 μ M.

Western blot analysis

The protein content of UGT1A1, UGT1A6, UGT1A7, total UGT1A and UGT2B were assessed in liver and kidney microsomes using polyclonal antibodies specific for rat UGT1A1, human UGT1A6, rat UGT1A7, rat UGT1A and UGT2B common region. Microsomal protein (15 μ g) was loaded onto an 8.5% SDS-PAGE gel and resolved under constant voltage (70V) for 2.5 hours at room temperature. The resolved protein samples and molecular weight marker were transferred to a nitrocellulose membrane (Bio-Rad, CA). The nitrocellulose membranes were blocked with 5% nonfat dry milk in TBS-Tween buffer and immunoblotted with primary antibody for specific UGT isoform and secondary antibody coupled with horseradish peroxidase (sheep anti-mouse IgG from Dr J.K Ritter.). The membranes were detected by Western LightningTM Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Inc. MA). The optical density of Western blot bands was determined using ImageQuant 5.2 software (Molecular Dynamics, CA).

Statistical analysis

All results were reported as mean \pm standard deviation (SD). One-way analysis of variance (ANOVA) was used to evaluate differences between the groups at an alpha level of 0.05. If the differences were significant then a comparison of means between all groups was carried out using Tukey-Kramer's test. The statistical analysis were performed using JMP[®] statistical software V4.2.6 (SAS Institute, Cary, NC). Although data has not been included for the purposes of brevity, Michaelis-Menten parameters

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were estimated for each metabolic pathway using nonlinear regression method.

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RESULTS

Biomedical parameters and body weight in CPF and CRI rats

Based on the previously published data by our laboratory (Rege et al., 2003), serum creatinine and urea nitrogen levels were increased by 98% ($p < 0.01$) and 250% ($p < 0.01$), respectively, in the CRI animals as compared to the CPF and control group. At the end of week 5 of the study, food intake and body weight of the rats in the CRI groups were reduced by 37% ($p < 0.01$) and 35% ($p < 0.01$), respectively, compared to control rats. Rats in CPF group had similar decrease in body weight as CRI rats while being fed similar amounts of food.

Glucuronidation by recombinant rat UGT enzymes

UGT1A1 and UGT1A7 isoforms were involved in the glucuronidation of estradiol at 3-position as shown in Figure 1A. UGT1A1 had about 2-fold higher estradiol 3-glucuronidation activity (213.08 pmole/min/mg protein) than UGT1A7 (101.62 pmole/min/mg protein). UGT1A2, UGT2B2 and UGT2B3 showed glucuronidation activity towards estradiol at 17-position as shown in Figure 1B. Estradiol was glucuronidated at the 17- position with highest activity by UGT2B3 (695.29 pmole/min/mg protein) and to lesser extent by UGT1A2 (249.49 pmole/min/mg protein) and UGT2B2 (65.04 pmole/min/mg protein). All UGT1A isoforms tested showed glucuronidation activity towards 4-MU as shown in Figure 1C. 4-MU was glucuronidated largely by UGT1A7 (1857.39 pmole/min/mg protein) and to a much lesser extent by UGT1A1 (103.63 pmole/min/mg protein), UGT1A2 (229.71 pmole/min/mg protein) and UGT1A3 (11.17 pmole/min/mg protein). Morphine 3-glucuronidation was below the limit of detection and not measurable with any of the recombinant hepatic UGT enzymes

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investigated (data not shown).

Effect of CRI on renal and hepatic UGT catalytic activities

The rates of formation of estradiol, 4-MU and morphine glucuronide metabolites in liver and kidney microsomes from CRI, CPF and control rats are shown in Table 1. The formation of E2-17G and M3G was not detectable in the kidney microsomes from any animal. E2-3G activity was below the limits of quantitation in the CPF and CRI animals. There were no significant differences in glucuronidation activity of liver or kidney microsomes towards estradiol, 4-MU or morphine between CPF and CRI group. Although not statistically significant, hepatic estradiol 3-glucuronidation was increased by 24% ($p=0.09$) in the CRI group as compared with CPF group (Table 1). The hepatic E2-3G formation was similar between CPF (185.13 ± 31.35 pmol/min/mg) and the control group (174.67 ± 28.22 pmol/min/mg). Compared to the control rats, hepatic glucuronidation of 4-MU and estradiol at the 17- position and renal glucuronidation of 4-MU and estradiol at the 3- position was significantly ($p<0.05$) downregulated in both the CPF and CRI groups. Hepatic M3G formation, although not statistically significant, tended to be lower in the control group as compared to the CPF ($p=0.06$) and CRI ($p=0.07$) groups.

Effect of CRI on hepatic and renal UGT protein expression

Western blot analysis of total UGT2B and 1A protein levels is shown in Figure 2. Rat livers had much higher levels of UGT2B protein than UGT1A protein and these levels were much higher in the liver as compared to kidney (Figure 2). The immunoblot bands showed that UGT2B and UGT1A protein expression in the livers were not obviously altered in the CPF and CRI groups, as compared to the control group. The

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intensity of the UGT2B and UGT1A immunoblots appeared to be lower in the CPF and CRI kidneys compared to the levels in the control animals.

There was no significant difference in either hepatic or renal UGT1A1, UGT1A6, and UGT1A7 protein content between the CPF and CRI groups (Figure 3). UGT1A6 was significantly ($p<0.05$) downregulated in the liver as well as the kidney from CPF and CRI animals as compared to the control group. In the kidney, UGT1A1 and UGT1A6, but not UGT1A7, expression was downregulated ($p<0.05$) in CPF and CRI rats, as compared to control rats. The decreases in protein expression for renal UGT1A1 and UGT1A6 significantly correlated with decreases in renal E2-3G formation ($r^2=0.88$, $p<0.0001$) (Figure 4B) and renal 4-MUG formation ($r^2=0.77$, $p<0.0001$) (Figure 4D). The protein content of UGT1A1 and UGT1A6 exhibited weak correlation with E2-3G formation ($R^2=0.28$, $p=0.06$) (Figure 4A) and moderately correlated with 4-MUG formation ($R^2=0.43$, $p=0.02$) (Figure 4C) in rat liver. There was no correlation found between the amount of UGT1A7 and E2-3G protein or 4-MUG formation, either in the liver or the kidney (not shown).

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DISCUSSION

This study has demonstrated that CRI had no significant effect on either UGT catalytic activities or protein content in rat liver or kidney. CRI was successfully induced in rats as confirmed by elevated serum creatinine and serum urea nitrogen levels in the CRI animals as compared to the CPF and control group. Furthermore, some degree of malnutrition was observed in the CRI rats based on reduced food intake and body weight as compared to normal rats. Since malnutrition itself is known to alter drug metabolism [Krishnaswamy and Naidu, 1977; Walter-Sack and Klotz, 1996; Mandl et al., 1995], control pair-fed (CPF) rats were included. The CPF rats lost a similar amount of body weight as the CRI rats, which suggests that the reduced body weight of the CRI rats was caused by decreased food intake.

Recently, more evidence is emerging that not only renal elimination, but also hepatic clearance of drugs could be impaired in patients with renal diseases [Touchette and Slaughter, 1991; Gibson, 1986]. One possible mechanism underlying this phenomenon could be a change in hepatic intrinsic clearance in CRI. CRI induced in rats has been shown to downregulate specific CYP450 enzymes in the liver [Leblond et al., 2000; Leblond et al., 2001, Rege et al., 2003]. In the present study, we examined the effect of CRI on UGT enzymes in rat liver and kidney using estradiol, 4-MU, and morphine as probe substrates for UGT1A1, UGT1A6, and UGT2B1, respectively. UGT1A1 plays an important physiological role in glucuronidation of endogenous substrates, such as bilirubin, and estrogens, as well as flavonoids [Senafi et al., 1994]. Rat and human UGT1A1 have been shown to be functionally identical [King et al., 1996]. UGT1A6 is a major UGT1A enzyme that is predominantly expressed in the liver

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but also found in the kidney and other extrahepatic tissues [Ritter , 2000]. UGT1A6 is responsible for glucuronidation of xenobiotic substrates such as simple and complex phenols, coumarins, and primary amines [Tukey et al., 2000]. UGT2B7 is a major human UGT2B isoform that is highly expressed in the liver/extrahepatic tissues and glucuronidates various compounds, including androgens, estrogens, catechol estrogens, and opioid compounds [Turgeon et al., 2001]. UGT2B1 is the corresponding isoform for UGT2B7 in rats [Tukey et al., 2000].

Estradiol metabolism. Estradiol is glucuronidated at the 3-position by UGT1A1 and at the 17-position by several UGT2B enzymes [Lepine et al., 2004]. By screening a panel of recombinant rat UGT enzymes, we found that in addition to UGT1A1, UGT1A7 was active in E2-3G formation (Figure 1A). UGT2B3 is the major isoform responsible for E2-17G formation while UGT2B2 and UGT1A2 also had a minor role (Figure 1B). In the present study we used estradiol 3-glucuronidation as a probe for UGT1A1 and E2-17G formation as a surrogate probe for UGT2B in the same sample (Table 1).

There was no difference in hepatic estradiol 17-glucuronidation or 3-glucuronidation between the CPF and CRI group, which indicates that CRI did not influence hepatic UGT1A1 and UGT2B activity. Hepatic E2-3G formation trended to be higher in the CPF and CRI group, perhaps to compensate for the decreased E2-17G formation. These results also indicate that estradiol is glucuronidated at the 3- and 17- position by different enzyme systems in the liver, which might be regulated separately in the liver of CPF and CRI rats.

Renal E2-3G formation, on the other hand, was significantly downregulated in CPF and CRI rats, as compared to control rats (only measurable in normal control rats).

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The lack of detectable E2-17G metabolites in the renal tissue of all animals suggests that UGT2B3 and UGT1A2 are probably not expressed in the rat kidney.

The western blot results revealed that the expression of UGT1A1 and UGT1A7 proteins (Figure 3), similar to the catalytic activity, were not affected by CRI in either the liver or the kidney. The downregulated UGT1A1 but not UGT1A7 protein expression suggests that the reduced E2-3G formation in the kidney of CPF and CRI rats was due to decreased UGT1A1 protein amount, which was further verified by the high correlation between the UGT1A1 protein level and E2-3G formation in the kidney (Figure 4). Since the isoform specific antibody for rat UGT2B was not available, only total UGT2B protein content was measured using the antibody for the UGT2B common region. The total UGT1A protein content was determined as well. The band density suggests that the protein amount of UGT2B in rat liver could potentially be higher than that of UGT1A. The Western blot results also revealed that UGT2Bs could be expressed at a lower level in the liver than in the kidney (Figure 2), which is consistent with the relative mRNA expression level of UGT2Bs in rat liver and kidney reported by Shelby M.K., et al (2002). This observation is also supported by E2-17G formation suggesting UGT2B is the main enzyme responsible for this metabolite. The role of other UGT enzymes cannot be ruled out however. Since the CPF group did not undergo renal surgery and the common treatment effect between the two groups was reduced food intake, it appears that food restriction may affect hepatic and renal glucuronidation of estradiol.

4-MU metabolism. To examine UGT1A6 catalytic activity, glucuronidation of 4-methylumbelliferone (4-MU), a probe substrate for human or rat UGT1A6 [Narayanan et al., 2000; Hanioka et al., 2001], was determined (Table 1). When comparing the CRI

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group to the CPF group, CRI itself did not have an effect on 4-MU glucuronidation in either the liver or the kidney. Interestingly, food restriction seemed to reduce both hepatic and renal formation of 4-MUG (40%-54% decrease, $p < 0.05$) in CPF and CRI group, as compared to the control group. 4-MU glucuronidation activity was also determined using the same set of recombinant rat UGT enzymes (Figure 1C). UGT1A7 turned out to have high 4-MU glucuronidation activity, . The western blot results showed that the protein content of UGT1A6 and UGT1A7 was not different between the CPF and CRI groups, which is similar to the activity results. Protein expression of UGT1A6, but not UGT1A7, was downregulated in the liver and kidney of CPF and CRI group, as compared to the control group. It suggests that even though 4-MU is glucuronidated by both UGT1A6 [Iwano et al., 1999] and UGT1A7 (Figure 1C), only UGT1A6 contributes to the downregulation of 4-MU glucuronidation by food restriction. This was further confirmed by a significant correlation between 4-MU glucuronidation activity and UGT1A6, but not UGT1A7, protein expression (Figure 4). The formation of 4-MUG was better correlated with UGT1A6 protein levels in the kidney than in the liver, suggesting that UGT1A6 is the primary isoform responsible for 4-MUG formation in the kidney however other isoforms cannot be ruled out.

Morphine metabolism. Morphine is a known substrate for both rat UGT2B1 and human UGT2B7 [King et al., 2001]. Morphine is metabolized predominantly to two glucuronide isomers: M3G and M6G [Ishii et al., 1997]. Only M3G is efficiently formed in both humans and rats [King et al., 1996]. In this study, the formation of M3G was determined (Table 1). There was no measurable M3G formation in kidney microsomal incubations, which is consistent with the results reported by Brunelle and Verbeeck. (1996). CRI did

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not affect hepatic glucuronidation of morphine at the 3- position, while the formation of M3G was decreased by ~24% in the CPF ($p=0.06$) and the CRI ($p=0.07$) group, as compared to the control group. Although not statistically significant, the trend suggests that food restriction is associated with reduced M3G formation. Morphine is known to be glucuronidated by rat UGT1A1 and UGT2B1 at low and high rates, respectively [King et al., 1997; Ishii et al., 1997]. In our study, none of the recombinant rat UGT enzymes, including UGT1A1, showed measurable glucuronidation activity towards morphine. While the role of UGT2B1 in M3G formation could not be confirmed in this study, the role of UGT2B is implied from the observation that protein expression of UGT2B enzymes, while similar to morphine glucuronidation activity, was nonstatistically changed in the liver of CPF and CRI rats, compared to control rats.

In summary our results suggest that neither hepatic nor renal UGT enzymes were affected directly by renal insufficiency. However food restriction appeared to be responsible for reduction in the rate of formation of hepatic E2-17G (UGT2B3) and 4-MUG (UGT1A6), along with renal E2-3G (UGT1A1) and 4-MUG. The downregulated glucuronidation activities were accompanied by corresponding reductions in protein content of specific UGT isoforms. Since food restriction was found to selectively downregulate hepatic and/or renal UGT enzyme protein repression, it can be speculated that the modified hepatic drug glucuronidation in some CRI patients could be due to the malnutrition arising from compromised renal function. These observations need to be verified in a well-controlled study.

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Reference:

- Ali M, Nicholls PJ, Yoosuf A.. (1979) The influence of old age and of renal failure on hepatic glucuronidation in the rat. *Br J Pharmacol.*: 498P-499P.
- Anders M.W. (1980) Metabolism of drugs by the kidney. *Kidney Int.* 18: 636-647.
- Balant L, Francis RJ, Tozer TN, Marmy A, Tschopp JM, Fabre J.. (1980) Influence of renal failure on the hepatic clearance of bufuralol in man. *J Pharmacokinet Biopharm.* 8 (5): 421-438.
- Brunelle F.M. and Verbeeck R.K. (1996) Glucuronidation of diflunisal in liver and kidney microsomes of rat and man. *Xenobiotica.* 26(2): 123-131.
- Coughtrie MW, Burchell B, Bend JR. (1987) Purification and properties of rat kidney UDP-glucuronosyltransferase. *Biochem Pharmacol.* 36(2): 245-251.
- Falany C.N. and Tephly T.R. (1983) Separation, purification and characterization of three isoenzymes of UDP-glucuronyltransferase from rat liver microsomes. *Arch Biochem Biophys.* 227(1): 248-258.
- Fisher MB, Paine MF, Strelevitz TJ, Wrighton SA.. (2001) The role of hepatic and extrahepatic UDP-glucuronosyltransferase in human drug metabolism. *Drug Metab Rev.* 33(3&4): 273-297.
- Gibson T.P. (1986) Renal disease and drug metabolism: an overview. *Am J Kidney Dis.* VIII (1): 7-17.
- Hanioka N, Jinno H, Tanaka-Kagawa T, Nishimura T, Ando M.. (2001) Determination of UDP-glucuronosyltransferase UGT1A6 activity in human and rat liver microsomes by HPLC with UV detection. *J Pharm Biomed Anal.* 25: 65-75.
- Ishii Y, Tsuruda K, Tanaka M, Oguri K.. (1994) Purification of a Phenobarbital-inducible morphine UDP-glucuronyltransferase isoform, absent from guinea rat liver. *Arch Biochem Biophys.* 315(2): 345-351.
- Iwano H, Yokota H, Ohgiya S, Yuasa A.. (1999) The significance of amino acid residue Asp446 for enzymatic stability of rat UDP-glucuronosyltransferase UGT1A6. *Arch Biochem Biophys.* 363(1): 116-120.
- Jansen PL, Mulder GJ, Burchell B, Bock KW. (1992) New developments in glucuronidation research: report of a workshop on "glucuronidation, its role in health and disease". *Hepatology*, 15(3): 532-44.
- King C, Finley B, Franklin R. (2000) The glucuronidation of morphine by dog liver microsomes: identification of morphine-6-O-glucuronide. *Drug Metab Dispos.* 28: 661-663.

DMD # 6601

King CD, Green MD, Rios GR, Coffman BL, Owens IS, Bishop WP, Tephly TR.. (1996) The glucuronidation of exogenous and endogenous compounds by stably expressed rat and human UDP-glucosyltransferase 1.1. *Arch Biochem Biophys*. 332(1): 92-100.

Krishnaswamy K, Naidu AN.. (1977) Microsomal enzymes in malnutrition as determined by plasma half life of antipyrine. *Br Med J*. 26: 538-540.

Leblond F, Guevin C, Demers C, Pellerin I, Gascon-Barre M, Pichette V.. (2001) Downregulation of hepatic cytochrome P450 in chronic renal failure. *J Am Soc Nephrol*. 12: 326-332.

Leblond FA, Giroux L, Villeneuve JP, Pichette V. (2000) Decreased in vivo metabolism of drugs in chronic renal failure. *Drug Metab Dispos*. 28: 1317-1320.

Le Guellec C, Lacarelle B, Villard PH, Point H, Catalin J, Durand A.. (1995) Glucuronidation of propofol in microsomal fractions from various tissues and species including humans: effect of different drugs. *Anesth Analg*. 81: 855-61.

Lepine J, Bernard O, Plante M, Tetu B, Pelletier G, Labrie F, Belanger A, Guillemette C.. (2004) Specificity and regioelectivity of the conjugation of estradiol, estrone, and their catecholestrogen and methoxyestrogen metabolites by human uridine diphospho-glucuronosyltransferases expressed in endometrium. *J Clin Endocrinol Metab*. 89(10): 5222-32.

Mandl J, Banhegyi G, Kalapos MP, Garzo T. (1995) Increased oxidation and decreased conjugation of drugs in the liver caused by starvation. Altered metabolism of certain aromatic compounds and acetone. *Chem Biol Interact*. 96: 87-101.

Miles KK, Stern ST, Smith PC, Kessler F, Ali S, Ritter J. (2005) An investigation of human and rat liver microsomal mycophenolic acid glucuronidation: evidence for a principal role of UGT1A enzymes and species differences in UGT1A specificity. *Drug Metab Dispos*. 2005 Jul 20.

Miners J.O. and Mackenzie P.I. (1991) Drug glucuronidation in humans. *Pharmacol Ther*, 51(3): 347-69.

Monteith DK, Michalopoulos G, Strom SC. (1990) Conjugation of chemical carcinogens by primary cultures of human hepatocytes. *Xenobiotica*. 20(8): 753-63.

Narayanan R, LeDuc B, Williams DA. (2000) Determination of the kinetics of rat UDP-glucuronosyltransferases (UGTs) in liver and intestine using HPLC. *J Pharm Biomed Anal*. 22: 527-

DMD # 6601

540.

Peters W.H.M. and Jansent P.L.M. (1988) Immunocharacterization of UDP-glucuronosyltransferases isoenzymes in human liver, interstine and kidney. *Biochem Pharmacol.* 37(3): 564-567.

Pritchard M, Fournel-Gigleux S, Siest G, Mackenzie P, Magdalou J. (1993) A recombinant Phenobarbital-inducible rat liver UDP-glucuronosyltransferase (UDP-glucuronosyltransferase 2B1) stably expressed in V79 cells catalyzes the glucuronidation of morphine, phenols, and carboxylic acids. *Mol Pharmacol.* 45: 42-50.

Rege B, Krieg R, Gao N, Sarkar MA. (2003) Down-regulation of hepatic CYP3A in chronic renal insufficiency. *Pharm Res.* 20 (10): 1600-1606.

Ritter J.K. (2000) Role of glucuronidaiton and UDP-glucuronosyltransferases in xenobiotic bioactivation reactions. *Chem Biol Interact.* 129: 171-193.

Ritter JK, Kessler FK, Thompson MT, Grove AD, Auyeung DJ, Fisher RA. (1999) Expression and inducibility of the human bilirubin UDP-glucuronosyltransferase UGT1A1 in liver and cultured primary hepatocytes: evidence for both genetic and environmental influences. *Hepatology* 30: 476-484.

Sarkar MA, Hunt C, Guzelian PS, Karnes HT. (1992) Characterization of human liver cytochromes P-450 involved in theophylline metabolism. *Drug metab dispos.* 20: 31-37.

Shelby MK, Cherrington NJ, Vansell NR, Klaassen CD. (2002) Tissue mRNA expression of the rat UDP-glucuronosyltransferase gene family. *Drug Metab Dispos.* 31(3):326-33.

Senafi SB, Clarke DJ, Burchell B. (1994) Investigation of the substrate specificity of a cloned expressed human bilirubin UDP-glucuronosyltransferase: UDP-sugar specificity and involvement in steroid and xenobiotic glucuronidation. *Biochem J.* 303: 233-240.

Singlas E, Pioger JC, Taburet AM, Colin JN, Fillastre JP. (1989) Zidovudine disposition in patients with severe renal impairment: influence of hemodialysis. *Clin Pharmacol Ther.* 46(2): 190-7.

Tephly T.R. and Burchell Brian. (1990) UDP-glucuronosyltransferases: a family of detoxifying enzymes. *Trends pharmacol Sci.* 11(7): 276-279.

Touchette M.A. and Slaughter R.L. (1991) The effect of renal failure on hepatic drug clearance. *DICP.* 25: 1214-24.

DMD # 6601

Tukey R.H and Strassburg C.P. (2000) Human UDP-glucuronosyltransferases: metabolism, expression, and disease. *Annu Rev Pharmacol Toxicol.* 40: 581-616.

Turgeon D, Carrier JS, Levesque E, Hum DW, Belanger A. (2001) Relative enzymatic activity, protein stability, and tissue distribution of human steroid-metabolizing UGT2B subfamily members. *Endocrinology.* 142(2): 778-787.

Walter-Sack I and Klotz U. (1996) Influence of diet and nutritional status on drug metabolism. *Clin Pharmacokinet.* 31(1): 47-64.

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FOOTNOTES

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Legends of Figures:

Figure 1. Glucuronidation of estradiol and 4-MU by recombinant rat UGT enzymes.

Estradiol 3- and 17-, and 4-MU activities were measured at 150 μ M estradiol and 1000 μ M 4-MU concentration using cell lysates from HepG2 cells expressing individual rat UGT isoform. HepG2 cells not expressing any UGT enzyme were included as negative control.

Figure 2. Protein expression of UGT1A and UGT2B in the liver and kidney of control (n=4), CPF (n=5), CRI (n=4) rats.

Fifteen micrograms microsomal protein was loaded onto an 8.5% SDS gel. Western blot analysis of total UGT1A and UGT2B was performed using polyclonal antibodies specific for the common region of rat UGT1A and UGT2B as outlined in Materials and Methods.

Figure 3. Protein expression of UGT1A1, UGT1A6, and UGT1A7 in the liver and kidney of control (n=4), CPF (n=5), CRI (n=4) rats.

*Fifteen micrograms microsomal protein was loaded onto an 8.5% SDS gel for Western blot analysis as described in Materials and Methods section. The results are expressed as mean \pm SD for each group. Liver (open bars), kidney (solid bars). * $p < 0.05$, compared to control group.*

Figure 4. Correlation between protein expression levels of UGT and glucuronidation activities.

The band densities of protein expression were plotted against glucuronidation activities for all control, CPF, and CRI samples. (A) E2-3G formation and UGT1A1 protein expression in liver; (B) E2-3G formation and UGT1A1 protein expression in kidney (rate of E2-3G formation below 33.33pmole/min/mg protein (LLOQ) and above

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9.99pmole/min/mg protein (LOD) were plotted); (C) 4-MUG formation and UGT1A6 protein expression in liver; (D) 4-MUG formation and UGT1A6 protein expression in kidney

Table 1: Renal and hepatic UGT catalytic activities in control, CPF and CRI rats.

Rate of E2-3G, E2-17G, 4-MUG, M3G formation was determined in the liver and kidney microsomes of control (n=4), control pair-fed (CPF) (n=5), and chronic renal insufficiency (CRI) (n=4) rats as described under *Materials and Methods*. Data represent means \pm SD of each group. N.D. indicates that glucuronide formation was not measurable (lower limit of quantification was 33.33 pmole/min/mg protein for E2-3G and E2-17G formation, 0.83 nmole/min/mg protein for 4-MUG formation, 0.07 nmole/min/mg protein for M3G formation). * $p < 0.05$, compared to control group.

		Glucuronide formation			
		E2-3G (pmole/min/mg protein)	E2-17G (pmole/min/mg protein)	4-MUG (nmole/min/mg protein)	M3G (nmole/min/mg protein)
Liver	Control	174.67 \pm 28.22	1785.85 \pm 178.94	47.66 \pm 9.32	11.39 \pm 2.35
	CPF	185.13 \pm 31.35	982.24 \pm 281.57 *	29.97 \pm 4.75 *	8.69 \pm 1.17
	CRI	229.96 \pm 36.78	847.96 \pm 245.87 *	29.00 \pm 4.98 *	8.73 \pm 0.62
Kidney	Control	89.54 \pm 22.24	N.D.	24.46 \pm 3.27	N.D.
	CPF	N.D. *	N.D.	13.19 \pm 5.41 *	N.D.
	CRI	N.D. *	N.D.	11.29 \pm 3.71 *	N.D.

Figure 1

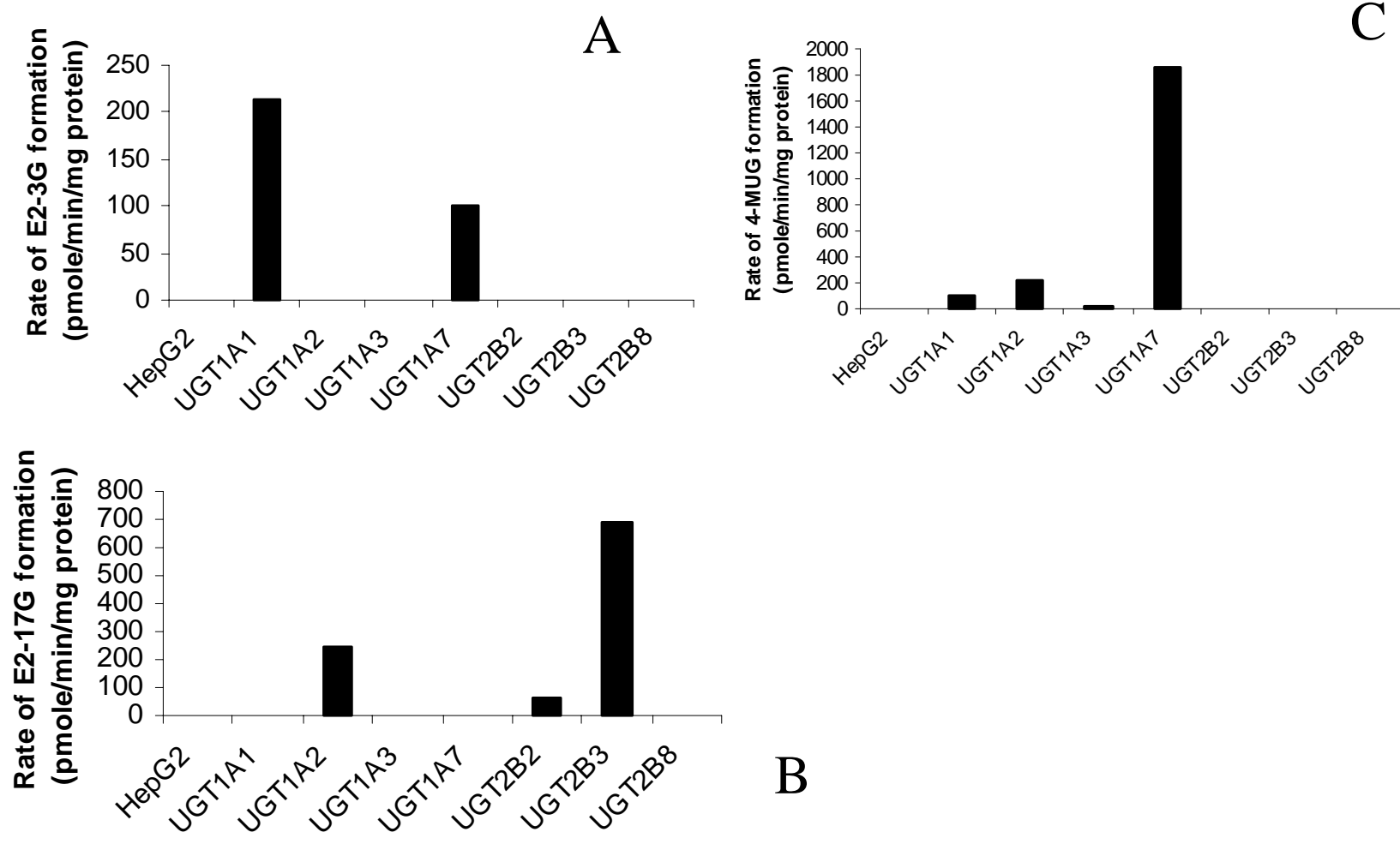


Figure 2

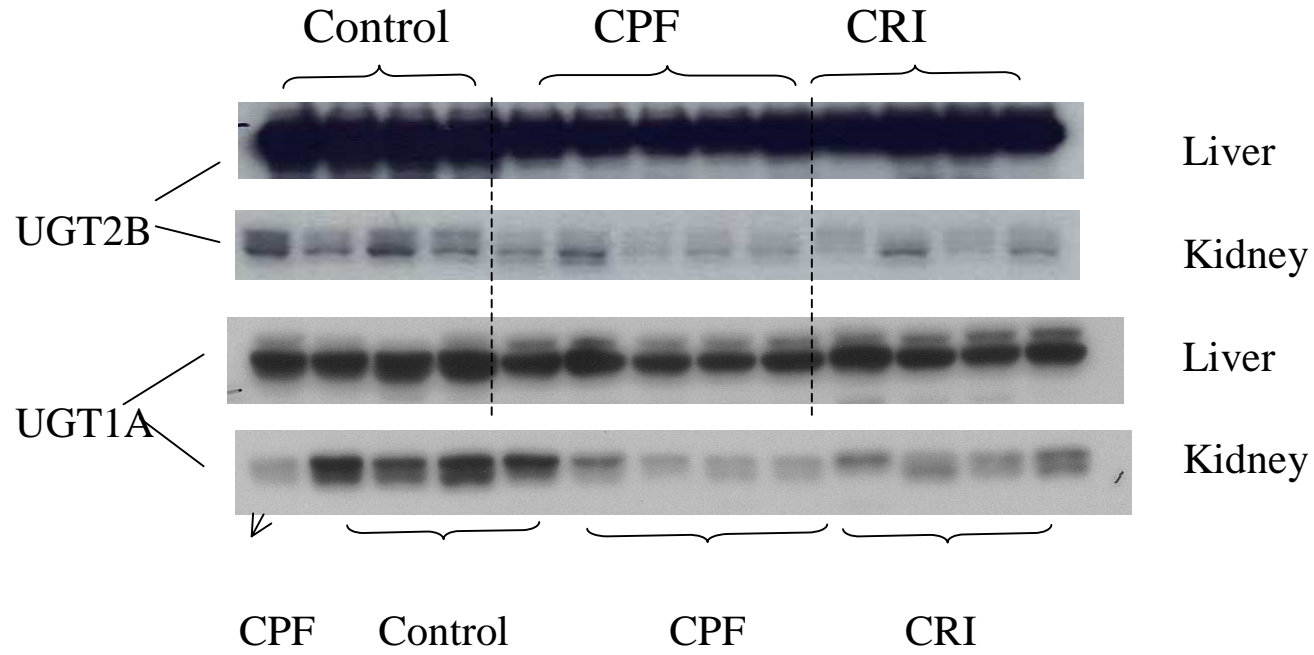


Figure 3

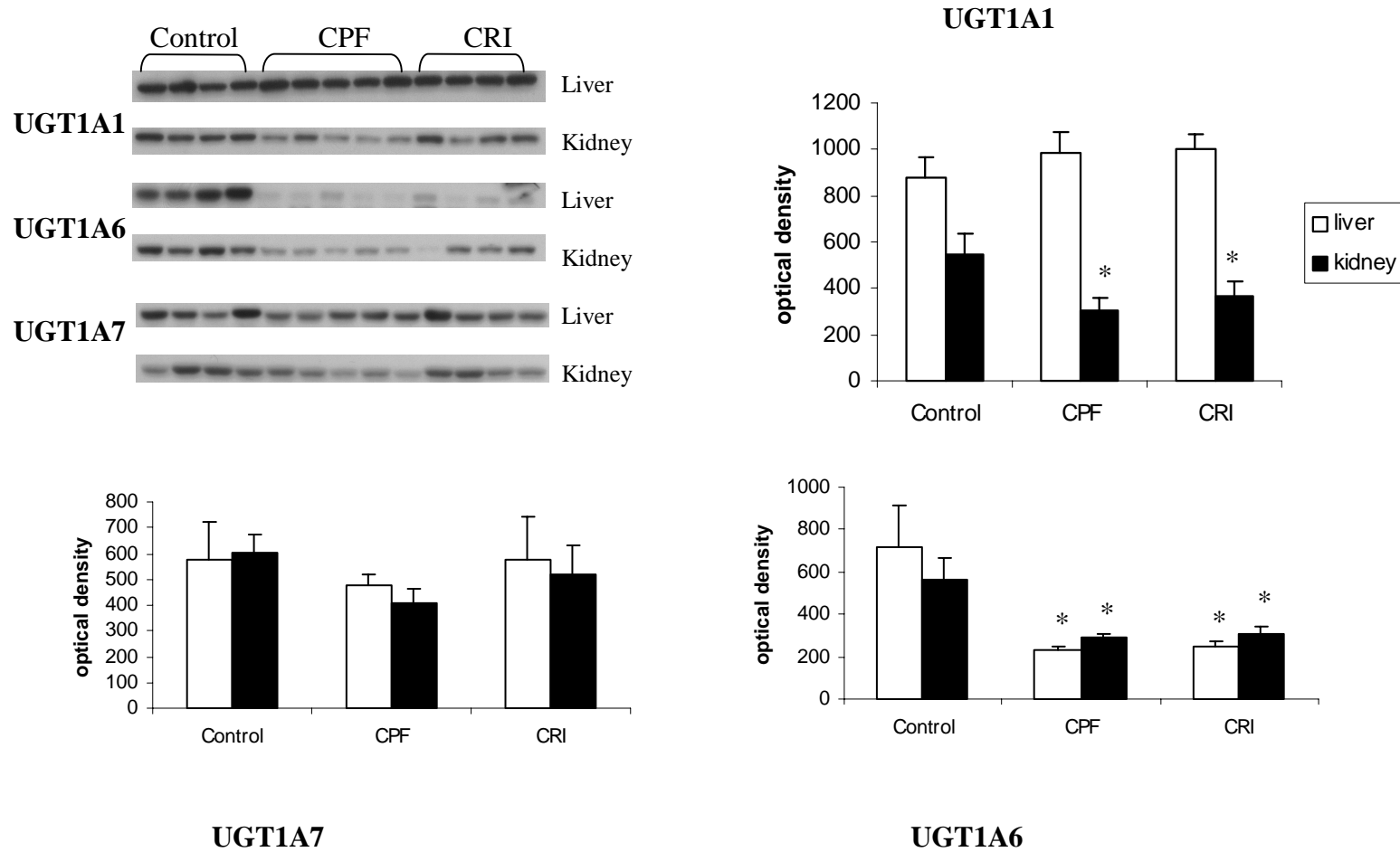


Figure 4

