DIABETES MELLITUS REDUCE ACTIVITY OF HUMAN UDP-GLUCURONOSYLTRANSFERASE 2B7 (UGT 2B7) IN LIVER AND KIDNEY LEADING TO DECREASED FORMATION OF MYCOPHENOLIC ACID ACYL-GLUCURONIDE METABOLITE

Miroslav Dostalek, Michael H. Court, Suwagmani Hazarika, Fatemeh Akhlaghi

Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island, 125 Fogarty Hall, 41 Lower College Road, Kingston, RI 02881 (M.D., F.A.); Comparative and Molecular Pharmacogenomics Laboratory, Department of Pharmacology and Experimental Therapeutics, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111 (M.H.C., S.H.).
Running title: UGT2B7 downregulation by diabetes

Correspondence: Fatemeh Akhlaghi, PharmD, PhD, Clinical Pharmacokinetics Research Laboratory and Center for Pharmacogenomics and Molecular Therapy, University of Rhode Island, 125 Fogarty Hall, 41 Lower College Road, Kingston, RI 02881, USA. Phone: (401) 874 9205. Fax: (401) 874 5787. Email: fatemeh@uri.edu.

Non-standard abbreviations used in text: AcMPAG, acyl glucuronide of mycophenolic acid; AZT, zidovudine or 3’-azido-3’-deoxythymidine; GI, gastrointestinal; HKM, human kidney microsomes; HLM, human liver microsomes; MMF, mycophenolate mofetil; MPA, mycophenolic acid; MPAG, 7-O-glucuronide of mycophenolic acid; TBARS, thiobarbituric acid reactive substances; UGT, uridine diphosphate-glucuronosyltransferase.
Abstract

Mycophenolic acid (MPA) is an immunosuppressive agent commonly used after organ transplantation. Altered concentrations of MPA metabolites have been reported in diabetic kidney transplant recipients, although the reason for this difference is unknown. We aimed to compare MPA biotransformation and UDP-glucuronosyltransferases (UGTs) expression and activity between liver (n=16) and kidney (n=8) from diabetic and non-diabetic donors. Glucuronidation of MPA, as well as the expression and probe substrate activity of UGTs primarily responsible for MPA phenol glucuronide (MPAG) formation (UGT1A1, 1A9), and MPA acyl glucuronide (AcMPAG) formation (UGT2B7), was characterized. We have found both diabetic and non-diabetic human liver microsomes (HLM) and kidney microsomes (HKM) formed MPAG with similar efficiency; however, AcMPAG formation was significantly lower in diabetic samples. Supporting this finding, markedly lower glucuronidation of the UGT2B7 probe zidovudine (AZT), UGT2B7 protein and UGT2B7 mRNA was observed in diabetic tissues. UGT genetic polymorphism did not explain this difference since UGT2B7*2 or *1c genotype were not associated with altered microsomal UGT2B7 protein levels or AcMPAG formation. Furthermore, mRNA expression and probe activities for UGT1A1 or UGT1A9, both forming MPAG but not AcMPAG, were comparable between diabetic and non-diabetic tissues suggesting the effect may be specific to UGT2B7 mediated AcMPAG formation. These findings suggest that diabetes mellitus is associated with significantly reduced UGT2B7 mRNA expression, protein level, and enzymatic activity of human liver and kidney, explaining in part the relatively low circulating concentrations of AcMPAG in diabetic patients.
Introduction

Mycophenolic acid (MPA) is an immunosuppressive agent widely used to prevent rejection following organ transplantation. Most of the administered dose (87-94%) ultimately appears in the urine as the pharmacologically inactive phenolic 7-O-glucuronide of MPA (MPAG) with small percentages reported to be biotransformed to either pharmacologically active acyl glucuronide (AcMPAG) or inactive glucoside conjugates (Shipkova et al., 1999). It has been suggested that AcMPAG may be the culprit for some of the adverse side effects of MPA, including gastrointestinal (GI) toxicity (Wieland et al., 2000). MPA exhibits prominent pharmacokinetic features consisting of a secondary peak observed in the MPA concentration-time profile. The latter is considered to result from hepatic MPA glucuronidation, followed by biliary excretion, hydrolysis in the intestines to MPA, and subsequent re-absorption of parent MPA. This drug and its metabolites are also transported by organic anion transporters (OAT), organic anion-transporting polypeptide (OATP), and by multi-drug resistance-associated protein 2 (MRP2) (Barraclough, 2010).

The uridine-5'-diphosphate-glucuronosyltransferases (UGTs) are a superfamily of membrane bound enzymes that catalyze glucuronidation at nucleophilic functional groups in xenobiotics and endogenous compounds leading to the formation of more hydrophilic derivatives for excretion in bile and/or urine. UGTs are classified based on the similarity in gene sequence. So far, all UGTs involved in the metabolism of marketed drugs are originated from the UGT 1A, 2A, and 2B subfamilies, and include 19 distinct catalytically active UGTs in humans (Mackenzie et al., 2005). Various studies have utilized recombinantly expressed enzymes to identify the specific UGT enzymes involved in the glucuronidation of MPA, with some disagreement between reports. Mackenzie initially reported that UGTs 1A8, 1A9, and 1A10 were capable of forming MPAG using enzymes transiently expressed in COS-7 cells (Mackenzie, 2000). However, the authors also reported a lack of detectable MPA glucuronidation activity for UGTs 1A1, 1A3, 1A6, 2B4, and 2B7. Shipkova et al. (Shipkova et
al., 2001) then reported that recombinant UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A10, 2B4, 2B7, and 2B15 expressed in insect cells were all capable of forming MPAG. However, Basu and coworkers (Basu, 2004) using COS-1 expressed UGTs, and Bernard and Guillemette (Bernard and Guillemette, 2004) using HEK293 expressed UGTs suggested that UGT1A7, 1A8, 1A9, and 1A10 were the main UGTs that could produce MPAG. Finally, a study by Picard et al. (Picard et al., 2005) suggested UGT1A9 and 1A10 are the major contributors to MPAG production. These discrepancies in data could result from different experimental conditions and cell systems used for UGT expression. However, taking into consideration all studies published so far, it appears that UGT1A9, which is mainly expressed in liver and kidney, and UGT1A10, which is mainly expressed in intestines, may be the main enzymes responsible for phenolic glucuronidation of MPA. Although less studied, UGT2B7 which is mainly expressed in liver, kidney and intestines, appears to be the most important UGT involved in the production of AcMPAG (Bernard et al., 2006).

The pharmacokinetics of MPA and its metabolites show high variability in various transplant subpopulations (Ensom et al., 2002). In previous clinical pharmacokinetics studies in kidney transplant recipients, we have described significantly higher MPAG to AcMPAG plasma concentration ratios in diabetic versus non-diabetic patients (Akhlaghi et al., 2006; Patel et al., 2007). This observation suggests that diabetes may influence UGT enzymes responsible for the formation of MPAG and/or AcMPAG or may alter other mechanisms governing the circulating concentration of these metabolites. However, the underlying molecular mechanisms are not yet known.

The purpose of the current study was to determine whether diabetes mellitus is associated with altered rates of microsomal MPAG and/or AcMPAG formation ex vivo using liver and kidney microsomes obtained from both diabetic donors and non-diabetic controls. We also evaluated whether the identified differences were correlated with differences in the activity
and expression of selected UGTs involved in the biotransformation of MPA to MPAG (UGT1A9) or to AcMPAG (UGT2B7).
Materials and Methods

Chemicals – AcMPAG, MPA, and MPAG were kindly donated by Roche Pharmaceuticals (Palo Alto, CA). 3-Acetamidophenol, estradiol, acetophenetidin (phenacetin), phenolphthalein glucuronic acid, propfol, and thymol were purchased from Sigma Aldrich (St. Louis, MO). Zidovudine (AZT), AZT-glucuronide, estradiol-3-β-glucuronide, estradiol-17-β-glucuronide and propofol-glucuronide were purchased from TRC (Toronto, ON, Canada), while HPLC-grade acetonitrile and methanol were obtained from Pharmco Products Inc. (Brookefield, CT). All other reagents and solvents were obtained from general commercial suppliers. All chemicals were used without further purification.

In vitro study

Tissues and microsomal preparation. Diabetic and non-diabetic human liver and kidney samples were obtained from XenoTech, LLC (Lenexa, KS). The tissues were selected to match, as best as possible, according to demographic characteristics between diabetic and non-diabetic donors (Table 1). Microsomal fractions were prepared as described previously (Guengerich and Bartleson, 2001) and were stored at -80°C until use. Protein concentrations were estimated using a bicinchoninic acid method (Pierce-Fisher, Rockford, IL). To measure the extent of oxidative stress, malondialdehyde, a product of lipid peroxidation, was measured in tissues using thiobarbituric acid assay (Ernster and Nordenbrand, 1967). The fraction of drug unbound in the microsomal fraction was determined by equilibrium dialysis against 0.1 M phosphate buffer, pH 7.4 using Slide-A-Lyzer Dialysis Cassettes, 2K MWCO, 0.5 mL, according to manufacturer’s manual (Pierce-Fisher, Rockford, IL).

UGTs Western blot analyses. Amounts of UGT2B7 in HLMs and HKMs were determined by quantitative Western immunoblotting by modifying a method used for cytochrome P450 as described previously (Guengerich et al., 1982). Rabbit anti-human UGT2B7 antisera with recombinant UGT2B7 standard were obtained from BD Biosciences (San Jose, CA). Protein levels were measured in duplicate and mean values were used for quantification. Expressed
UGT2B7 (BD Biosciences, San Jose, CA) was used as a reference standard. Known amounts of expressed UGT2B7 characterized by 7-hydroxy-4-trifluoromethylcoumarin (pmol/(min x mg protein)) were used on each gel and the results were quantified using densitometry. Each sample was quantified separately based on the concentration curve and used for statistical analysis.

**UGT activity assays.** Incubations (100 µl total volume) were performed in 50 mM phosphate buffer (pH 7.4) at 37°C, 4.0 mM MgCl₂, 4.0 mM UDPGA, and alamethicin (50 µg/mg of protein). Microsomal fractions were pre-incubated on ice for 15 min with alamethicin, which was found to maximally activate microsomal UGT activity. Probes and MPA were added to the reaction in 5% (v/v) final methanolic concentration. No change in the UGT2B7 activity was observed following the addition of 5% (v/v) methanol. After equilibrating incubation tubes at 37°C for 5 min, reactions were initiated by the addition of MgCl₂ and UDPGA and were allowed to proceed at 37°C for 90 min in a shaking water bath. Reactions were stopped by the addition of 100 µL of ice-cold acetonitrile with internal standard. Tubes were placed on ice for 10 min and then centrifuged at 10,000xg for 10 min at 4°C to remove the precipitate. The supernatant was transferred to HPLC tubes and partially dried down in a centrifugal evaporator at room temperature before analysis by HPLC. Acidification of acyl-glucuronides is very important for post reaction handling (Shipkova et al., 2000). However, no differences in rate of change were found when microsomal incubations with and without acidification from diabetic and non-diabetic subjects were compared.

**HPLC quantitation.** MPA and metabolites were quantified by HPLC-UV as described previously (Patel and Akhlaghi, 2006). HPLC assays used for quantification of estradiol (UGT1A1), propofol (UGT1A9) and their glucuronide metabolites have been given in detail previously (Court, 2005). The chromatographic separation was performed on HPLC Hitachi D-7000 series instrument (San Jose, CA). Data from the detector were collected and analyzed with HPLC system manager for Hitachi D-7000 software. Assay for quantification of
AZT (UGT2B7) and its glucuronide metabolite was described previously (Engtrakul et al., 2005). Chromatography was performed on a LC-MS/MS that included a binary pump and autosampler Shimadzu (Shimadzu, Kyoto, Japan) coupled to a AB Sciex triple quadrupole mass spectrometric detector API 3200 (Toronto, Ontario, Canada), equipped with Turbo V source electrospray ionization (ESI) probe. The column was heated to 50°C using Flatron Systems TC-50 temperature controller and CH-30 column heater (ASTEC, Whippany, NJ). The chromatographic data were collected and analyzed using Analyst® package (version 1.4.1., AB Sciex). Calculated rates of formation of MPAG, AcMPAG, AZT-glucuronide, 3β- and 17β-estradiol glucuronides, and propofol glucuronide were normalized to incubation time and total protein content.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Total RNA was isolated with RNA-Bee system (TEL-TEST, INC., Friendswood, TX) according to manufacturer’s manual and as described previously (Yang et al., 2007). Levels of UGT1A1, UGT1A9 and UGT2B7 mRNA were determined by real-time PCR (Model 7300, Applied Biosystems, USA) with Sybr green detection as previously described with minor modifications (Manevski et al., 2009). Quantitative PCR reactions (25 µL) included Sybr Green 2X master mix (Applied Biosystems, USA), 10 µL of diluted cDNA, and 100 nM of each primer (200 nM for 18S RNA). Primer pair sequences were as follows: CCC CTC GAT GCT CTT AGC TGA GTG T (18S-rRNA-forward), CGC CGG TCC AAG AAT TTC ACC TCT (18S-rRNA-reverse), GCT TTT GTC TGG CTG TTC CCA CT (UGT1A1-forward), TCG AAG GTC ATG TGA TCT GAA TGA GA (UGT1A1-reverse), GGA GCC ACT GGT TCA CCA TGA G (UGT1A9-forward), AGA TCC TCC AGG GTA TAT GAA GTT GAA (UGT1A9-reverse), TTT CAC AAG TAC AGG AAA TCA TGT CA A TAT (UGT2B7-forward), CAG CAG CTC ACT ACA GGG AAA AAT (UGT2B7-reverse).
**UGT2B7 genotyping.** Liver samples were genotyped for single nucleotide polymorphisms (SNPs) in the UGT2B7 exon 2 region by direct sequencing of genomic DNA as described previously (Court et al., 2003). SNPs included rs7439366 (c.802C>T; H268Y; UGT2B7*2) and rs28365062 (c.735A>G; UGT2B7*1c) that have been previously associated with altered AZT glucuronidation *in vitro* and *in vivo* (Kwara et al., 2009).

**Enzyme kinetic data analysis.** Data were fitted to appropriate models by non-linear least-squares regression using GraphPad Prism (version 5.00 for Windows, GraphPad Software, San Diego, CA). For AcMPAG and MPAG formation from MPA or AZT-glucuronide formation from AZT, a model incorporating substrate inhibition equation \[Y=V_{\text{max}}S/(K_{\text{m}}+S*(1+S/K_{\text{si}}))\] with \( S \) representing the concentration of substrate. The estimated parameters were maximum rate of formation \( V_{\text{max}} \) and Michaelis-Menten constant \( K_{\text{m}} \). Uncompetitive substrate inhibition constant \( K_{\text{si}} \) was fixed to a nominal value for each substrate, and intrinsic clearance was calculated as \( V_{\text{max}}/K_{\text{m}} \).

**Statistical analysis.** All statistical analyses were performed with SPSS (version 16, Chicago, IL). Kolmogorov-Smirnov’s test was used to test for normal distribution of data. The differences between diabetic and non-diabetic groups were determined using \( t \)-test and \( P \) values less than 0.05 were considered to be statistically significant. All data are expressed as mean ± SE.
Results

Demographic data for the liver (n=16) and kidney (n=8) donors are presented in Table 1. For livers, there were no differences between the two groups with respect to age, gender, ethnicity, and liver fat content (Table 1). Kidney samples were slightly dissimilar with respect to donor gender and ethnicity (Table 1).

Increased oxidative stress is a widely accepted contributor in the development and progression of diabetes mellitus. Diabetes is usually accompanied by an increased production of free radicals, thus increasing oxidative stress in body. The rate of formation of thiobarbituric acid reactive substances (TBARS) was measured as a marker of diabetes-associated oxidative stress. Formation of TBARS was significantly higher in HLMs (p<0.001) but not in HKMs (p=0.053) from diabetic donors (Table 1).

MPA glucuronidation by diabetic and non-diabetic liver and kidney. To characterize MPA-glucuronidation activities of HLM and HKM, individual liver and kidney samples were assayed for MPA UGT activity. MPA shows negligible binding to HLM, as the ratios of MPA concentration in the buffer and microsomal compartments of dialysis cassette were 1.02:1, 0.96:1, and 0.97:1 for diabetic and 1.04:1, 0.99:1, and 0.97:1 for non-diabetic HLM samples for added 10.0 µM, 100 µM, and 1 mM of MPA, indicating an absence of nonspecific binding. All microsomal preparations from liver and kidney samples, either from diabetic or non-diabetic donors, were capable of forming both MPAG and AcMPAG. MPAG and AcMPAG formation followed substrate inhibition kinetics in both tissues (Figures 1A, B, C, D). The kinetic constants for the two metabolites are summarized in Table 2. Interestingly, the $V_{\text{max}}$ for MPAG formation was higher with non-diabetic HKMs as compared to HLMs from non-diabetic donors. However, the $K_m$ values were similar for these microsomal preparations. Extrapolated whole organ $V_{\text{max}}/K_m$ values were estimated at 31.3 ± 9.9 for liver (40 mg.g⁻¹, organ weight is 1500 g) and 38.8 ± 2.6 for kidney (6 mg.g⁻¹, organ weight is 300 g). Thus, the
formation of MPAG occurs predominantly in the liver tissue. Furthermore, kidney microsomes were more capable than liver microsomes to form AcMPAG as the $K_m$ value was significantly higher for non-diabetic HKMs as compared with non-diabetic HLMs ($p<0.001$).

**UGT2B7, UGT1A1 and UGT1A9 probe activities in diabetic and non-diabetic liver and kidney.** To assess the effect of diabetes on liver and kidney UGT2B7 activity, we examined the rate of AZT-glucuronidation. Rates of AZT glucuronidation were significantly lower ($p<0.001$) in both HLMs (**Table 3, Figure 2D**) and HKMs (**Table 3, Figure 3D**) from diabetic donors as compared with those from non-diabetic donors. Moreover, the correlation coefficient between AZT and AcMPAG $V_{max}$ values was 0.95 indicating a good agreement in UGT2B7 activity assessed using two markers. AZT shows negligible binding to HLM, free fraction for AZT was $f_u=0.98$, 0.96, and 0.99 for non-diabetic and $f_u=0.99$, 0.94, and 0.96 for diabetic samples for added 1.00, 10.0, and 100 µM of AZT, indicating an absence of nonspecific binding. To investigate the effect of diabetes on liver and kidney UGT1A1 and UGT1A9 activity, we examined the rate of 3β- and 17β-estradiol and propofol glucuronidation, respectively. Both estradiol (**Figures 5C, D**) and propofol (**Figures 6C, D**) glucuronidation rates were not significantly different in HLMs and HKMs from diabetic versus non-diabetic donors.

**UGT2B7 protein content of diabetic and non-diabetic liver and kidney.** Immunoquantitation of UGT2B7 protein content in microsomes revealed significantly lower UGT2B7 levels in diabetic HLMs ($p<0.001$) (**Figure 2B**) and HKMs ($p=0.009$) (**Figure 3B**) as compared with those from non-diabetic donors.

**UGT2B7, UGT1A1, and UGT1A9 mRNA levels in diabetic and non-diabetic liver and kidney.** The level of UGT2B7 mRNA was significantly lower in diabetic liver ($p=0.001$) (**Figure 2C**).
and kidney (p=0.034) (Figure 3C). The mRNA level of both UGT1A1 (Figures 5A, B) and UGT1A9 (Figures 6A, B) was not significantly different in diabetic liver or kidney as compared with those from non-diabetic tissues, with large inter-individual variability in both groups.

Influence of liver UGT2B7 genotype on AcMPAG formation. DNA extracted from liver tissue was genotyped for common UGT2B7 polymorphisms previously associated with altered UGT2B7 expression and activity. Genotyping for the UGT2B7*1c allele (c.735 A>G) identified 11 A/A homozygous individuals, 4 A/G heterozygous individuals, and no G/G homozygous individuals. Similarly, assay of the UGT2B7*2 allele (c.802 C>T) identified 2 C/C homozygous individuals, 7 C/T heterozygous individuals, and 6 T/T homozygous individuals. Genotypes could not be determined in one liver sample. There were no differences in AcMPAG intrinsic clearance with respect to UGT2B7*1c (Figures 4A) or UGT2B7*2 (Figures 4B) genotypes.
Discussion

The liver is quantitatively the most important site of glucuronidation for most compounds (McGurk et al., 1998). The overall contribution of extrahepatic tissues to glucuronidation is generally lower than that of the liver (McGurk et al., 1998). However, several studies highlighted the fact that the kidney may significantly contribute to MPA biotransformation based on comparison of urinary clearance of MPAG after oral or intravenous administration of MMF and on the impact of hepatic impairment on MPAG clearance (Bullingham et al., 1996), (Parker et al., 1996). Zucker et al. (Zucker et al., 1999) provided additional confirmation and reported that purified kidney extracts contained higher amounts of UGT enzymes involved in MPAG formation than those from liver samples. In our experiments, the HKMs were shown to have the highest intrinsic clearance for glucuronidation of MPA to its principal metabolite, MPAG. In contrast, the highest AcMPAG formation rate was found in HLMs. However, when these results extrapolated to the whole organ, observations from our ex vivo experiment suggest that liver is the organ primarily responsible for the systemic clearance of MPA, with some contribution from the kidney. Therefore, our data on MPA glucuronidation are similar to those published previously (Shipkova et al., 2001), (Bowalgaha and Miners, 2001).

Previous clinical pharmacokinetic studies conducted by our group (Akhlaghi et al., 2006), (Patel et al., 2007) reported a higher MPAG to AcMPAG concentration ratio (or lower AcMPAG to MPAG concentration ratio) in stable kidney transplant recipients with diabetes. In parallel to our clinical observations, MPAG concentration was not significantly different when MPA incubated with both HLM and HKM samples from diabetic donors. In our experiment, data from immunoblotting using a non-specific antibody showed higher protein level of UGT1As in diabetic HLMs compared with non-diabetic HLMs (unpublished data). However, no differences were found in the activity of liver and kidney UGT1A9 using propofol as a specific probe. Moreover, MPAG is also formed by other UGTs present in liver tissue such as UGT 1A1, 1A3, 1A6 (Shipkova et al., 2001) and UGT1A1 was identified as a principal
UGT underlying MPAG formation in rat liver (Miles et al., 2005). In our experiment, no change in UGT1A1 activity was observed in diabetic samples using estradiol as a specific probe.

The formation of AcMPAG was markedly lower in both HLM and HKM samples from diabetic donors. Using incubation with AZT, a specific substrate of UGT2B7 (Court et al., 2003), immunological blotting, and real-time PCR, we demonstrated that diabetes significantly decreases mRNA expression, protein level, and enzymatic activity of liver and kidney UGT2B7. However, the magnitude of decrease in \textit{ex vivo} production of AcMPAG in diabetic tissue is much higher than the somewhat smaller changes in MPAG to AcMPAG concentration ratio observed in our clinical studies (Akhalghi et al., 2006), (Patel et al., 2007).

At least two main explanations can be proposed to explain the discrepancy between \textit{in vitro} data with those obtained clinically, (i) extrahepatic tissues might contain UGTs other than UGT2B7 capable of forming AcMPAG but are unaffected affected by diabetes. For example, UGT1A8 which is primarily expressed in extrahepatic tissue, is also capable of forming AcMPAG (Bernard et al., 2006). (ii) The excretion of AcMPAG appears to depend on MRP2 (Westley et al., 2006), OAT or other transporters that potentially can influence the pharmacokinetics of AcMPAG. For a better understanding of the underlying changes of AcMPAG in diabetic patients, elucidation of the expression of transporter proteins is warranted in diabetic tissues.

The impact of UGT2B7 polymorphism on the glucuronidation activity and on MPA exposure remains controversial (Kagaya et al., 2007). Previously published studies demonstrated that UGT2B7*2 polymorphism may affect UGT2B7 protein level or enzymatic activity (Barbier et al., 2000). However, various other studies have failed to show an effect of UGT2B7*2 on UGT2B7 expression or activity (Bhasker et al., 2000) (Court et al., 2003). On the other hand, UGT2B7*1c was recently associated with increased UGT2B7 protein levels and activity in
human livers, and higher AZT clearance in human subjects (Kwara et al., 2009). We did not observe any significant association between AcMPAG formation and polymorphism in UGT2B7, suggesting that the observed differences between diabetic and non-diabetic livers are independent of UGT2B7 polymorphism. However, the relatively small number of samples analyzed in the current study limits our ability to discern any genotype effect except to exclude it as a cause of the differences in AcMPAG formation between diabetic and non-diabetic livers.

Of all the immunosuppressant drugs currently available, MPA is the only compound that has been thoroughly investigated with regards to possible mechanism of action for causing GI toxicities. It has been reported that effect of MPA-associated GI toxicity appears independent of the route of administration (Pescovitz et al., 2000). Moreover, it has been suggested that this toxicity may be caused by local GI concentration of AcMPAG rather than MPA or MPAG (Wieland et al., 2000), (Picard et al., 2005). However, the exact mechanism is still unknown. Several possible mechanisms have been proposed, such as (i) changes in purine synthesis and replication of epithelial cells due to inhibition of IMPDH activity (Shipkova et al., 2003). However, epithelial cells in the GI tract may not be wholly dependent on de novo purine synthesis, and may be permeable to purines that are released into the intestine during digestion (Wilson and Wilson, 1962). (ii) formation of adducts with plasma proteins that can directly interfere with cell function or trigger the immune system, leading to hypersensitivity and autoimmune reaction (Wieland et al., 2000). Up-regulation has been described for polymeric immunoglobulin receptor, glutathione-catalase, and CCAAT/enhanced-binding protein (Shipkova et al., 2004); (iii) decreases glucuronidation activity leading to lower AcMPAG concentration resulted in fewer GI toxicity (Yang et al., 2009).

Diabetes is associated with a decrease prevalence of MPA-associated GI toxicity when MPA is prescribed as an enteric coated formulation of mycophenolate sodium than immediate
released formulation of mycophenolate mofetil (Result of MyGain study, unpublished communication with Novartis). Therefore, we speculate that down-regulation of UGT2B7 may decrease the formation of AcMPAG, and thereby could decrease local irritation of the GI tract lumen and result in a lower incidence of GI toxicity in diabetic patients.

The present study has several limitations including the ex vivo nature of the observations, small sample size and the question about quality of commercially obtained liver and kidney samples. To confirm the observation on UGT2B7 down-regulation by diabetes, a clinical pharmacokinetics study using a well-known UGT2B7 substrate is warranted in diabetic patients closely matched with non-diabetic controls. Lack of tissue with known disease state such as diabetes is an important problem in characterizing ex vivo biotransformation in human. Typically, most tissue banks in the United States lack extensive information about the medical history of the donor, disease state (i.e. diabetes status or type) or medications used by the donors. Hence despite our best effort, only a small number of tissues with known diabetes status could be obtained. It will be important to verify our observations in a larger number of tissues from donors with a more comprehensive medical history (i.e. type of diabetes, presence of obesity, insulin vs. oral hypoglycemic agent therapy).

In conclusion, the findings of the present study provide evidence that diabetes significantly reduces the mRNA expression, protein level, and activity of human liver and kidney UGT2B7. In addition, the activity of UGT2B7 in the GI tract of diabetic patients would provide better insight about AcMPAG formation in the gut but it was not studied in the current study. A major limitation of this study was the limited amount of information available regarding liver or kidney donors. Moreover, many questions remain unanswered concerning molecular mechanisms underlying the changes in UGT2B7 activity in diabetic patients.
ACKNOWLEDGEMENTS

We are grateful to Dr. Haiyan Xu (Division of Biology and Medicine, Brown University, Providence, RI, USA) for the quantification of UGT 2B7 protein levels.
AUTHORSHIP CONTRIBUTIONS

Participated in research design: Dostalek, Akhlaghi, Court

Conducted experiments: Dostalek, Hazarika, Court

Contributed new reagents or analytic tools: Akhlaghi

Performed data analysis: Court, Hazarika, Dostalek, Akhlaghi

Wrote or contributed to the writing of the manuscript: Dostalek, Akhlaghi, Court, Hazarika

Other: Akhlaghi and Court acquired funding for the research.
REFERENCES


FOOTNOTES

1. F. Akhlaghi was supported by the American Heart Association [#0855761D] and an investigator initiated grant from Novartis Pharmaceuticals. Use of the RI-INBRE core facility funded by the National Center for Research Resources [P20RR016457], a component of the National Institutes of Health, is gratefully acknowledged. M. H. Court was supported by the National Institute of General Medical Sciences [R01GM061834], National Institutes of Health. The content is solely the responsibility of the authors and does not necessarily represent the official views of the funding organizations.

2. The authors indicate no potential conflict of interest exists with any commercial entity whose products are described in the manuscript.
FIGURE LEGENDS

Figure 1 Formation rate of MPAG and AcMPAG from MPA in diabetic and non-diabetic HLM (8 in each group) and HKM (4 in each group) samples

The rate of formation of liver MPAG (A) and AcMPAG (C); kidney MPAG (B) and AcMPAG (D) when HLM or HKM samples from non-diabetic (solid line) and diabetic (dashed line) donors incubated with MPA. All data are expressed as mean ± SE.

Figure 2 Effect of diabetes on UGT2B7 expression and activity in human liver

(A) Representative western blot of UGT2B7 protein level in non-diabetic and diabetic liver samples. (B) UGT2B7 protein level in diabetic (n=8) (□) and diabetic (n=8) (■) HLMs quantified based on recombinant 7-hydroxy-4-trifluoromethylcoumarin glucuronidation activity (see methods). (C) The level of UGT2B7 mRNA expressed relatively to this in non-diabetic (considered as 1). (D) The rate of formation of zidovudine (AZT) glucuronide when HLMs from non-diabetic (solid line) and diabetic (dashed line) donors incubated with AZT. All data are expressed as mean ± SE.

Figure 3 Effect of diabetes on UGT2B7 expression and activity in human kidney

(A) Representative western blot of UGT2B7 protein level in non-diabetic and diabetic kidney samples. (B) UGT2B7 protein level in diabetic (n=4) (□) and diabetic (n=4) (■) HKMs quantified based on recombinant 7-hydroxy-4-trifluoromethylcoumarin glucuronidation activity (see methods). (C) The level of UGT2B7 mRNA expressed relatively to this in non-diabetic (considered as 1). (D) The rate of formation of zidovudine (AZT) glucuronide when HKMs from non-diabetic (solid line) and diabetic (dashed line) donors incubated with AZT. All data are expressed as mean ± SE.

Figure 4 Effect of liver UGT2B7 genotype on AcMPAG formation
Effect of liver UGT2B7*1c allele (c.735 A>G) (A) and UGT2B7*2 allele (c.802 C>T) (B) on AcMPAG formation in HLMs from non-diabetic (○) and diabetic donors (●). Values of intrinsic clearance ($V_{\text{max}}/K_m$) for each liver are shown.

Figure 5 Effect of diabetes on liver and kidney UGT1A1 mRNA and activity
UGT1A1 mRNA level in liver (n=8) (A) and kidney (n=4) (B) samples from non-diabetic (○) and diabetic (●) donors expressed relatively to this in non-diabetic (considered as 1). The rate of formation of estradiol 3-β-glucuronide (3-β-EDG) (mediated primarily by UGT1A1) and estradiol 17-β-glucuronide (17-β-EDG) (mediated in part by UGT2B7) in HLMs (C) and HKMs (D) from non-diabetic (○) and diabetic (●) donors incubated with estradiol. All data are expressed as mean ± SE.

Figure 6 Effect of diabetes on liver and kidney UGT1A9 mRNA and activity
UGT1A9 mRNA level in liver (n=8) (A) and kidney (n=4) (B) samples from non-diabetic (○) and diabetic (●) donors expressed relatively to this in non-diabetic (considered as 1). The rate of formation of propofol glucuronide (probe for UGT1A9) when HLMs (C) and HKMs (D) from non-diabetic (○) and diabetic (●) donors were incubated with propofol. All data are expressed as mean ± SE.
Table 1 Demographic characteristics of human liver and kidney samples from diabetic and non-diabetic donors

<table>
<thead>
<tr>
<th>Characteristics of liver samples from diabetic and non-diabetic donors</th>
<th>Diabetic</th>
<th>Non-diabetic</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender [male/female]</td>
<td>4/4</td>
<td>4/4</td>
<td>-</td>
</tr>
<tr>
<td>Age [years]</td>
<td>50.1 ± 5.2</td>
<td>48.5 ± 6.0</td>
<td>NS</td>
</tr>
<tr>
<td>Ethnicity</td>
<td>8C</td>
<td>8C</td>
<td>-</td>
</tr>
<tr>
<td>Liver fat content, macro [%]</td>
<td>27.0 ± 8.0</td>
<td>19.3 ± 5.1</td>
<td>NS</td>
</tr>
<tr>
<td>Liver fat content, micro [%]</td>
<td>14.0 ± 5.4</td>
<td>10.8 ± 4.3</td>
<td>NS</td>
</tr>
<tr>
<td>Cause of death</td>
<td>A(3), CVA(4), HT(1)</td>
<td>A(3), CVA(3), HT(1), MI(1)</td>
<td>-</td>
</tr>
<tr>
<td>TBARS [nmol/mg protein]</td>
<td>0.30 ± 0.05</td>
<td>0.19 ± 0.01</td>
<td>p=0.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Characteristics of kidney samples from diabetic and non-diabetic donors</th>
<th>Diabetic</th>
<th>Non-diabetic</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender [male/female]</td>
<td>3/1</td>
<td>2/2</td>
<td>-</td>
</tr>
<tr>
<td>Age [years]</td>
<td>60.5 ± 4.5</td>
<td>62.3 ± 0.9</td>
<td>NS</td>
</tr>
<tr>
<td>Ethnicity</td>
<td>3C/1AA</td>
<td>4C</td>
<td>-</td>
</tr>
<tr>
<td>Cause of death</td>
<td>A(1), CVA(3)</td>
<td>CVA(4)</td>
<td>-</td>
</tr>
<tr>
<td>TBARS [nmol/mg protein]</td>
<td>0.21 ± 0.02</td>
<td>0.15 ± 0.01</td>
<td>P=0.053</td>
</tr>
</tbody>
</table>

A, anoxia; AA, African American; C, Caucasian; CVA, cerebrovascular aneurysm; HT, head trauma; MI, myocardial infarction; TBARS, formation rate of thiobarbituric acid reactive substances; NS, non-significant at p>0.05; plus-minus values are mean ± SE.
Table 2 Enzyme kinetic parameters of the formation of MPAG and AcMPAG from MPA

Apparent $K_m$, $V_{max}$ and $K_{si}$ values for the formation of metabolites when MPA was incubated with diabetic and non-diabetic HLMs and HKMs, respectively.

<table>
<thead>
<tr>
<th></th>
<th>Non-diabetic</th>
<th>Diabetic</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HLM incubations with MPA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>MPAG</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{max}$ [pmol/min/pmol P450]</td>
<td>10.6 ± 1.1</td>
<td>15.7 ± 0.54</td>
<td>p=0.046</td>
</tr>
<tr>
<td>$K_m$ [µM]</td>
<td>454.3 ± 95</td>
<td>481.6 ± 35.1</td>
<td>NS</td>
</tr>
<tr>
<td>$V_{max}/K_m$ [pl/min/pmol P450]</td>
<td>22.9± 7.3</td>
<td>31.3± 9.2</td>
<td>NS</td>
</tr>
<tr>
<td>$K_{si}$ [µM]</td>
<td>1.5</td>
<td>1.5</td>
<td>-</td>
</tr>
<tr>
<td><strong>AcMPAG</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{max}$ [pmol/min/pmol P450]</td>
<td>2.43 ± 0.03</td>
<td>0.76 ± 0.40</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>$K_m$ [µM]</td>
<td>601 ± 35</td>
<td>502.3 ± 24.1</td>
<td>NS</td>
</tr>
<tr>
<td>$V_{max}/K_m$ [pl/min/pmol P450]</td>
<td>4.08 ± 0.05</td>
<td>1.51 ± 0.01</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>$K_{si}$ [µM]</td>
<td>2.0</td>
<td>2.0</td>
<td>-</td>
</tr>
<tr>
<td><strong>HKM incubations with MPA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>MPAG</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{max}$ [pmol/min/pmol P450]</td>
<td>26.4 ± 0.7</td>
<td>38.8 ± 2.6</td>
<td>p=0.035</td>
</tr>
<tr>
<td>$K_m$ [µM]</td>
<td>1009 ± 140</td>
<td>1158 ± 160</td>
<td>NS</td>
</tr>
<tr>
<td>$V_{max}/K_m$ [pl/min/pmol P450]</td>
<td>27.6 ± 5.2</td>
<td>34.1 ± 2.4</td>
<td>NS</td>
</tr>
<tr>
<td>$K_{si}$ [µM]</td>
<td>1.5</td>
<td>1.5</td>
<td>-</td>
</tr>
<tr>
<td><strong>AcMPAG</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{max}$ [pmol/min/pmol P450]</td>
<td>1.17 ± 0.001</td>
<td>0.43 ± 0.015</td>
<td>p=0.001</td>
</tr>
<tr>
<td>$K_m$ [µM]</td>
<td>3015 ± 210</td>
<td>3431 ± 310</td>
<td>NS</td>
</tr>
<tr>
<td>$V_{max}/K_m$ [pl/min/pmol P450]</td>
<td>0.41 ± 0.07</td>
<td>0.12 ± 0.03</td>
<td>p=0.001</td>
</tr>
<tr>
<td>$K_{si}$ [µM]</td>
<td>2.0</td>
<td>2.0</td>
<td>-</td>
</tr>
</tbody>
</table>

plus-minus values are mean ± SE.
Table 3 Enzyme kinetic parameters of the formation of zidovudine (AZT) glucuronide

Apparent $K_m$ and $V_{max}$ for the formation of metabolites when AZT was incubated with diabetic and non-diabetic HLMs and HKMs, respectively.

<table>
<thead>
<tr>
<th></th>
<th>Non-diabetic</th>
<th>Diabetic</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HLM incubations with AZT</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{max}$ [pmol/min/pmol P450]</td>
<td>1214 ± 37</td>
<td>274 ± 16</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>$K_m$ [μM]</td>
<td>330 ± 20</td>
<td>307 ± 43</td>
<td>NS</td>
</tr>
<tr>
<td>$V_{max}/K_m$ [pmol/min/pmol P450]</td>
<td>3.67 ± 0.30</td>
<td>0.87 ± 0.08</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>$K_{si}$ [μM]</td>
<td>200</td>
<td>200</td>
<td>-</td>
</tr>
</tbody>
</table>

| **HKM incubations with AZT** |              |          |         |
| $V_{max}$ [pmol/min/pmol P450] | 20.5 ± 0.5   | 6.52 ± 0.45 | p<0.001 |
| $K_m$ [μM]                  | 519 ± 49     | 519 ± 31  | NS      |
| $V_{max}/K_m$ [pmol/min/pmol P450] | 0.040 ± 0.004 | 0.013 ± 0.001 | p=0.010 |
| $K_{si}$ [μM]              | 150          | 150      | -       |

plus-minus values are mean ± SE.
Figure 2

A

non-diabetic

diabetic

B

liver microsomes

UGT2B7 protein level expressed as an activity [pmol/mg protein/min]

C

liver microsomes

Relative UGT2B7 mRNA level

D

liver microsomes

pmol AZT-glucuronide/ min/mg protein vs AZT concentration [mM]

ND D

ND D

*** **
Figure 4

(A) UGT 2B7*1c allele (c.735 A>G)

(B) UGT 2B7*2 allele (c.802 C>T)
Figure 5

(A) Relative UGT1A1 mRNA level in liver microsomes.

(B) Relative UGT1A1 mRNA level in kidney microsomes.

(C) pmol 3-β-ESG or 17-β-ESG/min/μg protein in liver microsomes.

(D) pmol 3-β-ESG or 17-β-ESG/min/μg protein in kidney microsomes.

Legend:
- ND: Normal Diet
- D: Diets
- #: Significant difference

Estradiol concentration, 100 μM
Figure 6

(A) Relative UGT1A9 mRNA level in liver microsomes with and without N-methyl-D-aspartate (NMDA) receptor agonist (D). ND: No Drug, D: Drug

(B) Relative UGT1A9 mRNA level in kidney microsomes with and without NMDA receptor agonist (D). ND: No Drug, D: Drug

(C) pmol propofol glucuronide/min/µg protein in liver microsomes with and without NMDA receptor agonist (D). ND: No Drug, D: Drug

(D) pmol propofol glucuronide/min/µg protein in kidney microsomes with and without NMDA receptor agonist (D). ND: No Drug, D: Drug