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RESEARCH PAPER

Significantly reduced cytochrome P450 3A4 expression and activity in liver from humans with diabetes mellitus

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BACKGROUND AND PURPOSE
Patients with diabetes mellitus require pharmacotherapy with numerous medications. However, the effect of diabetes on drug biotransformation is not well understood. Our goal was to investigate the effect of diabetes on liver cytochrome P450 3As, the most abundant phase I drug-metabolizing enzymes in humans.

EXPERIMENTAL APPROACH
Human liver microsomal fractions (HLMs) were prepared from diabetic (n = 12) and demographically matched nondiabetic (n = 12) donors, genotyped for *CYP3A4*1B* and *CYP3A5*3* polymorphisms. Cytochrome P450 3A4, 3A5 and 2E1 mRNA expression, protein level and enzymatic activity were compared between the two groups.

KEY RESULTS
Midazolam 1′- or 4-hydroxylation and testosterone 6β-hydroxylation, catalyzed by P450 3A, were markedly reduced in diabetic HLMs, irrespective of genotype. Significantly lower P450 3A4 protein and comparable mRNA levels were observed in diabetic HLMs. In contrast, neither P450 3A5 protein level nor mRNA expression differed significantly between the two groups. Concurrently, we have observed increased P450 2E1 protein level and higher chlorzoxazone 6-hydroxylation activity in diabetic HLMs.

CONCLUSIONS AND IMPLICATIONS
These studies indicate that diabetes is associated with a significant decrease in hepatic P450 3A4 enzymatic activity and protein level. This finding could be clinically relevant for diabetic patients who have additional comorbidities and are receiving multiple medications. To further characterize the effect of diabetes on P450 3A4 activity, a well-controlled clinical study in diabetic patients is warranted.

Abbreviations
1′-OH MDZ, 1′-hydroxymidazolam; 4-OH MDZ, 4-hydroxymidazolam; 6-OH CZ, 6-hydroxychlorzoxazone; 6β-OH TST, 6β hydroxytestosterone; CZ, chlorzoxazone; HLM, human liver microsomes; K_{si}, substrate inhibition constant; MDZ, midazolam; P450 3A, cytochrome P450 3A subfamily; P450, cytochrome P450 (also termed haeme-thiolate P450); rP450, recombinant cytochrome P450; STZ, streptozotocin-induced diabetes model; TBARS, thiobarbituric acid reactive substances; TST, testosterone

Introduction

Much of the variability in the plasma concentration of a drug among patients receiving the same dosage is caused by the interindividual variations in drug biotransformation. This results mainly from variability in the expression of cytochrome P450 (Lin and Lu, 2001). Cytochrome P450 (P450) enzymes are a superfamily of haemoproteins that mediate the biotransformation of both endogenous and exogenous compounds. Among these, the P450 1, 2 and 3 families include the principal enzymes responsible for the biotransformation of approximately 96% of xenobiotics (Guengerich, 1999). The P450 3A subfamily is comprised of P450 3A4, 3A5, 3A7 and 3A43, which have similar substrate specificities but different expression patterns (Guengerich, 1999). The cytochrome P450 3A subfamily contributes to the biotransformation of ~55% of marketed medications (Guengerich, 1999) as well as many endogenous substrates (cortisol, estradiol, progesterone and testosterone) (Anzenbacher and Anzenbacherova, 2001).

Cytochrome P450 3A4 is the most abundant P450 in the liver and intestine. This enzyme accounts for as much as 60% of the total P450 in the human liver and P450 3A4 protein level can vary 40-fold among individuals (Guengerich, 1999). Highly polymorphic P450 3A5 has been considered less important than P450 3A4 because of its relatively low levels of hepatic and intestinal expression. However, recent data suggest that P450 3A5 may account for more than 50% of the P450 3A content in approximately 30% of human livers (Lin *et al.*, 2002). Cytochrome P450 3A7 is a fetal form of P450 3A, and little is known about the function of P450 3A43.

The human *CYP450* genes are highly polymorphic. The different alleles are summarized at the human *CYP450* allele nomenclature committee home page (http://www.cypalleles.ki.se). Although numerous allelic variants for the *CYP3A4* have been studied, few variants lead to altered enzyme activity (Ingelman-Sundberg, 2004). Inconsistent results have been observed between the C*YP3A4*1B* allele and the pharmacokinetic variability of some P450 3A4 substrates (Wandel *et al.*, 2000). The *CYP3A5*3* variant is the most common defective *CYP3A5* allele with an allele frequency of about 90% in Caucasians. This variant is associated with a marked reduction in both *in vitro* and *in vivo* clearance of several P450 3A substrates (Krishna and Shekar, 2005). Considerable interindividual variation in *CYP3A* gene expression can be seen, which is largely the result of the transcriptional regulation of P450 3A through the nuclear receptors pregnane X receptor (PXR) and constitutive androstane receptor (CAR) (Krishna and Shekar, 2005). Thus, drugs that are ligands of PXR and CAR transcriptionally modulate expression of P450 3A (Krishna and Shekar, 2005). Moreover, it has been shown that the expression of P450 is modulated not only by exposure to various chemicals but also by pathophysiological conditions such as diabetes (Favreau and Schenkman, 1988; Shimojo *et al.* 1993).

The literature is very contradictory concerning the effect of diabetes on P450 activity in humans, potentially because of a variety of factors including age, gender, disease duration and degree of diabetes control (Wang *et al.*, 2007). Moreover, the molecular mechanisms that underlie these changes in drug biotransformation have not been fully characterized yet. Minimal data exist on the effect of diabetes on drug metabolizing enzymes in humans. The current study was designed to investigate the effect of diabetes on the activity of the P450 3A subfamily *ex vivo* in human liver samples obtained from diabetic and nondiabetic donors, using midazolam (MDZ) and testosterone (TST) as probes. In addition, the effects of diabetes on human liver P450 3A4 and 3A5 protein levels and mRNA expression were studied. Most human studies examining the effect of diabetes on P450 have focused on the modulation of P450 2E1 activity and protein level. Thus, P450 2E1 expression and enzyme activity as characterized by biotransformation of chlorzoxazone (CZ) to 6-hydroxychlorzoxazone (6-OH CZ) has been selected as a positive control and an indicator of the quality of human livers.

Methods

Chemicals

MDZ, 1′-OH MDZ, 4-OH MDZ, D_5-MDZ, CZ, 6-OH CZ and D_3-CZ were purchased from Toronto Research Chemicals (Toronto, ON, Canada). TST, 6β-OH TST and acetaminophen were obtained from Sigma Aldrich (St. Louise, MO, USA). HPLC-grade acetonitrile and methanol were obtained from Pharmco Products Inc. (Brookefield, CT, USA). All other reagents and solvents were obtained from general commercial suppliers. All chemicals were used without further purification.

Tissue collection and microsomal preparation of diabetic and nondiabetic livers

Diabetic (n = 12) and nondiabetic (n = 12) human livers (1 g) were obtained from XenoTech LLC (Lenexa, KS, USA) (for more details, see Table 1) and were carefully selected based on their similarity in demographic data. Unfortunately, the type of diabetes and diabetes treatment were unknown for most donors. The diabetic samples were identified as such by the supplier XenoTech LLC, who obtained this information from the organ procurement organizations providing the donated tissue. Microsomes were prepared from the livers as described previously (Guengerich and Bartleson, 2001) and stored at –80°C. Total protein concentrations were estimated using a bicinchoninic acid method (Pierce-Fisher, Rockford, IL, USA). Concentrations of total P450 were determined following the protocol of Omura and Sato (1964). To determine the extent of oxidative stress, malondialdehyde, a product of lipid peroxidation, was measured using thiobarbituric acid assay as described previously (Ernster *et al.*, 1968).

Incubation of various substrates in human liver microsomes

Incubation mixtures (100 µL, 100 mM potassium phosphate buffer, pH 7.4) contained either 50 $\mu g \cdot mL^{-1}$ microsomal proteins or rP450 3A4 or 3A5 (containing nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome P450 reductase) (5 nM) (BD Bioscience, San Jose, CA, USA), various concentrations of MDZ (0.001, 0.0025, 0.005, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10, 25, 50, 100, 250 µM), TST (0.5, 1.0, 2.5, 5.0, 10, 25, 50, 100, 250, 500, 1000 µM), or CZ

Table 1

Demographic characteristics of human liver samples from diabetic and nondiabetic donors

	Diabetic	Nondiabetic	*P*-value
Gender (male/female)	6/6	6/6	–
Age (years)	50.2 ± 14.0	52.7 ± 15.3	NS
Ethnicity	11C/1AA	11C/1AA	–
Liver fat content, macro (%)	23.3 ± 14.3	20.5 ± 13.9	NS
Liver fat content, micro (%)	15.7 ± 6.4	13.7 ± 6.9	NS
Cause of death	A(6), CVA(5), HT(1)	A(4), CVA(5), HT(1), MI(1), ICH(1)	–
Total P450 (pmol·mg^{-1} protein)	134 (93–280)	239 (181–321)	0.035
TBARS (nmol·mg^{-1} protein)	0.31 ± 0.05	0.18 ± 0.02	<0.001

A, anoxia; AA, African American; C, Caucasian; CVA, cerebrovascular aneurysm; HT, head trauma; ICH, intracranial haemorrhage; MI, myocardial infarction; NS, non-significant, $P > 0.05$; TBARS, thiobarbituric acid reactive substances. Plus-minus data are mean ± SD; total P450 levels are expressed as median and interquartile range.

(single concentration, 100 μM), and NADPH-regenerating system. Microsomal incubations were run in duplicate. Previously published data have demonstrated differential effects of cytochrome b_5 based on the activity of rP450 3A4 compared with rP450 3A5 (Yamazaki *et al.*, 2002). In our study, we used rP450 3A4 and 3A5, both without cytochrome b_5. Liver microsomes and a NADPH-regenerating system (Guengerich and Bartleson, 2001) were preincubated for 5 min at 37°C before the addition of the substrate of interest. The reactions were carried out at 37°C, in a shaking water bath, for 20 min for MDZ or 30 min for TST and CZ. Reactions were stopped by cooling on ice and adding 1.0 mL ice-cold acetonitrile (extracted twice, with separation each time by centrifugation at 20 000× *g* for 10 min at 4°C). D_5-MDZ, acetaminophen, or D_3-CZ in acetonitrile was added as an internal standard. The combined extracts were transferred to fresh centrifuge tubes and dried at room temperature. The residues were redissolved in 100 μL of mobile phase, vortex-mixed and then centrifuged; 5 μL for MDZ and CZ, or 50 μL for TST were injected onto the analytical column followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) or HPLC-UV analysis. MDZ unbound fraction (f_u) in the microsomal fraction was determined by equilibrium dialysis against 0.1 M phosphate buffer, pH 7.4 according to a previously published method (Wang *et al.*, 2007).

LC-MS/MS analysis

The chromatographic separation of MDZ, CZ and their metabolites was performed on a LC-MS/MS system, comprised of a Shimadzu binary pump and autosampler (Shimadzu, Kyoto, Japan) coupled to a AB Sciex triple quadrupole mass spectrometric detector API 3200 (Toronto, Ontario, Canada), equipped with a Turbo V source electrospray ionization (ESI) probe. The column was heated using a Flatron Systems TC-50 temperature controller and a CH-30 column heater (ASTEC, Whippany, NJ, USA). Chromatographic data were collected and analysed using Analyst® package (version 1.4.1., AB Sciex). Samples were analysed for 1′- and 4-OH MDZ as published previously (Dostalek *et al.*, 2010). The method used for analysis of 6-OH CZ was adapted from Lee *et al.* (2006).

HPLC-UV analysis

The chromatographic separation of TST and its 6β-OH metabolite was performed on a HPLC Hitachi D-7000 series instrument (San Jose, CA, USA) using acetaminophen as an internal standard. Data from the detector were collected and analysed by Hitachi D-7000 software. A slightly modified method from Purdon and Lehman-McKeeman (1997) was used for the analysis of 6β-OH TST. These analytes were separated on a Thermo ODS-2 Hyperosil (4.6 mm × 150 mm, 5.0 μm) analytical column (Thermo Electron Corporation, Waltham, MA, USA). Analysis was performed using Mobile Phase A (HPLC-grade water) and Mobile Phase B (HPLC-grade methanol). The mobile phase was comprised of solvent mixtures: A:B 90:10 (v/v) for 0.0–5.0 min; A:B 25:75 (v/v) for 5.0–30.0 min; A:B 10:90 (v/v) for 30.0–35.0 min; A:B 90:10 (v/v) for 35.0–40.0 min. Flow rate was 1.0 mL·min^{-1}. The retention times for acetaminophen, 6β-OH TST and TST, were 4.5, 25.3 and 32.0 min, respectively. Concentrations used to construct the standard curve were 0.01, 0.1, 1.0, 10, 100, 1000 and 5000 μM of TST or 6β-OH TST with an average r^2 value of 0.984 for TST and 0.976 for the metabolite. The lower limit of quantification (LLOQ) was 0.5 μM for TST and 0.1 μM for 6β-OH TST.

Western blot analysis

The amounts of P450 3A4 and 3A5 in HLMs were determined by quantitative immunoblotting as described previously (Guengerich *et al.*, 1982). Protein levels were measured in duplicate, and mean values were reported for each liver. Expressed P450 3A4 and 3A5 (BD Biosciences) were used as reference standards. Known amounts of P450s of interest were used on each gel (0.1–500 pmol of P450 3A4, 0.01–50 pmol of P450 3A5), and the results were quantified using densitometry. Rabbit anti-human P450 3A5 was obtained

from Abcam Inc. (Cambridge, MA, USA) and BD Biosciences (San Jose, CA, USA), rabbit anti-human P450 3A4 and 2E1 were prepared in Dr Bingfang Yan's laboratory (University of Rhode Island, Kingston, RI, USA). The antibodies against P450 3A4 or 2E1 were raised with isoform-specific peptide, conjugated with keyhole limpet haemocyanin. The sequence of P450 3A4 peptide was N-CVKRMKESRLEDTQKHR VDFLQ-C; and the sequence of P450 2E1 peptide was N-CVIPRS-C. The preparation of the anti-P450 3A4 antibody was as described previously (Zhu *et al.*, 2000). Western immunoblotting for P450 2E1 was performed without the quantification of protein level.

Reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated with a RNA-Bee system (TEL-TEST, INC., Friendswood, TX, USA) according to the manufacturer's instructions (Yang *et al.*, 2007). TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA, USA) identification numbers were P450 2E1: Hs00559370; P450 3A4: Hs00430021; P450 3A5: Hs01070905; RNA polymerase II (DNA directed) polypeptide (POLR A2): Hs00172187. Amplification and quantification were done with the Applied Biosystems 7500 PCR System. For each test sample, PCR reactions were run in duplicate.

*Determination of CYP3A4*1B and CYP3A5*3 genotypes*

Liver samples were genotyped for single nucleotide polymorphisms (SNPs) in the P450 3A4 gene promoter (rs2740574; *CYP3A4*: -392A>G) and the P450 3A5 gene intron (rs776746; *CYP3A5:* 6986A>G). The *CYP3A4*1B* SNP genotype was determined by PCR amplification and direct sequence analysis as described previously (He *et al.*, 2005). The *CYP3A5*3* SNP was determined by Taqman® Allelic Discrimination Assay on an ABI 7300 PCR instrument (Assay C_26201809-30; Applied Biosystems). DNA was isolated from liver tissue samples using commercially available DNAzol according to the manufacturer's instructions (Invitrogen Corporation, Carlsbad, CA, USA).

Nonlinear regression of kinetic data

For enzyme kinetic studies, MDZ and TST hydroxylation data from individual liver samples were fitted to appropriate models by non-linear least-squares regression. Rates of formation of 1′-OH MDZ, 4-OH MDZ and 6β-OH TST were normalized by total P450 level. Model selection was based on the empiric goodness of fit data. After fitting reactions, we generated a corresponding Akaike information criterion (AIC) to identify the most plausible model based on the quality of the fits. The formation rates of 1′-OH MDZ, 4-OH MDZ and 6β-OH TST were fitted to a Michaelis–Menten model with uncompetitive substrate inhibition (Lin *et al.*, 2001; Patki *et al.*, 2003). Estimated enzyme kinetic parameters were maximum rate of formation (V_{max}), Michaelis–Menten constant (K_m) and enzyme efficiency V_{max}/K_m. Substrate inhibition constant (K_{si}) was fixed to a nominal value for each substrate and the choice of that value was based on goodness of fit criteria. Calculation of all kinetic parameters in HLMs was performed using GraphPad Prism (version 5.00 for Windows, GraphPad Software, San Diego, CA, USA).

Statistical analysis

All statistical analyses were carried out with SPSS (version 16, Chicago, IL, USA). For all variables, Kolmogorov–Smirnov's test was used to confirm normal distribution. Natural logarithmic transformation was carried out as needed for non-normal distributed data before performing parametric statistical tests. The differences between diabetic and nondiabetic groups were determined using a *t*-test, and *P*-values < 0.05 were considered statistically significant. All data are expressed as the geometric mean ± SEM or mean ± SD.

Results

Demographic data of the liver donors are presented in Table 1. Age, gender, ethnicity and liver fat content were comparable between diabetic and nondiabetic livers.

Hepatic P450 content in diabetic and nondiabetic HLMs

Total spectrally determined P450 content in HLM from diabetic subjects was significantly lower as compared with those from nondiabetics ($P = 0.035$) (Table 1). Immunoquantification of the P450 3A4 using sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis technique employing rabbit antibody against P450 3A4 revealed that the P450 3A4 protein level was significantly lower ($P = 0.015$) in diabetic livers. Moreover, a wide variability in P450 3A4 protein levels was observed, with levels ranging from 14.2 to 116 pmol·mg^{-1} microsomal protein (Figure 1A, B). In contrast, the P450 3A5 protein level was not different in HLM from diabetic and nondiabetic subjects ($P = 0.079$) (Figure 2A, B). Due to interindividual variability, P450 3A5 protein levels varied from 0.14 to 15% of the P450 3A4 protein levels. Furthermore, the protein level for P450 2E1 was significantly increased in diabetic HLMs ($P < 0.001$) (Figure 3A, B). Western blots depicted in Figures 1 and 2 were run concurrently with recombinant P450 3A4 or 3A5, as positive controls, and also in Figure 3 concurrently with recombinant P450 2E1. The P450 3A4 blot indicated that the P450 3A4 antibody was specific without cross-reacting with 3A5. The commercially obtained P450 3A5 antibody, on the other hand, appeared to slightly cross-react with P450 3A4, as shown in Figure 2A.

Thiobarbituric acid reactive substances (TBARS)

An elevated level of oxidative stress accompanied by increased production of free radicals is commonly associated with the development and progression of diabetes. Thus, the rate of formation of TBARS was measured as a marker of diabetes-associated oxidative stress. The formation of TBARS was significantly higher in diabetic HLMs ($P < 0.001$) (Table 1).

Real-time quantitative PCR analysis of P450 3A4, 3A5 and 2E1

The level of P450 3A4 mRNA was similar in HLMs from diabetic donors as compared with nondiabetic donors ($P =$

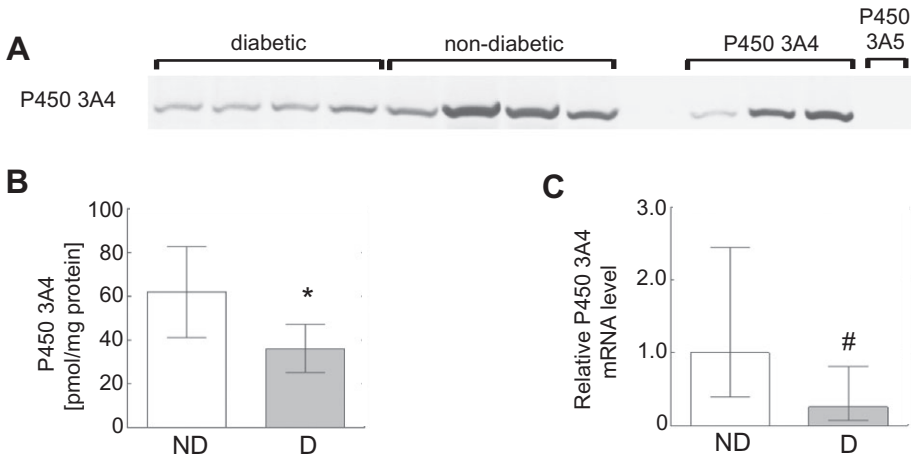

Figure 1

Effect of diabetes on P450 3A4 protein level and mRNA expression. (A) Representative Western blot traces for selected diabetic (lines 1–4) and nondiabetic (lines 5–8) livers, recombinant P450 3A4 at different concentrations (lines 10–12) and recombinant P450 3A5 (line 13). (B) P450 3A4 protein level in nondiabetic ($n = 12$) (ND) and diabetic ($n = 12$) (D) HLMs. (C) The level of P450 3A4 mRNA expressed as a relative value to that in nondiabetic (considered as 1). All data are presented as the mean ± SEM and statistical significance related to control (#, nonsignificant; *$P < 0.05$).

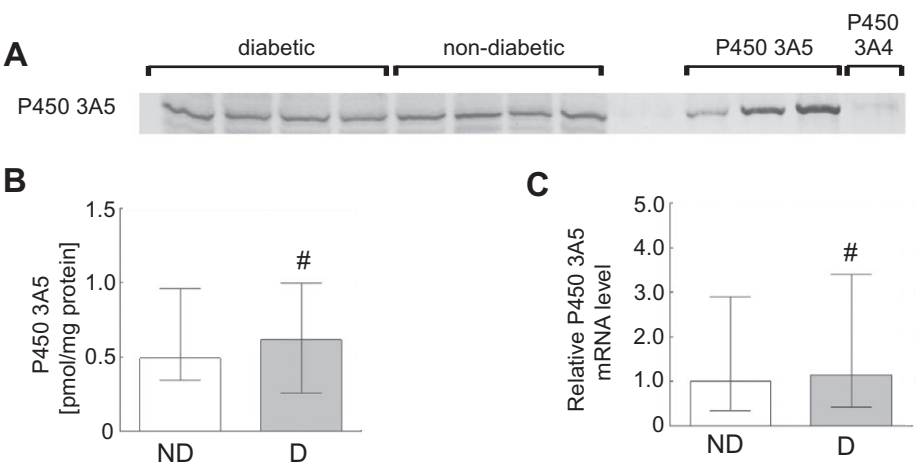

Figure 2

Effect of diabetes on P450 3A5 protein level and mRNA expression. (A) Representative Western blot traces for selected diabetic (lines1–4) and nondiabetic (lines 5–8) livers, recombinant P450 3A5 at different concentrations (lines 10–12) and recombinant P450 3A4 (line 13). (B) P450 3A5 protein level in nondiabetic ($n = 12$) (ND) and diabetic ($n = 12$) (D) HLMs. (C) The level of P450 3A5 mRNA expressed as a relative value to that in nondiabetic (considered as 1). All data are presented as the mean ± SEM (#, nonsignificant).

0.097) (Figure 1C). The mRNA levels of P450 3A5 ($P = 0.916$) (Figure 2C) and 2E1 ($P = 0.560$) (Figure 3C) were not significantly different between the two groups.

Biotransformation of MDZ by diabetic and nondiabetic HLMs

To evaluate the effect of diabetes on P450 3A activity in human liver, we examined the rate of MDZ hydroxylation. Both 1′- and 4-OH MDZ formation rates were analysed in this study. Because 1′-OH MDZ is the major hydroxy metabolite of MDZ, its formation rates were used to represent hepatic MDZ hydroxylation activity. However, the results did not differ if 4-OH MDZ formation rates were used for the analysis. V_{max}/K_m values for hepatic MDZ 1′-hydroxylation ($P < 0.001$) (Figure 4A) or 4-hydroxylation ($P < 0.001$) (Figure 4B) were significantly lower in all diabetic HLM samples (Table 2). Furthermore, no significant gender difference was observed

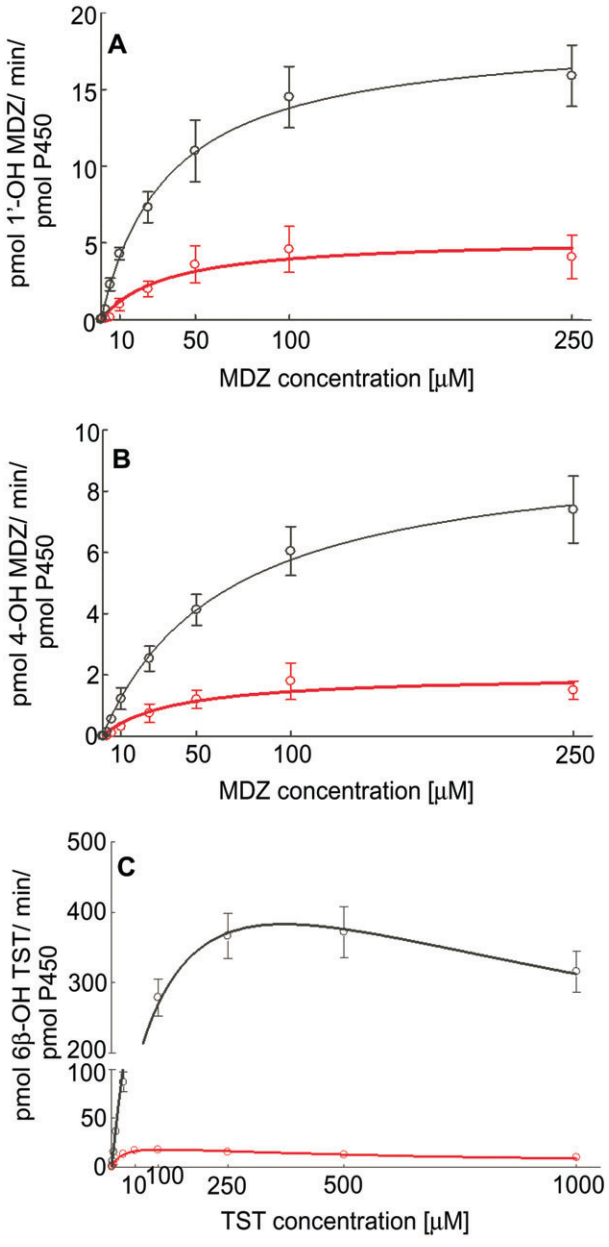

Figure 3

Effect of diabetes on P450 3A activity characterized by midazolam (MDZ) and testosterone (TST) biotransformation. The rate of formation of 1′-OH MDZ (A), 4-OH MDZ (B), 6β-OH TST (C) when HLMs from nondiabetic (black line) and diabetic donors (red line) are incubated with MDZ and TST, respectively. All data are presented as the mean ± SEM.

in V_{max}/K_m values for MDZ 1′-OH-hydroxylation in both diabetic (0.04 ± 0.05 vs. 0.07 ± 0.03 pL·min^{-1}·pmol^{-1} P450, female vs. male, P = 0.437) and nondiabetic (0.48 ± 0.10 vs. 0.32 ± 0.17 pL·min^{-1}·pmol^{-1} P450, female vs. male, P = 0.071) HLMs. MDZ hydroxylation kinetic parameters for rP450 3A4 and 3A5 are shown in Table 2. The ratio of unbound to bound MDZ in the microsomal fraction was 0.84:1, 0.85:1 and 0.84:1 for nondiabetic and 0.86:1, 0.88:1 and 0.85:1 for diabetic samples for added 1.0, 10.0 and 100 μM of MDZ.

Biotransformation of TST by diabetic and nondiabetic HLMs

Mean kinetic parameters for TST 6β-hydroxylation, in diabetic and nondiabetic HLMs, are shown in Table 3 and Figure 3C. Estimates of V_{max}/K_m for TST 6β-hydroxylation were significantly lower in diabetic HLMs as compared with nondiabetic HLMs (P < 0.001). No significant gender difference was observed in V_{max}/K_m values for TST 6β-hydroxylation in both diabetic (1.5 ± 0.3 vs. 2.3 ± 0.2 pL·min^{-1}·pmol^{-1} P450, female vs. male, P = 0.057) and nondiabetic (14.1 ± 1.9 vs. 9.3 ± 2.1 pL·min^{-1}·pmol^{-1} P450, female vs. male, P = 0.068) HLMs. Based on the results of recombinant P450 3A4 and 3A5 incubations, TST was primarily hydroxylated by P450 3A4 (Table 3), whereas MDZ was hydroxylated by both P450 3A4 and 3A5 (Table 2). Consequently, a greater difference was observed in TST 6β-hydroxylation, between diabetic and nondiabetic HLMs.

Determination of P450 3A4 and P450 3A5 polymorphism

DNA extracted from liver tissue was genotyped for common P450 3A4 and 3A5 polymorphisms previously associated with altered protein expression and enzyme activity. Genotyping for the *CY3A4*1B* allele identified 16 A/A homozygous individuals, 2 A/G heterozygous individuals (one African American, one Caucasian) and 0 G/G homozygous individuals. The genotype could not be determined for six of the liver samples (two diabetic, four nondiabetic donors). Similarly, assay of the *CYP3A5*3* allele identified no homozygous *1/*1 individuals, 6 *1/*3 heterozygous individuals and 18 *3/*3 homozygous individuals. There were no differences in MDZ 1′- and 4-hydroxylation and TST 6β-hydroxylation V_{max}/K_m with respect to *CYP3A4*1B* (Figure 5A, B) or *CYP3A5*1/3* (Figure 5C, D) genotypes.

Biotransformation of CZ by diabetic and nondiabetic HLM samples

We also examined whether diabetes affects P450 2E1 activity. The formation of 6-OH CZ was significantly higher in diabetic HLMs compared with that of nondiabetics (P = 0.015) (Figure 4D). Therefore, as anticipated, diabetes results in an increase in P450 2E1 activity leading to a higher formation of 6-OH CZ.

Discussion and conclusions

Chemically induced diabetes in laboratory animals can cause changes in expression, protein level and enzymatic activity of various P450s (Wang *et al.*, 2007). Although the effect of diabetes on animal models of diabetes has been studied extensively, minimal data are available on the effect of diabetes on human P450 (Wang *et al.*, 2007). Thus, the objective of this study was to determine whether diabetes can affect expression, protein level and enzymatic activity of human P450 3As. It was observed that diabetes significantly decreases

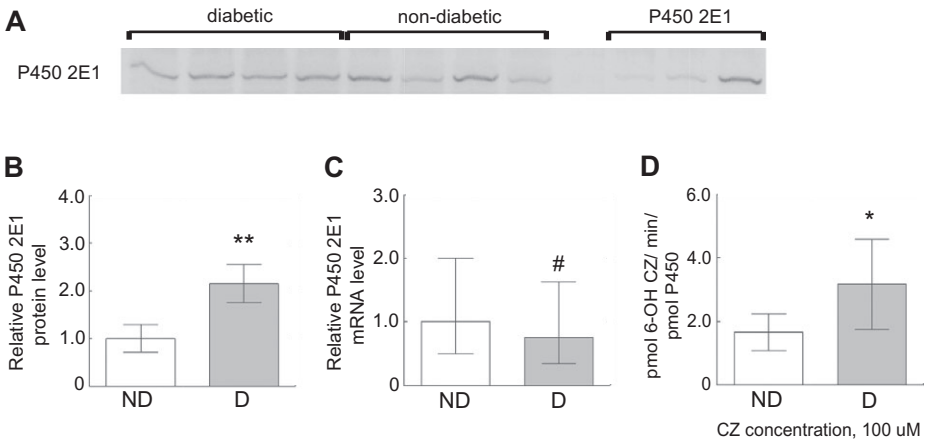

Figure 4

Effect of diabetes on 2E1 protein level, mRNA expression and enzymatic activity. (A) Representative Western blot traces for selected diabetic (lines 1–4) and nondiabetic (lines 5–8) livers, recombinant P450 2E1 at different concentrations (lines 10–12). (B) P450 2E1 protein level in nondiabetic ($n = 12$) (ND) and diabetic ($n = 12$) (D) HLMs (mean of protein level for nondiabetic HLMs were considered as 1). (C) The level of P450 mRNA expressed as a relative value to that in nondiabetic (considered as 1). (D) The rate of formation of 6-hydroxy chlorzoxazone when HLM from nondiabetic and diabetic incubated with CZ. All data are presented as the mean ± SEM and statistical significance related to control (#, nonsignificant; $^{**}P < 0.01$, $^{*}P < 0.05$).

Table 2

Enzyme kinetic parameters of the formation of 1′-hydroxymidazolam (1′-OH MDZ) and 4-hydroxymidazolam (4-OH MDZ)

	Nondiabetic	Diabetic	*P*-value
HLM incubations with MDZ			
1′OH MDZ			
V_{max} (pmol·min^{-1}·pmol^{-1} P450)	19.4 ± 2.8	3.5 ± 1.0	<0.001
K_m (mM)	52.1 ± 17.3	65.7 ± 19.9	NS
V_{max}/K_m (pL·min^{-1}·pmol^{-1} P450)	0.380 ± 0.029	0.056 ± 0.010	<0.001
K_{si} (mM)	50	50	–
4-OH MDZ			
V_{max} (pmol·min^{-1}·pmol^{-1} P450)	9.4 ± 2.1	1.9 ± 0.5	<0.001
K_m (mM)	95.1 ± 34.9	143.7 ± 20.0	NS
V_{max}/K_m (pL·min^{-1}·pmol^{-1} P450)	0.100 ± 0.004	0.015 ± 0.003	<0.001
K_{si} (mM)	5.0	5.0	–
Recombinant protein incubations with MDZ			
	1′OH MDZ	**4-OH MDZ**	
Recombinant P450 3A4			
V_{max} (pmol·min^{-1}·pmol^{-1} P450)	98.1 ± 5.2	29.1 ± 2.1	–
K_m (mM)	272.4 ± 23.4	51.6 ± 10.3	–
V_{max}/K_m (pL·min^{-1}·pmol^{-1} P450)	0.360	0.460	–
Recombinant P450 3A5			
V_{max} (pmol·min^{-1}·pmol^{-1} P450)	9.4 ± 0.8	10.1 ± 1.7	–
K_m (mM)	46.7 ± 10.8	115.1 ± 51.0	–
V_{max}/K_m (pL·min^{-1}·pmol^{-1} P450)	0.200	0.090	–

Apparent K_m and V_{max} for the formation of metabolites when midazolam (MDZ) was incubated with diabetic and nondiabetic human liver microsomes (HLM) (top part) and recombinant P450 3A4/5 (bottom part).
Data are expressed as mean ± SEM calculated based on the total concentration of midazolam. Mean ± SD of kinetic parameters is reported for recombinant P450s.

Table 3

Enzyme kinetic parameters of the formation of 6β hydroxytestosterone (6β-OH TST)

	Nondiabetic	Diabetic	*P*-value
HLM incubations with TST			
6β-OH TST			
V_{max} (pmol·min^{-1}·pmol^{-1} P450)	385.6 ± 30.5	19.8 ± 1.9	<0.001
K_m (mM)	34.9 ± 2.9	11.4 ± 1.5	<0.001
V_{max}/K_m (pL·min^{-1}·pmol^{-1} P450)	11.5 ± 1.3	1.8 ± 0.2	<0.001
K_{si} (mM)	200	200	
Recombinant P450s incubation with TST			
	6β-OH TST		
Recombinant P450 3A4			
V_{max} (pmol·min^{-1}·pmol^{-1} P450)	1197.0 ± 95.6	–	–
K_m (mM)	29.5 ± 4.7	–	–
V_{max}/K_m (pL·min^{-1}·pmol^{-1} P450)	40.5	–	–
Recombinant P450 3A5			
V_{max} (pmol·min^{-1}·pmol^{-1} P450)	148.2 ± 15.1	–	–
K_m (mM)	41.1 ± 8.4	–	–
V_{max}/K_m (pL·min^{-1}·pmol^{-1} P450)	3.6	–	–

Apparent K_m and V_{max} for the formation of metabolite when testosterone (TST) was incubated with diabetic and nondiabetic human liver microsomes (HLM) (top part) and recombinant P450 3A4/5 (bottom part). Data are expressed as mean ± SEM calculated based on the total concentration of testosterone. Mean ± SD of kinetic parameters is for recombinant P450s.

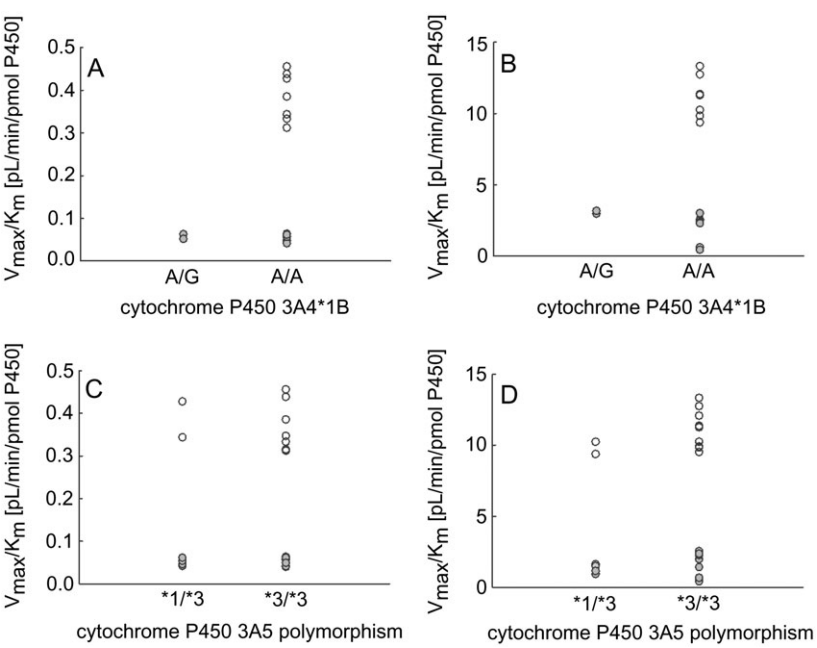

Figure 5

Effect of *CYP3A4*1B* allele on MDZ 1′-hydroxylation (A) and TST 6β-hydroxylation (B) activity and *CYP3A5*3* MDZ 1′-hydroxylation (C) and TST 6β-hydroxylation (D) activity in nondiabetic (n = 12) (open symbols) and diabetic (n = 12) (solid symbols) HLMs.

protein level and enzymatic activity of P450 3A4. However, no changes in P450 3A5 mRNA expression or protein level were found in HLM from diabetic donors. In contrast, we observed an increase in CZ 6-hydroxylation in diabetic HLMs indicating enhanced P450 2E1 activity.

MDZ hydroxylation yielding the 1′-OH and 4-OH MDZ and TST hydroxylation yielding the 6β-OH TST are well-established markers of human P450 3A-mediated biotransformation (Guengerich, 1999). The decreased activity of these markers paralleled a decrease in the hepatic P450 3A4 protein level as assessed by immunoblot cross-reactivity of P450 3A4 with a particular primary antibody. Moreover, a trend towards lower P450 3A4 mRNA expression indicates that changes in P450 3A4 activity appear to be pretranslation. The nonsignificant change in P450 3A4 mRNA expression may be the result of the small sample size as well as the high variability in the P450 3A4 mRNA expression.

In previous studies, hepatic P450 3A was found to be decreased in male and increased in female streptozotocin-induced (STZ) diabetic rats (Thummel and Schenkman, 1990; Gawronska-Szklarz *et al.*, 2003), and Shimojo *et al.* (1993) reported that hepatic P450 3A2 expression and TST 6β-hydroxylation were significantly increased in STZ rats. Barnett *et al.* (1994) showed that N-demethylation of ethylmorphine was significantly increased in STZ male rats, and Raza *et al.* (1996) reported that hepatic P450 3A1 protein level was significantly increased in STZ rats. Conversely, alprazolam biotransformation was not different between STZ and control mice (Kudo *et al.*, 2010). Also, Zaluzny *et al.* (1990) reported that TST 6β-hydroxylation was decreased in male type 2 diabetic Zucker *fa/fa* rats. However, rodents are not favourable models for investigating altered biotransformation of P450 3A substrates because eight different P4503A genes are expressed in mice but only four in humans. Moreover, several typical substrates of human P450 3A are not biotransformed by rat or mice P450 3A, and rat orthologous P450 3A1 is not induced by rifampicin, a classic human P450 3A inducer (Guengerich, 1999).

In addition, Moises *et al.* (2008) showed that the apparent clearance of lidocaine (a nonvalidated P450 3A4 marker) was lower in pregnant women with gestational diabetes. Also, in another study, using lidocaine in nonpregnant hypertensive women, it was found that those with type 2 diabetes had reduced P450 3A4 activity (Marques *et al.*, 2002). Collectively, it is evident that marked species-related differences exist in the modulation of P450 3A activity in response to diabetes.

Although P450s 3A4 and 3A5 are 84% identical in their primary sequence (Guengerich, 1999), their functional differences suggest that key differences in their active site and regulation may exist. Cytochrome P450 3A4 is believed to be more susceptible to mechanism-based inhibition than P450 3A5. Moreover, it has been demonstrated that P450 3A4 expression, protein level and enzymatic activity are selectively changed in chronic diseases such as liver cirrhosis (Yang *et al.*, 2003) or hepatocellular tumours of the non-cirrhotic liver (Haas *et al.*, 2009). Thus, different mechanisms could be proposed to account for the observed decrease in hepatic P450 3A4 protein level and activity in diabetic livers. These include the effects of pro-inflammatory cytokines, non-cytokine components, oxidative stress and the presence of obesity.

Elevated concentrations of interleukin (IL)-1β, IL-6 and tumour necrosis factor-α (TNF-α) have been found in both types of diabetes (Morgan, 1997). Expression of P450 3A4 in human hepatocytes was down-regulated by IL-1β, IL-6 (Sunman *et al.*, 2004), interferon-γ (INF-γ) (Donato *et al.*, 1997) and hepatocyte growth factor (HGF) (Donato *et al.*, 1998). A potent repressive effect of elevated cAMP levels on gene expression of P450 3A has been reported using primary rat hepatocyte cultures (Sidhu and Omiecinski, 1995). Ample evidence from experimental and clinical studies suggests that oxidative stress plays a significant role in the pathogenesis of diabetes. Thus, P450 down-regulation could be a survival mechanism to protect the cells from the deleterious effects of reactive oxygen/nitrogen species. The effect of obesity on P450 appears to be P450-specific, mainly affecting the activity of P450 3A4 (Kotlyar and Carson, 1999). Thus, obesity may play a role in regulating P450 3A4 in obese diabetic patients, although this remains to be further studied.

No association between the kinetic parameters of P450 3A substrates and polymorphism of *CYP3A4* and *CYP3A5* genes was observed. This would suggest that the differences we observed between diabetic and nondiabetic livers are independent of *CYP3A4* and *CYP3A5* polymorphism. However, the relatively small number of samples analysed limits our ability to discern any genotype effect, except to exclude it as a cause of the differences in P450 3A4 activity between diabetic and nondiabetic livers.

A major limitation of characterizing *ex vivo* biotransformation in human liver is the lack of availability of tissue from subjects with known clinical characteristics and disease state (i.e. diabetes). Most commercial or nonprofit liver banks in the United States do not have extensive information about the medical history of each donor. All information including donor demographics, medication use and disease status was provided by the Organ Procurement Organizations (OPO), which acquire donated human tissues for research purpose. However, the information on medication use was incomplete for most donors. It must be noted that the liver received by these banks are often those that are not used by liver transplant teams, and this may raise some questions about the overall quality of the tissue. To address this potential concern and to determine whether the decreased in P450 3A4 in diabetic livers was specific among the P450s, we have characterized hepatic content and activity of P450 2E1, the only P450 that was previously well-characterized in diabetes. We found increased P450 2E1 protein levels and CZ 6-hydroxylation activity in HLMs from diabetic donors, which is consistent with the earlier observations in diabetic liver or patients (Lucas *et al.*, 1998; Wang *et al.*, 2003). Thus, the change in P450 3A4 was not part of a generalized decrease in all hepatic P450. Furthermore, the level of oxidative stress was higher in diabetic livers as characterized by *in vitro* formation of TBARS.

Contrary to previous reports, we did not observe changes in P450 2E1 mRNA expression. However, controversy exists regarding altered 2E1 mRNA expression and its relevance to enzyme induction. In contrast, increased lymphocyte 2E1 mRNA expression from diabetic patients has been observed previously (Wang *et al.*, 2003). Another study showed lymphocyte P450 2E1 mRNA expression was unchanged (Pucci *et al.*, 2005). This may be because of the different

regulatory mechanism of P450 2E1 gene expression in mononuclear cells than in hepatocytes. Thummel and Schenkman showed that growth hormone administration to male rats failed to reverse the effect of diabetes on P450 expression (Thummel and Schenkman, 1990). Moreover, Johansson *et al*. (1991) found that the addition of insulin to primary rat hepatocytes stabilized P450 2E1 protein. Thus, more than one mechanism is likely to be important in the induction of P450 2E1 by diabetes.

The findings of the present study suggest that diabetes significantly reduces the activity and protein level of human liver P450 3A4. The liver is the most important site of drug biotransformation; therefore, reduced P450 3A4 activity may increase bioavailability and prolong elimination half-life of P450 3A4 substrates. This finding can be clinically significant for diabetic patients who have additional comorbidities and are receiving multiple medications with narrow therapeutic range. Moreover, down-regulation in P450 3A4 may reduce testosterone oxidation; this would decrease the amount of biologically active form of dihydrotestosterone, an androgen critical for regulation of prostate growth. However, many questions remain unanswered, particularly those concerning the molecular mechanisms underlying the changes in drug biotransformation in liver and/or extrahepatic tissue of diabetic patients.

In vitro or *ex vivo* approaches of studying P450 3A activity have the advantage that experimental conditions can be more closely controlled than *in vivo* studies. In contrast, the selected conditions may not sufficiently reflect those present *in vivo*; thus experimental findings cannot be readily extrapolated. In addition, limited clinical information from the donors of liver tissue was available including diabetes type, agents used to treat diabetes or presence of obesity. Without such information, it is difficult to conclude whether the observed differences in P450 3A4 are because of diabetes, degree of hyperglycaemia or agents used to treat diabetes. Thus, a well-controlled clinical study is warranted to evaluate the relationship between diabetes and P450 3A4 activity.

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Conflicts of interest

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