

ORIGINAL ARTICLE

Modulation of CYP3a expression and activity in mice models of type 1 and type 2 diabetes

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Introduction

CYP3A4, the most abundant cytochrome P450 (CYP450) enzyme in the human liver and small intestine, is responsible for the metabolism of about 50% of all mar-

Abstract

CYP3A4, the most abundant cytochrome P450 enzyme in the human liver and small intestine, is responsible for the metabolism of about 50% of all marketed drugs. Numerous pathophysiological factors, such as diabetes and obesity, were shown to affect CYP3A activity. Evidences suggest that drug disposition is altered in type 1 (T1D) and type 2 diabetes (T2D). The objective was to evaluate the effect of T1D and T2D on hepatic and intestinal CYP3a drug-metabolizing activity/expression in mice. Hepatic and intestinal microsomes were prepared from streptozotocin-induced T1D, *db/db* T2D and control mice. Domperidone was selected as a probe substrate for CYP3a and formation of five of its metabolites was evaluated using high performance liquid chromatography. Hepatic CYP3a protein and mRNA expression were assessed by Western blot and reverse-transcription quantitative polymerase chain reaction respectively. Hepatic microsomal CYP3a activity was significantly increased in both T1D and T2D groups versus control group. Intestinal CYP3a activity was also significantly increased in both T1D and T2D groups. Moreover, significant increases of both hepatic CYP3a mRNAs and protein expression were observed in both T1D and T2D groups versus control group. Additional experiments with testosterone further validated the increased activity of CYP3a under the effect of both T1D and T2D. Although differences exist in the pathophysiological insults associated with T1D and T2D, our results suggest that these two distinct diseases may have the same modulating effect on the regulation of CYP3a, ultimately leading to variability in drug response, ranging from lack of effect to life-threatening toxicity.

Abbreviations

ANOVA, analysis of variance; CAR, constitutive androstane receptor; CYP450, cytochrome P450; EDTA, ethylenediaminetetraacetic acid; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; PXR, pregnane X receptor; SRS, substrates recognition site; T1D, type 1 diabetes; T2D, type 2 diabetes; TSOD, Tsumura Suzuki Obese Diabetes.

keted drugs and many endogenous molecules (Danielson 2002). Changes in physiological and pathophysiological conditions including diabetes can affect drug metabolism and disposition (Lam et al. 2010). Several studies have shown that uncontrolled diabetes mellitus alters the

disposition of a number of drugs (Zysset and Wietholtz 1988), potentially increasing the risk for drug toxicity and side effects, or decreasing drug efficacy. Diabetes mellitus may be defined as a commonly occurring disease in which plasma glucose control is defective because of insulin deficiency or decreased target-cell responsiveness to insulin (Watkins and Sanders 1995). Type 1 diabetes (T1D), which accounts for about 5–10% of all cases of diabetes, is the consequence of pancreatic β -cells destruction and usually leads to absolute insulin deficiency (Daneman 2006). Type 2 diabetes (T2D) is a metabolic disorder characterized by insulin resistance mostly related to lifestyle (obesity and sedentary behavior) with a weaker genetic component (Ripsin et al. 2009). In 2011, over 350 million people worldwide were affected by this widespread chronic disease and its prevalence is expected to increase to approximately 550 million by 2030 (Whiting et al. 2011). Both T1D and T2D are known to modulate CYP3A activity and/or expression in humans and in animal models (Goldstein et al. 1990; Dong et al. 2009; Kudo et al. 2009). Streptozotocin-induced diabetes is a widely used mouse model of the disease, as this chemical selectively destroys pancreatic β -islet cells, further leading to a corresponding increase in blood glucose and pathological changes commonly seen in human with T1D (Gandhi et al. 2012). The *db/db* mouse is a routinely used animal model characterized by a mutation of the leptin receptor leading to a metabolic disturbance closely resembling T2D (Lam et al. 2010). In the present study, these two mice models of T1D and T2D have been used to better understand the effect of these two different diseases on the hepatic and intestinal metabolism of domperidone, a specific substrate of CYP3As with a complex metabolic profile (Simard et al. 2004; Appendix). An additional functional hepatic CYP3a-specific assay with testosterone was performed. Hepatic transcription and expression of CYP3a were also measured.

Materials and Methods

Animals

Male 7 weeks-old BKS.Cg-m *+/+* *Lepr^{db}/J* mice (*db/db*: T2D) and control C57BLKS/J wild-type mice were purchased from The Jackson Laboratory (Bar Harbor, ME). In a subgroup of wild-type mice, T1D was induced after one week (8 weeks-old) with intraperitoneal injections of streptozotocin (Sigma-Aldrich, St. Louis, MO) 50 mg kg⁻¹ day⁻¹ during 10 days. The animals were housed under a constant 12 h dark/light cycle and were given standard chow and water ad libitum. At 12 weeks-old, blood samples were collected via the submandibular vein and the animals were sacrificed by cervical dislocation. Livers and small intestine were quickly harvested, washed

with ice-cold phosphate-buffered saline (PBS), snap-frozen in liquid nitrogen and kept at -80°C . These experiments were carried out in accordance with the Declaration of Helsinki and with the Guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care.

Microsome preparation

The liver microsomes were prepared by differential centrifugation as described previously (Inaba and Kovacs 1989). The intestinal microsomes were extracted based on Bruyère's method (Bruyère et al. 2009), with modifications. Briefly, small intestines from two mice were pooled and homogenized in 5 mL of PBS containing 20% (v/v) glycerol, 0.1% protease inhibitor cocktail (Sigma-Aldrich), 5 mmol/L histidine, 0.25 mol/L sucrose, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), and then centrifuged at 10,000 g for 15 min, 4°C . The supernatant was collected and then centrifuged for 60 min at 100,000 g, 4°C . Microsomal pellets were reconstituted in a 50 mmol/L phosphate buffer (pH 7.0) containing 20% glycerol, 0.1% protease inhibitor cocktail, aliquoted and stored at -80°C . The protein content was determined with the DCTM protein assay (Bio-Rad, Mississauga ON, Canada).

Domperidone metabolism assay

Standard incubations and high performance liquid chromatography (HPLC) measurement of domperidone and its metabolites were performed as previously described (Patoine et al. 2013) with modification of the mobile phases as follows: A- H₂O and B- 20 mmol/L phosphate buffer, acetonitrile, methanol, triethylamine (62:23:15:0.1); final pH 7.0. The elution gradient used was the following: 0–6 min, 10% A; 7–26 min, 100% B. Variable flow-rates were imposed: 0–10 min, 1 mL/min, 11–13 min, 1.6 mL/min, 13–26 min, 1.7 mL/min.

Testosterone metabolism assay

Standard incubations containing testosterone 200 $\mu\text{mol/L}$ were performed. 11- β -OH-testosterone was used as internal standard. The measurement of testosterone, 6- β -OH-testosterone and 11- β -OH-testosterone was performed by a slight modification of a reported method (Mohri and Uesawa 2001). Mobile phase consisting of H₂O, methanol, acetonitrile (31.6:58.9:9.5) containing 0.05% of phosphoric acid was used.

RNA extraction and cDNA synthesis

Total RNA was extracted from 100 mg of frozen hepatic tissue using Trizol[®] reagent (Invitrogen, Burlington ON,

Canada). Purified RNA was treated with DNase (DNA-free kit: Invitrogen). cDNA was prepared with 1 µg of total RNA using the iScript™ cDNA synthesis kit (Bio-Rad).

Reverse transcription quantitative polymerase chain reaction

The level of gene expression in tissues was evaluated with the iQ SYBR green supermix (Bio-Rad). Gene-specific primers were designed with the VectorNTI software (Invitrogen) to amplify CYP3a11, 3a13, 3a25, 3a44, CAR, PXR (pregnane X receptor) and two reference genes; RPLP0 and β-Actin. The primers and PCR conditions used for amplifications were as described in Table 1. Primers were optimized and validated with standard curves to obtain efficiencies between 90–105%. Amplicons were sequenced to confirm the identity of the fragment. A dissociation curve protocol was linked to every PCR assay to ensure the specificity of the PCR product. Relative quantification was calculated with methods that correct for differences in amplification efficiency (Pfaffl 2001) and allow the use of multiple reference genes (Vandesompele et al. 2002).

Western Blot

Western blots were performed as previously described (Patoine et al. 2013) to assess CYP3a protein expression. Briefly, samples of frozen liver were homogenized in an ice-cold lysis buffer (10 mmol/L Tris-HCl [pH 7.4], 0.32 mol/L sucrose, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, 0.1% of a protease inhibitor cocktail from

Sigma-Aldrich). Western blot analysis of 5 µg of proteins was used to assess the levels of CYP3a protein in the livers. The membrane was incubated overnight at 4°C with anti-rat-CYP3a2 polyclonal antibody (1:1000; EMD Millipore, Billerica MA). The membrane was washed and then incubated with a secondary antibody conjugated with horseradish peroxidase. Immunodetection was done with the Luminata™ Crescendo Western HRP Substrate (EMD Millipore). Normalization against the total lane density of loaded proteins was used with TGX Stain-Free gels (Bio-Rad) according to the newly published methods (Taylor et al. 2013; Taylor and Posch 2014).

Data analysis

One-way analysis of variance (ANOVA) followed by a Tukey test or a Mann–Whitney test (when the normality test failed) were used to evaluate the statistical differences between groups (SigmaPlot 12.5, Jandel Scientific Software, San Rafael, CA). All the results were expressed as mean ± SEM. Level of statistical significance was set at $P < 0.05$.

Results and Discussion

The aim of the present study was to determine the effect of T1D and T2D on CYP3a in mouse. To this end, two different mice models of T1D and T2D were used. To confirm phenotypes, weight and a number of blood parameters were measured (Table 2). Figure 1 shows hepatic (A) and intestinal (B) CYP3a activities using domperidone and hepatic (C) CYP3a activity using testosterone. Figure 2 shows hepatic steady-state levels of

Table 1. Primer sequences used for the RT-qPCR analysis and conditions.

Gene	Primer sequence (5' → 3')	Product size (bp)	PCR conditions	
			Denaturation	Annealing
CYP3a11-F	GCC ATT TTT AGG CAC TGT GCT GA	124	95°C, 15 sec	60°C, 30 sec
CYP3a11-R	TGT GAC AGC AAG GAG AGG CGT			
CYP3a13-F	GAC CTG ATC CCA AAC TTT TCC	211	95°C, 15 sec	60°C, 30 sec
CYP3a13-R	TCC TTC TCC TAA TCC CTG CC			
CYP3a25-F	TGG GCA TAC TCA ACT TTC C	148	95°C, 15 sec	60°C, 30 sec
CYP3a25-R	CCA CAA GAA TCA GAA GGT			
CYP3a44-F	CTG AGC TTT CTC AGT GTC TGT G	102	95°C, 15 sec	60°C, 30 sec
CYP3a44-R	GAT CCC ATG AGA AAC GGT GAA G			
Beta-actin-F	TGT TAC CAA CTG GGA CGA CA	165	95°C, 15 sec	60°C, 30 sec
Beta-actin-R	GGG GTG TTG AAG GTC TCA AA			
CAR-F	CTC AAG GAA AGC AGG GTC AG	168	95°C, 15 sec	60°C, 30 sec
CAR-R	GGT CTC CAC ACA CCA CAC AG			
PXR-F	AGG GGG AAA TCC AAC AAA AG	184	95°C, 15 sec	60°C, 30 sec
PXR-R	ACA CGG CAG ATT TGA AGA CC			
RPLP0-F	ATC ACA GAG CAG GCC CTG CA	96	95°C, 15 sec	57°C, 30 sec
RPLP0-R	CAC CGA GGC AAC AGT TGG GT			

Table 2. Body weight and biochemical parameters at sacrifice (12 weeks-old). (** $P < 0.01$, *** $P < 0.001$ vs. control)

	Control	T1D	T2D
Weight (g)	23.5 ± 0.5 ($n = 17$)	19.9 ± 0.3*** ($n = 17$)	39.8 ± 1.2*** ($n = 20$)
Glycemia (mmol/L)	8.2 ± 0.7 ($n = 17$)	28.3 ± 0.9*** ($n = 17$)	31.3 ± 0.6*** ($n = 17$)
Insulinemia (ng/mL)	0.67 ± 0.06 ($n = 17$)	0.18 ± 0.04*** ($n = 12$)	3.90 ± 0.50*** ($n = 18$)
HDL-C (mmol/L)	2.10 ± 0.05 ($n = 16$)	3.54 ± 0.45*** ($n = 15$)	3.18 ± 0.13*** ($n = 15$)
Triglyceridemia (mmol/L)	1.67 ± 0.07 ($n = 16$)	2.58 ± 0.47*** ($n = 17$)	2.33 ± 0.22** ($n = 17$)
Cholesterolemia (mmol/L)	2.32 ± 0.17 ($n = 16$)	4.40 ± 0.45*** ($n = 17$)	3.77 ± 0.11*** ($n = 17$)

CYP3a (A), PXR and CAR (B) mRNAs and protein expression (C).

Type 1 diabetes

Mice with T1D displayed blood parameters with typical increases of glycemia, cholesterolemia, and triglyceridemia as well as about 75% decrease of insulinemia versus controls (Table 2). Hepatic CYP3a activity monitored through metabolism of domperidone and testosterone was increased (Fig. 1A and C). These results are consistent with many other studies. Sakuma et al. (2001) found increases in CYP3a activity and mRNA expression in streptozotocin-induced T1D in mice, both at 2 and 4 weeks after induction. Increased drug-metabolizing activity of CYP3a1 was also shown in rats with either alloxan- or streptozotocin-induced T1D (Kim et al. 2005; Lee et al. 2007). Similar rises of the CYP3A-related metabolism (Kudo et al. 2010) and mRNA transcription were also described and proved to be CAR-mediated (Dong et al. 2009). Interestingly, this is also consistent with our results showing significantly increased mRNA transcription of CAR and PXR in T1D (Fig. 2B). As it turned out, in the present study, increases in transcription of CYP3a11, 3a13 and 3a44 (but not 3a25) were observed (Fig. 2A). It has been reported that each CYP3a isoenzyme has a different sensitivity to diabetes and that these isoforms are not controlled by uniform regulatory mechanisms (Sakuma et al. 2001), which is consistent with our CYP3a25 mRNA transcription results. Moreover, western blot experiments revealed increase in CYP3a protein expression (Fig. 2C) which fairly correlates with the previously observed increase in CYP3a activity and mRNA transcription induced by T1D. As seen for liver metabolism, intestinal CYP3a activity was significantly increased (Fig. 1B).

Type 2 diabetes

Mice with T2D had severe obesity, hyperglycemia, hypercholesterolemia, hypertriglyceridemia, and hyperinsulinemia. Even if insulin levels at 12 weeks-old were still

elevated, they were reduced sixfold compared to those at 8 weeks-old (22.57 ± 2.94 vs. 3.90 ± 0.50 ng mL⁻¹, $P < 0.001$) indicating that diabetes had evolved toward a more severe stage. A significant increase in hepatic formation of two of the five CYP3A-related metabolites of domperidone (M2 and M4), as well as a significant decrease in the formation of M5, were observed (Fig. 1A). This is consistent (for M2 and M4) with the increase in protein expression of CYP3a (Fig. 2C). In human, it is recognized that CYP3A4 has six substrates recognition site (SRS) (Roussel et al. 2000; Domanski et al. 2001; Xue et al. 2001). It is possible that these SRS are differently modified by diabetes, thereby explaining the nonuniform effect on the formation of the metabolites. Moreover, a compensatory effect cannot be ruled out. Metabolism of testosterone to 6- β -OH-testosterone was also significantly increased (Fig. 1C), and correlates

Figure 1. (A) Hepatic microsomal CYP3a activity: This figure shows peak-height ratios of metabolites of domperidone to internal standard for the three groups. Formation of four of the five metabolites (M1-4) were significantly increased in the T1D group (M1: 0.417 ± 0.071 , M2: 0.199 ± 0.011 , M3: 0.818 ± 0.117 , M4: 0.201 ± 0.019 , M5: 0.178 ± 0.018 ; $n = 17$) when compared to the control group (M1: 0.158 ± 0.014 , M2: 0.090 ± 0.003 , M3: 0.384 ± 0.033 , M4: 0.109 ± 0.005 , M5: 0.152 ± 0.011 ; $n = 14$). In the T2D group, M2 and M4 were significantly increased while M5 was significantly decreased (M1: 0.209 ± 0.029 , M2: 0.171 ± 0.007 , M3: 0.487 ± 0.065 , M4: 0.147 ± 0.011 , M5: 0.093 ± 0.006 ; $n = 18$). Metabolites of domperidone were not formally identified. (B) Intestinal microsomal CYP3a activity: Formation of the four metabolites was significantly increased in both T1D (M1: 0.113 ± 0.013 , M2: 0.054 ± 0.007 , M3: 0.389 ± 0.040 , M4: 0.077 ± 0.007 ; $n = 8$) and T2D (M1: 0.093 ± 0.017 , M2: 0.032 ± 0.010 , M3: 0.338 ± 0.065 , M4: 0.075 ± 0.011 ; $n = 6$) groups when compared to the control group (M1: 0.004 ± 0.004 , M2: 0.002 ± 0.002 , M3: 0.039 ± 0.013 , M4: 0.015 ± 0.006 ; $n = 8$), except for M4 in the T2D group. (C) Hepatic microsomal CYP3a activity: This figure shows peak-height ratios of the CYP3a-catalyzed metabolite of testosterone to internal standard for the three groups. Formation of 6- β -OH-testosterone was significantly increased in both T1D and T2D groups when compared to control group (0.588 ± 0.063 , 0.661 ± 0.098 vs. 0.277 ± 0.018 ; $n = 15, 17, 16$, respectively)

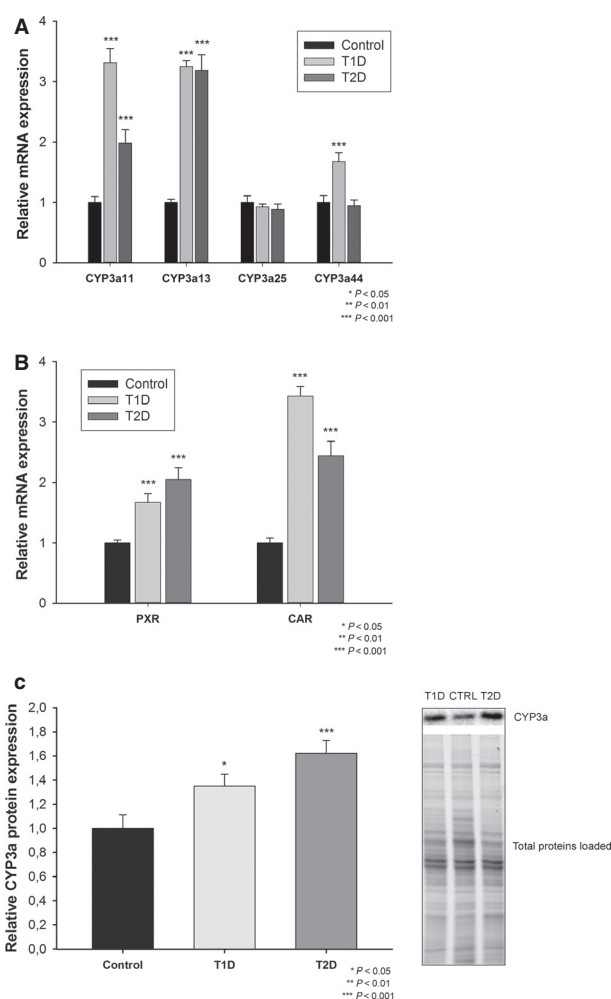
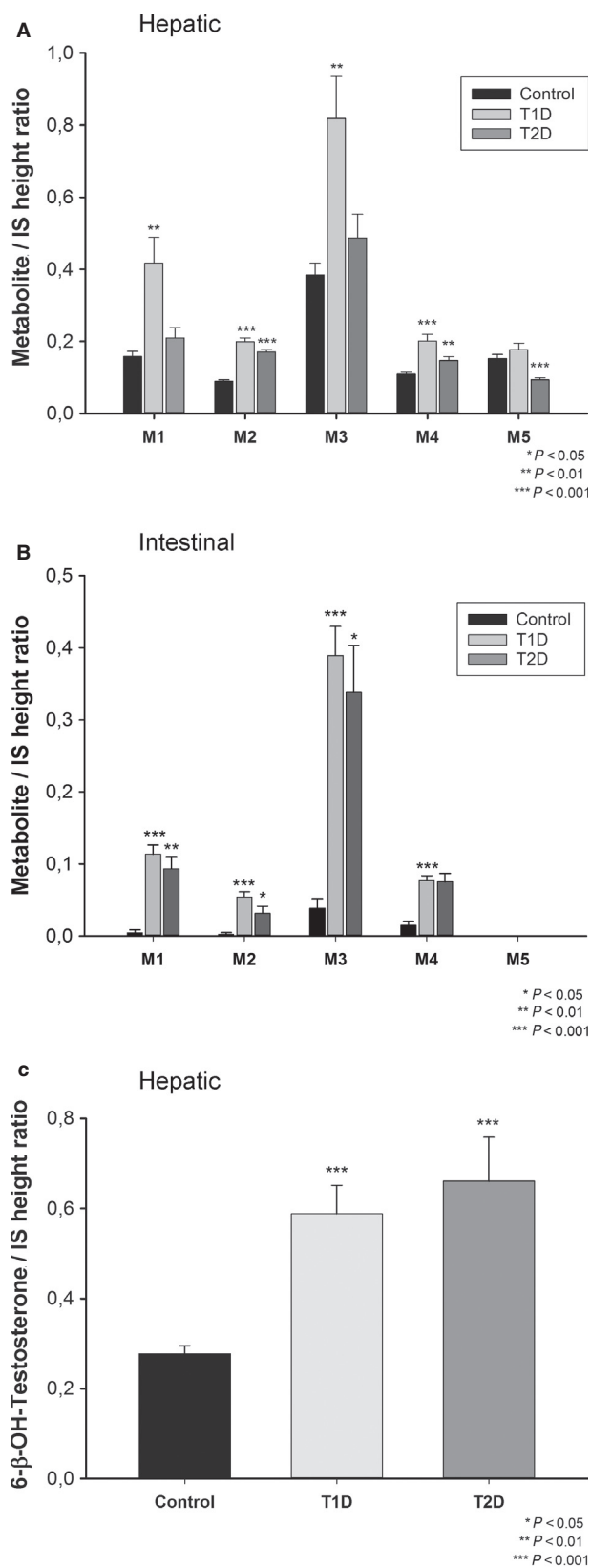


Figure 2. (A) Relative steady-state levels of CYP3a mRNAs in mouse livers. A significant increase of CYP3a11 and CYP3a13 was observed for both T1D and T2D groups when compared to the control group (3a11: 3.31 ± 0.24 , 1.99 ± 0.22 vs. 1.00 ± 0.10 ; $n = 17, 20, 18$ respectively) (3a13: 3.25 ± 0.10 , 3.19 ± 0.26 vs. 1.00 ± 0.05 ; $n = 17, 20, 17$ respectively), and for CYP3a44 in T1D group (1.68 ± 0.14 , 0.95 ± 0.10 vs. 1.00 ± 0.11 ; $n = 17, 20, 17$ respectively). No significant difference was observed for CYP3a25 (0.93 ± 0.05 , 0.89 ± 0.09 vs. 1.00 ± 0.11 ; $n = 17, 19$ respectively). (B) Relative steady-state levels of PXR and CAR mRNAs in mouse livers. A significant increase of PXR and CAR was observed in both T1D and T2D groups when compared to the control group (PXR: 1.67 ± 0.14 , 2.04 ± 0.20 vs. 1.00 ± 0.05 ; $n = 17, 19, 17$ respectively) (CAR: 3.43 ± 0.16 , 2.44 ± 0.24 vs. 1.00 ± 0.08 ; $n = 17, 15$ respectively). (C) Relative expression of CYP3a proteins in mouse livers. On the left, a significant increase was observed in both T1D and T2D groups when compared to the control group (1.35 ± 0.106 , 1.62 ± 0.11 vs. 1.00 ± 0.11 ; $n = 17, 18, 17$ respectively). On the right are the representative images of the ChemiBlot of CYP3a and the total proteins loaded for the three groups.

with the observed increase in CYP3a protein expression induced by T2D. Similar results were obtained by other groups. Indeed, increased CYP3a-mediated drug-metabolizing activity was also shown in rats with T2D induced by a combination of streptozotocin and high-fat diet (Chen et al. 2011), in Tsumura Suzuki Obese Diabetes (TSOD) mice with T2D and obesity (Kudo et al. 2009), and in C57BL/6J mice with T2D (Koide et al. 2011). Interestingly, mRNA of CAR, PXR, CYP3a11, and 3a13 were significantly increased while 3a25 and 3a44 were unchanged (Fig. 2A–B). Intestinal CYP3a activity was also increased (Fig. 1B) in *db/db* mice. In vitro studies have shown that hypertonicity of ambient osmotic environment in cultured intestinal human cells increases expression of CYP3A (Chuang and Ito 2010). Furthermore, after spontaneous ingestion of solid food with free access to water, rodent intestinal osmolality may reach 600–800 mOsm kg⁻¹, which is at least a twofold increase compared with the measured plasma osmolality of 300 mOsm kg⁻¹ (Osaka et al. 2001). Given that both mice models are characterized by robust increases in food intake, one due to classical T1D-associated hyperphagia and the other due to a *db/db* mutation that suppresses leptin receptor signaling, we speculate that this constant hyperosmolar stimulation may be in part responsible for the augmentation of CYP3a intestinal metabolism.

Contradictions exist in the literature regarding the effect of diabetes on P450 activity in humans and in animal models, potentially due to a number of factors including age, gender, disease duration and degree of diabetes control (Wang et al. 2007). The molecular mechanisms underlying these changes in drug biotransformation have not been fully characterized yet (Dostalek et al. 2011). Different laboratories have reported either decreased (Ma et al. 1989; Thummel and Schenkman 1990; Yamazoe et al. 1989) or increased (Barnett et al. 1990; Shimojo et al. 1993; Verrecchia and Guaitani 1993) CYP3A protein expression following STZ treatment. Cheng and Morgan (Cheng and Morgan 2001) proposed that these conflicting data on diabetic regulation of CYP3A proteins could be due to different specificities of the antibodies used for the Western blot assays, coupled with differential regulation of CYP3A family members. It has been shown that CYP3A4 expression and activity is significantly reduced in livers from diabetic donors as compared to nondiabetic counterparts (Dostalek et al. 2011), and that diabetes mellitus reduces clearance of both atorvastatin lactone (Dostalek et al. 2012) and cyclosporine (Akhlaghi et al. 2012), two typical CYP3A4 substrates in humans. Collectively, it is evident that marked species-related differences exist in the modulation of P450 3A activity in response to diabetes. Obviously, acting as an additional confusing effect, is the

fact that most people with diabetes end up being treated with oral hypoglycemic agents or insulin, which can modulate their pathophysiological state, along with ‘disease-modifying’ interventions such as diet and exercise (Cheng and Morgan 2001).

Overall, this study demonstrates that, although differences exist in the pathophysiological insults associated with T1D and T2D, these two distinct diseases may have similar effects on CYP3a regulation, ultimately leading to variability in endogenous substrates metabolism and drug response. Given the severity of the comorbidities associated with T1D and T2D, considerable medical and research resources have been allocated in an effort to minimize these debilitating complications. Yet, important and potentially life-threatening complications specifically resulting from drug-related toxicity or inefficacy in diabetic patients have passed under the radar. To fully elucidate the molecular mechanism(s) by which diabetes and other pathophysiological factors affect drug metabolism, further and complete characterization of inflammation mediators, nuclear receptors, adipokines, insulin signaling pathway, apoptosis, and liver metabolic dysregulation will be required.

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Disclosures

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Figure S1 shows the percentage of control activity of four of the five metabolites (M1–4) in pooled mouse liver microsomes using standard inhibitors such as ketoconazole (CYP3A), furafylline (CYP1A), sulfaphenazole (CYP2C), ciprofloxacin (CYP1A) and quinidine (CYP2D). Strong inhibition was observed only with ketoconazole.

Figure S2. Figure S2 shows the effect of ketoconazole on domperidone metabolism in pooled mouse liver microsomes. Strong inhibition of the formation of the five metabolites of domperidone was observed with ketoconazole 10 μ M. The inhibitory effect was also confirmed by a weaker biotransformation of domperidone.

Appendix: To further confirm the major involvement of CYP3A in domperidone metabolism, we performed inhibition study with our mice microsomes using ketoconazole, furafylline, sulfaphenazole, ciprofloxacin, and quinidine. Figure S1 demonstrates strong inhibition of domperidone metabolism only with ketoconazole. We also performed inhibition study and included a fifth metabolite of domperidone; M5. In Figure S2, we can see that the formation of M5 is also strongly inhibited by ketoconazole.