SHORT COMMUNICATION

Decreased CYP3A expression and activity in guinea pig models of diet-induced metabolic syndrome: Is fatty liver infiltration involved?


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Running title: Decreased CYP3A expression/activity in metabolic syndrome

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List of nonstandard abbreviations:
BMI: body mass index; CYP450: cytochrome P450; FFA: free fatty acid; HDL-C: high density lipoprotein cholesterol; HFHF: High Fat High Fructose diet; HFHS: High Fat High Sucrose diet; HPLC: high performance liquid chromatography; IL-1β: interleukin 1 beta; IL-6: interleukin 6; LDL-C: low density lipoprotein cholesterol; NAFLD: nonalcoholic fatty liver disease; PBS: phosphate-buffered saline; RT-qPCR: reverse transcription quantitative polymerase chain reaction; TNF-α: Tumor necrosis factor alpha
Abstract

**Background:** In humans, cytochrome P450 3A drug-metabolizing enzyme subfamily is the most important. Numerous pathophysiological factors, such as diabetes and obesity were shown to affect CYP3A activity. Often considered a precursor state for Type II diabetes, we hypothesized that metabolic syndrome exerts a modulating role on CYP3A. **Objective:** To evaluate the effect of metabolic syndrome on CYP3A drug-metabolizing activity/expression in guinea pigs.

**Methods:** Hepatic microsomes were prepared from male Hartley guinea pigs fed with a control, a High Fat High Sucrose (HFHS) or a High Fat High Fructose diet (HFHF). Domperidone was selected as a probe substrate of CYP3A and formation of four of its metabolites was evaluated using HPLC. CYP3A protein and mRNA expression were assessed by Western blot and RT-qPCR respectively. Hepatic fatty infiltration was evaluated using standard Oil Red-O staining. Triglyceride and free fatty acid liver content were also quantified. **Results:** Microsomal CYP3A activity was significantly decreased in both HFHS and HFHF diet groups VS the control diet group. Significant decreases of CYP3A mRNA and protein expression were observed in both HFHS and HFHF diet groups. Oil Red-O staining showed a massive liver fatty infiltration in the HFHS and HFHF diet groups, which was not observed in the control diet group. Both triglyceride and free fatty acid liver content were significantly increased in the HFHS and HFHF diet groups.

**Conclusion:** Diet-induced metabolic syndrome decreases CYP3A expression/activity in guinea pigs. This may ultimately lead to variability in drug response, ranging from lack of effect to life-threatening toxicity.
**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 marketed drugs (Danielson, 2002). There is a considerable variability in the expression and activity of CYP3A4 among the general population (10-100-fold), which cannot be solely explained by genetic factors (Elsens et al., 2011). There are evidences that diseases or pathological states may modulate human and animal CYP3A (Dostalek et al., 2011; Yoshinari et al., 2006). The variability in CYP3A4-associated drug metabolism is known as a major determinant of drug response and/or toxicity (Lamba et al., 2002). Currently, more than 30% of the US population has a body mass index (BMI) >30 kg/m², that puts those people in the obese category (Ogden et al., 2006). Ninety percent of obese patients present histologically proven fatty liver infiltration-related abnormalities (Moretto et al., 2003). Some pharmacokinetic studies performed in obese populations reported a significant decrease in the clearance of drugs, particularly when it is CYP3A-mediated (Abernethy et al., 1984; Caraco et al., 1995). Diabetes is a widespread chronic disease, which was affecting over 350 million people worldwide in 2011 (Whiting et al., 2011). Common comorbidities associated with diabetes are hyperlipidemia, cardiovascular disease, kidney disease, nonalcoholic fatty liver disease (NAFLD), and obesity. Diabetes is also known to decrease CYP3A activity and/or expression in humans and in animal models (Dostalek et al., 2011; Patoine et al., 2012). According to the National Cholesterol Education Program’s Adults Treatment Panel III (NCEP-ATP III), patients with metabolic syndrome must carry at least three components out of these: hyperglycemia, hypertension, hypertriglyceridemia, low high density lipoprotein cholesterol (HDL-C) and increased abdominal circumference and/or body mass index (BMI) >30 kg/m² (NCEP, 2002). Although a number of studies have described the impacts of
type II diabetes and obesity on CYP3A-mediated drug metabolism, much less is known about the
effect of the metabolic syndrome, frequently recognized as a precursor state of type II diabetes.
Considering the overlap on the criteria defining obesity, diabetes and metabolic syndrome, it is
reasonable to hypothesize that metabolic syndrome has an impact on CYP3A. NAFLD is a
metabolic disorder characterized by hepatic accumulation of free fatty acids (FFA) and
triglycerides (McCullough, 2002). It has become one of the most common liver disorders in
developed countries with a prevalence (USA) of approximately 5% in the general population and
up to 25% to 75% in patients with obesity and type II diabetes mellitus (McCullough, 2002). It is
also regarded as an hepatic manifestation of metabolic syndrome (Takahashi et al., 2012).
Recently, alterations of CYP450 were reported in human presenting NAFLD (Fisher et al., 2009).
In guinea pigs, 3 major CYP3A enzymes have been identified: CYP3A14, 3A15 and 3A17 (Mori
et al., 1997). The amino acid sequences of these enzymes show ~70% homology with human
CYP3A4. In the present study, guinea pig models have been used to elucidate the effect of
metabolic syndrome on the hepatic metabolism of domperidone, a specific substrate of CYP3As
in human liver microsomes (Simard et al., 2004), which however, has not been formally shown to
be specific to guinea pig CYP3A. Moreover, hepatic transcription and expression of CYP3A as
well as liver fatty infiltration were measured. Considering the widespread use of sucrose and
fructose (particularly in the form of high fructose corn syrup in the US), we decided to expose
our guinea pigs to the HFHS or the HFHF diet, to mimic metabolic syndrome and potentially
cause alterations of drug biotransformation. As opposed to other rodents, the guinea pig is an
excellent animal model for studying drug-induced QT prolongation (Bryant et al., 1998). This
animal is also useful to investigate the hypocholesterolemic effects of drugs since it carries the
majority of its cholesterol in the LDL form. It also develops atherosclerosis when challenged
with hypercholesterolemic diets (West and Fernandez, 2004). Of note, these pathological states also involve the use of a variety of CYP3A4-metabolized drugs.

**Material and Methods**

**Animals:** Guinea pig models of diet-induced metabolic syndrome were made as described previously (Caillier et al., 2012). 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:** Guinea pig liver microsomes were prepared by differential centrifugation as described previously (Inaba and Kovacs, 1989). The protein content was determined with the DC™ protein assay (Bio-Rad, Mississauga ON, Canada).

**Domperidone assay:** Standard incubations were made of 5 µl of domperidone solution (final concentration: 100 µM), 0.4 mg of microsomes and a NADPH-generating system in a final volume of 500 µl. Incubations were performed for 60 min at 37°C. The enzymatic process was stopped with 500 µl ice-cold acetonitrile and the internal standard (propranolol) was added. Following a 20-min centrifugation (16,000 g), 10 µl were injected at room temperature in a complete Shimadzu HPLC system (Columbia MD). An Ultrasphere ODS 5 µm column, 250 mm X 4.6 mm (Phenomenex, Torrance CA) and a µ-Bondapak C18 guard column (Waters, Milford MA) were also used. The mobile phase was composed of citrate-phosphate buffer (pH 3.5) and methanol (60:40) containing 0.4% (v/v) triethylamine (Sigma-Aldrich, St. Louis MO) and 0.3% (v/v) PIC® B-5 (Waters); final pH 3.0. Flow-rate was set at 1 ml/min, and the excitation/emission wavelengths were set at 282/328 nm.

**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<sup>™</sup> cDNA synthesis kit (Bio-Rad).

**RT-qPCR**: The level of gene expression in liver tissue was evaluated with the iQ SYBR green supermix (Bio-Rad). Gene-specific primers (Invitrogen) were designed with the VectorNTI software (Invitrogen) to amplify a highly conserved sequence in three CYP3A isoforms (3A14, 3A15 and 3A17) and two reference genes (calnexin and 18S). Relative quantification was obtained by an adaptation of the comparative threshold cycle (ΔΔCT) method which allows normalization with multiple reference genes (Vandesompele et al., 2002).

**Western Blot**: Samples of frozen liver were homogenized in an ice-cold lysis buffer (10 mM Tris-HCl [pH 7.4], 0.32 M sucrose, 1 mM EDTA, 1 mM 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-CYP3A polyclonal antibody (1:3000; Santa Cruz Biotechnology, Santa Cruz CA) and anti-α-tubulin monoclonal antibody (1:2000; Abcam Inc, Cambridge MA). The membrane was washed and then incubated with a secondary antibody conjugated with horseradish peroxidase. Densitometric analysis was done with the SuperSignal® West Dura Extended Duration Substrate (Thermo Scientific, Rockford IL). The expression of CYP3A protein was normalized against α-tubulin.

**Evaluation of liver fatty infiltration**: Frozen O.C.T.-embedded liver sections were stained using standard oil Red-O technique. Hepatic triglycerides were quantified using the GPO-PAP kit (Roche, Laval QC, Canada). Hepatic FFA were quantified using the Free Fatty Acid Quantification Kit from BioVision (San Francisco CA).
Data Analysis: One-way ANOVA followed by a Tukey test was used to determine the statistical differences in parameters between the control and the HFHF and HFHS diet groups. All the results were expressed as mean ± S.E.M. A multivariate analysis was performed to assess the relationships between the hepatic formation of CYP3A-related metabolites of domperidone and triglyceridemia, cholesterolemia, hepatic triglyceride content, hepatic FFA content, CYP3A protein and CYP3A mRNA expression. Spearman correlation coefficients were calculated using JMP software (Cary NC). 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 metabolic syndrome on CYP3A in guinea pigs. To this end, CYP3A activity (Fig. 1), steady-state levels of mRNAs (Fig. 2A) and protein expression (Fig. 2B) were measured. Reductions in transcription and expression of CYP3A were observed in both HFHS and HFHF diet groups compared to the control diet group. These results are consistent with the decrease in microsomal CYP3A activity observed with metabolism of domperidone. Oil Red-O staining showed a massive centrilobular liver fatty infiltration in the HFHS and HFHF diet groups, not observed in the control diet group (Fig. 3). Moreover, both triglyceride and FFA liver contents were significantly increased in the HFHS and HFHF diet groups (Fig. 4A and 4B). Yoshinari et al. reported decreases in hepatic CYP3a11 activity, transcription and expression in mice with high fat diet-induced obesity (Yoshinari et al., 2006). Our results are also similar to those of Ghose et al., who reported that high fat diet reduced CYP3a expression, transcription and activity in mouse (Ghose et al., 2011). It is known that pathophysiology of NAFLD involves insulin resistance (McCullough, 2002), which is related to inflammation more than obesity itself (Solinas et al., 2007). It has been previously demonstrated that increases of pro-inflammatory cytokines (IL-1β, TNF-α, IL-6) were associated
with reduced expression of drug-metabolizing enzymes (Aitken et al., 2006). Moreover, recent evidences have demonstrated mechanistic links between fatty acids, inflammation and hepatic insulin resistance, which is an important underlying feature of metabolic syndrome (Ghose et al., 2011). Indeed, hepatocytes exposed to FFAs showed fat overloading and lower activity, mRNA and protein level values of CYP3A4 enzymes, suggesting implication of fat in the phenomenon (Donato et al., 2006). Leclercq et al. (Leclercq et al., 1998) found a correlation between fat liver accumulation and decreased CYP3A in force-fed ducks. All these findings are consistent with the current results, since our models present a clear phenotype of NAFLD (massive fatty infiltration, high triglycerides and FFA liver content) associated with decrease of CYP3A activity, mRNA and protein expression. Interestingly, the sucrose versus fructose diet content did not impact the metabolic syndrome phenotype, as no significant difference was observed between HFHS and HFHF diet groups for CYP3A activity, mRNA, protein expression, triglyceride and FFA hepatic content.

The Spearman analyses (Table 1), showed good inverse correlations of triglyceride liver content, FFA, cholesterolemia and HDL-C with CYP3A activity, mRNA and protein expression. In contrast, triglyceridemia did not correlate with drug metabolism. More interesting is the strong inverse correlation between levels of blood LDL-C and the hepatic formation of CYP3A-related metabolites of domperidone. Despite the fact that the exact mechanism(s) involved is (are) unknown and even if this correlation derives from a direct association or an indirect consequence, it is suggesting that in this model, LDL-C is a good predictive marker of CYP3A activity. In conclusion, we have demonstrated that diet-induced metabolic syndrome in the guinea pig decreases CYP3A activity, mRNA and protein expression. Considering the narrow association between obesity, NAFLD, insulin resistance, metabolic syndrome, inflammation and diabetes,
further and complete characterization of inflammation mediators, nuclear receptors, adipokines, insulin signaling pathway, apoptosis and liver metabolic dysregulation will be required to fully elucidate the molecular mechanism(s) by which metabolic syndrome affects drug metabolism.
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Authorship Contributions

Participated in research design: Simard, Drolet

Conducted experiments: Patoine, Levac, Pilote

Performed data analysis: Patoine, Levac, Pilote

Wrote or contributed to the writing of the manuscript: Patoine, Levac, Pilote, Drolet, Simard
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Footnotes

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The first two authors contributed equally to this work.
Figure legend

Figure 1. Microsomal CYP3A activity: This figure shows peak-height ratios of metabolites of domperidone to internal standard for the three diet groups. Formation of the four metabolites was significantly decreased (p<0.05) in both HFHS (M1: 0.22±0.04; M2: 0.17±0.02; M3: 0.80±0.13; M4: 0.23±0.04) and HFHF (M1: 0.24±0.05; M2: 0.25±0.04; M3: 0.62±0.15; M4: 0.24±0.05) diet groups when compared to the control diet group (M1: 0.67±0.12; M2: 0.37±0.05; M3: 1.70±0.24; M4: 0.48±0.07). Metabolites of domperidone were not formally identified.

Figure 2. Panel A: Relative steady-state levels of CYP3A mRNAs in guinea pig livers. A significant decrease was observed for both HFHS and HFHF diet groups (27.6±12.0% and 11.7±6.7% of the control, p<0.01). Panel B: Relative expression of CYP3A proteins in guinea pig liver. A significant decrease was observed in both HFHS and HFHF diet groups when compared to the control group (45.6±8.2% and 58.8±8.9% of the control diet group, respectively, both p<0.01). Bottom insets show representative sample Western blots for the three diet groups.

Figure 3. Representative Oil Red-O staining on frozen liver sections. A. Control diet group, B. HFHS diet group, C. HFHF diet group. A massive centrilobular liver fatty infiltration was observed in the HFHS and HFHF diet groups, but not in the control diet group.

Figure 4. Panel A: Hepatic triglyceride content. A significant increase was observed in the HFHF diet group (2.59±0.80 µmole/mg) and in the HFHS diet group (2.21±0.32) vs the control diet group (0.42±0.02), both p<0.001. Panel B: Hepatic FFA content. A significant increase in FFA content was observed in the HFHF diet group (0.80±0.17 nmole/mg) and in the HFHS diet group (1.00±0.22) vs the control diet group (0.13±0.03), both p<0.01.
Table 1. Spearman correlations table. $\rho$ of Spearman (between -1 and 1) is a rank correlation coefficient. It is a non parametric measure of the strength of association between two variables. The sign of the Spearman correlation coefficient indicates the direction of association (direct or inverse) between each variable; 1 or -1 indicating a perfect correlation.
Figure 1

Metabolite / IS ratio (Height)

- **M1**
  - Control
  - HFHS
  - HFHF

- **M2**
  - Control
  - HFHS
  - HFHF

- **M3**
  - Control
  - HFHS
  - HFHF

- **M4**
  - Control
  - HFHS
  - HFHF

* p < 0.05
** p < 0.01
Figure 2

(A) Relative steady-state levels of mRNAs (%)

(B) Relative protein expression

** p < 0.01

Control HFHS HFHF

CYP3A α-Tubulin

** p < 0.01
Figure 4

(A) Triglyceride liver content (μmol/mg)

- Control
- HFHS
- HFHF

(B) Free fatty acid (nmol/mg liver)

- Control
- HFHS
- HFHF

** p < 0.001