SHORT COMMUNICATION

Calorie Restriction Increases P-glycoprotein and Decreases Intestinal Absorption of Digoxin in Mice

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Running Title: Calorie restriction decreases digoxin absorption

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List of Abbreviations:
Abcb1: ATP-Binding Cassette, Sub-Family B, Member 1; BSA: Bovine Serum Albumin; CR: Calorie Restriction; CYP: Cytochrome P450; MDR1: Multidrug Resistance Protein 1; P-gp: P-glycoprotein, OATP: Organic Anion-Transporting Polypeptide; PXR: Pregnane-X Receptor.
Abstract

There is wide variation in how patients respond to therapeutics. Factors that contribute to pharmacokinetic variation include disease, genetics, drugs, age, and diet. The purpose of this study was to determine the effect of calorie restriction on the expression of Abcb1a in the intestine and whether calorie restriction can alter the absorption of an Abcb1a substrate, digoxin, in mice. Ten-week old C57BL/6 mice were given either an ad libitum diet or a 25% calorie restricted diet for 3 weeks. To determine digoxin absorption, mice were administered [3H]-labeled digoxin by oral gavage. Blood and intestine with contents were collected at 1, 2, 4, and 12 hrs after digoxin administration. Concentrations of [3H]-digoxin in plasma and tissues were determined by liquid scintillation. Calorie restriction decreased plasma digoxin concentrations (about 60%) at 1, 2, and 4 hrs after administration. Additionally, digoxin concentrations in the small intestine of calorie restricted mice were elevated at 4 and 12 hrs after administration. Furthermore, calorie restriction increased Abcb1a transcripts in the duodenum (4.5 fold) and jejunum (12.5 fold). To confirm a role for Abcb1a in calorie restricted-induced altered digoxin pharmacokinetics, the experiment was repeated in Abcb1a/b-null mice 4 hrs after drug administration. No difference in intestine or plasma digoxin concentrations were observed between ad libitum fed and calorie restricted Abcb1a/b-null mice. Thus, these findings support the hypothesis that calorie restriction increases intestinal Abcb1a expression leading to decreased absorption of digoxin in mice. Because Abcb1a transports a wide variety of therapeutics, these results may be of important clinical significance.
Introduction

The absorption of many therapeutics can be affected by the expression and activity of drug transporters in the intestine. The first drug transporter described was P-glycoprotein (P-gp, also referred to as MDR1 or ABCB1) (Juliano and Ling, 1976; Ueda et al., 1986) and is encoded by the Abcb1a and Abcb1b genes in mice (Schinkel et al., 1994). P-gp is an ATP-dependent efflux pump that actively transports xenobiotics from cells (Gottesman and Pastan, 1993). P-gp transports a wide range of structurally different therapeutics including colchicine, tacrolimus, quinidine, chemotherapeutic drugs (such as etoposide, doxorubicin, and vinblastine), cardiac glycosides (such as digoxin), glucocorticoids (such as dexamethasone), and HIV-type 1 antiretroviral therapy drugs (Aller et al., 2009). Overexpression of Abcb1a and -1b can confer drug resistance by increasing efflux of these drugs from cells (Dhir et al., 1990; Raymond et al., 1990) and is a well-established cause of resistance to cancer chemotherapeutic drugs. Abcb1a is mainly expressed in gastrointestinal tract, testis, and capillaries within the brain (Cui et al., 2009). In humans, the ABCB1 gene encodes P-gp, which has a similar tissue distribution pattern, as mice (Cordon-Cardo et al., 1989; Thiebaut et al., 1987; Thiebaut et al., 1989). Thus, it is thought that P-gp functions to protect the body, limiting xenobiotic intestinal absorption and limiting the distribution to the central nervous system and germ cells. In enterocytes, P-gp is located on the apical surface where it transports xenobiotics back into the intestinal lumen, resulting in limited xenobiotic absorption (Croop et al., 1989; Takano et al., 2006). Because P-gp is known to transport a variety of therapeutics, altering its intestinal expression or activity may alter the bioavailability of a large range of therapeutics.

There is wide variation in the response of humans to therapeutics, both beneficially and adversely. A factor that is often proposed to contribute to this variation is diet (Boullata
and Hudson, 2012; Otles and Senturk, 2014; Won et al., 2010). Until recently, knowledge regarding food-drug interactions was primarily based on anecdotal accounts. But recent studies have now proven examples of foods that can alter the pharmacokinetics of drugs. For example, grapefruit juice inhibits Cytochrome P450 3A4 (CYP3A4), which results in increased bioavailability of drugs such as saquinavir, cyclosporine, and felodipine (Seden et al., 2010). Phytochemicals in grapefruit juice, such as bergamottin and quercetin, and green tea catechins inhibit the cellular efflux of P-gp substrates in vitro (Zhou et al., 2004). Furthermore, the amount of protein in the diet can influence drug metabolism and glomerular filtration. For example, in human volunteers, a protein-restricted diet resulted in a 70% decrease in the clearance of oxipurinol and uric acid (Berlinger et al., 1985).

There is a lack of knowledge regarding the effect of decreased calorie consumption on the pharmacokinetics of drugs. However we previously reported that mice on a calorie restricted diet, had a profound effects on gene expression in liver (Renaud et al., 2014). More specifically, we found that calorie restriction substantially increased hepatic Abcb1a transcripts. These results raised the question of whether calorie restriction alters the expression of Abcb1a in the intestine, where this transporter is known to have a major impact on xenobiotic absorption. In this report, we reveal that calorie restriction causes decreased absorption of digoxin – likely through a mechanism of calorie restriction-induced expression of P-gp. These results support the hypothesis that diet is an important regulator of drug absorption.
Materials and Methods

Chemicals

Digoxin (0.25 mg/ml) was purchased from Westward Pharm Corp (Eatontown, NJ), RNA-Bee RNA Isolation Reagent was from Tel-Test Inc. (Friendswood, TX), the High Capacity Reverse Transcriptase kit and SYBR® green were from Applied Biosystems (Foster City, CA), [3H]-digoxin and Ultima Gold counting fluid were from PerkinElmer (Shelton, CT), and Bovine Serum Albumin (BSA) was purchased from Amresco (Solon, OH). Unless otherwise stated, all other chemicals were purchased from Sigma-Aldrich.

Animals

C57BL/6 male mice, eight weeks of age, were purchased from Charles River Laboratories, Inc. (Wilmington, MA). Abcb1a/b-null mice were purchased from Taconic Inc. (Hudson, NY) and were back-crossed into the C57BL/6 background (>99% congenicity). Mice were bred and housed in a light-, temperature-, and humidity-controlled environment in an Association for Assessment and Accreditation of Laboratory Animal Care accredited animal housing facility at the University of Kansas Medical Center. All studies were approved by the University of Kansas Medical Center’s Institutional Animal Care and Use Committee.

Calorie Restriction

After 2 weeks acclimatization, mice were housed individually and given Laboratory Rodent Chow 8604 (Harlan, Madison, WI) either ad libitum, or 75% of the feed consumed by ad libitum feeding (calorie restriction). The average ad libitum daily consumption of feed was 4 g per mouse (determined using the average daily intake from 10 mice). Thus, mice in the calorie restriction group were given approximately 2.7–3.0 g of feed per day. Mice remained
on these diets for 3 weeks, after which they were either used for RNA studies or digoxin absorption studies.

**Tissue Collection for RNA Studies**

In the morning (8:00–10:00 A.M.) mice were euthanized with pentobarbital and their small intestines were collected. Fecal matter was flushed from the small intestines using a saline solution. Small intestines were divided into 3 equal parts – duodenum, jejunum, and ileum then immediately frozen in liquid nitrogen and stored at -80°C until further analysis.

**RNA Extraction**

RNA was isolated from frozen intestine using RNA Isolation Reagent RNA-Bee following the manufacturer’s protocol. Using a NanoDrop1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE), RNA concentrations were quantified at a wavelength of 260nm.

**Messenger RNA Quantification (RT-qPCR)**

To perform real-time PCR, we first reverse transcribed RNA to cDNA using an Applied Biosystems High Capacity Reverse Transcriptase kit. In brief, equal volumes of 2X reverse transcriptase, 50 ng/µl RNA, and random primers were mixed and placed in a Mastercycler (Eppendorf, Hauppauge, NY) under the following conditions: 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. With the resulting cDNA, quantitative PCR (qPCR) was performed as detailed below. Primers for qPCR of Abcb1a (GenBank Accession No. NM_011076) were designed using Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast) (Forward – GCGACTCCGATACATGGTTT; Reverse – ACCCTGTAGCCCCTTTCACT) and were synthesized by Integrated DNA Technologies (Coralville, IA). For qPCR the following was
contained per reaction in a 384-well plate (Applied Biosystems): 2.5 µl of 3 µM forward and reverse primer mix, 5 µl of Applied Biosystems SYBR® green PCR master mix, 0.5 µl RNase-free H₂O, and 2 µl of 2 ng/µl cDNA. Fluorescence was quantified with an Applied Biosystems 7300 Real Time PCR System (Foster City, CA) using the following conditions: 50°C for 2 min, 95°C for 10 min, (95°C for 15 sec, 60°C for 1 min) X 40 cycles. To ensure primer specificity, melt curves were performed for every reaction. To determine the relative mRNA expression, the comparative ΔΔCt method was applied using 18S as a reference transcript. Values from calorie restricted mice were normalized to values from mice fed ad libitum.

**Digoxin absorption experiments**

The protocol followed for these experiments was adapted from an earlier study (Mayer et al., 1996). Each mouse was given 0.2 mg/kg digoxin labeled with [³H]-digoxin (1 µCi/30g body weight) by oral gavage. Wild-type mice were euthanized 1, 2, 4, or 12 hrs after digoxin administration, and Abcb1a/b-null mice were euthanized 4 hrs after digoxin administration. Blood was collected by orbital bleeding into heparinized tubes and centrifuged for 10 min at 2000 xg to isolate plasma. Plasma (100 µl) was transferred to 4 ml of Ultima Gold high counting efficiency scintillation cocktail. The entire intestine from stomach to rectum (including fecal matter) was also collected, weighed, and homogenized in 4% (w/v) BSA. Thus, all intestine samples include the fecal matter. From here on in we will refer to the intestine + contents samples as “intestine”. Two-hundred µl of intestine homogenate was added to 4 ml Ultima Gold high counting efficiency scintillation cocktail. Radioactivity was quantified by liquid scintillation counting using a Packard Tri-Carb 2100TR Liquid Scintillation Analyzer (Packard Instrument Company, Meriden, CT). Concentrations of digoxin were calculated from a standard curve.
Statistics

Statistical differences between *ad libitum* and calorie restriction were determined using an unpaired, two-tailed Student’s *t*-test. For the RNA expression data (Figure 1), the individual values were log-transformed to obtain normal distribution before performing the *t*-test. Comparisons resulting with *p*<0.05 were considered statistically significant. All data are presented as means ± SEM.

Results

**Calorie restriction increases Abcb1a mRNA expression in the small intestine.**

The effect of calorie restriction on mRNA expression of Abcb1a in small intestines of mice was assessed by RT-qPCR (Fig. 1). Calorie restriction significantly (*p*<0.05) increased expression of Abcb1a mRNA in the duodenum (4.5 fold) and jejunum (12.5 fold) compared to mice fed the *ad libitum* diet (control). Expression of Abcb1a was also increased in the ileum; however, it was not statistically different from controls.

**Calorie restriction alters absorption of digoxin in mice.**

To determine whether calorie restriction alters absorption of drugs transported by Abcb1a in the intestine, mice were given [³H]-labeled digoxin and euthanized 1, 2, 4, and 12 hrs after administration. Time courses of intestine-digoxin and plasma-digoxin concentrations are presented Fig. 2. The intestine of calorie restricted mice contained 1.5-fold more digoxin at 4 and 12 hrs than the intestine of *ad libitum* fed mice. In contrast to the intestine, the plasma concentrations of digoxin were 61%, 69% and 57% lower in calorie restricted than in control mice at 1, 2, and 4 hrs, respectively.
**Calorie restriction does not alter digoxin absorption in Abcb1a/b-null mice.**

In mice fed *ad libitum*, compared to wild-type mice, Abcb1a/b-null mice had lower digoxin concentrations in their intestines (974 vs 265 ng digoxin/gram intestine) and higher digoxin concentrations in plasma (0.035 vs 0.100 mean ng digoxin/μl plasma) 4 hrs after digoxin administration (Figure 2 and 3). This result was as predicted because Abcb1a-null mice do not have the capacity to efflux digoxin via P-gp, thus resulting in increased digoxin absorption.

No statistical differences in the concentrations of digoxin in intestine or plasma were observed between *ad libitum* fed and calorie restricted Abcb1a/b-null mice (Fig. 3). Thus, these results support the findings that decreased digoxin absorption in calorie restricted wild-type mice is likely due to increased intestinal P-gp expression.

**Discussion**

In clinical practice, a challenging problem in of drug therapy is individual variations in patient response. Environmental, genetic, and pathophysiologic factors are known contributors to variation in drug responses and adverse effects; however, little attention has been given to the impact of food and diet on drug disposition. Thus, the results in this report help further our knowledge of how diet impacts drug absorption. Specifically, the current work revealed that calorie restriction can increase the expression of intestinal *Abcb1a*, and decrease drug absorption. Because Abcb1a transports a wide variety of therapeutics, these results in mice beg the question of whether this phenomenon might also occur in humans.
The gastrointestinal tract functions to digest and absorb nutrients from the diet. However, this organ is also exposed to ingested xenobiotics, and thus also functions as a defense barrier, expressing many metabolic enzymes and efflux transporters. Modulation of efflux transporter expression or function in the intestinal tract can lead to altered systemic and local xenobiotic concentrations (Huang et al., 2010; Murakami and Takano, 2008). The best characterized efflux transporter is P-gp. It is located apically on enterocytes and transports substrates back into the intestinal lumen, resulting in lowered systemic drug concentrations. Increased intestinal P-gp function or expression can markedly affect drug pharmacokinetics leading to decreased therapeutic effect. In humans, intestinal \textit{MDR1} mRNA is inversely correlated with oral tacrolimus concentrations (Masuda et al., 2004), cyclosporine pharmacokinetics (Masuda and Inui, 2006), and talinolol pharmacokinetics (Bernsdorf et al., 2006). Additionally, xenobiotics that alter the function of P-gp can cause drug-drug interactions; for example, St. John's Wort induces intestinal P-gp leading to decreased talinolol AUC in human subjects (Schwarz et al., 2007). There are many known therapeutics that inhibit P-gp leading to drug-drug interactions including cyclosporine A, ketoconazole, quinidine, ritonavir, verapamil, and reserpine, to name a few (http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DevelopmentResources/DrugInteractionsLabeling/ucm093664.htm). However, little is known about how nutrients in the diet or amount of calories consumed affect P-gp activity or expression.

Factors in the diet currently suspected to affect P-gp activity include certain flavonoids. \textit{In vitro} studies have shown that the flavonoids morin, biochanin A, silymarin, and phloretin all have the ability to increase intracellular daunomycin (a P-gp substrate) concentrations in human P-gp positive cells, but not in P-gp negative cells. Furthermore, the increase in daunomycin accumulation by these flavonoids is dependent on both flavonoid concentration
and P-gp expression, suggesting that these flavonoids inhibit P-gp activity (Zhang and Morris, 2003). Thus one would anticipate that these flavonoids would increase the absorption of drugs, however, in vivo, biochanin A failed to alter the pharmacokinetics of orally administered P-gp substrates (Zhang et al., 2010). The authors conclude that the disconnect between the in vitro and in vivo data may be due to poor bioavailability and rapid clearance of biochanin A in vivo. It remains to be determined whether flavonoids have the ability to inhibit P-gp in vivo.

Grapefruit juice and orange juice are known to inhibit CYP3A4 and also suspected to alter P-gp activity (Reviewed in Won, et al., 2010). Grapefruit juice is well known to increase plasma concentrations of drugs by decreasing CYP3A4 and possibly also by inhibiting P-gp and organic anion-transporting polypeptide (OATP) activity. Thus, considering that many P-gp substrates are also CYP3A substrates, it is difficult to distinguish the contribution of each factor to grapefruit juice-induced altered drug pharmacokinetics.

The molecular mechanism of how calorie restriction causes an induction of P-gp remains unknown at the present time. In vitro studies using cells derived from intestinal tissues have indicated the pregnane-X receptor (PXR) as a key player in drug-induced P-gp expression (Kim et al., 2015; Maier et al., 2007). For example, rifampin induces P-gp via casein kinase 2-mediated phosphorylation of heat shock protein 90β, and subsequent stabilization of PXR (Kim, et al., 2015). Thus, increased activation of PXR might be a good candidate to investigate as a possible mechanism of calorie restriction-induced P-gp expression.

The present study used the P-gp substrate digoxin as an indicator of P-gp activity. This drug is commonly used to evaluate P-gp function in mice because the pharmacokinetic attributes of digoxin are highly P-gp-dependent. Additionally, another advantage of using
digoxin is that it is not significantly metabolized in mice (Kawahara et al., 1999; Mayer et al., 1997; Schinkel et al., 1997; Schinkel et al., 1995). Although it is possible that digoxin is transported by other transporters (Taub et al., 2011), our observation that calorie restricted Abcb1a/b-null mice did not have decreased plasma concentration or increase the digoxin content of intestine compared to ad libitum Abcb1a/b-null mice. This suggests that the altered digoxin concentrations observed in calorie restricted wild-type mice is most likely due to altered P-gp expression. Additionally, digoxin is almost exclusively excreted by the gut mucosa (Mayer, et al., 1996), thus it is not likely that the decrease in plasma digoxin concentrations is due to altered P-gp expression in kidney or liver.

In rats, protein-calorie malnutrition suppresses the hepatic expression of P-gp causing reduced canaliculir excretion of the P-gp substrate daunomycin (Lee et al., 2003). These results are opposite of what we observed in intestinal tissue (present study) and hepatic tissue (Renaud, et al., 2014). However, the two studies are difficult to compare due to species and diet differences. The protein-calorie restricted diet in the study by Lee et al. (2003) was iso-caloric to the control diet, but contained only 20% of the protein (qualitative malnutrition). However, in the present study and our previous study (Renaud, et al., 2014), the calorie restricted mice received 25% less of the entire diet compared to the amount consumed by mice fed ad libitum (quantitative malnutrition). Nevertheless, it is evident that the quantity and the composition of the diet are certainly capable of modulating P-gp expression in both rats and mice.

A calorie restricted diet can arise out of choice (weight loss strategy), or from complications of an illness or drug therapy. Appetite loss leading to a calorie restricted diet often occurs in cancer and AIDS patients, which is particularly relevant to this study because P-gp transports many chemotherapeutics as well as HIV-antiviral therapies. Thus, the results
from this study showing that calorie restriction can alter the absorption of a P-gp substrate have implications for pharmacokinetic research and clinical practice.

In summary, this paper indicates that a calorie restricted diet can affect the absorption of P-gp substrates in mice is probably due to induced expression of intestinal P-pg. This study further underscores the importance of evaluating the influence of diet on drug disposition. Determining how the diet can influence drug pharmacokinetics is prudent to improving our understanding of inter-individual differences in response to therapeutic agents.

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**Authorship Contribution**

Participated in research design: Renaud, Klaassen, Csanaky

Conducted experiments: Renaud, Csanaky

Performed data analysis: Renaud, Csanaky

Wrote or contributed to the writing of the manuscript: Renaud, Klaassen, Csanaky
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Cellular localization of the multidrug-resistance gene product p-glycoprotein in normal human


Footnotes

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Legends for Figures

Figure 1. Mean mRNA of Abcb1a (± SEM) in mice on an *ad libitum* diet or calorie restriction (CR). Asterisks (*) denote data values statistically significant from control (*ad libitum*) (p< 0.05 as determined by a student’s t-test, n=3).

Figure 2. Mean concentrations of digoxin (± SEM) in intestine and plasma over time in mice fed an *ad libitum* diet or calorie restriction. Asterisks (*) denote data values statistically significant from the same time point *ad libitum* control (p< 0.05 as determined by student’s t-test, n=4 or 5).

Figure 3. Mean concentrations of digoxin (± SEM) 4 hours after administration in intestine and plasma of Abcb1a/b-null mice fed an *ad libitum* diet or calorie restriction (CR) (n=5).
FIGURE 1

Abcb1a

mRNA (relative to Ad Libitum)

\[ * \]

Ad Libitum
CR

Duodenum Jejunum Ileum
FIGURE 3

Abcb1a/b-null Intestine

ng digoxin/g intestine

Abcb1a/b-null Plasma

ng digoxin/μl plasma

Ad Libitum  CR