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Effect of nine diets on mRNAs of phase-II conjugation enzymes in livers of mice

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Abstract

1. Phase-II enzymes are important in metabolizing many xenobiotics including prescription drugs and chemical carcinogens. Whereas it is known that diet can alter the expression of phase-II conjugation enzymes, the previous studies are limited in using only two or three diets and examining only a few enzymes.
2. Adult male C57BL6 mice were fed one of 9 diets for 3 weeks. Of the 87 genes encoding major hepatic phase-II enzymes, approximately one-half (43) were altered by at least one diet. Diet restriction altered the hepatic expression of the most genes encoding phase-II enzymes (27), followed by lab chow (15), atherogenic diet (13), high-fat diet (10), western diet (7), high-fructose diet (5), and essential fatty acid-deficient diet (3), whereas the low n-3 fatty acid diet had no effect on the hepatic expression of these phase-II enzymes.
3. This comprehensive study provides detailed information on which conjugation enzymes are changed by these diets, and these data can be used to further investigate the mechanism for these changes in messenger RNAs, and whether these changes result in alterations in enzyme activity and drug action.

Keywords

Diet; phase-II conjugation enzyme; mRNA expression; microarray

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Introduction

Xenobiotics with functional groups or those metabolized by phase-I enzymes to obtain a functional group such as a hydroxyl (-OH), amino (NH₂), carboxyl (-COOH), and sulfhydryl (-SH) groups are often conjugated with glutathione (GSH), sulfate, glycine, or glucuronic acid by phase-II conjugation enzymes. Major phase-II drug metabolizing enzymes including uridine diphosphate-glucuronosyltransferases (Ugt), sulfotransferases (Sult), glutathione *S*-transferases (Gst), *N*-acetyltransferases (Nat), and amino-acid conjugation enzymes play important roles in making xenobiotics more water soluble to enhance their excretion. The addition of these groups by the phase-II enzymes usually decreases the pharmacological activity and toxicity of the chemical, and increases the hydrophilicity of the compounds, which enables the metabolites to be actively transported out of cells and be excreted into urine and bile (Jancova *et al.*, 2010). In general, phase-II conjugation reactions proceed more rapidly than phase-I reactions (mainly catalyzed by cytochrome P450 enzymes). It is reported that 40–70% of drugs are metabolized by phase-II enzymes (Xu *et al.*, 2005).

Phase-II drug metabolizing enzymes consist of families and subfamilies. These enzymes have different substrate specificity and expression patterns in various organs (Lu *et al.*, 2013), and some are known to be induced and inhibited by xenobiotics. The promoter regions of numerous genes encoding phase-II enzymes have *cis*-acting regulatory elements, including the antioxidant response element/electrophile response element and xenobiotic-responsive element binding sites in their 5'-flanking regulatory region, which bind to transcription factors such as aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), nuclear factor (erythroid-derived 2)-like 2 (Nrf2), pregnane X receptor (PXR), and vitamin D receptor (VDR) (Wen *et al.*, 2013; Xu *et al.*, 2005).

Many dietary chemicals have been reported to affect phase-II enzymes (Jancova *et al.*, 2010). For example, grapefruit juice, orange juice, green tea, black tea, and oolong tea inhibit recombinant SULT1A1 and 1A3; the polyphenol epigallocatechin gallate in green tea is an inhibitor of catechol-*O*-methyl transferase (Comt); the genistein in soybean products induce SULT1A1 and SULT2A1 mRNA and protein expression in HepG2 and Caco-2 cells; caffeic acid, esculetin, quercetin, kaemferol, and genistein inhibit NAT1 activity; scopuletin and coumarin inhibit NAT2 activity; major components of garlic (diallyl sulfide and diallyl disulfide) alter NAT activity in human colon tumor cells and human promyelocytic leukemia cells (Jancova *et al.*, 2010); and many naturally occurring flavonoids, benzo[a]pyrene, coumarin, α -angelicalactone, ellagic acid, and ferulic acid induce phase-II enzymes (Felton and Malfatti, 2006). In addition, a few different diets fed to laboratory animals have been reported to alter the expression of these phase-II genes (Bouchard-Mercier *et al.*, 2013; Fu and Klaassen, 2014; Ghose *et al.*, 2011; Xu *et al.*, 2012). However, these studies used only two or three diets and different animal models, and thus it is difficult to compare the effects of diets on various phase-II drug metabolizing enzymes. Therefore, in the current study, 9 experimental diets, which are often used for laboratory animal studies, were given to mice for 3 weeks, the livers were removed and mRNA of major phase-II enzymes in the C57BL/6 mice were compared after microarray analysis of the mRNA.

Materials and Methods

Animals

Male C57BL/6 mice (22±2 g, 8-weeks old, n=5 in each group) were obtained from Charles River Laboratories, Inc. (Wilmington, MA). Animals were housed in a temperature-, light-, and humidity-controlled environment. Lab chow (Teklad #8604; 14% calories from fat), essential fatty acid (EFA) deficient diet (#84224), high-fat diet (#97070; 59.9% calories from fat), western diet (#88137, 42% calories from fat, and 0.2% cholesterol), atherogenic rodent diet (#02028, 42.6% calories from fat; cholesterol 1.3%, 0.5% cholic acid), high-fructose diet (#89247 60% fructose), AIN-93M purified diet (#94048; 10.2% calories from fat), and a low n-3 FA diet (#00235 + 7% sunflower oil) (Levant *et al.*, 2006) were all purchased from Harlan Laboratories (Madison, WI), and diet restriction was performed by providing 75% of the #8604 diet consumed by *ad libitum* feeding (Varady *et al.*, 2007) were given to mice for 3 weeks, and water *ad lib*. The *ad libitum* daily feed intake was approximately 4 g/mouse. Therefore, for diet restriction, each mouse was given approximately 2.7–3.0 g of food per day.

The general characteristics of each diet were described previously (Renaud *et al.*, 2014). All mice were euthanized in the morning (8:00–10:00 A.M.) when liver samples were collected. Mice were not fasted before liver sample isolation. Livers were collected and frozen in liquid nitrogen, and stored at –80°C before use. These mouse livers were previously used to examine the influence of diets on expression of genes involved in lipid metabolism, oxidative stress, and inflammation (Renaud *et al.*, 2014), as well as transporters (Guo *et al.*, 2015a) and phase-I drug metabolizing enzymes (Guo *et al.*, 2015b). The housing facility was an American Animal Association Laboratory Animal Care-accredited facility at the University of Kansas Medical Center, and all procedures were approved by the Institutional Animal Care and Use Committee.

Total RNA isolation

Total liver RNA was isolated using RNeasy lysis reagent (Qiagen, Crawfordsville, IN) per the manufacturer's protocol, and concentrations were quantified with a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE) at 260 nm. Formaldehyde-agarose gel electrophoresis was used to evaluate the integrity of these total RNA samples, which were confirmed by visualization of the 18s and 28s rRNA bands.

Microarray and data analysis

Gene expression in livers of mice fed the various diets was determined using Affymetrix Mouse 430.20 arrays at the KUMC Microarray Core Facility. The cRNAs of three individual mice fed each diet were separately hybridized to an individual array. Raw data CEL files were imported into the 'R' program using the "Affy" package, normalized by the Robust Multichip Averaging (GCRMA) package, with the output data being log₂ transformed. The probes with intensities higher than log₂100 in at least one group were selected for further analysis. Gene annotations were obtained using GeneSpring (Agilent Technologies, Santa Clara, CA), and gene symbols were obtained from the mouse 4302 package (Wu *et al.*, 2012). The "pheatmap" package was used to show the average values of three replicates per

diet by colored squares. Accession number of the microarray data is GSE51885 (Gene Expression Omnibus database).

Statistical analysis

A standard AIN-93M purified diet was used as the control to keep the results comparable among different studies. Differential expression of microarray data was determined using the limma package in 'R' program (compared to data from mice fed AIN purified diet, $P < 0.05$). All values are expressed as mean $\pm$ S.E.M. Hierarchical clustering of mRNAs of the phase-II enzymes in livers of mice that exhibited changes in at least 1 diet was compared to the AIN-93M purified diet. High mRNA abundance on the heat map is represented in red, whereas low mRNA abundance is in blue.

Results

Global expression of phase-II enzymes in livers of male C57BL/6J mice fed 9 diets

Genes of importance in phase-II drug metabolizing enzymes include uridine diphosphate-glucuronosyltransferases (Ugt), sulfotransferases (Sult), glutathione *S*-transferases (Gst), catechol-*O*-methyl transferases (Comt), *N*-acetyltransferases (Nat), arsenic (+3 oxidation state) methyltransferases (As3mt), nicotinamide *N*-methyltransferases (Nnmt), thiopurine *S*-methyltransferases (Tpmt), bile acid-CoA ligases (Bal), and bile acid-CoA:amino acid *N*-acyltransferases (Baat). In addition, we also quantified the mRNA of the enzymes important for the synthesis of the co-substrates required for the conjugation reactions, which include glycine-*N*-acyltransferase (Glyat), γ -glutamyltransferase (Ggt), cysteine conjugate- β -lyase (Ccb1), UDP glucose 6-dehydrogenase (Ugdh), UDP-glucose pyrophosphorylase (Ugp), 3'-phosphoadenosine 5'-phosphosulfate synthase (Papss), glutamate-cysteine ligase (Gcl) catalytic subunit (Gclc) and the modifier subunit (Gclm), and methionine adenosyltransferase (Mat). The expression of 43 major phase-II enzyme genes were changed by at least one diet in livers of the mice, whereas the other 44 genes had no significant change.

Effect of 9 various diets on the Ugt mRNAs in livers of mice

Seven of the 21 mRNAs of the Ugt enzymes, including Ugt2b and 3a subfamilies were altered in livers of the mice fed the 9 diets (Fig. 1A). Ugt2b1 mRNA decreased in livers of mice fed the restricted diet (58.8%) and the atherogenic diet (30.3%), whereas the mRNAs of other members of the Ugt2b subfamily, including Ugt2b5, 2b34, 2b35 and 2b36 were increased by 32.5%, 39.8%, 80.4% and 27.5% in livers of mice fed the lab chow. Ugt3a1 mRNA decreased 54.8% in livers of mice fed the restricted diet, whereas it increased 57.6% in livers of mice on the high-fat diet. The mRNA of Ugt3a2 in livers exhibited a small increase in mice fed the high-fat diet (35.5%) and western diet (44.4%). None of the 9 diets altered hepatic mRNAs of 14 Ugt enzymes including Ugt1a1, 1a2, 1a5, 1a6a, 1a6b, 1a7c, 1a9, 1a10, 2a1, 2a2, 2a3, 2b37, 2b38 and 8a (data not shown).

Effect of 9 various diets on the Sult mRNAs in livers of mice

The mRNAs of the Sult superfamily in livers of male C57BL/6J mice changed markedly by feeding the various diets (Fig. 1B). Sult1a1 mRNA increased (121.4%) in mice on diet

restriction, but decreased (39.1%) in mice fed the atherogenic diet. Sult1b1 mRNA also increased (71.5%) in livers of mice fed the restricted diet but decreased in livers of mice fed the high-fructose diet (45.5%), atherogenic diet (54.6%) and EFA-deficient diet (42.9%). Sult1d1, Sult1e1 and Sult3a1 only changed in livers of mice on diet restriction. More specifically, Sult1d1 mRNA increased (160.2%), whereas the mRNAs of Sult1e1 and Sult3a1 dramatically increased 145.6 and 74.9 fold in livers of the diet-restricted mice. Sult5a1 mRNA decreased 78.1% in livers of mice fed the atherogenic diet. The mRNAs of Sult1c1, 1c2, 2a2, 2b1, 4a1 and 6a1 (6 genes) had no changes in livers of mice fed the various diets (data not shown).

Effect of 9 various diets on the Gst mRNAs in livers of mice

The expression of 14 Gst mRNAs were changed by at least one diet in livers of mice (Fig. 2). The alterations of hepatic mRNA for Gsta2 and Gsta4 were similar. Gsta2 mRNA was increased moderately in mice on the western diet (2.1 fold) and high-fat diet (2.7 fold), but markedly in livers of mice fed the restricted diet (19.5 fold), lab chow (10.7 fold) and atherogenic diet (9.8 fold). Gsta4 mRNA also increased in livers of mice on the restricted (7.2 fold), lab chow (4.0 fold), atherogenic (5.8 fold) and high-fat diet (1.2 fold). Gstk1 is the only member in the Gstk family that was altered with diet, with a moderate increase in livers of mice on the high-fat diet (79.2%). The mRNAs of 5 members of the Gstm family were altered in mice on the various diets. Gstm1 mRNA was increased in livers of mice fed the atherogenic (2.0 fold), restricted diet (1.5 fold) and lab chow (1.1 fold). Moreover, Gstm3 mRNAs also increased in mice on these three diets with more marked changes (diet restriction, 17.0 fold; atherogenic diet, 5.7 fold; lab chow, 3.0 fold). Gstm2 mRNA was only increased 1.4 fold in mice on the restricted diet. Gstm4 and Gstm6 had of an increase in mice on lab chow (Gstm4, 1.2 fold) and the high-fat diet (Gstm6, 92.7%). The mRNA of Gsto1 was moderately increased in livers of mice fed the restricted diet (1.1 fold), lab chow (86.2%), atherogenic diet (58.4%) and high-fructose diet (52.0%), whereas hepatic expression of Gstp1 mRNA decreased 22.1% in mice on the restricted diet. The mRNAs of Gstt2 and Gstt3 are two members that changed in the Gstt family. The mRNA of Gstt2 increased dramatically in mice fed the restricted diet (7.7 fold) and moderately in mice fed the lab chow (1.4 fold), high-fat (1.0 fold) and EFA-deficient diet (78.4%). Similar to Gstt2, hepatic expression of Gstt3 increased markedly in mice on the restricted diet (7.8 fold) and moderately in mice fed the lab chow (1.4 fold), high-fat (1.0 fold), and EFA-deficient diet (78.4%). Gstz1 had a moderate increase (58.7%) in livers of mice fed the high-fat diet, and mGst3 mRNA had a 3.1-fold increase in mice on the restricted diet, a 1.1-fold increase in mice on lab chow, but a 48.3% decrease in mice fed the EFA-deficient diet. In addition, hepatic expression of Gsta1, a3, m5, m7, o2, t1, t4, mGst1, and t2 (9 genes) were not altered by these various diets (data not shown).

Effects of 9 various diets on mRNAs of Nat, methyltransferase, amino acid-conjugating enzymes, and enzymes responsible for synthesis of cosubstrates for conjugation reactions in livers of mice

Nat2 and Nat8 were the two members in the Nat superfamily whose hepatic expression were changed with diet (Fig. 3A). Nat2 slightly increased in mice on the western diet (44.1%),

whereas *Nat8* decreased in mice on the restricted diet (54.2%). *Nat1*, 3, 6, 8l, 9, 10, 14 and 15 (8 genes) were not significantly altered by the various diets (data not shown).

Comt, *As3mt*, and *Tpmt* were the three methyltransferases whose mRNAs in livers of mice were altered by the various diets (Fig. 3B). *Comt* mRNA decreased in mice on the restricted diet (48.3%), whereas *As3mt* mRNA increased in livers of mice on the restricted diet (1.1 fold), but decreased in mice fed the western diet (39.7%) and atherogenic diet (33.5%). The expression of *Tpmt* increased in mice on the western diet (43.9%). However, *Nnmt* was not altered by these various diets (data not shown).

The expression of the mRNAs for the amino acid-conjugating enzymes in liver were relatively similar in mice fed the various diets (Fig. 3C). *Baat* mRNA was slightly decreased in mice fed the high-fructose diet (26.1%). The mRNA expression of *Bal* also had a small increase in mice with the restricted diet (29.3%), whereas glycine-*N*-acyltransferase (*Glyat*) mRNA was decreased 22.5% in mice fed the restricted diet, but increased 23.2% in mice with the western diet. The mRNAs of γ -glutamyltransferase 6 (*Ggt6*) and cysteine conjugate- β -lyase 2 (*Ccbl2*) (Fig. 3D) were increased in mice fed the atherogenic diet (1.3 fold) and the restricted diet (77.0%), respectively. Hepatic expression of *Ggt1*, 5, 7 (3 genes) and *Ccbl1* were not altered by these diets (data not shown).

Hepatic expression of enzymes for the synthesis of co-substrates, namely UDP glucose 6-dehydrogenase (*Ugdh*), 3'-phosphoadenosine 5'-phosphosulfate synthase (*Papss*), and glutamate-cysteine ligase modifier subunit (*Gclm*) mRNAs are shown in Fig. 4A. *Ugdh* mRNA was increased in mice fed the lab chow (1.6 fold), the atherogenic (89.2%), restricted (85.6%), and high-fat diet (81.6%), whereas *Papss2* was only increased in mice on the restricted diet (1.3 fold), and *Papss1* was not altered by any of these various diets (data not shown). The mRNA of *Gclm* was increased in mice fed the atherogenic diet (87.1%), lab chow (61.4%), and western diet (51.3%), whereas the hepatic mRNA of *Gclc* did not change (data not shown). The three major members of the methionine adenosyltransferase (*Mat*) superfamily were less affected by these various diets (Fig. 4B). *Mat1a* was increased 83.5% and *Mat2b* was increased 40.0% in mice fed the restricted diet, whereas *Mat2a* was decreased 37.1% in mice on the high-fructose diet.

Hierarchical cluster analysis of hepatic mRNAs encoding phase-II enzymes in livers of mice fed 9 various diets

Compared to mice on the AIN-93M purified diet, mice fed the low n-3 FA diet had the most similar expression pattern of genes encoding phase-II enzymes in livers of the mice, followed by the high-fructose diet, and the EFA-deficient diet. The expression of phase II enzymes in mice fed the high-fat diet, western diet, lab chow, and the atherogenic diet patterned into a different cluster. The restricted diet had the most obvious differential expression pattern (Fig. 5). Diet restriction altered the expression of the most number of genes encoding phase-II enzymes (27 genes), followed by lab chow (15), the atherogenic diet (13), high-fat diet (10), western diet (7), high-fructose diet (5), and EFA-deficient diet (3), whereas the low n-3 FA diet had little or no effect on the hepatic expression of these phase-II enzymes.

The mRNAs that were quantitatively increased the most in mice fed the various diets were 145.6 fold for Sult1e1 and 74.9 fold for Sult3a1 in mice on the restricted diet, followed by 19.5 fold for Gsta2 and 17.0 fold for Gstm3 in mice on the restricted diet, 10.7 fold for Gsta2 in mice on lab chow, 9.8 fold for Gsta2 in mice on the atherogenic diet, 7.7 fold for Gstt3 and 7.6 fold for Gstt2, as well as a 7.2 fold for Gsta4 in mice on the restricted diet, 5.8 fold for Gsta4 in mice on the atherogenic diet, 5.7 fold for Gstm3 in mice on the atherogenic diet, and 4.0 fold for Gsta4 in mice on the lab chow (the fold change below 4.0 are not detailed here). The mRNAs of the phase II enzymes that were quantitatively decreased the most in the current study were 58.8% decrease of Ugt2b1 in mice on the restricted diet, followed by 54.8% decrease for Ugt3a1 in mice on the restricted diet, 54.6% decrease of Sult1b1 in mice on the atherogenic diet, 54.2% decrease for Nat8 in mice on the restricted diet, 48.3% decrease for Comt in mice on the restricted diet, 48.3% decrease for mGst3 in mice on the EFA-deficient diet, 45.5% decrease for Sult1b1 in mice on the high-fructose diet, 42.9% decrease for Sult1b1 in mice on the EFA-deficient diet, 39.7% decrease for As3mt in mice on the western diet, 39.1% decrease for Sult1a1 in mice on the atherogenic diet, 37.1% decrease for Mat2a in mice on the high-fructose diet, 33.5% decrease for As3mt in mice on the atherogenic diet, 30.3% decrease for Ugt2b1 in mice on the atherogenic diet (a decrease less than 30% are not noted here).

Discussion

The present study comprehensively characterized the mRNA profiles for genes encoding major phase-II enzymes involved in glucuronidation, sulfation, GSH conjugation, acetylation, methylation, amino-acid conjugation, as well as synthesis of cosubstrates for phase-II conjugation reactions in livers of mice fed 9 different diets. Compared with the AIN-93M purified diet, changes in the hepatic expression of these genes in livers of mice fed 8 laboratory diets including lab chow, diet-restriction, high-fructose, high-fat, atherogenic, western, low n-3, and EFA-deficient diet were examined simultaneously, resulting in more comparable information on these genes in diverse nutritional conditions than in previous reports (Bouchard-Mercier *et al.*, 2013; Fu and Klaassen, 2014; Ghose *et al.*, 2011).

Ugts are integral membrane proteins located in the endoplasmic reticulum with the catalytic domains facing the lumen. They catalyze the transfer of the glucuronic acid component of UDP-glucuronic acid to small hydrophobic molecules that are structurally diverse, which include steroid hormones, bile acids, biogenic amines, plant and bacterial metabolites, carcinogens, and many therapeutic drugs (King *et al.*, 2000). As shown in Fig. 1A, the most marked changes for the Ugt superfamily was the more than a 50% decrease of Ugt2b1 and Ugt3a1 mRNAs in livers of mice fed the restricted diet, while a moderate 30.3% decrease of Ugt2b1 in mice on the atherogenic diet was also observed. Specifically, Ugt2b1 is known as one of the isoforms primarily responsible for bisphenol A glucuronidation, and this enzyme has broad substrate specificity, including morphine, phenols, carboxylic acids, and bile acids, whereas Ugt3a1 is unique in the Ugt superfamily because it uses *N*-acetylglucosamine as the preferred sugar donor (Meech *et al.*, 2012), and transfers it from UDP *N*-acetylglucosamine to ursodeoxycholic acid and other UGT substrates (Lu *et al.*, 2013). Thus, mice on diet restriction and the atherogenic diet might have decreased rates of conjugation of these substrates.

Sult enzymes localize in the cytoplasm of liver cells, and transfer a sulfo moiety to substrates to catalyze the sulfate conjugation of many hormones, neurotransmitters, drugs, and xenobiotics (Alnouti and Klaassen, 2008). Specifically, Sult1e1 is known as the estrogen sulfotransferase that metabolizes and inactivates estrogen in the circulation by conjugating a sulfate group at the 3-hydroxyl position of estrogens to make it less effective as an estrogen receptor activator in the circulation, however, the sulfated estrogen can provide the precursor for estrogen deconjugation and activation in the target organs (Sueyoshi *et al.*, 2011). Sult3a1 is the only isoform that predominantly catalyzes *N*-sulfonation, rather than *O*-sulfonation (Yoshinari *et al.*, 1998), and plays an important role in the detoxification of phenyltetrahydropyridine, a potent neurotoxin that induces neurodegenerative diseases. In the current study, the most striking observation was the tremendous increase of Sult1e1 and Sult3a1 in mice on the restricted diet. As is shown in Fig. 1B, Sult1e1 and 3a1 mRNAs are almost undetectable in livers of male mice fed the 8 various diets, but increased markedly (145.6 and 74.9 fold) in livers of mice on the restricted diet. It has been reported that estrogen regulates the expression of Sult1e1 (Nash *et al.*, 1988) and 3a1 (Alnouti and Klaassen, 2011; Klaassen *et al.*, 1998), therefore, diet restriction may have an effect on estrogen disposition and the expression of genes regulated by it. Another important alteration in the mRNAs of the Sult superfamily in livers of mice with these diets was the marked decrease of Sult1b1 in mice on the atherogenic (54.6%), high-fructose (45.5%), and EFA-deficient diet (42.9%) (Fig. 1B). Sult1b1 catalyzes the sulfo-conjugation of biosynthetic precursors of catecholamines including dopamine and tyrosine (Alnouti and Klaassen, 2011), and has the highest affinity for thyroid hormones to decrease its biological activity. Therefore, these 3 diets may affect functions of the nervous and endocrine systems. In addition, the 39.1% decrease in Sult1a1, a major Sult with broad substrate specificity (Hempel *et al.*, 2007) in mice on the atherogenic diet indicates that the sulfation of certain chemicals in liver might be affected by an atherogenic diet.

Gsts enzymes are best known for their primary role to detoxify xenobiotics though catalyzing the nucleophilic attack of GSH on electrophilic carbon, sulfur, or nitrogen atoms of nonpolar substrates, thus preventing their interaction with crucial cellular proteins and nucleic acids (Eaton and Bammler, 1999). GSTs also bind nonsubstrate ligands to inhibit the function of kinases involved in the MAPK pathway, which regulates cell proliferation and death (Laborde, 2010). Moreover, induction of Gsts is a potential mechanism for blockade of the early stages of carcinogenesis and to improve lipid hydroperoxide detoxification (Bhattacharjee *et al.*, 2007; Knight *et al.*, 2008). In the present study (Fig. 2), compared with the AIN-93M purified diet, diet restriction had the most marked increase (19.5–7.2 fold) in the mRNA expression of cytosolic Gsts (Gsta2, Gsta4, Gstm3, Gstt2 and Gstt3) in livers of mice, and the atherogenic diet and lab chow also upregulated Gsta2, Gsta4 and Gstm3 dramatically (10.7–4.0 fold), which emphasizes the marked effects of these diets. The alpha-class Gsts have been reported to play an important role as antioxidant enzymes modulating stress-induced apoptosis pathways (Yang *et al.*, 2001) and are associated with “immunity and defense” (Yun *et al.*, 2009). Gstm3 regulates apoptosis- and proliferation-related pathways and was identified as a tumor suppressor (Sun *et al.*, 2014), whereas theta-class Gsts (Gstt2 and 3) are critical in the early response induced by toxicants, such as D-galactosamine and 1-menaphthyl sulphate, due to altered oxidative stress (Coggan *et al.*,

2002; Yun *et al.*, 2010). Therefore, mice on the restricted diet, atherogenic diet, and lab chow might be more resistant to carcinogens and by-products of oxidative stress, compared to mice on the AIN-93M purified diet. Additionally, the mRNA of a microsomal Gst (mGst3), which might possess GSH-dependent peroxidase activity and has been implicated in the synthesis of leukotriene C4 and prostaglandin E from arachidonic acid (Jakobsson *et al.*, 1997; Lu *et al.*, 2013), was increased 1.1 fold and 3.1 fold in mice on the lab chow and diet restriction, respectively, whereas it was decreased markedly (48.3%) in mice on the EFA-deficient diet. Thus, compared to mice on the AIN-93M purified diet, mice on the diet restriction, lab chow, and EFA-deficient diet might have altered arachidonic acid metabolism as well as inflammatory and allergic reactions.

Acetylation of endobiotics is catalyzed by arylamine *N*-acetyltransferases (Nat). Nat8 is a newly identified enzyme for mercapturic acid (a condensation product formed from the coupling of cysteine with aromatic compounds) synthesis via acetylation of the cysteine *S*-conjugates (Veiga-da-Cunha *et al.*, 2010). As shown in Fig. 3, Nat8 decreased markedly (54.2%) in mice on the restricted diet, which indicates that the detoxification of aromatic compounds might decrease in these mice compared to mice on the control diet. Two methyltransferases decreased markedly in mice on these diets, more specifically, Comt decreased 48.3% in mice on the restricted diet, and As3mt decreased 39.7% and 33.5% in mice on the western and atherogenic diet, respectively. Comt plays a vital role in the metabolism of hormones (e.g., catecholamines and estrogens) and a large number of dietary phenolic compounds (Zhu, 2002). The restricted diet had decreased dietary intake, which might lead to a corresponding decrease of the needs for this enzyme in the organism. As3mt is the arsenic methyltransferase that converts inorganic arsenic to the methylated metabolite (Dodmane *et al.*, 2013), and down-regulation of this enzyme in mice on the western and atherogenic diet might alter the disposition and retention of arsenic in arsenate-treated mice. Methionine adenosyltransferase (Mat) catalyzes the synthesis of the major methyl donor *S*-adenosylmethionine in mammals, and increase risk of liver pathogenesis has been reported in extreme Mat deficiency in humans and mouse models (Wang and Chiang, 2012). In the current study (Fig. 3), there was a moderate decrease of hepatic mRNA expression for Mat2a (33.5%) in mice on the high-fructose diet, thus, this diet might lead to low delivery of *S*-adenosylmethionine from the liver to plasma and tissues, and increase the susceptibility to liver injury and/or promote the progression of existing liver diseases (Wang and Chiang, 2012).

Different transcriptional pathways are involved in phase-II gene regulation. Nrf2 is an important transcriptional factor involved in the activation of ARE-mediated phase-II gene induction, and results from our previous study revealed that the hepatic expression of Nrf2 mRNA only had a moderate increase (73%) in mice on the atherogenic diet compared to AIN-93M purified diet (Guo *et al.*, 2015b), however, it was reported that the activation of Nrf2 is mainly due to posttranscriptional modification and translocation to the nucleus (Mota *et al.*, 2015; Rushmore and Kong, 2002). Many phase-II enzymes are induced with graded Nrf2 activation though decreased Kelch-like ECH associating protein 1 expression (Wu *et al.*, 2012) and Nrf2 activators (Alnouti and Klaassen, 2008), and in the current study some of the Nrf2 target genes including Ugt2b35, 2b36, Sult1b1, Gsts (Gsta2, a4, m1, m2, m3, m6, p1, t3 and mGst3) and Ugdh were up-regulated in livers of mice fed the restricted diet,

moreover, among those phase-II genes, *Gstm3* that was increased the most by graded Nrf2 activation (Wu *et al.*, 2012) also had the largest fold changes by diet-restriction, which indicates the role that Nrf2 might play in the restricted diet. In addition, some other genes that were induced by Nrf2 graded activation were not changed (*Ugt1a6a*, *1a9*, *2b5*, *Gsta3*) by the restricted diet. CAR, PXR as well as peroxisome proliferator-activated receptor- α (PPAR α) were up-regulated in livers of mice on the restricted diet (Guo *et al.*, 2015b). Therefore, multiple nuclear receptors and other transcription factors may be involved in the induction of phase-II genes by diet restriction, which is also consistent with the dramatic increase of their target genes (*Sult1d1*, *1e1*, *3a1* and *Papss2*)(Alnouti and Klaassen, 2008).

One intriguing finding in this study is the markedly higher expression levels of many (15) phase-II genes in the lab chow-fed mice compared to mice fed the AIN-93M purified diet. The #8604 lab chow contains high levels of soybean isoflavones (350–575 mg/kg), whereas the isoflavones content is likely much lower in the AIN-93M purified diet. Soy isoflavones are metabolized by phase-II enzymes, whereas there are conflicting reports on whether isoflavones can induce Phase-II enzymes in mice and humans (Mann *et al.*, 2009). The marked differences in hepatic expression of phase-II enzymes between #8604 lab chow and AIN-93M, the two common "control diets", suggest that the selection of basal diet has a major impact on the basal Phase-II gene expression, and thus may greatly affect the inducibility of phase-II enzymes by dietary interventions in animals and humans.

The current study gives clues to explain some clinical phenomena at a transcriptional level and will enable one to consider corresponding alterations in study design and interpretation of the data in the further studies using these individual diets. For instance, in a comprehensive analysis of polymorphisms in UGT enzymes in patients with non-small-cell lung cancer treated with irinotecan, the authors suggest that certain UGT genotypes may be associated with irinotecan efficacy and/or toxicity, as they regulate the rate of glucuronidation of SN-38, the active metabolite of irinotecan (Han et al., 2006). However, dietary cruciferae such as broccoli, citrus fruits or onions contain chemicals that can increase the activity of UGT isoenzymes (Peterson et al., 2005). These two findings, when juxtaposed, should alert oncologists to the possibility of pharmacokinetic interactions between dietary cruciferae and irinotecan, which seem worthy of clinical evaluation (D'Incalci et al., 2007). However, caution should be exercised when making generalization from the mRNA to enzyme activities, however, performing experiments in mice with these diets and administered substrates for each phase-II enzyme to assess their role in the pharmacokinetics of drugs and xenobiotics would be an arduous undertaking (Harris *et al.*, 2003). In addition, for many of the enzymes there are no specific substrates or specific antibodies to determine the similarities of the changes in mRNA, enzyme amounts and activities. The effect of the diets on activities of phase-II enzymes and pharmacokinetics as well as pharmacodynamics of compounds metabolized by phase-II enzymes should be considered in studies in humans.

Conclusion

This comprehensive study indicates that phase-II enzymes are altered by diets, and provide detailed information on which conjugation enzymes are changed by certain diet. This

information can be used to further investigate the mechanism for the changes in mRNAs and whether the changes in mRNA result in similar alteration in enzyme activity and affect drug action as well as adverse effects.

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Abbreviations

AhR	aryl hydrocarbon receptor
As3mt	arsenic (+3 oxidation state) methyltransferase
Baat	bile acid-CoA:amino acid <i>N</i> -acyltransferase
Bal	bile acid-CoA ligase
BPA	bisphenol A
CAR	constitutive androstane receptor
Ccbl	cysteine conjugate- β -lyase
Comt	catechol <i>O</i> -methyltransferase
EFA	essential fatty acid
Gclc	glutamate-cysteine ligase catalytic subunit
Gclm	glutamate-cysteine ligase modifier subunit
Ggt	γ -glutamyltransferase
Glyat	glycine- <i>N</i> -acyltransferase
GSH	glutathione
Gst	glutathione <i>S</i> -transferase
Mat	methionine adenosyltransferase
mGst	microsomal Gst
Nat	<i>N</i> -acetyltransferase
Nnmt	nicotinamide <i>N</i> -methyltransferase
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
Papss	3'-phosphoadenosine 5'-phosphosulfate synthase
PCR	polymerase chain reaction
PXR	pregnane X receptor

Sult	sulfotransferase
Tpmt	thiopurine <i>S</i> -methyltransferase
Ugdh	UDP glucose 6-dehydrogenase
Ugp	UDP-glucose pyrophosphorylase
Ugt	uridine diphosphate-glucuronosyltransferase
VDR	vitamin D receptor.

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Figure 1.

Effect of 9 various diets on the Ugt and Sult mRNAs in livers of mice. Seven of the 21 mRNAs of the Ugt enzymes, including Ugt2b and 3a subfamilies were altered in livers of the mice fed the 9 diets (Fig. 1A); The mRNAs of the Sult superfamily in livers of male C57BL/6J mice changed markedly by feeding the various diets (Fig. 1B). * represent $P < 0.05$. Data are presented as Mean $\pm$ S.E.M. "AIN-93M purified" represents AIN-93M purified diet (#94048); "Lab chow" represents tekla rodent diet (#8604); Diet restriction performed by providing 75% of the diet (#8604) consumed by ad libitum feeding; "high fructose" represents 60% fructose diet (#89247); "high fat" represents high-fat diet (#97070); "atherogenic" represents atherogenic rodent diet (#02028); "western" represents adjusted calories diet (western diet, #88137); "low n-3 FA" represents DHA deficient diet (#00235 + 7% sunflower oil); "EFA deficient" represents EFA-deficient diet (#84224). The same abbreviations used in all the figures of the current study.

Figure 2.

Effect of 9 various diets on the Gst mRNAs in livers of mice. The expression of 14 Gst mRNAs were changed by at least one diet in livers of mice. * represent $P < 0.05$. Data are presented as Mean $\pm$ S.E.M.

Figure 3.

Effects of 9 various diets on mRNAs of Nat, methyltransferase, AA-conjugating enzymes, Nat2 and Nat8 were the two members in the Nat superfamily whose hepatic expression changed with diet (Fig. 3A); Comt, As3mt, and Tpm were the three methyltransferase mRNAs in livers of mice that were altered by the various diets (Fig. 3B); The expression of the mRNAs for the AA-conjugating enzymes in liver were relatively similar in mice fed the various diets (Fig. 3C); The mRNAs of γ -glutamyltransferase (Ggt) and cysteine conjugate- β -lyase 2 (Ccbl2) were altered in mice fed various diets (Fig. 3D). * represent $P < 0.05$. Data are presented as Mean $\pm$ S.E.M.

Figure 4.

Effects of 9 various diets on mRNAs of enzymes responsible for synthesis of cosubstrates for conjugation reactions in livers of mice. The hepatic expression of UDP glucose 6-dehydrogenase (Ugdh), 3'-phosphoadenosine 5'-phosphosulfate synthase (Papss), and glutamate-cysteine ligase modifier subunit (Gclm) mRNAs are shown in Fig. 4A; All three major members of the methionine adenosyltransferase (Mat) superfamily had slightly altered hepatic mRNAs in mice fed these various diets (Fig. 4B). * represent $P < 0.05$. Data are presented as Mean $\pm$ S.E.M.

Figure 5.

Hierarchical cluster analysis of hepatic mRNAs encoding phase-II enzymes in livers of mice fed 9 various diets. Compared to the mice on the AIN-93M purified diet, the mice fed the low n-3 FA diet had the most similar expression pattern of genes encoding phase-II enzymes in livers of the mice, followed by the high-fructose diet, and the EFA-deficient diet. The expression of phase II enzymes in mice fed the high-fat diet, western diet, lab chow, and the atherogenic diet patterned into a different cluster. The restricted diet had the most obvious differentially expression pattern. Average values of three replicates per diet are given by colored squares. High mRNA abundance is represented in red, whereas low mRNA abundance is in blue.