INTERACTIONS BETWEEN GENETIC BACKGROUND AND DIET: EFFECTS ON THE GLUTATHIONE REDOX SYSTEM

by

KATHLEEN MARIE NORRIS

(Under the direction of Robert Pazdro)

ABSTRACT

The glutathione (GSH) antioxidant system governs cellular protection against oxidative stress, which is characterized by depletion of GSH and subsequent accumulation of the dimer glutathione disulfide (GSSG). Previous research indicates a negative effect of a high-fat diet on GSH redox status (GSH/GSSG) and a possible protective effect of certain flavonoids such as anthocyanins. Although tissue levels of GSH, GSSG, and GSH/GSSG are also heritable, it is unclear whether genetic background and diet interact to regulate the GSH system. The current work tested the hypothesis that a high-fat diet regulates GSH homeostasis in a manner dependent on genetic background. Additionally, it tested the hypothesis that anthocyanin-induced alterations in GSH homeostasis are genetically determined. Both hypotheses were confirmed, indicating that a high-fat diet and anthocyanin supplementation interact with genetic background to regulate the GSH system and subsequent chronic disease risk.

INDEX WORDS: Oxidative stress, Glutathione, Redox status, Obesity, Anthocyanins, Cyandin-3-*O*-β-glucoside, Polyphenols, Phytochemicals, Antioxidants

INTERACTIONS BETWEEN GENETIC BACKGROUND AND DIET: EFFECTS ON THE GLUTATHIONE REDOX SYSTEM

by

KATHLEEN MARIE NORRIS

B.S., The University of Georgia, 2014

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2016

INTERACTIONS BETWEEN GENETIC BACKGROUND AND DIET: EFFECTS ON THE GLUTATHIONE REDOX SYSTEM

by

KATHLEEN MARIE NORRIS

Major Professor: Robert Pazdro

Committee: Arthur Grider
Joan Fischer

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
August 2016

TABLE OF CONTENTS

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Purpose of the Study

This work seeks to understand whether diet and genetic background interact to control the endogenous glutathione (GSH) antioxidant system. It is known that GSH levels and redox status are influenced by certain dietary components such as a high-fat diet. Additionally, it is known that GSH levels and redox status are genetically determined. Taken together, the present work tested the hypothesis that diet and genetic background interact to modulate GSH homeostasis. Due to the essential role of the GSH system in reducing oxidative stress and preventing disease, the findings of the studies included within this thesis improve understanding of mechanisms by which chronic diseases are inherited.

GSH Biochemistry and Its Role in Health and Oxidative Stress

GSH is a ubiquitous, thiol tripeptide that governs cellular defense against oxidative stress. The protective capacity of this molecule is attributable to its capacities to directly neutralize reactive oxygen species (ROS) and to serve as a cofactor for ROS metabolizing enzymes [1]. The body produces ROS, such as peroxides, as a normal byproduct of cellular metabolism. While ROS are important for cell signaling, excessive ROS production can cause cellular damage to lipids, membranes, proteins, and DNA [2, 3]. Processes that generate ROS are typically kept in balance by antioxidant systems such as GSH when a cell is under normal physiological conditions and not stressed [1]. However, if ROS are overproduced, they can overwhelm the endogenous antioxidant defense system and thereby induce cellular damage. This damage occurs either

through direct physical damage to the cell or an interruption of intracellular signaling pathways [1]. Thus, prolonged exposure to oxidative stress can subsequently lead to many degenerative diseases. Elevated ROS levels are a key finding in inflammation, cardiovascular disease, neurodegenerative diseases, cancer, and diabetes mellitus [1, 4].

GSH concentrations within the cell are a function of the balance between depletion, regeneration, and de novo synthesis [5]. De novo synthesis of GSH is a two-step process. The first and rate-limiting step combines glutamate and cysteine to yield γ-glutamylcysteine through action of the enzyme glutamate cysteine ligase (GCL) [1]. GCL is a heterodimer with two subunits: a catalytic subunit (GCLC) and a modifier subunit (GCLM). The catalytic subunit contains all the enzymatic activity and substrate binding sites of the enzyme, while the modifier subunit modulates the activity of the catalytic subunit [5]. Both subunits are highly inducible, and the relative levels of the subunits are a major determinant of cellular GCL activity and GSH biosynthetic capacity [6]. In the second and last step of GSH synthesis, γ-glutamylcysteine is combined with glycine to yield GSH through action of the enzyme glutathione synthetase [5].

GSH is a very stable electrophile that is able to donate its extra electron to ROS in order to stabilize them. GSH is also able to stabilize ROS through the activity of certain enzymes. For example, GSH acts as a cofactor for the enzyme glutathione peroxidase (GPx), which catalyzes the reduction of many peroxides within the cell including hydrogen peroxide (H_2O_2) [1]. GPx enzymes are also involved in the removal of lipid hydroperoxides, thereby terminating lipid peroxidation chain reactions and protecting biological membranes [1]. In the process of stabilizing ROS, whether directly or through the action of an enzyme, GSH becomes oxidized to glutathione disulfide (GSSG). Once GSH has been oxidized to GSSG, it can then be reduced back to GSH by the enzyme glutathione reductase (GR) [1]. Failure to replenish depleted GSH

stores, however, compromises cellular redox balance and cell viability. As GSH oxidation is coupled to the generation of the dimer GSSG, the subsequent decrease in GSH/GSSG serves as a quantitative measure of oxidative stress.

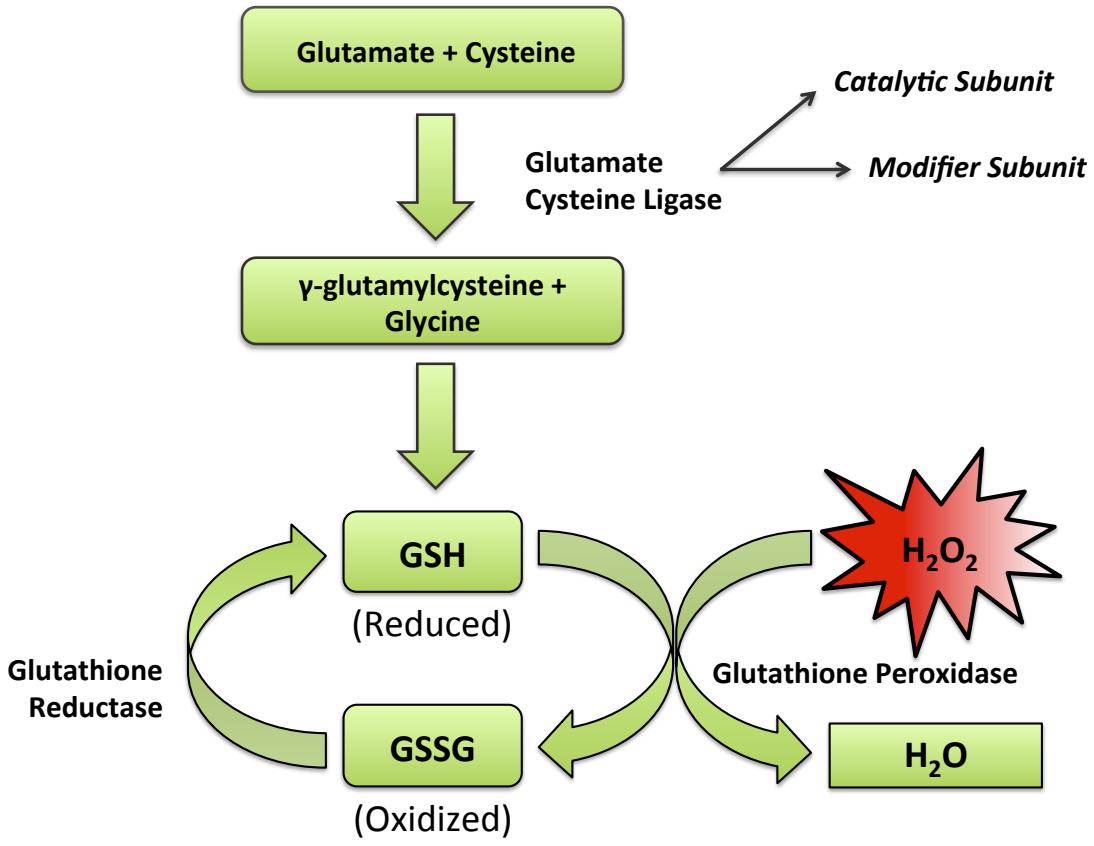

Figure 1.1 GSH Biosynthetic Pathways and Redox Cycling

Dietary Effects on the GSH System

Due to the integral role of GSH in health and disease, many researchers have investigated the potential of various dietary components to influence the GSH system. Such data indicate that a

high-fat diet and subsequent obesity have a negative effect on GSH redox status, while anthocyanins exhibit a possible protective effect.

The Effects of a High-Fat Diet on GSH

More than one third of adults in the United States are now obese. The medical costs of obesity continue to climb, having reached $147 billion in the U.S. in 2008. The pathogenesis of comorbidities associated with obesity, such as metabolic syndrome [7, 8], cardiovascular disease [9-12], renal disease [11, 13], and certain types of cancer [14] are promoted by oxidative stress. Importantly, alleviation of oxidative stress protects against many of these outcomes. For example, the drug epalrestat protects against diabetic peripheral neuropathy by alleviating oxidative stress in obese animals [15], and similar effects have been observed for insulin resistance [16] and liver tumorigenesis [17] using other treatments. As the worldwide obesity epidemic continues to grow, it is important to clarify the mechanisms that drive obesity-associated oxidative stress.

Epidemiological studies indicate the primary role of a high-fat diet in promoting obesity [18], so evaluation of the effects of a high-fat diet on the GSH antioxidant defense system is highly important. Studies have generally shown that diet-induced obesity perturbs GSH homeostasis, as illustrated by a decrease in GSH/GSSG and altered activity of GSH-related enzymes [19-24]. For example, Li et al. reported that a high-fat diet increases hepatic GSSG levels and decreases GSH/GSSG ratios in mice [25]. These alterations in GSH were also associated with an increased risk of nonalcoholic fatty liver disease. Jarukamjorn et al. similarly reported that high-fat induced imbalances in GSH redox status and enzyme capacity were associated with increased risk and progression of nonalcoholic liver disease [19]. Interestingly, compromised GSH/GSSG in response to a high-fat diet appear to be rescued by supplementation

with certain plant compounds such as rosemary extract [26]. Thus, many biomedical researchers suggest the potential to use supplementation of these molecules as therapeutic medical treatments.

The Effects of Anthocyanins on GSH

In the search for therapeutic strategies for obesity and its related comorbidities, anthocyanins have been a topic of particular interest. Anthocyanins are flavonoids that contribute to the blue, purple, and red color of many fruits and vegetables, such as blueberries, blackberries, and purple corn [27]. Inclusion of foods rich in these compounds appears to modulate chronic disease risk. In models of diabetes [13], obesity [28, 29], cancer [30, 31], and metabolic syndrome [32], anthocyanins decrease markers of oxidative stress. This effect is attributable, in part, to their strong antioxidant activity and ability to scavenge free radicals [33-37]. In 2005, Moskaug et al. reported that flavonoids might also concurrently increase cellular levels of GSH by increasing expression of γ-glutamylcysteine synthetase in vitro [38]. This effect has now been shown in vivo; cyanidin-3-*O*-β-glucoside (C3G), a commonly consumed anthocyanin, increased hepatic GSH levels nearly threefold in a mouse model of type 2 diabetes [13]. In the study, *db/db* mice were supplemented with 100 mg/kg C3G for 8 weeks. With C3G supplementation, significantly higher concentrations of GSH were found in the liver in comparison to controls. Additionally, levels of oxidative stress determined by lipid peroxidation, neutrophil infiltration, and hepatic steatosis were significantly attenuated in the C3G supplemented group. This paper provides valuable insight into some of the potential mechanisms through which anthocyanins impact health, but this study only assessed changes in GSH levels in the liver. Additionally, while GSH/GSSG redox ratios were significantly improved with C3G treatment in vitro, GSH/GSSG ratios in vivo were not reported in this study. Although GSH/GSSG redox data in response to

anthocyanin intake has not commonly been reported in vivo, many other studies support the findings of Zhu et al. in regard to the GSH biosynthetic capacity of anthocyanins. For example, a study conducted by Jiang et al. demonstrated that anthocyanin pretreatment inhibits alcohol-induced GSH depletion in the liver concurrent with decreased oxidative stress and liver injury [39]. Similarly, Hou et al. reported that anthocyanin treatment restores GSH levels in alcohol-treated rats as well as activity of antioxidant enzymes such as GPx [40].

Genetic Effects on GSH

In addition to diet, genetic background is a critical determinant of tissue GSH levels and GSH/GSSG [41, 42]. In a panel of 30 mouse strains, Zhou et al. demonstrated that genetic background induces about a threefold difference in hepatic and renal GSH/GSSG in the most divergent strains [41]. Additionally, GSH antioxidant responses to various challenges have been shown to vary with genetic background. For example, Tsuchiya et al. reported that GSH levels and GSH/GSSG differed across strains in response to alcohol-induced liver injury [42], and Ahotupa et al. reported strain differences in altered GSH enzyme functions in response to phenobarbital [43].

Several studies have utilized knockout mice to assess the role of certain genes on GSH homeostasis and subsequent chronic disease risk. For example, nuclear factor erythroid 2-related factor 2 (Nrf2) knockout rats exhibit decreased expression of GSH and Gr in cardiac fibroblasts compared to wild type controls [44], reflecting reduced antioxidant capacity of these mice. Furthermore, double-knockout mice (GPx-1(-/-) and ApoE(-/-)) have been shown to develop increased oxidative stress and exacerbated atherosclerosis compared to apolipoprotein E-deficient controls [45]. Importantly, it has also been shown that red blood cell GSH/GSSG may be heritable in humans [46], and polymorphisms in GSH-related genes are associated with the

development of certain diseases in human populations as well. For example, Ruperez et al. found an association between Gpx variants and obesity in Spanish children [47]. Additionally, Gpx variants were associated with an increased risk of type 2 diabetes in a South Indian population [48]. Overall, these findings indicate the ability of genetic variation to drive divergent GSH-related phenotypes and resultant chronic disease risk.

Conclusions

Protection from obesity-associated chronic diseases may be provided by the alleviation of oxidative stress. Oxidative stress is attenuated by GSH, a potent antioxidant, but most research shows that GSH redox status is compromised by obesity and a high-fat diet. Potential therapeutic strategies for the comorbidities of obesity have been extensively researched, and one area of particular interest is anthocyanin supplementation. Anthocyanins reduce oxidative stress in many animal models of disease, and it has been suggested that these protective effects are partially due to increased GSH production.

In addition to diet, genetic background is known to regulate tissue GSH levels and GSH/GSSG. There is a lack of knowledge, however, about potential interaction between diet and genetic background in regulation of the GSH system. Thus, the current work sought to investigate this relationship. We predict that dietary components, such as high fat and anthocyanin supplementation, modulate GSH antioxidant defenses and subsequent disease risk, but that the effects are dependent on genetic background. This hypothesis is divided into two chapters within this thesis. Chapter 2 addresses interactions of genetic background with high-dietary fat on the GSH system, while chapter 3 addresses interactions of genetic background with anthocyanin supplementation on the GSH system. Lastly, chapter 4 provides overall conclusions of our work.

CHAPTER 2

A HIGH-FAT DIET DIFFERENTIALLY REGULATES HEPATIC GLUTATHIONE HOMEOSTASIS IN THE OBESITY-PRONE MOUSE STRAINS DBA/2J, C57BL/6J, AND AKR/J[1]

[1] Norris, K.M., W. Okie, W.K. Kim, R. Adhikari, S. King, and R. Pazdro. Submitted to *Nutrition Research*, 05/13/16.

Abstract

Obesity is a major risk factor for chronic diseases such as cardiovascular disease and cancer. The mechanisms that couple obesity and chronic disease have not been entirely elucidated, but oxidative stress appears to be integral to the relationship. Oxidative stress is characterized by depletion of the ubiquitous antioxidant, glutathione (GSH), and subsequent accumulation of the dimer GSSG. Tissue levels of GSH, GSSG, as well as GSH/GSSG are heritable, but it is unclear whether genetic background and obesity interact to regulate the GSH system. The current study tested the hypothesis that diet-induced obesity regulates GSH homeostasis in a manner dependent on genetic background. Mice representing three obesity-prone inbred strains — C57BL/6J (B6), DBA/2J (D2), and AKR/J (AKR) — were randomly assigned to consume a control (10% energy from fat) or high-fat (60% energy from fat) diet for ten weeks. Tissue GSH levels, GSSG levels, and GSH/GSSG were quantified, and hepatic expression of GSH-related enzymes was evaluated by qPCR. The high-fat diet caused a decrease in hepatic GSH/GSSG in D2 mice. In contrast, B6 mice exhibited a decrease in GSSG levels in liver and kidney, as well as a resultant increase in renal GSH/GSSG. GSH levels and GSH/GSSG were unaffected by diet in AKR mice. Finally, the high-fat diet induced a divergent gene expression response in D2 mice compared to B6 and AKR. These data indicate that high dietary fat regulates the GSH system in a strain-dependent manner and that D2 mice are an appropriate model for diet-induced hepatic redox disruption.

Keywords: Obesity, high-fat diet, oxidative stress, glutathione, antioxidant, redox status

Introduction

Oxidative stress contributes to the pathogenesis of comorbidities associated with obesity, such as metabolic syndrome [7, 8], cardiovascular disease [9-12], renal disease [11, 13], and certain types of cancer [14]. Alleviation of oxidative stress protects against many of these outcomes. For example, the carotenoid astaxanthin prevents liver tumorigenesis [17] in obese animals, and reducing oxidative stress also modulates symptoms of diabetic peripheral neuropathy [15] and insulin resistance [16]. Oxidative stress mediates many of the deleterious effects associated with obesity, and the knowledge of oxidative stress regulation will become increasingly critical as the global obesity epidemic grows.

Cellular defenses against oxidative stress are governed by the ubiquitous thiol tripeptide, glutathione (GSH). The protective capacity of this molecule is attributable to its abilities to directly neutralize reactive oxygen species (ROS) and to serve as a cofactor for ROS metabolizing enzymes [1]. GSH oxidation is coupled to the generation of the glutathione dimer (GSSG), and the subsequent decrease in GSH/GSSG serves as a quantitative measure of oxidative stress. A high-fat diet has been shown to increase hepatic GSSG levels and decrease GSH/GSSG ratios, and the effects are associated with the onset of nonalcoholic fatty liver disease in mice [25]. These findings align with the data from other studies, which have generally shown that diet-induced obesity perturbs GSH homeostasis, illustrated by a decline in GSH/GSSG and altered activity of GSH-related enzymes [19-24].

In addition to diet, genetic background is a critical determinant of tissue GSH levels and GSH/GSSG [41, 42]. However, it is unclear whether genetic background and diet-induced obesity intersect to regulate GSH homeostasis. Our hypothesis predicted that the effects of a high-fat diet on the GSH system vary by genetic background. To test this hypothesis, we

designed a study involving three mouse strains that develop obesity on a high-fat diet: C57BL/6J (B6), DBA/2J (D2), and AKR/J (AKR). The strains have been thoroughly characterized with regard to obesity-related physiology [49-55], which supports their inclusion in this study. B6, D2, and AKR mice were fed a control or high-fat diet for 10 weeks. GSH levels and GSH/GSSG were quantified in livers and kidneys, and expression of hepatic GSH-related enzymes was determined by qPCR. D2 mice fed a high-fat diet exhibited lower hepatic GSH/GSSG than controls. In contrast, B6 mice exhibited a decrease in GSSG levels on the high-fat diet; a similar effect was observed in the kidney, which caused an increase in renal GSH/GSSG. Diet-induced obesity did not alter GSH levels of AKR mice in either tissue. B6 and AKR mice exhibited increased hepatic expression of Gpx-1 on the high-fat diet, while D2 mice exhibited a divergent pattern of gene expression. In all, these data indicate that high dietary fat regulates GSH homeostasis in a strain-dependent manner. Future studies will clarify the mechanisms by which genotype and a high-fat diet interact to control the GSH antioxidant system.

Methods

Animals

Female C57BL/6J (B6; JAX #000664), DBA/2J (D2; JAX #000671), and AKR/J (AKR; JAX #000648) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Five mice from each strain were randomly assigned to consume a control diet and an additional five mice were assigned to a high-fat diet. Mice were housed in an animal room on a 12 h light/dark cycle. At the conclusion of the study, mice were humanely euthanized by cervical dislocation, and tissues were collected for analyses. All methods and experimental procedures for this study were approved by the University of Georgia Institutional Animal Care and Use Committee (A2013-08-011).

Diet

Mice were fed diets containing either 10% energy from fat (*control*; TestDiet 58Y2) or 60% energy from fat (*high-fat*; TestDiet 58Y1), which contains extra fat from lard. The composition of the experimental diets is described in Table 2.1. Dietary interventions were initiated when mice reached four months of age and were sustained for ten weeks. During the study period, mice were fed *ad libitum* and given unrestricted access to water. Food intake and weights of the mice were measured on a weekly basis.

Assessment of Total Glutathione, GSH, GSSG, and GSH/GSSG

Livers and kidneys were removed, rinsed in ice-cold phosphate-buffered saline (PBS), and flash-frozen in liquid nitrogen. Within 24 hours after collection, tissues were homogenized in PBS containing 10 mM diethylenetriaminepentaacetic acid (Sigma-Aldrich, St. Louis, MO), immediately acidified with perchloric acid (Sigma-Aldrich), and centrifuged. After centrifugation, supernatants were flash-frozen in liquid nitrogen and stored at -80°C until analysis. Concentrations of GSH and GSSG were measured by high performance liquid chromatography (HPLC) coupled with electrochemical detection (flow rate: 0.5 ml/min; Dionex UltiMate 3000, Thermo Scientific, Waltham, MA). Cells were set at 1600 mV with an additional cleaning potential of 1900 mV occurring between samples. The mobile phase consisted of 4.0% acetonitrile, 0.1% pentafluoropropionic acid, and 0.02% ammonium hydroxide. An injection volume of 2.0 μL was used for all samples. Data were quantified by the Chromeleon Chromatography Data System Software (Dionex Version 7.2, Thermo Scientific) after electrochemical detection. Total glutathione was calculated by the formula GSH + 2GSSG, and glutathione redox status was assessed by the ratio GSH/GSSG. Concentrations of GSH and GSSG were standardized to total protein, which was quantified by Pierce BCA Protein Assay

(Thermo Fisher Scientific, Rockford, IL).

Assessment of Endogenous Antioxidant Enzyme Expression

TRIzol reagent (Thermo Scientific) was used to extract mRNA from flash-frozen liver according to manufacturer's instructions. Synthesis of cDNA was completed using a High-Capacity cDNA Reverse Transcription Kit (Thermo Scientific). Primers were obtained from Integrated DNA Technologies (Coralville, IA). Relative gene expression levels of glutamate-cysteine ligase modifier subunit (Gclm), glutamate-cysteine ligase catalytic subunit (Gclc), glutathione peroxidase-1 (Gpx-1), and glutathione reductase (Gr) were determined using SYBR Green MasterMix (BIO-RAD Life Science Research, Hercules, CA). Target gene expression was normalized using β-actin as a reference, and all samples were run in triplicate. Primer sequences that were used for RT-qPCR are listed in Table 2.2. SYBR green fluorescence was detected by a LightCycler 480 II (Roche Life Science). The ΔΔCt method was used to calculate quantitative fold-changes, which are presented as the fold-change comparative to the B6 control.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism (Version 6.0, GraphPad Software Inc., La Jolla, CA, USA). Analyses of body weight (percent initial and end point) were completed using two-way ANOVA with Bonferroni adjustments. Food intake analyses were completed using one-way ANOVA with Bonferroni adjustments. Independent t-tests were used to detect differences in total glutathione levels, GSH and GSSG concentrations, GSH/GSSG ratios, and expression of GSH-related enzymes in response to high-fat dietary treatment. The level of statistical significance was defined at $P < 0.05$. Data are reported as means ± standard error of mean (SEM). GraphPad's Grubb's test was used to identify one significant outlier in the

PCR data using a 95% confidence interval, as previously reported [56-60]. The outlier existed in the D2 control group for expression of *Gr*, and it was subsequently removed from data analysis.

Results

Food Intake and Body Weight

B6 mice consumed the high-fat diet in a manner that generated excessive waste, as signified by food remnants scattered throughout the cage. A similar effect has recently been observed in NONcNZO10/LtJ mice [61], which prevented accurate measurement of food consumption. Therefore, intake of high-fat food by B6 mice was not quantified, but all other experimental groups were assessed. High-fat fed AKR and D2 mice consumed the same amount of food as their respective controls (Table 2.3). As expected, B6 and AKR strains gained significantly more weight than their respective controls on a high-fat diet, but the high-fat diet did not induce significant weight gain in D2 mice until the last week of the study (Figure 2.1A). This difference only emerged when weight gain was expressed as a percentage of initial body weight. The true body weights, expressed in grams, did not demonstrate the same effect (Figure 2.1B). High-fat fed D2 mice also weighed significantly less than high-fat fed B6 and AKR mice at study termination (Figure 2.1B).

GSH and GSSG Levels, Total Glutathione, and Redox Status

No differences in total glutathione were found in response to a high-fat diet in B6, D2, or AKR mice (Figure 2.2A, 2.3A). Similarly, no differences were observed in GSH concentrations in any of the strains (Figure 2.2B, 2.3B). However, obese B6 mice exhibited significantly lower concentrations of GSSG in both livers ($P = 0.0133$; Figure 2.2C) and kidneys ($P = 0.0429$; Figure 2.3C) compared to controls. Additionally, the high-fat diet induced an approximate 49% increase in mean renal GSH/GSSG in B6 mice ($P = 0.0390$; Figure 2.3D). In contrast, the mean

hepatic GSH/GSSG was approximately 33% lower in D2 mice administered a high-fat diet versus control diet ($P = 0.0348$; Figure 2.2D). The AKR strain exhibited no diet-induced alterations of GSH levels or GSH/GSSG (Figures 2.2 and 2.3).

Hepatic Expression of GSH-Related Enzymes

In response to a high-fat diet, expression of Gpx-1 increased approximately two-fold in B6 ($P = 0.003$) and AKR ($P = 0.026$) mice (Figure 2.4A). The high-fat diet did not alter Gpx-1 levels in D2 mice. High-fat fed D2 mice exhibited decreased expression of Gr *(*$P = 0.012$*)* and increased expression of Gclm *(*$P = 0.027$*)* (Figure 2.4, panels B and C). The difference in Gclm expression between AKR mice fed control and high-fat diets approached significance, but did not achieve it ($P = 0.06$; Figure 2.4C). Hepatic expression of Gclc was unaffected by diet in B6, D2, and AKR mice (Figure 2.4D).

Discussion

Mice are an established model organism for dissecting the underlying genetic architecture of obesity, and strains such as B6, D2, and AKR are susceptible to the obesity-promoting effects of a high-fat diet [49-55]. These three strains gain significant amounts of weight on a high-fat diet, but their obese phenotypes are distinct. For example, obese D2 mice display hyperleptinemia while B6 mice do not [54]. Also, obese B6 mice develop hyperglycemia and impaired glucose tolerance, while AKR mice exhibit higher plasma insulin and more severe insulin resistance [62]. Overall, previous studies indicate that genetic background determines susceptibility to obesity and its associated physiology. In this study, we used a high-fat diet to induce obesity in the B6, D2, and AKR strains. Although all three strains gained more weight than their respective controls on the high-fat diet, B6 and AKR had a significantly higher body weight than D2 at study termination. These data deviated from previous findings by Andrikopoulos et al., which

showed that D2 mice gain more weight than B6 on a high-fat diet [63]. The conflict may have originated because Andrikopoulos et al. used male mice, which tend to exhibit more severe phenotypes related to obesity and insulin resistance. However, it was critical that the current study focus on female mice to ensure that the GSH data could be put into context of previous studies related to GSH heritability [41].

Because genetic background plays a prominent role in determining obesity risk and physiology, we predicted that genetic background would also determine the effects of obesity on GSH homeostasis. After B6, AKR, and D2 mice were fed a control or high-fat diet for ten weeks, we assessed total glutathione, levels of GSH and GSSG, and GSH/GSSG. We also evaluated expression levels of GSH-related enzymes. D2 was the only strain to exhibit a decline in hepatic GSH/GSSG in response to high-fat diet, despite the fact that these mice gained minimal weight compared to B6 and AKR. These data suggest that the effects of a high-fat diet on GSH homeostasis may be partly independent of obesity.

Previous studies have established that hepatic GSH/GSSG decreases in response to obesity and a high-fat diet [19, 21, 22]. However, the relationship between GSH/GSSG and obesity has not been entirely consistent. For example, Park et al reported that genetically obese *ob/ob* mice do not exhibit altered GSH/GSSG compared to their lean littermates [64], which reflects the results of the current study. Importantly, both projects share several experimental characteristics, including the particular HPLC method and the B6 genetic background. Specifically, the *ob/ob* mice contain a mutation in the leptin gene, which arose spontaneously and was backcrossed onto the B6 background, while we used diet to induce obesity in B6 mice. The current study also tested whether the relationship between a high-fat diet and GSH homeostasis is tissue-specific. Renal GSH homeostasis was assessed, and GSH/GSSG ratios

were found to be higher in obese B6 mice than controls. This finding does not completely coincide with results from Zhu et al., in which renal GSH homeostasis of B6 mice was unaffected by a high-fat diet [65]. B6 mice are resistant to high-fat diet-induced renal injury compared to other strains [66], so our discovery of renal GSH/GSSG increases may propose a mechanism that mediates renal protection under these conditions.

The overall impact of a high-fat diet on GSH-related enzymes is unclear. Some studies have highlighted that high-fat interventions upregulate hepatic GSH-peroxidase (Gpx) activity [19, 67], while others have shown decreased [20, 68] or unchanged [69] enzyme activity. The current study discovered Gpx-1 upregulation in obese AKR and B6 mice compared to their respective controls, and these findings are consistent with previously published data [67]. D2 mice exhibited a unique gene expression profile, including downregulation of Gr, which could explain the diet-induced decrease in GSH/GSSG. High-fat fed D2 mice also showed upregulation of Gclm and no effect on Gpx-1 levels. The biological relevance of these gene expression profiles should be carefully considered, especially with regard to Gpx-1. Upregulation of Gpx-1 is associated with insulin resistance and obesity [70, 71], while Gpx-1 deficiency enhances insulin sensitivity [71] and protects against diet-induced hepatic steatosis [72]. One may therefore predict that stability of Gpx-1 levels is essential for resistance to these deleterious effects in D2 mice. The rationale is supported by previous results showing that hepatic steatosis is more severe in the B6 strain compared to D2 [73], but conclusions cannot be drawn because constitutive Gpx-1 levels are higher in D2 mice compared to the other strains. The relative importance of basal versus upregulated levels of Gpx-1 is currently unknown.

Overall, this study demonstrates that a high-fat diet regulates the GSH system in a strain- and tissue- dependent manner. These findings propose a novel mechanism by which the risks for

comorbidities associated with obesity are inherited, and these relationships should be further explored. Our findings also suggest that the D2 strain is a suitable model for diet-induced hepatic redox disruption. Future studies will clarify the mechanisms by which genotype and dietary fat intersect to control the GSH system.

Acknowledgment

The authors gratefully acknowledge Anesia Reticker, Yang Zhou, Gene Rodrick, and Roger Nilsen for their contributions to this project.

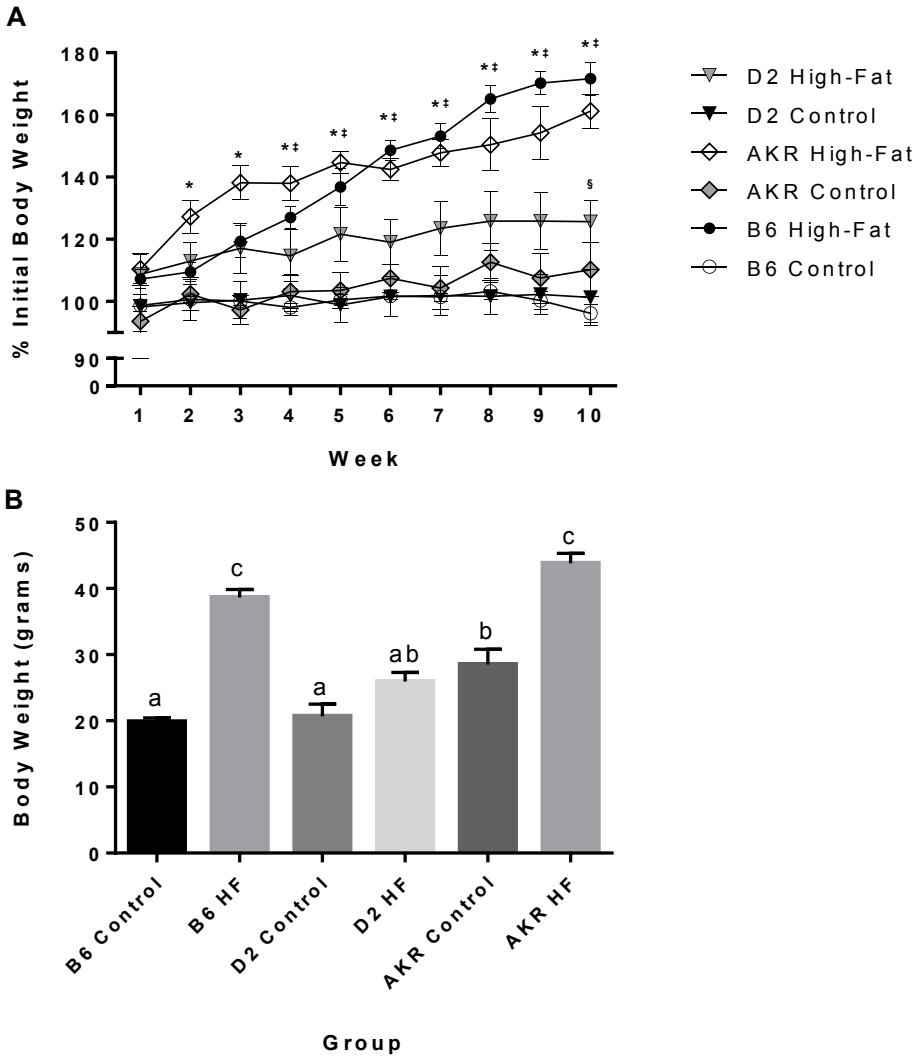

Figure 1. Percent initial body weight and end body weights of mice fed a control or high-fat diet for 10 wk. (A) Percent initial body weight (B) Body weights of mice after ten weeks of control or high fat feeding. Data are reported as means ± standard error of mean (SEM). Two-way ANOVA with Bonferroni adjustments were used to determine significant differences across all groups at each time point. For clarity, statistical differences are only noted between the control and high-fat group for each strain in panel A, * $P < 0.05$ for AKR, ‡ $P < 0.05$ for B6, and § $P < 0.05$ for D2. In panel B, means without a common letter differ, $P < 0.05$. Abbreviations: HF, high-fat.

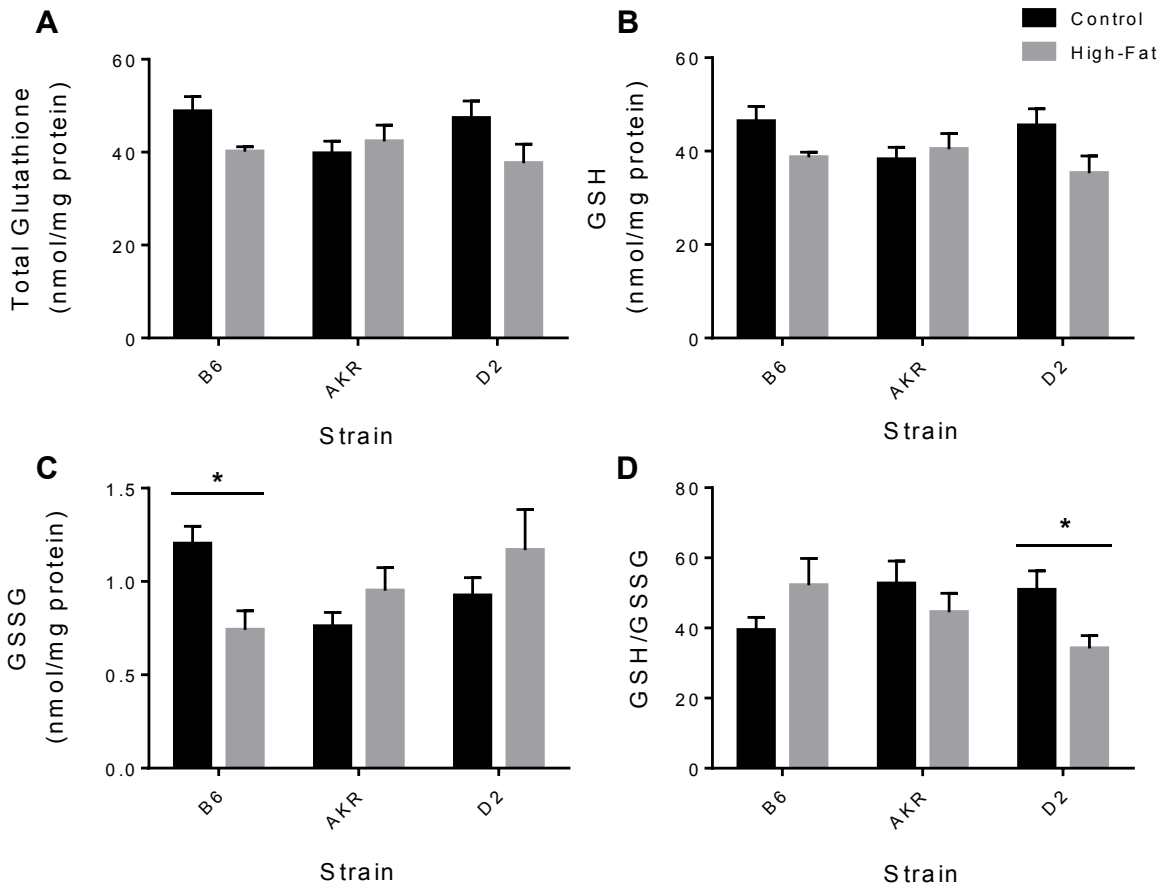

Figure 2.2. Hepatic glutathione homeostasis in mice fed a control or high-fat diet for 10 wk. (A) Liver total glutathione standardized to total protein (B) Liver GSH levels standardized to total protein (C) Liver GSSG levels standardized to total protein (D) Liver GSH/GSSG Ratio. Data are reported as means ± standard error of mean (SEM). Independent t-tests were conducted to determine significance within each strain in response to C3G treatment, * $P < 0.05$, ** $P < 0.01$.

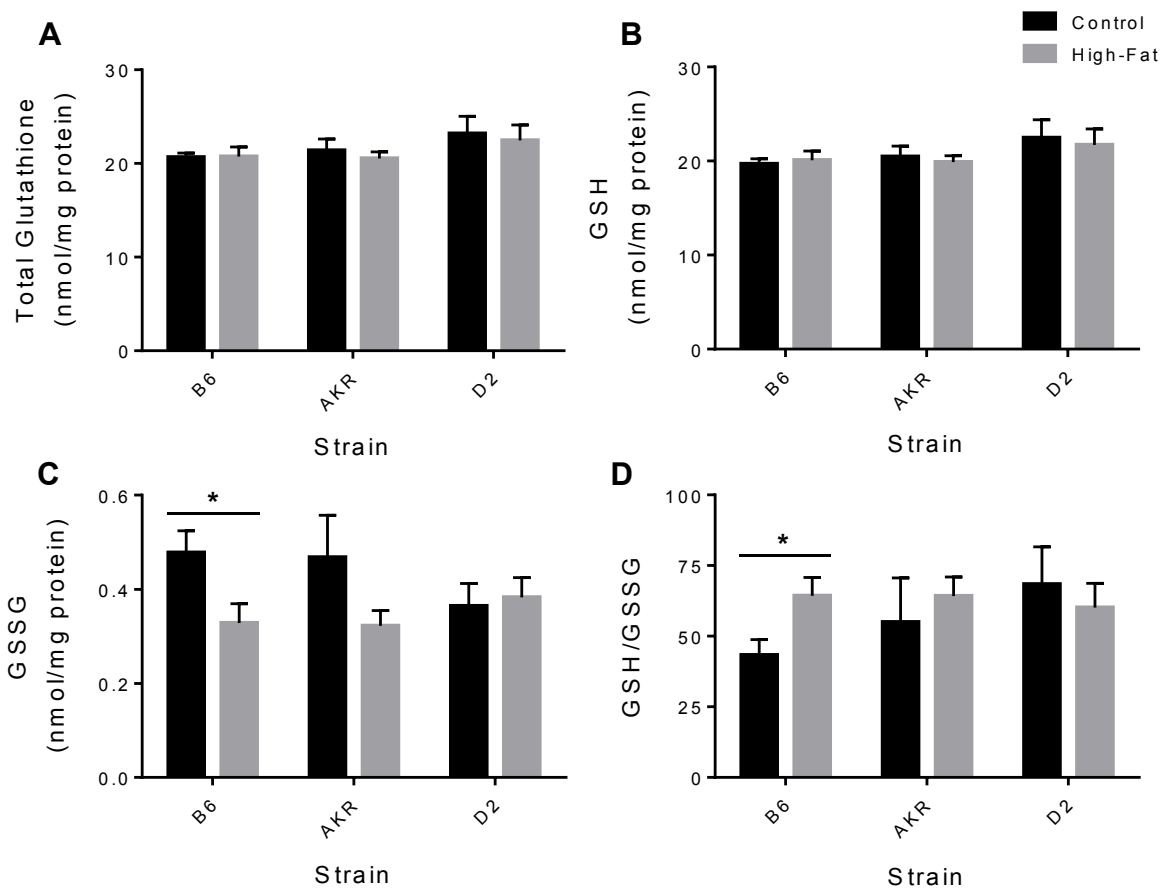

Figure 2.3. Renal glutathione homeostasis in mice fed a control or high-fat diet for 10 wk. (A) Kidney total glutathione standardized to total protein (B) Kidney GSH levels standardized to total protein (C) Kidney GSSG levels standardized to total protein (D) Kidney GSH/GSSG Ratio. Data are reported as means ± standard error of mean (SEM). Independent t-tests were conducted to determine significance within each strain in response to C3G treatment, * $P < 0.05$.

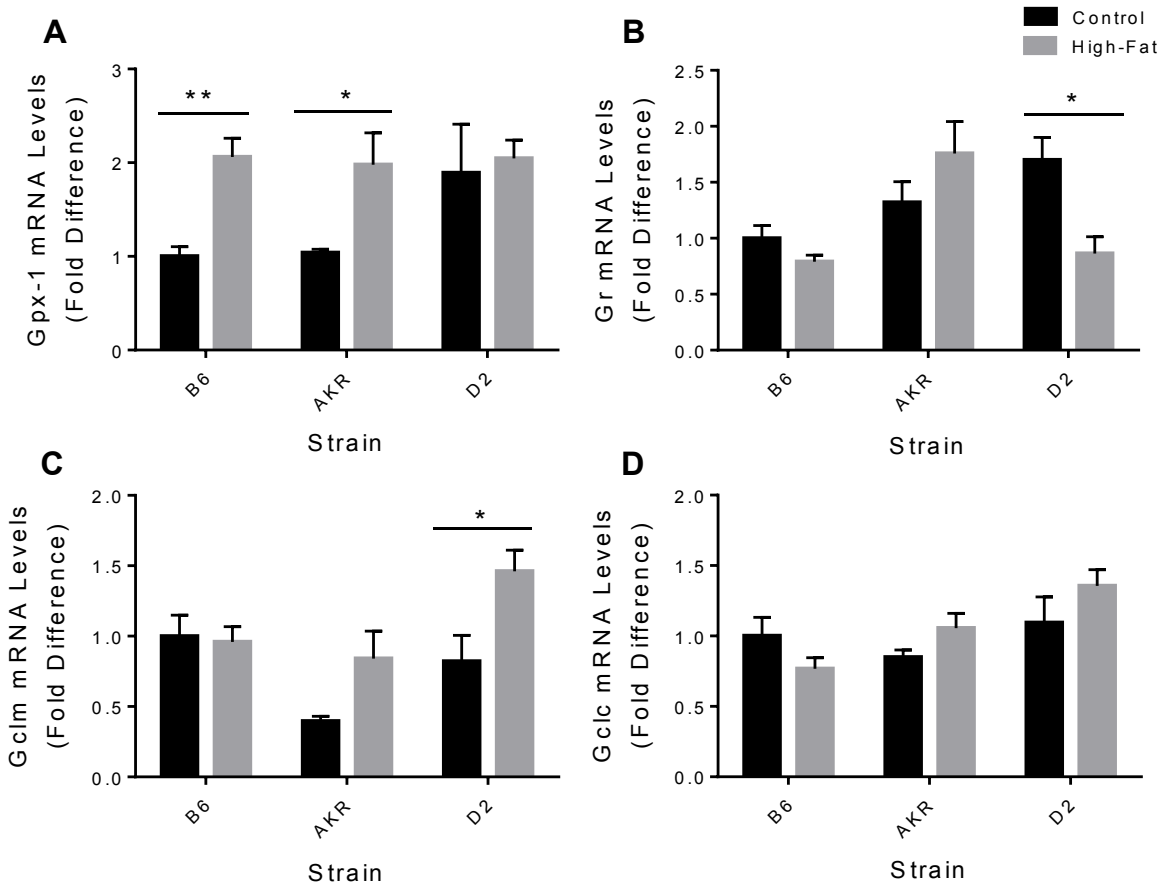

Figure 2.4. Hepatic expression of glutathione enzymes in mice fed a control or high-fat diet for 10 wk. (A) Gpx-1 mRNA (B) Gr mRNA (C) Gclm mRNA (D) Gclc mRNA. Data represent fold expression relative to the B6 control group and are reported as means ± standard error of mean (SEM). Independent t-tests were conducted to determine significance within each strain in response to C3G treatment, * $P < 0.05$.

Table 2.1. Composition of experimental diets. Values are reported as percent of ration, except where otherwise noted. Diets were purchased from TestDiet.

Ingredients (Values reported as % of ration)	**Control**	**High-fat**
Sucrose	33.129	8.847
Dextrin	29.856	---
Casein	18.956	25.845
Powdered Cellulose	4.739	6.461
Maltodextrin	3.317	16.153
Soybean Oil	2.37	3.231
Lard	1.896	31.66
Potassium Citrate, Tribasic Monohydrate	1.564	2.132
Calcium Phosphate	1.232	1.68
DIO Mineral Mix	0.948	1.292
AIN-76A Vitamin Mix	0.948	1.292
Calcium Carbonate	0.521	0.711
L-Cystine	0.284	0.388
Choline Bitartrate	0.19	0.258
Lipid Profile		
Saturated Fatty Acid	1.14	13.68
Monounsaturated Fatty Acid	1.3	14
Polyunsaturated Fatty Acid	1.77	5.54
Energy (kcal/g diet)	3.76	5.1
% Energy from Fat	10.2	61.6
% Energy from Carbohydrate	71.8	20.3
% Energy from Protein	18	18.1

Table 2.2. Primers used for real-time qPCR.

Gene	Forward Primer Sequence	Reverse Primer Sequence
Gclm	CACAATGACCCGAAAGAACTG	AGACTTGATGATTCCCCTGCT
Gclc	CCTCCTCCTCCAAACTCAGATA	CCACAAATACCACATAGGCAGA
Gpx-1	CCCGTGCAATCAGTTC	TTCGCACTTCTC AAACAA
Gr	GGTGGTGGAGAGTCACAAGC	ATCGTGCATGAATTCCGAGT
β-actin	AGCCATGTACGTAGCCATCC	CTCTCAGCTGTGGTGGTGAA

Table 2.3. Food intake of mice fed a control or high-fat diet for 10 wk. Data are reported as means ± standard error of mean (SEM). One-way ANOVA with Bonferroni adjustments were used to determine significant differences across all groups. Means without a common letter are statistically different, $P < 0.05$. Abbreviations: HF, high-fat.

Food Intake	B6 Control	B6 HF	D2 Control	D2 HF	AKR Control	AKR HF
(grams/mouse/day)	2.21 ± 0.229[a]	Irregular Food Behavior	2.41 ± 0.0588[abc]	2.10 ± 0.0529[a]	2.91 ± 0.113[bc]	3.02 ± 0.252[c]

CHAPTER 3

THE ANTHOCYANIN CYANIDIN-3-*O*-β-GLUCOSIDE MODULATES MURINE GLUTATHIONE HOMEOSTASIS IN A MANNER DEPENDENT ON GENETIC BACKGROUND[2]

[2] Norris, K.M., W. Okie, C.L. Yakaitis, and R. Pazdro. Submitted to *Redox Biology*, 06/30/16.

Abstract

Anthocyanins are phytochemicals that have generated considerable interest due to their reported health benefits. It has been proposed that commonly consumed anthocyanins, such as cyandin-3-*O*-β-glucoside (C3G), confer cellular protection by stimulating biosynthesis of glutathione (GSH), an endogenous antioxidant. It is unknown whether anthocyanin-induced health effects are subject to genetic differences. Thus, we tested the hypothesis that anthocyanin-induced alterations in GSH homeostasis vary by genetic background. Mice representing five genetically diverse inbred strains (A/J, 129S1/SvImJ, CAST/EiJ, C57BL/6J, and NOD/ShiLtJ) were assigned to a control or 100 mg/kg C3G diet (n=5/diet/strain) for six weeks. GSH and GSSG levels were quantified in liver, kidney, heart, pancreas, and brain using HPLC. The C3G diet promoted an increase in renal GSH concentrations, hepatic GSH/GSSG, and cardiac GSH/GSSG in CAST/EiJ mice. C3G treatment also induced an increase in pancreatic GSH/GSSG in C57BL/6J mice. In contrast, C3G did not affect GSH homeostasis in NOD/ShiLtJ mice. Surprisingly, the C3G-diet caused a decrease in hepatic GSH/GSSG in A/J and 129S1/SvImJ mice compared to controls. C3G-treated 129S1/SvImJ mice also exhibited lower total glutathione in the heart. To our knowledge, this study is the first to show that the biochemical effects of anthocyanins are determined by genetic background.

Keywords: Oxidative stress, anthocyanins, cyandin-3-*O*-β-glucoside, polyphenols, phytochemicals, antioxidants, glutathione, glutathione disulfide, redox status

Introduction

Anthocyanidins and their glycosides, anthocyanins, are flavonoids that contribute to the blue, purple, and red color of many fruits and vegetables, such as blueberries, blackberries, and purple corn [27]. Integration of foods rich in these compounds appears to modulate chronic disease risk in humans. High dietary anthocyanidin intakes have been associated with lower levels of C-reactive protein (CRP), a circulating predictor of cardiovascular disease (CVD) [74], as well as decreased risk of CVD-related mortality [75, 76]. However, evidence suggests that the relationship between anthocyanidin intake and health outcomes is not entirely consistent. For example, Mursu, et al., found no relationship between anthocyanidin intake and CVD mortality in a cohort of Finnish men [77]. To improve understanding of the relationships between anthocyanidins, related anthocyanins, and health, factors that contribute to inconsistent epidemiological data must be clarified.

Important insight regarding the relationship between anthocyanin intake and health has been gained from various disease models. In models of diabetes [13], obesity [28, 29], cancer [30, 31], and metabolic syndrome [32], anthocyanins decrease markers of oxidative stress. This effect is attributable, in part, to their strong antioxidant activity. These compounds are potent free radical scavengers [33-37] and concurrently increase cellular levels of glutathione (GSH), the most abundant endogenous thiol antioxidant. For example, cyanidin-3-*O*-β-glucoside (C3G), a commonly consumed anthocyanin, increases hepatic GSH levels nearly threefold in a mouse model of type 2 diabetes (T2D) [13]. Recent studies have demonstrated that, in addition to diet, genetic background regulates tissue GSH levels and redox status (GSH/GSSG) [41, 42]. We predict that the genetic and dietary regulation of the GSH system intersect, and specifically, that

the effects of C3G on the GSH system are genetically determined. Furthermore, this hypothesis implies that genetic variation confounds the results from previous epidemiological studies.

The current study tested whether anthocyanin effects on GSH redox status are influenced by genetic background. We predicted that C3G-induced increases in GSH levels and GSH/GSSG would vary by mouse strain, and furthermore, that that these redox effects would be concentrated to organs that metabolize and excrete C3G, notably liver and kidney. Mice representing five inbred strains (C57BL/6J, A/J, 129S1/SvImJ, NOD/ShiLtJ, and CAST/EiJ) were fed either control or 100 mg/kg C3G diets for six weeks. These five strains were chosen because of their relative genetic diversity, a characteristic that has been utilized in previous studies and during the generation of novel genetic reference populations [78]. Tissue concentrations of GSH and GSSG were quantified by HPLC, and expression of hepatic GSH-related enzymes was determined by qPCR. We discovered that C3G modulates the GSH system in a strain- and tissue-specific manner. We observed divergent responses to C3G; two strains exhibited increases in GSH levels while two others exhibited GSH depletion. Our findings demonstrate that genetic background is a critical determinant of redox effects related to anthocyanin-rich diets. Future studies will clarify the genetic mechanisms that mediate anthocyanin effects, which may inform differential responses to these compounds.

Methods

Animals

Female C57BL/6J (B6), A/J (A), 129S1/SvImJ (129), NOD/ShiLtJ (NOD), and CAST/EiJ (CAST) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Five mice from each strain were assigned to the control diet, and an additional five mice were assigned to the C3G diet (100 mg/kg). Mice were housed in an animal room on a 12 h light/dark cycle. At

approximately 18 weeks of age, the mice were humanely euthanized by cervical dislocation, and tissues were harvested for analyses. Prior to euthanasia, all mice were fasted for four hours. The University of Georgia Institutional Animal Care and Use Committee approved all methods and experimental procedures for this study (AUP # A2013 08-011).

Diet

Mice were fed a standard purified AIN-93M mouse diet (control) or AIN-93M plus C3G (100mg/kg). The C3G diet was generated as described previously [13]. Briefly, C3G was obtained from Polyphenols Laboratories AS (Sandnes, Norway), and provided to TestDiet (St. Louis, MO) for diet formulation. Control and C3G diets were pelleted. Dietary interventions were initiated when mice reached three months of age and were sustained for six weeks. During the study period, mice were fed *ad libitum* and given unrestricted access to water. Diets were stored at -20°C, and fresh food was provided weekly to maintain optimal stability of dietary C3G. Food intake and weights of the mice were also measured on a weekly basis.

Assessment of Total Glutathione, GSH, GSSG, and GSH/GSSG Ratios

Liver, kidneys, heart, pancreas, and whole brain were removed, rinsed in ice-cold phosphate-buffered saline (PBS), and flash-frozen in liquid nitrogen. Within 24 hours after collection, tissues were homogenized and immediately acidified. Following centrifugation, acidified supernatants were flash-frozen in liquid nitrogen and stored at -80°C until analysis. GSH and GSSG were quantified by high performance liquid chromatography (HPLC) coupled with electrochemical detection (Dionex UltiMate 3000, Thermo Scientific, Waltham, MA). The cells were set at 1600 mV with a cleaning potential of 1900 mV between samples. The mobile phase was composed of 4.0% acetonitrile, 0.1% pentafluoropropionic acid, and 0.02% ammonium hydroxide; a flow rate of 0.5 ml/min was set. An injection volume of 2.0 µL was used for liver

and kidney samples, while 3.0 μL was used for heart, pancreas, and brain samples. After electrochemical detection, data were quantified by the Chromeleon Chromatography Data System Software (Dionex Version 7.2, Thermo Scientific). Total glutathione was calculated as GSH + 2GSSG, and glutathione redox status was assessed by the ratio GSH/GSSG. GSH and GSSG concentrations were standardized to total protein, which was quantified by Pierce BCA Protein Assay (Thermo Fisher Scientific, Rockford, IL).

Assessment of Endogenous Antioxidant Enzyme Expression

Total RNA was extracted from flash-frozen liver using TRIzol reagent (Thermo Scientific), and cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Thermo Scientific) according to the manufacturer's instructions. SYBR Green MasterMix (BIO-RAD Life Science Research, Hercules, CA) was used to determine relative gene expression of glutamate-cysteine ligase modifier subunit (*Gclm*; Forward: CACAATGACCCGAAAGAACTG; Reverse: AGACTTGATGATTCCCCTGCT), glutamate-cysteine ligase catalytic subunit (*Gclc*; Forward: CCTCCTCCTCCAAACTCAGATA; Reverse: CCACAAATACCACATAGGCAGA), glutathione peroxidase-1 (*Gpx-1*; Forward: CCCGTGCAATCAGTTC; Reverse: TTCGCACTTCTC AAACAA), and glutathione reductase (*Gr*; Forward: GGTGGTGGAGAGTCACAAGC; Reverse: ATCGTGCATGAATTCCGAGT) using RT-PCR. SYBR green fluorescence was detected by a LightCycler 480 II (Roche Life Science). Target gene expression was normalized using β-actin (Forward: AGCCATGTACGTAGCCATCC; Reverse: CTCTCAGCTGTGGTGGTGAA) as a reference, and all samples were run in triplicate. Quantitative fold-changes were derived using the ΔΔCt method and are presented as the fold-change relative to the B6 control group.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism (Version 6.0, GraphPad Software Inc., La Jolla, CA, USA). Body weight and food intake analyses were completed using two-way ANOVA with Bonferroni adjustments. Independent t-tests were used to identify which strains exhibited altered total glutathione levels, GSH and GSSG concentrations, GSH/GSSG ratios, and expression of GSH-related enzymes in response to C3G treatment. The level of statistical significance was defined at $P < 0.05$. Data are reported as means ± standard error of mean (SEM).

Results

Food Intake and Body Weight

Throughout the six-week duration of the study, there was no significant difference in food intake between C3G-fed mice and their respective controls (Figure 3.1). However, a strain effect was observed. NOD mice consumed significantly more food than all other strains, but the NOD treatment groups were not significantly different from one another. Additionally, no differences in body weight changes were observed between groups assigned to the control and C3G diets (Table 3.1). CAST mice exhibited lower true body weights than the other strains (data not shown). These effects were expected, as CAST mice are wild-derived and are known to be smaller than classical inbred strains.

GSH and GSSG Levels, Total Glutathione, and Redox Status

In response to C3G treatment, CAST mice showed a twofold increase in hepatic GSH/GSSG ($P = 0.041$; Figure 3.2D) and a nearly fourfold increase in cardiac GSH/GSSG ($P = 0.028$; Figure 3D). Renal total glutathione ($P = 0.014$) and GSH concentrations ($P = 0.010$) increased in C3G-treated CAST mice (Figure 3.4A,B), and pancreatic levels of oxidized glutathione, GSSG, were lower in the C3G group compared to control CAST mice ($P = 0.021$) (Figure 3.5C). The

difference in cardiac GSH concentrations between control and C3G-treated CAST mice approached statistical significance ($P = 0.056$), but did not achieve it.

B6 mice were largely unresponsive to C3G treatment. The exception was B6 pancreas samples, where C3G-fed mice had higher GSH/GSSG than controls ($P = 0.042$; Figure 3.5D). However, this effect was modest, only accounting for a 12.5% increase in GSH/GSSG. NOD mice exhibited no phenotypic differences due to C3G treatment, and no significant differences in GSH levels were found within the brains of any of the five strains (Figure 3.6).

Surprisingly, the C3G diet caused a 40% decrease in hepatic GSH/GSSG in A mice ($P = 0.017$) and a 43% decrease in 129 mice ($P = 0.0066$) (Figure 3.2D). These effects appear to be driven by distinct mechanisms. In A mice, the C3G diet caused a decline in hepatic GSH concentrations ($P = 0.044$) while GSSG levels remained stable (Figure 3.2B,C). In contrast, C3G-fed 129 mice displayed increased hepatic GSSG levels ($P = 0.020$) while GSH levels were unaffected (Figure 3.2B,C). 129 mice also contained lower cardiac GSH concentrations ($P = 0.033$) and approximately 25% less total glutathione ($P = 0.034$) following the C3G dietary intervention (Figure 3.4A,B).

Hepatic Expression of GSH-Related Enzymes

Hepatic expression of *Gclc*, *Gclm*, and *Gr* was not significantly different in response to C3G treatment in any of the five strains (Figure 3.7A,B,C). A, CAST, B6, and NOD did not exhibit altered expression of *Gpx-1* in response to C3G supplementation (Figure 3.7D). C3G-treated 129 mice did exhibit a twofold increase in *Gpx-1* expression compared to controls ($P = 0.023$) (Figure 3.7D), which was the only significant change in antioxidant gene expression discovered in this study.

Discussion

Rationale for the current study was informed by conflicting evidence surrounding chronic disease risk and its relationship with phytochemical intake. For example, total flavonoid intake was found to exhibit an inverse association with stroke incidence [79] and mortality [76], while in other studies, flavonoid intake has exhibited no correlation with risk [80, 81] or mortality [82]. The effect of flavonoid intake on cancer risk has also shown disparate findings. No association was discovered between anthocyanidin consumption and gastric cancer risk in a Korean population [83], but a significant inverse correlation was found in European women [84]. The relationship between T2D and anthocyanidin/anthocyanin intake is similarly uncertain. One study on U.S. adults found an inverse correlation between T2D risk and anthocyanin consumption [85], while a European case-cohort study found no correlation with T2D risk and anthocyanidin intake [86]. Importantly, the inconsistencies highlighted in these studies have been captured in clinical intervention studies as well [87-98]. In both chronic and acute intervention trials, the effects of anthocyanin consumption on endogenous antioxidant enzyme activity, plasma antioxidant capacity, and DNA damage have been inconsistent [99]. We predict that genetic variation in part drives the variable responses to dietary anthocyanins, and we tested our hypothesis in the current study. We fed mice representing five genetically diverse strains a control or 100 mg/kg C3G diet. Overall food intake did not differ between controls and C3G-fed animals. Furthermore, NOD consumed the largest amounts of C3G diet, yet these mice did not respond in any of the assessments we measured. We therefore concluded that results from the current study were not confounded by strain-dependent differences in food intake.

We tested whether genetic background determines the extent to which C3G regulates GSH levels and redox status. The C3G diet increased pancreatic GSH/GSSG in B6 mice, but this

difference represented a relatively minor alteration. The C3G diet exerted no other effects on the GSH system of this strain. Upon initial review, our results appear to conflict with work by Zhu and colleagues, who showed that the same C3G diet increases hepatic GSH synthesis nearly threefold in the same genetic background [13]. However, it must be noted that Zhu, et al., employed *db/db* mice, which contain a spontaneous mutation on the B6 or C57BLKS/J background that drives a diabetic phenotype. In that study, GSH levels were compared between *db/db* mice fed control and C3G diets; unstressed wild-type B6 control mice were not included in the design. If unstressed, wild-type B6 mice had been evaluated, as in this study, we predict that a similar lack of effect would have been observed. Taken together, these results suggest that the C3G diet does not alter GSH levels in unstressed B6 mice, and may only rescue GSH levels in stressed, mutant B6 mice. Similarly, the relationship between flavonoid intake and disease risk in some human populations may require a stressor. Cutler, et al., found that flavanone intake was inversely correlated with lung cancer incidence among current and past smokers, but the relationship was not observed among individuals who had never smoked [100].

The effects of stress on the C3G-GSH paradigm must be further evaluated in the context of genetic background. Although C3G rescues GSH levels in diabetic B6 mice, the current study showed no effect of this diet on NOD mice, an established model of type 1 diabetes. In contrast, the most potent GSH-inducing effects were observed in CAST mice. CAST is not a model of a specific disease, but these mice appear to exhibit deficiencies within the GSH redox system. Our previous reports identified CAST as having among the lowest GSH levels and GSH/GSSG in a large panel of inbred strains [41]. C3G appears capable of rescuing redox deficiencies in B6 and CAST backgrounds, but it has no effect on the diabetic NOD mice. We predict that genetic background provides a platform on which stress and diet modulate GSH levels (Figure 3.8). We

also predict that these effects are independent of gene expression due to the minimal changes in hepatic GSH-related enzyme expression observed here.

This study demonstrated that GSH levels and GSH/GSSG can decrease in response to an established C3G-rich diet [13]. The C3G diet caused apparent disruptions in GSH homeostasis in 129 and A mice, and the effect was most apparent in the liver, suggesting oxidative stress and hepatotoxicity [101-104]. Several polyphenols are known to exert toxicity at high levels [105-107], and in the case of epigallocatechin gallate (EGCG), a polyphenol present in green tea, toxicity is determined by genetic background [107]. As use of dietary supplements continues to grow considerably in the United States, it will be critical to further characterize the genetic mechanisms that drive hepatotoxicity attributable to polyphenols such as EGCG and C3G. It will also be important to elucidate whether distinct mechanisms direct toxicity of each compound.

We tested the hypothesis that the redox effects of C3G would be limited to the liver and kidney due to the primary role of these organs in phytochemical metabolism and clearance. Our hypothesis was partially confirmed because the most significant effects were discovered in liver. The kidney, as well as the heart and pancreas, showed fewer and less pronounced effects on GSH homeostasis. The brain exhibited no effects, and we predicted this outcome based on the function of the blood-brain barrier. Overall, our data support a tissue-specific effect of anthocyanins on GSH homeostasis. To our knowledge, this is the first study to show that the biochemical effects of anthocyanins are determined by genetic background. Our long-term hypothesis predicts that anthocyanins differentially affect humans based on their genetics. If that hypothesis is correct, it may highlight the underlying reason for inconsistent findings in previous epidemiological and clinical studies. Furthermore, such findings would indicate that anthocyanin supplementation may cause toxicity in a highly susceptible subpopulation. Overall, our data will inform future

efforts to clarify genetic mechanisms that regulate differential responses to ingested anthocyanins.

Acknowledgments

The authors wish to thank Woo-Kyun Kim and Roshan Adhikary for their help with creating cDNA libraries. We also wish to thank Roger Nilsen for his technical assistance with PCR, Zachary Grunewald for his advice on PCR data analysis, and Erica Coe for her help with designing primers.

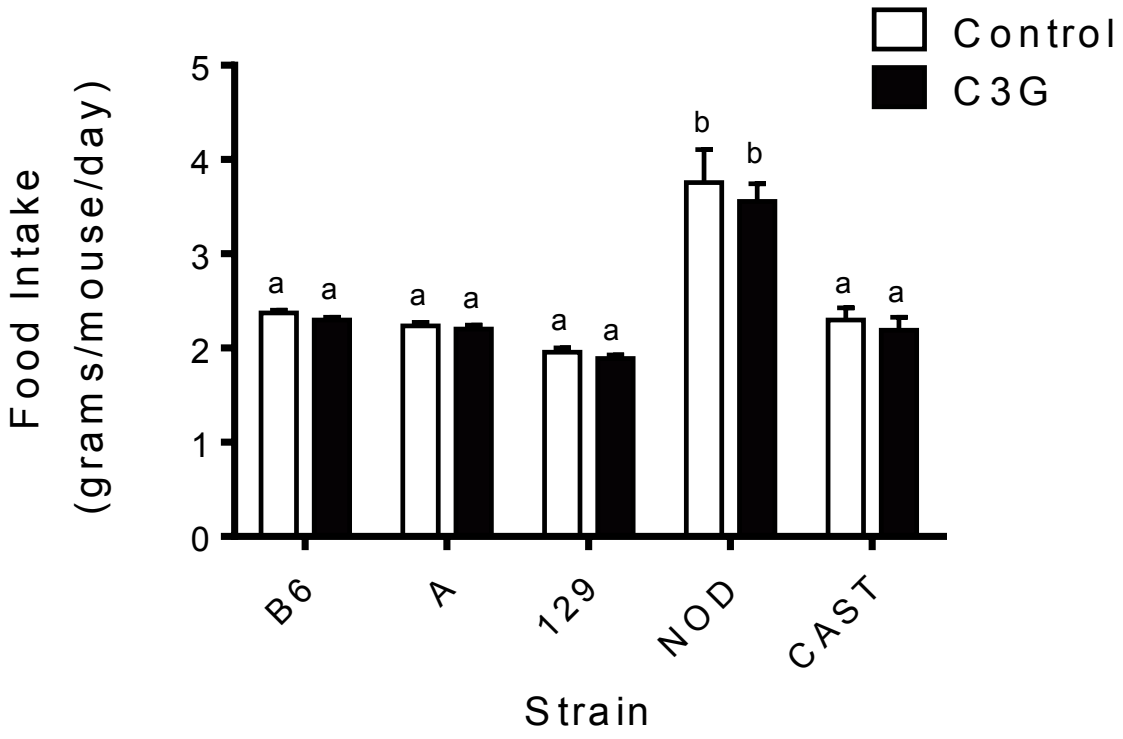

Figure 3.1. Food intake of mice fed a control or high-C3G diet for 6 wk. Data are reported as means ± standard error of mean (SEM). Two-way ANOVA with Bonferroni adjustments were used to determine significant differences across all groups. Means without a common letter are statistically different, $P < 0.05$.

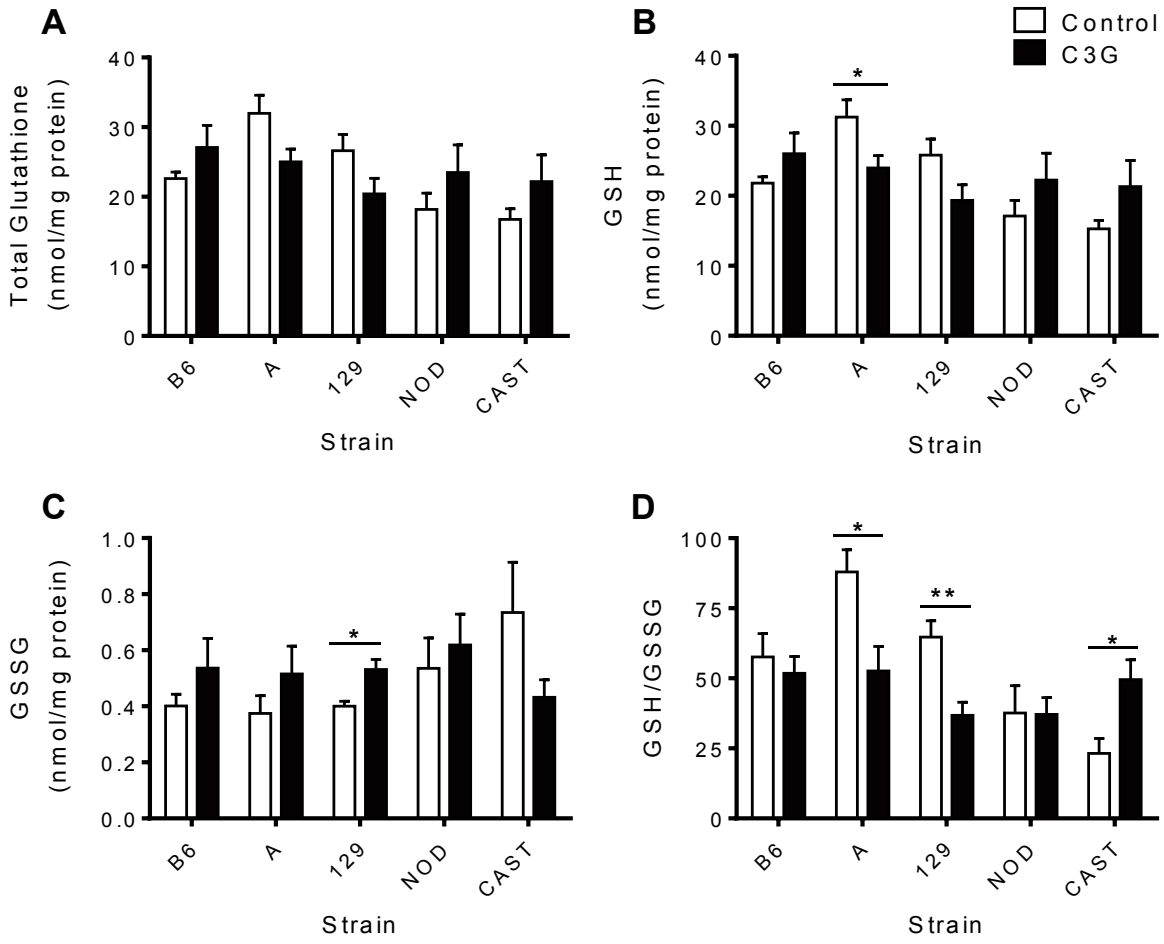

Figure 3.2. Hepatic glutathione homeostasis in mice fed a control or high-C3G diet for 6 wk. (A) Liver total glutathione standardized to total protein (B) Liver GSH levels standardized to total protein (C) Liver GSSG levels standardized to total protein (D) Liver GSH/GSSG Ratio. Data are reported as means ± standard error of mean (SEM). Independent t-tests were conducted to determine significance within each strain in response to C3G treatment, * $P < 0.05$, ** $P < 0.01$.

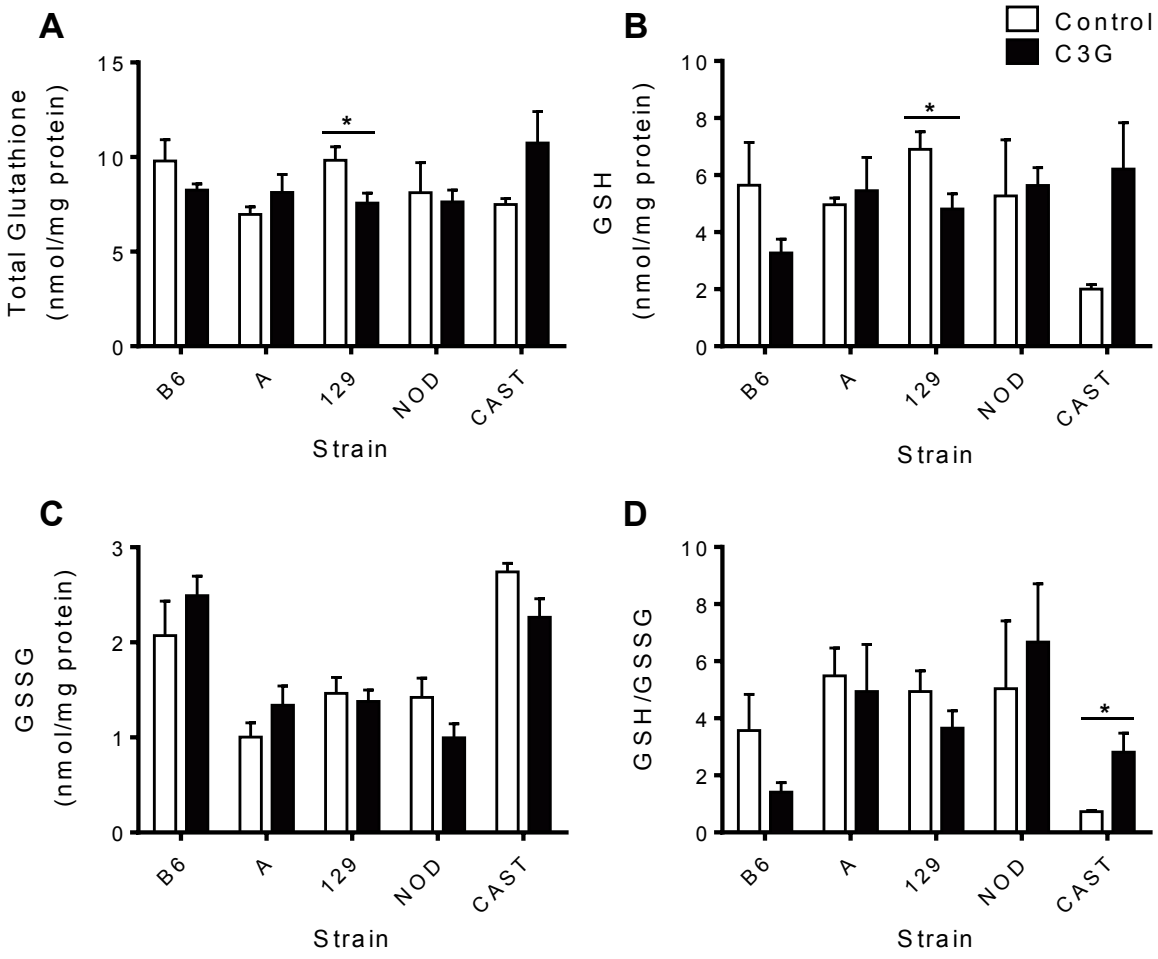

Figure 3.3. Glutathione homeostasis in hearts from mice fed a control or high-C3G diet for 6 wk. (A) Heart total glutathione standardized to total protein (B) Heart GSH levels standardized to total protein (C) Heart GSSG levels standardized to total protein (D) Heart GSH/GSSG Ratio. Data are reported as means ± standard error of mean (SEM). Independent t-tests were conducted to determine significance within each strain in response to C3G treatment, * $P < 0.05$.

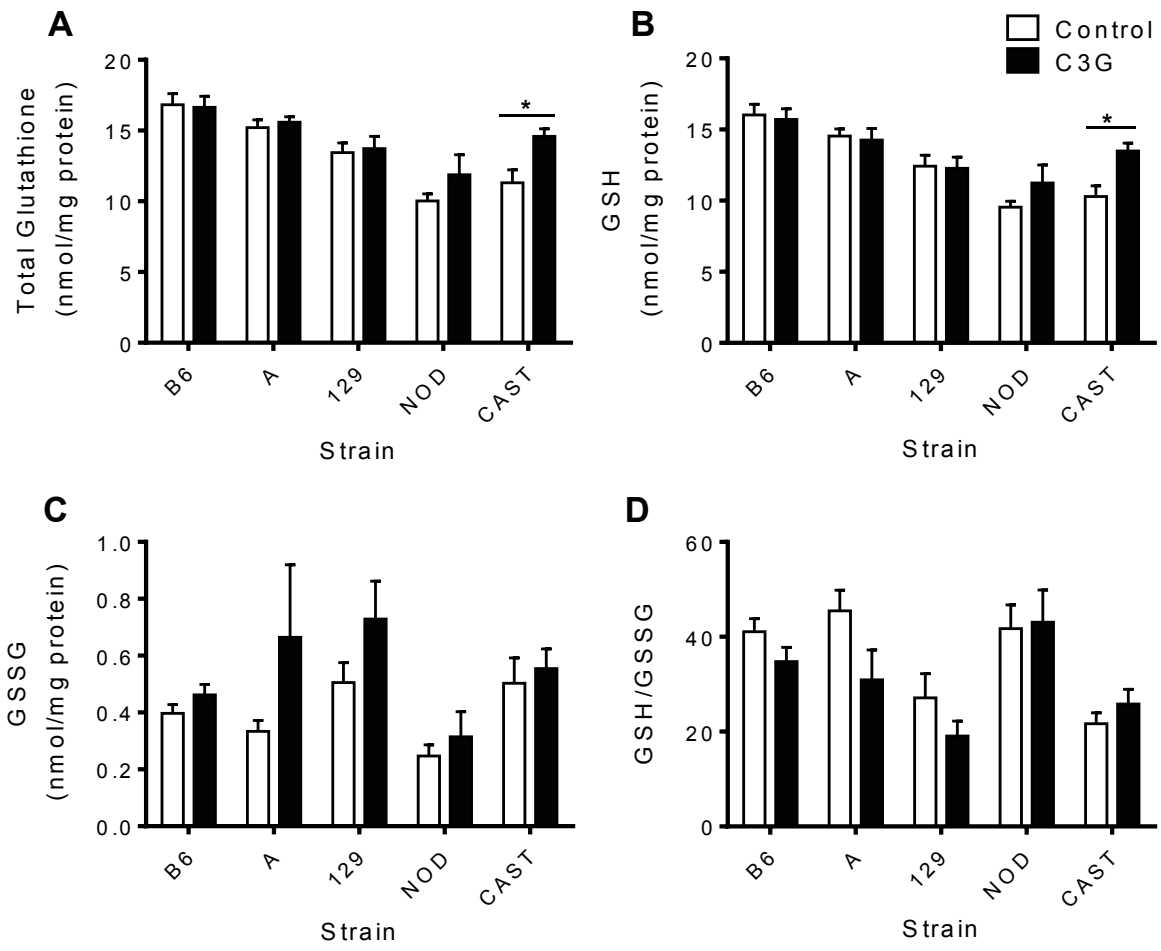

Figure 3.4. Renal glutathione homeostasis in mice fed a control or high-C3G diet for 6 wk. (A) Kidney total glutathione standardized to total protein (B) Kidney GSH levels standardized to total protein (C) Kidney GSSG levels standardized to total protein (D) Kidney GSH/GSSG Ratio. Data are reported as means ± standard error of mean (SEM). Independent t-tests were conducted to determine significance within each strain in response to C3G treatment, * $P < 0.05$.

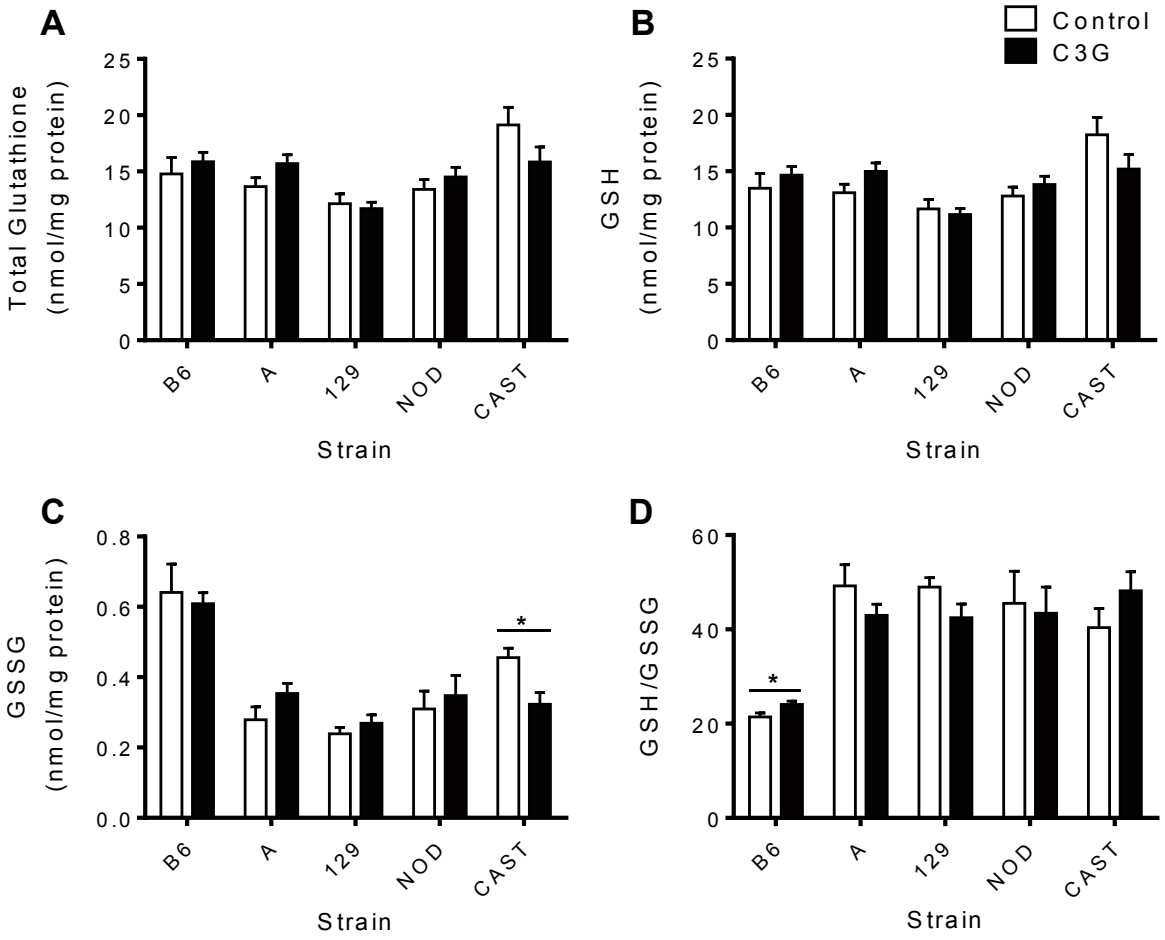

Figure 3.5. Pancreas glutathione homeostasis in mice fed a control or high-C3G diet for 6 wk. (A) Pancreas total glutathione standardized to total protein (B) Pancreas GSH levels standardized to total protein (C) Pancreas GSSG levels standardized to total protein (D) Pancreas GSH/GSSG Ratio. Data are reported as means ± standard error of mean (SEM). Independent t-tests were conducted to determine significance within each strain in response to C3G treatment, * $P < 0.05$.

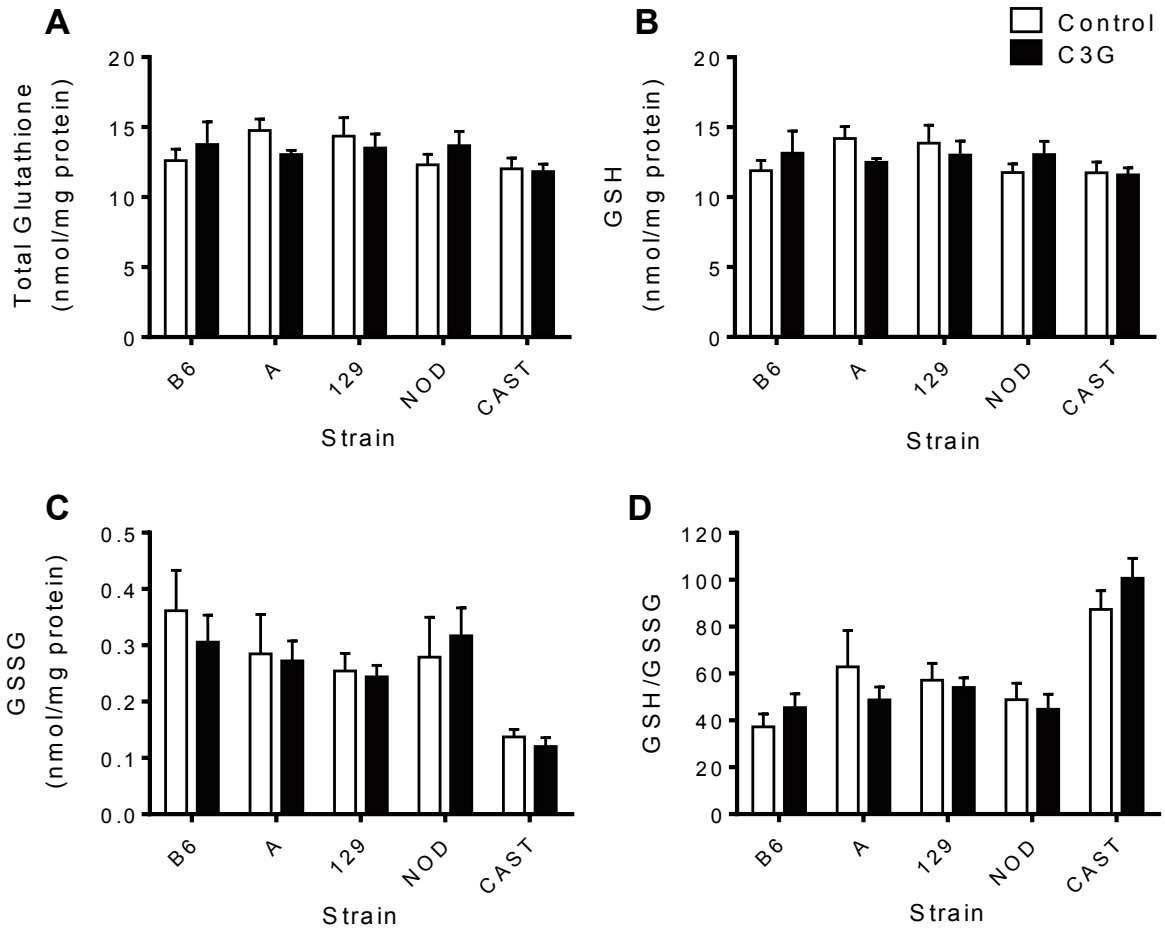

Figure 3.6. Whole brain glutathione homeostasis in mice fed a control or high-C3G diet for 6 wk. (A) Whole brain total glutathione standardized to total protein (B) Whole brain GSH levels standardized to total protein (C) Whole brain GSSG levels standardized to total protein (D) Whole brain GSH/GSSG ratio. Data are reported as means ± standard error of mean (SEM). Independent t-tests were conducted to determine significance within each strain in response to C3G treatment, * $P < 0.05$.

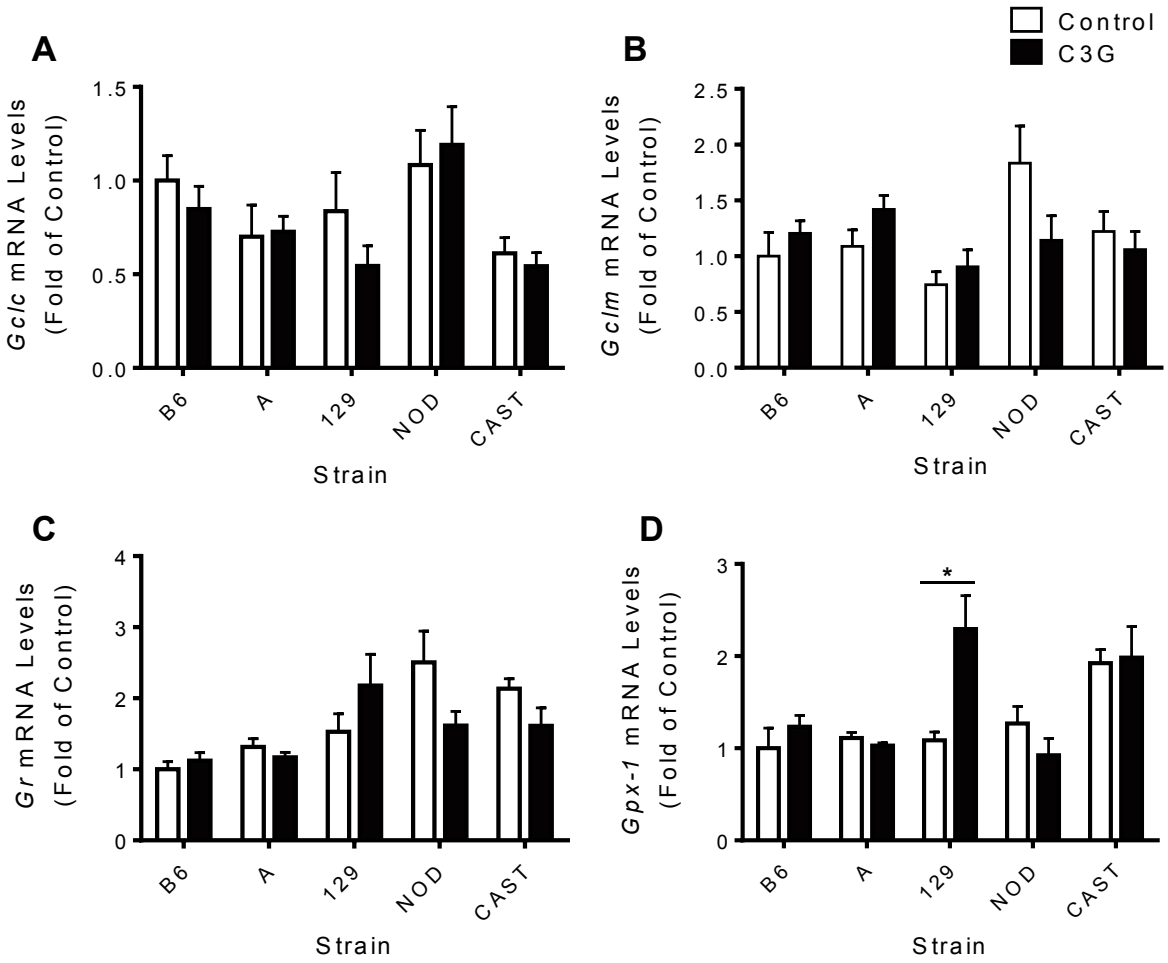

Figure 3.7. Hepatic expression of glutathione enzymes in mice fed a control or high-C3G diet for 6 wk. (A) *Gclc* mRNA (B) *Gclm* mRNA (C) *Gr* mRNA (D) *Gpx-1* mRNA. Data represent fold expression relative to the B6 control group and are reported as means ± standard error of mean (SEM). Independent t-tests were conducted to determine significance within each strain in response to C3G treatment, * $P < 0.05$.

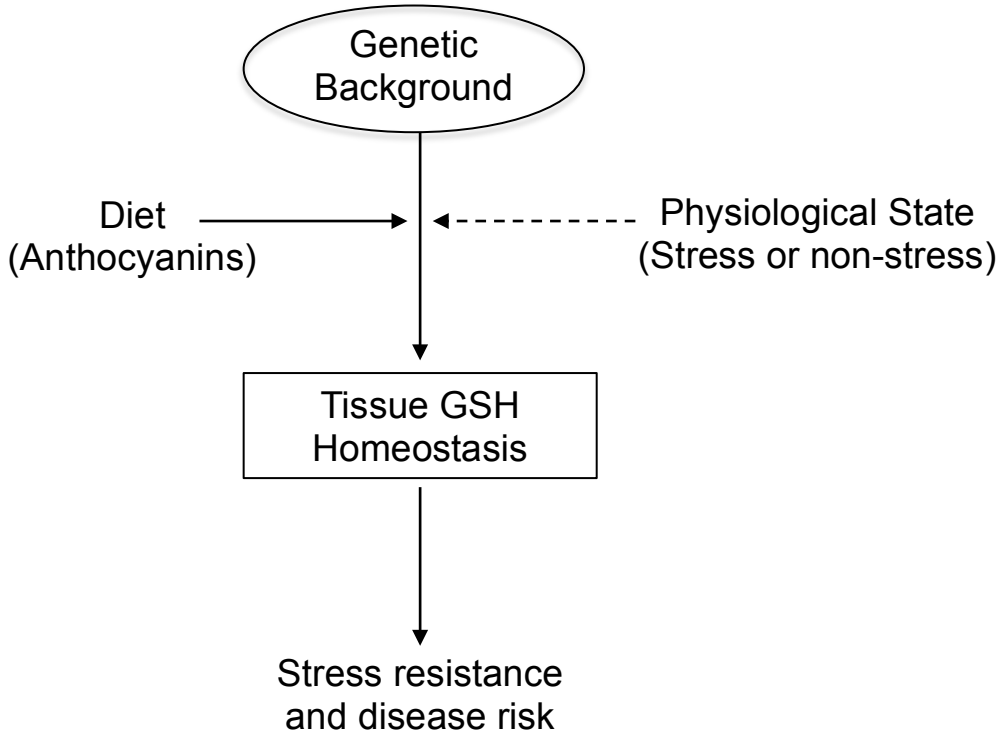

Figure 3.8. Model of genetic regulation of GSH. Genetic background directly regulates GSH homeostasis and determines the relative effects of diet and physiological stress on this system. Together, these interactions influence disease risk.

Table 3.1. Percent initial body weight of mice fed a control or high-C3G diet for 6 weeks.

Data are reported as means ± standard error of mean (SEM). Two-way ANOVA with Bonferroni adjustments were used to determine significant differences across all groups at each time point. Means without a common letter are statistically different, $P < 0.05$.

	% Initial Body Weight					
Group	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
B6 Control	96.0 ± 1.4[a]	96.9 ± 1.2[a]	96.7 ± 0.8[a]	96.5 ± 2.1[ab]	96.8 ± 1.6[a]	99.6 ± 0.7[a]
B6 C3G	97.0 ± 1.9[a]	96.8 ± 2.0[a]	97.3 ± 1.5[a]	97.7 ± 2.2[ab]	99.5 ± 3.2[a]	99.6 ± 1.6[a]
AJ Control	101.0 ± 2.6[a]	101.9 ± 2.4[a]	104.4 ± 2.6[a]	108.4 ± 3.2[a]	106.9 ± 3.7[a]	106.8 ± 3.0[a]
AJ C3G	101.6 ± 4.0[a]	103.1 ± 4.5[a]	106.8 ± 4.5[a]	106.1 ± 4.7[ab]	107.2 ± 4.3[a]	105.9 ± 4.1[a]
129 Control	96.8 ± 5.9[a]	98.8 ± 6.0[a]	103.2 ± 5.0[a]	102.1 ± 5.6[ab]	105.4 ± 5.5[a]	108.2 ± 5.6[a]
129 C3G	93.9 ± 5.3[a]	98.1 ± 5.7[a]	99.3 ± 6.0[a]	99.5 ± 5.8[ab]	101.2 ± 6.1[a]	105.7 ± 6.3[a]
NOD Control	91.9 ± 2.6[a]	96.8 ± 2.8[a]	99.7 ± 2.5[a]	95.5 ± 4.2[ab]	95.8 ± 6.2[a]	89.5 ± 8.1[a]
NOD C3G	96.0 ± 2.6[a]	100.9 ± 4.2[a]	98.8 ± 5.2[a]	96.3 ± 7.4[ab]	93.4 ± 9.4[a]	91.4 ± 10.0[a]
CAST Control	92.0 ± 0.6[a]	91.6 ± 0.4[a]	91.3 ± 0.6[a]	94.8 ± 1.1[ab]	97.9 ± 1.4[a]	102.8 ± 1.7[a]
CAST C3G	90.2 ± 0.9[a]	87.8 ± 1.0[a]	89.3 ± 1.2[a]	87.7 ± 2.2[b]	92.9 ± 2.1[a]	100.2 ± 2.3[a]

CHAPTER 4

CONCLUSIONS

GSH plays a prominent role in attenuating oxidative stress and reducing risk of chronic disease. Although the effects of genetic background, a high-fat diet, and anthocyanin intake on GSH levels and redox status (GSH/GSSG) have been independently established, there is a lack of knowledge about potential interaction between diet and genetic background in regulation of the GSH system. Thus, the current work tested the hypothesis that diet and genetic background interact to modulate GSH homeostasis.

Both chapters of this thesis support the hypothesis that interactions between diet and genetic background determine modulation of the GSH redox system. Furthermore, our data demonstrate that for both a high-fat diet and anthocyanin supplementation, these effects are tissue-specific. We conclude that genetic background directly regulates GSH homeostasis and determines the relative effects of diet and physiological stress on this system in a tissue specific manner. Together, these interactions thereby influence disease risk. We believe our findings highlight the need to strongly consider genetic background in study design. Additionally, we predict that a high fat diet and anthocyanin intake affect humans differently based on their genetics.

One limitation of our findings is the inability to compare the effects of a high-fat diet and anthocyanin supplementation across strains, as mostly different strains were used for the two studies. While it would have been insightful for the same strains to be used in both studies, the mouse strains were chosen to best fit each study design. For example, in the high-fat study, it

was important to compare mouse strains that are known to be susceptible to diet-induced obesity. Alternatively, in the anthocyanin study, it was crucial to compare mouse strains that are more genetically diverse. In future research, however, it may be advantageous to observe D2 and AKR mice on a high anthocyanin diet and to observe 129 and A mice on a high-fat diet. Additionally, a combined study in which different mouse strains received both a high-fat diet and anthocyanin supplementation would be insightful. Ultimately, however, a major goal would be to establish if the interaction of diet and genetic background in regulation of the GSH system translates to humans.

Future work will first seek to identify the genes and alleles that drive divergent GSH responses to dietary components. This can be done using a forward genetics approach, in which mice with variant phenotypes are intercrossed and genetically mapped. Knowing the responsible genes for variable phenotypes can clarify their biological basis. In turn, this information could then be useful for identification of individuals at risk for developing certain diseases and for the development of therapeutic treatments.

In conclusion, our findings demonstrate that interactions between diet and genetic background determine modulation of the GSH redox system in a tissue specific manner. Due to the essential role of the GSH antioxidant system in reducing oxidative stress and augmenting health, our findings improve understanding of how chronic diseases are inherited and provide rationale for further research. Future work will seek to clarify genetic mechanisms that regulate differential responses of the GSH system to diet and may have important implications for chronic disease prevention.

REFERENCES

[1] Lushchak VI. Glutathione homeostasis and functions: potential targets for medical interventions. J Amino Acids. 2012;2012:736837.

[2] Martinon F. Signaling by ROS drives inflammasome activation. Eur J Immunol. 2010;40:616-9.

[3] Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol. 2007;39:44-84.

[4] Rahman K. Studies on free radicals, antioxidants, and co-factors. Clin Interv Aging. 2007;2:219-36.

[5] Dickinson DA, Levonen AL, Moellering DR, Arnold EK, Zhang H, Darley-Usmar VM, et al. Human glutamate cysteine ligase gene regulation through the electrophile response element. Free Radic Biol Med. 2004;37:1152-9.

[6] Zhu W, Jia Q, Wang Y, Zhang Y, Xia M. The anthocyanin cyanidin-3-O-beta-glucoside, a flavonoid, increases hepatic glutathione synthesis and protects hepatocytes against reactive oxygen species during hyperglycemia: Involvement of a cAMP-PKA-dependent signaling pathway. Free Radic Biol Med. 2012;52:314-27.

[7] Liu J, Han L, Zhu L, Yu Y. Free fatty acids, not triglycerides, are associated with non-alcoholic liver injury progression in high fat diet induced obese rats. Lipids Health Dis. 2016;15:27.

[8] Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, et al. Increased oxidative stress in obesity and its impact on metabolic syndrome. J Clin Invest. 2004;114:1752-61.

[9] Pashkow FJ. Oxidative Stress and Inflammation in Heart Disease: Do Antioxidants Have a Role in Treatment and/or Prevention? Int J Inflam. 2011;2011:514623.

[10] Zhang ZH, Zhou SH, Qi SS, Li XP. [Oxidative stress and inflammation with angiographic morphology of coronary lesions in patients with coronary heart disease]. Zhong Nan Da Xue Xue Bao Yi Xue Ban. 2006;31:556-9.

[11] Agarwal R, Campbell RC, Warnock DG. Oxidative stress in hypertension and chronic kidney disease: role of angiotensin II. Semin Nephrol. 2004;24:101-14.

[12] Araujo M, Wilcox CS. Oxidative stress in hypertension: role of the kidney. Antioxid Redox Signal. 2014;20:74-101.

[13] Tang J, Yan H, Zhuang S. Inflammation and oxidative stress in obesity-related glomerulopathy. Int J Nephrol. 2012;2012:608397.

[14] Yang XD, Jiang S, Wang G, Zhang R, Zhang J, Zhu JS. Link of obesity and gastrointestinal cancer: crossroad of inflammation and oxidative stress. J Biol Regul Homeost Agents. 2015;29:755-60.

[15] Li QR, Wang Z, Zhou W, Fan SR, Ma R, Xue L, et al. Epalrestat protects against diabetic peripheral neuropathy by alleviating oxidative stress and inhibiting polyol pathway. Neural Regen Res. 2016;11:345-51.

[16] Liu Y, Zhai M, Guo F, Shi T, Liu J, Wang X, et al. Whole Body Vibration Improves Insulin Resistance in db/db Mice: Amelioration of Lipid Accumulation and Oxidative Stress. Appl Biochem Biotechnol. 2016.

[17] Ohno T, Shimizu M, Shirakami Y, Miyazaki T, Ideta T, Kochi T, et al. Preventive effects of astaxanthin on diethylnitrosamine-induced liver tumorigenesis in C57/BL/KsJ-db/db obese mice. Hepatol Res. 2016;46:E201-9.

[18] Golay A, Bobbioni E. The role of dietary fat in obesity. Int J Obes Relat Metab Disord. 1997;21 Suppl 3:S2-11.

[19] Jarukamjorn K, Jearapong N, Pimson C, Chatuphonprasert W. A High-Fat, High-Fructose Diet Induces Antioxidant Imbalance and Increases the Risk and Progression of Nonalcoholic Fatty Liver Disease in Mice. Scientifica (Cairo). 2016;2016:5029414.

[20] Noeman SA, Hamooda HE, Baalash AA. Biochemical study of oxidative stress markers in the liver, kidney and heart of high fat diet induced obesity in rats. Diabetol Metab Syndr. 2011;3:17.

[21] Salazar DE, Sorge CL, Jordan SW, Corcoran GB. Obesity decreases hepatic glutathione concentrations and markedly potentiates allyl alcohol-induced periportal necrosis in the overfed rat. Int J Obes Relat Metab Disord. 1994;18:25-33.

[22] Sastre J, Pallardo FV, Llopis J, Furukawa T, Vina JR, Vina J. Glutathione depletion by hyperphagia-induced obesity. Life Sci. 1989;45:183-7.

[23] Sverdlov AL, Elezaby A, Behring JB, Bachschmid MM, Luptak I, Tu VH, et al. High fat, high sucrose diet causes cardiac mitochondrial dysfunction due in part to oxidative post-translational modification of mitochondrial complex II. J Mol Cell Cardiol. 2015;78:165-73.

[24] Wang X, West JA, Murray AJ, Griffin JL. Comprehensive Metabolic Profiling of Age-Related Mitochondrial Dysfunction in the High-Fat-Fed ob/ob Mouse Heart. J Proteome Res. 2015;14:2849-62.

[25] Li L, Zhang GF, Lee K, Lopez R, Previs SF, Willard B, et al. A Western diet induced NAFLD in LDLR mice is associated with reduced hepatic glutathione synthesis. Free Radic Biol Med. 2016;96:13-21.

[26] Zhao Y, Sedighi R, Wang P, Chen H, Zhu Y, Sang S. Carnosic acid as a major bioactive component in rosemary extract ameliorates high-fat-diet-induced obesity and metabolic syndrome in mice. J Agric Food Chem. 2015;63:4843-52.

[27] He J, Giusti MM. Anthocyanins: natural colorants with health-promoting properties. Annual review of food science and technology. 2010;1:163-87.

[28] Kwon SH, Ahn IS, Kim SO, Kong CS, Chung HY, Do MS, et al. Anti-obesity and hypolipidemic effects of black soybean anthocyanins. Journal of medicinal food. 2007;10:552-6.

[29] Takahashi A, Shimizu H, Okazaki Y, Sakaguchi H, Taira T, Suzuki T, et al. Anthocyanin-rich Phytochemicals from Aronia Fruits Inhibit Visceral Fat Accumulation and Hyperglycemia in High-fat Diet-induced Dietary Obese Rats. Journal of oleo science. 2015;64:1243-50.

[30] Pratheeshkumar P, Son YO, Wang X, Divya SP, Joseph B, Hitron JA, et al. Cyanidin-3-glucoside inhibits UVB-induced oxidative damage and inflammation by regulating MAP kinase and NF-kappaB signaling pathways in SKH-1 hairless mice skin. Toxicology and applied pharmacology. 2014;280:127-37.

[31] Sorrenti V, Vanella L, Acquaviva R, Cardile V, Giofre S, Di Giacomo C. Cyanidin induces apoptosis and differentiation in prostate cancer cells. International journal of oncology. 2015;47:1303-10.

[32] Bhaswant M, Fanning K, Netzel M, Mathai ML, Panchal SK, Brown L. Cyanidin 3-glucoside improves diet-induced metabolic syndrome in rats. Pharmacological research. 2015;102:208-17.

[33] Chorfa N, Savard S, Belkacemi K. An efficient method for high-purity anthocyanin isomers isolation from wild blueberries and their radical scavenging activity. Food chemistry. 2016;197 Pt B:1226-34.

[34] De Rosso VV, Moran Vieyra FE, Mercadante AZ, Borsarelli CD. Singlet oxygen quenching by anthocyanin's flavylium cations. Free radical research. 2008;42:885-91.

[35] Jang YP, Zhou J, Nakanishi K, Sparrow JR. Anthocyanins protect against A2E photooxidation and membrane permeabilization in retinal pigment epithelial cells. Photochemistry and photobiology. 2005;81:529-36.

[36] Yamasaki H, Uefuji H, Sakihama Y. Bleaching of the red anthocyanin induced by superoxide radical. Archives of biochemistry and biophysics. 1996;332:183-6.

[37] Youdim KA, Shukitt-Hale B, MacKinnon S, Kalt W, Joseph JA. Polyphenolics enhance red blood cell resistance to oxidative stress: in vitro and in vivo. Biochimica et biophysica acta. 2000;1523:117-22.

[38] Moskaug JO, Carlsen H, Myhrstad MC, Blomhoff R. Polyphenols and glutathione synthesis regulation. The American journal of clinical nutrition. 2005;81:277S-83S.

[39] Jiang Z, Chen C, Wang J, Xie W, Wang M, Li X, et al. Purple potato (Solanum tuberosum L.) anthocyanins attenuate alcohol-induced hepatic injury by enhancing antioxidant defense. J Nat Med. 2016;70:45-53.

[40] Hou Z, Qin P, Ren G. Effect of anthocyanin-rich extract from black rice (Oryza sativa L. Japonica) on chronically alcohol-induced liver damage in rats. J Agric Food Chem. 2010;58:3191-6.

[41] Zhou Y, Harrison DE, Love-Myers K, Chen Y, Grider A, Wickwire K, et al. Genetic analysis of tissue glutathione concentrations and redox balance. Free Radic Biol Med. 2014;71:157-64.

[42] Tsuchiya M, Ji C, Kosyk O, Shymonyak S, Melnyk S, Kono H, et al. Interstrain differences in liver injury and one-carbon metabolism in alcohol-fed mice. Hepatology. 2012;56:130-9.

[43] Ahotupa M, Bereziat JC, Mantyla E, Bartsch H. Dietary fat- and phenobarbital-induced alterations in hepatic antioxidant functions of mice. Carcinogenesis. 1993;14:1225-8.

[44] Zhu H, Itoh K, Yamamoto M, Zweier JL, Li Y. Role of Nrf2 signaling in regulation of antioxidants and phase 2 enzymes in cardiac fibroblasts: protection against reactive oxygen and nitrogen species-induced cell injury. FEBS Lett. 2005;579:3029-36.

[45] Torzewski M, Ochsenhirt V, Kleschyov AL, Oelze M, Daiber A, Li H, et al. Deficiency of glutathione peroxidase-1 accelerates the progression of atherosclerosis in apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol. 2007;27:850-7.

[46] van 't Erve TJ, Wagner BA, Ryckman KK, Raife TJ, Buettner GR. The concentration of glutathione in human erythrocytes is a heritable trait. Free Radic Biol Med. 2013;65:742-9.

[47] Ruperez AI, Olza J, Gil-Campos M, Leis R, Mesa MD, Tojo R, et al. Association of genetic polymorphisms for glutathione peroxidase genes with obesity in Spanish children. J Nutrigenet Nutrigenomics. 2014;7:130-42.

[48] Ramprasath T, Murugan PS, Kalaiarasan E, Gomathi P, Rathinavel A, Selvam GS. Genetic association of Glutathione peroxidase-1 (GPx-1) and NAD(P)H:Quinone Oxidoreductase 1(NQO1) variants and their association of CAD in patients with type-2 diabetes. Mol Cell Biochem. 2012;361:143-50.

[49] West DB, Boozer CN, Moody DL, Atkinson RL. Dietary obesity in nine inbred mouse strains. Am J Physiol. 1992;262:R1025-32.

[50] West DB, Waguespack J, McCollister S. Dietary obesity in the mouse: interaction of strain with diet composition. Am J Physiol. 1995;268:R658-65.

[51] Smith BK, Andrews PK, West DB. Macronutrient diet selection in thirteen mouse strains. Am J Physiol Regul Integr Comp Physiol. 2000;278:R797-805.

[52] Surwit RS, Feinglos MN, Rodin J, Sutherland A, Petro AE, Opara EC, et al. Differential effects of fat and sucrose on the development of obesity and diabetes in C57BL/6J and A/J mice. Metabolism. 1995;44:645-51.

[53] Watson PM, Commins SP, Beiler RJ, Hatcher HC, Gettys TW. Differential regulation of leptin expression and function in A/J vs. C57BL/6J mice during diet-induced obesity. Am J Physiol Endocrinol Metab. 2000;279:E356-65.

[54] Tortoriello DV, McMinn J, Chua SC. Dietary-induced obesity and hypothalamic infertility in female DBA/2J mice. Endocrinology. 2004;145:1238-47.

[55] Alexander J, Chang GQ, Dourmashkin JT, Leibowitz SF. Distinct phenotypes of obesity-prone AKR/J, DBA2J and C57BL/6J mice compared to control strains. Int J Obes (Lond). 2006;30:50-9.

[56] Craig SS, Craig SA, Ganio MS, Maresh CM, Horrace G, da Costa KA, et al. The betaine content of sweat from adolescent females. J Int Soc Sports Nutr. 2010;7:3.

[57] Sanchez CL, Van Swearingen AE, Arrant AE, Biskup CS, Kuhn CM, Zepf FD. Simplified dietary acute tryptophan depletion: effects of a novel amino acid mixture on the neurochemistry of C57BL/6J mice. Food Nutr Res. 2015;59:27424.

[58] Tyburczy C, Brenna ME, DeMari JA, Kothapalli KS, Blank BS, Valentine H, et al. Evaluation of bioequivalency and toxicological effects of three sources of arachidonic acid (ARA) in domestic piglets. Food Chem Toxicol. 2011;49:2320-7.

[59] Costanzo MT, Yost RA, Davenport PW. Standardized method for solubility and storage of capsaicin-based solutions for cough induction. Cough. 2014;10:6.

[60] Aggarwal K, Choe LH, Lee KH. Quantitative analysis of protein expression using amine-specific isobaric tags in Escherichia coli cells expressing rhsA elements. Proteomics. 2005;5:2297-308.

[61] Reifsnyder PC, Doty R, Harrison DE. Rapamycin ameliorates nephropathy despite elevating hyperglycemia in a polygenic mouse model of type 2 diabetes, NONcNZO10/LtJ. PLoS One. 2014;9:e114324.

[62] Rossmeisl M, Rim JS, Koza RA, Kozak LP. Variation in type 2 diabetes--related traits in mouse strains susceptible to diet-induced obesity. Diabetes. 2003;52:1958-66.

[63] Andrikopoulos S, Massa CM, Aston-Mourney K, Funkat A, Fam BC, Hull RL, et al. Differential effect of inbred mouse strain (C57BL/6, DBA/2, 129T2) on insulin secretory function in response to a high fat diet. J Endocrinol. 2005;187:45-53.

[64] Park HJ, Mah E, Bruno RS. Validation of high-performance liquid chromatography-boron-doped diamond detection for assessing hepatic glutathione redox status. Anal Biochem. 2010;407:151-9.

[65] Zhu X, Jiang S, Hu N, Luo F, Dong H, Kang YM, et al. Tumour necrosis factor-alpha inhibition with lenalidomide alleviates tissue oxidative injury and apoptosis in ob/ob obese mice. Clin Exp Pharmacol Physiol. 2014;41:489-501.

[66] Wicks SE, Nguyen TT, Breaux C, Kruger C, Stadler K. Diet-induced obesity and kidney disease - In search of a susceptible mouse model. Biochimie. 2016;124:65-73.

[67] Hong JH, Lee IS. Effects of Artemisia capillaris ethyl acetate fraction on oxidative stress and antioxidant enzyme in high-fat diet induced obese mice. Chem Biol Interact. 2009;179:88-93.

[68] Shao F, Gu L, Chen H, Liu R, Huang H, Ren G. Comparation of Hypolipidemic and Antioxidant Effects of Aqueous and Ethanol Extracts of Crataegus pinnatifida Fruit in High-Fat Emulsion-Induced Hyperlipidemia Rats. Pharmacogn Mag. 2016;12:64-9.

[69] Vukovic R, Blazetic S, Orsolic I, Heffer M, Vari SG, Gajdos M, et al. Impact of ovariectomy, high fat diet, and lifestyle modifications on oxidative/antioxidative status in the rat liver. Croat Med J. 2014;55:218-27.

[70] McClung JP, Roneker CA, Mu W, Lisk DJ, Langlais P, Liu F, et al. Development of insulin resistance and obesity in mice overexpressing cellular glutathione peroxidase. Proc Natl Acad Sci U S A. 2004;101:8852-7.

[71] Loh K, Deng H, Fukushima A, Cai X, Boivin B, Galic S, et al. Reactive oxygen species enhance insulin sensitivity. Cell Metab. 2009;10:260-72.

[72] Merry TL, Tran M, Stathopoulos M, Wiede F, Fam BC, Dodd GT, et al. High-fat-fed obese glutathione peroxidase 1-deficient mice exhibit defective insulin secretion but protection from hepatic steatosis and liver damage. Antioxid Redox Signal. 2014;20:2114-29.

[73] Asai A, Chou PM, Bu HF, Wang X, Rao MS, Jiang A, et al. Dissociation of hepatic insulin resistance from susceptibility of nonalcoholic fatty liver disease induced by a high-fat and high-carbohydrate diet in mice. Am J Physiol Gastrointest Liver Physiol. 2014;306:G496-504.

[74] Chun OK, Chung SJ, Claycombe KJ, Song WO. Serum C-reactive protein concentrations are inversely associated with dietary flavonoid intake in U.S. adults. The Journal of nutrition. 2008;138:753-60.

[75] Mink PJ, Scrafford CG, Barraj LM, Harnack L, Hong CP, Nettleton JA, et al. Flavonoid intake and cardiovascular disease mortality: a prospective study in postmenopausal women. The American journal of clinical nutrition. 2007;85:895-909.

[76] McCullough ML, Peterson JJ, Patel R, Jacques PF, Shah R, Dwyer JT. Flavonoid intake and cardiovascular disease mortality in a prospective cohort of US adults. The American journal of clinical nutrition. 2012;95:454-64.

[77] Mursu J, Voutilainen S, Nurmi T, Tuomainen TP, Kurl S, Salonen JT. Flavonoid intake and the risk of ischaemic stroke and CVD mortality in middle-aged Finnish men: the Kuopio Ischaemic Heart Disease Risk Factor Study. The British journal of nutrition. 2008;100:890-5.

[78] Iancu OD, Darakjian P, Walter NA, Malmanger B, Oberbeck D, Belknap J, et al. Genetic diversity and striatal gene networks: focus on the heterogeneous stock-collaborative cross (HS-CC) mouse. BMC Genomics. 2010;11:585.

[79] Keli SO, Hertog MG, Feskens EJ, Kromhout D. Dietary flavonoids, antioxidant vitamins, and incidence of stroke: the Zutphen study. Archives of internal medicine. 1996;156:637-42.

[80] Sesso HD, Gaziano JM, Liu S, Buring JE. Flavonoid intake and the risk of cardiovascular disease in women. The American journal of clinical nutrition. 2003;77:1400-8.

[81] Cassidy A, Rimm EB, O'Reilly EJ, Logroscino G, Kay C, Chiuve SE, et al. Dietary flavonoids and risk of stroke in women. Stroke; a journal of cerebral circulation. 2012;43:946-51.

[82] Yochum L, Kushi LH, Meyer K, Folsom AR. Dietary flavonoid intake and risk of cardiovascular disease in postmenopausal women. American journal of epidemiology. 1999;149:943-9.

[83] Woo HD, Lee J, Choi IJ, Kim CG, Lee JY, Kwon O, et al. Dietary flavonoids and gastric cancer risk in a Korean population. Nutrients. 2014;6:4961-73.

[84] Zamora-Ros R, Agudo A, Lujan-Barroso L, Romieu I, Ferrari P, Knaze V, et al. Dietary flavonoid and lignan intake and gastric adenocarcinoma risk in the European Prospective Investigation into Cancer and Nutrition (EPIC) study. The American journal of clinical nutrition. 2012;96:1398-408.

[85] Wedick NM, Pan A, Cassidy A, Rimm EB, Sampson L, Rosner B, et al. Dietary flavonoid intakes and risk of type 2 diabetes in US men and women. The American journal of clinical nutrition. 2012;95:925-33.

[86] Zamora-Ros R, Forouhi NG, Sharp SJ, Gonzalez CA, Buijsse B, Guevara M, et al. The association between dietary flavonoid and lignan intakes and incident type 2 diabetes in European populations: the EPIC-InterAct study. Diabetes care. 2013;36:3961-70.

[87] Rosenblat M, Volkova N, Attias J, Mahamid R, Aviram M. Consumption of polyphenolic-rich beverages (mostly pomegranate and black currant juices) by healthy subjects for a short term increased serum antioxidant status, and the serum's ability to attenuate macrophage cholesterol accumulation. Food & function. 2010;1:99-109.

[88] Ruel G, Pomerleau S, Couture P, Lamarche B, Couillard C. Changes in plasma antioxidant capacity and oxidized low-density lipoprotein levels in men after short-term cranberry juice consumption. Metabolism. 2005;54:856-61.

[89] Ruel G, Pomerleau S, Couture P, Lemieux S, Lamarche B, Couillard C. Low-calorie cranberry juice supplementation reduces plasma oxidized LDL and cell adhesion molecule concentrations in men. The British journal of nutrition. 2008;99:352-9.

[90] Basu A, Du M, Leyva MJ, Sanchez K, Betts NM, Wu M, et al. Blueberries decrease cardiovascular risk factors in obese men and women with metabolic syndrome. The Journal of nutrition. 2010;140:1582-7.

[91] Henning SM, Seeram NP, Zhang Y, Li L, Gao K, Lee RP, et al. Strawberry consumption is associated with increased antioxidant capacity in serum. Journal of medicinal food. 2010;13:116-22.

[92] Lee IT, Chan YC, Lin CW, Lee WJ, Sheu WH. Effect of cranberry extracts on lipid profiles in subjects with Type 2 diabetes. Diabetic medicine : a journal of the British Diabetic Association. 2008;25:1473-7.

[93] Burton-Freeman B, Linares A, Hyson D, Kappagoda T. Strawberry modulates LDL oxidation and postprandial lipemia in response to high-fat meal in overweight hyperlipidemic men and women. Journal of the American College of Nutrition. 2010;29:46-54.

[94] Basu A, Wilkinson M, Penugonda K, Simmons B, Betts NM, Lyons TJ. Freeze-dried strawberry powder improves lipid profile and lipid peroxidation in women with metabolic syndrome: baseline and post intervention effects. Nutrition journal. 2009;8:43.

[95] Ruel G, Lapointe A, Pomerleau S, Couture P, Lemieux S, Lamarche B, et al. Evidence that cranberry juice may improve augmentation index in overweight men. Nutr Res. 2013;33:41-9.

[96] Alvarez-Suarez JM, Giampieri F, Tulipani S, Casoli T, Di Stefano G, Gonzalez-Paramas AM, et al. One-month strawberry-rich anthocyanin supplementation ameliorates cardiovascular

risk, oxidative stress markers and platelet activation in humans. The Journal of nutritional biochemistry. 2014;25:289-94.

[97] Duthie SJ, Jenkinson AM, Crozier A, Mullen W, Pirie L, Kyle J, et al. The effects of cranberry juice consumption on antioxidant status and biomarkers relating to heart disease and cancer in healthy human volunteers. European journal of nutrition. 2006;45:113-22.

[98] Kuntz S, Kunz C, Herrmann J, Borsch CH, Abel G, Frohling B, et al. Anthocyanins from fruit juices improve the antioxidant status of healthy young female volunteers without affecting anti-inflammatory parameters: results from the randomised, double-blind, placebo-controlled, cross-over ANTHONIA (ANTHOcyanins in Nutrition Investigation Alliance) study. The British journal of nutrition. 2014;112:925-36.

[99] Del Bo C, Martini D, Porrini M, Klimis-Zacas D, Riso P. Berries and oxidative stress markers: an overview of human intervention studies. Food & function. 2015;6:2890-917.

[100] Cutler GJ, Nettleton JA, Ross JA, Harnack LJ, Jacobs DR, Jr., Scrafford CG, et al. Dietary flavonoid intake and risk of cancer in postmenopausal women: the Iowa Women's Health Study. International journal of cancer Journal international du cancer. 2008;123:664-71.

[101] Tien YH, Chen BH, Wang Hsu GS, Lin WT, Huang JH, Lu YF. Hepatoprotective and anti-oxidant activities of Glossogyne tenuifolia against acetaminophen-induced hepatotoxicity in mice. The American journal of Chinese medicine. 2014;42:1385-98.

[102] Noh JR, Kim YH, Hwang JH, Gang GT, Kim KS, Lee IK, et al. Davallialactone protects against acetaminophen overdose-induced liver injuries in mice. Food Chem Toxicol. 2013;58:14-21.

[103] Zhang W, Xue J, Ge M, Yu M, Liu L, Zhang Z. Resveratrol attenuates hepatotoxicity of rats exposed to arsenic trioxide. Food Chem Toxicol. 2013;51:87-92.

[104] Sentellas S, Morales-Ibanez O, Zanuy M, Alberti JJ. GSSG/GSH ratios in cryopreserved rat and human hepatocytes as a biomarker for drug induced oxidative stress. Toxicology in vitro : an international journal published in association with BIBRA. 2014;28:1006-15.

[105] Dunnick JK, Hailey JR. Toxicity and carcinogenicity studies of quercetin, a natural component of foods. Fundamental and applied toxicology : official journal of the Society of Toxicology. 1992;19:423-31.

[106] Murakami A. Dose-dependent functionality and toxicity of green tea polyphenols in experimental rodents. Archives of biochemistry and biophysics. 2014;557:3-10.

[107] Church RJ, Gatti DM, Urban TJ, Long N, Yang X, Shi Q, et al. Sensitivity to hepatotoxicity due to epigallocatechin gallate is affected by genetic background in diversity outbred mice. Food Chem Toxicol. 2015;76:19-26.