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The anthocyanin cyanidin-3-O- β -glucoside modulates murine glutathione homeostasis in a manner dependent on genetic background

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ABSTRACT

Anthocyanins are a class of phytochemicals that have generated considerable interest due to their reported health benefits. It has been proposed that commonly consumed anthocyanins, such as cyanidin-3-O- β -glucoside (C3G), confer cellular protection by stimulating biosynthesis of glutathione (GSH), an endogenous antioxidant. Currently, it is unknown whether the health effects of dietary anthocyanins are genetically determined. We therefore 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 samples 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. Overall, we discovered that C3G modulates the GSH system in a strain- and tissue-specific manner. To our knowledge, this study is the first to show that the redox effects of anthocyanins are determined by genetic background.

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1. 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 [1]. 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) [2], as well as decreased risk of CVD-related mortality [3,4]. 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 [5]. To improve understanding of the relationships between anthocyanidins, their corresponding 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 [6], obesity [7,8], cancer [9,10], and metabolic syndrome [11], anthocyanins decrease markers of oxidative stress. This effect is attributable, in part, to their strong antioxidant activity. These compounds are potent free radical scavengers [12–16], and they 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) [6]. Such a significant change in GSH levels is noteworthy, as a more robust GSH system has been associated with stress resistance [17]; conversely, depletion of GSH promotes the onset of morbidities such as impaired glucose tolerance [18], cardiomyopathy [19], and

Abbreviations: GSH, Glutathione; GSSG, Glutathione Disulfide; C3G, Cyanidin-3-O- β -Glucoside

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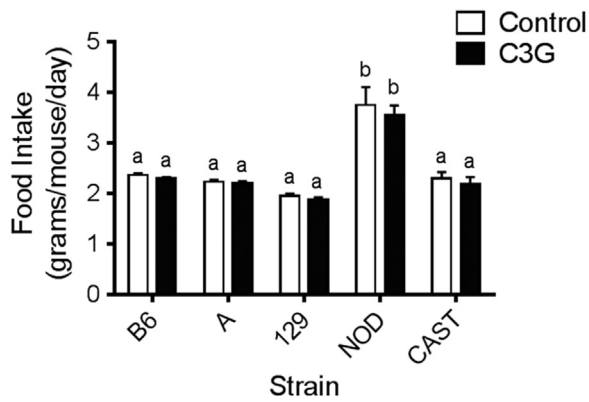


Fig. 1. Food intake of mice fed a control or C3G diet for 6 weeks. Data are reported as means \pm standard error of mean (SEM). Two-way ANOVA with Bonferroni adjustments were used to determine significant differences across all groups. Means without a common letter are statistically different, $P < 0.05$.

carcinogenesis [20].

Recent studies have demonstrated that, in addition to diet, natural genetic variation regulates tissue GSH levels and redox status (GSH/GSSG) [21,22]. We predicted that the genetic and dietary regulation of the GSH system intersect, and specifically, that C3G effects on GSH levels and GSH/GSSG would vary by mouse strain. We also expected that these redox effects would be most prominent in organs that metabolize and excrete C3G, notably liver and kidney. To test our hypothesis, 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, and the 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 [23]. We observed that these strains exhibited 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.

2. Material and methods

2.1. 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). The mice arrived at our facility at 4–6 weeks of age, depending on availability, and the mice were

housed in an animal room on a 12 h light/dark cycle in SPF conditions. At three months of age, 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). After six weeks of dietary intervention, 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), and all procedures aligned with the National Institutes of Health guide for the care and use of laboratory animals.

2.2. Diet

Mice were fed a standard purified AIN-93M mouse diet (control) or AIN-93M plus C3G (100 mg/kg). The C3G diet was generated as described previously [6]. 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.

2.3. 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 h after collection, tissues were homogenized and immediately acidified with perchloric acid. 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. External GSH and GSSG standards were prepared in a 1:1 solution of PBS containing 10 mM DTPA and 10% perchloric acid containing 1 mM DTPA to create the same chemical condition as the samples. After electrochemical detection, data were quantified by the Chromeleon Chromatography Data System Software (Dionex Version 7.2, Thermo Scientific). Total glutathione

Table 1

Percent initial body weight of mice fed a control or C3G diet for 6 weeks. Data are reported as means \pm 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, $P < 0.05$.

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

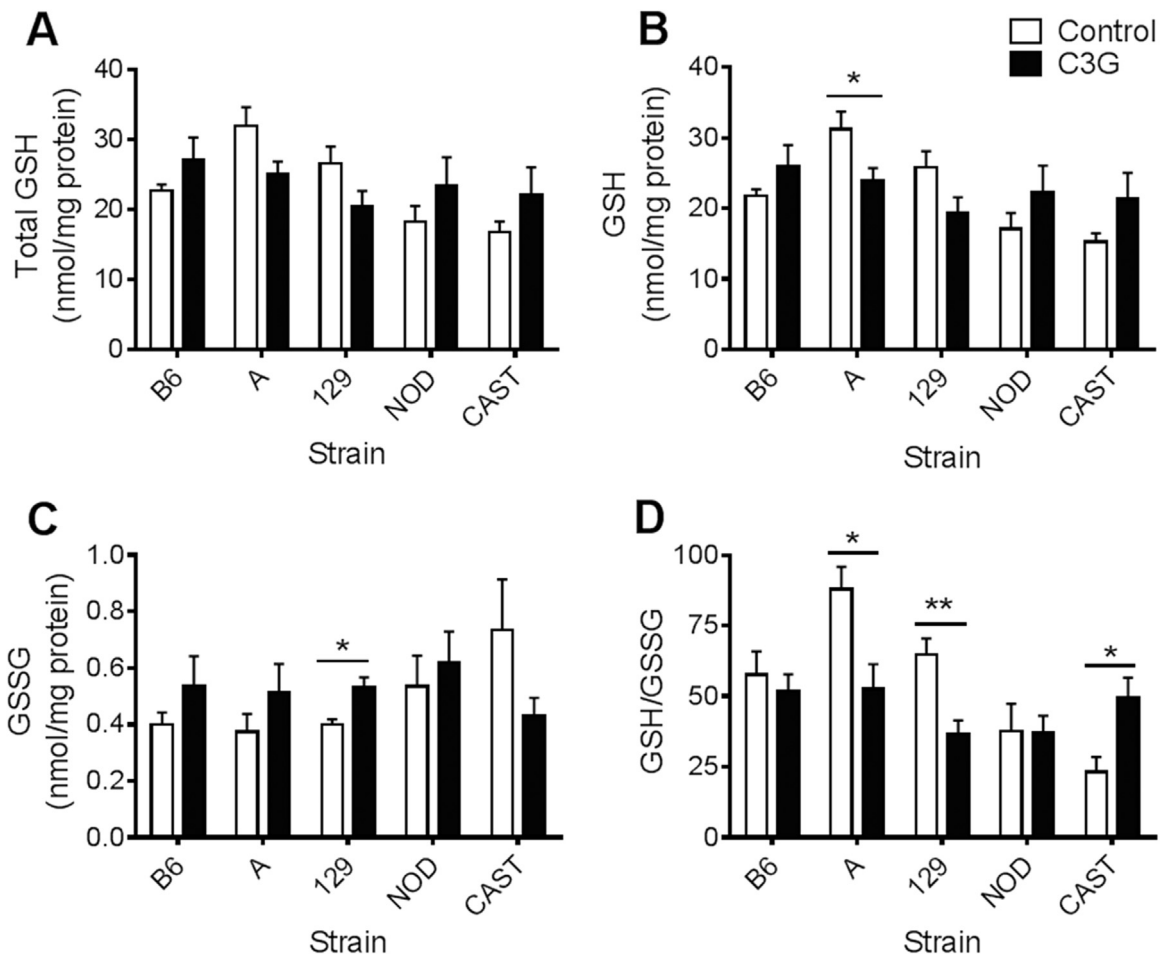


Fig. 2. Hepatic glutathione concentrations and redox status in mice fed a control or C3G diet. (A) Liver total glutathione, standardized to total protein; (B) GSH levels; (C) GSSG levels; and (D) GSH/GSSG. Data are reported as means \pm standard error of mean (SEM). Independent *t*-tests were conducted to determine significance within each strain in response to C3G treatment, **P* < 0.05, ***P* < 0.01.

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).

2.4. Assessment of GSH redox potential

GSH redox potentials (E_h) were calculated in liver, kidney, heart, pancreas, and brain samples using the Nernst equation, $E_h = E_o + RT/nF \ln [\text{disulfide}]/([\text{thiol}]^2)$. E_o represents the standard potential for the redox couple, R is the gas constant, T is temperature, n is 2 for the number of transferred electrons, F is Faraday's constant, and molar concentrations of GSH and GSSG were used. The standard E_o used for GSH/GSSG was -264 mV (mV) for pH 7.4 [24].

2.5. 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: CACAATGACCCGAAAGAAGCTG; Reverse: AGACTTGATGATTCCCCTGCT), glutamate-cysteine ligase catalytic subunit (*Gclc*; Forward: CCTCTCTCCAAACTCAGATA;

Reverse: CCACAAATACCACATAGGCAGA), glutathione peroxidase-1 (*Gpx-1*; Forward: CCCGTGCAATCAGTTC; Reverse: TTCGCACTTCTCAAACAA), and glutathione reductase (*Gr*; Forward: GGTGGTGGAGAGTCAACAAG; Reverse: ATCGTGCATGAATCCGAGT) 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 $\Delta\Delta Ct$ method and are presented as the fold-change relative to the B6 control group.

2.6. Statistical analysis

Statistical analyses were performed using GraphPad Prism (Version 6.0, GraphPad Software Inc., La Jolla, CA, USA) and SPSS Statistics (Version 24, IBM, Armonk, NY, 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, GSH redox potentials, and expression of GSH-related enzymes in response to C3G treatment. Correlations between relative hepatic enzyme expression and other GSH phenotypes were separately tested among controls, C3G-fed mice, and all mice pooled together using Pearson's coefficient. Groups were first tested for normality using the Shapiro-Wilk test, and skewed distributions were transformed using the square root (*Gclc*, *Gclm*, *Gpx-1*, and GSSG)

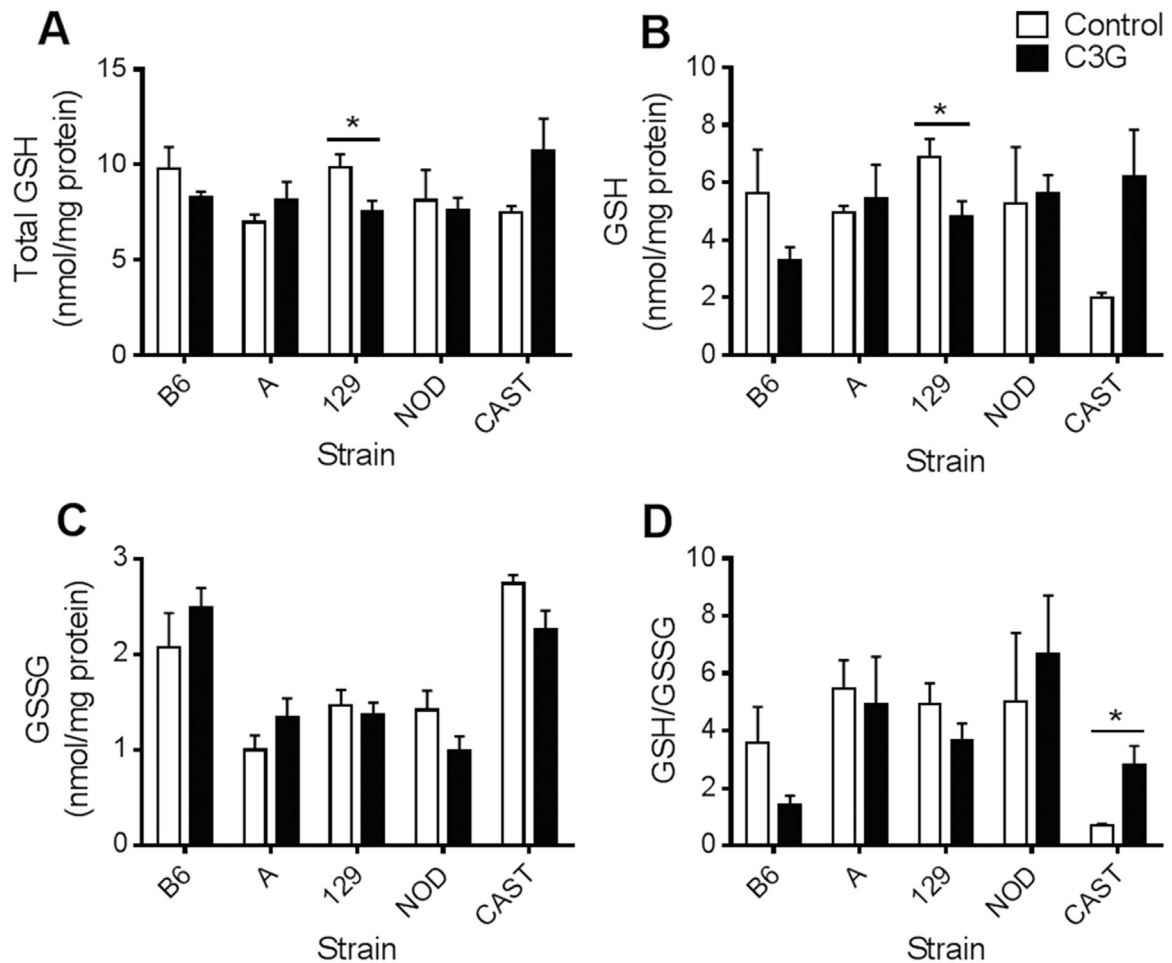


Fig. 3. Glutathione concentrations and redox status in hearts isolated from mice fed a control or C3G diet. (A) Heart total glutathione, standardized to total protein; (B) GSH levels; (C) GSSG levels; and (D) GSH/GSSG. Data are reported as means \pm standard error of mean (SEM). Independent *t*-tests were conducted to determine significance within each strain in response to C3G treatment, * $P < 0.05$.

or reciprocal square root (*Gr*) function. The level of statistical significance was defined at $P < 0.05$. Data are reported as means \pm standard error of mean (SEM).

3. Results

3.1. 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 (Fig. 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 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.

3.2. GSH and GSSG levels, total glutathione, redox status, and redox potential

In this study, baseline GSH levels and GSH/GSSG redox statuses reflected the values that have been previously published [21,25]. In response to C3G treatment, CAST mice showed a twofold increase in

hepatic GSH/GSSG ($P = 0.041$; Fig. 2D) and a nearly fourfold increase in cardiac GSH/GSSG ($P = 0.028$; Fig. 3D). Renal total glutathione ($P = 0.014$) and GSH concentrations ($P = 0.010$) increased in C3G-treated CAST mice (Fig. 4A, B), and pancreatic levels of oxidized glutathione, GSSG, were lower in the C3G group compared to control CAST mice ($P = 0.021$) (Fig. 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 ($P = 0.042$; Fig. 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 (Fig. 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 ($P = 0.0066$) (Fig. 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 (Fig. 2B, C). In contrast, C3G-fed 129 mice displayed increased hepatic GSSG levels ($P = 0.020$) while GSH levels were unaffected (Fig. 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 (Fig. 3A, B).

GSH redox potentials were calculated for each tissue (Table 2), and the values were similar to those that have been reported

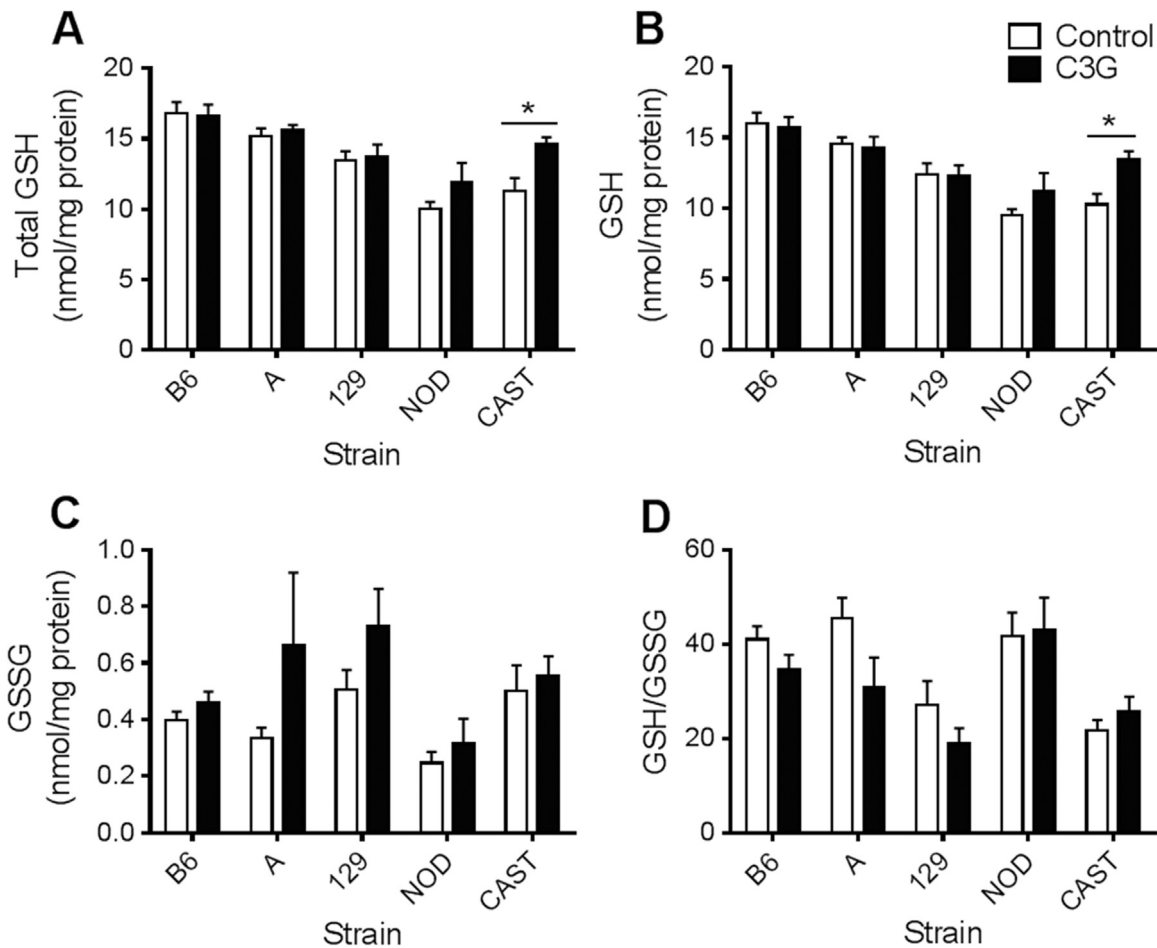


Fig. 4. Renal glutathione concentrations and redox status in mice fed a control or C3G diet. (A) Kidney total glutathione, standardized to total protein; (B) GSH levels; (C) GSSG levels; and (D) GSH/GSSG. Data are reported as means \pm standard error of mean (SEM). Independent *t*-tests were conducted to determine significance within each strain in response to C3G treatment, * $P < 0.05$.

[24,26]. In these analyses, less negative redox potentials reflect a more oxidizing environment. The C3G diet induced oxidation of the hepatic GSH redox potentials of A mice ($P=0.003$) and 129 mice ($P=0.012$). In contrast, the C3G diet promoted a more reducing hepatic redox potential in CAST mice ($P=0.031$). CAST mice also exhibited more reducing cardiac ($P=0.004$) and pancreatic ($P=0.025$) redox potentials on the C3G diet compared to controls. B6 mice exhibited a similar reduction of pancreatic redox potential ($P=0.040$). No changes were found in redox potential of the kidney or brain as a result of the C3G diet.

3.3. Hepatic expression of GSH-related enzymes

Hepatic expression levels of *Gclc*, *Gclm*, and *Gr* were not significantly different in response to C3G treatment in any of the five strains (Fig. 7A, B, C). Similarly, A, CAST, B6, and NOD did not exhibit altered expression of *Gpx-1* in response to C3G supplementation (Fig. 7D). C3G-treated 129 mice did exhibit a twofold increase in *Gpx-1* expression compared to controls ($P=0.023$) (Fig. 7D), which was the only significant change in antioxidant gene expression discovered in this study.

3.4. Correlations between expression of GSH-related enzymes and hepatic GSH phenotypes

Relationships between GSH-related enzyme expression levels and GSH phenotypes were tested in the liver (Table 3). After

pooling data from control and C3G-treated mice, significant correlations were discovered between *Gr* and: 1) total glutathione levels ($P=0.001$); 2) GSH levels ($P < 0.001$); 3) GSH/GSSG ($P=0.003$); and 4) GSH redox potential ($P < 0.001$; Supplemental Figure 1). Significant correlations were also found between *Gpx-1* and: 1) total glutathione levels ($P=0.042$); 2) GSH levels ($P=0.037$); 3) GSH/GSSG ($P=0.049$); and 4) GSH redox potential ($P=0.014$; Supplemental Figure 2). Interestingly, the relationships between *Gpx-1* and GSH phenotypes emerged within the control group, yet disappeared in the C3G-treated mice, suggesting that C3G alters the relationship between *Gpx-1* expression and GSH homeostasis. In all, higher expression levels of *Gr* were associated with a more reducing GSH redox potential, while increased expression of *Gpx-1* was associated with a more oxidizing redox potential. No significant correlations were present between *Gclc* or *Gclm* expression and GSH phenotypes in the pooled data. However, a direct relationship was found between expression of *Gclm* and GSSG levels in controls ($P=0.049$), while an inverse relationship was found between *Gclm* and GSSG levels in C3G-fed mice ($P=0.026$).

4. 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

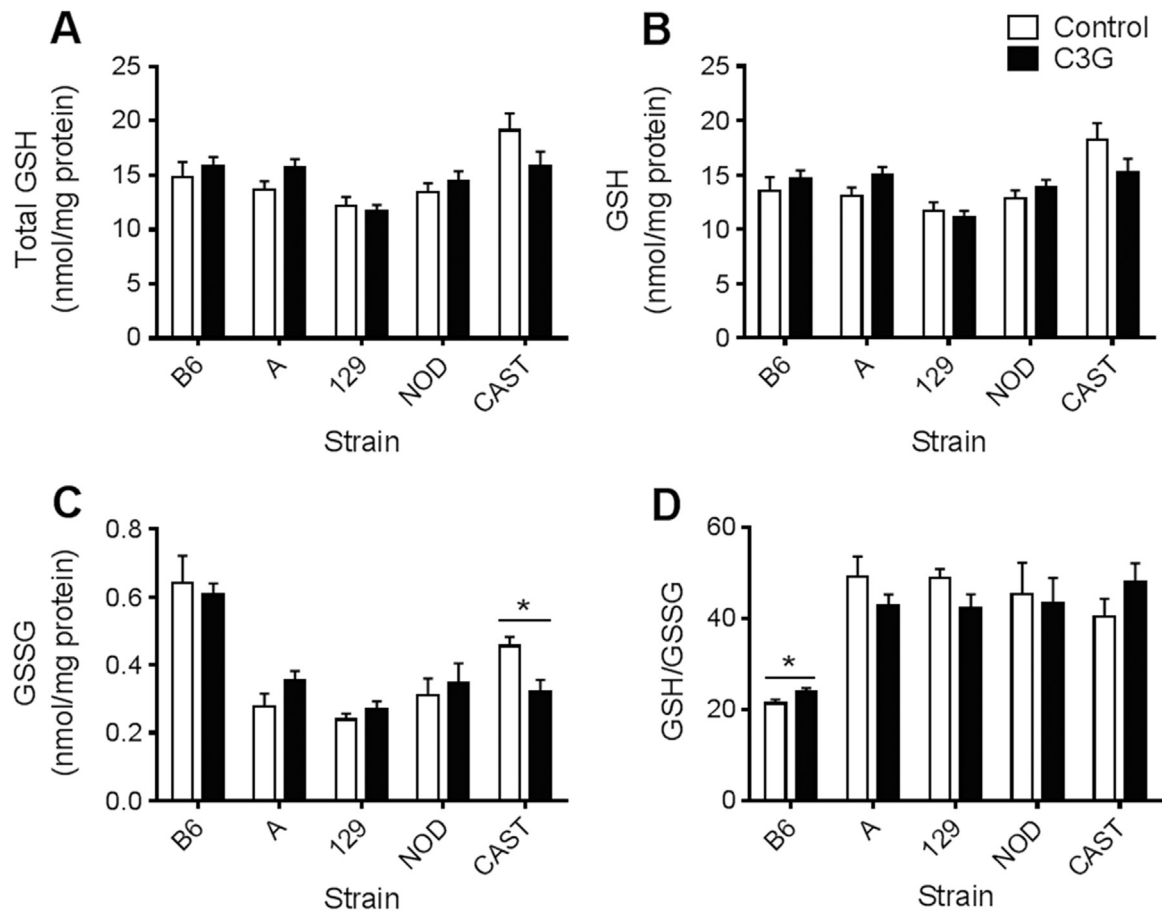


Fig. 5. Pancreas glutathione concentrations and redox status in mice fed a control or C3G diet. (A) Pancreas total glutathione, standardized to total protein; (B) GSH levels; (C) GSSG levels; and (D) GSH/GSSG. Data are reported as means \pm standard error of mean (SEM). Independent *t*-tests were conducted to determine significance within each strain in response to C3G treatment, **P* < 0.05.

was found to exhibit an inverse association with stroke incidence [27] and mortality [4], while in other studies, flavonoid intake has exhibited no correlation with risk [28,29] or mortality [30]. 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 [31], but a significant inverse correlation was found in European women [32]. 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 anthocyanidin consumption [33], while a European case-cohort study found no correlation with T2D risk and anthocyanidin intake [34]. Importantly, the inconsistencies highlighted in these studies have been captured in clinical intervention studies as well [35–46]. 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 [47]. 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 [6]. 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 [48].

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

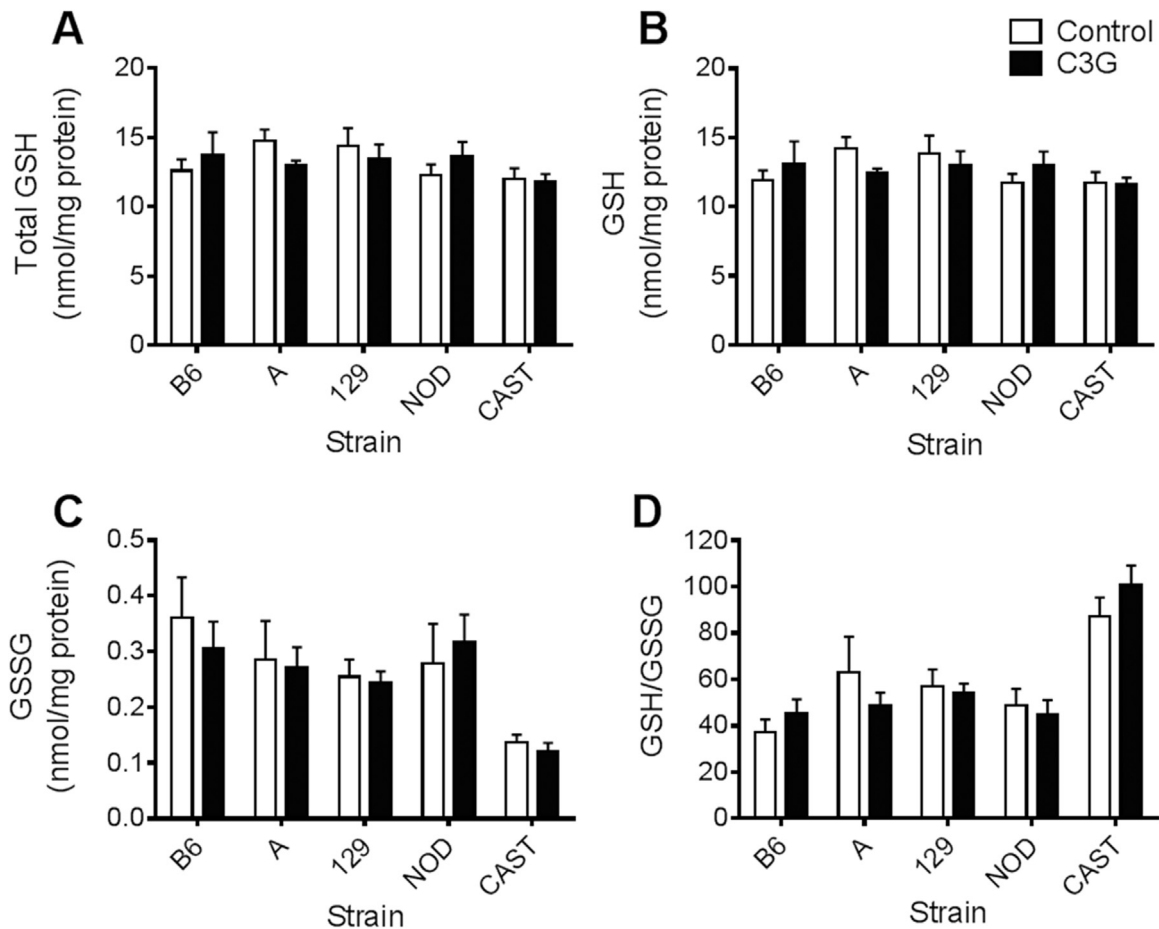


Fig. 6. Whole brain glutathione concentrations and redox status in mice fed a control or C3G diet. (A) Whole brain total glutathione, standardized to total protein; (B) GSH levels; (C) GSSG levels; and (D) GSH/GSSG. Data are reported as means \pm standard error of mean (SEM). Independent *t*-tests were conducted to determine significance within each strain in response to C3G treatment, **P* < 0.05, ***P* < 0.01 from control.

Table 2
GSH redox potentials. Redox potentials (expressed in mV) were calculated in liver, kidney, heart, pancreas, and whole brain samples using the Nernst equation. Data are reported as means \pm 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 from control.

Group	Liver	Kidney	Heart	Pancreas	Brain
B6 Control	-215.8 \pm 2.0	-204 \pm 1.0	-140.1 \pm 10.0	-188.1 \pm 0.7	-199.5 \pm 1.8
B6 C3G	-215.4 \pm 1.5	-201 \pm 1.4	-125.6 \pm 4.6	-190.4 \pm 0.7*	-200.6 \pm 2.5
AJ Control	-228.7 \pm 0.9	-204 \pm 2.0	-151.8 \pm 2.0	-197.1 \pm 1.4	-204 \pm 4.0
AJ C3G	-218.2 \pm 2.4**	-196.4 \pm 4.7	-146.9 \pm 6.2	-197 \pm 0.7	-201.7 \pm 1.7
129 Control	-220.1 \pm 1.1	-194.5 \pm 3.1	-152.5 \pm 3.4	-196 \pm 1.8	-204.8 \pm 2.1
129 C3G	-209.4 \pm 2.7*	-189.2 \pm 2.7	-146.8 \pm 3.6	-194 \pm 1.2	-203.9 \pm 1.7
NOD Control	-207.3 \pm 3.6	-200.6 \pm 1.8	-138.3 \pm 10.0	-192.2 \pm 1.8	-201 \pm 2.4
NOD C3G	-211 \pm 3.6	-201.8 \pm 2.1	-154.1 \pm 2.9	-193.6 \pm 1.7	-201.7 \pm 1.6
CAST Control	-197.7 \pm 1.8	-187.3 \pm 1.2	-116.3 \pm 1.2	-180.1 \pm 0.3	-208.9 \pm 1.5
CAST C3G	-213.3 \pm 4.5*	-191.2 \pm 2.6	-141.3 \pm 5.3**	-183.8 \pm 1.1*	-210.8 \pm 1.0

lowest GSH levels and GSH/GSSG in a large panel of inbred strains [21]. 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 (Fig. 8). We initially predicted that these effects are largely independent of gene expression due to the minimal changes in hepatic GSH-related enzyme expression observed here. However, subsequent statistical analyses revealed correlations between GSH phenotypes and expression of GSH-

related enzymes, indicating that basal expression levels may play a role in the redox effects outlined in this study. It is important to note that additional factors may have also influenced GSH homeostasis beyond what was assayed, such as glutathione transferase activity, activity of GSH efflux pumps, NAD(P)H supply, as well as composition of the gut microbiome, which could affect C3G metabolism, absorption, and bioactivity.

This study demonstrated that GSH levels and GSH/GSSG can decrease in response to an established C3G-rich diet [6]. The C3G

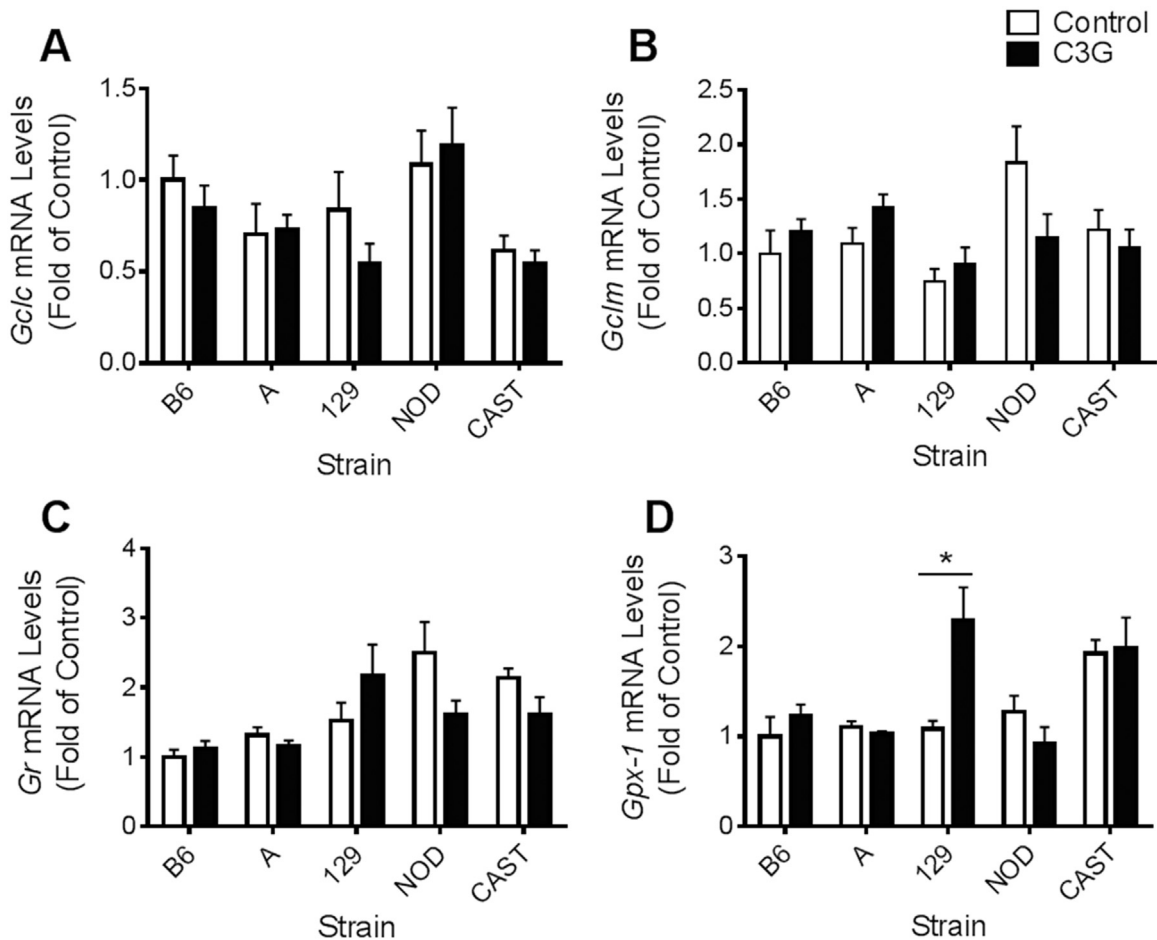


Fig. 7. Hepatic expression of glutathione enzymes in mice fed a control or high-C3G diet for 6 weeks. (A) Relative *Gclc* mRNA levels; (B) *Gclm* mRNA levels; (C) *Gr* mRNA levels; and (D) *Gpx-1* mRNA levels. Data represent fold expression relative to the B6 control group and are reported as means \pm standard error of mean (SEM). Independent *t*-tests were conducted to determine significance within each strain in response to C3G treatment, * $P < 0.05$.

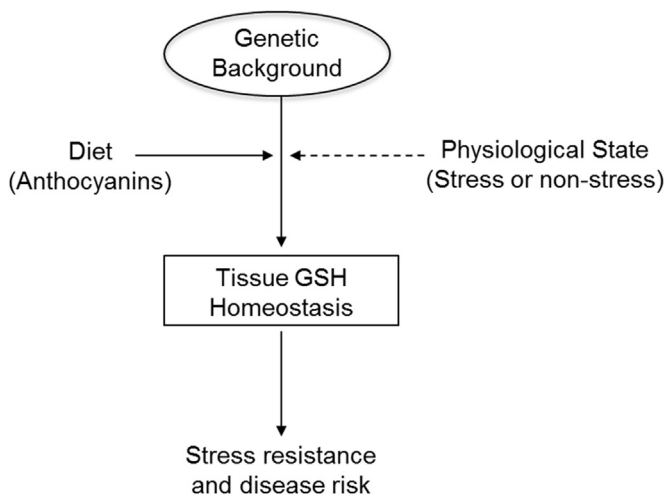


Fig. 8. Model of genetic regulation of GSH. Genetic background directly regulates GSH homeostasis and determines the relative effects of diet and physiological stress on this system. Together, these interactions influence disease risk.

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 [49–52]. Several polyphenols are known to exert toxicity at high levels [53–55], and in the case

of epigallocatechin gallate (EGCG), a polyphenol present in green tea, toxicity is determined by genetic background [55]. 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. 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 redox 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.

Table 3
Pearson correlation coefficients for GSH-related enzyme expression and GSH phenotypes in the liver. Correlations were separately tested in controls, C3G-fed mice, and all mice pooled together. The Pearson correlation coefficient is expressed as *r*, and the *P* value for the correlation is provided. Statistically significant relationships are indicated in bold, and their corresponding significant *p*-values are marked with an asterisk.

		Control	C3G	Pooled
Gclc and Total Glutathione	<i>r</i>	−0.092	−0.100	−0.096
	<i>p</i> -value	0.699	0.658	0.545
Gclc and GSH	<i>r</i>	−0.081	−0.102	−0.090
	<i>p</i> -value	0.735	0.650	0.572
Gclc and GSSG	<i>r</i>	−0.190	−0.025	−0.132
	<i>p</i> -value	0.422	0.911	0.404
Gclc and GSH/GSSG	<i>r</i>	0.041	−0.082	0.031
	<i>p</i> -value	0.864	0.717	0.843
Gclc and GSH Redox Potential	<i>r</i>	−0.017	0.161	0.043
	<i>p</i> -value	0.942	0.474	0.786
Gclm and Total Glutathione	<i>r</i>	−0.228	−0.182	−0.209
	<i>p</i> -value	0.334	0.417	0.185
Gclm and GSH	<i>r</i>	−0.252	−0.161	−0.214
	<i>p</i> -value	0.284	0.474	0.173
Gclm and GSSG	<i>r</i>	0.445	−0.473	0.053
	<i>p</i> -value	0.049*	0.026*	0.738
Gclm and GSH/GSSG	<i>r</i>	−0.365	0.388	−0.102
	<i>p</i> -value	0.113	0.075	0.519
Gclm and GSH Redox Potential	<i>r</i>	0.311	−0.157	0.152
	<i>p</i> -value	0.182	0.486	0.335
Gr and Total Glutathione	<i>r</i>	0.486	0.565	0.518
	<i>p</i> -value	0.030*	0.008*	0.001*
Gr and GSH	<i>r</i>	0.498	0.575	0.528
	<i>p</i> -value	0.025*	0.006*	< 0.001*
Gr and GSSG	<i>r</i>	−0.272	0.124	−0.089
	<i>p</i> -value	0.246	0.591	0.580
Gr and GSH/GSSG	<i>r</i>	0.526	0.418	0.451
	<i>p</i> -value	0.017*	0.059	0.003*
Gr and GSH Redox Potential	<i>r</i>	−0.579	−0.533	−0.551
	<i>p</i> -value	0.007*	0.013*	< 0.001*
Gpx−1 and Total Glutathione	<i>r</i>	−0.358	−0.312	−0.319
	<i>p</i> -value	0.122	0.169	0.042*
Gpx−1 and GSH	<i>r</i>	−0.386	−0.305	−0.327
	<i>p</i> -value	0.093	0.179	0.037*
Gpx−1 and GSSG	<i>r</i>	0.549	−0.262	0.103
	<i>p</i> -value	0.012*	0.252	0.521
Gpx−1 and GSH/GSSG	<i>r</i>	−0.565	−0.045	−0.310
	<i>p</i> -value	0.009*	0.846	0.049*
Gpx−1 and GSH Redox Potential	<i>r</i>	0.585	0.229	0.383
	<i>p</i> -value	0.007*	0.318	0.014*

Conflict of interest and funding disclosure

The authors declare that there are no conflicts of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.redox.2016.08.014.

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