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Diet-relevant phytochemical intake affects the cardiac AhR and nrf2 transcriptome and reduces heart failure in hypertensive rats

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Abstract

Intake of phytochemical-rich diets is inversely related to hypertension. Phytochemicals alter *in vitro* aryl hydrocarbon receptor (AhR) and NF-E2 related factor (nrf2) transcription factor activity and related genes pertinent to antioxidant defense. However, it is unknown if these molecular effects occur in the heart with dietary intake of physiologically-relevant phytochemicals and if this correlates with reduced hypertension-associated heart failure. This extended feeding study used whole grapes as a model of a phytochemical-rich food and hypertensive heart failure-prone rats to assess mechanisms of effect. Grape intake reduced cardiac hypertrophy and fibrosis and improved diastolic function. At the development of diastolic dysfunction, hypertensive rats show reduced AhR activity, reduced expression of AhR-regulated genes, reduced glutathione, and reduced activity of glutathione-regulating proteins. However, grape intake significantly increased cardiac AhR and nrf2 activity, Phase I/II gene transcripts and protein activity related to antioxidant defense. Heart failure is the leading cause of morbidity and mortality in the aged and the intake of phytochemicals from fruits and vegetables decreases with age. Concentrated antioxidant nutrient trials have failed to affect heart failure. However, this study demonstrates that diet-relevant intake of non-nutrient phytochemicals significantly reduces heart failure progression. Therefore, this study suggests that higher intake of phytochemical-containing foods may achieve cardiac benefits that isolated antioxidant supplements may not. In summary, intake of diet-relevant phytochemicals altered the cardiac antioxidant transcriptome, antioxidant defense, oxidative damage, and fibrosis. Regular phytochemical intake may therefore increase cardiac resistance to cardiac pathology instigated by prolonged hypertension.

Keywords

heart failure; AhR; nrf2; phytochemicals; antioxidant; cardiac

1. Introduction

While antioxidant supplements have shown limited efficacy against heart disease, antioxidant-rich diets consistently correlate with reduced hypertension. The Dietary Approaches to Stop Hypertension (DASH) trials indicated that diets rich in fruits and

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vegetables, low-fat dairy, and whole grains reduced blood pressure[1], but the effects of diet upon heart failure pathogenesis are poorly understood. Over 90% of heart failure cases are preceded by prolonged hypertension[2]. It was recently shown that DASH-style diets reduced the incidence of heart failure in women[3]. Given the complexity of the DASH diet, multiple mechanisms of cardioprotection are likely. The clinically-studied DASH diet contained significantly more phytochemicals compared to the control diet[4]. However, in hypertensive rats, an attempt to experimentally duplicate the DASH diet nutrients with altered vitamins, minerals, fiber, fats, proteins, and carbohydrates failed to reduce blood pressure[5]. This null result could support the value of the non-nutritive phytochemicals from foods for altering hypertension and its pathologic sequelae.

Grapes contain a broad array of commonly consumed flavonoids including anthocyanins, flavan-3-ols (e.g. catechin, epicateachin, proanthocyanins), and flavonols (e.g. quercetin, kaempferol, isorhamnetin)[6]. As a whole food, grapes may serve as a simple experimental model for a phytochemical-rich diet. We previously demonstrated that regular intake of whole grape powder reduced hypertension-associated diastolic dysfunction in the Dahl Salt-Sensitive (Dahl-SS) rat. Furthermore, grape-associated benefits exceeded those of the vasodilator drug, hydralazine, indicating that a depressor effect does not entirely explain the scope of grape benefit[7]. However, the cardiac-specific mechanisms associated with these grape-related benefits are unknown. Beyond a systemic depressor effect, grape-related changes in cardiac tissue may be vital to reduced cardiac oxidative damage and fibrosis that contribute to adverse changes in cardiac geometry and hemodynamic function.

As with hypertension-associated human heart failure, Dahl-SS rat hearts show reduced cardiac antioxidant defense and reduced glutathione[7–8]. Among endogenous antioxidant defenses, glutathione (GSH) is the most prominent and is found at millimolar concentrations in most cells. Intracellular glutathione exists in both a reduced form (GSH) and an oxidized form (GSSG) and may also be covalently bound to proteins[9–10]. The ratio of GSH to GSSG impacts the overall redox state of the cell, and GSH is significantly reduced in failing human hearts[11–13]. *In vitro*, phytochemicals can increase glutathione and the transcription and translation of glutathione-regulating proteins. However, these in vitro studies typically phytochemicals at high concentrations and with chemical identities not found in vivo. It is currently unknown if diet-relevant phytochemical intake can alter cardiac glutathione dynamics in hypertensive, at-risk rats and if this mechanism is actually associated with reduced cardiac pathology.

Numerous glutathione-regulating genes contain response elements called xenobiotic response elements (XREs) and antioxidant response elements (AREs)[14–15]. Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that binds to XREs and can be activated in vitro by several dietary phytochemicals[16]. Another transcription factor of interest is NF-E2 related factor (nrf2) that binds to AREs and is also activated in vitro by phytochemicals[14–15]. AhR and nrf2 activation increase the transcription/translation of genes related to Phase I/II metabolism that affect cellular responses to xenobiotics and to oxidative stress. Bioavailable phytochemicals and their metabolites may alter cellular kinase and/or transcription factor activity that could then impact antioxidant gene regulation. Specifically, grape phytochemicals and their *in vivo* metabolites may activate cardiac AhR, nrf2, or both to ultimately alter cardiac glutathione dynamics and antioxidant defense. The current effort explored the effects of physiologically-relevant intake of phytochemicals upon AhR- and nrf2–associated cardiac antioxidant defense and cardiac pathology in hypertensive Dahl-SS rats with diastolic heart failure.

2. MATERIALS AND METHODS

2.1 Animal model and diets

Five week old Dahl-Rapp Salt-Sensitive rats (Dahl-SS, Harlan, Indianapolis, IN) were acclimated for one week on AIN-76a powdered diet (Research Diets, New Brunswick, NJ). Afterwards, each rat was randomly assigned to one of four treatments (n = 12 each): low-salt diet (LS, AIN-76a with 0.17% NaCl and 2.8% added carbohydrate [glucose:fructose 1:1]); low-salt diet + grape powder (LSG, AIN-76a with 0.17% NaCl and 3.0% w/w added grape powder); high-salt diet with 6% added NaCl (HS, AIN-76a with 2.8% w/w added carbohydrate); or high-salt diet + grape powder (HSG, AIN-76a with 3.0% w/w added grape powder). Diet preparation and diet storage are as we described previously[7] and in the Online Supplement Table S1. Grape powder phytochemical content is shown in Table 1. Animals were fed 20g of powdered diet/day to match average Dahl-SS rat *ad libitum* intake as we determined from our previous studies[7] to ensure complete daily consumption. With regards to grape "dose" justification, the dose of grape powder given per day was made relative to body weight. One human serving of fresh grapes is ¾ cup, or approximately 126g. With loss on drying, one human serving of freeze dried whole grape powder equals 23g. The intent of the study was to model 8–9 servings/day of phytocyhemical-containing fruits and vegetables as used in the DASH diet clinical trials[1]. For calculations, adult human male body weight was 75 kg while adult male rat weight averaged 225 g. The rat body weight equivalent of 8–9 servings of grape/day then averaged 600 mg of grape powder/day, or 3% of the daily diet. Rats were housed in 12h light:12h dark cycles, and water was provided *ad libitum*. This project was approved by the Animal Care and Use Committee at the University of Michigan.

2.2 Blood pressure and echocardiography

During the 18 week study, blood pressure was measured bi-monthly in conscious, restrained rats by the IITC Mark 12 photoelectric/oscillometric tail cuff system (IITC Life Sciences, Woodland Hills, CA) using the unit and method we previously described[7](see Online Supplement) and validated against telemetric approaches[17]. A run was accepted if at least six of eight repeated measures were adequate (having detectable pulses and free of gross artifacts). The average was then calculated as the mean value for that time point.

Echocardiography was performed monthly. Anesthesia was induced with 4% isoflurane and maintained with 1% isoflurane. Two-dimensionally guided M-mode recordings and Doppler tissue imaging were acquired as we described previously[17](see Online Supplement). All measurements were made in accordance with the conventions of the American Society of Echocardiography and were conducted by the same trained, blinded research animal sonographer.

2.3 Cardiac fibrosis and oxidative damage

After 18 weeks, rats were anesthetized with 4% isoflurane and sacrificed by guillotine. Hearts were harvested, washed in phosphate-buffered saline, blotted, and weighed. The left ventricle is the main working chamber of the heart and is thus most impacted by oxidative stress and fibrosis during heart failure pathogenesis. The left ventricle was minced, flash frozen in liquid nitrogen, and stored at −80°C. Collagen component hydroxyproline was measured in left ventricle homogenates as a quantitative index of total tissue fibrosis using a method we described previously[7](see Online Supplement). The amount of hydroxyproline was calculated using the standard curve and expressed as μg/mg total protein. Oxidative damage was revealed by the cardiac content of malonyldialdehyde (MDA) as assayed by the conjugation ability of MDA with thiobarbituric acid. MDA was measured using a

commercial kit (Cayman Chemical, Ann Arbor, MI) according to manufacturers' instructions.

2.4 Transcription factor DNA binding activity

Frozen cardiac tissue was fractionated to obtain nuclear and cytosolic homogenates using the method of Li *et al.*[18] with modifications using the NE-PER Nuclear Extraction Kit (Pierce, Rockford, IL) as we described previously[19](see Online Supplement). Once successful fractionation was confirmed, nrf2 DNA binding was measured in nuclear fractions using the TransAM™ ELISA (Active Motif, Carlsbad, CA) according to manufacturers' instructions. The 96-well plate is coated with a proprietary oligonucleotide that contained several ARE sequences. Nrf2 in the nuclear extract (loaded at 5ug total protein/well) binds to the ARE-containing oligonucleotides. The provided primary antibody is then targeted to nrf2 (1:100), and a horseradish peroxidase(HRP)-conjugated secondary antibody (1:10,000) enables colorimetric detection at 450nm. AhR DNA-binding activity was measured using a custom sandwich ELISA. A 96-well plate (Pierce® High Sensitivity NeutrAvidin®) was coated with a fabricated biotinylated, oligonucleotide that contained three XRE sequences (5′-TCGACATTGCCACGCCAGCTCACGCTGCTACACGCTTAGCGCTACT-3′) with XRE consensus sequences underlined. AhR in the nuclear extract (loaded at 10μg total protein/well) then binds to the XRE-containing oligonucleotides. Primary antibody is then targeted to AhR (1:100), and a HRP-conjugated secondary antibody (1:10,000) enables colorimetric detection at 450nm.

2.5 RT-PCR/PCR Array

Total RNA from minced left ventricle was isolated with the RNeasy™ Fibrous Tissue Midi Kit (Qiagen, Valencia CA, USA) following the manufacturer's protocol. From the twelve animals per group, four representative RNA samples were obtained by randomly combining equimolar amounts of RNA from three rats. The relative abundance of mRNA transcripts was then compared using a RT^2 Profiler PCR Array™ (SABiosciences, Frederick MD). The array contains pre-optimized, rat-specific primer sets for controls (housekeeping genes, genomic DNA control, reverse transcription control, and positive PCR control) and the experimental genes of interest. cDNA was prepared using the RT^2 First Strand Kit. Relative expression was determined by the ΔΔCT method as described by Livak[20]. ΔCt for each transcript is normalized relative to average ΔCt of four housekeeping genes that were unaffected by treatment (P1 large ribosomal protein, hypoxanthine guanine phosphoribosyl transferase, ribosomal protein L13A, and lactate dehydrogenase).

2.6 Cardiac glutathione and enzymatic activity assays

The glutathione-related assays were conducted on homogenates derived from frozen left ventricle. This tissue was added (1:20 w:v) to T-PER™ tissue lysis buffer (Pierce, Rockford IL, USA) according to manufacturer's protocol, with added 5% metaphosphoric acid to limit auto-oxidation. The tissue fragment and extraction buffer were pulsed for thirty seconds with a Polytron homogenizer (Brinkmann) and either placed on ice for immediate assay or flash-frozen for later analysis. Total protein content was measured using the BCA assay (Pierce). Cardiac GSH/GSSG, glutathione peroxidase activity, and glutathione reductase activity were measured using commercial kits (Oxis International, Beverly Hills, CA) according to manufacturers' instructions.

2.7 Statistics

mRNA transcript pair-wise comparisons were accomplished using the ΔΔCT method as described by Livak[20] using the PCR Array data analysis web portal of SABiosciences

(http://www.sabiosciences.com/pcr/arrayanalysis.php). All other endpoints were assessed using SPSS version 16.0 (SPSS, Chicago, IL). Data for Western Blot relative abundance, transcription factor ELISA, GSH/GSSG ratio, and enzyme activity were compared using a two-way ANOVA with salt and grape as factors. Pair-wise comparisons were accomplished with Bonferroni *post-hoc* tests. For all measures, a p value < 0.05 was considered statistically significant.

3. Results

3.1 Cardiac pathology

For all measured parameters, baseline values were not significantly different among groups. Grape intake significantly reduced systolic blood pressure in hypertensive rats (Table 2) which was a sustained effect first observed at the week four measurement (data not shown). Salt intake was associated with cardiac hypertrophy and increased cardiac hydroxyproline content, an index of collagen content and fibrosis. These effects were attenuated with grape intake (HSG group)(Table 2). In contrast, there were no differences between the LSG group and LS groups. Grape intake reduced blood pressure only in hypertensive rats. The high-salt group (HS) had increased relative wall thickness (RWT), but HSG reduced this effect. Mild or early diastolic dysfunction can be characterized by altered ventricular filling velocities; this parameter is reflected the ratio of early filling velocity (E wave) to late filling velocity (A wave). HS showed increased E/A elevation which was significantly attenuated in HSG. Prolonged isovolumetric relaxation time (IVRT) indicates increased myocardial stiffness due to fibrosis [21] which is further supported by increased hydroxyproline content. IVRT and cardiac hydroxyproline content increased in HS, but HSG reduced this effect. Ejection fraction was not altered by high-salt feeding which is expected in this rat model of diastolic dysfunction. Cardiac index reflects cardiac contractile efficiency by measuring the volume of blood pumped per minute (stroke volume x heart rate) per unit of body weight, and therefore reflects both diastolic and systolic function. Cardiac index was lower in HS but was improved in HSG. Collectively, these findings indicate that changes in blood pressure, cardiac geometry, diastolic parameters, and cardiac function were improved by physiologically-relevant phytochemical intake.

3.2 Transcription factor DNA binding

Results for nuclear fraction AhR activity are shown in Figure 1A. Compared to LS, LSG had enhanced AhR activity. In contrast, HS had reduced AhR activity. Compared to HS, HSG showed enhanced AhR activity. Results for nrf2 DNA binding activity are shown in Figure 1B. Compared to LS, LSG showed increased nrf2 activity. HS also showed enhanced nrf2 activity, which is expected given the stimulating effect of oxidative stress of nrf2 activity. However, compared to HS, HSG showed further enhanced nrf2 activity. Collectively, these findings indicate that both salt and grape affected AhR and nrf2 activity.

3.3 RT-PCR

Transcript abundance is compared in Table 3 which includes the gene name, fold regulation, and whether the gene of interest contains XRE sequences (5′-TnGCGTG-3′) or ARE sequences (5′-TGACTCAG-3′) indicating AhR or nrf2 activation, respectively. Results include the pair-wise fold-regulation comparisons of: 1) effect of salt alone (LS v HS); 2) effect of grape in healthy rats (LSG v LS); and 3) effect of grape in diseased rats (HSG v HS).

Effect of salt—Compared to LS, HS showed reduced AhR mRNA and its nuclear chaperone ARNT. In accordance with these effects, HS also reduced mRNA related to XRE activation including CYP1A1, CYP1B1, and UGT1A6. In addition, HS had reduced mRNA

for several glutathione-S-transferase (GST) isoforms that contain both XRE and ARE elements and would be responsive to both AhR and nrf2 activation. HS did not reduce nrf2 mRNA but reduced ARE-related mRNAs for γ-glutamylcysteine synthetase (γ-GCS), the rate limiting enzyme in glutathione synthesis, and for glutathione reductase (GR), the protein responsible for reducing GSSG to GSH. Results of salt effect upon ARE-related glutathione peroxidase (GPx) isoforms appear mixed with decreased GPx3 mRNA but unaffected GPx4 mRNA. The results therefore indicate that in Dahl-SS rats, both ARE and XRE-affected mRNAs are impacted by salt intake.

Effect of grape in healthy rats—Compared to LS, LSG showed increased mRNA for AhR mRNA and CYP1A1 mRNA. LSG was also associated with increased GST isoforms mu 2 and 3 mRNA. Amongst ARE-related transcripts, LSG was associated with significantly increased γ-GCS mRNA. The results therefore indicate that both ARE and XRE-affected mRNAs are increased by grape intake in healthy rats.

Effect of grape in salt-fed rats—Compared to HS, HSG showed attenuated salt-related changes. HSG showed increased AhR mRNA and ARNT mRNA, and XRE-related mRNAs like nrf2, CYP1A1 and CYP1B1. In addition, HSG showed increased GST isoforms mu 2, 3, and 4. Amongst ARE-related transcripts, HSG showed increased γ-GCS and GR mRNA and mixed effects upon GpX isoform mRNA. The results therefore indicate that in salt-fed Dahl-SS rats, both ARE and XRE-affected mRNAs are increased by grape intake.

3.4 Cardiac glutathione and enzymatic activity assays

Figure 2 demonstrates that LSG and HSG showed increased glutathione (GSH) relative to their respective controls. It is then prudent to measure the activity of two proteins that impact the GSH:GSSG ratio - glutathione reductase and glutathione peroxidase. Grape intake increased the activity of glutathione reductase (Figure 3A) which would favor for GSH formation. Glutathione peroxidase activity (Figure 3B) was not significantly altered by salt or by grape. However, this assay was not isoform-specific; it is possible that individual isoforms of glutathione peroxidase have altered activity; this may be important given the mixed effects of salt intake on GPx mRNA. On balance, increased GR activity and null effect on GPx would contribute to elevated GSH/GSSG.

4. Discussion

Glutathione is the most abundant cellular antioxidant in the heart, and deficiency in cardiac and systemic glutathione relates to heart failure progression in animal models, including the Dahl-SS rat as described here. Glutathione deficiency is also observed in human heart failure[12–13] and may serve as a marker of early heart failure. The association of glutathione levels and disease was demonstrated in cardiac patients of different New York Heart Association (NYHA) functional classes and with different cardiac structural heart diseases[22]. Glutathione was measured in venous blood samples of healthy volunteers and cardiac patients rated on the revised NYHA functional class I to IV scale and undergoing cardiac surgery for coronary artery disease, aortic stenosis or terminal cardiomyopathy. Glutathione was also quantified in right atrial appendages. Compared to healthy controls, plasma glutathione was decreased by 21% in patients of NYHA class I with structural cardiac disease, and by 40% in patients of NYHA class II to IV. In addition, significant depletion in glutathione occurred before detectable elevation in plasma TNF-α, a marker of symptomatic heart failure severity. Atrial tissue glutathione was severely depleted (−58%) in NYHA class IV patients compared to NYHA class I patients. This study provided evidence that both cardiac and systemic glutathione deficiency are related to functional status and to structural cardiac abnormalities of patients with cardiac diseases of varied etiology. If

glutathione levels correlate with heart failure pathogenesis[22], methods to enhance this abundant antioxidant may affect the trajectory and degree of cardiac remodeling and dysfunction.

Because oxidative stress is strongly correlated with heart failure pathogenesis, numerous trials have attempted to assess the clinical value of antioxidant supplements in cardiac patients. However, clinical trials with isolated, antioxidant nutrients have failed to show consistent and significant clinical benefits[23–25]. It is therefore possible that consumption of antioxidant rich whole foods rather than isolated nutrients alone could confer a superior cardiovascular benefit.

4.1 Phytochemical benefits – direct versus indirect mechanisms

Direct antioxidant effects from phytochemicals in heart tissue would require tissue bioavailability of the phytochemicals and/or their metabolites. The grape compounds and their metabolites that are bioavailable to the heart may react directly with free metals or with reactive chemical species, forming products with much lower reactivity, or may act indirectly by affecting cardiac cell signaling and/or gene transcription. Because the tissue levels of these compounds are likely low as compared to endogenous antioxidants, bioavailable phytochemical metabolites likely perform an indirect antioxidant benefit on the Dahl-SS rat heart by altered cell signaling and related gene transcription. For example, grape phytochemicals may act as xenobiotics and thereby alter several genes related to Phase I/II metabolism proteins and antioxidant defense. Studies in animal and cell culture models show that phytochemical-rich diets activate the expression of Phase I/II metabolism in varied body tissues[26–28]. This effect of diet could be achieved by ligand interaction or by altering kinase signaling cascades that then trigger transcription factor activation. Two transcription factors of interest for the present work are AhR and nrf2.

4.2 Phytochemical intake increased AhR activity

AhR is a ligand-activated transcription factor that is classically activated by synthetic xenobiotics like dioxin but is also activated by an array of dietary phytochemicals[16]. Owing to their aromatic chemical structure, several phytochemicals like flavone, catechin, and quercetin display *in vitro* agonist properties towards AhR. In the cytoplasm, AhR remains bound to heat shock protein HSP90. The molecular mechanisms of AhR activation by polyphenols are unknown, but likely involve kinase-mediated dissociation with HSP90 to allow nuclear translocation. AhR binds with AhR nuclear translocator (ARNT) to allow binding to XRE regions in the genome. In adult tissue, the consequences of AhR activation primarily involve induced transcription of Phase I enzymes like CYP1A1, CYP1A2, CYP1B1, NADPH:quinone oxioreductase-1(NQO-1), GST, aldehyde dehydrogenase 3A1, UGT1A1 and 1A6, and nrf2[29].

In the current study, salt-fed rats showed reduced AhR activity and reduced mRNA for AhR and of ARNT. These results are supported by other models of pathologic cardiac hypertrophy. For example, AhR-null mice display cardiac hypertrophy[30], elevated blood pressure, and elevated plasma angiotensin II and endothelin I[31]. However, grape intake increased AhR activity. Both LSG and HSG rats showed significantly increased AhR activity and CYP1A1 mRNA, a hallmark of AhR activation. HSG also showed increased ARNT mRNA, which could contribute to HSG-enhanced AhR activity. Given the complexity of the whole food model, it is inappropriate to utilize a reductionist approach to determine which grape phytochemicals are responsible for enhanced AhR activity. However, the current results of increased AhR activity and AhR-related transcripts are in agreement with studies using phytochemicals found in whole grapes including quercetin[32–33], kaempherol[34], catechins[35–36], and resveratrol[33–37].

4.3 Phytochemical intake increased nrf2 activity

Nrf2 is a basic leucine zipper transcription factor that can be activated by kinases induced by oxidative stress or by xenobiotics like phytochemicals. *In vitro*, phytochemicals and phytochemical-rich extracts activate signaling kinases that enhance nrf2 nuclear translocation and activity[38–40]. For example, phytochemicals could alter kinase modification of the Nrf2 chaperone Keap1. Kinase-mediated phosphorylation of Keap1 would stearically alter the nrf2/Keap1 complex, allowing the release of nrf2 and subsequent binding to genomic AREs. The consequences of nrf2 activation primarily involve increased mRNA for nrf2, NQO1, numerous GST isoforms, γ-GCS, UGT1A6, and antioxidant defense enzymes like thioredoxin, metallothionein-1/2, and heme-oxygenase-1.

4.4 Phytochemical intake improves glutathione reserve

While there are several proteins involved in antioxidant defense, the current work focused on glutathione due to its abundance in the heart and its correlation with cardioprotection and heart failure. HS rats showed reduced mRNA to support glutathione formation (e.g. γ-GCS), glutathione conjugation (e.g. GST isoforms), and glutathione reduction to GSH (glutathione reductase). We also observed reduced GSH/GSSG and reduced glutathione reductase activity. However, grape intake increased glutathione reserve. In both LSG and HSG rats, grape-mediated nrf2 activation is supported by significantly increased γ-GCS mRNA which would correlate with the observed increases in tissue GSH. Additionally, the HSG rats showed enhanced glutathione reductase that would also favor an elevated GSH/GSSG ratio.

As stated before, it is inappropriate to utilize a reductionist approach to determine which grape phytochemicals are responsible for enhanced nrf2 activity. However, the results here that show enhanced nrf2 activity are in agreement with studies using select phytochemicals found in whole grapes including quercetin[39-41-42], kaempherol[43], catechins[38-39-44], and resveratrol[39-40-45]. In addition, previous studies support the ability of flavonoids to increase glutathione-related enzymes and glutathione synthesis[15-26-46]. As shown here, enhanced tissue GSH was observed using a whole food model of purple corn[47–48]. Prolonged feeding of healthy rats with purple corn (20% of the daily diet) enhanced cardiac GSH and enhanced protection against *in vivo* and *ex vivo* cardiac ischemia/reperfusion injury. However, the molecular mechanisms of this effect were not explored as conducted here.

4.5 Possible advantages of concurrent cardiac AhR and nrf2 activation

The current study shows that grape intake induces both cardiac AhR and nrf2 activity. Enhanced CYP enzyme activity by AhR often generates low levels of reactive oxygen species that can then activate redox-sensitive nrf2[49–50]. It is now believed that cross-talk between AhR and nrf2 provides an evolutionary advantage by allowing the upregulation of Phase I enzymes (via AhR/XRE binding) and Phase II enzymes (via nrf2/ARE binding) in succession to address xenobiotic metabolism[49] and to limit oxidative stress. Therefore, dual activation of AhR and nrf2 by grape may allow greater cardiac antioxidant defense than either alone.

4.6 Limitations and alternative hypotheses

In addition to AhR and nrf2 activation, other potential mechanisms may be considered. In HSG rats, grape effects on glutathione may be indirect as well as direct. For example, grape intake may be associated with reduced oxidative stress that would indirectly favor preserved GSH levels. However, the data from the healthy LSG group support the direct effect of grape upon AhR and nrf2 activity and glutathione dynamics independent of hypertension-associated oxidative stress[7]. Also, grape intake may indirectly alter the formation of

endogenous ligands of AhR like lipid peroxidative products. However, we previously observed that grape intake reduced cardiac lipid oxidative damage marker malonyldialdehyde [7]. This would suggest that grape actually lowered endogenous ligands for AhR activation, and that grape-relate AhR agonism is more likely due to direct effects of the bioavailable phytochemicals. Finally, the current study cannot exclude that the depressor effect of grape indirectly contributes to the observed genetic differences in the HSG group. However, given our previous observation that comparable blood pressure reduction by vasodilator hydralazine fails to match the cardioprotective effects of grape[7], the data suggest that the benefits of grape intake extend beyond blood pressure reduction.

In summary, grape intake altered genes that affect glutathione dynamics that on balance, would favor elevated glutathione. In addition, grape intake was associated with elevated activity of glutathione reducatse that would favor elevated GSH relative to GSSG. Importantly, these changes were conserved in grape-fed, healthy rats, further supporting the direct genetic mechanisms of grape intake on glutathione dynamics. The grape-related mechanisms of cardiac AhR and nrf2 activity may be vital to the diets' cardioprotective effects in hypertensive rats. In addition to lowering blood pressure as observed in the DASH trials, phytochemical-rich diets could alter the cardiac transcriptome in ways that favor greater resistance to prolonged hypertension and concomitant oxidative stress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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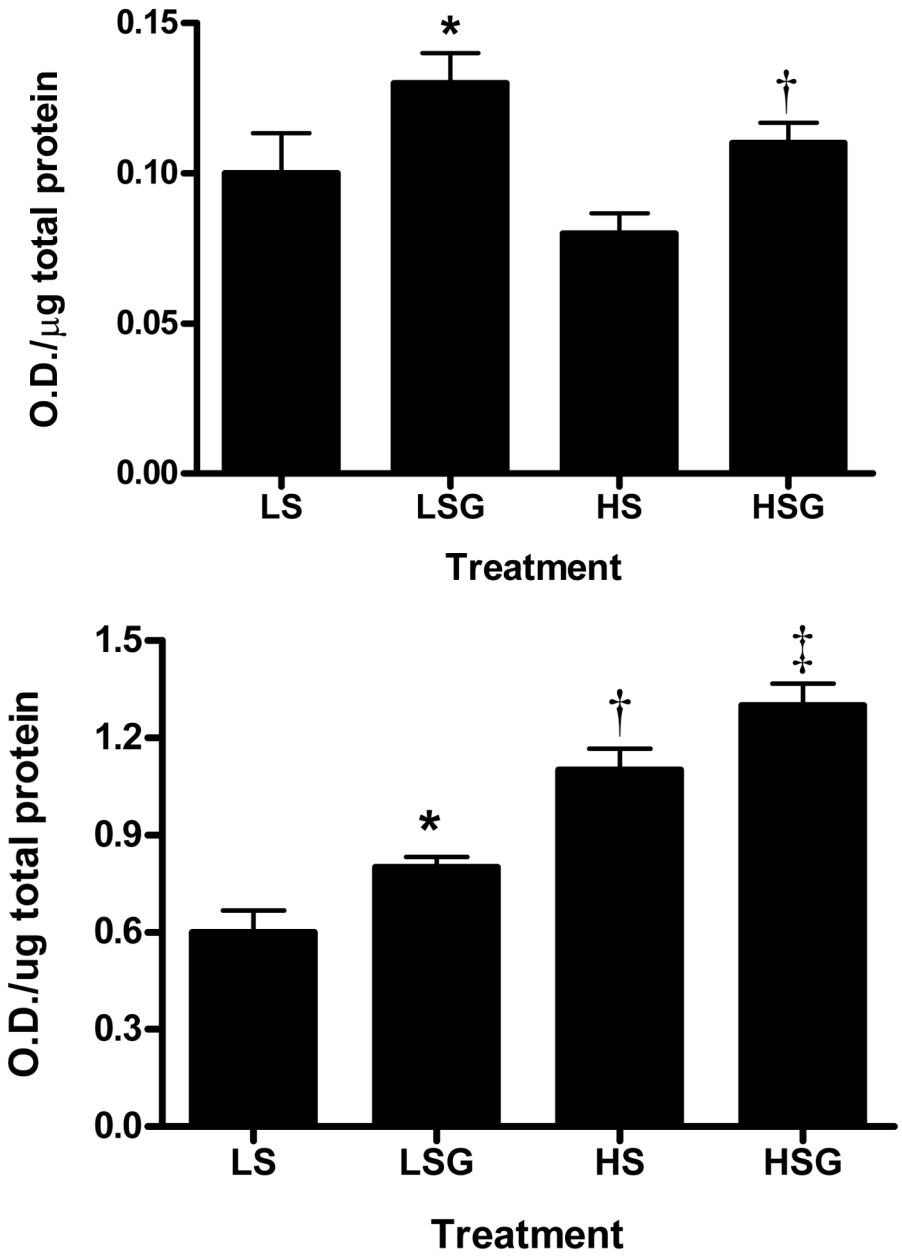


Figures 1A–B. Cardiac AhR and nrf2 Activity

n = 12 each. (LS) low-salt diet; (LSG) low salt + grape powder diet; (HS) high-salt diet; (HSG) high salt + grape powder diet. **p* < 0.05 vs LS, †*p* < 0.05 vs LS, LSG, ††*p* < 0.05 vs HS.

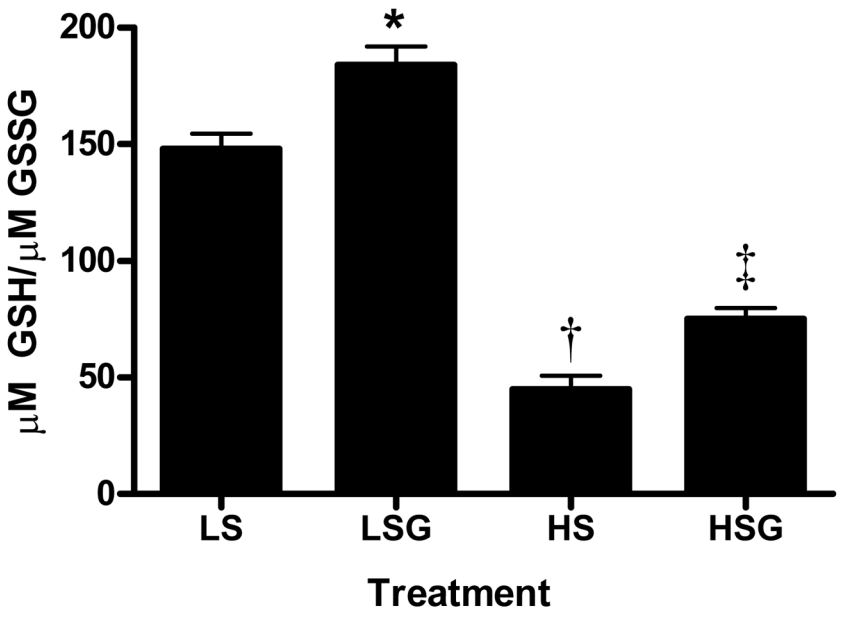
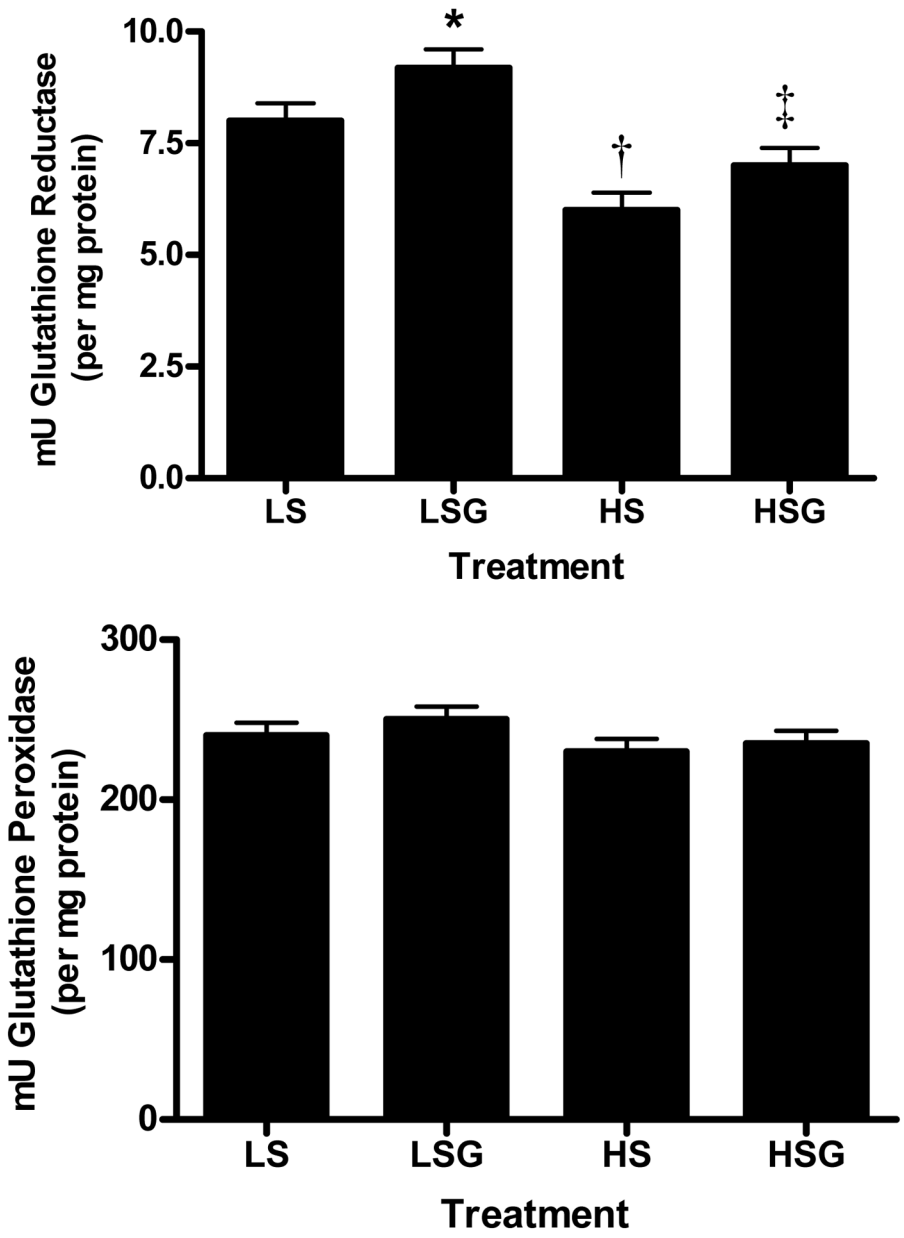


Figure 2. Cardiac GHS/GSSG

n = 12 each. (LS) low-salt diet; (LSG) low salt + grape powder diet; (HS) high-salt diet; (HSG) high salt + grape powder diet. **p* < 0.05 vs LS. †*p* < 0.05 vs LS, LSG. ††*p* < 0.05 vs HS.



Figures 3A–B. Cardiac GR Activity and GPx Activity

n = 12 each. (LS) low-salt diet; (LSG) low salt + grape powder diet; (HS) high-salt diet; (HSG) high salt + grape powder diet. **p* < 0.05 vs LS, †*p* < 0.05 vs LS, LSG, ††*p* < 0.05 vs HS.

Table 1

Grape powder phytochemical content

Anthocyanins	
Cyanidin	124.6 mg/kg
Malvidin	142.2 mg/kg
Peonidin	31.7 mg/kg
Monomeric Flavanols	
Catechin	19.7 mg/kg
Epicatechin	12.6 mg/kg
Flavonols	
Quercetin	32.6 mg/kg
Kaempferol	5.6 mg/kg
Isorhamnetin	6.8 mg/kg
Stilbenes	
Resveratrol	1.75 mg/kg
Total Phenols	513 mg/100g

Analyzed by the National Food Laboratories, Inc. on behalf of the California Table Grape Commission.

Table 2

Cardiac structure, function, fibrosis, and oxidative damage

Endpoints	LS	LSG	HS	HSG
% Change BW	379.1±13	381.2±15	336.5±11*	354.5±10†
Systolic BP	151 ± 16	141 ± 12	244 ± 15*	221 ± 13†
HW/TL	0.34±0.01	0.33±0.02	0.42±0.1*	0.32±0.01
Malonyldialdehyde	0.4±0.02	0.3±0.02	1.4±0.2	1.0±0.1
Hydroxyproline	5.2±0.3	5.1±0.2	9.2±0.2*	7.4±0.3†
RW th	0.4±0.02	0.4±0.03	0.7±0.02*	0.5±0.03†
IVRT	21.1±2	20.4±3	31.5±3*	24.3±1†
E/A	2.6±0.3	2.5±0.1	6.2 ±0.3*	3.8 ±0.13†
% Eject. Fraction	72.1±4	72.1±4	70.1±4	71.1±5
Cardiac Index	437±22	436±19	333±26*	375±21†

% Change Body Weight (BW) from day zero. Blood Pressure (BP) in mm Hg as measured the final week before sacrifice. HW (gram heart weight) relative to tibial length (TL) in centimeters. Cardiac hydroxyproline as milligrams/grams of total protein. Echocardiography measures included RW th (relative wall thickness, mm), IVRT (Isovolumetric Relaxation Time), E/A (E Wave to A Wave), and % Ejection Fraction (% Eject. Fraction). Cardiac index is ml of blood/pumped per minute/g body weight. Data presented as mean ± SEM, $n = 12$ per group.

* at least $p < 0.05$ vs. LS and LSG;

† $p < 0.05$ vs. LS, LSG, and HS.

Table 3

PCR Array results

DNA Response Element	Gene	Name	Fold Regulation by Salt	Fold Regulation by Grape	
			HS (v LS)	LSG (v LS)	HSG (v HS)
	AhR	Aryl Hydrocarbon Receptor	−1.389*	1.206*	1.244*
	ARNT	AhR Nuclear Translocator	−2.437*	1.042	1.922*
XRE	Nrf2	nuclear factor erythroid-derived 2	1.095	1.285*	1.568*
XRE	CYPA1	Cytochrome P450 1A1	−2.692*	1.955*	1.329*
XRE	CYPB1	Cytochrome P450 1B1	−1.496*	1.226	1.522*
XRE/ARE	GST M2	Glutathione S-Transferase mu 2	−1.726*	1.252*	1.348*
XRE/ARE	GST M3	Glutathione S-Transferase mu 3	−2.495*	1.232*	1.417*
XRE/ARE	UGT1A6	UDP-glucuronosyltransferase1A6	−1.284*	1.173	1.126
ARE	GST A1	Glutathione S-Transferase A1	−1.115	1.002	1.036
ARE	GCS	γ-glutamylcysteine synthetase	−1.422*	2.965*	5.617*
ARE	GR	Glutathione Reductase	−2.220*	1.152	1.967*
ARE	GPx3	Glutathione Peroxidase 3	−1.318*	1.056	1.309*
ARE	GPx4	Glutathione Peroxidase 4	1.057	−1.182	−1.144

$N = 4$ per group. Pair-wise comparison via ΔΔCT method.

*p at least < 0.05 versus respective control (LS or HS) (LS) low-salt diet; (LSG) low salt +grape powder diet; (HS) high-salt diet; (HSG) high salt + grape powder diet.