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PATHOGENESIS AND CARDIAC GENE  
TRANSCRIPTION/TRANSLATION

presented by

E Mitchell Seymour

has been accepted towards fulfillment  
of the requirements for the

Doctoral degree in Human Nutrition

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**GRAPE PHYTOCHEMICAL INTAKE ALTERS HEART FAILURE PATHOGENESIS  
AND CARDIAC GENE TRANSCRIPTION/TRANSLATION**

**By**

**E Mitchell Seymour**

**A DISSERTATION**

**Submitted to  
Michigan State University  
In partial fulfillment of the degree requirements  
for the degree of**

**DOCTOR OF PHILOSOPHY**

**Human Nutrition**

**2009**

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## **ABSTRACT**

### **GRAPE PHYTOCHEMICAL INTAKE ALTERS HEART FAILURE PATHOGENESIS AND CARDIAC GENE TRANSCRIPTION/TRANSLATION**

By

E Mitchell Seymour

High blood pressure or hypertension is a prevalent and significant contributor to morbidity and mortality from heart failure. The DASH (Dietary Approaches to Stop Hypertension) clinical trials provided evidence that diets rich in fruits and vegetables reduced blood pressure. Animal models of hypertension may permit mechanistic appraisals of the interaction of diet and disease. One recent study in hypertensive rats modeled the DASH diet using added vitamins and minerals, but failed to detect an effect on blood pressure. Therefore, a whole foods model may be more appropriate to assess the effect of diet on hypertension, versus elevated vitamins and minerals, alone.

It is currently unknown if intake of phytochemical-rich whole foods affects hypertension-associated heart failure. This proposal uses whole table grapes as a model of a phytochemical-rich food. We first tested the hypothesis that a grape-enriched diet would impact the development of hypertension-related cardiac pathology in the Dahl Salt-Sensitive (Dahl-SS) rat model. Whole table grape powder (3% of diet) significantly lowered blood pressure, cardiac hypertrophy and cardiac oxidative damage. In addition, grape-fed rats displayed improved diastolic function and cardiac output. However, cardiac-specific mechanisms of these effects remain unknown.

Bioavailable grape phytochemicals may have reduced heart failure pathogenesis by altered cardiac cell signaling and gene transcription/translation. One project arm focuses on transcription factor peroxisome proliferator-activating receptor (PPAR).

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During heart failure pathogenesis, cardiac PPAR isoforms are down-regulated while cardiac pro-inflammatory transcription factor NF $\kappa$ B activity is elevated. Importantly, PPAR activation directly reduces NF- $\kappa$ B activation. Phytochemicals can activate PPARs in varied experimental models. If grape powder diet altered cardiac PPAR activity, it could also limit NF- $\kappa$ B activity and thereby reduce inflammation and fibrosis.

Bioavailable grape phytochemicals could also activate the phenol-responsive, aryl-hydrocarbon receptor (AhR), which binds to genomic xenobiotic response elements (XREs) and stimulates the transcription of mRNA related to antioxidant defense. Bioavailable grape phytochemicals could also activate NF-E2 p45-related factor (Nrf2), a transcription factor which binds genomic antioxidant-responsive elements (AREs) and like AhR, stimulates the transcription of genes related to antioxidant defense. While AhR/XRE and Nrf2/ARE interactions have been shown *in vitro* with select phytochemicals, it is uncertain if physiologically-relevant doses of grape powder could confer a similar effect *in vivo*.

Four groups were studied: low salt diet + grape, high salt + grape, and their respective low-salt and high-salt, carbohydrate-equivalent controls. Grape diets enhanced cardiac PPAR and reduced NF- $\kappa$ B activity, NF- $\kappa$ B-related mRNA, and TNF- $\alpha$  and TGF- $\beta$  expression. Grape diets also enhanced cardiac AhR and Nrf2 activation, related mRNA, and glutathione dynamics. Importantly, the majority of these effects were conserved between low salt grape and high salt grape groups, indicating specific effects from the grape powder treatment. In summary, consumption of whole table grape powder reduced Dahl-SS rat cardiac hypertensive pathology, and altered gene transcription/translation related to inflammation, fibrosis, and glutathione antioxidant defense.

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## **DEDICATION**

I wish to dedicate this effort to my friends and martial art instructors, Tammy Williams and Dan Timlin. You give selflessly, providing me with your patience, your guidance, a safe place to learn, and a soft place to fall.

You exemplify a brave and determined pursuit of excellence, in times of both fortune and adversity. Dan, despite your diagnosis of heart failure in the prime of your life, you adapted and thrived. Your attitude continues to inspire me in my own life with chronic illness. In return, I hope that my research efforts in heart failure can contribute something of value - in your honor.

Your faith in me is a gift that I will cherish always. You have honored my strengths and counseled my weaknesses, gently supporting the evolution of a better human being. You are teachers in the highest sense, and friends of the highest character. In respect and gratitude, forever yours in “Black Belt Excellence”.

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I wish to thank my Michigan State University doctoral committee members for their time and effort, and especially for their flexibility and their private counsel – Drs. Les Bourquin, Maija Zile, Kathleen Hoag, Stephanie Watts, and Maurice Bennink. I also wish to thank my University of Michigan lab colleagues Drs. Ara Kirakosyan and Peter Kaufman, who provided daily support, scientific mentorship, and camaraderie. Finally, I wish to thank my lab director Dr. Steven Bolling, for generously supporting and facilitating my continued education and professional development.

I extend a special thanks to my advisor Maurice Bennink, who has provided both professional and personal inspiration through his intellectual curiosity, his balanced work ethic, his gentle patience, and his genuine concern for his students. As a role model, Dr. Bennink has not only impacted my own work, but also the way in which I supervise and mentor other young scientists. In this manner, he truly “touches beyond his reach”. Dr. Bennink has been a valued mentor and friend, and I am truly honored to pursue this degree under his guidance.

Finally, I wish to thank my partner Kristin and my family. Kristin encouraged me to re-enroll in my PhD program following my earlier departure due to chronic illness, and she continues to provide encouragement and inspiration. And to my family, I’ve finally earned my long-time moniker, “Dr Buff”. I’m looking forward to that vanity plate. Thank you for your continued love and support!

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$\alpha$  - A

$\beta$  - B

$\delta$  - D

$\gamma$  - G

$\kappa$  - K

AhR

AIN

cGMP

CYP1A1

CYP1B1

DASH

DHF

E/A

E Dec t

FS

GCS

GPx

GSH

GST

## **KEY TO SYMBOLS and ABBREVIATIONS**

### **SYMBOLS**

$\alpha$  - Alpha

$\beta$  - Beta

$\delta$  - Delta

$\gamma$  - Gamma

$\kappa$  - Kappa

### **ABBREVIATIONS**

|                |                                         |
|----------------|-----------------------------------------|
| <b>AhR</b>     | Aryl hydrocarbon receptor               |
| <b>AIN</b>     | American Institute of Nutrition         |
| <b>cGMP</b>    | Cyclic guanosine monophosphate          |
| <b>CYP1A1</b>  | Cytochrome P450 1A1                     |
| <b>CYP1B1</b>  | Cytochrome P450 1B1                     |
| <b>DASH</b>    | Dietary Approached to Stop Hypertension |
| <b>DHF</b>     | Diastolic Heart Failure                 |
| <b>E/A</b>     | E Wave to A wave                        |
| <b>E Dec t</b> | E Wave Deceleration time                |
| <b>FS</b>      | Fractional Shortening                   |
| <b>GCS</b>     | $\gamma$ -glutamylcysteine synthetase   |
| <b>GPx</b>     | Glutathione Peroxidase                  |
| <b>GSH</b>     | Reduced Glutathione                     |
| <b>GST</b>     | Glutathione S-Transferase               |

GSSG

GR

HS

HSG

HSH

ICAM

I $\kappa$ B $\alpha$

IL-1 $\beta$

IL-6

IWHS

IVRT

Keap1

LC-MS/N

L-NAME

LS

LSG

LV

LVEDD

LVESD

LV/BW

MCP-1

MDA

MTS

|                                                 |                                                |
|-------------------------------------------------|------------------------------------------------|
| <b>GSSG</b>                                     | Oxidized Glutathion                            |
| <b>GR</b>                                       | Glutathione Reductase                          |
| <b>HS</b>                                       | High salt diet                                 |
| <b>HSG</b>                                      | High salt diet + grape                         |
| <b>HSH</b>                                      | High salt diet + hydralazine                   |
| <b>ICAM</b>                                     | Intercellular Adhesion Molecule                |
| <b>I<math>\kappa</math>B<math>\alpha</math></b> | Inhibitor kappa B $\alpha$                     |
| <b>IL-1<math>\beta</math></b>                   | Interleukin-1 $\beta$                          |
| <b>IL-6</b>                                     | Interleukin-6                                  |
| <b>IWHS</b>                                     | Iowa Women's Health Study                      |
| <b>IVRT</b>                                     | isovolumetric relaxation time                  |
| <b>Keap1</b>                                    | Kelch-associated protein 1                     |
| <b>LC-MS/MS</b>                                 | liquid chromatography tandem mass spectroscopy |
| <b>L-NAME</b>                                   | L-N <sup>6</sup> -Nitroarginine methyl ester   |
| <b>LS</b>                                       | Low Salt                                       |
| <b>LSG</b>                                      | Low Salt + Grape Diet                          |
| <b>LV</b>                                       | Left Ventricle                                 |
| <b>LVEDD</b>                                    | Left ventricular end-diastolic dimension       |
| <b>LVESD</b>                                    | Left ventricular end-systolic dimension        |
| <b>LV/BW</b>                                    | Left ventricle mass/body weight                |
| <b>MCP-1</b>                                    | Monocyte chemotactic protein-1                 |
| <b>MDA</b>                                      | Malonyldialdehyde                              |
| <b>MTS</b>                                      | Masson-Trichrome Stain                         |

NF- $\kappa$ B

NHAN

NIH-N

NQO-1

NO

nrf2

NYHA

PDE-5

PGC-1 $\alpha$

PPAR

RAAS

RAS

RNS

ROS

RW<sub>th</sub>

SHE

TNF- $\alpha$

TGF- $\beta$

UGT1A6

XREs

|                   |                                                                            |
|-------------------|----------------------------------------------------------------------------|
| <b>NF-κB</b>      | Nuclear factor-κB                                                          |
| <b>NHANES III</b> | National Health and Nutrition Examination Survey III                       |
| <b>NIH-NHLBI</b>  | National Institutes of Health – National Heart Lung and Blood<br>Institute |
| <b>NQO-1</b>      | NADPH:quinone oxoreductase-1                                               |
| <b>NO</b>         | Nitric Oxide                                                               |
| <b>nrf2</b>       | NF-E2 p45-related factor                                                   |
| <b>NYHA</b>       | New York Heart Association                                                 |
| <b>PDE-5</b>      | Phosphodiesterase-5                                                        |
| <b>PGC-1α</b>     | PPAR-γ coactivator 1α                                                      |
| <b>PPAR</b>       | Peroxisome proliferator-activating receptor                                |
| <b>RAAS</b>       | Renin-angiotensin-aldosterone-system                                       |
| <b>RAS</b>        | Renin-angiotensin-system                                                   |
| <b>RNS</b>        | Reactive nitrogen species                                                  |
| <b>ROS</b>        | Reactive oxygen species                                                    |
| <b>RW th</b>      | Relative wall thickness                                                    |
| <b>SHF</b>        | Systolic heart failure                                                     |
| <b>TNF-α</b>      | Tumor necrosis factor-α                                                    |
| <b>TGF-β</b>      | Transforming growth factor-β                                               |
| <b>UGT1A6</b>     | UDP-glucuronosyltransferase 1A6                                            |
| <b>XREs</b>       | Xenobiotic response elements                                               |

# **INTRODUCTION**

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Heart failure syndromes are frequently fatal illnesses in which the heart loses its ability to pump effectively in response to the body's needs. Heart failure is typically a chronic disease, with progressive deterioration of ventricular function occurring over a period of years or even decades. Patients may become incapable of performing even the simplest activities of daily living, and are at very high risk of medical complications and death.

Currently, there are more than six million patients living with heart failure in the United States, with over 750,000 new diagnoses and over 300,000 deaths each year. Heart failure treatment has a profound economic impact, as well. Heart failure is the #1 diagnosis in the Medicare system based upon patient volume, the #1 discharge diagnosis in patients over 62, and the #1 cause of hospital readmission(1). Over 90% of heart failure diagnoses are preceded by prolonged hypertension, and hypertension is associated with two to three times higher risk for developing heart failure(2). Therefore, experimental models of hypertension-induced heart failure are of particular value to examine interventions which may alter heart failure pathogenesis – including dietary approaches.

The influence of nutrition on heart failure phenotypes is poorly understood, although epidemiologic and experimental evidence has emerged that suggests that alterations in cardiac nutrient metabolism may play a critical role in the development and progression of the disease. This implies that dietary modifications might reduce the risk of developing heart failure, or might delay disease progression to a more severe and intractable condition. Little guidance is available at present regarding optimal nutritional

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approaches for advanced heart failure, but dietary management represents a potentially cost-effective means of improving clinical outcomes for these patients. In addition, little is known about the impact of nutrition upon the trajectory of early heart failure pathogenesis, which would be vital to informed, preventive cardiology approaches.

Hypothesis-testing research is needed to identify the roles of specific foods, nutrients, and dietary patterns in the development and progression of heart failure. Compared with nutrition guidelines for other cardiovascular conditions, those for heart failure are minimal. More information is needed regarding dietary macronutrient composition, overall dietary patterns, and the usage of dietary supplements amongst heart failure patients. In addition, more research is needed to identify plausible benefits of nutrition-based interventions and their biological mechanisms of effect.

The Dietary Approaches to Stop Hypertension (DASH) clinical trials assessed the effect of dietary patterns with higher intakes of fruits, vegetables, and low-fat dairy products on hypertension. Results revealed that intake of an averaged 8.6 servings/day of fruit and vegetables reduced blood pressure as compared to the control group, which averaged 2.6 servings/day. The addition of low-fat dairy products to the diet further reduced blood pressure(3-5). However, the effects of DASH diet adherence upon heart failure incidence or mortality are not adequately understood. A recent large, prospective, observational study assessed the correlation of heart failure incidence with the intake of a DASH-style diet(6). Of the over 36,000 participants, those in the top quartile of the DASH diet food guidelines had a 37% lower rate of heart failure. Importantly, this effect was sustained after multivariate adjustments for age and other risk factors for heart

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failure. This important study provided the first compelling evidence that DASH-style diets could reduce the incidence of heart failure.

The specific mechanisms of the DASH-style diet upon hypertension-related pathology and heart failure are unknown, but studies in relevant animal models may permit such assessments. One recent study in spontaneously hypertensive rats attempted to nutritionally model the DASH diet. With detailed effort to match the control and DASH diet macronutrient and micronutrient profiles (vitamins, minerals, fats, carbohydrates, proteins, and fiber), the treatment failed reduce blood pressure(7). However, the semi-purified diets were not based upon whole-foods, and lacked phytochemicals, which could support the importance of these non-nutritive factors in the DASH diet benefits. In the DASH clinical trials, phytochemical intake from fruits, vegetables, and grains was distinctly different between the DASH-diet group and the control group(8). Phytochemicals elevated in the DASH diet included flavonols, flavanones, flavan-3-ols,  $\beta$ -carotene,  $\beta$ -cryptoxanthin, lycopene, lutein, zeaxanthin, and phytosterols. Therefore, it may be necessary to use a whole food model to mechanistically examine the benefits of DASH-style diets for hypertension and its related pathologic sequelae.

This dissertation is focused upon modeling a fruit and vegetable-enriched diet and its effects upon hypertension-related diastolic heart failure. Specifically, the studies examine the impact of diet upon key cardiac transcription factors and genes in both healthy and failing hearts. The approach includes the Dahl-Salt Sensitive rat as a model of hypertension and diastolic heart failure, and diets supplemented with table grapes as a

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model of a phytochemical-containing diet. Finally, the limitations of the chosen experimental approaches and possible directions of future research are discussed.

## **CHAPTER ONE**

### **LITERATURE REVIEW**

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## **A. HEART FAILURE**

### ***A1. Normal Cardiac Geometry and Function***

The normal heart is a strong muscle that beats about 120,000 times a day to pump blood through the body. The heart itself is made up of four chambers - two atria and two ventricles. De-oxygenated blood returns to the right side of the heart via the venous circulation. It is pumped into the right ventricle and then to the lungs where carbon dioxide is released and oxygen is absorbed. The oxygenated blood then travels back to the left side of the heart into the left atria, then into the left ventricle, from where it is pumped into the aorta and arterial circulation.

The pressure created in the arteries by the contraction of the left ventricle is the systolic blood pressure. Once the left ventricle has fully contracted, it begins to relax and refill with blood from the left atria. The pressure in the arteries falls whilst the ventricle refills. This is the diastolic blood pressure. Blood travels from right side of the heart to left side of the heart via the lungs. However, the chambers themselves work together. The two atria contract simultaneously, and the two ventricles contract simultaneously. The period during ventricular relaxation and blood filling is known as diastole, while the period during ventricular contraction and blood ejection is termed systole.

The cardiovascular system is made up of the heart, lungs, arteries and veins, and it is under the control of the autonomic nervous system (sympathetic and parasympathetic). In a healthy individual with a healthy heart, heart rate is dictated by the body's needs. When the body is at rest, the organs, muscles and tissues require a reduced amount of blood and oxygen, resulting in reduced blood pressure, heart rate and respirations. When

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(DHF) c

the body is active, then the organs, muscles and tissues require an increasing amount of blood and oxygen, resulting in increased blood pressure, heart rate and respirations. These responses are all involuntary, under the direct control of the autonomic nervous system. If the individual remains reasonably healthy with no cardiac complications, then the cardiovascular system will continue to meet the demands of the body.

## ***A2. Heart Failure Definition and Etiology***

Heart failure is defined by the inability of the heart to adequately meet oxygen demands of the body. Specifically, this failure is characterized by the inefficient systolic and/or diastolic actions of the heart chambers and valves. Heart failure can be initiated by acute cardiac injury, such as a heart-targeted immune response or infarction which destroys functional heart tissue. More commonly, heart failure develops from prolonged work load induced by hypertension and/or increased vascular resistance from atherosclerosis. Over 90% of heart failure diagnoses are preceded by prolonged hypertension(2). Therefore, experimental models of hypertension-induced heart failure are of particular value to examine interventions which may alter heart failure pathogenesis, including dietary approaches.

It is generally agreed that patients with chronic heart failure can be divided into two groups based on changes in cardiac structure and function. Patients with systolic heart failure (SHF) are characterized by eccentric remodeling of the left ventricle with progressive left ventricular dilation, and by predominant abnormalities in left ventricular and myocardial systolic properties. By contrast, patients with diastolic heart failure (DHF) often have normal systolic parameters, but are characterized by concentric

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remodeling of the left ventricle and by abnormalities in left ventricular and myocardial diastolic properties. The heart can contract normally, but is stiff and less compliant when it is relaxing and filling with blood. This change impedes blood filling into the heart, and may produce symptoms of heart failure.

A distinction between DHF and SHF is important because DHF has a strong association with “normal aging”, and is far more common than SHF in patients older than 75 years of age, especially in women with hypertension. While DHF is associated with better long-term survival than SHF, morbidity and quality of life is still greatly impacted. These two forms of heart failure, which may co-exist, may require different therapeutic approaches. Despite the high prevalence and economic impact of DHF, it has received far less attention than its systolic counterpart. Numerous clinical trials have documented the benefits of treatment for SHF; however, the optimal treatment for DHF has not yet been defined.

### **A3. *Heart Failure Epidemiology***

As a chronic disease, heart failure compromises patient quality of life and significantly promotes morbidity and mortality. Currently, there are more than six million patients living with heart failure in the United States, with over 750,000 new diagnoses and over 300,000 deaths each year. Heart failure treatment also has a profound economic impact. Over \$40 billion of the US health care budget is spent on heart failure annually. Heart failure is the #1 diagnosis in Medicare system based upon patient volume, the #1 discharge diagnosis in patients over 62, and the #1 cause of hospital readmission(1). Heart failure is therefore a significant and growing public health burden. Once

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diagnosed, heart failure shows a average five-year mortality rate of 60%(9). Therefore, it is clear that prevention of heart failure, rather than treatment, would provide the greatest public health impact.

#### ***A4. Interactive Roles of Cardiac Oxidative Stress, Inflammation, and Fibrosis in Heart Failure.***

*A4.1. Oxidative Stress.* Heart failure involves both systemic and cardiac-specific oxidative stress. Oxidative stress caused by reactive oxygen species (ROS) and reactive nitrogen species (RNS) contributes to the initiation and progression of heart failure. In the initiation phase, aberrant pressure and volume adjustment by the vasculature involves the generation of free radicals. Intracellular oxidative stress is generated by increased renin-angiotensin-aldosterone system activation and from increased exposure to norepinephrine. Oxidative stress may also be generated from local inflammation. In addition, pressure overload increases cardiac metabolic demand and involves greater activity of mitochondrial respiratory chain enzymes. High mitochondrial flux increases the opportunity for lost free electrons which can then generate reactive oxygen species. During disease progression, extended pressure overload leads to cardiac hypertrophy and fibrosis. Increased fibrosis in the heart muscle reduces its compliance, which leads to contractile insufficiency, especially apparent under exertion. If not quenched by endogenous antioxidants, (catalase, superoxide dismutase, glutathione, etc.) reactive oxygen species damage local lipids, proteins, and DNA, leading to sub-optimal cardiac function and cell death. Accumulating evidence suggests that there is a significant

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correlation between oxidative stress and indices of functional capacity, such as peak exercise oxygen consumption(10-13) and the severity of heart failure(14-16). Not surprisingly, biochemical markers of oxidative stress are markedly elevated in patients with heart failure(13,17).

*A4.2. Inflammation.* In addition to oxidative stress, heart failure pathogenesis also involves progressive local and systemic inflammation. ROS such as superoxide, hydroxy radical, and hydrogen peroxide, and RNS such as nitric oxide and peroxynitrite are widely implicated in inflammatory processes. Oxidative stress can activate redox-regulated transcription factors, such as NF $\kappa$ B and AP-1, which regulate genes related to inflammation including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ) and interleukin-6 (IL-6). It is established that heart failure involves early and sustained redox-regulated transcription factor activation(18,19). In addition, long-term antioxidant provision to animal model of heart failure reduces cardiac redox factor activation and the resultant cardiac inflammation, cardiac hypertrophy and cardiac dysfunction(20,21).

*A4.3 Fibrosis.* Prolonged oxidative stress also contributes to cardiac fibrosis. Fibrosis can be a beneficial short-term response to inflammation and wound healing, but prolonged fibrosis can result in various degrees of tissue remodeling. Abnormal cardiac remodeling is characterized by structural rearrangements that involve myocyte hypertrophy, fibroblast hyperplasia, and disproportionate increases in extracellular matrix collagen deposition which collectively lead to myocardial fibrosis(22). Extracellular matrix collagen is an important determinant of myocyte shape and alignment, and plays

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regulatory roles in transduction of cardiac contractile force. Thus, remodeling of myocardial collagen matrix is critical in the development of ventricular diastolic and systolic dysfunctions(23). As in inflammation, redox factor activation triggers growth factors and the expansion of the extracellular matrix. Reduced redox factor activation could reduce both inflammation and fibrosis, key players in heart failure pathogenesis.

## **B. SALT-SENSITIVE HYPERTENSION AND HEART FAILURE**

### ***B1. Salt-Sensitive Hypertension – Definition, Epidemiology and Etiology***

*B1.1 Definition and Epidemiology.* Excessive salt intake is one of the most important environmental contributors to the high prevalence of hypertension and cardiovascular disease in developed countries. In humans, the link between salt intake and blood pressure has been established in cross-sectional and longitudinal epidemiological studies. However, it is also true that the blood pressure response to changes in salt intake varies from one individual to another, a phenomenon known as “salt sensitivity”.

Salt sensitivity affects approximately 50% of hypertensive patients and 20% of normotensive patients(24). The onset of salt sensitive hypertension increases with age. In addition, salt sensitive hypertension is more prevalent in blacks, a population at higher risk for disease related to hypertension including renal dysfunction, heart failure, and stroke(25). Greater understanding of the pathology and sequelae of salt-sensitive hypertension is critical to reducing the public health burden of hypertension.

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*B1.2 The Etiology of Salt-Sensitive Hypertension.* The etiology of salt-sensitive hypertension is likely pleiotropic and is not completely understood. The kidneys appear to play a central role in the functional disturbances that link salt intake to arterial blood pressure(26). Earlier studies on renal transplantation in hypertensive patients(27), as well as in experimental animal models of hypertension(28), provide evidence that the kidneys are ultimately responsible for salt-sensitive increases in blood pressure. Furthermore, it is likely that renal injury resulting from prolonged hypertension further contributes to impaired natriuresis and plasma volume regulation.

While it has long been accepted that sodium retention tends to be associated with hypertension, the mechanisms involved have been debated for some time. Sodium retention causes extracellular volume expansion. Extracellular volume expansion does not directly increase blood pressure, but it could serve to increase cardiac output, which would raise tissue perfusion to levels exceeding the metabolic needs(29,30). The relative surplus of blood supply may then trigger an ‘autoregulatory’ response in the tissues leading to vasoconstriction, increased peripheral vascular resistance, and higher blood pressure.

## **C. FRUIT AND VEGETABLE INTAKE AND HYPERTENSION PATHOLOGIES**

*C1. The Effects of Nutrients Versus Whole Foods on Hypertension and Heart Disease*

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It is clear from observational population studies that the intake of fruits and vegetables is inversely related to cardiovascular morbidity and mortality. However, the benefits of additional nutrients alone cannot likely explain the benefits of increased fruit and vegetable intake. This hypothesis is supported by both observational and intervention studies showing that vitamin and mineral supplementation studies do not confer consistent benefit for reduced hypertension(31-34). In addition, clinical trials have largely failed to demonstrate a beneficial effect of antioxidant supplements on cardiovascular disease morbidity and mortality. At this time, the American Heart Association endorses the consumption of a diet high in food sources of antioxidants and other “cardioprotective” whole foods such as fruits, vegetables, whole grains, and nuts, instead of antioxidant supplements, to reduce risk of heart disease(35,36).

The Dietary Approaches to Stop Hypertension (DASH) clinical trials assessed the effect of dietary patterns with higher intakes of fruits, vegetables, and low-fat dairy products on hypertension. In addition, the DASH diet includes whole grains, poultry, fish and nuts, while limiting fats, red meat, sweets, and sugar-containing beverages. Results revealed that a mean intake of 8.6 servings/day of fruit/vegetables reduced blood pressure as compared to the control group, which averaged 2.6 servings/day(3-5,37).

The content of phytochemicals was distinctly different between the DASH-diet and control diet group(8), and may be a vital part of the DASH diet benefit. Phytochemicals elevated in the DASH diet included flavonols, flavanones, flavan-3-ols, beta-carotene, beta-cryptoxanthin, lycopene, lutein+zeaxanthin, and phytosterols. The importance of these non-nutritive components may be reflected in one very relevant animal study in spontaneously hypertensive rats. In this study, DASH-style diets were

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achieved with altered nutrients using semi-purified diets. However, the results failed to show a benefit for blood pressure reduction as compared to rats fed the control diet(7). The semi-purified diets employed lacked phytochemicals, which could support the importance of these non-nutritive factors. Therefore, it may be necessary to use a whole food model to mechanistically examine the benefits of fruits and vegetable intake for hypertension and its downstream pathologies.

One observational, prospective study examined whether a greater concordance with the DASH diet was associated with reduced incidence of self-reported hypertension and mortality from cardiovascular disease(38). Subjects included 20,993 women in the Iowa Women's Health Study (IWHS), initially aged 55 to 69 years, followed for a period of six years. Adjusted for age and energy intake, the incidence of hypertension was inversely and significantly associated with the degree of concordance with the DASH diet. There were also inverse associations between better DASH diet concordance and mortality from coronary heart disease and "all cause" cardiovascular disease. However, the correlation between DASH diet and reduced mortality was not sustained after an additional level of multivariate adjustment, which included education, body mass index, waist to hip ratio, smoking status and frequency, estrogen use, alcohol intake, physical activity, and multivitamin use. These results suggest that DASH diet benefits for reduced heart disease may be specific to select patient groups with defined risk factors.

However, numerous clear and potentially important differences between the IWHS study and the DASH diet clinical trials need mention. The two-month DASH trial provided food to study participants. In contrast, the IWHS observational study assessed the agreement of the participants' typical diet agreed with the DASH diet guidelines.

Also, many of the IWHS participants had normal blood pressure levels, not above-normal levels as did the DASH trial participants. It is likely that the long-term effect of the DASH diet differs by initial level of blood pressure. In addition, the IWHS cohort was predominantly Caucasian, whereas the DASH trials over-represented African Americans who are at greater risk for hypertension. Additional design differences may also have contributed to study outcomes. In the IWHS, dietary intake was measured by a single, semi-quantitative food frequency questionnaire, in which diet assessment is typically imprecise and energy intake is often underestimated. Another shortcoming of IWHS was the reliance upon self-reports of hypertension; blood pressure was not actually measured, which limits quantitative assessment of group differences. In summary, this study and its conclusions may not be a logical extension of the DASH trials to assess diet effects upon hypertension and eventual heart disease.

Another recent, prospective study evaluated the association between the DASH-style diet and mortality in hypertensive adults(39). Subjects included 5,532 participants from the Third National Health and Nutrition Examination Survey (NHANES III), who were followed for eight years. Like the IWHS, diet was assessed using a single assessment method, though with 24-hour food recall versus a food frequency questionnaire. Unlike IWHS, the subjects were hypertensive, and were from a broader racial demographic. Only 7.1% of these freely living NHANES III subjects consumed a DASH-like diet as determined by their study-specific criteria. The subjects were thus divided and compared as two groups (DASH diet n=391, non-DASH diet n=5,141). While diastolic blood pressure was lower in the DASH group ( $p<0.05$ ), systolic blood pressure was not ( $p=0.85$ ). Results demonstrated inverse associations between the

DASH-style diet and mortality from coronary heart disease, stroke, and all cardiovascular disease. After multivariate analysis, a DASH-style diet was still associated with lower all-cause mortality, but specific mortality from cardiovascular disease, ischemic heart disease, and stroke no longer reached statistical significance. The results from these observational studies in freely living subjects suggest a cardioprotective effect, but the specific diet impact upon heart failure morbidity and mortality was not examined.

## ***C2. The DASH-style Diet and Heart Failure***

Despite the epidemiologic evidence for the benefits of fruit and vegetable intake, little is known about the effect of diet on heart failure pathogenesis. Instead, because this diet pattern reduces hypertension, it is assumed that this hypotensive effect could reduce hypertension-associated heart failure.

To date, one study has assessed the correlation of a DASH-style diet and heart failure. The Swedish Mammography Cohort(6) is a prospective, observational study, whose database contains information on subject diet and disease. Of the 36,000 participants, those in the top quartile of the DASH diet food guidelines had a 37% lower rate of heart failure after multivariate adjustment for age, physical activity, energy intake, education status, family history of myocardial infarction, cigarette smoking, post-menopausal hormone use, living alone, hypertension, high cholesterol, body mass index, and incident myocardial infarction. This important study provided the first compelling evidence that DASH-style diets reduced the incidence of heart failure, across a broad scope of confounding variables.

The specific mechanisms of the DASH-style diet upon hypertension-related pathology and heart failure are unknown, but studies in relevant animal models may permit such assessments. It is possible that fruit and vegetable intake would exert tissue-specific effects which may alter heart failure pathogenesis. Animal models of heart failure would allow specific diet manipulation and simultaneous assessment of tissue-specific effects.

## **D. CARDIOVASCULAR EFFECTS OF GRAPE PRODUCT CONSUMPTION**

### ***D1. Grape Intake and Cardiovascular Epidemiology***

Observational studies show that men and women with moderate alcohol consumption are substantially less likely to die of a heart attack than non-drinkers. Specifically, low cardiac mortality rates are observed in countries with a high wine consumption. However, these same populations also consume higher fat diets, exercise less, and smoke more cigarettes than neighboring countries. This seemingly illogical finding has been coined the 'French Paradox'(40). Wine consumption, particularly red wine, is speculated to play a role in the protective association of the French Paradox. The cardioprotective constituents in wine are unknown, but numerous studies suggest that the polyphenolic compounds in grapes may play a causative role.

The cardiovascular effects of grape product consumption are broad. Clinical trials and animal studies with purple grape juice suggest cardioprotective effects(41) through enhanced vasodilation(42-44), reduced platelet aggregation(45-47), reduced oxidation of

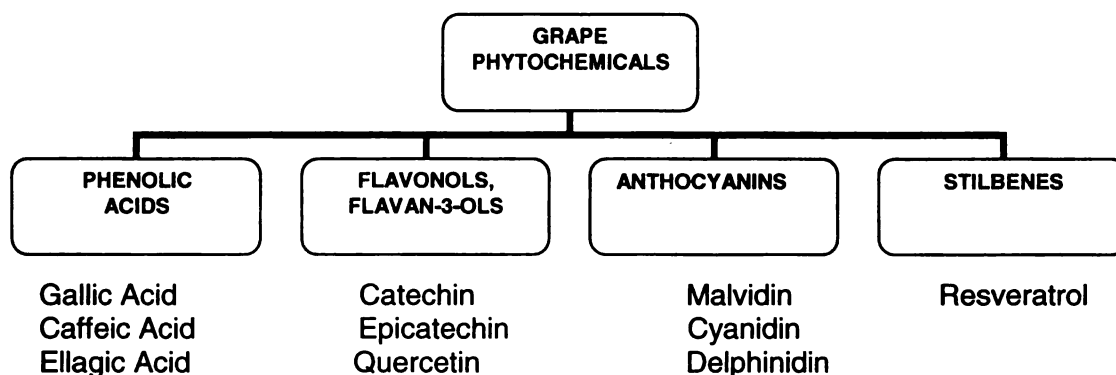
plasma lipids(48-50), DNA(49), and protein(49), and enhanced plasma antioxidant capacity(49,50). The fact that both wine and grape juice experimentally confers cardiovascular benefits supports that some critical polyphenols are shared between the two grape products. The grape constituents responsible for the health benefits remain unclear. Furthermore, differences in polyphenol content exist between grape components (juice, pomace, seed), different varieties of grape, and different geographic regions of origin(51).

## **D2. Table Grape Phytochemicals**

Table grapes are the primary *Vitis vinifera* variety of whole grapes sold as produce. Table grapes are available in green, red, and black varieties, which vary in their phytochemical content depending on regional differences in growth conditions, including climatic and soil differences. Phenolic compounds in table grapes can arbitrarily be divided into four groups: simple phenols, flavonols and flavan-3-ols, anthocyanins, and stilbenes. Grape phytochemicals are often called polyphenols because of their common phenolic acid group. Differences in the degree of oxidation and hydroxylation of the phenolic rings lead to a large family of structures with essential differences in biological behavior, bioavailability, and efficacy.

Grape polyphenols of potential interest for common dietary intake are found within the skins and seeds. Grape phenols and polyphenols exist as free compounds, as sugar polymers, or as part of larger molecular weight oligomeric chains or structures(52). The most common polyphenols in grape skin and pomace include phenolic compounds, including simple phenols, phenolic acids, cinnamic acids, stilbenes, flavonoids, flavans,

flavonols, and anthocyanins. **Figure 1.1** illustrates these groups, and lists some representative compounds present in table grapes.



**Figure 1.1. Subclasses of table grape phytochemicals**

Of interest to dietary intake, these components are found in various concentrations in fresh grapes, grape juice, wine, and grape skin extract. However, it remains unknown which grape constituents or their *in vivo* metabolites offer greater biologic/health effects, or if these components act synergistically.

## **E. RATIONALE FOR CURRENT STUDIES**

### ***E1. Rationale for Grape Product Efficacy Against Hypertension-Related Pathology.***

This project will assess diet effects on heart failure using a whole foods model to simulate a phytochemical-rich diet. For modification of heart failure pathogenesis, the potential antioxidant, anti-inflammatory, and vasodilatory effects of grape product intake

are of great interest. The following *in vitro* and *in vivo* studies demonstrate that grape products can confer these biologic effects.

*E1.1 Antioxidant Effects.* Grape products limit tissue and systemic oxidative stress in several animal models. Proposed mechanisms of this effect include direct scavenging of free radicals and improved endogenous antioxidant defense. If able to provide an *in vivo* antioxidant effect in humans, grape phytochemical intake may modify diverse contributors to cardiovascular morbidity and mortality. The antioxidant effects of grape product ingestion can be measured directly by enhanced plasma antioxidant capacity, or indirectly by reduced plasma oxidative stress and/or reduced oxidative damage of biomolecules within tissues. Several grape products are capable of providing *in vivo* and *ex vivo* antioxidant effects; they have demonstrated *in vivo* capacity to enhance plasma antioxidant capacity(49,53,54), to decrease oxidation of LDL(53-58), and to lower 8-isoprostane(49,59), a lipid oxidation product which serves as a systemic marker of oxidative stress. In addition, effects were demonstrated in both healthy subjects and at-risk subjects.

*E1.2. Anti-Inflammatory Effects.* Inflammation is a key contributor to the progression of many forms of heart disease. Local inflammation directly damages target tissue and generates reactive oxygen species which can damage neighboring tissue. *In vitro* studies and animal studies suggest that grape polyphenols have the potential to modulate eicosanoid metabolism(60). In particular, the 5-lipoxygenase pathway is an important target because it is involved in the synthesis of leukotrienes which contribute to inflammation. *In vitro*, the polyphenols quercetin and resveratrol prove to be effective

inhibitors of pro-inflammatory lipooxygenase pathways(61). In addition, red wine extracts reduce adhesion of monocytes to the endothelial surface and block cytokine-induced expression of endothelial adhesion molecules(62). Red wine and select grape phytochemicals inhibit activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) activity and production of pro-inflammatory factors in endothelial cells and immune cells(62,63). Incubation of monocytes with catechin decreased their adhesion to endothelial cells(64). Relevant polyphenols also inhibit NF- $\kappa$ B activity in T lymphocytes(65,66). Resveratrol has also demonstrated anti-inflammatory effects, including inhibition of adhesion molecule expression and reduced responses to cytokines(67-71). Also, resveratrol inhibits the release of degradative enzymes by neutrophils and downregulates neutrophil surface expression of adhesion-dependent, pro-thrombogenic proteins(72).

*In vivo* studies examining anti-inflammatory effects of grape products have been more limited(54,73-75). In humans, treatment with table grape powder for four weeks was associated with a reduction in plasma TNF- $\alpha$ , but not C-reactive protein or IL-6(59). Wine consumption for four weeks also reduced systemic markers of inflammation in healthy men(76). Collectively, these studies show that diverse grape products are able to lower systemic and local markers of inflammation.

*E1.3. Vasodilatory Effects.* The vascular endothelium plays a central role in the regulation of vascular tone, thrombosis, local inflammation, and cell proliferation by producing paracrine factors that act on the arterial wall and on blood cells. *In vitro* studies demonstrate the favorable effects of grape products on endothelial function. In cultured endothelial cells, wine, grape juice, grape seed extract, and specific polyphenols increase

the activity of the endothelial isoform of nitric oxide synthase and stimulate nitric oxide (NO) production(77,78). NO is formed from the guanidine-nitrogen terminal of L-arginine by nitric oxide synthases. Endothelium-derived NO plays a crucial role in the homeostasis of the vascular tone by acting as a vasodilator. As a dynamic mediator of vascular compliance, NO is a freely diffusible gas that can act as an intracellular and intercellular messenger molecule. Most of the cellular actions of NO are explained by the activation of the cytosolic enzyme soluble guanylate cyclase, which catalyzes the formation of cyclic guanosine monophosphate (cGMP). Increased cGMP activates protein kinase G, which in turn phosphorylates a number of proteins involved in vasodilation. In addition to the depressor effect of vasodilation, NO inhibits platelet adherence to the endothelium. Enhanced NO production or availability would thereby enhance vasodilation and lower blood pressure.

*In vitro* studies indicate that NO degradation by phosphodiesterase-5 is delayed by exposure to grape phytochemicals,(79) which would prolong NO availability. In addition, incubating arterial rings in a tissue bath containing diluted grape juice increased endothelial-dependent vasorelaxation by a NO-dependent mechanism(80). In the short term, polyphenols stimulate endothelial NO synthase phosphorylation via protein kinases phosphatidylinositol-3-hydroxy kinase and Akt(81). Longer term exposure to red wine extracts or resveratrol increases nitric oxide synthase enzyme expression and activity(77,78). Human studies support a benefit of grape beverages on endothelial function(82). In patients with coronary artery disease, consumption of purple grape juice improved endothelium-dependent brachial artery flow(44,48). In healthy subjects, de-alcoholized wine also improved brachial artery flow-mediated dilation(83).

In addition to the effects on NO, grapes have important effects on other factors that influence vascular function. For example, flavonoid-containing beverages increase endothelial production of prostacyclin and suppress production of endothelin-1, a potent endothelium-derived vasoconstrictor(84,85). Also, NO activity is negatively impacted by oxidative stress, because NO can be oxidized to non-dilatory peroxynitrite. An antioxidant effect of grape intake could reduce the conversion of NO to peroxynitrite and thus prolong NO action and vasodilation.

In summary, these studies show that diverse grape products have the capacity to improve vascular reactivity, and perhaps lower blood pressure. It remains unknown if these temporal changes can then extend to sustained reductions in blood pressure, or confer greater resistance to hypertension-related pathologies.

## ***E2. The Disease Model – The Dahl Salt Sensitive Rat***

The model employed in this proposal is the Dahl-Salt Sensitive (Dahl-SS) rat. Dahl-SS rats are used as a model of human salt-sensitive hypertension. This model was first characterized by Louis K. Dahl in the early 1970s, as a spontaneous mutation in Sprague-Dawley rats conferring salt-sensitivity. Kidney transplant studies in Dahl-SS rats indicate that the kidneys are the primary source of pathology due to aberrant volume/solute regulation(86-88).

Dahl-SS rats follow a very predictable course from hypertension and renal hypertrophy to cardiac hypertrophy, followed by cardiac insufficiency and diastolic heart failure. Dahl-SS rats develop only mild systolic impairment; the rats typically die of renal failure before the onset of overt systolic dysfunction. Diastolic heart failure develops

within 15-20 weeks of high-salt feeding, depending on the amount of salt provided. Unlike surgical models of heart failure induction, high-salt fed animals show excellent correlation with one another in disease course, enhancing statistical power. The NIH-NHLBI Program for Genomic Applications funded a study to characterize disease progression in the Dahl-SS rat, fed a 6.0% NaCl diet, the model and salt intake included in this proposal. Because of its highly reproducible and predictable pathogenesis, the Dahl-SS rat has been used for pharmaceutical studies of angiotensin converting enzyme inhibitors, angiotensin II receptor blockers,  $\beta$ -blockers, and other common drugs for heart failure. However, the effect of diet on heart failure development is poorly understood.

As with human heart failure, the Dahl-SS rat pathogenesis involves oxidative stress. In addition, Dahl-SS rats have compromised antioxidant defenses when given a high salt diet. Of significance for this proposal, salt-fed Dahl-SS rat hearts show significantly decreased glutathione peroxidase activity and reduced glutathione(89), which leads to sustained exposure to ROS. Synthetic antioxidants and concentrated antioxidant vitamins provide substantial benefit to the Dahl-SS rat, supporting the impact of oxidative stress upon pathogenesis. For example, daily treatment with superoxide dismutase mimetic Tempol improves Dahl-SS renal function, reduces oxidative stress, and improves heart function(90-98). Vitamin E limits Dahl-SS hypertension, hypertensive nephropathy, renal oxidative stress, and improves glomerular filtration rate and renal perfusion in the Dahl-SS rat. Taken together, these findings suggest that in the Dahl-SS rat, higher salt diets are associated with increased oxidative stress, and that antioxidant intake can counter these adverse effects and reduce hypertension-related

pathologies. However, it is unclear if an antioxidant-rich whole food would provide a similar benefit or alter cardiac pathology.

While antioxidant treatment limits these early phenotypes of Dahl-SS hypertension, the current gap in knowledge concerns whether these accumulated effects of diet will affect the eventual development of heart failure. Antioxidant-rich diets may pay dividends beyond short-term depressor effects by limiting the degree of irreversible organ damage caused by prolonged hypertension.

### ***E3. The Diet Intervention Model – Whole Table Grape Powder***

For greater relevance to normal dietary intake, as opposed to dietary supplementation, this project will utilize a whole food model. The freeze-dried grape powder used in this study is a composite of green, red, and black table grapes grown in California (supplied by the California Table Grape Commission, processed and chemically characterized by National Food Laboratories, Inc). As detailed earlier, table grape powder is a source of diverse phytochemicals including phenols, simple phenolic acids, cinnamic acids, stilbenes, flavonoids, flavans, flavonols, and anthocyanins.

*E3.1 Table Grape Powder – Demonstrated Efficacy.* Importantly, this standardized, whole grape powder has been shown by other investigators to reduce both plasma and tissue markers of oxidative stress *in vivo*(55,59,99). This protection was afforded by grape powder delivered by three different mechanisms [gavage(59), diet(99), drinking water(55)] and in multiple models [mouse(55), gerbil(99), human(59)]. Protection

occurred at both a tissue and a systemic level; protection was associated with reduced oxidative stress in plasma(55,59), in isolated leukocytes(55) and in tissues(99-101).

*E3.2 Table Grape Powder - Cardiac Bioavailability.* Of specific interest for this project, the cardiac-specific benefits of table grape powder have already been demonstrated. Cui et al. (100,101) employed the same table grape powder (provided by the California Table Grape Commission) that we use in the current studies. Healthy male rats were gavaged with table grape powder for three weeks. Afterwards, hearts were excised and perfused *ex vivo* with an oxygenated buffer. Beating hearts were then made ischemic for thirty minutes followed by two hours of oxygenated buffer reperfusion. In this blood-free model, grape powder intake provided significant cardioprotection as evidenced by improved post-ischemic ventricular recovery and reduced degree of myocardial infarction. Grape powder intake also reduced the post-reperfusion cardiac malonyldialdehyde content, indicating reduction of oxidative damage to cardiac lipids. The results demonstrate a direct cardioprotective role of regular intake of table grape powder; the model was saline-perfused and blood-free, so treatment effect was associated with a tissue-specific mechanism. In addition, the results are more reflective of the chronic effects of grape intake rather than acute exposure to plasma metabolites of the ingested table grape powder.

In summary, numerous *in vitro* and *in vivo* studies suggest protective effects from grape products. Of interest to this proposal, *ex vivo* cardiac studies indicate that beneficial components and/or metabolites of the whole table grape powder provided greater resistance to cardiac oxidative stress(100). As such, bioavailable grape phytochemical

metabolites could confer health benefits against diseases which involve oxidative stress such as hypertension-associated heart failure.

#### ***E4. Rationale and Specific Aims of Dissertation***

Bioavailable grape phytochemicals may reduce heart failure pathogenesis and alter cardiac cell signaling, resulting in reduced inflammation and fibrosis. The current project focuses on cardiac signaling related to the transcription factor peroxisome proliferator-activating receptor (PPAR). Although the effects of cardiac PPAR agonism are diverse, including altered metabolism and cell differentiation, the inverse relation between PPAR activity and nuclear factor kappa B (NF- $\kappa$ B)-related inflammation is of particular interest for this proposal. Cardiac PPAR isoforms are down-regulated with Dahl-SS rat heart failure, while cardiac pro-inflammatory transcription factor NF- $\kappa$ B activity is elevated. PPAR activation reduces NF- $\kappa$ B activation, because PPAR activation increases transcription of a cytoplasmic inhibitor of NF- $\kappa$ B activation. Phytochemical-rich extracts can activate PPARs in varied experimental models. If the grape diet alters cardiac PPAR activity, it could also limit NF- $\kappa$ B activity and thereby reduce inflammation and fibrosis.

Bioavailable grape phytochemicals may also reduce heart failure pathogenesis by improved cardiac antioxidant defense. Grape phytochemicals could activate the phenol-responsive, aryl-hydrocarbon receptor (AhR), which binds to genomic xenobiotic response elements (XREs) and stimulates the transcription of mRNA related to antioxidant defense. Polyphenols can also interact with sulfhydryl moieties on kinase proteins and alter their secondary structure and activity. This type of effect is observed in

NF-E2 p45-related factor (nrf2) activation, a transcription factor which binds genomic antioxidant-responsive elements (AREs) and like AhR, stimulates the transcription of genes related to antioxidant defense. While AhR/XRE and nrf2/ARE interactions have been shown *in vitro*, it is uncertain if physiologically-relevant doses of grape powder could confer a similar effect *in vivo*.

The **central hypothesis** of this dissertation is that whole grape powder supplementation increases Dahl-SS rat cardiac PPAR-activity and decreases NF- $\kappa$ B activity and related genes/proteins, and increases the activation of cardiac AhR and nrf2 and the expression of antioxidant defense genes. The first phase of the doctoral project is to test the efficacy of the table grape powder against diastolic heart failure progression in the Dahl-SS rat, as described in Chapter Two. Five groups are studied: low salt diet + grape, high salt diet + grape, high salt diet + vasodilator drug hydralazine, and low-salt and high-salt, carbohydrate-equivalent controls. The second phase will then examine potential cardiac-specific mechanisms of effect, as detailed in Chapters Three and Four. Archived left ventricular tissue from the Chapter Two efficacy studies will be analyzed by the following two Aims:

**AIM 1.** Compare Diet Effect on Cardiac PPARs and Cardiac NF- $\kappa$ B signaling.

The working hypothesis is that grape-fed rats have enhanced PPAR and reduced NF- $\kappa$ B activity and NF- $\kappa$ B-related protein expression. Examine changes in cardiac nuclear PPAR- $\alpha$ , PPAR- $\gamma$ , and NF- $\kappa$ B DNA binding and downstream genomic targets and proteins related to inflammation and fibrosis.

**AIM 2.** Compare Diet Effect on Cardiac Endogenous Antioxidant Defense. The working hypothesis is that grape-fed animals have enhanced AhR and nrf2 activation and

related protein expression. Examine changes in cardiac AhR and nrf2 DNA binding and downstream expression of select XRE and ARE-related genomic targets related to antioxidant defense.

Several trials show that intake of grape juice and red wine cause transient vasodilation. However, it is unknown if and how chronic intake of physiologically relevant, phytochemical-rich grape products could affect chronic disease related to high blood pressure. The proposed studies are innovative because they go beyond transient effects of grape product intake and examine the accumulated, cardiac-specific effects of a physiologically relevant, phytochemical-enriched diet on both healthy and diseased hearts. The study is significant because findings could improve knowledge of the value of dietary approaches to limit hypertension-associated cardiac pathology.

Finally, Chapter Five will discuss alternative approaches and future directions.

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## **CHAPTER TWO**

### **CHRONIC INTAKE OF A PHYTOCHEMICAL-ENRICHED DIET REDUCES CARDIAC FIBROSIS AND DIASTOLIC DYSFUNCTION CAUSED BY PROLONGED SALT-SENSITIVE HYPERTENSION**

## **A. ABSTRACT**

Salt-sensitive hypertension is common in the aged. Increased fruit and vegetable intake reduces hypertension, but its effect upon eventual diastolic dysfunction is unknown. This relationship is tested in the Dahl-Salt Sensitive (Dahl-SS) rat model of salt-sensitive hypertension and diastolic dysfunction. Table grape powder contains phytochemicals that are relevant to human diets. For 18 weeks, male Dahl-SS rats were fed one of five diets: Low Salt (LS), a Low Salt + grape powder (LSG), High Salt, or a High Salt + grape powder (HSG), or High Salt + vasodilator hydralazine (HSH). Compared to HS, HSG diet lowered blood pressure and improved cardiac function, reduced systemic inflammation, reduced cardiac hypertrophy, fibrosis, and oxidative damage, and increased cardiac glutathione. HSH similarly reduced blood pressure but did not reduce cardiac pathogenesis. LSG reduced cardiac oxidative damage and increased cardiac glutathione. In conclusion, physiologically relevant phytochemical intake reduced salt-sensitive hypertension and diastolic dysfunction.

## **B. INTRODUCTION**

In humans, the link between salt intake and blood pressure has been established in cross-sectional and longitudinal epidemiological studies. However, blood pressure response to changes in salt intake can vary from one individual to another, a phenomenon known as “salt sensitivity”. Salt sensitivity affects approximately 50% of hypertensive patients and 20% of normotensive patients(1), and its incidence increases linearly with age. Greater understanding of the pathology and sequelae of salt-sensitive hypertension is then critical to reducing the public health burden of hypertension and its associated pathologies.

Prolonged hypertension frequently contributes to the development of heart failure. Heart failure is defined by the inability of the heart to adequately meet oxygen demands of the body, characterized by inefficient systolic and/or diastolic actions of the heart chambers and valves. Over 90% of heart failure cases are preceded by prolonged hypertension(2). Heart failure is a significant and growing problem in our aging population. Heart failure is the #1 diagnosis in the Medicare system based upon patient volume, the #1 discharge diagnosis in patients over 62, and the #1 cause of hospital readmission(3). As such, preventive approaches which address risk factors for heart failure could impact public health burden.

Our group is focused on the effects of diet upon hypertension-associated cardiac pathogenesis. The Dietary Approaches to Stop Hypertension (DASH) clinical trial revealed that diets rich in fruits and vegetables reduced blood pressure(4,5). One recent animal study carefully modeled the DASH diet nutrients to assess effects on hypertension in spontaneously hypertensive rats. However, the findings failed to reveal an anti-

hypertensive effect(6). Importantly, this study did not include any non-nutritive phytochemicals contained in fruits and vegetables. The DASH diet phytochemical profile was distinctly different than the control group(7), and these compounds may be vital to the diet benefits.

The Dahl Salt-Sensitive (Dahl-SS) rat is a model which provides insight into the pathology and treatment of salt-sensitive hypertension. When fed a higher salt diet, the Dahl-SS rat predictably and gradually develops the clinically relevant sequelae of hypertension, renal hypertrophy, renal dysfunction, cardiac hypertrophy, and diastolic dysfunction. Many animal models of induced heart failure depend upon infarct induction or surgical modification of the vasculature, which impose rapid morbidity and higher mortality. In contrast, the hypertension-induced model described here develops pathology over several months, allowing one to serially test for the more gradual effects of diet modification. As such, we propose that the Dahl-SS rat is a valuable model of diet effects on aging-related salt sensitive hypertension and its associated cardiac pathologies.

The current study examines the cumulative cardiac effects of a diet supplemented with phytochemical-rich whole table grape powder. Although the use of one food source may be a simplified approach, table grapes are a relevant model to human diets because they are a widely available and affordable produce, and because they contain the major classes of commonly consumed, produce-derived flavonoids, including anthocyanins, flavanols (e.g. catechin, epicatechin, proanthocyanins), and flavonols (e.g. quercetin, kaempferol, isorhamnetin)(8). In addition, the table grape powder employed in this study has already been shown to reduce other pathologies(9-13), evidence which supports the *in vivo* efficacy and bioavailability of the grape powder constituents. Using the Dahl-SS

rat model, we tested the hypothesis that table grape powder-enriched diets could lower hypertension-associated cardiac pathology and diastolic dysfunction. Furthermore, because grape product consumption is known to impart acute vasodilation(14-18), we compared grape treatment effects to those of hydralazine, a well-characterized vasodilator which has been shown at the selected dose to lower blood pressure in the Dahl-SS rat(19-21).

## **C. MATERIALS AND METHODS**

### ***C1. Animal Care and Diets***

Five week old Dahl-Rapp Salt-Sensitive rats (Harlan, Indianapolis, IN) were acclimated for one week on AIN-76a powdered diet (Research Diets, New Brunswick, NJ). Afterwards, each rat was randomly assigned (n = 12 each) to one of five treatments. Low Salt diet (LS, AIN-76a with 2.8% added carbohydrate, glucose:fructose 1:1), Low Salt diet + grape powder (LSG, AIN-76a with 3.0% w/w added grape powder), High Salt diet with 6% added NaCl (HS, AIN-76a with 2.8% w/w added carbohydrate), High Salt Diet + grape powder (HSG, AIN-76a with 3.0% w/w added grape powder), or High Salt Diet + hydralazine (20mg/kg body weight/day, in drinking water). Hydralazine dose was selected based upon previously published findings in the Dahl-SS rat(19-21), to obtain a similar % reduction in systolic blood pressure as that observed with our grape powder. Diet nutrient content is described in **Table 2.1**, while grape powder phytochemical content is described in **Table 2.2**. The freeze-dried table grape powder was obtained from the California Table Grape Commission as a composite of green, red, and black

California table grapes, processed and chemically characterized by the National Food Laboratory, Inc (Dublin, CA). Grape powder or added carbohydrate was mixed weekly into the AIN-76A base diet in-house using a commercial baking blender, and then stored in vacuum-sealed bags (Deni Magic Vac, Buffalo NY) at 4°C.

**Table 2.1.** Diets and estimated nutrient content

|                                                                                                                                                                            | <b>LS</b>        | <b>LSG</b>       | <b>HS</b>        | <b>HSG</b>       | <b>HSB</b>       |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------|------------------|------------------|------------------|------------------|
| Total Protein                                                                                                                                                              | 20               | 21               | 20               | 21               | 20               |
| Total Carb                                                                                                                                                                 | 68               | 68               | 68               | 68               | 68               |
| Total Fat                                                                                                                                                                  | 5                | 5                | 5                | 5                | 5                |
| Total Fiber                                                                                                                                                                | 5                | 5                | 5                | 5                | 5                |
| kcal/gram of diet                                                                                                                                                          | 3.9              | 4                | 3.9              | 4                | 3.9              |
|                                                                                                                                                                            | <b>g/kg diet</b> | <b>g/kg diet</b> | <b>g/kg diet</b> | <b>g/kg diet</b> | <b>g/kg diet</b> |
| Casein                                                                                                                                                                     | 198              | 198              | 198              | 198              | 198              |
| Protein from Grape                                                                                                                                                         | 0                | 1.1              | 0                | 1.1              | 0                |
| Corn Starch                                                                                                                                                                | 150              | 150              | 150              | 150              | 150              |
| Sucrose                                                                                                                                                                    | 500              | 500              | 500              | 500              | 500              |
| Sugar from Grape                                                                                                                                                           | 0                | 27.7             | 0                | 27.7             | 0                |
| Dextrose                                                                                                                                                                   | 14               | 0                | 14               | 0                | 14               |
| Fructose                                                                                                                                                                   | 14               | 0                | 14               | 0                | 14               |
| Cellulose                                                                                                                                                                  | 50               | 50               | 50               | 50               | 50               |
| Fiber from Grape                                                                                                                                                           | 0                | 0.02             | 0                | 0.02             | 0                |
| Corn Oil                                                                                                                                                                   | 50               | 50               | 50               | 50               | 50               |
| AIN76a Vitamin Mix                                                                                                                                                         | 10               | 10               | 10               | 10               | 10               |
| AIN76a Mineral Mix                                                                                                                                                         | 35               | 35               | 35               | 35               | 35               |
| Vitamin C (Grape)                                                                                                                                                          | 0                | 0.001            | 0                | 0.001            | 0                |
| Potassium (Grape)                                                                                                                                                          |                  | 0.3              | 0                | 0.3              | 0                |
| Vitamin A (Grape)                                                                                                                                                          | 0                | 99.6 IU          | 0                | 99.6 IU          | 0                |
| Nutrient content of grape powder was analyzed by National Food Laboratory, Inc. (Dublin, CA) Nutrient content of the base AIN76a diet was provided by Research Diets, Inc. |                  |                  |                  |                  |                  |

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**Table 2.2.** Grape Powder Phytochemical Analysis

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| <b>Grape Powder Analysis</b><br>(per kg of grape powder)                               |          |
|----------------------------------------------------------------------------------------|----------|
| <b>Anthocyanins</b>                                                                    |          |
| Cyanidin                                                                               | 380.0 mg |
| Malvidin                                                                               | 170.3 mg |
| Peonidin                                                                               | 33.5 mg  |
| <b>Monomeric Flavanols</b>                                                             |          |
| Catechin                                                                               | 19.1 mg  |
| Epicatechin                                                                            | 12.5 mg  |
| <b>Flavonols</b>                                                                       |          |
| Quercetin                                                                              | 49 mg    |
| Kaempferol                                                                             | 5.7 mg   |
| Isorhamnetin                                                                           | 4.4 mg   |
| <b>Stilbenes</b>                                                                       |          |
| Resveratrol                                                                            | 36.0 mg  |
| Phytochemical analysis (per kg of grape powder) by<br>National Food Laboratories, Inc. |          |

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Hydralazine-fortified drinking water was made every two days, with concentration adjusted dynamically based upon changing water intake and body weight over the course of the study. Animals were fed 20g of powdered diet/head/day. *Ad libitum* intake of AIN diet averages 19-21 grams of AIN powder/day in the Dahl-SS rat(22), so provision of 20 grams/day ensured complete daily consumption. For the high salt diets, NaCl was added directly to the food hopper and mixed carefully with the daily ration of powdered diet. Rats were housed three/cage 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.

## ***C2. Blood Pressure and Echocardiography Measures***

During the 18 week study, blood pressure was measured bi-monthly by the IITC Mark 12 photoelectric/oscillometric tail cuff system (IITC Life Sciences, Woodland Hills, CA) using the unit and method we described in detail previously and validated against telemetric approaches(22). Using preconditioned, conscious, restrained animals, the first two of ten measures were universally discarded due to acclimation to tail cuff pressure and to operational noise. A run was accepted if at least six of the eight measures were adequate (having detectable pulses and free of gross artifacts). When the requisite determinations were obtained, the average was calculated and used as the mean heart rate and the mean systolic value for that session.

Echocardiography of all animals was performed at 0, 8 and 18 weeks of diet treatment, following the predicted Dahl-SS rat development of compensated cardiac hypertrophy and of diastolic dysfunction, respectively. All measurements were made by a trained research animal sonographer who was unaware of treatment assignment. Animals were anesthetized by 4% isoflurane and maintained with 1% isoflurane. Two-dimensionally guided M-mode recordings and Doppler tissue imaging were acquired as we described previously(22). Equations for each derived parameter are as described by Boluyt(23), with the exception of mid-wall fractional shortening. In the Dahl-SS rat, endocardial fractional shortening overestimates LV systolic function; mid-wall fractional shortening has been determined to be a more appropriate index of LV systolic function(24-26). Mid-wall fractional shortening was calculated according to the two-shell cylindrical model of Shimizu et al(27).

### ***C3. Terminal Plasma Analysis***

At sacrifice, conscious rats were decapitated and trunk blood was collected. Whole blood was collected into an EDTA vacutainer, (BD Vacutainer Systems, Franklin Lakes, NJ) then spun at 4°C, 5,000 x g for 20 minutes. The plasma was stored at -80°C until further analysis. Plasma TNF- $\alpha$  and IL-6 were measured by enzyme immunoassay kits (R&D Systems, Minneapolis, MN) according to manufacturers' instructions.

### ***C4. Organ Weights and Cardiac Hydroxyproline Content***

At sacrifice, the heart, kidneys, and lungs were harvested, blotted and weighed. Organ weights were compared to tibial length rather than to body weight, due to the variable weight loss from cachexia. The left ventricle (LV) was isolated and minced, then flash frozen and stored in aliquots in liquid nitrogen. Collagen component hydroxyproline was measured in LV homogenates as a quantitative index of fibrosis. Frozen LV tissue was homogenized in ice-cold phosphate-buffered saline containing a Complete Protease Inhibitor Mini-Tab cocktail (Roche, Indianapolis, IN). The tissue was homogenized with a 30 second pulse of a Polytron (Brinkmann) tissue homogenizer and hydrolysis of the sample solution was carried out with 6 N HCl at 100°C for 24 hours. The hydrolyzed samples were dried under a stream of nitrogen. Hydroxyproline standard solutions were prepared in a range from 2.0-10.0  $\mu\text{g/mL}$ , and 0.5 mL of each standard and cardiac homogenates were placed in glass tubes with 1.0 mL of isopropanol and vortexed. To this solution, 0.5 mL of oxidant (0.35 g chloramine T in 5.0 mL water and 20.0 mL citrate buffer) was added, vortexed, and allowed to stand at room temperature for 4 minutes. Next, 3.25 mL of Ehrlich's reagent (3.0 mL Ehrlich's reagent in 15.0 mL

isopropanol) was added, and the tubes were kept at 25°C for 18 hours. The intensity of red coloration was measured using a spectrophotometer at 560nm. Total protein content was assessed using the BCA assay (Pierce, Rockford, IL). The amount of hydroxyproline was calculated using the standard curve and expressed as µg/mg total protein.

#### ***C5. Cardiac Histology Area of Fibrosis***

Four hearts from each group were utilized for histology determination of cardiac fibrosis. A transverse section of the left ventricle was fixed in 10% neutral buffered formalin. Tissue sections were prepped and mounted, then stained with Masson-Trichrome stain (MTS) for determination of fibrosis. Digital images were acquired with a Olympus BX40 digital microscope camera mounted on a Nikon DN100 light microscope. MTS-stained cross sections of the heart were captured at x200 magnification. The fibrotic areas stained blue with the MTS. True-color image analysis was performed using Bioquant image analysis software (BIOQUANT Life Science, Nashville, TN). Perivascular fibrosis was determined from ten random measures around ten distinct vessels. The area of fibrosis value was derived from the total area encompassing the vessel lumen plus the fibrotic ring divided by the area of the vessel lumen.

#### ***C6. Cardiac Oxidative Damage and Cardiac GSH/GSSG***

Frozen LV tissue was homogenized in ice-cold phosphate-buffered saline containing a Complete Protease Inhibitor Mini-Tab cocktail (Roche), and 0.01% volume of antioxidant 0.1M butylated hydroxytoluene in acetonitrile to limit auto-oxidation during sample processing. The tissue was homogenized with a 30 second pulse of a tissue

homogenizer (Polytron), then centrifuged at 4°C for 10 minutes at 3,000 x g. The supernatant was collected and stored at -80°C until further analysis. Total protein content was assessed using the BCA assay (Pierce). Malonyldialdehyde (MDA) detection was accomplished using the Biotech LPO-586 kit (Oxis Research, Portland, OR) according to manufacturers' instructions and expressed relative to total protein. Determination of the cardiac reduced/oxidized glutathione (GSH/GSSG) ratio was performed by using the Bioxytech GSH/GSSG-412 kit (Oxis Research) according to manufacturers' instructions.

#### ***C7. Statistical Methods***

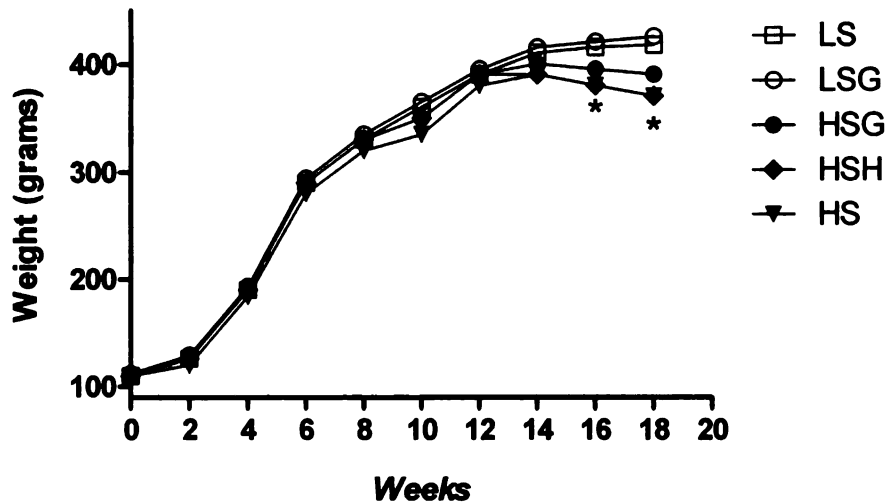
All results are expressed  $\pm$  SEM. Groups were compared using one-way ANOVA. If the interaction was significant, between-group comparisons were conducted by the Bonferroni post-hoc test. Analysis was conducted using GraphPad PRISM 4 (La Jolla, CA). A p value  $<0.05$  was considered statistically significant.

### **D. RESULTS**

#### ***D1. Body Weight and Blood Pressure***

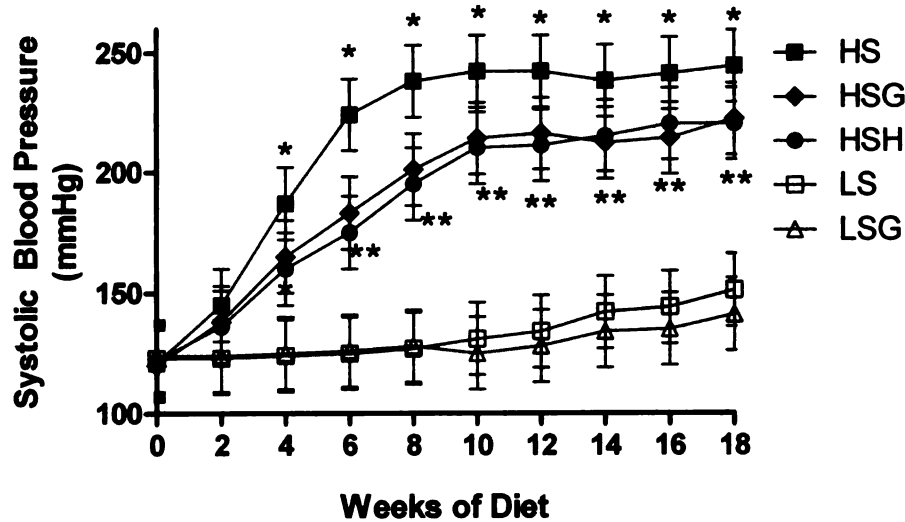
No significant differences were observed between LS and LSG groups in body weight gain during the course of the study. Cachexia is characteristic of human heart failure and is positively correlated with disease severity and mortality. Dahl-SS rats also develop cachexia as heart failure progresses, so it was expected that body weight would decrease in salt-fed groups at the later time points of the study. The results in **Figure 2.1** show that by 18 weeks of diet, body weight fell 22% in the HS group and by 19% in the

HSH group relative to the LS group, but only fell 12% in the HSG group. However, this difference from HSG only approached significance ( $p < 0.08$ ).



**Figure 2.1. Serial body weight.** Each value given is the mean from 12 rats per group. Shown without error bars to preserve visualization. Both the high salt (HS) and high salt + vasodilator hydralazine (HSH) groups were significantly affected, \*  $p < 0.05$  vs low salt (LS) group. (LSG) low salt + grape powder diet group; (HSG) high salt + grape powder diet group.

As shown in **Figure 2.2**, HSG did not prevent the development of hypertension, but the HSG diet significantly reduced systolic blood pressure relative to HS diet. For both HSG and HSH, the first statistically significant decrease versus HS was detected at six weeks of treatment. The LSG group trended to have slightly lower systolic blood pressure versus the LS group, but the difference was not statistically significant at any time point.



**Figure 2.2. Systolic blood pressure.** Each value given is an average from 12 rats per group  $\pm$  standard error of the mean. \* At least  $p < 0.05$  vs low salt (LS) group; \*\*  $p < 0.05$  vs high salt (HS) group. (HSG) high salt + grape powder diet group; (HSH) high salt + vasodilator hydralazine diet group; (LSG) low salt + grape powder diet group.

## D2. Echocardiography

Cardiac geometry and function were measured at study baseline (0 weeks), compensated hypertrophy (8 weeks), and diastolic dysfunction (18 weeks). For changes in cardiac geometry, **Table 2.3** shows that HS rats showed greater left ventricular end diastolic dimension (LVEDD) at 18 weeks. This remodeling was reduced with the HSG group but not in the HSH group. In the Dahl-SS rat, increasing relative wall thickness (RWT, or  $2 \times$  posterior wall thickness during diastole/LVEDD) is found to correlate strongly with contractile failure, more so than increasing LV mass(28). HS diets increased wall thickness, changes first evident at the 8 week compensated hypertrophy stage. When measured at 18 weeks, HSG reduced RWT and left ventricular mass/body weight. This effect was not observed in the HSH group at any time point.

**Table 2.3. Serial Changes in Cardiac Geometry**

| <b>Cardiac Geometry</b> | <b>LS</b>  | <b>LSG</b> | <b>HS</b>   | <b>HSG</b> | <b>HSB</b>  |
|-------------------------|------------|------------|-------------|------------|-------------|
| <b>LVEDD</b>            |            |            |             |            |             |
| 0 w                     | 7.2 ± 0.4  | 7.0 ± 0.4  | 7.3 ± 0.4   | 7.1 ± 0.5  | 7.1 ± 0.3   |
| 8 w                     | 7.8 ± 0.5  | 7.7 ± 0.3  | *7.2 ± 0.3  | 7.6 ± 0.5  | *7.0 ± 0.4  |
| 18 w                    | 8.1 ± 0.3  | 8.0 ± 0.5  | *8.9 ± 0.5  | 8.3 ± 0.3  | *8.8 ± 0.3  |
| <b>LVESD</b>            |            |            |             |            |             |
| 0 w                     | 3.8 ± 0.2  | 3.7 ± 0.2  | 3.7 ± 0.1   | 3.5 ± 0.3  | 3.8 ± 0.2   |
| 8 w                     | 4.0 ± 0.2  | 4.2 ± 0.3  | 3.8 ± 0.2   | 3.6 ± 0.2  | 3.7 ± 0.3   |
| 18 w                    | 4.7 ± 0.3  | 4.6 ± 0.3  | 4.9 ± 0.3   | 4.9 ± 0.4  | 4.8 ± 0.3   |
| <b>RW th</b>            |            |            |             |            |             |
| 0 w                     | 0.3 ± 0.01 | 0.3 ± 0.03 | 0.3 ± 0.02  | 0.3 ± 0.02 | 0.3 ± 0.03  |
| 8 w                     | 0.3 ± 0.02 | 0.3 ± 0.03 | *0.6 ± 0.02 | 0.4 ± 0.02 | *0.6 ± 0.04 |
| 18 w                    | 0.4 ± 0.02 | 0.4 ± 0.03 | *0.7 ± 0.02 | 0.5 ± 0.03 | *0.8 ± 0.03 |
| <b>LV Mass</b>          |            |            |             |            |             |
| 0 w                     | 2.5 ± 0.1  | 2.4 ± 0.1  | 2.5 ± 0.1   | 2.4 ± 0.2  | 2.6 ± 0.1   |
| 8 w                     | 2.6 ± 0.1  | 2.5 ± 0.2  | *3.5 ± 0.2  | 2.7 ± 0.2  | *3.6 ± 0.3  |
| 18 w                    | 2.8 ± 0.1  | 2.7 ± 0.2  | *4.3 ± 0.4  | 3.1 ± 0.2  | *4.4 ± 0.3  |

Echocardiography measures at 0 weeks of diet (baseline), 8 weeks of diet, and 18 weeks of diet. LVEDD (left ventricular end-diastolic dimension, mm), LVESD (left ventricular end systolic dimension, mm), RW th (relative wall thickness, mm), and LV/BW (gram left ventricular mass/gram body weight). Mean ±SEM. n=12 per group. \* at least p<0.01 vs LS, LSG, HSG.

In addition to grape-associated changes in cardiac geometry, diastolic parameters were also positively affected by HSG diets (**Table 2.4**). Changes in diastolic parameters were assessed by using M-Mode echocardiography and Doppler tissue imaging. Mild or early diastolic dysfunction is commonly characterized by altered filling velocities, which are measured by the ratio of peak early filling velocity (E wave) to late filling velocity (A wave). The E/A ratio falls at compensated hypertrophy stage, indicating an abnormal relaxation pattern or early diastolic dysfunction. However, E/A sharply rises with cardiac decompensation, indicating increased LV end diastolic pressures and pseudonormalized mitral valve inflow. During compensated hypertrophy at 8 weeks, HS-fed rats displayed a lower E/A ratio indicating a relaxation abnormality. This effect was attenuated in the

HSG group, but not in the HSH group. At 18 weeks, HS showed the expected E/A elevation which was also significantly attenuated by HSG, but not in the HSH group. Isovolumetric relaxation time (IVRT) of the left ventricle increased significantly between 8 and 18 weeks. Prolonged IVRT can be considered an indicator of increased myocardial stiffness due to fibrosis(29-32), but HSG significantly reduced IVRT. This effect was not observed in the HSH group. Collectively, these findings indicate that diastolic parameters are improved by the grape-containing diet, but not by the vasodilator hydralazine.

**Table 2.4.** Serial Changes in Diastolic Parameters

| Diastolic Function | LS       | LSG      | HS        | HSG        | HSH       |
|--------------------|----------|----------|-----------|------------|-----------|
| <b>E/A</b>         |          |          |           |            |           |
| 0 w                | 2.7 ±0.2 | 2.7 ±0.3 | 2.6 ±0.2  | 2.6 ±0.3   | 2.4 ±0.3  |
| 8 w                | 2.9 ±0.1 | 2.8 ±0.3 | *2.0 ±0.3 | †2.4 ±0.2  | *2.2 ±0.2 |
| 18 w               | 2.6 ±0.3 | 2.5 ±0.1 | *6.2 ±0.3 | **3.8 ±0.1 | *5.8 ±0.3 |
| <b>E Dec time</b>  |          |          |           |            |           |
| 0 w                | 43.6 ±3  | 44.0 ±4  | 43.3 ±6   | 43.2 ±5    | 43.1 ±4   |
| 8 w                | 42.1 ±3  | 41.7 ±5  | *48.3 ±3  | 45.7 ±4    | *47.2 ±4  |
| 18 w               | 44.0 ±2  | 43.0 ±4  | *34.2 ±3  | †39.9 ±3   | *35.6 ±3  |
| <b>IVRT</b>        |          |          |           |            |           |
| 0 w                | 17.7 ±3  | 17.3 ±2  | 18.8 ±2   | 16.7 ±1    | 18.1 ±3   |
| 8 w                | 18.3 ±2  | 18.1 ±3  | 20.5 ±2   | 20.1 ±3    | 19.5 ±2   |
| 18 w               | 21.1 ±2  | 20.4 ±3  | *31.5 ±3  | †24.3 ±1   | *30.1 ±3  |

Echocardiography measures at 0 weeks of diet (baseline), 8 weeks of diet, and 18 weeks of diet. (E/A) E Wave to A wave, (E Dec t, in milliseconds) E Wave Deceleration time, (IVRT, in milliseconds) Isovolumetric Relaxation Time. Mean ±SEM. n=12 per group. \*at least p<0.05 vs LS, LSG. †p<0.05 vs LS, LSG, HS, HSH.

Regarding systolic function (**Table 2.5**), the % mid-wall fractional shortening and ejection fraction were not significantly altered by high-salt feeding, which is expected in this rat model; the Dahl-SS rat is a model of diastolic dysfunction rather than systolic dysfunction. However, cardiac index reflects cardiac contractile efficiency by measuring the volume of blood moved per minute (stroke volume x heart rate), per unit of body

weight. As such, cardiac index can reflect both diastolic and systolic function. The 8 week measures in all groups did not show significantly impaired cardiac index, which is expected during compensated hypertrophy. However, at 18 weeks, cardiac index was significantly lower in the HS fed group but was significantly improved by HSG. This effect was not observed in the HSH group.

**Table 2.5.** Serial Changes in Systolic Parameters

| <b>Systolic Function</b>                                                                                                                                                                                                                                                                   | <b>LS</b> | <b>LSG</b> | <b>HS</b> | <b>HSG</b> | <b>HSH</b> |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|------------|-----------|------------|------------|
| <b>% Mid-Wall FS</b>                                                                                                                                                                                                                                                                       |           |            |           |            |            |
| 0 w                                                                                                                                                                                                                                                                                        | 21.2 ±2   | 21.2 ±2    | 22.2 ±2   | 20.3 ±3    | 21.7 ±3    |
| 8 w                                                                                                                                                                                                                                                                                        | 20.4 ±3   | 20.4 ±3    | 18.3 ±2   | 21.2 ±2    | 17.9 ±2    |
| 18 w                                                                                                                                                                                                                                                                                       | 19.2 ±2   | 19.2 ±2    | 17.1 ±2   | 18.3 ±3    | 16.9 ±2    |
| <b>% Ejection Fraction</b>                                                                                                                                                                                                                                                                 |           |            |           |            |            |
| 0 w                                                                                                                                                                                                                                                                                        | 73.0 ±4   | 73.0 ±4    | 71.3 ±5   | 72.2 ±6    | 72.1 ±4    |
| 8 w                                                                                                                                                                                                                                                                                        | 75.0 ±8   | 75.0 ±8    | 69.4 ±5   | 70.3 ±5    | 68.6 ±6    |
| 18 w                                                                                                                                                                                                                                                                                       | 72.1 ±4   | 72.1 ±4    | 70.1 ±4   | 71.1 ±5    | 70.4 ±5    |
| <b>Cardiac Index</b>                                                                                                                                                                                                                                                                       |           |            |           |            |            |
| 0 w                                                                                                                                                                                                                                                                                        | 444 ±32   | 440 ±27    | 440 ±23   | 438 ±33    | 438 ±17    |
| 8 w                                                                                                                                                                                                                                                                                        | 435 ±31   | 434 ±25    | 431 ±24   | 432 ±31    | 426 ±22    |
| 18 w                                                                                                                                                                                                                                                                                       | 437 ±22   | 436 ±19    | *333 ±26  | †375 ±21   | *339 ±23   |
| <b>Heart Rate</b>                                                                                                                                                                                                                                                                          |           |            |           |            |            |
| 0 w                                                                                                                                                                                                                                                                                        | 386 ±22   | 394 ±21    | 392 ±20   | 382 ±19    | 391 ±18    |
| 8 w                                                                                                                                                                                                                                                                                        | 397 ±17   | 410 ±19    | 412 ±35   | 410 ±21    | 417 ±25    |
| 18 w                                                                                                                                                                                                                                                                                       | 385 ±23   | 404 ±25    | 423 ±14   | 418 ±23    | 415 ±24    |
| Echocardiography measures at 0 weeks of diet (baseline), 8 weeks of diet, and 18 weeks of diet. FS = fractional shortening. Cardiac index is ml of blood/minute/g body weight. Heart rate is beats/minute. Mean ±SEM. n= 12 per group. *p<0.05 vs LS and LSG. †p<0.05 vs LS, LSG, HS, HSH. |           |            |           |            |            |

Heart rate was not affected by treatment, so changes in cardiac index likely reflect changes in cardiac geometry and functionality independent of sympathetic outflow. As observed with diastolic function values, LSG did not confer benefits for systolic function over the LS group.

### ***D3. Cardiac Hypertrophy, Histology and Hydroxyproline Content***

Cardiac hypertrophy correlates with increased blood pressure, increased fibrosis and collagen deposition, and reduced cardiac function. Importantly, cardiac hypertrophy precedes the development of more advanced, irreversible pathogenesis such as heart failure. Compared to HS diet, HSG was associated with significantly lower cardiac and renal hypertrophy (**Table 2.6**). Strikingly, the HSG cardiac weights were similar in weight to that of LS and LSG group. HSH reduced renal weight but did not reduce cardiac weight. LSG did not impact cardiac weight relative to the LS group. LSG trended to reduce kidney weight relative to LS group, but the difference was not statistically significant. Hydroxyproline is a component of collagen and a quantitative index of fibrosis. Collagen accumulation occurs in the heart during heart failure and contributes to stiffening of the heart walls, impaired relaxation, impaired filling, and reduced cardiac output. As shown in Table 2.6, HSG had significantly reduced cardiac hydroxyproline and perivascular area of fibrosis content relative to HS, which was not observed in the HSH group.

**Table 2.6.** Changes in Organ Weight and Cardiac Fibrosis

|                                  | LS         | LSG        | HS         | HSG        | HSH        |
|----------------------------------|------------|------------|------------|------------|------------|
| <b>Heart<sup>1</sup></b>         | 0.34 ±0.01 | 0.33 ±0.02 | *0.42±0.1  | †0.32±0.01 | *0.43±0.3  |
| <b>Kidney<sup>1</sup></b>        | 0.71 ±0.04 | 0.65 ±0.04 | *0.98±0.05 | †0.82±0.04 | †0.83±0.06 |
| <b>Lung<sup>1</sup></b>          | 0.42 ±0.04 | 0.40 ±0.04 | 0.54±0.06  | 0.43±0.05  | 0.52 ±0.04 |
| <b>Hydoxproline</b>              | 5.2 ±0.3   | 5.1 ±0.2   | *9.2±0.2   | †7.4±0.3   | *9.1 ±0.3  |
| <b>Area Fibrosis<sup>2</sup></b> | 1.2 ±0.04  | 1.2 ±0.02  | *1.45±0.1  | †1.3 ±0.03 | *1.40 ±0.4 |

<sup>1</sup>Organ weights in grams/cm tibial length. Hydroxyproline is µg/mg total protein. Mean ±SEM. n=12 per group. <sup>2</sup>Determined from ten random measures around ten distinct vessels \*at least p<0.05 vs LS and LSG, †p<0.05 vs LS, LSG, HS.

#### ***D4. Cardiac GSH/GSSG, Oxidative Damage, and Plasma Inflammation***

Malonyldialdehyde (MDA) is a by-product of the oxidation of lipids, and serves as a marker of oxidative stress. Data in **Table 2.7** indicates that HSG diet was associated with significantly lower MDA content relative to HS, but HSH did not provide this effect. Although MDA was relatively low in the healthy LS rat hearts, LSG still conferred a significant treatment effect versus LS. Cardiac reduced glutathione (GSH) is decreased in the salt-fed Dahl-SS rat heart relative to oxidized glutathione (GSSG)(33,34). HSG diet significantly improved the GHS/GSSG ratio over HS group (Table 2.7), reflecting improved antioxidant defense. This effect was not observed in the HSH group. Interestingly, this effect was also observed in LSG rats as compared to LS rats, indicating that grape powder provision improved cardiac antioxidant defense even in the absence of concurrent disease. Also in Table 2.7, ELISA for plasma markers of inflammation indicated that HSG diet was associated with significantly reduced plasma IL-6 and TNF- $\alpha$  relative to the HS group. This effect was not observed in the HSH group. While LSG trended to reduce IL-6 and TNF- $\alpha$ , the results were not statistically significant.

**Table 2.7.** Cardiac GSH/GSSH, Cardiac MDA, and Plasma Inflammation

|                                       | LS             | LSG            | HS             | HSG             | HSH            |
|---------------------------------------|----------------|----------------|----------------|-----------------|----------------|
| <b>Cardiac GSH/GSSG</b>               | 148 $\pm$ 7    | *184 $\pm$ 9   | *45 $\pm$ 3    | †75 $\pm$ 5     | *53 $\pm$ 6    |
| <b>Cardiac MDA</b>                    | 0.4 $\pm$ 0.02 | 0.3 $\pm$ 0.02 | *1.4 $\pm$ 0.1 | †1.0 $\pm$ 0.06 | *1.3 $\pm$ 0.2 |
| <b>plasma TNF-<math>\alpha</math></b> | 1.4 $\pm$ 0.02 | 1.1 $\pm$ 0.03 | *6.8 $\pm$ 0.3 | †4.3 $\pm$ 0.3  | *6.3 $\pm$ 0.4 |
| <b>plasma IL-6</b>                    | 0.9 $\pm$ 0.02 | 0.7 $\pm$ 0.02 | *5.4 $\pm$ 0.3 | †3.7 $\pm$ 0.2  | *5.1 $\pm$ 0.4 |

GSH in  $\mu$ M relative to GSSG in  $\mu$ M. MDA is expressed as mg/per g total protein. TNF- $\alpha$  and IL-6 are in pg/mL. Mean  $\pm$ SEM. n=12 per group. \* at least  $p < 0.05$  vs LS, †  $p < 0.05$  vs LS, LSG, HS, HSH.

## **E. DISCUSSION**

The current results demonstrate the broad effects of a phytochemical-enriched diet on the gradual development of hypertension-associated diastolic dysfunction. The focus on diastolic pathogenesis is of great significance in the aged. Systolic dysfunction primarily concerns the heart's reduced ejection capacity, where diastolic dysfunction concerns the heart's reduced filling capacity. While systolic failure has a higher mortality rate, diastolic heart failure has a strong association with normal aging and is more common than systolic heart failure in the elderly(35,36). Importantly, numerous clinical trials have documented the benefits of pharmacologic treatment for systolic heart failure; however, the optimal treatment for diastolic heart failure has not yet been defined. Diastolic dysfunction develops over a prolonged period of time and is largely reversible, so the effects of diet patterns on disease course are of great interest for both preventive and interventional cardiology. The descriptive approach employed here is intended to reveal the breadth of phytochemical-rich diet effects on many phenotypes relevant to hypertension and to diastolic heart failure pathogenesis.

The mechanisms behind the treatment effects are likely complex and involve the interaction amongst a number of organ systems. For example, grape-related benefits may be derived in part from reduced blood pressure, and blood pressure is regulated dynamically by interactions amongst the kidney, brain, vasculature, and heart. In the HSG group, reduced systolic blood pressure was observed early and was sustained throughout the study. The lack of depressor effect in the LSG group as compared to LS suggests that grape-related depressor effects are largely observed in hypertensive as opposed to normotensive animals. The mechanisms of grape-associated vasodilation are

not completely understood, but some studies have indicated that grape-product consumption may improve the availability of the vasodilator nitric oxide. However, hydralazine afforded a similar reduction in systolic blood pressure throughout the study, yet failed to impact eventual cardiac pathology, suggesting that reduced hypertension alone is not sufficient for cardioprotection. Hydralazine limits calcium release from smooth muscle sarcoplasmic reticulum, resulting in arterial and arteriolar relaxation. In the Dahl-SS rat and in other hypertensive rat models, hydralazine consistently reduces arterial pressure but does not impact cardiac hypertrophy or fibrosis(19-21), results which support our current findings. Hydralazine can elicit a reflex sympathetic stimulation at higher doses, but the dose provided here did not cause elevated heart rate or cardiac output. It is possible that differing routes of vasodilation lead to different protective phenotypes, but it is clear that reduced blood pressure alone does not protect against cardiac fibrosis or hypertrophy in this model.

The current results therefore imply that additional mechanisms beyond vasodilation are participating in grape-mediated cardioprotection. Hypertension contributes to cardiac oxidative stress, and grape-enrichment may confer antioxidant effects. In the heart, unquenched reactive oxygen and reactive nitrogen species damage local lipids, proteins, and DNA, leading to cardiomyocyte death and sub-optimal cardiac function. Although a specific relationship between oxidative stress and ventricular performance has not been clearly established, there is considerable association between oxidative stress and underlying components of cardiac pathogenesis including systemic inflammation, cardiomyocyte apoptosis, cardiac remodeling, mechanoenergetic uncoupling, and endothelial dysfunction. Furthermore, accumulated evidence suggests a

significant correlation between oxidative stress and clinical indexes of cardiac functional capacity, such as New York Heart Association class and peak exercise oxygen consumption(37,38). By reducing oxidative stress, antioxidant-rich diets may thus impact the pathogenesis or severity of cardiac dysfunction.

Direct antioxidant effects in the heart tissue would require cardiac bioavailability of the grape phytochemicals. The predominant bioavailable components would likely include enterohepatic metabolites such as sulfate conjugates, glucuronides, and *O*-methylated forms, with very low levels of non-conjugated, parent compounds. However, these enterohepatic and intracellular metabolites have a reduced ability to donate hydrogen, and are less effective scavengers of radicals as compared to their parent compounds. Also, concentrations of these metabolites in the plasma or in tissues are lower (nanomolar, low micromolar) than those recorded *in vivo* antioxidants such as ascorbate, uric acid, glutathione, and Vitamin E(39). Consequently, bioavailable grape phytochemicals are unlikely to supersede these antioxidants for radical scavenging effects, and thus direct antioxidant action of grape metabolites in cardiac tissue may be relatively minor.

Instead, accumulating evidence suggests that the tissue antioxidant effects of phytochemicals may be mediated indirectly by their interactions with intracellular signaling cascades and with altered gene expression. For example, bioavailable phytochemicals may stimulate the transcription and translation of endogenous antioxidants in the heart. Polyphenols like those found in grape can activate response elements in the genome which regulate the transcription of glutathione-regulating enzymes. In the current study, both LSG and HSG were associated with elevated

GSH/GSSG relative to their controls LS and HS, respectively. Bioavailable grape phenolic phytochemicals may activate cardiac genes which modify glutathione dynamics, like glutathione peroxidase and glutathione-S-transferase(40).

Finally, grape-related benefits may derive in part from indirect effects on reduced cachexia and systemic inflammation. Cachexia is a catabolic state characterized by weight loss and muscle wasting and occurs frequently in patients with heart failure, and is a strong independent risk factor for heart failure-related mortality(41). Cachexia is also characteristic of Dahl-SS pathogenesis, and appeared in our rats after 14 weeks of diet. The onset of cachexia is associated with elevations in pro-inflammatory mediators, including IL-6 and TNF- $\alpha$ , both of which correlate with advancing heart failure(22,42). Grape diet effect on body weight loss approached significance, and significantly reduced plasma TNF-  $\alpha$  and IL-6. Thus, limited cachexia may contribute to grape-associated benefits.

The phytochemical model presented here is limited by the use of only one fruit. We expect that treatment effect could be amplified were we to use a more complex mix of phytochemical-containing whole foods. Rationale for grape selection is supported from several studies showing a depressor effect of grape juice and wine consumption(14-18). Results found here may not extend to the dietary supplement grape seed extract, which contains higher levels of high molecular weight tannins of questionable bioavailability, and which lacks anthocyanins. Instead, table grape powder derived from grape skin, flesh and seed contains a broader phytochemical profile that is more relevant to that observed in fruit/vegetable-rich human diets. Our intent was to use a model food that has modest antioxidant potential and has demonstrated efficacy. This standardized,

whole grape powder used here has been shown by other investigators to reduce both plasma and tissue markers of oxidative stress *in vivo* and *ex vivo*(9-13). These studies indicate that beneficial components of the whole table grape powder are bioavailable to tissues, and could confer health benefits against diseases which involve oxidative stress such as hypertension-associated heart failure. The simplified model presented here may thus serve as a precursor to studies which model more complex dietary patterns and their effects upon cardiac pathogenesis.

With regards to grape “dose” justification, allometric scaling or the bioequivalence between rodents and man is unknown. As such, 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 126 grams. With loss on drying, one human serving of freeze dried whole grape powder equals 23 grams. The rat body weight equivalent of nine servings of grapes/day then averaged 600 milligrams/day, or 3% of the daily diet. In this manner, the dose used here attempted to model the nine servings/day of fruit/vegetables in the DASH diet trials(4). Other methods of estimating bioequivalence could lead to different dose justifications, including adjustments made relative to metabolic rate, food intake, food intake relative to body weight, differences in body surface area, or target organ weight relative to body weight. Regardless of the approach to estimate bioequivalence, the level of whole fruit powder employed here is likely to be physiologically relevant to human diets.

Although we controlled for macronutrient and calorie intake in the current design, we cannot conclusively exclude any benefit from the additional micronutrients from grape (as described in Table 2.1). However, the 3% w/w grape powder enrichment

supplied a modest six milligram increase in potassium and a 0.02 milligram increase in vitamin C intake per day. Previous studies in the Dahl-SS rat suggest that greater potassium (43) (5-10x higher than provided here) and greater Vitamin C (44) (5,000x higher than provided here) is required to reduce blood pressure. Further studies may be warranted to ascertain the specific contribution of micronutrients in the absence and presence of phytochemicals. Clinical trials in patients with heart failure have failed to detect benefits from antioxidant micronutrient supplementation and indeed, some have observed adverse effects(45,46). In contrast to dietary supplements, whole food models allow synergistic interaction between micronutrients and phytochemicals which may improve their bioavailability or potency. Therefore, whole foods approaches may confer both increased efficacy and safety versus dietary supplements for the prevention or treatment of heart failure.

In summary, the diet incorporation of grape-derived phytochemicals improved cardiac glutathione reserve and reduced experimental hypertension-induced cardiac fibrosis and diastolic dysfunction in the Dahl-SS rat. This benefit correlated with reduced cardiac oxidative damage and improved cardiac antioxidant reserve. The findings support the efficacy of phytochemical-enriched diets against hypertension-associated cardiac pathology. This may have particular importance to our aging population, which has reduced intake of both fruit and vegetables. The 2000 edition of the Dietary Guidelines for Americans(47) revealed that in individuals over age 60, only 35% of women and 39% of men met the two servings/day objective for fruits, and only 6% of both women and men met the three servings/day objective for vegetables. Because grape supplementation occurred at the onset of the study, this model examines preventive

effects versus interventional effects. Ongoing studies in our lab are assessing the effect of diet change after the development of hypertension and compensated hypertrophy, respectively. In this manner, we may reveal if the further value of phytochemical-enriched diets to interventional cardiology.

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### **CHAPTER THREE**

#### **A PHYTOCHEMICAL-ENRICHED DIET IMPACTS CARDIAC PPAR AND NF- $\kappa$ B ACTIVITY, FIBROSIS, AND CYTOKINE EXPRESSION IN RATS WITH DIASTOLIC HEART FAILURE**

## A. ABSTRACT

Prolonged hypertension is the leading cause of heart failure. Failing hearts show reduced peroxisome proliferator-activating receptor (PPAR) activity and enhanced nuclear factor kappa B (NF- $\kappa$ B) activity, which modify cardiac inflammation and fibrosis. *In vitro* studies suggest that phytochemicals alter PPAR and NF- $\kappa$ B activity, but effects of a phytochemical-rich diet are less understood. Grapes contain an array of commonly consumed dietary phytochemicals and may serve as a model of a phytochemical-rich diet. In Dahl-Salt Sensitive hypertensive rats, we previously showed(1) that dietary provision of 3% w:w whole table grape powder for 18 weeks reduced blood pressure, cardiac hypertrophy, and diastolic dysfunction. The working hypothesis is that in rats, phytochemical provision from whole grape powder impacts cardiac PPAR and NF- $\kappa$ B activity and their related gene transcripts. In rat hearts, we measured PPAR and NF- $\kappa$ B DNA binding activity, mRNA related to PPAR and NF- $\kappa$ B activation, NF- $\kappa$ B target TNF- $\alpha$  and TGF- $\beta$ 1 expression, and histology-determined perivascular fibrosis. Grape-fed groups had enhanced PPAR- $\alpha$  and PPAR- $\gamma$  activity and reduced NF- $\kappa$ B activity. RT-PCR indicated grape-associated up-regulation of PPAR- $\alpha$  mRNA, PPAR- $\gamma$  co-activator-1, PPAR- $\gamma$ , and NF- $\kappa$ B inhibitor I $\kappa$ B $\alpha$ . RT-PCR also indicated significant, grape-associated down-regulation of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1). Finally, grape intake was associated with significantly reduced cardiac TNF- $\alpha$  and TGF- $\beta$ 1 protein expression and reduced perivascular fibrosis. In the Dahl-SS rat, chronic intake of grapes altered cardiac transcripts related to PPAR and NF- $\kappa$ B, which may be significant to the observed diet-associated cardioprotection.

## **B. INTRODUCTION**

Prolonged hypertension is a prevalent and significant contributor to morbidity and mortality from heart failure. The DASH (Dietary Approaches to Stop Hypertension) clinical trials provided evidence that diets rich in fruits and vegetables reduced blood pressure(2,3). Animal models of hypertension may permit mechanistic appraisals of the interaction of diet and disease. One recent study in hypertensive rats modeled the DASH diet using added nutrients, but failed to show an effect on blood pressure(4). Therefore, a whole foods approach rather than altered nutrients alone may be more appropriate to assess the effect of diet on hypertension and related pathologies.

Previous studies by Seymour et al(1). showed that in the Dahl Salt-Sensitive (DSS) rats fed a high salt diet, the incorporation of freeze-dried whole table grape powder (3% of diet) significantly lowered blood pressure, cardiac hypertrophy and cardiac lipid peroxide formation. In addition, grape-fed rats displayed improved diastolic function and cardiac output. Furthermore, the benefits were not entirely related to blood pressure reduction, because comparable blood pressure reduction by hydralazine failed to match the cardioprotective effects of grape diet(1). However, cardiac-specific mechanisms of these effects remain unknown.

The current project focuses on cardiac cell signaling related to transcription of PPAR and NF- $\kappa$ B. PPARs are nuclear receptor transcription factors which intimately impact cell metabolism, cell differentiation, and inflammation. PPAR agonist drugs are used clinically to address hyperlipidemia and/or insulin resistance, but ancillary anti-inflammatory affects have also been observed. Importantly, PPARs are deficient in failing hearts(5,6), and PPAR agonism is beneficial in experimental heart failure

models(7-10). The risks and benefits of PPAR-targeted drugs in human heart failure are controversial(11-13) and are of current clinical interest given the widespread use of PPAR agonist drugs in cardiac patients.

Transcription factor NF- $\kappa$ B is exquisitely sensitive to oxidative stress, and engages in inflammatory responses by activating the transcription of various pro-inflammatory genes including cell adhesion molecules, inflammatory cytokines, and chemokines. Cardiac NF- $\kappa$ B activity is positively correlated with heart failure progression(14-16), and inhibition of NF- $\kappa$ B limits heart failure progression(17-21). The relation between PPAR activity and NF- $\kappa$ B-related inflammation can be described as bidirectional antagonism. That is, PPAR activation reduces NF- $\kappa$ B activation, and NF- $\kappa$ B reduces PPAR/DNA binding. In heart failure models, PPAR agonism with drugs experimentally reduces cardiac NF- $\kappa$ B activity and reduces morbidity and mortality(22,23). However, the effect of diet upon cardiac PPAR and NF- $\kappa$ B activity is poorly understood.

In the current research model, bioavailable grape phytochemicals may have altered cardiac PPAR and/or NF- $\kappa$ B activity. Grapes are a source of diverse phytochemicals, but are particularly rich in pigment-conferring anthocyanins. *In vitro* studies show that anthocyanin-rich extracts can activate PPARs in varied experimental models(24-30). If the grape diet altered cardiac PPAR activity, it could also limit cardiac NF- $\kappa$ B activity and associated cardiac inflammation and fibrosis. This project then tests the hypothesis that 3% dietary grape powder supplementation, which reduces Dahl-SS rat diastolic heart failure pathogenesis(1), is also associated with increased cardiac PPAR

activity and decreased NF- $\kappa$ B activity, and with reduced cardiac expression of cytokines and growth factors relevant to heart failure pathogenesis.

## **C. MATERIALS AND METHODS**

### ***C1. Animal Care and Diets***

Five week old Dahl-Rapp Salt-Sensitive rats (Harlan, Indianapolis, IN) were acclimated for one week on AIN-76a powdered diet (Research Diets, New Brunswick, NJ). Afterwards, each rat was randomly assigned ( $n = 12$  each) to one of four treatments. Low Salt diet (LS, AIN-76a with 2.8% added carbohydrate, glucose:fructose 1:1), Low Salt diet + grape powder (LSG, AIN-76a with 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). Grape powder content, diet preparation, and diet storage are as presented in Chapter Two, Section C.1. Animals were fed 20 grams of powdered diet/head/day. *Ad libitum* intake of AIN diet averages 19-21 grams of AIN powder/day in the Dahl-SS rat(31), so provision of 20 grams/day ensured complete daily consumption. For the high salt diets, NaCl was added directly to the food hopper and mixed carefully with the daily ration of powdered diet. Rats were housed three/cage 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.

### ***C2. Cardiac Tissue Sub-cellular Fractionation and Western Blot.***

Rats were sacrificed by guillotine, then hearts were harvested, washed in phosphate-buffered saline (pH 7.4), blotted, and weighed. The left ventricle was minced and flash frozen in liquid nitrogen, then stored at -80°C until further use. Frozen cardiac tissue was fractionated to obtain nuclear and cytosolic homogenates using the method of Li et al.(32) with modifications, using a NE-PER Nuclear Extraction Kit (Pierce, Rockford, IL). Frozen cardiac fragments were powdered using a mortar and pestle cooled by a cocoon of dry ice. The powder was then added to kit buffer cytoplasmic extraction reagent I (CER I) at 50 mg powder/ml CER I buffer, and subjected to four, 10-second, low speed pulses of a Brinkmann Polytron homogenizer (Kinematica, Bohemia, NY). The resulting homogenate was incubated on ice for ten minutes, followed by the addition of cytoplasmic extraction reagent Buffer II (CERII, 55 µl/ml of CER I buffer volume). The sample was briefly vortexed and centrifuged for five minutes at 14,000 x g (at 4°C). The resulting supernatant was considered the cytosolic fraction. The pellet was then resuspended in the nuclear extraction reagent (NER, 500µl/ml of CER I buffer volume), and incubated on ice for a total of 40 minutes with brief vortexing (10 seconds) at 10-minute intervals. The sample was then centrifuged for 10 minutes at 16,000 x g (at 4°C). The resulting supernatant was considered the nuclear fraction.

Nuclear and cytosolic fractions (50 µg each) were mixed with SDS sample buffer, denatured for five minutes at 95°C, resolved on pre-cast NuPAGE™ 10% Bis-Tris polyacrylamide gels (Invitrogen, Carlsbad, CA, USA) by electrophoresis using a Novex Mini-Cell (Invitrogen), and subsequently transferred onto PVDF membranes. Blocking and antibody incubation steps were accomplished using the vacuum-based, SNAP i.d. Protein Detection System (Millipore, Billerica, MA, USA) using the ECL-specific

blocking reagents and ECL-chemiluminescence detection system (GE Healthcare, Piscataway, NJ, USA). Successful fractionation was verified by immunodetection of  $\alpha$ -tubulin (1° antibody 1:1000, SantaCruz Biotechnology, CA) and lamin B (1° antibody 1:200, SantaCruz Biotechnology) for cytosolic and nuclear fractions, respectively. Membranes were exposed to CL-XPosure film (Pierce, Rockford, IL, USA) and band densities were analyzed using UN-SCAN-IT Gel software version 6.1 (Silk Scientific, Orem, UT, USA).

### ***C3. Transcription Factor DNA Binding Assays.***

Once successful fractionation was confirmed, PPAR- $\alpha$ , PPAR- $\gamma$ , and NF- $\kappa$ B activity were determined in nuclear extracts using Transcription Factor DNA Binding assays (Cayman Chemical, Ann Arbor MI) according to manufacturers' instructions. A specific, proprietary oligonucleotide containing PPAR response elements (PPREs) or  $\kappa$ B responsive elements was immobilized onto the bottom of the wells of a 96-well plate. If present in the nuclear extract (loaded at 10 $\mu$ g/well), PPAR isoforms and NF- $\kappa$ B-element p65 bind to the well-bound oligonucleotide PPREs or  $\kappa$ Bs, respectively. Binding was then detected by addition of specific primary antibodies directed against the individual PPAR isoforms or against p65 subunit of NF- $\kappa$ B. A secondary antibody conjugated to horseradish peroxidase was added to enable colorimetric detection by reaction with substrate TMB/hydrogen peroxide and measurement of color development at 450 nm. Values were expressed as optical density relative to total protein in the respective nuclear extract as determined by the BCA Assay(Pierce).

#### ***C4. RT-PCR***

Total RNA from minced left ventricle was isolated with the RNeasy™ Fibrous Tissue Midi Kit (Qiagen, Valencia CA, USA) following the manufacturer's protocol. RNA quality and quantity (260/280 ratio >2.0, prominent 18S and 28S bands) were verified using the Agilent 2100 BioAnalyzer (Agilent Technologies, USA). From the twelve animals per group, four representative RNA samples were obtained by randomly combining equimolar amounts of RNA from three rats. First strand cDNA synthesis was accomplished with the RT<sup>2</sup> First Stand Kit (SABiosciences, Frederick MD). cDNA was then added to the RT<sup>2</sup> qPCR Master Mix, which contains SYBR Green and a reference dye. The relative abundance of eleven mRNA transcripts was then compared using a RT<sup>2</sup> Profiler PCR Array™ (SABiosciences). Relative expression was determined by the  $\Delta\Delta\text{CT}$  method as described by Livak(33), normalized relative to the average  $\Delta\text{Ct}$  of four housekeeping genes (P1 large ribosomal protein, hypoxanthine guanine phosphoribosyl transferase, ribosomal protein L13A, and lactate dehydrogenase).

#### ***C5. Histology Determined Fibrosis***

Four hearts from each group were utilized for histology determination of cardiac fibrosis. A transverse section of the left ventricle was fixed in 10% neutral buffered formalin, and sections were stained with Masson-Trichrome stain for determination of fibrosis. The fibrotic areas stain blue/purple, and the non-fibrotic areas stain red. Digital images were acquired with a Olympus BX40 microscope camera mounted on a Nikon DN100 light microscope. True-color image analysis was performed using Bioquant image analysis software (BIOQUANT Life Science, Nashville, TN).

#### **C6. *TNF- $\alpha$ and TGF- $\beta$ Enzyme-Linked Immunosorbent Assays (ELISA)***

ELISAs were conducted on cytosolic homogenates derived from frozen left ventricle. Total protein content was assessed using the BCA assay (Pierce). Cardiac TNF- $\alpha$  and TGF- $\beta$  were measured using commercial kits (R&D Systems, Minneapolis, MN) according to manufacturers' instructions. Results are expressed relative to total protein (BCA Assay, Pierce).

#### **C7. *Statistics***

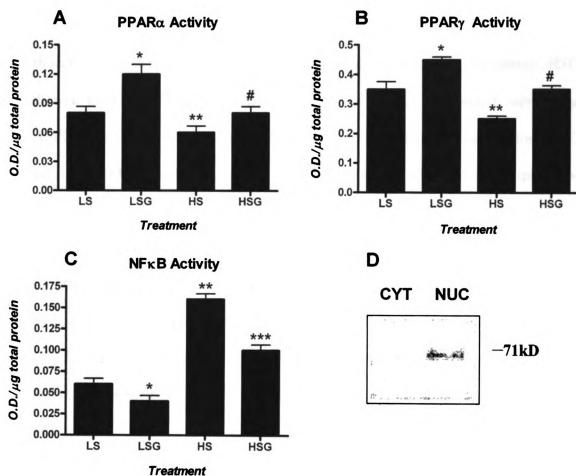
mRNA transcript pair-wise comparisons are determined  $\pm$  SD using the  $\Delta\Delta CT$  method as described by Livak(33), using the PCR Array data analysis web portal of SABiosciences (<http://www.sabiosciences.com/pcr/arrayanalysis.php>). All other endpoints were expressed  $\pm$  SEM and compared using a two-way ANOVA with salt and grape as independent factors. Given significant interaction, pair-wise comparisons were accomplished with Bonferonni post-hoc tests. Analysis was conducted with SPSS, version 16.0 (SPSS, Chicago, IL). For all measures, a p value < 0.05 was considered statistically significant.

### **D. RESULTS**

#### **D1. *Transcription Factor DNA Binding ELISA***

Results for the PPAR isoforms are in **Figure 3.1A and 3.1B**. In the LSG group, both PPAR- $\alpha$  and PPAR- $\gamma$  activity were increased as compared to the LS group. In contrast, the HS group showed reduced PPAR- $\alpha$  and PPAR- $\gamma$  activity. Finally, compared

to HS, the HSG group showed enhanced PPAR- $\alpha$  and PPAR- $\gamma$  activity. The conserved increase in nuclear extract PPAR binding in both LSG and HSG groups relative to their respective salt controls could suggest a specific effect of bioavailable grape phytochemicals and/or their metabolites upon PPAR activity. Results for NF- $\kappa$ B activity are shown in **Figure 3.2C**. Compared to LS rats, the LSG group showed reduced NF- $\kappa$ B activity. In contrast, the HS group showed sharply increased NF- $\kappa$ B activity. Finally, compared to HS, the HSG group showed reduced NF- $\kappa$ B activity.



**Figure 3.2A-D. Cardiac PPAR $\alpha$ , PPAR $\gamma$  and NF- $\kappa$ B Activity.** PPAR- $\alpha$ (A), PPAR- $\gamma$ (B), and NF- $\kappa$ B(C) activity. n=9 per group, \*p<0.05 v LS. \*\*p<0.05 v LS, LSG. # or \*\*\* indicate p<0.05 v HS. (D) Representative Immunoblot of nuclear v cytosolic Lamin B.

## **D2. RT-PCR**

mRNA transcript abundance is compared in **Table 3.1**, which includes the gene name and fold regulation derived from pair-wise comparisons, including the change from salt alone (LS v HS), from grape in healthy rats (LSG v LS), and from grape in diseased rats (HSG v HS). Compared to LS rats, LSG rats showed increased mRNA for PPAR- $\alpha$ , PPAR $\gamma$ , and PGC-1 $\alpha$  that would support the greater PPAR activity observed in the transcription factor ELISA. LSG also showed increased mRNA for I $\kappa$ B $\alpha$  and reduced mRNA for pro-inflammatory TNF- $\alpha$ . HS rats showed decreased PPAR- $\alpha$ , PGC-1 $\alpha$ , and I $\kappa$ B $\alpha$ , and increased mRNA for NF $\kappa$ B and for multiple pro-inflammatory cytokines and growth factors. These results are aligned with increased NF $\kappa$ B activity. In contrast, HSG rats showed increased PPAR- $\alpha$ , PPAR $\gamma$ , and PGC-1 $\alpha$  mRNA that would support the greater PPAR activity observed in the transcription factor ELISA. In addition, HSG showed increased I $\kappa$ B $\alpha$  mRNA and decreased mRNA related to multiple pro-inflammatory cytokines and growth factors, which supports the reduced NF $\kappa$ B activity.

The conserved transcriptional effects in both LSG and HSG groups suggest specific effects from bioavailable grape phytochemicals and/or their metabolites. The current data in Table 3.1 show that not all NF- $\kappa$ B-impacted transcripts were significantly ( $p < 0.05$ ) changed by grape intake. It is known that some of these genes are also regulated by other redox transcription factors such as AP-1 and ets-1, which may also impact the observed results.

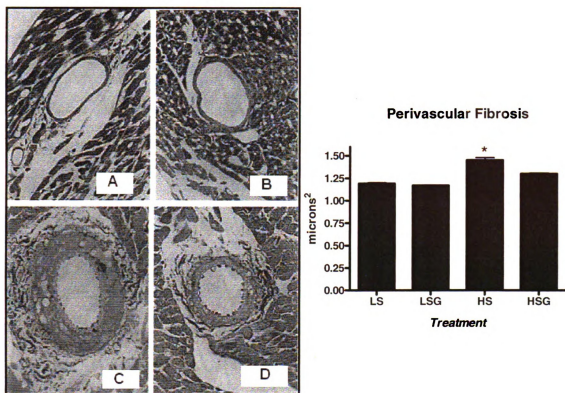
**Table 3.1. RT-PCR Results**

| Gene Symbol           | Gene Name                             | Fold Regulation by Salt | Fold Regulation by Grape |            |
|-----------------------|---------------------------------------|-------------------------|--------------------------|------------|
|                       |                                       | HS (v LS)               | LSG (v LS)               | HSG (v HS) |
| PPAR- $\alpha$        | PPAR- $\alpha$                        | -6.86*                  | 1.44*                    | 2.91*      |
| PPAR- $\gamma$        | PPAR- $\gamma$                        | 1.17                    | 1.52*                    | 1.71*      |
| PGC-1 $\alpha$        | PPAR- $\gamma$ coactivator 1 $\alpha$ | -3.62*                  | 1.56*                    | 3.65*      |
| NF- $\kappa$ B        | Nuclear factor $\kappa$ B             | 1.28                    | 1.02                     | 1.14       |
| I $\kappa$ B $\alpha$ | Inhibitor kappa B $\alpha$            | -2.31*                  | 1.3*                     | 3.48*      |
| TNF- $\alpha$         | Tumor necrosis factor- $\alpha$       | 3.64*                   | -1.56*                   | -1.92*     |
| IL-6                  | Interleukin-6                         | 1.23                    | 1.06                     | -1.75*     |
| IL-1 $\beta$          | Interleukin-1 $\beta$                 | 1.64*                   | -1.1                     | -5.06*     |
| TGF- $\beta$ 1        | Transforming growth factor- $\beta$ 1 | 2.62*                   | -1.21                    | -2.06*     |
| ICAM-1                | Intercellular Adhesion Molecule 1     | 1.26*                   | -1.17                    | -1.62*     |

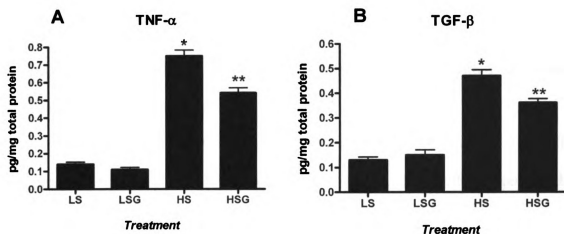
n=4 per group. Comparison via  $\Delta\Delta$ CT method. \*p at least <0.05 versus respective salt control.

### D3. Cardiac Fibrosis, TNF- $\alpha$ , and TGF- $\beta$ 1

Seymour et al.(1) previously demonstrated that hearts from HSG group had reduced cardiac homogenate hydroxyproline content, an index of collagen content and fibrosis(1). Histochemistry results in **Figure 3.2** support this finding, and indicate significantly greater perivascular fibrosis in the HS group relative to the HSG group. Results for TNF- $\alpha$  and TGF- $\beta$ 1 expression are shown in **Figure 3.3A** and **3.3B**. Compared to LS, the LSG group showed slightly reduced TNF- $\alpha$  expression which was not statistically significant. In contrast, HS rats showed sharply increased TNF- $\alpha$  and



**Figure 3.2. Masson-Trichrome Stain Determined Cardiac Fibrosis.** Representative images are of LS (A), LSG (B), HS (C), and HSG (D). The fibrotic areas stain blue/purple, and the non-fibrotic areas stain red. The perivascular area of fibrosis was the mean  $\pm$  SEM as determined from ten random measures around ten distinct vessels. The area of fibrosis equation: (total area encompassing the vessel lumen + total area of the fibrotic ring) / total area of the vessel lumen.



**Figure 3.3A-B. Cardiac TNF- $\alpha$  and TGF- $\beta$ 1.** Compared to HS, HSG significantly reduced cardiac TNF- $\alpha$  and TGF- $\beta$ . n=9 per group, \* $p<0.05$  versus LS, LSG. \*\* $p<0.05$  vs HS.

## E. DISCUSSION

Anthocyanins are the largest group of water-soluble pigments in the plant kingdom. They are widely distributed in the human diet through crops, beans, fruits, vegetables, and their derived products. Anthocyanins have been shown to modify PPAR activity[22-28]. While the grape powder contained several flavonoids, the grape diets may have impacted cardiac PPAR activity due in part to the grape powder content of anthocyanins, which average over 50% of the total flavonoids in table grapes(34). This hypothesis is supported by findings in other research models. We previously showed in Dahl-SS rats that anthocyanin-rich tart cherry powder-enriched diets (1% w:w) were associated with elevated PPAR- $\alpha$  and PPAR- $\gamma$  mRNA in the liver(27). In diabetic mice, diets enriched with anthocyanin-rich mulberry extract showed increased liver and adipose tissue expression of PPAR- $\gamma$ , PPAR- $\alpha$  and liver PPAR-transcript lipoprotein lipase(35). *In vitro*, prostate cancer cells treated with cyanidin showed increased PPAR- $\gamma$ (28), and

*ex vivo* macrophages treated with isolated anthocyanins showed increased PPAR- $\gamma$  expression and PPAR- $\gamma$  transcriptional activity(29). As such, the current results showing increased cardiac PPAR mRNA and activity are in agreement with others using anthocyanins, anthocyanin-rich extracts, or anthocyanin-rich foods.

The interaction of PPARs and inflammation is a focus of this present work. NF- $\kappa$ B is present in the cytosol in an inactive form, complexed to an inhibitory kappaB (I $\kappa$ B) monomer. Various stimuli, including ischemia, hypoxia, free radicals, and cytokines activate NF- $\kappa$ B by inducing phosphorylation of its cytoplasmic inhibitor I $\kappa$ B. NF- $\kappa$ B has been shown to regulate the expression of important genes/proteins for heart failure progression including tumor necrosis factor (TNF- $\alpha$ ), interleukins (IL-1 $\beta$ , IL-6). growth factors (TGF- $\beta$ 1), and adhesion molecules (ICAM-1) as select examples.

It is currently known that PPAR and NF- $\kappa$ B interact in several ways to oppose their respective activities. First, PPAR activation increases the transcription of I $\kappa$ B $\alpha$ (36). Furthermore, PPARs physically interact with NF- $\kappa$ B via its Rel homology domain which mediates interaction with I $\kappa$ B $\alpha$ (37). Finally, nuclear NF- $\kappa$ B can inhibit PPAR binding to genomic PPREs, thereby reducing PPAR transcriptional activity and the expression of PPAR-related transcripts(37).

Studies with PPAR agonists confirm this inverse association with NF- $\kappa$ B activity. Stroke-prone, spontaneously hypertensive rats treated with a PPAR $\gamma$  agonist drug showed reduced cardiac NF- $\kappa$ B activity, reduced cardiac fibrosis, and reduced expression of NF- $\kappa$ B-related TNF- $\alpha$  and various adhesion molecules(38). Treatment of Dahl-SS rats with PPAR- $\alpha$  agonist drug fibrate inhibited the cardiac hypertrophy, attenuated the diastolic relaxation abnormality and systolic dysfunction, and improved survival(10). In concert

with these phenotypes, PPAR- $\alpha$  agonism also decreased Dahl-SS cardiac NF- $\kappa$ B activity and the expression of NF- $\kappa$ B related target genes like adhesion molecules (VCAM-1, ICAM-1), cytokines (MCP-1), and growth factors (TGF- $\beta$ 1)(10).

The results presented here and previously in the current model(1) compare favorably with the earlier PPAR- $\alpha$  agonist fibrate study. After 18 weeks of high salt diet(1), the HSG group (as compared to the HS group) showed reduced left ventricle weight(-28%), surpassing that of the fibrate group(-19%). The HSG group showed reduced systolic blood pressure(-12%), superior to the fibrate group(-7%). We present here that the HSG showed reduced cardiac TGF- $\beta$ 1(-24%) and NF- $\kappa$ B activity(-27%), while the fibrate group showed reduced cardiac TGF- $\beta$ 1(-25%) and reduced NF- $\kappa$ B activity(-40%). Given the broad clinical use of PPAR agonists, the comparable observed benefits from a dietary modification are encouraging.

The HSG group showed reduced NF- $\kappa$ B transcripts, and reduced translation of TNF- $\alpha$  and TGF- $\beta$ 1. Human heart failure is correlated with increasing plasma levels of TNF- $\alpha$  and TGF- $\beta$ 1, and *ex vivo* analysis of explanted cardiac tissue in humans and in animal models show increased levels of cardiac TNF- $\alpha$ (39,40) and TGF- $\beta$ 1(41-43). TNF- $\alpha$ , a pro-inflammatory cytokine, may be directly involved in the progression of heart failure by exerting direct negative inotropic effects and by triggering apoptosis in cardiomyocytes(44), and elevated plasma TNF- $\alpha$  correlates with heart failure trajectory(45). TGF- $\beta$ 1 is up-regulated in the heart by increased work load, and suffices to provoke the hypertrophic program of cardiac gene expression(46), regulating cell growth, fibrosis and inflammation. In the HSG group, other genes affected by grape

include IL-6, IL-1 $\beta$ , and ICAM-1. Collectively, these changes would contribute to local inflammation and fibrosis.

The phenotypic sequelae of PPAR activation can depend upon which PPAR isoforms are activated. In hypertensive rats, PPAR- $\alpha$  activation reduces cardiac hypertrophy(10,22,47,48) improves diastolic and systolic function, and prolongs lifespan(10,49). In spontaneously hypertensive stroke-prone rats, PPAR- $\gamma$  activation did not reduce blood pressure or cardiac hypertrophy, but did reduce NF- $\kappa$ B activity, fibrosis and a cardiac inflammation(50). When isoform-specific agonists were directly compared in post-infarction rats, PPAR- $\alpha$  activation dose-dependently improved cardiac output, myocardial contractility, and diastolic relaxation while reducing cardiac hypertrophy and fibrosis. However, treatment with a PPAR- $\gamma$  agonist exacerbated cardiac dysfunction(49). Therefore, experimental models appear to support the premise that PPAR- $\alpha$  agonism is beneficial while the effects of PPAR- $\gamma$  agonism appear to vary.

In concurrence with animal studies, there is current controversy over the clinical safety of PPAR- $\gamma$  agonism in heart failure patients. While PPAR- $\alpha$  agonists are typically employed to reduce hyperlipidemia, PPAR- $\gamma$  agonists are prescribed to increase insulin sensitivity. However, PPAR- $\gamma$  agonism can cause edema and weight gain which can exacerbate heart failure(13,51). Seymour et al. previously reported in Dahl-SS rats that salt-fed groups displayed cachexia beyond study week 14 (of 18), with a final weight loss of ~25 % relative to the low-salt group(1). However, the HSG group lost only 12% body weight ( $p < 0.05$  vs HS). It is uncertain if this reduced weight loss was due to edema, reduced cachexia, or both. Seymour et al. also previously showed that grape intake significantly reduced cachexia-associated plasma TNF- $\alpha$ (1), so this result suggests that

3

grape intake reduced cachexia, itself. Finally, altered PPAR activity may have altered cardiac energy substrate utilization in the heart. It is known that cardiac energy substrate preferences change during heart failure pathogenesis, which can then affect cardiac performance(52). However, these metabolic disturbances are typically associated with systolic heart failure(52), rather than diastolic heart failure as observed in the Dahl-SS rat model.

An effect of grape phytochemicals upon cardiac transcription or cell signaling would likely require tissue availability. In similar long-term feeding studies, anthocyanins have demonstrated bioavailability in several tissues as measured by LC-MS/MS(53-55), including studies using a whole food model(27,56,57) as utilized here. For example, rats fed with whole blueberry powder (at 2% of diet), had diverse anthocyanins in the brain, including cyanidin (3-O- $\beta$ -galactoside, 3-O- $\beta$ -glucoside, 3-O- $\beta$ -arabinoside), malvidin (3-O- $\beta$ -galactoside, 3-O- $\beta$ -glucoside, 3-O- $\beta$ -arabinoside), and peonidin (3-O- $\beta$ -arabinoside and 3-O- $\beta$ -galactoside)(56). In addition, pigs fed 2% blueberry diets showed eleven intact anthocyanins in the liver, eye, brain(57), with some variation between tissues on the predominant anthocyanins and their glycosidic moieties. Differences in the anthocyanidin and sugar moieties among the different tissues may reflect selectivity of anthocyanin absorption, metabolism, and retention among tissues and amongst sites within tissues. Importantly, these animals were fasted at sacrifice and show no detectable plasma anthocyanins while showing diverse tissue deposition. For the heart, it was previously shown that isolated, buffer-perfused rat hearts can directly absorb and retain cyanidin-3-O- $\beta$ -glucoside in a dose-dependent manner, and show parallel reductions in oxidative damage following ischemia/reperfusion injury(58). It is

then possible that bioavailable anthocyanin metabolites in cardiac tissue participated in altered PPAR activity.

Regarding altered PPAR activity, bioavailable grape anthocyanins (and perhaps other phytochemicals) may modulate cell signaling pathways including cascades such as phosphoinositide 3-kinase, Akt/PKB, tyrosine kinases, protein kinase C, and MAP kinases. Bioavailable phytochemicals and/or their metabolites can bind to the ATP-binding sites of a large number of proteins(59), which causes three-dimensional structural changes and altered protein activity. In addition, bioavailable phytochemicals, their enterohepatic metabolites, and their intracellular metabolites may interact with sulfhydryl moieties on kinase proteins and alter secondary protein structure and activity. The exact kinase signaling pathways involved in the observed grape-related effects are unknown and require further investigation.

Regarding altered NF- $\kappa$ B activity, bioavailable grape phytochemicals, including non-anthocyanin compounds, may act directly as antioxidants and thereby reduce oxidative stress and NF- $\kappa$ B activity. However, conjugated phytochemical metabolites present in the heart have a reduced ability to donate hydrogen and to scavenge damaging radicals as compared to their non-conjugated or aglycone parent compounds. Also, concentrations of these conjugated metabolites in tissues are much lower than endogenous antioxidant compounds like glutathione, superoxide dismutase, or catalase. As such, grape diet effects upon NF- $\kappa$ B activity are likely indirect rather than direct, through altered kinase signaling and gene transcription/translation.

Several changes in gene transcription were conserved between LSG and HSG groups. However, diet-mediated protection in the HSG group may also involve reduced neurohormonal or biomechanical sources of cardiac oxidative stress. In early heart failure pathogenesis, aberrant pressure and volume adjustment by the vasculature involves the generation of free radicals. Local cardiac oxidative stress is also generated by increased renin-angiotensin-aldosterone system (RAAS) activation, increased norepinephrine, and from local inflammation. Furthermore, pressure/volume overload increases cardiac work and cardiac metabolism, increasing the opportunity for lost free electrons and oxidative stress.

Should grape intake impact local RAAS activation, tissue norepinephrine, or cardiac work, it may then indirectly affect redox-regulated NF- $\kappa$ B activity. For example, grape mediated vasodilation has been demonstrated in several models. However, Seymour et al.(1) previously showed in Dahl-SS rats that comparable vasodilation by hydralazine failed to mimic the benefits of grape, indicating that vasodilation alone is not wholly responsible for grape-associated benefits. As related to PPAR activity, grape intake may have indirectly altered the formation of endogenous PPAR ligands, such as free fatty acids or eicosanoids. Also, the current study did not explore the activation of PPAR- $\delta$ , another PPAR isoform of growing interest in cardiology. Finally, phytochemicals other than anthocyanins may be involved in altered cell signaling and transcription factor activities, or they may participate synergistically.

In conclusion, physiologically relevant whole grape powder intake increased Dahl-SS rat cardiac PPAR activity and decreased NF- $\kappa$ B activity. These changes are present with reduced hypertensive cardiac pathology, fibrosis, and cytokine expression,

and these biochemical and molecular mechanisms may be vital for the grape intake-associated cardioprotective effects.

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## **CHAPTER FOUR**

### **A PHYTOCHEMICAL-ENRICHED DIET IMPACTS CARDIAC AhR and nrf2 TRANSCRIPTION FACTOR ACTIVITY AND GLUTATHIONE DYNAMICS**

## **A. ABSTRACT**

Higher intake of phytochemical-rich fruits and vegetables is associated with reduced hypertension, but its effect upon hypertensive cardiac pathology is less understood. Phytochemicals can act as antioxidants, but they also alter *in vitro* transcription factor activity, including aryl hydrocarbon receptor (AhR) and NF-E2 related factor (nrf2), and alter related gene transcription pertinent to antioxidant defense. However, it is unknown if these molecular effects can occur *in vivo* with a phytochemical-rich food, and if they correlate with reduced cardiac pathology. We previously showed that grape intake was associated with reduced cardiac hypertrophy, improved cardiac diastolic function, and enhanced cardiac glutathione. The study reported here assessed potential molecular mechanisms, using male Dahl-Salt Sensitive rats, fed a high-salt or a low-salt diet with or without 3% (w:w) whole grape powder. After 18 weeks, left ventricle tissue was analyzed for genetic changes and enzyme activity as affected by salt and by grape intake. High salt fed rats show reduced AhR activity, reduced glutathione, and reduced activity of glutathione-regulating proteins. In contrast, grape-fed hypertensive rats showed enhanced cardiac AhR and nrf2 activity, up-regulated mRNA related to AhR and nrf2 activation, and enhanced the expression and activity of glutathione-regulating proteins. We conclude that phytochemical intake altered the cardiac transcriptome and altered cardiac glutathione dynamics, thereby improving cardiac antioxidant defense.

## **B. INTRODUCTION**

The effects of diet upon heart failure pathogenesis are poorly understood. Over 90% of heart failure cases are preceded by prolonged hypertension(1). The Dietary Approaches to Stop Hypertension (DASH) trials indicated that diets rich in fruits and vegetables, low fat dairy, and whole grains reduced blood pressure(2). Furthermore, 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 greater amounts of non-nutritive phytochemicals as compared to the control diet(4). An attempt to experimentally duplicate the DASH diet with altered nutrients (vitamins, minerals, fiber, fats, proteins, and carbohydrates) failed to reduce blood pressure in hypertensive rats(5). This null result could support the value of the non-nutritive phytochemicals from foods for altering hypertension and related pathologic sequelae.

Seymour et al.(6) demonstrated that regular intake of physiologically-relevant whole table grape powder reduced hypertension-associated diastolic dysfunction in the Dahl Salt-Sensitive (Dahl-SS) rat. Furthermore, the cardiac benefits from grape intake exceeded those of a vasodilator drug, hydralazine, indicating that a depressor effect does not entirely explain the scope of grape benefit. Whole grape intake also reduced cardiac oxidative damage and fibrosis(6). However, the cardiac-specific mechanisms associated with these grape-related benefits have not been explored. Beyond a systemic blood pressure reducing effect, grape-related changes in cardiac tissue may be vital to the observed local changes in cardiac oxidative damage and fibrosis that contribute to adverse changes in cardiac geometry and hemodynamic function.

As with hypertensive human heart failure, Dahl-SS rat hearts show reduced cardiac antioxidant defense(7). Among endogenous antioxidant defense enzymes, glutathione (GSH) is the most prominent, found at millimolar concentrations in most cells. Glutathione exists in cells in both a reduced form (GSH) and an oxidized form (GSSG), and may also be covalently bound to proteins(8,9). The ratio of GSH to GSSG impacts the overall redox state of the cell, and GSH is significantly reduced in failing human hearts(10-12). In Dahl-SS rats, Seymour et al. observed reduced cardiac GSH content in hearts with diastolic dysfunction, but significantly higher cardiac GSH in grape-fed rats(6). However, the mechanisms of altered glutathione dynamics are currently unknown.

Bioavailable phytochemicals and their metabolites may alter cellular kinase and/or transcription factor activity, which would then impact gene regulation. Numerous glutathione-regulating genes contain response elements called xenobiotic response elements (XREs) and antioxidant response elements (AREs)(13,14). Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor which binds to XREs and is activated by synthetic xenobiotics and an array of dietary phytochemicals(15). Another transcription factor of interest is NF-E2 related factor (nrf2), which binds to AREs and is also activated by phytochemicals(13,14). Bioavailable grape polyphenols may thus activate AhR, nrf2, or both to ultimately alter glutathione dynamics and antioxidant defense.

This project will now explore the effects of regular intake of grape phytochemicals upon AhR and nrf2-associated cardiac antioxidant defense in Dahl-SS rats fed a high-salt diet. The working central hypothesis is that grape provision is

associated with enhanced cardiac AhR and nrf2 nuclear translocation and the enhanced expression of genes/proteins related to enhanced glutathione.

## **C. MATERIALS AND METHODS**

### ***C1. Animal Model and Diets***

Five week old Dahl-Rapp Salt-Sensitive rats (Harlan, Indianapolis, IN) were acclimated for one week on AIN-76a powdered diet (Research Diets, New Brunswick, NJ). Afterwards, each rat was randomly assigned (n = 12 each) to one of four treatments. Low Salt diet (LS, AIN-76a with 2.8% added carbohydrate, glucose:fructose 1:1), Low Salt diet + grape powder (LSG, AIN-76a with 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). Grape powder content, diet preparation, and diet storage are as presented in Chapter Two, Section C.1. Animals were fed 20 grams of powdered diet/head/day. *Ad libitum* intake of AIN diet averages 19-21 grams of AIN powder/day in the Dahl-SS rat(16), so provision of 20 grams/day ensured complete daily consumption. For the high salt diets, NaCl was added directly to the food hopper and mixed carefully with the daily ration of powdered diet. Rats were housed three/cage 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.

### ***C2. Cardiac Tissue Sub-cellular Fractionation and Western Blot***

Rats were sacrificed by guillotine, then hearts were harvested, washed in phosphate-buffered saline (pH 7.4), blotted, and weighed. The left ventricle was minced and flash frozen in liquid nitrogen, then stored at -80°C until further use. Frozen cardiac tissue was fractionated to obtain nuclear and cytosolic homogenates using the method of Li et al.(17) with modifications, using a NE-PER Nuclear Extraction Kit (Pierce, Rockford, IL). Frozen cardiac fragments were powdered using a mortar and pestle cooled by a cocoon of dry ice. The powder was then added to kit-supplied buffer cytoplasmic extraction reagent I (CER I) at 50 mg powder/ml CER I buffer, and subjected to four, 10-second, low speed pulses of a Brinkmann Polytron homogenizer (Kinematica, Bohemia, NY). The resulting homogenate was incubated on ice for ten minutes, followed by the addition of cytoplasmic extraction reagent Buffer II (CERII, 55 µl/ml of CER I buffer volume). The sample was briefly vortexed and centrifuged for five minutes at 14,000 x g (at 4°C). The resulting supernatant was considered the cytosolic fraction. The pellet was then resuspended in the nuclear extraction reagent (NER, 500 µl/ml of CER I buffer volume), and incubated on ice for a total of 40 minutes with brief vortexing (ten seconds) at 10-minute intervals. The sample was then centrifuged for 10 minutes at 16,000 x g (at 4°C). The resulting supernatant was considered the nuclear fraction.

Nuclear and cytosolic fractions (50 µg each) were mixed with SDS sample buffer, denatured for five minutes at 95°C, resolved on pre-cast NuPAGE™ 10% Bis-Tris polyacrylamide gels (Invitrogen, Carlsbad, CA, USA) by electrophoresis using a Novex Mini-Cell (Invitrogen), and subsequently transferred onto PVDF membranes. Blocking and antibody incubation steps were accomplished using the vacuum-based, SNAP i.d. Protein Detection System (Millipore, Billerica, MA, USA) using the ECL-specific

blocking reagents and ECL-chemiluminescence detection system (GE Healthcare, Piscataway, NJ, USA). Successful fractionation was verified by immunodetection of  $\alpha$ -tubulin (1° antibody 1:1000, SantaCruz Biotechnology, CA) and lamin B (1° antibody 1:200, SantaCruz Biotechnology) for cytosolic and nuclear fractions, respectively. Membranes were exposed to CL-XPosure film (Pierce, Rockford, IL, USA) and band densities were analyzed using UN-SCAN-IT Gel software version 6.1 (Silk Scientific, Orem, UT, USA).

### ***C3. Transcription Factor ELISA***

Once successful fractionation was confirmed, nrf2 expression 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 which contains several ARE sequences. Nrf2 in the nuclear extract (loaded at 5  $\mu$ g total protein/well) binds to these AREs. 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 activity was measured using a sandwich ELISA designed in our laboratory. A 96-well plate (Pierce® High Sensitivity NeutrAvidin®) was coated with a fabricated biotinylated, oligonucleotide containing three XRE sequences (5'-TCGACATTGCCCACGCCAGCTCACGCTGCTACACGCTTTAGCGCTACT-3'), with XRE consensus sequences underlined. AhR in the nuclear extract (loaded at 10  $\mu$ g total protein/well) then binds to XREs. Primary antibody is then targeted to AhR (1:100, SantaCruz Biotechnology), and

a HRP-conjugated secondary antibody (1:10,000 SantaCruz Biotechnology) enables colorimetric detection at 450nm.

#### ***C4. RT-PCR/PCR Array***

Rats were sacrificed by guillotine, then the hearts were harvested, washed in phosphate-buffered saline pH 7.4, blotted and weighed. The left ventricle was minced and flash frozen in liquid nitrogen. Total RNA from minced left ventricle was isolated with the RNeasy™ Fibrous Tissue Midi Kit (Qiagen, Valencia CA, USA) following the manufacturer's protocol. RNA quality (260/280, 18S and 28S bands) was verified using the Agilent 2100 BioAnalyzer. 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 nineteen mRNA transcripts was then compared using a RT<sup>2</sup> Profiler PCR Array™ (SABiosciences, Frederick MD). First, cDNA was prepared using the RT<sup>2</sup> First Strand Kit. cDNA was then added to the RT<sup>2</sup> qPCR Master Mix, which contains SYBR Green and a reference dye. The array contains pre-optimized, species-specific primer sets for controls (housekeeping genes, genomic DNA control, reverse transcription control, and positive PCR control), and the experimental genes of interest. Relative expression was determined by the  $\Delta\Delta CT$  method as described by Livak(18).  $\Delta Ct$  for each transcript is normalized relative to average  $\Delta Ct$  of four housekeeping genes (P1 large ribosomal protein, hypoxanthine guanine phosphoribosyl transferase, ribosomal protein L13A, and lactate dehydrogenase).

#### ***C5. Cardiac Glutathione and Enzymatic Activity Assays***

The following assays were conducted on whole cell homogenates derived from frozen left ventricle. Frozen left ventricle 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 assessed using the BCA assay (Pierce). Cardiac GSH/GSSG, glutathione peroxidase activity, and glutathione reductase activity were measured in whole cell homogenates using commercial kits (Oxis International, Beverly Hills, CA) according to manufacturers' instructions.

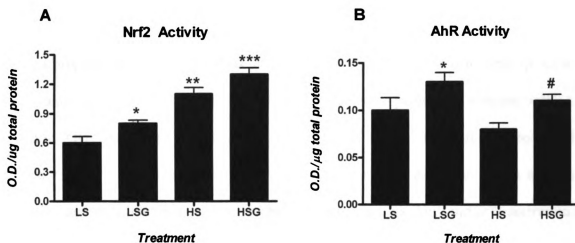
#### **C6. *Statistics***

mRNA transcript pair-wise comparisons are determined  $\pm$  SD using the  $\Delta\Delta CT$  method as described by Livak(18), 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). Western Blot relative abundance data, transcription factor ELISA data, GSH/GSSG, and enzyme activity data are expressed  $\pm$  SEM, and compared using a two-way ANOVA with salt and grape as independent factors. Pair-wise comparisons were accomplished with Bonferonni post-hoc tests. For all measures, a p value < 0.05 was considered statistically significant.

## **D. RESULTS**

#### D1. Transcription Factor ELISA

Results for nuclear fraction nrf2 activity are shown in **Figure 4.1A**. Compared to LS rats, LSG showed increased nrf2 activity. HS rats also showed enhanced nrf2 activity, which is expected given the effect of oxidative stress in nrf2 activation. Compared to HS rats, HSG rats showed further enhancement of nrf2 activity.



**Figures 4.1A-1B. Cardiac Nrf2 and AhR Activity.** n=12 each. \* $p < 0.05$  vs LS. \*\* $p < 0.05$  vs LS, LSG. \*\*\* $p < 0.05$  vs HS. # $p < 0.05$  vs HS.

Results for nuclear fraction AhR immunoreactivity are shown in **Figure 4.1B**. Compared to LS rats, LSG rats showed enhanced nuclear AhR. In contrast, HS rats showed reduced nuclear AhR ( $p$  value=0.08). Compared to HS rats, HSG rats showed enhanced nuclear AhR. The conserved increased in nuclear extract nrf2 and AhR binding in both LSG and HSG groups relative to their perspective controls could suggest a specific effect of bioavailable grape phytochemicals and/or their metabolites upon nrf2 and AhR activity.

## **D2. RT-PCR**

Transcript abundance is compared in **Table 4.1**, 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 salt alone (LS v HS), from grape in healthy rats (LSG v LS), and from grape in diseased rats (HSG v HS).

Compared to LS rats, HS rats showed reduced mRNA related to AhR ( $p$  value =0.11) and its nuclear chaperone ARNT ( $p < 0.05$ ), and significantly reduced mRNA related to XRE including CYP1A1, CYP1B1, and UGT1A6. In addition, HS group had reduced mRNA for several glutathione-S- transferase (GST) isoforms, which contain both XRE and ARE elements and would be responsive to both AhR and nrf2 activation. HS also reduced ARE-related mRNAs for  $\gamma$ -glutamylcysteine synthetase ( $\gamma$ -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 and unaffected GPx4.. The results therefore indicate that in healthy Dahl-SS rats, both ARE and XRE-affected mRNAs are impacted by salt intake.

Compared to LS rats, LSG rats showed increased mRNA for AhR and CYP1A1. LSG was associated with a increase in GST isoform mu 5. Amongst ARE-related transcripts, LSG was associated with significantly increased  $\gamma$ -GCS mRNA. The results therefore indicate that both ARE and XRE-affected mRNAs are impacted by grape intake.

Compared to HS rats, HSG rats showed a reverse trend to that shown in the HS samples. HSG showed significantly increased mRNA for AhR and ARNT, and for XRE-related mRNAs like nrf2, CYP1A1 and CYP1B1. In addition, HSG showed increased GST isoform mRNA mu 5. Amongst ARE-related transcripts, HSG showed significant increases in  $\gamma$ -GCS and GR mRNA, and mixed effects upon GpX mRNA. The results therefore indicate that in salt-fed Dahl-SS rats, both ARE and XRE-affected mRNAs are impacted by grape intake.

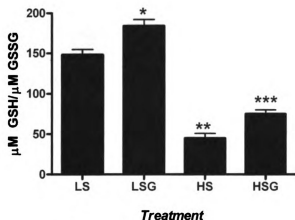
**Table 4.1. RT-PCR Results**

|             |        |                                       | Fold<br>Regulation<br>by Salt | Fold<br>Regulation<br>by Grape |               |
|-------------|--------|---------------------------------------|-------------------------------|--------------------------------|---------------|
| Gene        |        | Name                                  | HS<br>(v LS)                  | LSG<br>(v LS)                  | HSG<br>(v HS) |
|             | AhR    | Aryl Hydrocarbon Receptor             | -1.189                        | 1.206*                         | 1.244*        |
|             | ARNT   | AhR Nuclear Translocator              | -2.437*                       | 1.042                          | 1.922*        |
| XRE         | Nrf2   | nuclear factor-erythroid<br>derived 2 | 1.195                         | 1.185                          | 1.568*        |
| XRE         | CYP1A1 | Cytochrome P450 1A1                   | -2.692*                       | 1.955*                         | 1.329*        |
| XRE         | CYP1B1 | Cytochrome P450 1B1                   | -1.496*                       | 1.226                          | 1.522*        |
| XRE/<br>ARE | GST M4 | Glutathione S-Transferase mu 4        | -1.125                        | 1.167                          | 1.336*        |
| XRE/<br>ARE | GST M5 | Glutathione S-Transferase mu 5        | 2.175*                        | 1.497*                         | 1.414*        |
| XRE/<br>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    | $\gamma$ -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  $\Delta\Delta$ CT method. \*p at least <0.05 versus respective control (LS or HS)

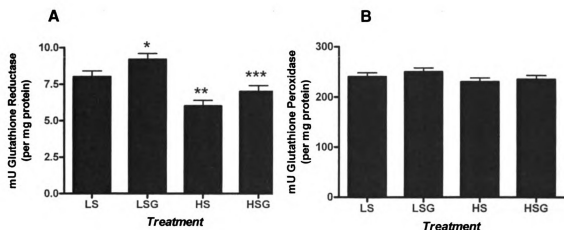
### D3. Cardiac Glutathione and Enzymatic Activity Assays

**Figure 4.2** shows that LSG and HSG increased the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG), relative to their respective controls.



**Figure 4.2. Cardiac GSH/GSSG.** n=12 each. \* $p < 0.05$  vs LS. \*\* $p < 0.05$  vs LS, LSG. \*\*\* $p < 0.05$  vs HS.

We then measured the activity of two proteins which impact the GSH:GSSG ratio - glutathione reductase and glutathione peroxidase. Grape intake increased the activity of glutathione reductase (**Figure 4.3A**), which would favor the formation of GSH. Glutathione peroxidase activity (**Figure 4.3B**) was not significantly altered by salt or grape. However, this assay was not isoform-specific; it is possible that individual isoforms of glutathione peroxidase have altered activity, which may be important given the mixed effects of treatment on GPx mRNA.



**Figures 4.3A-3B. Cardiac GR Activity and GPx Activity.** n=12 each. \* $p < 0.05$  vs LS. \*\* $p < 0.05$  vs LS, LSG. \*\*\* $p < 0.05$  vs HS.

## E. DISCUSSION

Glutathione is the most abundant cellular antioxidant, and deficiency in cardiac and systemic glutathione relates to heart failure progression in animal models and in humans. Glutathione deficiency is also observed in advanced heart failure(10-12), and may serve as a marker of early heart failure. The association between glutathione levels and disease was assessed in patients of different New York Heart Association (NYHA) functional classes and with different cardiac structural heart diseases(19). Glutathione was measured in venous blood samples obtained from healthy volunteers and patients rated on the revised NYHA functional class I to IV scale, undergoing cardiac surgery for coronary artery disease, aortic stenosis or terminal cardiomyopathy. Glutathione was also quantified in right atrial appendages. In atrial tissue, glutathione was severely depleted (-58%) in NYHA class IV patients compared to NYHA class I patients. Compared to healthy controls, blood glutathione was decreased in NYHA class I patients with structural cardiac disease (-21%), and in symptomatic patients of NYHA class II to IV (-

41%). Significant depletion in blood glutathione occurred before detectable elevation in plasma TNF- $\alpha$ , a marker of symptomatic heart failure severity. This study provided evidence that cardiac and systemic glutathione deficiency is related to the functional status and structural cardiac abnormalities of patients with cardiac diseases of varied etiology. If glutathione levels can serve as a valuable marker of heart failure pathogenesis, 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(20-22). It is therefore possible that consumption of antioxidant rich whole foods, rather than isolated nutrients alone, could confer a superior cardiovascular benefit. Direct antioxidant effects from phytochemicals in the heart tissue would require tissue bioavailability of the grape phytochemicals or their metabolites.

The grape compounds and their metabolites which are bioavailable to the heart may react directly with free metals or with reactive chemical species, forming products with much lower reactivity, or 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 and antioxidant defense.

Studies in animal and cell culture models show that phytochemical-rich diets activate the expression of Phase I and Phase II metabolism in varied body tissues(23-26). This effect of diet could be achieved by ligand interaction or by altering kinase signaling cascades, which then trigger transcription factor activation. Two transcription factors of interest here are AhR and nrf2.

AhR is a ligand-activated transcription factor which is classically activated by synthetic xenobiotics like dioxin, but also displays activation by an array of dietary phytochemicals(15). Owing to their aromatic chemical structure, several phytochemicals like flavone, catechin, and quercetin display *in vitro* agonist properties towards AhR(15,25,27-38). 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 from 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 oxoreductase-1(NQO-1), GST, aldehyde dehydrogenase 3A1, UGT1A1 and 1A6, and nrf2(39).

In the current study, salt-fed rats showed reduced AhR activity, and reduced expression of ARNT, which is supported by other models of pathologic cardiac hypertrophy(40-45). For example, AhR-null mice display cardiac hypertrophy(43), elevated blood pressure, and elevated plasma angiotensin II and endothelin I(44). Both LSG and HSG rats showed significantly increased AhR activity and mRNA of CYP1A1, a hallmark AhR activation. HSG also showed increased ARNT mRNA, which if

correlated with increased protein expression, would 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 compounds are responsible for the enhanced AhR activity. However, the results here are in agreement with studies using select phytochemicals found in table grapes, including quercetin(46-49), kaempferol(47), catechins(30,50-55), and resveratrol(32,34,48,56).

Nrf2 is a basic leucine zipper transcription factor that can be activated by kinases induced by oxidative stress or by xenobiotics like phytochemicals. Studies with phytochemicals and phytochemical-rich extracts indicate that signaling kinases become activated which enhances the nuclear translocation of nrf2(57-64). For example, phytochemicals could alter kinase modification of the Nrf2 chaperone Keap1. Kinase-mediated phosphorylation of Keap1 would sterically 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,  $\gamma$ -GCS, UGT1A6, and antioxidant defense enzymes like thioredoxin, metallothionein-1/2, and heme-oxygenase-1.

In the present study as related to glutathione dynamics, HS rats showed reduced mRNA to support glutathione formation (e.g.  $\gamma$ -GCS), glutathione conjugation (e.g. GST isoforms), and glutathione reduction to GSH (glutathione reductase). We also observe reduced GSH/GSSG and reduced glutathione reductase activity. In both LSG and HSG rats, grape-mediated nrf2 activation is supported by significantly increased mRNA for  $\gamma$ -GCS, which would correlate with the observed increases in tissue GSH. Additionally, the HSG rats showed enhanced glutathione reductase which would also favor a higher

GSH/GSSG ratio. As stated before, it is inappropriate to utilize a reductionist approach to determine which grape compounds are responsible for enhanced nrf2 activity. However, the results here are in agreement with studies using select phytochemicals showing enhanced nrf2 activity, including quercetin(57,60,61,63,65-67), kaempferol(61), catechins(51,58-60,62,68-72), and resveratrol(60,62,73-75). In addition, previous studies support the ability of flavonoids to increase glutathione-related enzymes and glutathione synthesis(14,23,76). Enhanced tissue GSH was also observed using a whole food model of purple corn(77,78). Prolonged diets of purple corn at 20% of the diet enhanced rat 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.

The current study shows that grape intake induces both cardiac AhR and nrf2 activity, which may allow greater antioxidant defense than either alone. For example, both AhR and nrf2 can enhance the transcription of UGT1A6 and nrf2. However, AhR activation leads to induction of CYP1 isoforms, while nrf2 activation does not. Enhanced CYP enzyme activity often generates low levels of reactive oxygen species, which can then activate redox-sensitive nrf2(51,54,79). It is now believed that cross-talk between AhR and nrf2 provides an evolutionary advantage, by allowing the upregulation of both Phase I enzymes (via AhR/XRE binding) and Phase II enzymes (via nrf2/ARE binding) in succession to address xenobiotic metabolism(51,54,79) and to limit oxidative stress. Nrf2 activation by grape in this model may then be achieved by higher CYP activity, direct action upon Keap1/nrf2 association, or both. Finally, although current results show

that HS was associated with reduced CYP1A1/1B1 mRNA, the expression of other cardiac CYPs may be induced by salt, and may be targets of diet effect.

In addition to AhR and nrf2 activation, other potential mechanisms can 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 which would indirectly favor preserved GSH levels. However, the data from the healthy LSG group support the direct effect of grape on AhR and nrf2 activity and glutathione dynamics independent from changes in oxidative stress(6). Also, grape intake may indirectly alter the formation of endogenous ligands of AhR, such as lipid peroxidative products. However, the reduction of lipid oxidative damage marker malonyldialdehyde suggests that grape was associated with lower lipid peroxidation(6), and perhaps lower candidates for endogenous AhR ligand formation. As such, AhR agonism by grape 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 genomic differences. However, given the previous observation that comparable blood pressure reduction by vasodilator hydralazine fails to match the cardioprotective effects of grape(6), the data suggest that the effects of grape extend beyond blood pressure reduction, alone.

In summary, grape provision altered gene expression involved in glutathione dynamics, with an overall effect favoring elevated glutathione. In addition, grape intake was associated with elevated activity of glutathione reductase, which would also favor higher elevations in GSH relative to GSSG. Importantly, these changes were conserved in

grape-fed, healthy rats. The grape-related mechanisms of cardiac AhR and nrf2 activity may be vital to the cardioprotective effects of the grape-enriched diet.

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## **CHAPTER FIVE**

### **SUMMARY, ALTERNATIVE APPROACHES, AND FUTURE DIRECTIONS**

## **A. SUMMARY**

The completed studies suggest that regular grape intake impacted both heart failure pathogenesis and cardiac gene transcription/translation. Specifically, grape diet in both healthy and diseased hearts was associated with increased activity of PPAR isoforms, AhR and nrf2, and reduced activity of pro-inflammatory NF- $\kappa$ B. Grape was also associated with enhanced transcription and activity of glutathione regulating enzymes, supporting enhanced antioxidant defense.

The relative importance of these mechanisms to altered heart failure pathogenesis is unknown. However, glutathione is the most prominent endogenous antioxidant in the heart, and even minor changes in glutathione dynamics could have a significant effect upon ROS-mediated damage. In addition, ROS impact diverse cell signaling pathways. Reduced cardiac ROS would affect cardiac redox-regulated NF- $\kappa$ B activity and related inflammation and fibrosis. As such, it is likely that enhanced glutathione dynamics would have a relatively greater impact upon heart failure pathogenesis than would altered PPAR activity.

## **B. ALTERNATIVE APPROACHES - GRAPE INTAKE MAY AFFECT PEROXYNITRITE FORMATION AND RENIN SYNTHESIS**

### ***B1. Nitric Oxide and Heart Failure.***

Chapter One first described the impact of grape phytochemicals upon nitric oxide (NO) availability and acute vasodilation. In heart failure, NO plays a vital role in the modulation of tissue perfusion(1). Reduced perfusion affects both myocardial and

skeletal muscle vascular beds, resulting in arrhythmias, myocardial ischemia, left ventricular dysfunction, systemic hypoperfusion, and exercise intolerance(2,3). In Dahl-SS rats, treatments which enhance NO production significantly improve hypertension pathologies. The amino acid L-arginine participates in NO synthesis, and dietary provision of L-arginine reverses Dahl-SS kidney pathogenesis(4-6). In contrast, nitric oxide synthase inhibitors like L-NAME perpetuate Dahl-SS kidney pathogenesis(7-9). As such, treatments which enhance NO availability affect both acute vasodilation and the sequelae of prolonged hypertension.

Oxidative stress during heart failure progression limits NO availability due to oxidation of NO to peroxynitrite, primarily by the ROS superoxide. Peroxynitrite damages local molecules and uncouples nitric oxide synthase, further reducing NO formation. The data here demonstrate that grape powder intake reduces cardiac oxidative stress. As such, grape powder intake may impact the cardiac NO dynamics by reducing the oxidation of NO to peroxynitrite.

## *B2. Grape Intake Could Reduce Dahl-SS Cardiac Peroxynitrite Formation.*

Similar to the current study, other studies have observed grape-reduced cardiac pathology and cardiac oxidative stress. In one study(10), fructose-fed rats were gavaged daily for six weeks with a solution containing red grape extracts enriched in either anthocyanins, procyanidins, or catechin oligomers, with the intent to discern which grape phytochemical constituents provided the most benefit. The results indicate that treatment with grape anthocyanins reduced hypertension, cardiac hypertrophy, and cardiac ROS production. Treatment with proanthocyanins reduced cardiac ROS but had only minor

effects on hypertension or hypertrophy. Finally, treatment with catechins matched that of anthocyanins; it reduced hypertension, cardiac hypertrophy, and cardiac ROS. All treatments reduced the cardiac expression of NADPH oxidase, which would likely limit superoxide formation, a key player in peroxynitrite formation.

In another study, spontaneously hypertensive rats were given high salt diet enriched with grape proanthocyanidins. Results showed that proanthocyanidins decreased hypertension and *in vitro* aortic superoxide production(11). Table grape powder contains anthocyanins, catechins, and proanthocyanins, therefore intake of table grape powder would likely reduce the production of cardiac ROS and reduce superoxide and peroxynitrite formation.

### *B3. Nitric Oxide and Renin Synthesis*

NO is important not only for vasodilation, but for negative feedback regulation of a vital neurohormonal system, the renin-angiotensin-system (RAS). RAS is present both locally (within tissue) and systemically (within plasma). Renin is a rate limiting upstream component in the production of the neurohormone angiotensin II (Ang II), and Ang II is a major effector of elevated blood pressure and hypertension-related pathogenesis. NO exhibits negative feedback upon renin synthesis(12-15), that is, more NO means less renin, and thereby less Ang II production.

Ang II plays several key roles in heart failure pathogenesis. In the kidney, Ang II is a critical mediator of adaptations to dietary sodium intake, affecting both tubular and glomerular function to regulate sodium excretion. In the heart, elevated Ang II promotes growth factor mediated hypertrophy, which is initially compensatory to handle the

increased volume/pressure load on the heart. However, prolonged growth signal stimulation leads to increased fibrosis and stiffened heart walls, culminating in reduced compliance and contractile efficiency(16). Tissue Ang II also activates NADPH oxidase, which produces superoxide. If not quenched by the antioxidant enzyme superoxide dismutase, excess superoxide can interact with NO to form peroxynitrite. There is thus an established vicious cycle which includes three main players - oxidative stress, peroxynitrite formation, and further Ang II production.

Dahl-SS hypertension is accompanied by increased Ang II in the kidney, vasculature, and the heart(17). Importantly, Dahl-SS rat pathology is reversed by the administration of inhibitors of Ang II production(16,18,19) or activity(16), highlighting the causative role of Ang II. In human heart failure, Ang II action is targeted by drugs which inhibit both its production and its receptor-mediated activity. If grape intake alters cardiac peroxynitrite formation, it would likely affect cardiac renin and cardiac Ang II.

#### *B4. Grape Intake Could Affect Renin Synthesis by Increasing Nitric Oxide Availability.*

In addition to grape-altered altered peroxynitrite formation and renin feedback, grape intake could have affected NO degradation. Rationale is provided by studies showing that ingestion of grape phytochemicals confers vasodilatory and depressor effects in humans(20-24). *In vitro* studies with rat(25) and human arteries(26) show that grape phytochemicals dose-dependently relaxed the aorta in a NO-dependent manner. Grape-enriched diets may enhance NO availability by inhibiting NO degradation. NO mediates vasodilation through cGMP, which is degraded by phosphodiesterases. *In vitro*

studies show that phytochemicals from red grape seeds enhance NO production(27) and can inhibit phosphodiesterase-5 (PDE-5)(28). NO mediates vasodilation through cGMP, which is degraded by PDEs. These studies suggest that grape-enriched diets lower blood-pressure in a NO-dependent manner, and increase tissue NO availability by decreasing its degradation.

In conclusion, it would be important to explore the effect of grape intake on the plasma and cardiac RAS-NO axis. This could include measures of cardiac renin, Ang II and peroxynitrite, as well as the other related proteins like NOS, PDE, and Ang II receptors.

### **C. FUTURE DIRECTIONS – DETERMINE CARDIAC BIOAVAILABILITY OF GRAPE PHYTOCHEMICALS**

#### ***C1. Define Plasma Metabolites of Grape Phytochemicals.***

Research on bioavailability is critical to the advancement of research in bioactive components from foods. For many years, little was known about the tissue bioavailability of phytochemicals. This was mostly due to difficulties in reliable quantification of the various food-derived phytochemicals and their metabolites in both biological fluids and tissues. The metabolism of several common phytochemicals is now reasonably well understood. Phytochemicals are extensively altered during metabolism, so that the molecular forms reaching the peripheral circulation and tissues are different from those present in whole foods. Here, the term “metabolism” describes the typical modifications that occur during or after absorption, which includes modifications made to

intracellular metabolites. In general, the resulting metabolites are conjugates (e.g, sulfates and glucuronates) of the parent aglycone, or conjugates of methylated parent aglycones.

Catabolism of phytochemicals in mammals generally occurs as a result of microbial activity in the large intestine(29). Most phytochemical glucosides are deglycosylated by  $\beta$ -glucosidases in the small intestine, namely, the broad-specificity cytosolic  $\beta$ -glucosidase and lactase phlorizin hydrolase; this step is requisite for the absorption of many of these phytochemicals (30). The small intestine appears to be the primary site responsible for glucuronidation; the major small intestine metabolites in the hepatic portal vein are glucuronides. After absorption, the conjugates may then travel to the liver and may be further methylated, glucuronidated, or sulfated. In the liver, polyphenols and their conjugates are metabolized by the Phase II drug-metabolizing enzymes, the glucuronosyl-transferases, sulfotransferases, and catechol-*O*-methyltransferases. The resulting molecules are glucuronate and sulfate conjugates, with or without methylation across the catechol functional group, and many are conjugated at multiple sites.

The predominance of phytochemical metabolites and conjugates over parent compounds has important consequences for biomedical research in this area. Phytochemical metabolites are chemically distinct from their parent compounds, differing in size, polarity, and ionic form. Consequently, their physiologic behavior is likely to be different from that of the parent compounds. Therefore, *in vitro* studies with the parent constituents will not likely yield useful information regarding possible disease-modifying effects afforded by grape product ingestion. This is a critical factor to keep in mind when

interpreting the possible clinical significance of *in vitro* studies using grape phytochemicals. Conversely, studies with physiologically relevant whole foods models such as that used here are an important and vital first step for studying the link between diet and disease.

C2. *Define Tissue Bioavailability of Grape Phytochemicals.*

For the tissue effects observed here, it is likely that tissue bioavailability of one of more grape-derived phytochemicals is required. The half-life of plasma phytochemical constituents and their metabolites is within hours, but constituents taken into tissues and cells can extend the time frame for exerting biologic effects. In support of this finding, many dietary supplementation studies demonstrate a biologic effect while not showing significant plasma presence of the predicted constituents. As a result, there is increasing speculation that enterohepatic conjugates, colonic metabolites, and intracellular metabolites of the parent compounds are actually responsible for the observed biologic effects(31).

In addition, many studies confirm the plasma and urine kinetics of flavonoids like catechins(32-37), anthocyanins (38-44), flavones(45-49), and the stilbene resveratrol(48,50,51). Grape proanthocyanidins of high molecular weight like tannins may have questionable bioavailability(52). From these larger molecules, it is likely that only the lowest molecular weight constituents (monomers, dimers) can be absorbed directly. An alternative hypothesis is that the proanthocyanidin polymers are metabolized by colonic microflora to alternate, absorbable phenolic acid compounds(53) which can be absorbed, distributed in plasma, and exert biologic activity in varied tissues.

Future directions would include identification of the grape constituents present in Dahl-SS rat hearts after prolonged feeding with the table grape powder. The tissue availability of individual grape phytochemicals like anthocyanins(41,44,54-57), quercetin(49,58-62), catechins(34,63-66), and resveratrol(51,67-70) has been demonstrated by others using chromatography with diode array detection coupled with tandem mass spectroscopy. The availability of authentic chemical standards and effective extraction techniques enables the identification and quantification of select phytochemicals in tissues.

#### **D. FUTURE DIRECTIONS – DETERMINE THE EFFECT OF DIET TIMING UPON DAHL-SS RAT HYPERTENSION PATHOLOGY**

Lastly, the current model explores the effects of a phytochemical rich diet during the onset and progression of hypertension and related cardiac pathology. However, it would be of interest to test diet effects upon established hypertension or compensated cardiac hypertrophy. This approach would have more clinical relevance, because medical professionals often counsel patients with established hypertension on the value of adopting new dietary approaches.

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