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The Acute And Chronic Effects Of Resveratrol On Renal Function And Blood Pressure

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THE ACUTE AND CHRONIC EFFECTS OF RESVERATROL ON RENAL FUNCTION AND BLOOD PRESSURE

by

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Advisor Date

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DEDICATION

To my loving departed parents I honor your memory with education and hard work,

To my wife Erin, a Hitchcock aficionado, for withstanding The Man Who Knew Too Much, To Stuart “frogs” never frightened me away from school,

To RIW and my gaming cohorts, thank you for the fun distractions,

To my family and friends thank you for the understanding.

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Lastly, thank you to my friend, Christine Cupps, who knows M&M's really do melt in your hand. Your administrative prowess is unmatched.

PREFACE

In the 1940's, the initial discovery of resveratrol by Japanese scientist, MJ Takaota, in the roots of the white hellebore plant, garnered little interest from the research community (164, 170). However, in the early 1990's resveratrol was also shown to be present in red wine from the skin of the grape. It was suggested resveratrol might explain the lower incidence of cardiovascular events associated with of red wine consumption (152). This observation sparked interest in resveratrol both from the research community and the public. Interest in resveratrol was increased when an association was made that there is a low incidence of heart disease and obesity among the French society despite their relatively high-fat diet (138). In contrast, a recent study disputes the cardiovascular benefit of resveratrol (148). Notwithstanding, resveratrol was compelling to a growing field of researchers. Resveratrol is found in two configurations (Figure 1). The trans-resveratrol isomer, the biologically active form, is stable for an extended time when protected from light, but in the presence of light it can undergo photoisomeric changes to the inactive cis-resveratrol isomer (173).

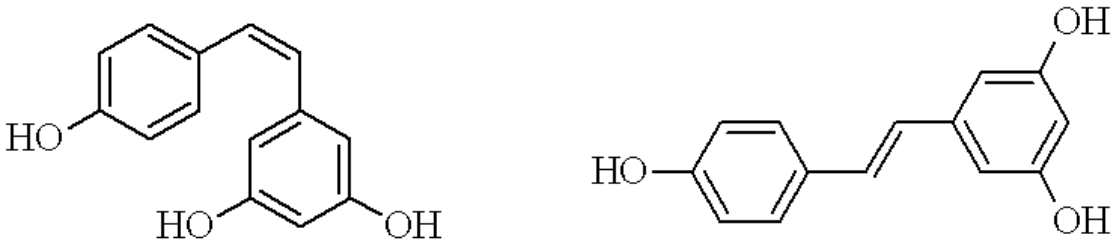

Figure 1: Chemical structure of resveratrol isomers: cis-resveratrol (left) and trans-resveratrol (right).

Resveratrol research crosses many disciplines ranging from anti-aging, cancer, inflammation to diabetes and cardiovascular research. This expansive field of research has yielded noteworthy results and controversy. Resveratrol was reported to activate

Sirt1 (22). Sirt1, known as NAD-dependent deacetylase sirtuin1, an enzyme found in both in yeast and humans, is linked to increased longevity possibly under states of caloric restriction (20). However, these results were drawn into question when it was shown experimental artifacts of florescent tags may have led to falsely reported Sirt1 activation (20). Further other groups also report resveratrol has no effect on Sirt1 activity (50). It remains to be seen if the *in vitro* effects of Sirt1 activation is germane to the biological effects of resveratrol *in vivo*.

Another area of resveratrol research is its potential effect to reduce inflammation. The inflammatory response is mediated in part by prostaglandins which are metabolic products of cyclooxygenase activity. Resveratrol has been shown to reduce inflammation in a model of colitis in rat by reducing NF-kappa beta activity (Martin). NF-kappa beta is found to be chronically active in many inflammatory diseases and is considered to be pro-inflammatory (109). NF-kappa beta is known to regulate interleukin-1 (IL-1), an inducer of pro-inflammatory cytokines (37). Resveratrol has been shown to reduce IL-1activity possibly through inhibition of NF-kappa beta (149). There is a growing association between inflammation and the development of hypertension. IL-1 levels are increased in individuals with pulmonary hypertension and IL-1 antagonists are shown to reduce pulmonary hypertension (30, 56).

Another area of resveratrol research is the effect of resveratrol treatment on osteoporosis. Resveratrol treatment in ovariectomized rats improves bone density (187). Further in ovariectomized rats, resveratrol treatment is shown to influence changes in mRNAs that are associated with bone degradation. Guo et al. (57) has shown resveratrol treatment diminishes osteoporosis by suppressing miR 338 3p in ovariectomized rats.

Despite these observations, it is apropos to address a major criticism underlying all of resveratrol research. The *in vivo* biological effects of resveratrol may be severely limited by low bioavailability. Although resveratrol is well absorbed and can readily enter the cell by diffusion, resveratrol is not maintained in high levels within the blood stream (4). Following absorption, under 4% of non-degraded resveratrol remains in the circulation while the majority of resveratrol is degraded into two main metabolites: resveratrol-3-sulfate and resveratrol-3-glucuronide (5, 105). The possible biological effects of these resveratrol metabolites has not been well described. Potentially, the resveratrol metabolites themselves could have biologic effects, especially after chronic administration of resveratrol. However, the field is dominated by a plethora of conflicting *in vitro* and *in vivo* studies with disparate results. This may in part be explained that *in vitro* studies are able to provide resveratrol doses not normally found during normal physiological conditions, in part due to the low resveratrol bioavailability. It is likely the interplay between biologically active resveratrol and possible effects of the metabolites will be the focus of future research.

Criticisms aside, resveratrol is fascinating to a renal physiologist for two reasons: surprisingly the acute and long term effects of resveratrol on renal function in a normal human or rat have not been characterized. Subsequently, resveratrol is known to stimulate pathways which may influence changes in basic renal physiology. It is unknown if resveratrol can modify changes in plasma renin activity (PRA), *in vivo* alterations in renal nitric oxide, free radical formation, renal hemodynamics, or sodium homeostasis. These are of particular interest because changes in these renal systems may influence changes in overall renal function, sodium balance and blood pressure.

Resveratrol as a biological ‘effector’ is particularly interesting due to numerous

reports that it increases nitric oxide (101, 185). Nitric oxide is produced by three isoforms of nitric oxide synthase (NOS): the neuronal nNOS, the inducible iNOS, and the endothelial (eNOS). Under physiological conditions, vascular nitric oxide is mainly produced by eNOS. This enzyme is constitutively expressed in the endothelium, is tonically active, and is activated by shear stress and by additional agonists (25). Nitric oxide derived from all 3 NOS isoforms contributes to the overall regulation of kidney function. Nitric oxide inhibition, in normal rats, has been shown to result in acute hypertension and renal vasoconstriction (14).

Studies have demonstrated that treatment of cultured human endothelial cells with resveratrol enhances the mRNA and protein expression of eNOS (177-178). An extensive review by Li and Förstermann (101, 185) summarized pathways by which resveratrol increased nitric oxide, including stimulation of eNOS enzymatic activity and reducing oxidative stress. The potential effects of nitric oxide on blood pressure via action in the kidney are multifactorial. Nitric oxide increases renal blood flow, enhances glomerular filtration rate, regulates renin secretion, and inhibits sodium transport in the nephron (46, 106, 145, 156). It is unknown whether resveratrol, acting through the actions of increased nitric oxide, may influences changes in renal hemodynamics, sodium homeostasis, and ultimately blood pressure.

Under normal physiological conditions, the kidneys (on average) receive 20-22% of total cardiac output (83). This represents a large amount of blood when considering that total kidney weight is relatively small when compared to total body weight. Thus, relatively small changes in renal hemodynamics can have profound effects on renal function. Nitric oxide inhibition in anesthetized rats significantly deceases blood flow to visceral organs (kidney, intestine, and lung), but overall has minimal effect on blood flow

to the brain, heart, or hindlimbs (153). In humans, nitric oxide synthase inhibition deceases renal blood flow, glomerular filtration rate and urinary sodium excretion (12). Agents that increase nitric oxide production may reduce renal vasoconstriction in pathological states associated with reduced renal perfusion (171). In particular, the renal vascular NO plays a role in balancing the effects of the endogenous vasoconstrictor Angiotensin II (153, 156). These data suggest that the kidney and renal hemodynamics are particularly sensitive to changes in nitric oxide. Moreover, changes in nitric oxide can impact renal function including transport of electrolytes and plasma renin activity.

Currently, there are no reports on the effect of resveratrol on plasma renin activity. The renin-angiotensin system is an important regulator of sodium homeostasis, extracellular fluid volume, systemic vascular resistance, and arterial blood pressure. Renin is produced within and secreted from juxtaglomerular cells (JG cells) of the afferent arteriole (41, 147). Control of renin secretion, the rate-limiting step in the formation of angiotensin II, is therefore essential for blood pressure regulation. Renin secretion is regulated by the second messenger cAMP (16). However, in contrast to most secretory cells, renin secretion is inversely related to extracellular and intracellular calcium concentrations (16). The inverse relation of calcium to renin is referred to as the “calcium paradox.” Three classic factors promote the release of renin from JG cells: activation of renal baroreceptor, the macula densa sensing decreased sodium chloride delivery, and increased sympathetic nerve activity (64). Resveratrol is a selective inhibitor of cyclooxygenase-1 (COX-1), but not COX-2 (78). The primary pathway for the stimulation of renin via to the macula densa pathway is due to an upregulation of COX-2 (63). PGE_2 is known to stimulate renin secretion (80). While resveratrol does

not inhibit COX-2, resveratrol has been shown to inhibit PGE_2 production (32, 36). It is unclear if resveratrol can modify plasma renin activity by influencing changes in renal hemodynamics, distal sodium delivery, or prostaglandins. Further it unclear whether resveratrol treatment, acting through changes in PRA and/or increasing vasodilation, is responsible for its reported effects to lower blood pressure in animal models.

In multiple models of hypertension in the rat resveratrol has been shown to reduce blood pressure. Bhatt et al (19) gave resveratrol (50 mg/L in drinking water) to SHR and reported that SHR given resveratrol had lower systolic blood pressure than non-treated SHR. Dolinsky et al (42) implanted mice with osmotic minipumps to deliver Ang II and placed mice on a diet containing resveratrol. After two weeks of resveratrol treatment, the Ang II plus resveratrol group had lower blood pressure when compared to the Ang II infused alone. While these studies and other similar reports show a blood pressure lowering effect of resveratrol (presumably due to enhanced nitric oxide synthesis or decreased oxidative stress), there are no renal investigators studying the influence of resveratrol on plasma renin activity, renal nitric oxide, sodium homeostasis, or renal hemodynamics.

My overall hypothesis is that the antihypertensive effects of resveratrol are mediated through increasing vasodilation, increasing NO synthesis, reducing oxidative stress and sodium retention, all of which would diminish hypertension. We intend to systematically investigate the acute and sustained effects of resveratrol on renal physiology in the normal rat and the chronic effects in two models of hypertension. Extrapolating from the existing literature, we anticipate resveratrol will increase renal blood flow and decrease sodium retention due to a nitric oxide-mediated mechanism, and gradually reverse increases in blood pressure.

A frequently asked question relates to how much resveratrol is found in red wine and how does this compare to doses employed in experimental research? The amount of resveratrol found in red wine varies greatly 0.1 to 14.3 mg/liter (9). The common wine bottle holds 750 ml and using high-end estimates would contain as much as 10.7 mg of resveratrol. Over-the-counter resveratrol supplements range in doses of 100 to 1200 mg. Walle et al (176) found that human subjects who consumed 25 mg of 14C-resveratrol (a supplement approximately equal to 2.5 bottles of red wine) showed plasma levels of resveratrol peak at 491± 90 ng/ml within the 1st hour (176). While a debate can be had on experimental resveratrol doses versus normal dietary consumption of resveratrol, in our studies we are solely concerned with resveratrol-induced changes in renal function. Thus, we choose a pharmacological approach, using higher levels of resveratrol than found in red wine to exaggerate the possible effects. This was intended to observe potential mechanisms that may be masked at lower resveratrol levels. Further, we choose to use resveratrol alone to separate out any confounding effects of alcohol.

To investigate the effects of resveratrol on renal physiology we will study: 1) the acute effects of resveratrol on renal hemodynamics by administering a single bolus resveratrol into systemic circulation in anesthetized rats, 2) the sustained effects of resveratrol on renal hemodynamics and renal function during continued intravenous resveratrol infusion, 3) effects of chronically ingested resveratrol on blood pressure and sodium retention in a model of mild Ang II-induced increases in systemic blood pressure, and finally 4) effects of chronically ingested resveratrol on blood pressure and sodium retention in a salt-sensitive fructose-induced model of hypertension.

Each subsequent chapter in this dissertation is written in the format of a stand-

alone manuscript. To the reader, please be advised there may be some recurrence of themes, particularly in the introductions.

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CHAPTER 1

RESVERATROL INDUCES ACUTE ENDOTHELIUM-DEPENDENT RENAL VASODILATION MEDIATED THROUGH NITRIC OXIDE AND REACTIVE OXYGEN SPECIES SCAVENGING

(This Chapter contains previously published material. See Appendix C)

Abstract

Resveratrol is suggested to have beneficial cardiovascular and renoprotective effects. Resveratrol increases endothelial nitric oxide synthase (eNOS) expression and nitric oxide (NO) synthesis. We hypothesized resveratrol acts as an acute renal vasodilator, mediated through increased NO production and scavenging of reactive oxygen species (ROS). In anesthetized rats, we found a bolus injection of 5.0 mg/kg body weight (bw) of resveratrol increased renal blood flow (RBF) by 8% [from 6.98±0.42 to 7.54±0.17 ml·min^{-1}·gram of kidney weight^{-1} (gkw); n = 8; P < 0.002] and decreased renal vascular resistance (RVR) by 18% from 15.00±1.65 to 12.32 ± 1.20 arbitrary resistance units (ARU; P < 0.002). To test the participation of NO, we administered 5.0 mg/kg bw resveratrol before and after 10 mg/kg bw of the NOS inhibitor *N*-nitro-l-arginine methyl ester (L-NAME). L-NAME reduced the increase in RBF to resveratrol by 54% (from 0.59±0.05 to 0.27±0.06 ml·min^{-1}·gkw^{-1}; n = 10; P < 0.001). To test the participation of ROS, we gave 5.0 mg/kg bw resveratrol before and after 1 mg/kg bw tempol, a superoxide dismutase mimetic. Resveratrol increased RBF 7.6% (from 5.91±0.32 to 6.36±0.12 ml·min^{-1}·gkw^{-1}; n = 7; P < 0.001) and decreased RVR 19% (from 18.83± 1.37 to 15.27±1.37 ARU). Tempol blocked resveratrol-induced increase in RBF (from 0.45±0.12 to 0.10±0.05 ml·min^{-1}·gkw^{-1}; n = 7; P < 0.03) and the decrease in RVR posttempol was 44% of the control response (3.56±0.34 vs. 1.57±0.21 ARU; n = 7; P < 0.006). We also tested the role of endothelium-derived prostanoids. Two days of

10 mg/kg bw indomethacin pretreatment did not alter basal blood pressure or RBF. Resveratrol-induced vasodilation remained unaffected. We conclude intravenous resveratrol acts as an acute renal vasodilator, partially mediated by increased NO production/NO bioavailability and superoxide scavenging but not by inducing vasodilatory cyclooxygenase products.

Introduction

In addition to maintaining water balance and electrolyte levels, the kidney must regulate renal vascular resistance (RVR) to maintain renal blood flow (RBF) and glomerular filtration rate. RBF is maintained by vessels constantly adjusting diameter and tone in response a variety of mechanisms including local shear stress, sympathetic, endocrine, and paracrine factors (97). Nitric oxide (NO), a vasodilator, is tonically produced in the vascular endothelium by the enzyme endothelial nitric oxide synthase (eNOS) and synthesized from the amino acid substrate l-arginine (130). In normotensive rats, NO inhibition results in acute hypertension and renal vasoconstriction (10, 14). The vascular endothelium maintains the balance between vasodilatation and vasoconstriction. In hypertension, renal failure, and diabetes mellitus, diseases characterized by endothelial dysfunction, pharmacological agents that increase nitric oxide production or reduce vasoconstriction may improve renal perfusion (171).

Resveratrol (3,5,4′-trihydroxystilbene), a naturally occurring polyphenol found in red wine and other dietary vegetation, is proposed to have cardioprotective effects as well as anti-inflammatory, antioxidant, and anticancer properties (9). Resveratrol increases eNOS expression and NO production (177,178). Resveratrol has been shown to increase vascular relaxation in endothelial-intact aortic strips and *N*-nitro-l-

arginine methyl ester hydrochloride (L-NAME), a NOS inhibitor, was able to block this effect (31). Bhatt et al. (19) reported resveratrol partially reversed the endothelial dysfunction found in spontaneously hypertensive rats (SHRs). While SHRs had significantly lower NO levels compared with control Wistar-Kyoto rats, resveratrol treatment increased NO content and increased eNOS protein expression. They also found vascular relaxation in phenylephrine-preconstricted mesenteric arterial rings was significantly improved in SHR by resveratrol.

In addition to stimulating NO, resveratrol is itself a reported antioxidant and scavenger of reactive oxygen species (ROS) (142, 99). Excessive production of ROS contributes to the development of hypertension, and the antihypertensive effect of (chronic) resveratrol may be in part through inhibition of ROS-producing NADPH and xanthine oxidase and through direct actions as a free radical-scavenging antioxidant (26, 88, 141). Resveratrol has also been shown to activate NAD(+)-dependent protein deacetylase SIRT1 (70), which stimulates the mitochondrial free radical scavenging enzyme superoxide dismutase (SOD2). Further, it has been shown that ROS are renal vasoconstrictors (85, 96) and their production in renal afferent arterioles is induced by increased luminal pressure (137). Tempol significantly attenuated this pressure-induced renal vasoconstriction (137). Lai et al. (96) have also shown that superoxide constricts the renal afferent arteriole.

Combined, these data suggest resveratrol may vasodilate by increasing the synthesis of NO and decreasing the vasoconstrictor ROS. Although cardiac hemodynamic effects of acute and chronic resveratrol have been studied, the effects of resveratrol on renal hemodynamics still remain unclear (160). The only published report of the effects of resveratrol on the renal artery are from Gojkovic-Bukarica et al (49),

which investigated the mechanism of resveratrol-induced vasorelaxation of the renal artery in normal and diabetic rats using an *in vitro* mounted isometric tension preparation.

Vasodilator arachidonic acid products such as prostaglandin E_2 (PGE_2) and PGI_2 are renal vasodilators (24, 77, 81). Resveratrol has a complex relationship with arachidonic acid metabolism. The anti-inflammatory actions of resveratrol are suggested to be due to its chronic suppression of cyclooxygenase (COX) expression, induction, activity, and prostanoid production (110). However, it has also been shown to selectively inhibit the COX-1 isoform but not COX-2 (163). Similarly, prostaglandin H synthase (PGHS), a primary enzyme in the biosynthesis of prostaglandins (65), is differentially isoenzyme-specific for resveratrol. The constitutive PGHS-1 is inhibited by resveratrol while PGHS-2 is activated by it (81). *In vitro* relaxation of porcine coronary arteries has been characterized as partially NO-dependent but unaffected by COX inhibition using indomethacin (102). In contrast, resveratrol has been shown to enhance the antiaggregatory actions of PGE_2 and PGI_2 (184). Thus it is not really clear if acute resveratrol-mediated renal vasodilation is in part a function of renal vascular prostanoid production. One might build a case for or against such a role, but no data exist in the literature for the renal response.

We hypothesized resveratrol would induce acute renal vasodilation and decrease RVR, mediated through an increase in endothelium-derived NO and a reduction of vasoconstrictive ROS including superoxide. We also tested the possibility that resveratrol may act through stimulation of vasodilatory prostanoids.

Materials and Methods

Male Sprague-Dawley rats (Charles River, Wilmington MA) of 225–250 g body wt

(bw) were fasted overnight but allowed free access to drinking water. On the day of the experiment, rats were anesthetized via intraperitoneal injection with thiobutabarbital (125 mg/kg bw; Inactin, Sigma-Aldrich, St. Louis, MO). Rats were placed on a heated surgical table to maintain constant body temperature (BrainTree Scientific, Braintree, MA). A tracheotomy was performed using PE-240 tubing to allow free breathing of room air. A femoral cut down was performed to cannulate the femoral artery and vein with PE-50 catheters (Becton Dickinson, Franklin Lakes, NJ). The arterial catheter was connected to an iWorx BP-102 probe with LabScribe2 software (iWorx, Dover, NH) for simultaneous recording of mean arterial pressure (MAP) and heart rate (HR). Pressure transducers were calibrated using a digital, mercury-free Traceable manometer (Fisher Scientific, Pittsburg, PA). The femoral venous catheter was used for a 1-ml supplement of 6% bovine serum albumin (Sigma-Aldrich), for a constant infusion physiologic saline at a rate of 38 µl/min using a Genie Plus micro-pump (Kent Scientific, Torrington, CT), and for bolus resveratrol administration. A mid-ventral abdominal incision was performed, and the intestines were wrapped in moist gauze and moved to the side of the peritoneal cavity to expose the left kidney. The renal artery and vein were carefully isolated and the renal artery was fitted with a Doppler flow probe (Transonic, Ithaca, NY) connected to a transit-time perivascular flow meter TS-420 model (Transonic) to record RBF via the iWorx system. The rat was draped and allowed to stabilize for 30 min before the running of the experimental protocols.

All protocols and surgical procedures employed in this study were reviewed and approved by Wayne State University and Henry Ford Health System Institutional Animal Care and Use Committee and were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* endorsed by the American Physiological Society in

accordance with National Institutes of Health guidelines.

Protocol 1: measurement of renal hemodynamics in response to resveratrol

We hypothesized resveratrol would act as acute renal vasodilator. To test this, we first ran a resveratrol dose response. Resveratrol (or vehicle) was given as an acute bolus injection (300 μl over 30 s to minimize infusion artifacts) intravenously via the femoral vein catheter. RBF, MAP, and HR were recorded. RVR was calculated by dividing MAP by RBF in units of $mmHg \cdot ml \cdot min^{-1} \cdot gram\ of\ kidney\ weight^{-1}$ (gkw) hereafter referred to as arbitrary resistance units (ARU). Any small infusion artifact found with the vehicle was subtracted from all paired responses to resveratrol in each experiment. The resveratrol (Sigma-Aldrich) doses were prepared daily. Fifteen milligrams of resveratrol (Sigma-Aldrich) were dissolved in DMSO and diluted with 0.9% saline to 300 μl. Resveratrol is reported to be photosensitive (38). The resveratrol was stored in light-protected Eppendorf tubes wrapped in aluminum foil and kept at 37°C until experimental use. Resveratrol doses of 0 mg/kg (vehicle control), 0.5, 2.0, and 5.0 mg/kg bw were each administered over 30 s. Following each bolus, a recovery period of 15 min was provided. At the conclusion of the protocol, animals were terminated by barbiturate overdose and aortic transection. The left kidney was removed, decapsulated, and weighed to allow for normalization of RBF per gram of kidney weight (n = 8).

Protocol 2: resveratrol and NO-mediated renal vasodilation

To investigate the role of endothelium-derived NO in mediating resveratrol-induced renal vasodilation, we used NOS inhibition via N_{ω}-nitro-l-arginine methyl ester hydrochloride (L-NAME; Sigma-Aldrich). We used a 5.0 mg/kg bw vasodilator dose of resveratrol, as determined in *Protocol 1*. The same surgical procedures were

performed as above. Only the vehicle and 5.0 mg/kg resveratrol dose was administered before and 10 min after 10 mg/kg bw L-NAME treatment. We have previously shown that L-NAME at this dose produces complete and sustained inhibition of systemic and renal endothelium-dependent vasodilation (153). L-NAME was administered intravenously, via the femoral vein. RBF, MAP, and HR were recorded. (*n* = 10).

Protocol 3: resveratrol renal vasodilation with AT_1 receptor blockade

To serve as a control for *Protocol 4*, we administered 5.0 mg/kg bw resveratrol before and after delivering 10 mg/kg bw losartan (Cayman Chemicals, Ann Arbor, MI) since two pharmacological treatments were used in *Protocol 4* (losartan and L-NAME). We measured RBF, MAP, HR, and RVR in response to resveratrol before and after treatment with losartan (*n* = 6).

Protocol 4: resveratrol and NO-mediated renal vasodilation after eliminating the L-NAME-induced changes in baseline hemodynamics

L-NAME given to an anesthetized rat produces significant renal vasoconstriction and acute hypertension due to the unbridled effect of elevated angiotensin II in the absence of NO (10). To minimize these hemodynamic effects of L-NAME in our anesthetized preparation but still evaluate the role of NO, we administered the angiotensin AT_1 receptor blocker losartan (Cayman Chemicals) after our control period but before L-NAME was given. This minimized the overall renal hemodynamic and pressor effects of L-NAME treatment to change the baseline. The same surgical procedures were performed as in *Protocol 2* with the exception of treatment with 10 mg/kg bw losartan given 5 min before L-NAME administration. We measured RBF, MAP, HR, and RVR in response to resveratrol before and after treatment with losartan and L-NAME. (*n* = 11).

Protocol 5: resveratrol and scavenging of superoxide anion

We hypothesized resveratrol-induced renal vasodilation may be mediated in part through NO scavenging of superoxide, reducing it's inherit vasoconstriction. The ROS superoxide anion reacts quickly with NO in the vasculature, producing peroxynitrite and depleting NO. To investigate the role of superoxide scavenging properties of resveratrol, we administered 5.0 mg/kg bw resveratrol before and after delivering 1.0 mg/kg bw tempol (Sigma-Aldrich), a superoxide dismutase mimetic. Thus if tempol scavenged superoxide before the resveratrol bolus, resveratrol-induced renal vasodilation should be diminished. (n = 7).

Protocol 6: resveratrol and vasodilatory prostanoids

To investigate whether resveratrol-induced vasodilation was mediated in part via vasodilator prostaglandins, rats were pretreated with the nonselective COX inhibitor indomethacin (Sigma-Aldrich); 4 mg/kg bw the day before the experiment, 4 mg/kg bw again on the day of the experiment, and 2 mg/kg bw just before the surgery as previously described (62). Indomethacin was dissolved in DMSO (Sigma-Aldrich). Surgical procedures were performed as above using only vehicle and 5.0 mg/kg resveratrol before and 10 min after 10 mg/kg L-NAME treatment in indomethacin-pretreated animals. RBF, MAP, and HR were recorded. RVR was calculated. (n = 10).

Analysis

For dose responses (*Protocol 1*), the changes in RBF minus any vehicle infusion artifact and the changes in RVR were analyzed using ANOVA for repeated measures using a Bonferroni adjustment of the P value as a post hoc test for multiple comparisons. All statistically significant responses are listed only as an adjusted $P < 0.05$ for Figure 2. For comparisons of the resveratrol response between untreated

controls and after treatment (*Protocols 2–6*), we used a paired Student's *t*-test with an α acceptance at 0.05, and *n* values were chosen to provide a power of at least 0.8.

Results

Protocol 1: measurement of renal hemodynamics in response to resveratrol

A significant increase in RBF was observed with 5.0 mg/kg resveratrol (Figure 2). At this dose RBF increased 8% from 6.98±0.42 to 7.54±0.17 ml·min^{-1}·gkw^{-1} (n = 8; P<0.05) and RVR decreased by 18% from 15.00 ± 1.65 to 12.32±1.20 ARU (P<0.05). Neither MAP nor HR was changed in response to resveratrol (Figure 3). The increase in RBF and decrease in RVR in response to resveratrol were transient responses, and resveratrol-induced changes in RBF and RVR returned to preinjection values. Thus we chose a bolus dose of 5.0 mg/kg bw for all subsequent protocols to investigate the mechanism of vasodilation.

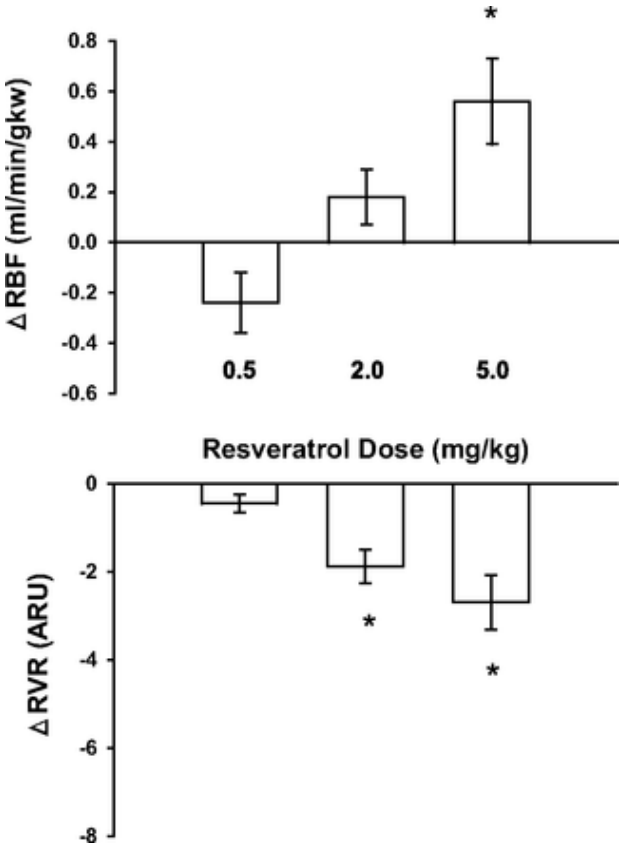


Figure 2: Effect of a dose response by resveratrol on renal blood flow (RBF) and renal vascular resistance (RVR); 5.0 mg/kg resveratrol increased RBF by 8% and decreased RVR by 18%. ARU, arbitrary resistance units; gkw, gram of kidney weight. *P<0.05, statistically significant responses.

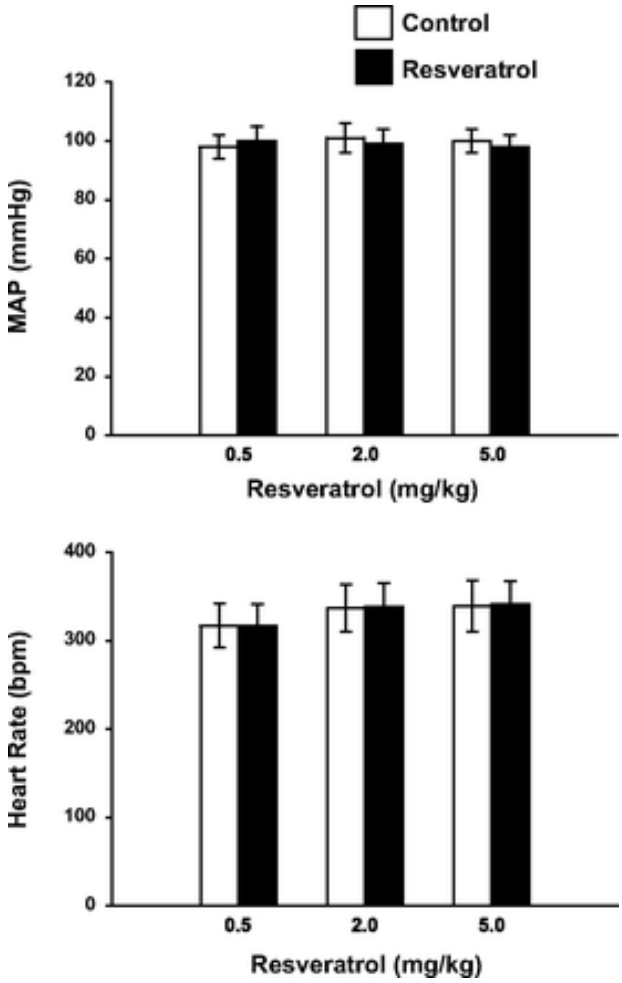


Figure 3: Effect of acute resveratrol on mean arterial pressure (MAP) and heart rate (HR). Acute resveratrol had no effect on MAP or HR; bpm, beats/min.

Protocol 2: resveratrol and NO-mediated renal vasodilation.

As in *Protocol 1*, 5.0 mg/kg resveratrol increased RBF 8% from 7.09±0.44 to 7.68±0.46 ml·min^{-1}·gkw^{-1} ($P < 0.001$). Likewise, RVR decreased 17% from 14.65±0.92 to 12.14±0.82 ARU ($P < 0.001$). MAP remained unchanged at 101±3 to 98±2 mmHg, and HR was unaffected [327±16 beats/min (bpm)]. Following administration of L-NAME, basal RBF decreased from 7.09±0.44 to 4.36±0.32 ml·min^{-1}·gkw^{-1} ($P < 0.0001$), RVR increased from 14.65±0.92 to 33.41±2.63 ARU ($P < 0.0001$), MAP increased from 101±3 to 139±6 mmHg ($P < 0.0001$), and HR decreased from 327±16 to 274±11 bpm ($P < 0.003$). L-NAME significantly diminished but did not eliminate resveratrol-induced renal vasodilation. Resveratrol-induced vasodilation increased RBF from 4.36±0.32 to

4.63±.32 ml·min^{-1}·gkw^{-1} (P < 0.001), an absolute change of 0.27±0.06 ml·min^{-1}·gkw^{-1}, which was only half the response seen before L-NAME (Figure 4). L-NAME-treated rats had a significant decrease in RVR with resveratrol from 33.41±2.63 to 26.72±2.27 ARU. After L-NAME administration, resveratrol did not affect HR, but it significantly decreased MAP from 139±6 to 131±6 mmHg (P < 0.003). Overall, we found L-NAME reduced resveratrol-induced vasodilation by 54% suggesting a significant component of resveratrol-induced vasodilation is mediated in part by NO.

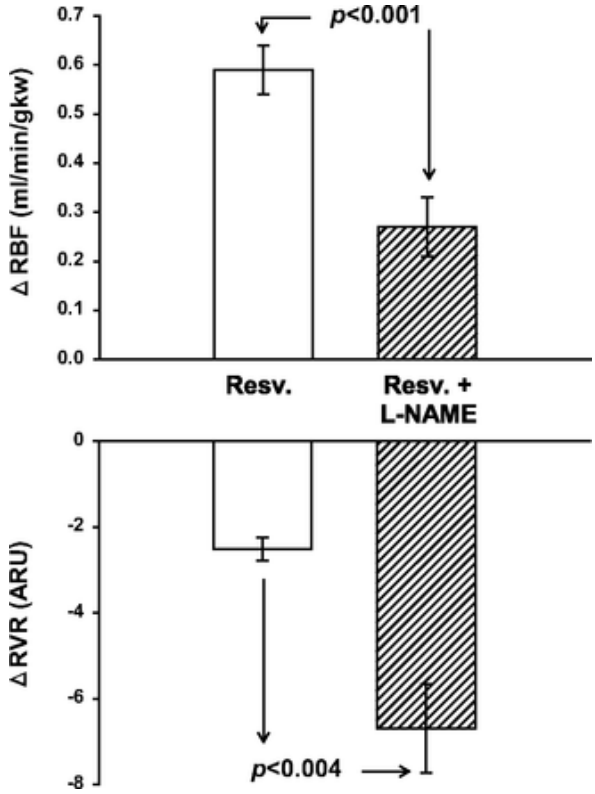


Figure 4: Effect of nitric oxide synthase (NOS) inhibition with *N*-nitro-l-arginine methyl ester (L-NAME) on RBF and RVR on resveratrol-induced vasodilation. L-NAME reduced the increase in RBF in response to resveratrol by 54% (P < 0.001) and significantly decreased the change in RVR by 20% (P < 0.004).

Protocol 3: resveratrol renal vasodilation with AT_1 receptor blockade.

We expected resveratrol-induced renal vasodilation would decrease due to the vasodilatory actions of losartan. 5.0 mg/kg resveratrol increased RBF 7.5% from 7.12±0.29 to 7.66±0.42 ml·min^{-1}·gkw^{-1} (n = 6, P < 0.01) and decreased RVR 21% from 15.46±85 to 12.24±0.84 ARU (P < 0.001; Figure 5). Following administration of 10

mg/kg bw losartan, baseline RBF increased 16% from 7.12±0.29 to 8.27 ± 0.32 $ml \cdot min^{-1} \cdot gkw^{-1}$ ($P < 0.0001$) and RVR decreased 24% from 15.46±0.85 to 11.80±0.74 ARU ($P < 0.0001$), MAP decreased 12% from 109±4 to 97±4 mmHg ($P < 0.0001$), and HR was unchanged from 312±19 to 309±23 bpm. Even after administration of losartan, 5.0 mg/kg resveratrol still significantly increased RBF 4.5% from 8.27±0.32 to 8.65±0.44 $ml \cdot min^{-1} \cdot gkw^{-1}$ ($P < 0.049$) and RVR decreased 14% from 11.80±0.74 to 10.10±0.90 ARU ($P < 0.003$).

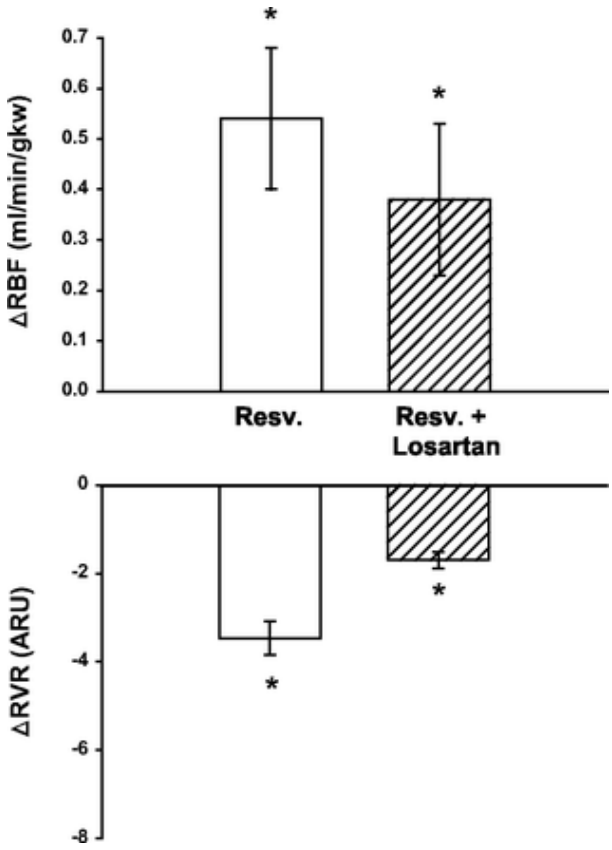


Figure 5: Effect of AT_1 receptor inhibition with losartan on resveratrol-induced renal vasodilation. With losartan treatment, resveratrol-induced vasodilation still increased RBF 4.5%.

Protocol 4: resveratrol and NO-mediated renal vasodilation after eliminating the L-NAME-induced changes in baseline hemodynamics

Under control conditions, resveratrol increased RBF (Figure 6) from 6.58±0.36 to 6.91±0.39 $ml \cdot min^{-1} \cdot gkw^{-1}$ ($P < 0.0001$) and RVR decreased from 18.27±1.44 to 14.47±1.10 ARU ($P < 0.0001$). Losartan diminished the renal hemodynamic changes

induced by L-NAME treatment (seen in *Protocol 2*) by preventing the significant decrease in RBF. Basal RBF was 6.58±0.36, and after both losartan and L-NAME, the baseline was 6.16±0.53 $ml \cdot min^{-1} \cdot gkw^{-1}$. MAP was unchanged 123±4 mmHg, but HR fell significantly from 304±12 to 263±14 bpm ($P < 0.001$). Similar to the results of *Protocol 2* with L-NAME alone, NO inhibition in the presence of losartan significantly reduced the resveratrol-induced renal vasodilation from an increase in RBF of 0.33±0.05 to just 0.21±0.04 $ml \cdot min^{-1} \cdot gkw^{-1}$ ($P < 0.02$). RVR decreased from 21.29± 1.72 to 18.70±2.13 ARU ($P < 0.03$; Figure 6). MAP and HR were unchanged in response to resveratrol. Overall, angiotensin receptor blockade prevented the hemodynamic and pressor effects associated with NOS inhibition. Resveratrol-induced renal vasodilation was significantly reduced by NOS inhibition, similar to the results of *Protocol 2*.

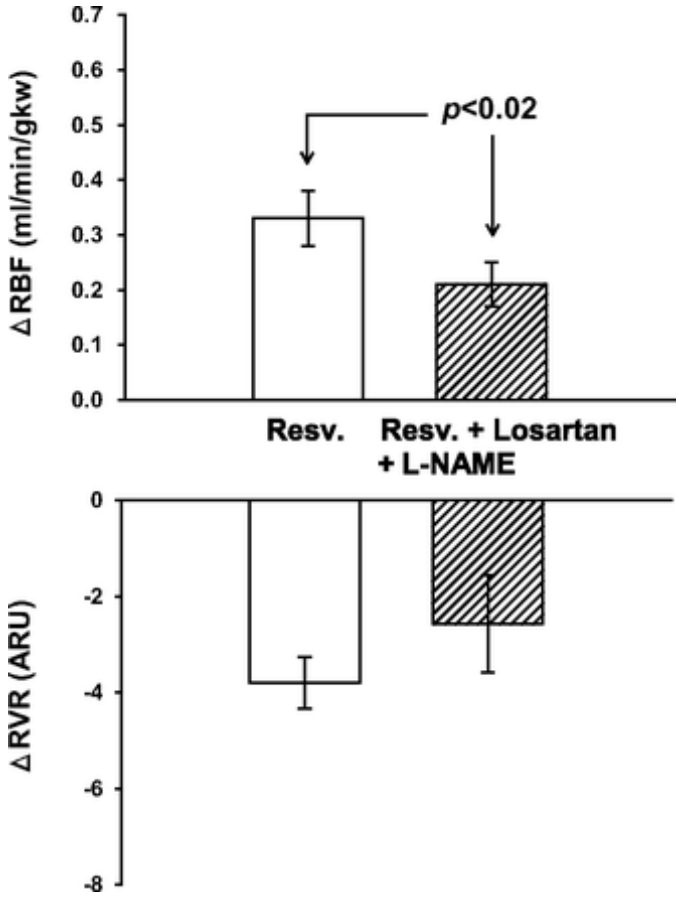


Figure 6: Effect of NOS inhibition with L-NAME after AT_1 receptor inhibition with losartan on resveratrol-induced renal vasodilation and RVR. Losartan and L-NAME reduced the resveratrol-induced increase in RBF by 36%.

Protocol 5: resveratrol and scavenging of superoxide anion

Resveratrol increased RBF 8% (from 5.91±0.32 to 6.36±0.12 ml·min^{-1}·gkw^{-1}; n = 7; P < 0.001) and decreased RVR 19% (from 18.83±1.37 to 15.27±1.37 ARU; Figure 7) under control conditions. Tempol alone increased RBF 9% (from 5.91±0.32 to 6.43±0.28 ml·min^{-1}·gkw^{-1}; P < 0.005), decreased RVR 20% (18.83±1.37 to 15.01±0.77 ARU P < 0.006), decreased MAP 14 mmHg (from 109±2 to 95±3 n = 7 P < 0.001), and decreased HR 15% (327±15 to 277±15 bpm; P < 0.003). Tempol treatment completely blocked the resveratrol-induced increase in RBF (a decrease of 78% in vasodilation from 0.45±0.12 to 0.10±0.05 ml·min^{-1}·gkw^{-1}; P < 0.03), and the decrease in RVR in response to resveratrol was only 44% that of the control response (3.56±0.34 vs. 1.57±0.21 ARU; P < 0.006). Thus by scavenging superoxide, the resveratrol-induced vasodilation was completely eliminated.

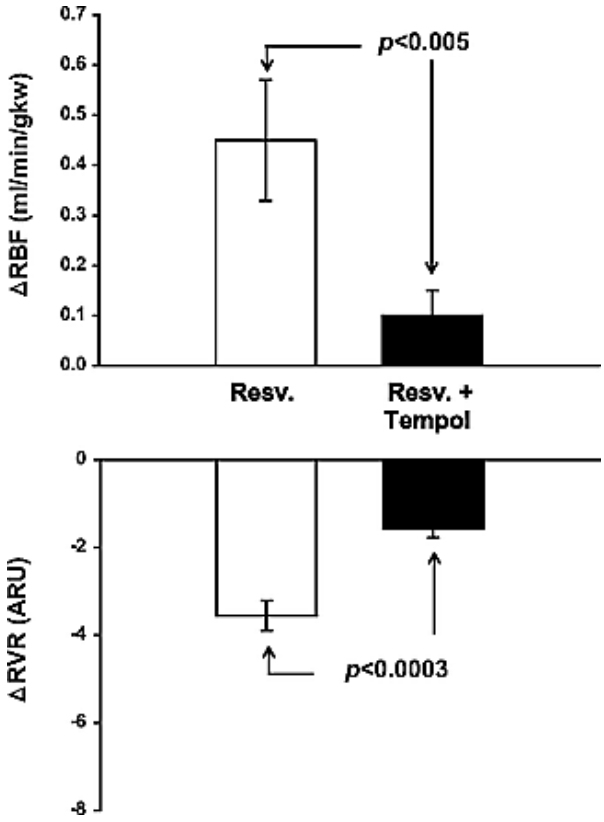


Figure 7: Effect of superoxide scavenging with tempol on resveratrol-induced renal vasodilation and RVR. Tempol eliminated resveratrol-induced vasodilation.

Protocol 6: resveratrol and vasodilatory prostanoids

In indomethacin-treated rats, a bolus of 5.0 mg/kg resveratrol resulted in a significant increase in RBF (Figure 8) from 6.77±0.18 to 7.17 ± 0.23 ml·min^{-1}·gkw^{-1} (P < 0.0004), similar to the change seen as in *Protocol 2*. Resveratrol also decreased RVR from 15.08±0.50 to 12.53±0.49 ARU (P < 0.0001). In indomethacin-treated rats, L-NAME increased MAP from 100±3 to 138±5 mmHg (P < 0.0001), decreased HR from 331±10 to 265±11 bpm (P < 0.001), and increased RVR from 15.08±0.50 to 31.77±3.81 ARU (P < 0.002). As in *Protocol 2*, L-NAME significantly blunted renal vasodilation seen in response to resveratrol by 44%, from 0.50±0.09 to 0.28±0.06 ml·min^{-1}·gkw^{-1} (n = 10; P < 0.044). Resveratrol also decreased RVR from 31.77±3.81 to 25.62±2.51 ARU (P < 0.007; Figure 8). In indomethacin-treated rats, resveratrol did not affect heart rate but significantly decreased MAP from 100±3 to 95±3 mmHg (P < 0.001) and after L-NAME decreased MAP from 138±5 to 131±6 mmHg (P < 0.003). Overall, COX inhibition did not change the renal hemodynamic response to resveratrol or its attenuation by L-NAME.

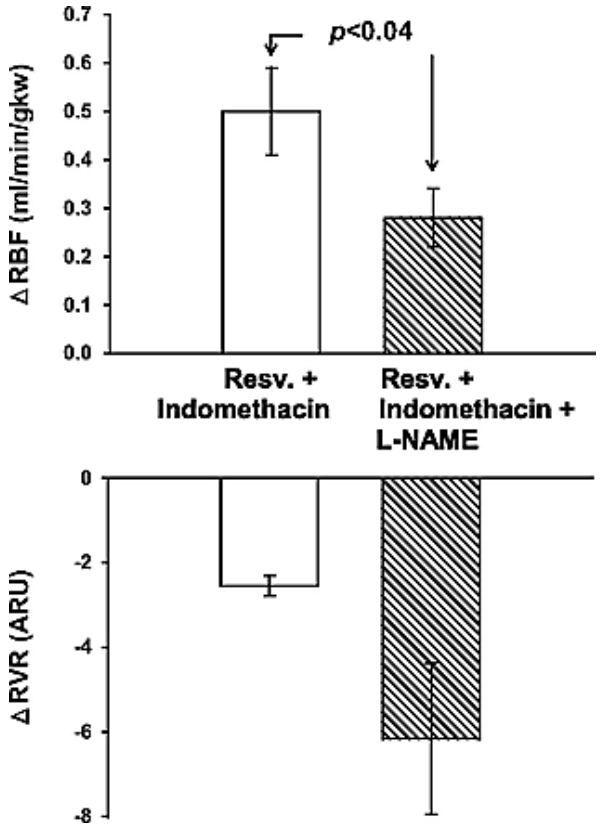


Figure 8: Effect of cyclooxygenase inhibition with indomethacin and NOS inhibition on resveratrol-induced renal vasodilation and RVR. Similar to controls, indomethacin did not change resveratrol-induced vasodilation. With indomethacin treatment, L-NAME reduced resveratrol-induced vasodilation by 44% (P < 0.04).

Discussion

Very little is known about the renal hemodynamic effects of resveratrol. In this study we present *in vivo* data to support our hypothesis that resveratrol is a renal vasodilator. In particular the mechanisms by which resveratrol functions as a renal vasodilator include *1*) increased NO production and/or NO availability, and *2*) subsequent reduction of vasoconstrictor ROS, presumably superoxide. Resveratrol treatment significantly increased RBF and with a concomitant decrease in RVR. Our findings are consistent with the literature that resveratrol acutely increases NO synthesis. Wallerath et al. (177, 179) demonstrated resveratrol increases eNOS expression and NO production in human umbilical vein endothelial cells. Here we demonstrate resveratrol is capable of inducing renal vasodilation in normal rats by an endothelial dependent, NO-mediated pathway. However, only about half of the resveratrol-induced renal vasodilation was blocked by competitive NOS inhibition. This is similar to the *in vitro* results of Li et al. (102) using isolated porcine coronary arteries. They found that either NOS inhibition or de-endothelization of the preparation only partially reversed the resveratrol-induced vascular relaxation.

One concern of blocking NOS using L-NAME in an *in vivo* protocol is the significant shift in the hemodynamic baselines. L-NAME given to an anesthetized rat produces significant renal vasoconstriction and acute hypertension due to the unbridled effect of elevated angiotensin II in the absence of NO (10). To serve as a control, in *Protocol 4*, we solely treated with losartan because in *Protocol 5* we treated with both losartan and L-NAME to minimize hemodynamic changes. In *Protocol 4*, we found both in the control and with losartan treatment, resveratrol elicited significant vasodilation, albeit, the vasodilation magnitude was decreased with losartan treatment, as would be

expected. In *Protocol 4*, we controlled for the hemodynamic and pressor effects of L-NAME. After the control period, we administered the AT_1 antagonist losartan (47). Previous work has shown losartan was able to diminish the decreased RBF due to L-NAME (155). Under these conditions, we replicated our results of diminished resveratrol-induced renal vasodilation after NOS inhibition with the inclusion of losartan eliminating gross changes in the hemodynamic baseline induced by L-NAME. Collectively, these data also support the results in *Protocol 2* that resveratrol-induced renal vasodilation is at least partially NO dependent, as evident during NOS inhibition.

Nitric oxide and superoxide interact and scavenge each other in a balance that controls endothelial function (86). With approximately half of resveratrol-induced renal vasodilation explained by an endothelial NO-dependent mechanism, we tested whether this renal vasodilation is mediated through scavenging of ROS by reducing the vasoconstrictor effects of superoxide. Besides the ROS scavenging of NO, resveratrol has been shown to directly scavenge ROS (99, 162). Resveratrol treatment has been shown to downregulate NOX4 expression (161). Since our protocol was an acute experiment, expression is unlikely to be a significant factor. Our idea was that resveratrol may be acting as an antioxidant, either through its stimulation of NO synthesis, or independently acting as a ROS scavenger, or both. When we delivered resveratrol after administration of the superoxide dismutase mimetic tempol, renal vasodilation was completely blocked. This finding suggests resveratrol may increase RBF through both increasing acute NO release as well as directly scavenging free radicals. This could explain why tempol was more efficacious in blocking the resveratrol-induced vasodilatory response than just NOS inhibition alone. Resveratrol-induced renal vasodilation mediated by scavenging ROS is a novel finding under normal

basal conditions. In our protocol, we do not have direct measurements of NO synthesis in the renal resistance vessels. Thus we do not really know if the resveratrol induces increased NO synthesis or alternatively exerts a NOS-dependent vasodilation resulting from a mechanism involving direct resveratrol scavenging of ROS, which in turn would allow increased NO bioavailability without actually increasing NOS activity or NO production (or both of these possibilities).

This scavenging and its effect on renal hemodynamics may have therapeutic potential. Increased oxidative stress and reactive oxygen species and the subsequent endothelial dysfunction are often found in hypertension (6) and other renal diseases (45).

Following tempol treatment in our protocol, we witnessed an increase in RBF. It should be noted that the involvement of ROS in regulation of renal hemodynamics during resting basal conditions is a controversial topic. Conflicting studies debate the role ROS contributes toward resting vasomotor tone in the kidney. Some studies support ROS in having a tonic vasoconstricting effect as evident in knockout and pharmacological inhibition experiments (61, 104, 107, 163). Conversely, different studies demonstrate tempol does not alter renal hemodynamics in normotensive rats (93, 143). Confounding this issue is different treatment dosages and RBF measurements taken over different time periods make direct comparisons difficult.

One final closing thought concerning the interpretation of tempol protocol data (*Protocol 5*) should be highlighted. It is straightforward to recognize when the vasculature is in a vasodilated state, this will decrease the dilator response to vasodilatory agent. In our data tempol increased basal RBF 9% and virtually abolished resveratrol-induced vasodilation. However, in *Protocol 4* we found losartan increased

basal RBF 16%, an amount greater than with tempol, and still resveratrol was able to significantly vasodilate following losartan treatment. In both protocols, the pharmacological treatments (losartan or tempol) increased basal RBF, but there were divergent vasodilatory outcomes in response to resveratrol. Overall, this suggests the reduced vasodilation in response to resveratrol during tempol treatment is not merely due to dilated state of the vessel.

Previously, we have shown acute NOS inhibition unmasked renal vasoconstriction with COX-2 inhibition, suggesting that the influence of COX-2-derived vasodilator eicosanoids is exaggerated to maintain renal perfusion, compensating for the acute loss of NO (15). However, *in vitro* fibroblast culture studies have shown resveratrol may inhibit phospholipase A_2 activity and PGE_2 synthesis (119). In a cancer line, resveratrol has been suggested to inhibit COX-2 (188). However, another study has shown resveratrol increases COX mRNA levels (23). Since resveratrol-induced renal vasodilation was not completely eliminated by NOS inhibition, we considered the possible influence of prostanoids in resveratrol-induced renal vasodilation by pretreating rats with indomethacin. However, our results suggest no involvement of prostanoids in acute resveratrol-induced vasodilation. *In vitro* relaxation of porcine coronary arteries has been characterized as partially NO dependent but similar to our data unaffected by COX inhibition using indomethacin (102). In contrast, resveratrol has been shown to enhance the anti-aggretory actions of PGE_2 and PGI_2 (184) suggesting some acute interaction with the actions of prostaglandin. There are no existing data for an interaction between resveratrol and vasodilator prostaglandins in the renal vasculature. Overall these data generally support our findings that resveratrol-induced renal vasodilation is not influenced by vasodilator prostanoids.

The acute renal vasodilation we observed in response to resveratrol administration was a transient event. Peak vasodilation took place within 30 s following the resveratrol bolus, and blood flow gradually returned to prebolus values within a few of minutes. In human subjects treated with oral ^{14}C-resveratrol (176), absorption of resveratrol is ~70%, and it has a plasma half-life of 9.2 ± 0.6h. Boocock et al (21) found peak plasma levels of resveratrol at 539±384 ng/ml, in healthy human volunteers, 1.5 h after consuming a 5-g dose of resveratrol. Our transient increase in RBF and decrease in RVR may be explained in part due the metabolism, distribution and possibly excretion of resveratrol.

We did not observe any significant changes in MAP with acute resveratrol administration in normal rats under control conditions. However, in both L-NAME and indomethacin plus L-NAME groups, we observed resveratrol significantly decreased MAP. Other groups have reported that resveratrol may reduce blood pressure. Thandapilly et al (169) reported grape powder in SHRs significantly decreased blood pressure (200±4 vs.190±2 mmHg with treatment). In diabetic patients, resveratrol supplements for 3 months (250 mg/day) lowered systolic pressure from 139±16 to 127±15 mmHg (18). Bhatt et al (19) showed that resveratrol treatment (5 mg/kg/day) attenuated hypertension development in SHR as indicated by lower MAP in resveratrol-treated SHR compared with control SHR (161±2 vs. 180±1 mmHg, respectively). These are, however, chronic studies and do not reflect the immediate changes we see. The explanation for why the treated animals responded but the controls did not remains elusive, though the changes in MAP we report are relatively small.

Although the data we present support either increased NO production and/or NO bioavailability, there may be a possible alternative which might explain NO-mediated

resveratrol induced renal vasodilation. Park et al (132) found resveratrol inhibits phosphodiesterase (PDE) isoforms 1, 3, and 4. PDE-4 is expressed in endothelial cells, and within the kidney several PDE isoforms are present including PDE-4 (122). In a dog model, Tanahashi et al. (165) observed that intracranial arterial infusion of rolipram, a selective phosphodiesterase IV inhibitor, increased renal blood flow. Dalaklioglu and Ozbey (40) found, in isolated corpus cavernosum, resveratrol produced a concentration-dependent relaxation that was significantly attenuated by removal of the endothelium, L-NAME, or the soluble guanylyl cyclase inhibitor 1H-[1, 2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), suggesting a cGMP-mediated effect (40). As in our results, the COX inhibitor indomethacin had no effect (40). El-Mowafy (44) found that in sheep coronary arteries resveratrol increased cGMP formation threefold, and this was not abrogated by the phosphodiesterase inhibitor IBMX. Further, resveratrol activated guanylyl cyclase in the particulate, but not in the soluble membrane fraction. He proposed that resveratrol increases cGMP in coronary arteries partially by activation of particulate guanylyl cyclase (44). Thus resveratrol could act via its second messenger cGMP through a direct effect on nitric oxide synthesis, through activation of particulate guanylyl cyclase, through inhibition of phosphodiesterase(s), or through some combinations of all these pathways. It remains to be shown if acute resveratrol increases renal vasodilation through one or all of these actions.

Because our study is based on acute and transient responses to a bolus injection of resveratrol, it does not lend itself to measuring any metric for NO or O_2^- levels in a meaningful manner. Both these molecules are short-lived and highly reactive. Thus we have not attempted to quantify such changes. Lastly, another possible limitation of this study is that tempol increased RBF. The resultant vasodilation by tempol may have

masked the actions of vasodilatory actions of resveratrol, yet with losartan treatment there was vasodilation and resveratrol still induced significant vasodilation.

In summary, our findings suggest acute resveratrol treatment influences renal hemodynamics inducing a significant increase in RBF and decreased RVR. The mechanism of renal vasodilation is partially mediated by either stimulating endothelial-NO production and/or increasing NO availability and through a reduction of endogenous reactive oxygen species; either due to the increased NO synthesis or a direct ROS scavenging by resveratrol itself. Resveratrol-induced renal vasodilation is not influenced by COX metabolism and vasodilatory prostanoids.

CHAPTER 2

SUSTAINED RESVERATROL INFUSION INCREASES NATRIURESIS INDEPENDENT OF RENAL VASODILATION

(This Chapter contains previously published material. See Appendix D)

Abstract

Resveratrol is reported to exert cardio-renal protective effects in animal models of pathology, yet the mechanisms underlying these effects are poorly understood. Previously, we reported an *i.v*. bolus of resveratrol induces renal vasodilation by increasing nitric oxide bioavailability and inhibiting reactive oxygen species. Thus, we hypothesized a sustained infusion of resveratrol would also increase renal blood flow (RBF), and additionally glomerular filtration rate (GFR). We infused vehicle for 30 min followed by 30 min resveratrol at either: 0, 0.5, 1.0, 1.5 mg/min, and measured RBF, renal vascular resistance (RVR), GFR, and urinary sodium excretion. At all three doses, blood pressure and GFR remained unchanged. Control RBF was 7.69±0.84 mL/min/gkw and remained unchanged by 0.5 mg/min resveratrol (7.88±0.94 mL/min/gkw, n = 9), but urinary sodium excretion increased from 2.19±1.1 to 5.07±0.92 μmol/min/gkw (n = 7, P < 0.01). In separate experiments, 1.0 mg/min resveratrol increased RBF by 17%, from 7.16±0.29 to 8.35±0.42 mL/min/gkw (P < 0.01, n = 10), decreased RVR 16% from 13.63±0.65 to 11.36±0.75 ARU (P < 0.003) and increased sodium excretion from 1.57±0.46 to 3.10±0.80 μmol/min/gkw (n = 7, P < 0.04). At the 1.5 mg/min dose, resveratrol increased RBF 12% from 6.76±0.57 to 7.58±0.60 mL/min/gkw (n = 8, P < 0.003), decreased RVR 15% (15.58±1.35 to 13.27±1.14 ARU, P < 0.003) and increased sodium excretion (3.99±1.71 to 7.80±1.51 μmol/min/gkw, n = 8, P < 0.04). We conclude that a constant infusion of resveratrol can induce significant renal vasodilation while not altering GFR or blood pressure. Also, resveratrol infusion

produced significant natriuresis at all doses, suggesting it may have a direct effect on renal tubular sodium handling independent of renal perfusion pressure or flow.

Introduction

Resveratrol (3,5,4′-trihydroxystilbene), a polyphenol found in red wine and other foods, is often attributed to providing cardioprotective effects through nitric oxide-mediated vascular relaxation and possible antioxidant properties (9). However, little is known about its effects on the kidney. Recently, we reported an i.v. bolus of resveratrol induces renal vasodilation by increasing nitric oxide (NO) availability and inhibiting generation of reactive oxygen species (52).

NO has an important role in the control of renal function and long-term regulation of blood pressure (135). NO has multiple effects within the kidney including increasing renal blood flow (RBF) (120), increasing glomerular filtration rate (GFR) (46), and inhibiting thick ascending limb (TAL) sodium reabsorption (67). NO, a vasodilator, is tonically produced in the vascular endothelium by the enzyme endothelial nitric oxide synthase (eNOS) and synthesized from the amino acid substrate L-arginine (131). Inhibition of NOS results in acute hypertension and renal vasoconstriction (10,14). Thus, the renal vascular endothelium maintains a tonic balance between vasodilatation and vasoconstriction.

The existing literature supports an interactive involvement between resveratrol and nitric oxide. Resveratrol increases eNOS expression and when incubated with human umbilical vein endothelial cells can acutely increase NO synthesis (177, 178). NO production is well-established as an endothelial-dependent vasodilator and *N*-nitro-L-arginine methyl ester hydrochloride (L-NAME), a NOS inhibitor, reverses this effect (31). At a molecular level, nanomolar concentrations of resveratrol phosphorylates

eNOS at serine 1177, thereby increasing eNOS activity (42). Consistent with our data in the kidney (52), these data suggest resveratrol vasodilates through NO dependent mechanisms.

Relatively few studies have investigated the effects of resveratrol on renal hemodynamics and glomerular filtration, especially in normal rat. NO synthesis within the kidney contributes toward basal tone, enhancing RBF, and GFR (95). In a rat model of acute gentamicin-induced kidney failure, 5 days of resveratrol treatment improved diminished RBF and improved GFR (118). Similarly, in a rat model of sepsis-induced acute kidney injury, resveratrol treatment increased RBF and partially restored diminished GFR (68). Also, resveratrol marginally improved glomerular filtration in an acute model of renal failure induced by cisplatin (89). Combined, these data suggest resveratrol, in various animal models of compromised renal function, may increase RBF and partially restore GFR after an insult. However, the effects of resveratrol on GFR in normal (uncompromised) rat kidneys remain unclear.

NO also plays a critical role in salt and water transport along the nephron (124). NO enhances natriuresis through inhibition of sodium reabsorption along segments of the nephron (113). eNOS generated NO inhibits sodium reabsorption in the thick ascending limb (TAL) (127). However, it is not known if resveratrol influences NO synthesis in these sites.

On the basis of these observations, we hypothesized a sustained resveratrol infusion would increase RBF and also GFR. Furthermore, we hypothesized resveratrol, independent of its hemodynamic effects, may increase sodium excretion.

Materials and Methods

All protocols and surgical procedures employed in this study were reviewed and

approved by Wayne State University and Henry Ford Health System Institutional Animal Care and Use Committee (IACUC) and were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* endorsed by the American Physiological Society in accordance with National Institutes of Health guidelines.

Male Sprague Dawley rats (Charles River, Wilmington, MA) of 300–400 g body weight (b.w.) were fasted overnight, but allowed free access to drinking water. On the day of the experiment, rats were anesthetized *via* intraperitoneal injection with thiobutabarbital, 125 mg/kg b.w. (Inactin, Sigma Aldrich, St. Louis, MO). Rats were placed on a heated surgical table to maintain constant body temperature (BrainTree Scientific, Braintree, MA). A tracheotomy was performed using PE-240 tubing to allow free breathing of room air. A femoral cut down was performed to cannulate the femoral artery and vein with PE-50 catheters (Becton Dickinson, Franklin Lakes, NJ). The arterial catheter was connected to an iWorx BP-102 probe with LabScribe2 software (iWorx, Dover, NH) for simultaneous recording of mean arterial pressure (MAP) and also used for blood collection. Pressure transducers were calibrated using a digital, mercury-free Traceable manometer (Fisher Scientific, Pittsburg, PA). The femoral venous catheter was used for a 1 mL postsurgical supplement of 6% bovine serum albumin (BSA) (Sigma Aldrich) and for constant infusion of either vehicle or resveratrol plus inulin. A midventral abdominal incision was performed and the intestines wrapped in moist gauze and moved to the right side of the peritoneal cavity to expose the left kidney. The left renal artery and vein were carefully isolated and the renal artery was fitted with a Doppler flow probe (Transonic, Ithaca, NY) connected to a transit-time perivascular flow meter TS-420 model (Transonic) and the iWorx data acquisition system to record renal blood flow (RBF). The bladder was exposed through a

suprapubic incision and was cannulated with a 23-gauge needle connected to PE-50 tubing. It was secured with Vetbond (3M, St. Paul, MN). The rat was draped and allowed to stabilize for 20–30 min prior to running the experimental protocols.

Protocol 1: Renal hemodynamics with resveratrol

We hypothesized sustained resveratrol would increase RBF and GFR. To test the effects of resveratrol on renal hemodynamics, we employed three different doses (0.5, 1.0, and 1.5 mg/min) using three separate groups of rats; one for each dose (n = 7, 10, and 7, respectively). Following surgical stabilization, we intravenously infused vehicle at a rate of 80 µl/min for 30 min followed by a 30 min resveratrol infusion. Recordings for the resveratrol infusion period began after readings stabilized between the changing of the infusion syringes. Data presented for vehicle or resveratrol periods are 30 min averages. FITC-inulin was infused throughout the protocols. RBF and mean arterial pressure (MAP) were recorded. Renal vascular resistance (RVR) was calculated by dividing MAP by RBF in units of mmHg·mL/min·gram of kidney/weight (gkw), hereafter referred to as arbitrary resistance units (ARU).

GFR was measured by the clearance of FITC-inulin (154). Arterial blood was sampled and urine was collected during both the vehicle and resveratrol infusions. Plasma and urine inulin fluorescence was assessed with a Synergy H1 Microplate Reader (BioTek, Winooski, VT) to calculate plasma and urine inulin concentrations.

Urinary sodium concentrations were measured with a Nova1 autoanalyzer (Nova Biomedical, Waltham, MA). Urinary sodium excretion was calculated from urine volume, collection time, and urine sodium concentration.

At the conclusion of the protocol, animals were terminated by barbiturate overdose and aortic transection. The kidneys were removed, decapsulated, blotted,

and weighed.

The resveratrol (Cayman Chemical, Ann Arbor, MI) solution was prepared daily. Resveratrol was dissolved in 4 mL of DMSO and diluted with 5.6 mL of 0.9% saline (final concentration of DMSO was approximately 42%). The resveratrol solution was wrapped in aluminum foil and kept at 37°C until it was used. Vehicle periods used the saline plus DMSO cocktail without resveratrol.

Protocol 2: Renal hemodynamics with vehicle

We performed a set of vehicle infusion controls to serve as controls for Protocol 1. Vehicle, as in *Protocol 1*, contained 4 mL of DMSO and 5.6 mL 0.9% saline. We infused vehicle at the same rate of 80 *μ*l/min plus inulin, but vehicle infusion was maintained throughout the entire protocol (without the addition of resveratrol as above). As above, the time course for vehicle controls was similar to the experimental protocol. Measurements were similar to those in *Protocol 1*.

Analysis

The resveratrol response was compared to matched vehicle periods using a paired Student's *t*-test with *α* acceptance at 0.05, and *n* values were chosen to provide a power of at least 0.8.

Results

Renal hemodynamics with resveratrol

MAP was unchanged with 0.5 mg/min resveratrol (Figure 9). RBF was also not changed by 0.5 mg/min resveratrol (7.69±0.84 vs. 7.88±0.94 mL/min/gkw, n = 9) (Figure 10), and thus RVR remained unchanged (Figure 11). In this group, GFR was 0.98 ± 13 mL/min/gkw (Figure 12) and was unchanged by the lower dose of resveratrol infusion. However, urine flow rate was increased by 60% (19.37 ± 4.97 to 30.89 ± 2.81

μL/min/gkw, [Figure 13] P < 0.006), and urinary sodium excretion increased by 2.3-fold, from 2.19 ± 1.1 to 5.07 ± 0.92 μmol/min/gkw (Figure 14, n = 7, P < 0.01).

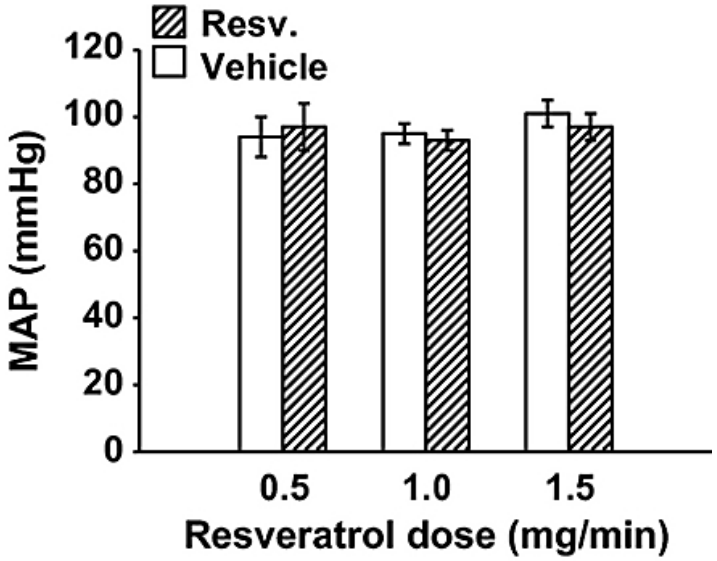


Figure 9: Mean arterial pressure (MAP) with intravenous vehicle (30 min) followed by resveratrol (Resv.) infusion (30 min) in three different groups (*n* = 7, 10, and 7, respectively) at three different doses. Resveratrol infusion had no effect on MAP.

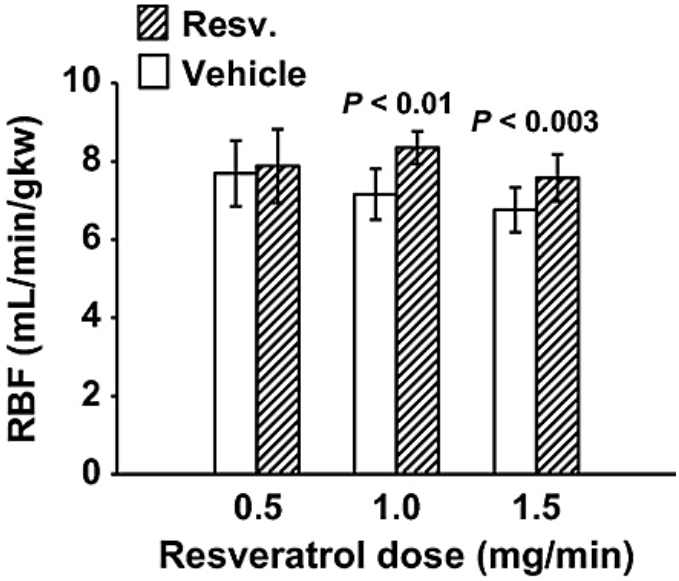


Figure 10: Renal blood flow (RBF) in response to vehicle (30 min) followed by resveratrol (Resv.) infusion (30 min). The lower dose of 0.5 mg/min had no effect on RBF, but both higher doses increased RBF by 17% and 12%, respectively.

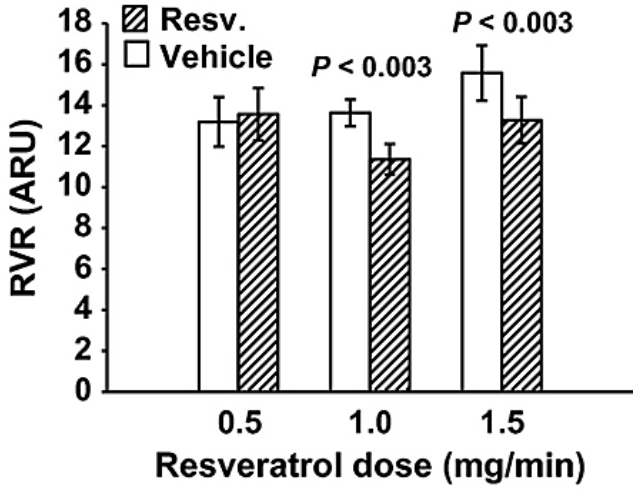


Figure 11: Renal vascular resistance (RVR) in response to vehicle (30 min) followed by resveratrol (Resv.) infusion (30 min). The lower dose of 0.5 mg/min had no effect on RVR, but both higher doses decreased RVR by 16% and 15%, respectively.

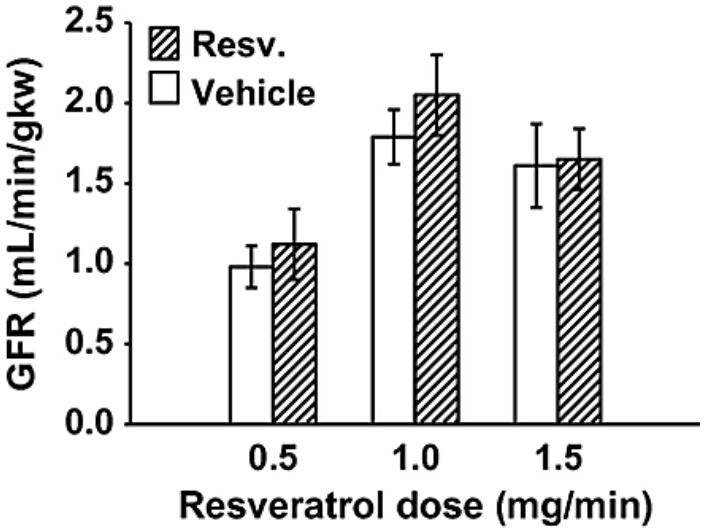


Figure 12: Glomerular Filtration Rate (GFR) during vehicle (30 min) followed by resveratrol (Resv.) infusion (30 min). Resveratrol infusion had no effect on GFR at any dose.

In separate experiments, 1.0 mg/min resveratrol had no effect on MAP (Figure 9), but RBF increased by 17%, from 7.16 ± 0.29 to 8.35 ± 0.42 mL/min/gkw (Figure 10, $P < 0.01$, $n = 10$) and RVR decreased 16% (Figure 11) from 13.63 ± 0.65 to 11.36 ± 0.75 ARU ($P < 0.003$). However, GFR remained unchanged (Figure 12, 1.79 ± 0.17 vs. 2.05 ± 0.25 mg/min/gkw). Urine flow rate doubled from 22.24 ± 5.47 to 45.07 ± 8.36

µL/min/gkw (Figure 13, n = 7, P < 0.002), and sodium excretion increased from 1.57 ± 0.46 to 3.10 ± 0.80 µmol/min/gkw (Figure 14, n = 7, P < 0.04).

In rats given the higher 1.5 mg/min dose of resveratrol, again there was no effect on MAP (Figure 9). Similar to the mid-dose, resveratrol increased RBF 12% from 6.76 ± 0.57 to 7.58 ± 0.60 mL/min/gkw (Figure 10, n = 8, P < 0.003) and thus RVR decreased 15% (Figure 11) from 15.58 ± 1.35 to 13.27 ± 1.14 ARU (P < 0.003). As before, GFR remained unchanged (Figure 12, 1.61 ± 0.26 vs. 1.65 ± 0.19 mL/min/gkw). Urine flow rate increased 70% (Figure 13) from 33.01 ± 6.48 to 56.53 ± 6.56 µL/min/gkw (n = 8, P < 0.005) and sodium excretion nearly doubled (Figure 14) from 3.99 ± 1.71 to 7.80 ± 1.51 µmol/min/gkw (n = 8, P < 0.04).

Protocol 2: Renal hemodynamics with vehicle

In vehicle time controls, (Table 1), MAP was unchanged and both RBF and RVR remained constant. With the vehicle infusion, urine flow rate increased 20% from 42.83 ± 5.36 to 53.08 ± 7.62 µL/min/gkw (P < 0.04), but sodium excretion remained unchanged. Our vehicle including DMSO had no overall effect on MAP, RBF, or RVR.

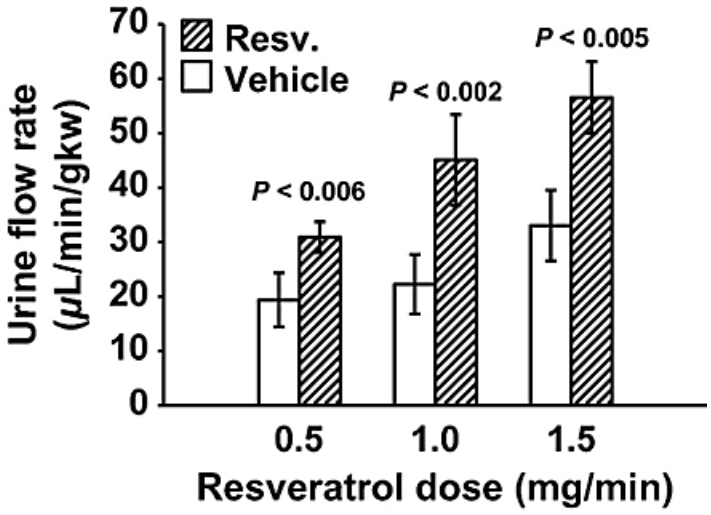


Figure 13: Urine Flow Rate in response to vehicle (30 min) followed by resveratrol (Resv.) infusion (30 min). Resveratrol infusion rate increased urine flow in all three groups by 59%, 103%, and 71%, respectively.

Figure 14: Urinary sodium excretion in response to vehicle (30 min) and resveratrol (Resv.) infusion (30 min). All three doses of resveratrol infusion increased sodium excretion, by 132%, 97%, and 95%, respectively.

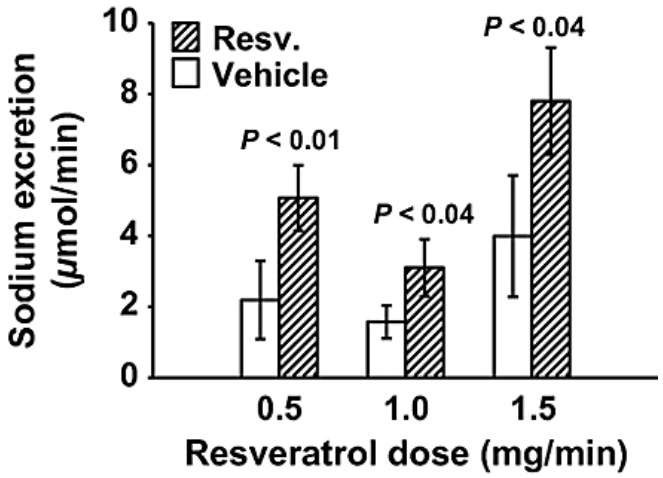


Table 1: Time Control renal Hemodynamics and excretion with vehicle.

Table 1. Time control renal hemodynamics and excretion with vehicle only.

	Vehicle period 1 (30 min)		Vehicle period 2 (30 min)
MAP (mL/min/gkw)	104 ± 8	N.S.	105 ± 8
RBF (mL/min/gkw)	7.25 ± 0.98	N.S.	7.02 ± 1.17
RVR (ARU)	14.76 ± 1.47	N.S.	15.79 ± 2.23
Urine flow rate (μL/min/gkw)	42.83 ± 5.36	$P < 0.04$	53.08 ± 7.62
Sodium excretion (μmol/min)	3.24 ± 1.10	N.S.	4.93 ± 1.62

Discussion

Very little is known about the effects of resveratrol on renal function, especially in the normal state in the absence of renal pathology. We previously reported an i.v. bolus of resveratrol produced an acute renal vasodilation that was mediated by increased NO bioavailability and scavenging of reactive oxygen free radicals. This study expands upon these observations, and addresses resveratrol-induced changes in renal function in response to sustained infusion. Notably, we still find significant renal vasodilation, but

surprisingly without changes in GFR. Additionally, we found resveratrol produced a significant diuresis and natriuresis, and this can occur independent of hemodynamic changes.

Resveratrol has been reported to lower blood pressure, but these observations are primarily in animal models of hypertension (33, 42, 169) or diabetes with hypertension as a comorbidity (140). Translational studies with human participants are sparse and offer mixed results (135, 183). Chronic resveratrol supplementation (75 mg/day) for 6 weeks in mildly hypertensive obese human subjects did not lower blood pressure (183) and another study using chronic resveratrol (500 mg 3×/day) for 4 weeks also did not reduce blood pressure in obese men with prehypertension (135). In our present acute protocol using different doses of sustained resveratrol infusion, our normotensive rats did not have any change in blood pressure.

In our previous work (52), we addressed responsible mechanisms for renal vasodilation. We found it was in part due to increased NO synthesis or availability and also involved a reduction in reactive oxygen species. In this study, we infused resveratrol hypothesizing it would increase RBF which would lead to increased GFR. Interactions between vasoconstriction and vasodilation (including nitric oxide) in the kidney plays a role in regulation of renal hemodynamics (11, 66, 153, 156). Our low dose of 0.5 mg/min resveratrol did not increase RBF or decrease RVR. However, the higher doses of resveratrol (1.0 and 1.5 mg/min) significantly increased RBF and decreased RVR, consistent with our previous findings (52). The present results show renal vasodilation in response to resveratrol is maintained during constant infusion at the mid and high dose. However, there appears to be a maximal limit to resveratrol-induced renal vasodilation in a normotensive rat, as infusion at the mid and high dose

both reduced renal vascular resistance by 16% and 15%, respectively; similar to the changes seen in response to an acute bolus (52). Resveratrol-induced increases in perfusion may be advantageous in compromised renal states when there is lower nitric oxide levels and increased oxidative stress as is often the case in hypertension (60) and other renal diseases (45).

Nonselective NO inhibition is found to increase RVR and decrease glomerular filtration rate (12, 46). We hypothesized that resveratrol-induced increases in RBF (due to increased NO) would lead to increased GFR, but this was not supported by our data, as all three resveratrol doses failed to increase GFR. It is not clear why the increased RBF is uncoupled from GFR in these studies. A possible explanation may lay in that while resveratrol reduced afferent arteriolar resistance and increased RBF, the efferent arterioles (in the normal rat) may have also dilated to maintain glomerular filtration at a constant rate. Thus, while resveratrol may rescue GFR in models of compromised renal function (68, 89, 118), it had no effect in a normal, intact kidney.

The most novel finding of these studies was the significant resveratrol-induced diuresis and natriuresis, even in the absence of hemodynamics changes. This increase in urine flow rate and urinary sodium excretion is also likely due to the effects of renal NO. An extensive review by Li and Förstermann (99) on resveratrol and endothelial function details numerous pathways in which resveratrol increases NO synthesis and bioavailability. Perez-Rojas et al (133) demonstrated (using NOS-3 knock-out mice) how NO within the kidney promotes water and sodium excretion. Resveratrol may possibly be acting as a natriuretic by stimulating NO production in the nephron as it does in the endothelium (52).

NO is important regulator of thick ascending limb (TAL) sodium transport where

approximately 20–30% of filtered sodium is reabsorbed (54). NO has been shown to inhibit sodium reabsorption within the TAL (123). Majid et al. (106) have shown when NO is inhibited in dogs, renal blood flow, urine flow, and sodium excretion are decreased, suggesting NO has both diuretic and natriuretic effects *in vivo*. Thus, NO changes within the TAL are physiologically relevant. Increased shear stress in primary culture of TAL cells has been shown to stimulate the production of NO by NOS 3 (28) and increased renal luminal flow also stimulates NO production within in the TAL (27). In our previous work (52), we demonstrated resveratrol increased renal vasodilation due to increased NO synthesis or availability and through reductions in reactive oxygen species including the possibility of superoxide formation. Changes in tubular flow-induced stretch and sodium chloride delivery stimulate superoxide production by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in the TAL. Three isoforms of NADPH oxidase are present within the TAL (111). When NADPH oxidase 4 (Nox4) was silenced superoxide production was decreased, suggesting Nox4 mediates flow-induced superoxide production in the TAL (69). Resveratrol, in addition to increasing NO, has been shown to decrease NOX activity (35) and NOX4 expression (161). Our whole animal studies do not allow us to pin-point the tubular site of action of the resveratrol-induced natriuresis, but our results are consistent with the literature suggesting NO acts within the TAL (69, 111). It remains unclear if the diuretic and natriuretic effects of resveratrol are due to increased NO availability, reduced superoxide production, or a combination of the above mechanisms as seen in the renal vasculature (52). Overall, the data presented in our study are consistent with resveratrol-induced tubular NO synthesis promoting diuresis and natriuresis.

In our study, the resveratrol-induced natriuretic effect was observed with all three

resveratrol doses. The increases in urinary sodium excretion were dissociated from changes in blood flow. Separate from the effects of NO, resveratrol may affect renal tubular sodium handling through direct inhibition of sodium transport. Weixel et al (180) has shown that resveratrol inhibits Epithelial Sodium Channel (ENaC) in mouse cortical collecting duct cells. It remains unknown if ENaC is being inhibited *in vivo*. However, this may explain how resveratrol may contribute to lowering blood pressure in animal models of hypertension, particularly when salt-sensitive hypertension is present (42, 98, 115, 140).

In summary our findings suggest a constant infusion of resveratrol induced significant renal vasodilation while not altering either GFR or blood pressure in normal rats. Additionally, resveratrol infusion produced significant natriuresis at all doses, independent of hemodynamic responses, suggesting it may have a direct effect on renal tubular sodium handling independent of perfusion pressure, RBF, or changes in RVR.

CHAPTER 3

CHRONIC RESVERATROL REVERSES A MILD ANGIOTENSIN II-INDUCED PRESSOR EFFECT IN A RAT MODEL

Abstract

Resveratrol is reported to reduce blood pressure in animal models of hypertension, but the mechanisms are unknown. We have shown resveratrol infusion increases sodium excretion. We hypothesized chronic ingestion of resveratrol would reduce Ang II-induced increases in blood pressure by decreasing sodium reabsorption through a NO-dependent mechanism. We infused rats with vehicle or 80 µg Ang II/day over 4 weeks. Vehicle or Ang II infused rats were individually housed, pair fed and placed on a diet of normal chow or normal chow plus 146 mg resveratrol/day. Groups included: 1) Control, 2) Resveratrol-fed., 3) Ang II-treated, and 4) Ang II plus resveratrol. Systolic blood pressure was measured by tail cuff. During the 4th week rats were placed in metabolic caging for urine collection. Ang II increased systolic blood pressure in the first week by +14 ±5 mmHg (p<0.05) in group 3 and +10 ±3 mmHg (p<0.05) in the group 4, respectively. Blood pressure was unchanged in groups 1 and 2. After 4 weeks, blood pressure remained elevated in group 3 rats with Ang II, (+9±3 mmHg, p<0.05), but in group 4 blood pressure was no longer elevated (+2±2 mmHg). We found no significant differences between groups in sodium excretion or cumulative sodium balance (18.49 ±0.12, 17.75 ±0.16, 17.97±0.17, 18.46±0.18 µEq Na+/7 days in groups 1-4 respectively). We conclude chronic resveratrol supplementation does not blunt Ang II-increased blood pressure and resveratrol may have mild depressor effects, but these do not seem to be due to natriuresis or enhanced kidney nitric oxide synthesis.

Introduction

Resveratrol is a polyphenol found in some foods including the skins of grapes, red wine, and peanuts. It is proposed to be responsible for the beneficial effects of red wine consumption contributing possibly to reductions in oxidative stress and blood pressure (9, 18). We were interested in whether a possible renal mechanism may explain how resveratrol lowers blood pressure in a rat model. Resveratrol has been reported to lower blood pressure primarily in animal models of hypertension (169). Dolinsky et al. (42) showed that resveratrol was able to reduce increases in systolic pressure in spontaneously hypertensive rats (SHR) and mice infused with Ang II via osmotic minipumps. When resveratrol was removed, SHR blood pressure increased to the same level of that to control SHR. The existing literature supports an interactive involvement between resveratrol and nitric oxide (NO). Defects in NO production or lack of NO have been implicated in hypertension (112, 113). Resveratrol has been shown to increase NO synthesis in multiple tissues including human umbilical vein endothelial cells and also in isolated preparations of thick ascending limb (50, 177, 178). Consistent with this, our previous studies in the kidney show resveratrol acts as a renal vasodilator (52). Renal vasodilation was achieved partly through a NO-dependent mechanism and also through a reduction in reactive oxygen species and/or increasing NO bioavailability (52).

Nitric Oxide plays a critical role in regulation of blood pressure along with salt and water transport along the nephron (60, 124). NO enhances natriuresis through inhibition of sodium reabsorption along segments of the nephron (113). Endothelial nitric oxide synthase generated NO inhibits sodium reabsorption in the thick ascending limb (127). Gonzelez-Vincente et al (50) have shown resveratrol increases NO

production in thick ascending limbs via a Ca^{2+}/calmodulin dependent mechanism. Recently, we published that resveratrol infusion resulted in renal vasodilation and also increased sodium excretion (53). The recognizable limitation to this study was resveratrol was being directly infused into the bloodstream. It is unknown if consumed dietary resveratrol can increase sodium excretion.

Angiotensin II (Ang II) has widely been recognized as a potent pressor and regulator of cardiovascular and renal function. Ang II infusion is frequently used as a model of experimental hypertension in animals. In addition to increasing total peripheral resistance and blood pressure (60), Ang II directly stimulates sodium reabsorption within the kidney and via the indirect actions of aldosterone (48). Increased sodium reabsorption is linked to salt-sensitive increases in blood pressure. Rhaleb et al (139) demonstrated Ang II at a concentration of 80 µg/day, delivered i.p. via osmotic minipump in control Wistar rats over a period of two weeks, induced a mild increase in systolic blood pressure of approximately 15-25 mmHg.

Increased oxidative stress can result in vasoconstriction and may contribute to hypertension. Low, subpressor, doses of Ang II (10 ng/kg/min over 28 days) in normotensive pigs has been shown to increase the concentration of freely circulating isoprostane levels within the plasma (59). Ortiz et al (122) showed the pressor doses of Ang II increased isoprostane levels in Sprague Dawley rats. Further the development of hypertension and the increased isoprostane levels were blunted with the addition of Ang II type I receptor antagonists (122). Resveratrol treatment has been shown to decrease isoprostane production within the aged rat brain and in heart in a model of diabetic cardiomyopathy. (6, 24) Resveratrol treatment has also been shown to decrease urinary excretion of isoprostane in a mouse model of diabetes (90).

On the basis of these observations, we hypothesized chronic ingestion of resveratrol would reduce Ang II-induced increases in blood pressure by decreasing sodium reabsorption through a NO-dependent mechanism.

Materials and Methods

Male Sprague Dawley Rats weighing 200 to 225 grams were housed in standard caging. Rats were fed normal chow containing 0.4% sodium (Harlan Teklad, Madison, WI) and allowed free access to distilled water. Prior to the beginning of the experiment, rats were pre-trained on a non-invasive tail cuff plethsmography multi-channel system (Kent Scientific, Torrington, CT) three times a week for two weeks to measure systolic pressure. All procedures were approved by Wayne State University and Henry Ford Health System Institutional Animal Care and Use Committee (IACUC) and adhered to the guiding principles in the care and use of experimental animals in accordance with the National Institute of Health (NIH) guidelines. Henry Ford Hospital operates an AALAC-certified animal care facility.

Diet Assignment

After the training period, rats were moved to individually housed caging and assigned to one of four groups: 1) control, consuming normal rat chow diet supplemented with 3 grams dark chocolate frosting vehicle (Duncan Hines, Cherryhill, NJ) (*n*=7); 2) normal rat chow supplemented with 3 grams chocolate frosting containing 146 mg of resveratrol (Cayman Chemical, Ann Arbor, MI) (*n*=8), 3) normal rat chow with frosting vehicle and administered Ang II (80µg/day) (Sigma-Aldrich, St. Louis, MO) *i.p*. via an osmotic minipumps (implantation methodology outlined below) (*n*=7), 4) Ang II (80µg/day) *i.p*. supplemented with 146 mg resveratrol mixed into 3 grams of chocolate frosting (*n*=8). Rats were pair-fed. Rat chow was rationed and increased over time to

accommodate for growth. Effort was taken to ensure food/caloric consumption was equal between groups. Rats not infused with Ang II (Groups 1 and 2) were implanted with osmotic minipumps containing vehicle solution (0.01 N acetic acid in sterile 0.9% NaCl). Dark frosting was used as a vehicle for the resveratrol because the rats consumed it all voraciously, the resveratrol otherwise has a bitter taste the rats do not like, and the dark opaque color prevented photo-degradation of the resveratrol.

Implantation of Osmotic Minipumps

After two weeks of tail cuff training and at the beginning of dietary assignments, Model 2002 osmotic minipumps (Alzet, Cupertino, CA) were primed overnight according to the manufacturer's instructions. Pumps were loaded with vehicle or Ang II (80µg/day) dissolved in sterile 0.9% NaCl plus 0.01N acetic acid. Rats were anesthetized with 50 mg/kg body weight Nembutal (Ovation Pharmaceuticals, Deerfield, IL) via intraperitoneal injection. The surgical procedure was performed using aseptic techniques on a heating pad to maintain constant body temperature. The incision site was shaved, cleaned with 70% isopropyl alcohol, and a topical antiseptic bactericide, Betadine was applied (Purdue Products, Stamford, Ct). An incision was made between the scapulae, and the minipumps were implanted subcutaneously. The incision site was closed with surgical staples. Buprenex (2.5 mg/kg) was administered to reduce pain (Reckitt Benckiser Healthcare, Parsippany, NJ). Rats were allowed to regain consciousness and recover on the hot pad before being returned to their cages.

Sodium Balance Studies

Daily consumption of rat chow and frosting was monitored to measure sodium intake. After three weeks on the diets/treatments, rats were moved from regular caging to metabolic caging for the final 7 days and continued on their previously assigned diets.

Rats were allowed to acclimate to the new environment for one day prior to the collection of urine. Urine from each cage drained into clean 50 ml conical centrifuge tubes and was collected every twenty four hours over seven days. Urine volumes were recorded daily. Samples were transferred to Eppendorf tubes, spun at 10K rpm for 5 minutes to remove sediment, and placed in a -80 freezer. Daily sodium excretion values were calculated from sodium concentrations using a Nova Biomedical pHOX ultra (Waltham, MA). Urine sodium concentrations were multiplied by the twenty four hour volume to achieve the twenty four hour sodium excretion. Total cumulative sodium balance was calculated by summing the sequential daily differences between intake and total sodium excretion and expressed in μEq ± SEM over a period of seven days (13).

Plasma Renin Activity (PRA)

After four weeks, rats were euthanized via decapitation using a rat guillotine (Harvard Bioscience, Cambridge, MA). Promptly after decapitation, the first 3 seconds of trunk blood were collected for PRA analyses in 15 ml tubes containing 200 μl of 6% EDTA in 0.9% NaCl. This collection method avoids the influences of the renal baroreceptor reflex and anesthesia, which otherwise increase PRA. Additional blood samples were taken using either 200 μl of 6% EDTA (Sigma-Aldrich, St. Louis, MO), or 100 μl of sodium heparin (Sagent Pharmaceuticals, Schaumberg, IL) as anticoagulants. Blood samples were spun at 1,000 *g* at 4°C for 10 min, and the plasma was separated and stored at −80°C until further analysis. PRA was analyzed by generation of ANG I (ng ANG I·ml^{-1}·h^{-1}·min^{-1}) using a Gamma Coat RIA kit (DiaSorin, Stillwater, MN) as previously described, and according to the manufacturer's instructions. Following blood collection, the gross physical appearance of the kidneys were assessed. Kidneys displaying hydronephrosis were discarded. Kidneys were excised, capsules removed,

blotted, and weighed.

Creatinine Clearance

Creatinine was measured in the 24-hour urine collections and in the plasma during terminal sampling of trunk blood to accommodate the large volume of blood needed without the confounding influence of anesthesia. Blood samples were taken using either 200 µl of 6% EDTA in 0.9% NaCl (Sigma-Aldrich, St. Louis, MO) or 100 µl of sodium heparin (Sagent Pharmaceuticals, Schaumberg, IL) as anticoagulants. Blood samples were spun at 1,000 *g* at 4°C for 10 min, and the plasma was separated and stored at −80°C until creatinine analysis. Creatinine clearance was calculated by multiplying the concentration of urinary creatinine by the twenty four hour urine volume, dividing by the plasma creatinine concentration, and then correcting to units of ml/min and for kidney weight (kw). Creatinine clearance was calculated from the urine collected during the last two days while the rats were in metabolic caging. Clearance values were expressed as ml/min/gkw. Following blood collection, the gross physical appearance of the kidneys were assessed. Renal hydronephrosis was an exclusion criteria. Kidneys were excised, capsules removed, blotted, and weighed. (Groups 2 and 4 *n*=8, Groups 1 and 3 *n*=7)

Urinary Nitric Oxide Assessment

At Day 27, a 24 hour urinary excretion of nitric oxide metabolites (NO_2/NO_3) was measured across groups while rats were housed in metabolic caging. 200 µl of Anti-Anti, an antibiotic and antimycotic, was placed in the urine collection tubes prior to urine collection to prevent degradation of the samples. NO concentration was measured using a Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI) via the Griess Reaction. NO excretion was calculation by multiplying the concentration by

twenty four hour urine volume. Units are expressed as μM/24 hr (*n*=3/group).

Urinary Isoprostane Assessment

At Day 26, urinary excretion of 8-Isoprostane, a marker of oxidative stress, was measured across groups. 100 μl of 0.005, BHT, 3,5-Di-*tert*-4-butylhydroxytoluene was added to urine collection tubes to prevent degradation of the samples. An 8-Isoprostane EIA kit was used to measure urinary concentrations (Cayman Chemical, Ann Arbor, MI). Urinary concentrations were multiplied by urine volumes to reach isoprostane excretion values, expressed as pg/12 hr (*n*=3/group).

Statistics

When multiple comparisons between groups with one differing factor were performed, one-way ANOVA with a Student-Newman-Keuls post hoc test was performed. When comparing two groups, an unpaired student's *t*-test was performed. When comparing changes within a group, a paired student's *t*-test was used. All data are presented as mean ± SE. In all cases, $p < 0.05$ was considered statistically significant.

Results

Blood Pressure

In this study, four groups of rats were fed a standard rat chow containing 0.4% NaCl. The groups included: 1) Control group fed rat chow plus frosting vehicle, 2) a group fed rat chow diet, but supplemented with 146 mg resveratrol in vehicle, 3) a group fed rat chow diet, but administered Ang II (80μg/day) plus vehicle only, and 4) a group fed rat chow diet supplemented with 146 mg resveratrol in vehicle. Blood pressure in Group 1, the control group, was unchanged from 121±2 to 123±1 mmHg over the course of four weeks (Figure 15). In Group 2, normal chow plus resveratrol, blood

pressure also remained unchanged; 122±2 to 126±5 mmHg over four weeks. In groups 3 and 4, blood pressure significantly and similarly increased in the first week following the implantation of Ang II osmotic minipumps. In Group 3 (Ang II), blood pressure increased from 118±3 to 132±4 mmHg $p<0.04$, whereas in Group 4 (Ang II plus resveratrol) blood increased from 122±1 to 132±3 mmHg, $p<0.01$). However, by the end of four weeks, blood pressure in Group 3 remained significantly elevated at 128±2 mmHg (Figure 16), while in Group 4 (Ang II plus resveratrol), the increase in blood pressure was reversed between weeks 3 and 4. After 4 weeks, group 3 (Ang II) was the only one in which blood pressure remained elevated (Figure 16).

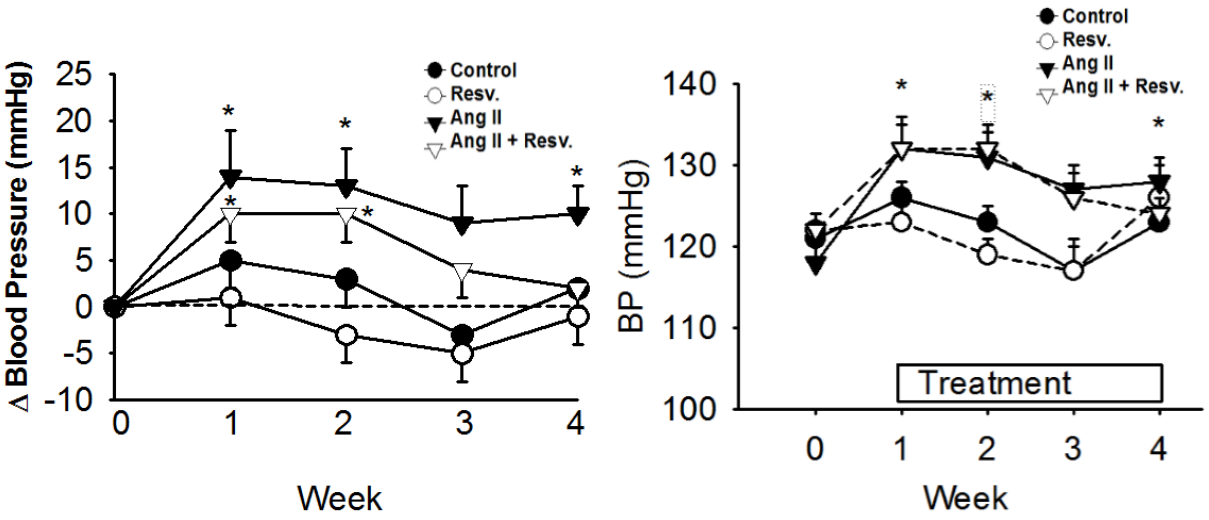


Figure 15: The change in systolic blood pressure over four weeks. Systolic blood pressure remained unchanged in the control and resveratrol treated groups (Groups 1 and 2). Blood pressure initially increased with Ang II infusion (Groups 3 and 4), but between weeks 3 and 4, the increase in systolic blood pressure was reversed in the Ang II plus resveratrol group (Group 4). * $p<0.05$ vs. week 0 control. Left panel: delta blood pressure over four weeks. Right panel: actual blood systolic pressure

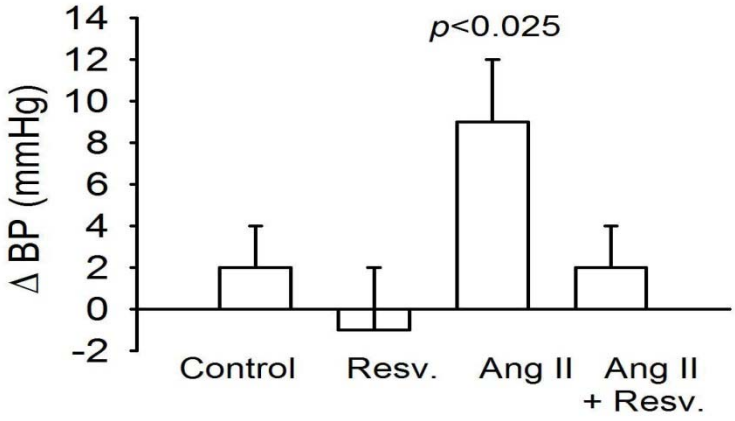


Figure 16: Change in systolic blood pressure at end of four weeks. Only Ang II treatment alone increased systolic blood pressure (p<0.025) after four weeks

Body Weight:

Effort was made to ensure the four groups of rats had similar caloric intake. There were no significant differences in body weight between the four groups at the beginning of the experiment: Groups 1) 243±5; 2) 248±8; 3) 243±7; and 4) 253±8 g. Similarly, there were no differences in body weight after 4 weeks between the four groups: 1) 360±7; 2) 338±10; 3) 345±10; and 4) 355±9 g, so that growth rate was similar in all 4 groups (Figure 17).

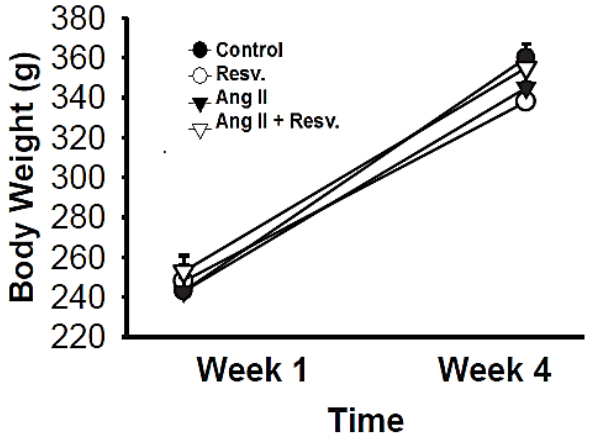


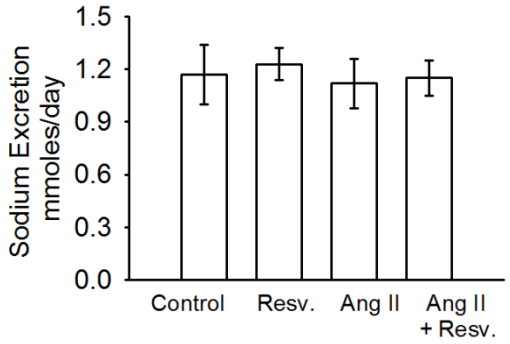
Figure 17: The change in body weight measured over four weeks. Body weight in all four groups significantly increased over four weeks (p<0.05), but no differences in growth was seen between the four groups.

Sodium Excretion and Cumulative Sodium Balance

To examine whether resveratrol consumption can increase sodium excretion and reduce positive cumulative sodium balance, we measured daily sodium intake, excretion, and cumulative sodium balance during the final week. Between Day 20 and Day 27 there were no differences between daily sodium excretion between groups: 1)

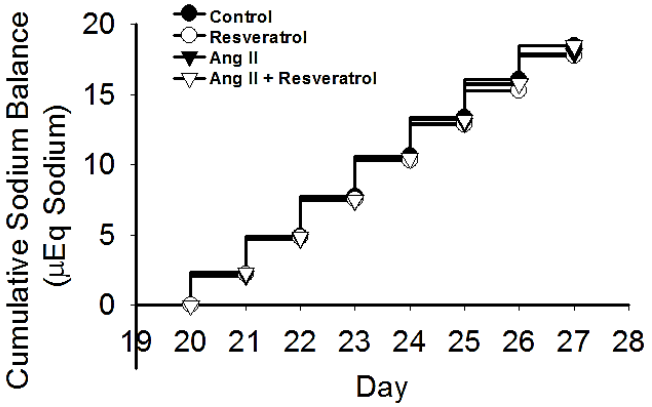
1.17±0.17, 2) 1.23±0.09, 3) 1.12±0.14, and 4) 1.15±0.10 mmoles Na/day (Figure 18).

Figure 18: Mean 24 hour urinary sodium excretion measured over the final 7 days of the protocol. There were no differences in sodium excretion between the four groups.



Between Day 20 and Day 27 there were no differences in cumulative sodium balance among all four groups: 1) 18.49±0.12, 2) 17.75±0.16, 3) 17.91±0.17, and 4) 18.46±0.18 µEq NaCl (Figure 19). Overall, there were no differences in sodium excretion or cumulative sodium balance in the control or Ang II groups with or without resveratrol supplementation over the final 7 days when the Ang II induced pressor response was reversed.

Figure 19: Cumulative sodium balance measured over the final 7 days of the protocol. There were no differences in cumulative sodium balance between the four groups.



Plasma Renin Activity (PRA)

PRA for the four groups: 1) 3.55±0.29, 2) 2.19±0.39, 3) 2.94±0.36, and 4) 2.57±0.26 ng AI/ml of plasma/hour (Figure 20). Group 2 (resveratrol-fed group) had a significantly lower value when compared to the Group 1 control (p<0.03). No other groups were significantly different.

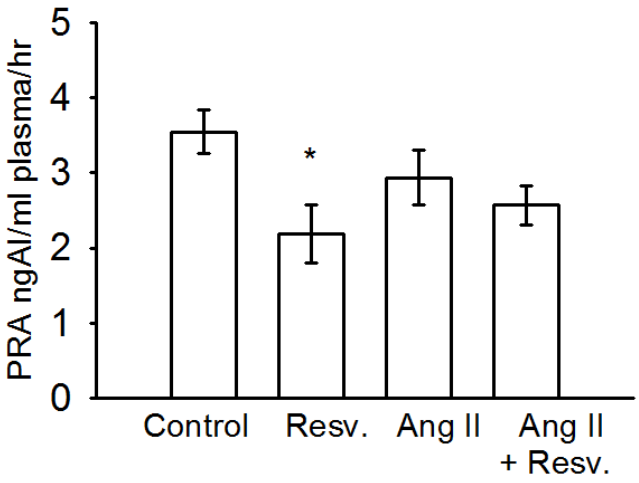
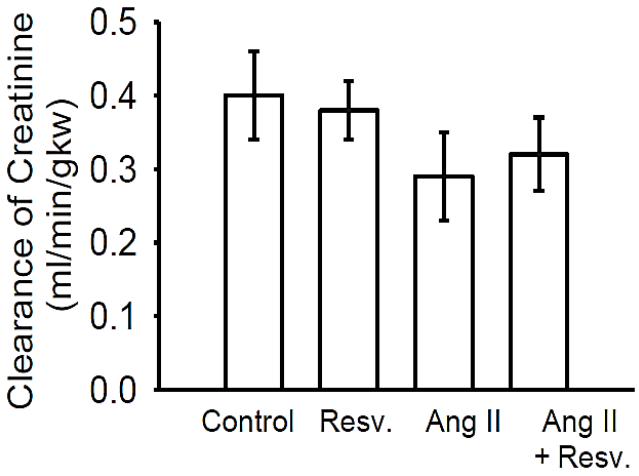


Figure 20: Plasma Renin Activity: Resveratrol group along had lower PRA.

Clearance of Creatinine, Urinary Excretion of NO Metabolites (NO_2/NO_3) and 8-Isoprostane

We measured clearance of creatinine, urinary excretion of NO metabolites (NO_2/NO_3), and urinary excretion of 8-isoprostane at the conclusion of the study. There were no differences in clearance of creatinine between the four groups: 1) 0.40±0.06 (*n*=7), 2) 0.38±0.04 (*n*=8), 3) 0.29±0.06 (*n*=7), and 4) 0.32±0.05 ml/min/gkw (Figure 21).

Figure 21: Clearance of Creatinine: There were no differences in clearance of creatinine between the four groups.



To test if sodium excretion was influenced by increased nitric oxide production, we sampled urinary excretion of NO_2/NO_3 in the four groups and obtained values of: 1) 1631±207, 2) 1045±236, 3) 1490±161, and 4) 609±17 µM/day (Figure 22). Group 4 (Ang II plus resveratrol) total nitrate/nitrite excretion was decreased compared to Group

1 (p<0.05) and Group 3 (p<0.05, ANOVA adjusted for multiple comparisons).

Values for urinary excretion of 8-Isoprostane excretion were: 1) 9669±1969, 2) 11218±3004, 3) 7992±1376, and 4) 14516±2139 pg/12 hr (Figure 23). There were no significant differences between the 4 groups.

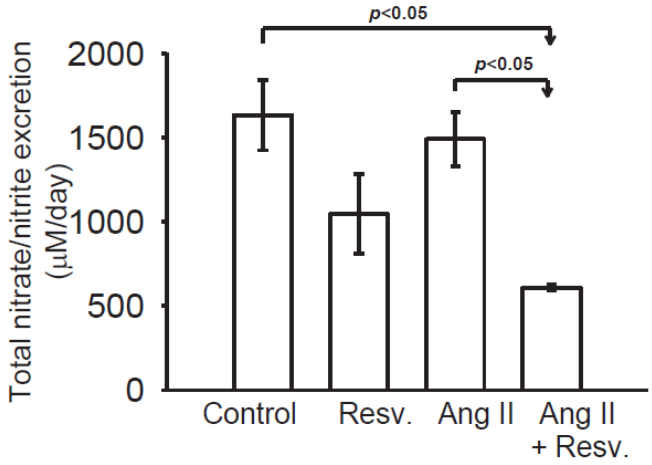
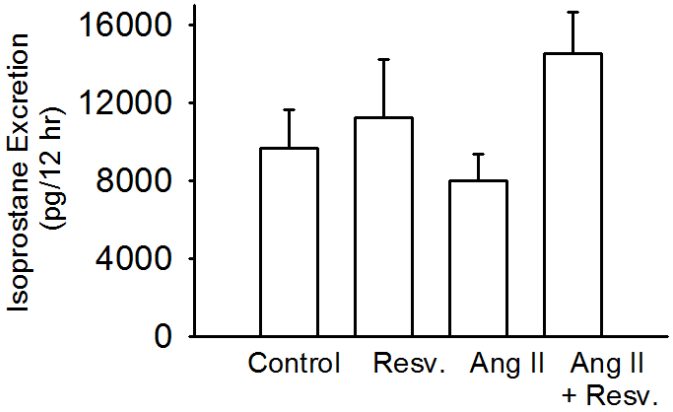


Figure 22: Urinary Excretion of Nitrate/Nitrite. Resveratrol did not increase excretion of nitric oxide metabolites. NO_2/NO_3 excretion was decreased in Ang II plus resveratrol group.

Figure 23: Urinary Excretion of 8-Isoprostane. Resveratrol did not decrease renal oxidative stress, as no differences were observed between groups in urinary excretion of 8-Isoprostane.



Discussion

We hypothesized chronic ingestion of resveratrol (over 4 weeks) would reverse Ang II increases in blood pressure by decreasing sodium reabsorption (and thus cumulative sodium balance) through a NO-dependent mechanism. To test our hypothesis, we specifically selected a mild dose of Ang II infusion (139) to allow for possible resveratrol-induced reductions in blood pressure. We found resveratrol

treatment was unable to prevent Ang II-induced increases in blood pressure. However, with extended resveratrol consumption, the Ang II-induced increases in blood pressure were reversed.

Resveratrol has been reported to lower blood pressure (42, 169). Bhatt et al (19) used resveratrol dissolved in drinking water (50 mg/L) in spontaneously hypertensive rats (SHR) and reported that SHR given resveratrol had lower systolic blood pressure than non-treated SHR (221±2 vs. 196±3 mmHg). Overall, the existing literature referencing resveratrol and Ang II in whole animal studies is surprisingly sparse. Polydatin, a resveratrol glucoside, was delivered in an Ang II-induced model of hypertension by Zhang et al (186) who found the chronic infusion of Ang II significantly increased systolic blood pressure from 120±3 to 162±7 mmHg with Ang II, and oral administration of polydatin for five weeks did not decrease the pressor effect of Ang II. Dolinsky et al (42) implanted mice with osmotic minipumps to deliver Ang II (1.4 mg/kg/day) and placed mice on a diet containing resveratrol (using approximately 320 mg/kg/day resveratrol). Following two weeks of resveratrol treatment, the Ang II plus resveratrol group had lower blood pressure when compared to the Ang II infused alone. They also showed that resveratrol-treated SHR maintained lower systolic blood pressures than control SHR. However, even with resveratrol treatment, the SHR still had systolic blood pressures above 160 mmHg (42). Inanaga et al (74), using C57/B6 mice, showed that resveratrol treatment blunted Ang II-induced increases in systolic blood pressure. However, their systolic blood pressure values using tail cuff measurements were unusually low.

During the course of our 4 week study, the control and normal chow plus resveratrol groups (Groups 1 and 2) showed no increase in blood pressure. However,

both Ang II infused groups (Groups 3 and 4) saw similar, significant increases in systolic blood pressure in the first week. Resveratrol treatment was unable to prevent the pressor effects of mild Ang II infusion. Blood pressure remained elevated in our Ang II group, but blood pressure in the Ang II plus resveratrol group returned to control levels between weeks 3 and 4. Javkhedkar et al (79) using SHR and normotensive controls treated for 9 weeks with resveratrol (50 mg/L) or vehicle in their drinking water. They reported that SHR plus resveratrol group had lower mean arterial pressure during weeks 7-12, but not to normotensive levels. These data corroborates our findings that resveratrol treatment is unable to prevent the onset of hypertension, but there may be a long-term, temporal effect of resveratrol reversing increased blood pressure after a few weeks of treatment.

In our previous studies (52) we showed resveratrol acted as an acute renal vasodilator working through increasing NO and decreasing reactive oxygen species. We also found (53) that sub-vasodilatory doses of sustained resveratrol administration could induce natriuresis without affecting glomerular filtration rate. This suggested a direct tubular action of resveratrol (53). We wanted to test whether a dietary resveratrol supplement would increase sodium excretion, ultimately leading to a decreased positive sodium balance. Our approach of metabolic studies to measure sodium intake, sodium excretion, and sodium balance in resveratrol-treated rats is a novel approach to determine possible tubular sodium effects of resveratrol.

Resveratrol is rapidly degraded within the body and later cleared by the kidney. Juan et al (84) demonstrated rats given an intravenous administration of 15 mg/kg resveratrol distributed at the highest concentrations in kidney. It has been shown 100 μM of resveratrol can increase NO in thick ascending limb suspensions (50). NO is

important regulator of thick ascending limb sodium transport (54). NO has been shown to inhibit sodium reabsorption within the thick ascending limb of the loop of Henle (126). Majid et al (108) have shown when NO is inhibited in dogs, sodium excretion is decreased. Their results suggest NO contributes towards diuresis and natriuresis *in vivo.* We found resveratrol consumed in the diet did not increase sodium excretion in Group 2 (normal rat chow plus resveratrol) nor did it increase sodium excretion in Group 4. No differences in urinary sodium excretion or cumulative sodium balance existed between any of the groups. Contrary to our hypothesis, we conclude that resveratrol had no effect in increasing sodium excretion or diminishing positive sodium balance. Thus, the mechanism by which resveratrol lowers blood pressure does not appear to be linked to sodium handling within the nephron. However, we did not measure sodium excretion during the first weeks, though there were no differences in systolic pressure during this period. Notably, we did find an Ang II-induced positive cumulative sodium balance in our model, at least during the final week of treatment which would be consistent with the lack of any effect of resveratrol on natriuresis.

Oxidative stress is determined by the balance between the generation of reactive oxygen species and opposing antioxidant systems (91). The renal medulla is the primary site of superoxide in the kidney, and also the location of thick ascending limbs of juxtamedullary nephrons. In our previous work (52), we showed that resveratrol-induced renal vasodilation was both nitric oxide dependent and due to a possible reduction in reactive oxygen species and/or increase in nitric oxide bioavailability. To test if resveratrol reduced sodium reabsorption through a nitric oxide or superoxide dependent mechanism, we measured urinary excretion of nitric oxide metabolites and urinary 8-isoprostane, a reliable index of renal oxidative stress.

Resveratrol treatment has been shown to lower 8-isoprostane levels in diabetic rats (116). Bertelli et al (17) in a model of acute kidney failure induced by ischemia showed rats pretreated with resveratrol raised urinary excretion of cGMP, a marker for nitric oxide synthase activity. We did not find that resveratrol stimulated an increase in urinary excretion of nitric oxide metabolites. Contrary to our expectations, resveratrol treatment significantly decreased excretion of nitric oxide metabolites. This finding is clearly contrary to our hypothesis and an obvious explanation is lacking. We also measured urinary excretion of 8-isoprostane to test if resveratrol would potentially lower oxidative stress. We saw no decrease in 8-isoprostane excretion with resveratrol treatment. Thus, we conclude resveratrol treatment at this dose was not able to stimulate measurable NO within the nephron or decrease oxidative stress. Our measurements of these metrics was only done at the end of the protocol, but that is also the period in which we observed a depressor effect of resveratrol.

To evaluate the effect of Ang II and resveratrol on renal function we measured clearance of creatinine at the conclusion of the protocol. Ang II induced hypertension is associated with decreased glomerular filtration rate (179). In our study, the clearance of creatinine in the Ang II group (Group 3) was not significantly lower when compared to the non-Ang II treated groups. There were no differences in clearance of creatinine between the four groups after 4 weeks.

In summary, our findings suggest resveratrol is unable to prevent increases blood pressure due to Ang II. Rigorous metabolic studies show that resveratrol supplementation does not increase urinary sodium excretion, decrease cumulative sodium balance, or diminish sodium-sensitive increases in blood pressure. However, we did observe a reversal of the Ang II increase in blood pressure with chronic dietary

resveratrol treatment following 3-4 weeks of treatment. In view of our data, chronic supplementation with resveratrol may have mild depressor effects, but these do not seem to be due to natriuresis or chronically enhanced nitric oxide synthesis.

CHAPTER 4

CHRONIC RESVERATROL DOES NOT BLUNT FRUCTOSE-INDUCED SALT SENSITIVE INCREASE IN BLOOD PRESSURE IN A RAT MODEL

Abstract

Resveratrol is suggested to reduce blood pressure and oxidative stress in rodent models of hypertension, but mechanisms are poorly understood. Previously, we have shown resveratrol infusion induces natriuresis. We hypothesized chronic ingestion of resveratrol would reduce fructose-induced salt sensitive increases in blood pressure by decreasing sodium retention through a NO-dependent mechanism. Rats were placed in metabolic caging for 3 weeks to measure sodium balance and groups included: 1) Control, 2) 20% fructose and resveratrol, (146 mg/day) 3) 20% fructose and high salt (4%NaCl in the food), and 4) 20% fructose, high salt, and resveratrol. We measured blood pressure and urinary excretion of NO_2/NO_3 and 8-isoprostane. During week 1 all rats received normal rat chow diet 20% fructose and resveratrol was introduced during week 2, and high salt during week 3. Systolic blood pressure was unchanged in the control group over three weeks as well as the 20% fructose-treated groups (Groups 2, 3, and 4) (121±1 to 125±1 mmHg). However, blood pressure significantly increased in Group 3 (124±1 to 138±2 mmHg $p<0.001$), and Group 4 (126±2 to 142±3 mmHg $p<0.003$) with high salt. Increases in blood pressure were coincident a significant retention of dietary sodium. Resveratrol did not increase urinary excretion of sodium or NO_2/NO_3. Fructose treatment, independent of blood pressure or resveratrol, increased 8-isoprostane excretion with 2 weeks ($p<0.003$). Overall, we conclude 20% fructose coupled with high salt intake can induce elevated blood pressure in as little as 2 weeks. Resveratrol did not blunt salt-sensitive increases in blood pressure nor did resveratrol

have an effect on sodium retention or reduce oxidative stress.

Introduction

Resveratrol is a polyphenol found in some foods including the skins of grapes, red wine, and peanuts (9). It is purported to contribute to reductions in oxidative stress and blood pressure in various models of hypertension (58, 169). Resveratrol has been shown to increase nitric oxide (NO) synthesis in multiple tissues including human umbilical vein endothelial cells and also in isolated preparations of renal thick ascending limb (50, 178). Our previous studies (52) show resveratrol acts as a renal vasodilator. Renal vasodilation was mediated through a NO-dependent mechanism, and also through a reduction in reactive oxygen species and/or increasing NO bioavailability (52). We also have shown (53) that resveratrol can induce natriuresis, independent of changes in renal hemodynamics. NO enhances natriuresis through inhibition of sodium reabsorption along multiple segments of the nephron (113). Because of the salt-sensitivity in fructose-induced hypertension (29), we used this model to test our hypothesis that resveratrol-stimulated natriuresis could blunt increases in blood pressure. On the basis of these observations, overall we hypothesized that chronic ingestion of resveratrol would reduce fructose-induced salt sensitive increases in blood pressure by decreasing sodium reabsorption and overall cumulative sodium balance through a NO-dependent mechanism. We were interested in whether a possible renal mechanism may explain the effect of resveratrol lowering blood pressure in a salt-sensitive model of hypertension.

Fructose is increasingly implicated as a causal factor in the development of hypertension, diabetes, and obesity (73, 76, 168). However, the exact mechanism of fructose-induced hypertension is not fully understood. High intake of fructose (60%)

can induce insulin resistance, hypertriglyceridemia, and changes in endothelial function within two weeks (73). A strong association has been shown that Type 2 diabetes rates are twenty percent higher in countries where fructose is consumed the most, compared to disease rates in countries with low fructose consumption (51).

Within the kidney, a diet containing 60% fructose in the drinking water can induce renal hypertrophy, afferent arteriolar thickening, glomerular hypertension, and vasoconstriction (144). Fructose intake can easily overwhelm hepatic fructose metabolism and eventually escapes into the systemic circulation and thus enters the renal circulation (174). Fructose consumption may contribute to hypertension by abnormally increasing sodium reabsorption within the intestine and kidney (158). Fructose is shown to stimulate sodium hydrogen exchange 3 (NHE3) activity within the proximal tubule (29). However, high experimental levels of fructose do not adequately reflect average fructose intake within the American diet (82). A diet containing 20% fructose is more akin to the upper end of the American diet as percentage of their total caloric intake. Importantly, low doses of fructose (with the addition of salt) can induce salt-sensitive increases in blood pressure with two weeks (29). Elevations in blood pressure in this fructose model occur prior to the development of diabetes and metabolic syndrome. Increases in plasma glucose and insulin have been reported with 10% and 20% fructose starting beyond 6 weeks of fructose treatment suggesting the cumulative effects of enhanced dietary fructose on hypertension and eventually metabolic syndrome are both concentration and time dependent (39).

Materials and Methods

Male Sprague Dawley Rats weighing 200 to 225 grams were housed in standard caging. Rats were fed normal chow containing 0.4% sodium (Harlan Teklad, Madison,

WI) and allowed free access to distilled water. Prior to the beginning of the protocol, rats were pre-trained on a non-invasive tail cuff plethsmography multi-channel system (Kent Scientific, Torrington, CT) three times a week for two weeks to measure systolic pressure. All procedures were approved by Wayne State University and Henry Ford Health System Institutional Animal Care and Use Committee (IACUC) and adhered to the guiding principles in the care and use of experimental animals in accordance with the National Institute of Health (NIH) guidelines. Henry Ford Hospital operates an AALAC-certified animal care facility.

Diet Assignment

After the blood pressure training period, rats were moved to metabolic caging and allowed to acclimate to the new environment for one day. During week 1 (days 1 to 5) all rats were pair-fed normal rat chow containing 0.4% NaCl (Harlan Teklad, Madison, WI) and allowed free access to distilled water. During week 2 (days 6-12) rats were assigned to one of four dietary groups: 1) control, normal rat chow supplemented with 3 grams of fructose-free chocolate frosting vehicle (Duncan Hines, Cherryhill, NJ) with access to distilled water (*n*=9); 2) 20% fructose (in the drinking water) (Sigma-Aldrich, St. Louis, MO), normal rat chow supplemented with 3 grams chocolate frosting containing 146 mg of resveratrol (Cayman Chemical, Ann Arbor, MI) (*n*=9), 3) 20% fructose, normal rat chow supplemented with 3 grams chocolate frosting vehicle (n=9), and 4) 20% fructose, normal rat chow supplemented with 3 grams chocolate frosting containing 146 mg of resveratrol (*n*=9). During week 3 (days 13-19) groups 1 and 2 were maintained on the same diet regime as week 2. However, groups 3 and 4 were switched from normal rat chow to a high salt diet (4% NaCl rat chow, Harlan Teklad, Madison, WI).

Effort was taken to ensure food/caloric consumption was equal between groups by pair feeding of rat chow. During the course of the 3 week protocol, rat chow was rationed equally to all groups, and increased over time to accommodate for growth. Rat chow was restricted across all four groups when a rat did not consume the daily rat chow allotment in entirety. Dark frosting was used as a vehicle for the resveratrol because the rats consumed it all voraciously, the resveratrol otherwise has a bitter taste the rats do not like, and the dark opaque color prevented photo-degradation of the resveratrol.

Sodium Balance Studies

Daily consumption of rat chow and frosting was recorded to measure sodium intake. Urine from each cage drained into clean 50 ml conical centrifuge tubes and was collected every twenty four hours over 19 days. Urine volumes were recorded daily. Urine samples were transferred to Eppendorf tubes, spun at 10K rpm for 5 minutes to remove sediment, and placed in a -80 freezer. Daily sodium excretion values were calculated from 24 hour urine volumes and sodium concentrations measured by a Nova Biomedical 1 electrolyte analyzer (Waltham, MA). Total cumulative sodium balance was calculated by summing the sequential daily differences between intake and total sodium excretion and expressed in μEq ± SEM over a period of 19 days (2). Volume of consumed distilled water or 20% fructose was also recorded.

Fecal Sodium Excretion

Fecal samples were collected in a subset within the four groups (n=3) to measure fecal sodium excretion as a percent of total sodium excretion (urinary and fecal sodium excretion). Fecal samples were transferred to 50 ml conical centrifuge tubes and placed in a -80 freezer until further analysis. Fecal samples were thawed to room

temperature and total fecal weight was recorded. One gram of feces was combined with 4ml of distilled water, and homogenized with a Tissuemiser (Fisher Scientific, Pittsburgh, PA). The fecal slurry was transferred to a 1.5 ml Eppendorf, spun down at 1,000 *g* at 4°C for 5 minutes, the supernatant pipetted into a new Eppendorf. Daily fecal sodium excretions were calculated from sodium concentrations using a Nova Biomedical 1 Electrolyte Analyzer (Waltham, MA) and total fecal mass to achieve a twenty four hour fecal sodium excretion.

Urinary Nitric Oxide Assessment

At Day 19, a 24 hour urinary excretion of nitric oxide (NO) metabolites (NO_2/NO_3) was measured as an index of renal NO production across groups while rats were housed in metabolic caging. 200 µl of Anti-Anti, an antibiotic and antimycotic, (Life Technologies, Grand Island, NY) was placed in the urine collection tubes prior to urine collection to prevent growth of bacteria. NO_2/NO_3 concentration was measured using a Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI) via the Griess Reaction. NO_2/NO_3 excretion was calculation by multiplying the concentration by twenty four hour urine volume. Units are expressed as µM/24 hr (*n*=9/group).

Urinary Isoprostane Assessment

At Day 19, a portion of the collected rat urine samples were subdivided to measure urinary excretion of 8-Isoprostane, a marker of oxidative stress. 0.005%, BHT, 3,5-Di-*tert*-4-butylhydroxytoluene (Sigma-Aldrich, St. Louis, MO) was added to urine collection tubes to prevent degradation of 8-Isoprostane. An 8-Isoprostane EIA kit was used to measure urinary concentrations (Cayman Chemical, Ann Arbor, MI). Urinary concentrations were multiplied by urine volumes to reach isoprostane excretion values, expressed as pg/24 hr (*n*=7-9/group).

Plasma Renin Activity (PRA)

After three weeks, rats were euthanized via decapitation. Promptly after decapitation, the first 3 seconds of trunk blood were collected for plasma renin activity (PRA) analyses in 15 ml tubes containing 200 µl of 6% EDTA in 0.9% NaCl. This collection method avoids the influences of the renal baroreceptor reflex and anesthesia on PRA. Additional blood samples were collected using either 200 µl of 6% EDTA (Sigma-Aldrich, St. Louis, MO) or 100 µl of sodium heparin (Sagent Pharmaceuticals, Schaumberg, IL) as anticoagulants. Blood samples were spun at 1,000 *g* at 4°C for 10 min, and the plasma was separated and stored at −80°C until further analysis. PRA was analyzed by generation of ANG I (ng ANG I·ml^{-1}·h^{-1}) using a Gamma Coat RIA kit (DiaSorin, Stillwater, MN) as previously described and according to the manufacturer's instructions. Following blood collection, the gross physical appearance of the kidneys were assessed. Renal hydronephrosis was an exclusion criterion. Kidneys were excised, decapsulated, blotted, and weighed.

Plasma glucose, insulin levels, glucose tolerance test (GTT), and Renal Hypertrophy

To confirm that fructose-induced, salt-sensitive hypertension preceded diabetes and metabolic syndrome, we measured plasma glucose and insulin levels at the conclusion of the protocol. Following euthanasia, blood droplets were collected in the four groups of rats to measure fasting plasma glucose levels. Plasma glucose was measured with a True Result Blood Glucose Monitor (CVS, Woonsocket, RI). The additional plasma samples (containing 6% EDTA) stored at -80 were thawed and insulin levels were measured using a rat insulin EIA kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s instructions.

In a separate protocol, glucose tolerance tests (GTT) were performed in three groups of rats: 1) control 2) 20% fructose (20% fructose in water for two weeks), and 3) 20% fructose plus high salt (4%) rat chow fed over the final 7 days to demonstrate that only 14 days of fructose-feeding was not (yet) initiating metabolic syndrome. Male Sprague Dawley rats (Charles River, Wilmington MA) of 300 to 500 grams body weight (b.w.) were fasted overnight but allowed free access to drinking water. On the day of the experiment, rats were anesthetized *via* intraperitoneal injection with thiobutabarbital, 125 mg/kg b.w. (Inactin, Sigma Aldrich, St. Louis, MO). Rats were placed on a heated surgical table to maintain constant body temperature (BrainTree Scientific, Braintree, MA). A tracheotomy was performed using PE-240 tubing to allow free breathing of room air. A femoral cut down was performed to cannulate the femoral artery with PE-50 catheters. Arterial blood was collected and plasma glucose measured with a True Result Blood Glucose Monitor (CVS, Woonsocket, RI). Plasma glucose was measured at baseline and following an intragastric bolus by gavage of glucose (3mg/kg bw), sampling at 5, 10, 15, 30, 60, 120 minutes after gavage (n=3-5/group).

To quantify any renal hypertrophic response to fructose and high salt in the presence or absence of resveratrol, we calculated total kidney weight/body weight (mg/g) ratios in the four groups of rats at the end of the 3 week dietary protocol.

Confirmation of Resveratrol Activity

As a negative control for the activity of resveratrol, we performed a systemic injection bolus of resveratrol (5 mg/kg) in an anesthetized rat to assess if it induced renal vasodilation. Procedure for bolus injection is found in Chapter 1.

Statistics

When multiple comparisons between groups with one differing factor were

performed, one-way ANOVA with a Student-Newman-Keuls post hoc test was performed. When comparing two groups, an unpaired student's *t*-test was performed. When comparing changes within a group, a paired student's *t*-test was used. All data are presented as mean ± SE. In all cases, $p < 0.05$ was considered statistically significant.

Results

Blood Pressure

During the initial 5 days of normal diet, there were no significant changes in systolic blood pressure (Figure 24). Systolic blood pressure in Group 1, the control group, was unchanged from 121±2 to 124±3 mmHg from week 1 to week 2. Collectively, in Groups 2, 3, and 4, with the addition of 20% fructose blood pressure remain unchanged from week 1 to week 2 (121±1 to 125±1 mmHg) (Figure 24). From week 2 to week 3, blood pressure remained unchanged in Group 1, the control group (124±3 to 122±2 mmHg), as well as in Group 2 (20% fructose and resveratrol, 126±2 to 128±1 mmHg). However, in week 3, with the addition of high salt diet to Groups 3 and 4, blood pressure significantly increased (Figure 24). In Group 3 (20% fructose and high salt), blood pressure increased from 124±1 to 138±2 mmHg ($p<0.001$), and in Group 4 (20% fructose, high salt, and resveratrol) blood pressure also increased from 126±2 to 142±3 mmHg ($p<0.003$). Thus, resveratrol did not diminish the salt-sensitive hypertension in the presence of fructose.

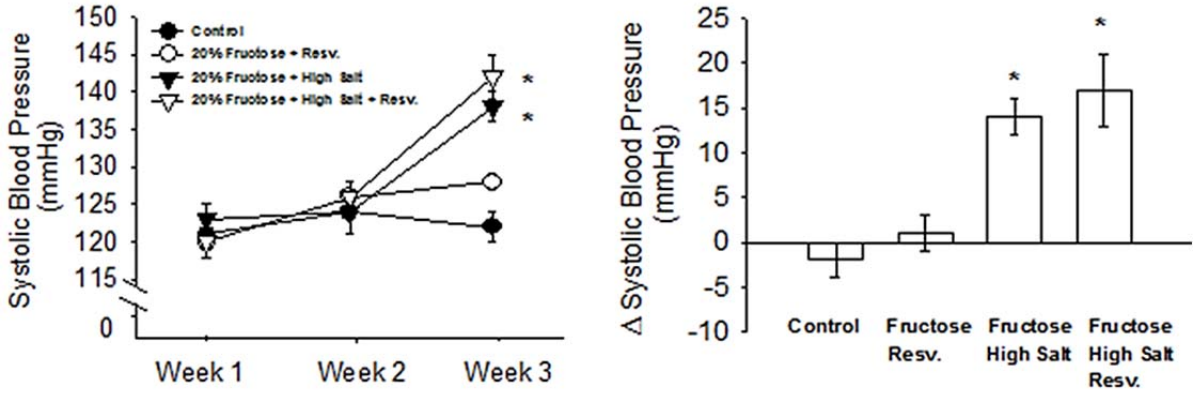


Figure 24: Systolic Blood Pressure over 3 weeks. High salt diet to Groups 3 and 4 significantly increase blood pressure in the presence of fructose. Resveratrol had no effect. Left panel: actual blood pressure over 3 weeks. Right panel: delta blood pressure from week 2 to week 3.

Body Weight

Effort was made to ensure the four groups of rats had similar caloric intake. There were no significant differences in body weight between the four groups at the beginning of the experiment (Table 2). By the end of three weeks, as expected, all four groups of rats significantly increased in body weight. However, by the end of three weeks, there were differences in growth rates between Group 1 compared to Groups 2, 3, and 4 (p<0.0001) (Figure 25). Pair-feeding of rat chow was maintained across all four groups, but Group 1 lacked the 20% fructose in its water and its accompanying calories.

Table 2: Body Weights (g)

	Beginning	End of Week 1	End of Week 2	End of Week 3
Control	**323±8**	**332±9**	**351±6**	**353±8**
F+R	**337±10**	**342±11**	**382±10**	**418±9**
F+HS	**330±9**	**335±10**	**384±8**	**407±10**
F+HS+R	**332±7**	**341±9**	**381±8**	**402±9**

F = fructose, HS = high salt, and R = resveratrol

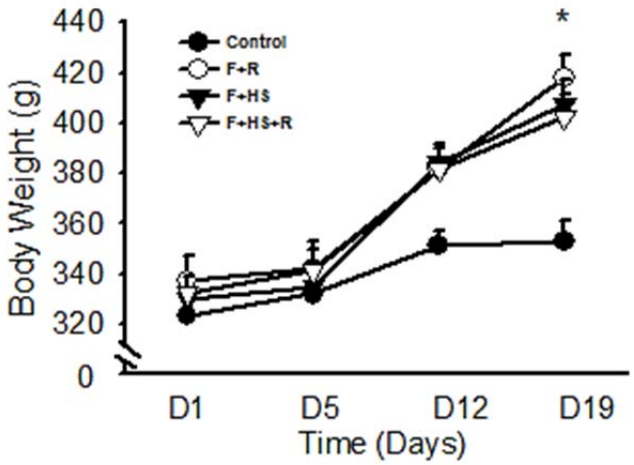


Figure 25: Body Weight over 3 weeks. Fructose-fed rats grew significant greater rate.

Food Intake/Sodium Consumption

Rats were pair fed to ensure consumption of rat chow (and its sodium content) was similar across groups. During week 1 through week 3, there were no differences in chow consumption across all four groups (Table 3). However, with the addition of fructose to the drinking water in the beginning of week 2, food consumption decreased as fructose replaced calories from rat chow. Food consumption decreased on average 20% ($p<0.0001$) when 20% fructose was added the diet. Food consumption further decreased on average 26% with the addition of high salt during week 3 in groups 3 and 4 ($p<0.0001$) (Table 3). The frosting vehicle was normally consumed daily in all groups in entirety.

During the first two weeks of the protocol there were no differences in sodium intake across all four groups, but sodium intake decreased as expected with decreased rat chow consumption by some 13%. However, during week 3, with the addition of high salt diet to only Groups 3 and 4, sodium intake significantly increased by nearly 6-fold. (Table 4)

Table 3: Food Consumption/Day (g)

	Week 1	Week 2	% Change	*p* value	Week 3	% Change	*p* value
Control	**23.1±0.5**	**19±0.5**	**-17**	**0.0001**	**14.5±0.5**	**-24**	**0.0001**
F+R	**23.0±0.5**	**17.8±0.5**	**-23**	**0.0001**	**14.2±0.4**	**-20**	**0.0001**
F+HS	**23.1±0.5**	**18.7±0.5**	**-19**	**0.0001**	**13.5±1.0**	**-28**	**0.0001**
F+HS+R	**22.8±0.5**	**18.2±0.5**	**-20**	**0.0001**	**13.2±1**	**-27**	**0.0001**

Week 1: All rats on normal rat chow, Week 2: Introduction of fructose and resveratrol, and Week 3: Introduction of high salt. F = fructose, HS = high salt, and R = resveratrol. Represents food intake alone.

Table 4: Sodium Intake/Day(µEq NaCl)

	Week 1	Week 2	% Change	*p* value	Week 3	% Change	*p* value
Control	**4.00±0.09**	**3.64±0.08**	**-9**	**0.0001**	**2.68±0.08**	**-26**	**0.0001**
F+R	**3.97±0.07**	**3.14±0.08**	**-21**	**0.0001**	**2.81±0.07**	**-11**	**0.0001**
F+HS	**4.00±0.08**	**3.57±0.09**	**-11**	**0.0001**	**23.71±1.33**	**+564**	**0.0001**
F+HS+R	**3.96±0.07**	**3.48±0.05**	**-12**	**0.0001**	**23.14±1.31**	**+565**	**0.0001**

Week 1: All rats on normal rat chow, Week 2: Introduction of fructose and resveratrol, and Week 3: Introduction of high salt. F = fructose, HS = high salt, and R = resveratrol. Represents total sodium from food and frosting.

Water Consumption/Urine Excretion:

During Week 1, there were no differences in the amount of water consumed across groups (Table 5a). In week 2, the control group 1 deceased water consumption by 22% ($p<0.003$) while water consumption remained constant in all fructose-fed groups 2-4. During week 3, water consumption in the control group decreased marginally. In group 2 water consumption increased by 10% ($p<0.015$), and with the addition of high salt water intake substantially increased in group 3 ($p< 0.002$) and group 4 ($p<0.005$). There were no differences in water intake with or without resveratrol supplementation (Table 5a). There were no differences in urine volumes between groups during weeks 1 or 2. However, with the addition of high salt during week 3, as water consumption

increased, so also urine volumes increased in groups 3 (p<0.0003) and 4 (p<0.007). (Table 5b)

Table 5a: Water Consumption (ml/24 hr)

	Week 1	Week 2	% Change	*p* value	Week 3	% Change	*p* value
Control	**45±2**	**35±2**	**-22**	**0.003**	**33±2**	**-6**	**0.046**
F+R	**41±2**	**41±4**	**0**	**n.s.**	**45±4**	**10**	**0.015**
F+HS	**41±3**	**44±2**	**-7**	**n.s.**	**56±4**	**27**	**0.002**
F+HS+R	**38±1**	**38±2**	**0**	**n.s.**	**52±3**	**37**	**0.005**

F = fructose, HS = high salt, and R = resveratrol

Table 5b: Daily Urine Excretion (ml/24 hr)

	Week 1	Week 2	Week 3	*p*-value
Control	**11.7±1.4**	**9.5±1.3**	**10.3±1.4**	**n.s.**
F+R	**11.9±1.3**	**10.1±1.9**	**14.6±2.3**	**n.s.**
F+HS	**13.4±2.2**	**13.3±1.5**	**21.5±2.4**	**0.001**
F+HS+R	**10.6±1.1**	**9.4±1.0**	**17.6±1.9**	**0.007**

F = fructose, HS = high salt, and R = resveratrol

***Sodium Excretion and Cumulative Sodium Balance**:*

To examine whether resveratrol treatment can increase sodium excretion and reduce sodium retention, we measured sodium excretion and cumulative sodium balance over all 19 days of the diet manipulations. During week 1, there were no significant differences in mean urinary sodium excretion between the four groups while on normal rat chow (Table 6). Mean urinary sodium excretion did not change in Group 1 (control) from week 1 to week 2. However, sodium excretion within Groups 2, 3, and 4, significantly decreased (29%, 15%, 27%, respectively) (p<0.05) with the addition to 20% fructose to their diets, as chow and sodium consumption decreased (Table 3).

During week 3, with the addition of the high salt diet, sodium excretion

significantly increased in both Groups 3 and Group 4 ($p<0.001$). Overall, there were no differences in sodium excretion between Group 1 (control) and Group 2 (20% fructose and resveratrol). Further, we found no differences in sodium excretion between Groups 3 and 4 during the high salt diet, with or without resveratrol supplementation. Resveratrol had no measurable effect on sodium excretion in any experimental period.

Table 6: Mean Sodium Excretion (μEq/Day)

	Week 1	Week 2	% change	*p*-value	Week 3	% change	*p*-value
Control	**1.35±0.1**	**1.31±0.09**	**-3**	**n.s.**	**1.13±0.07**	**-14**	**0.01**
F+R	**1.56±0.08**	**1.11±0.13**	**-29**	**0.02**	**1.20±0.10**	**+8**	**n.s.**
F+HS	**1.61±0.07**	**1.40±0.09**	**-15**	**0.02**	**5.63±0.26**	**+402**	**0.001**
F+HS+R	**1.38±0.10**	**1.10±0.10**	**-27**	**0.02**	**5.04±0.32**	**+458**	**0.001**

Week 1: All rats on normal rat chow, Week 2: Introduction of fructose and resveratrol, and Week 3: Introduction of high salt. F = fructose, HS = high salt, and R = resveratrol. N.S.= not significant. Represents total sodium from food and frosting.

There were no differences in cumulative sodium balance between groups over the first week (Table 7). Further, by the end of week 2 there were no differences between Group 1 (control) and the fructose-treated groups, with or without resveratrol supplementation. However, by the end of the week 3, there were significant differences in cumulative sodium balance (Figure 26). The cumulative sodium balance in both fructose plus high salt groups (with or without resveratrol) increased significantly, 3.8-fold greater than the normal sodium diet groups (1 and 2), suggesting increased sodium retention. However, resveratrol treatment had no effect in reducing cumulative sodium balance. The increase in positive sodium balance representing sodium retention during week 3 in the 20% fructose and high salt groups with or with resveratrol (Groups 3 and 4), corresponds directly to the increases in blood pressure (Figure 24).

Table 7: Cumulative Sodium Balance (µEq NaCl):

	Day 1	Day 5	Day 7	Day 19	p-value
Control	**2.59±0.19**	**13.23±0.79**	**29.32±1.37**	**41.45±2.03**	
F+R	**2.27±0.13**	**12.10±0.62**	**28.16±1.54**	**39.52±2.31**	
F+HS	**2.37±0.16**	**11.97±0.51**	**26.88±1.39**	**153.26±8.46**	***p*<0.001 vs. control**
F+HS+R	**2.52±0.11**	**12.89±0.64**	**29.57±1.41**	**156.25±8.75**	***p*<0.001 vs. control**

F = fructose, HS = high salt, and R = resveratrol

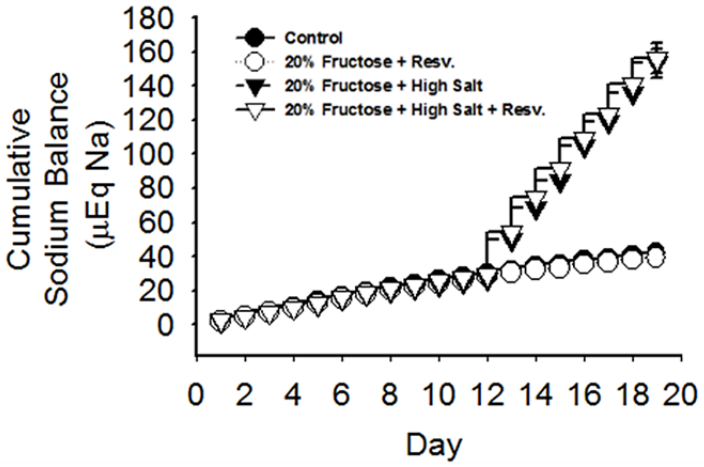


Figure 26: Sodium Balance over 3 weeks. The cumulative sodium balance in both fructose plus high salt groups (with or without resveratrol) increased significantly suggesting increased sodium retention. Resveratrol treatment had no effect in reducing cumulative sodium balance.

Fecal Sodium Excretion

To examine the effects of normal diet, 20% fructose, and high salt diet with and without resveratrol on fecal sodium excretion as a percent of total sodium excretion, we measured fecal sodium over the course of 3 weeks (Table 8). There were no differences in fecal sodium excretion across all groups during week 1 with normal rat chow as a percent of total sodium excretion. Again, during week 2, the addition of 20% fructose with or without resveratrol supplementation produced no differences in percent of fecal sodium excretion as a percent of total sodium excretion. During week 3, when high salt was added to the diet (in Groups 3 and 4), fecal sodium excretion decreased as a percent of total sodium excretion from 3.3±0.4 and 5.1±2.2% to 0.4±0.2 and 0.4±0.1% in Groups 3 and 4, respectively ($p<0.001$). Overall, fecal sodium excretion across groups was consistent with or without resveratrol supplementation and

represented a trivial, but consistent amount of total sodium excretion. Interestingly, with the addition of high salt diet, excess sodium intake did not pass through the digestive system to increase fecal sodium excretion.

Table 8: Fecal Sodium Excretion as a Percent of Total Sodium Excretion (%)

	Week 1	Week 2	Week 3
Control	**3.8±0.3**	**4.0±0.3**	**2.5±0.3**
F+R	**3.4±0.5**	**4.2±0.4**	**2.7±0.2**
F+HS	**4.0±0.6**	**3.3±0.4**	**0.4±0.2**
F+HS+R	**3.8±0.8**	**5.1±2.2**	**0.4±0.1**

F = fructose, HS = high salt, and R = resveratrol

Urinary Excretion of NO metabolites (NO_2/NO_3) and 8-Isoprostane

We measured urinary excretion of NO metabolites (NO_2/NO_3) and urinary excretion of 8-isoprostane on the concluding day of the study. To test if sodium excretion was influenced by resveratrol-stimulated nitric oxide production we sampled urinary excretion of NO_2/NO_3 in the four groups. NO_2/NO_3 excretion in the four groups were: 1) 1579±126, 2) 1452±175, 3) 2375±242 and 4) 1902±242 µM/24 hr (Figure 27). Collectively, the high salt groups (Group 3 and 4) had significantly higher nitrate/nitrite excretion (p<0.04) than normal salt groups 1 and 2. Resveratrol had no effect on nitrate/nitrite excretion, either with a normal salt diet or in groups with a high salt diet.

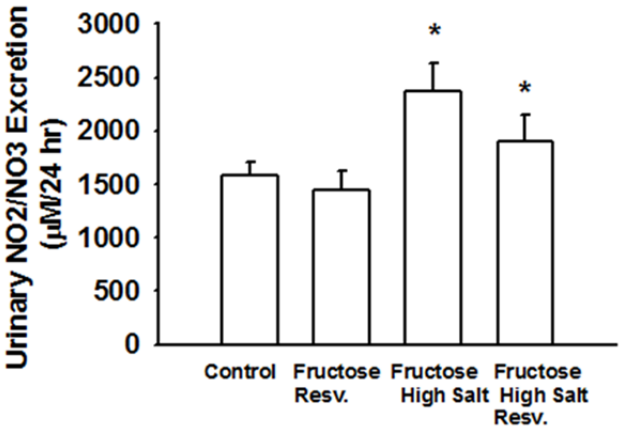


Figure 27: Urinary Excretion of NO_2/NO_3. Urinary excretion of NO_2/NO_3 was significantly higher in fructose and high salt groups. Resveratrol did not increase NO_2/NO_3 excretion.

To evaluate if resveratrol reduced oxidative stress, we measured urinary excretion of 8-Isoprostane. 8-Isoprostane was significantly higher in all fructose-treated groups compared to control: 1) 16381±1303, 2) 32176±3894, 3) 29387±2848, and 4) 33063±4305 pg/24 hr (p<0.003) (Figure 28). Resveratrol supplementation had no effect on fructose-induced oxidative stress in the presence of fructose or fructose and high salt.

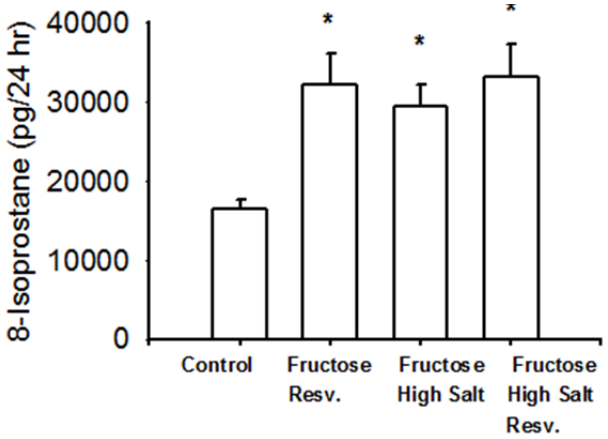


Figure 28: Urinary Excretion of 8-Isoprostane. 8-Isoprostane excretion increased in all fructose-treated groups. Resveratrol did not decease oxidative stress.

Plasma Renin Activity (PRA)

Plasma renin activity (PRA) for the four groups were: 1) 3.27±0.58, 2) 3.48±0.45, 3) 2.05±0.27 and 4) 2.66±0.43 ng AI/ml/hour. PRA was suppressed in both high salt diet groups (groups 3 and 4) vs. normal salt groups 1 and 2 (p<0.026). There were no differences in PRA between the resveratrol-treated and non-treated groups (Figure 29).

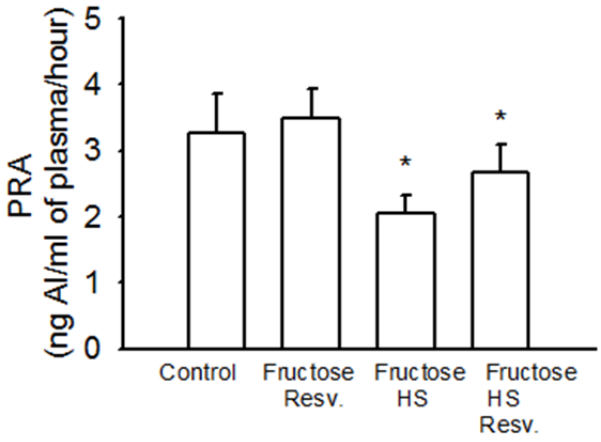


Figure 29: Plasma Renin Activity: High Salt diet decreased PRA. Resveratrol had no effect.

Plasma Glucose, Insulin, Glucose Tolerance Test and Renal Hypertrophy

Plasma glucose was measured at the conclusion of the study to evaluate if the onset of hypertension preceded the development of diabetes (Figure 30). Plasma glucose levels at the end of week 3 were: 1) 64±2, 2) 72±2, 3) 74±2, and 4) 68±1 mg/dl. Plasma levels were significantly higher in the three fructose treated groups, yet remained in the normal glycemic range of a normal fasted rat (p<0.002). Plasma glucose levels were unaffected by resveratrol treatment.

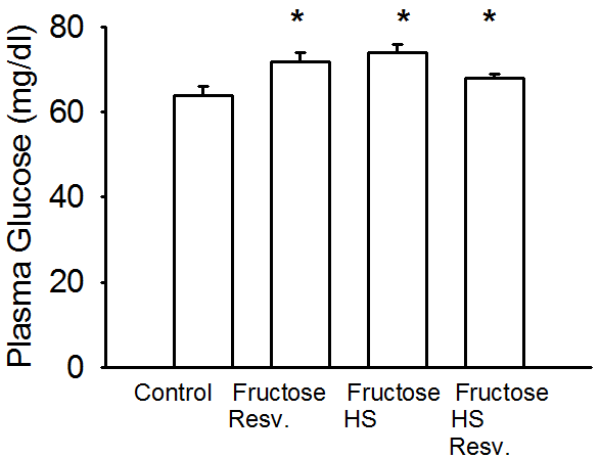


Figure 30: Fasted Plasma Glucose: Plasma glucose were elevated in all fructose-fed groups. Resveratrol had no effect on plasma glucose.

Plasma insulin was measured to assess whether the development of fructose-induced salt sensitive hypertension was independent from the onset of insulin resistance (Figure 31). Plasma insulin levels in the four groups were: 1) 1.09±0.18, 2) 2.18±0.43, 3) 2.39±0.37, and 4) 1.94±0.19 ng/ml. There were no differences in insulin levels among the fructose-treated groups (Groups 2, 3, and 4). However, insulin levels were higher in fructose-treated group as compared to Group 1, control (p<0.005). Resveratrol treatment had no effect on plasma insulin levels (Figure 31).

Glucose tolerance tests were run rats on a control diet, after 2 weeks of either 20% fructose-feeding or 20% fructose plus high salt to assess the possible development of insulin resistance or diabetes. While the fructose-fed rats had a higher baseline

plasma glucose (Figure 32), all three groups increased to a similar blood glucose levels, then returned to their baseline in a similar fashion.

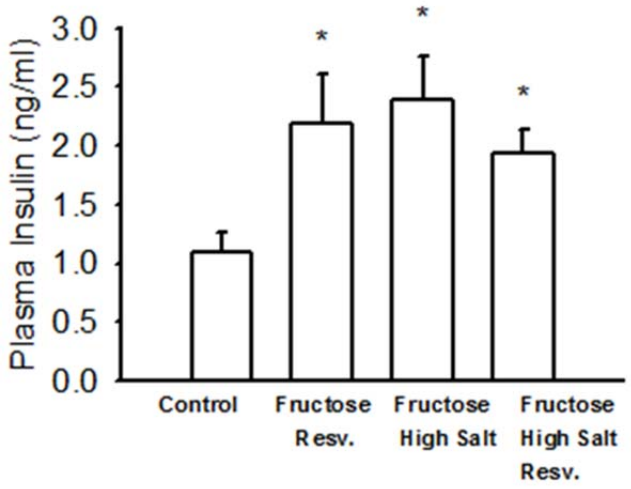


Figure 31: Fasted Plasma Insulin: Insulin levels were elevated in all fructose-fed groups. Resveratrol had no effect on insulin levels.

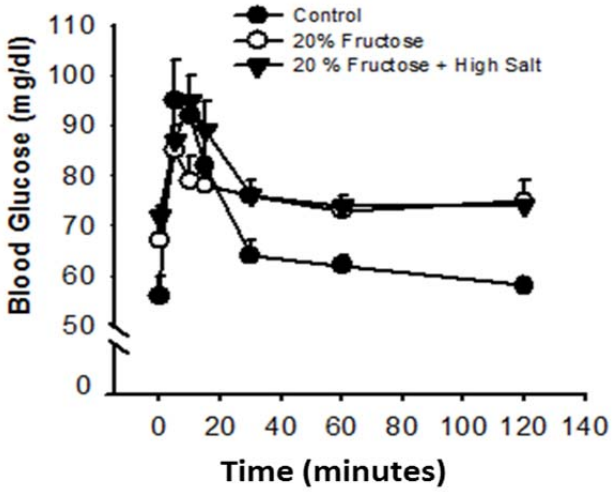


Figure 32: Glucose Tolerance Tests in Control, 20% Fructose (treated for 2 weeks), and 20% Fructose plus high salt.

To assess possible renal hypertrophy, total kidney weight/body weight ratios were calculated and these values for the four groups were: 1) 6.89, 2) 6.29, 3) 6.60, and 4) 6.55. The ratios were not different between the four groups suggesting no renal hypertrophy.

Negative Control: Bolus Injection of Resveratrol

We performed a bolus intravenous injection of resveratrol as we had previously performed (see chapter 1) is to confirm resveratrol is biologically active and still induced

renal vasodilation. Since we observed no effect of resveratrol on systolic blood pressure (Figure 24), we wanted to confirm it was still biologically active. We found a resveratrol bolus increased renal blood flow from 8.32 ml/min/gkw to 9.22 ml/min/gkw and decreased renal vascular resistance from 13.10 ARU to 10.85 ARU. Post-injection renal hemodynamics returned to baseline. This suggests the resveratrol was still biologically active.

Discussion

The prevailing literature suggests that resveratrol, acting through the kidney, may induce natriuresis and this may play a role in lowering blood pressure. However, this idea has not been studied. The kidney plays an essential role in blood pressure regulation by controlling short-term and long-term NaCl reabsorption and water balance. The known mechanisms of resveratrol act to increase nitric oxide and decrease superoxide production suggest that resveratrol may work to decrease sodium reabsorption. Resveratrol has been shown to increase endothelial nitric oxide synthase expression and increase nitric oxide synthesis (177, 178, 185). Resveratrol is found to reduce superoxide production through NADPH (nicotinamide adenine dinucleotide phosphate-oxidase) oxidase inhibition (58). Reductions in superoxide and oxidative stress can increase nitric oxide availability. Relevant to our fructose-induced salt-sensitive model, fructose is shown to increase Angiotensin II (Ang II) in fructose-fed rats. Ang II stimulates NADPH oxidase through its actions on AT1 receptors and increases superoxide (166). NADPH oxidase inhibition in aortic strips in fructose-fed rats has been shown to reduce superoxide production (150). Superoxide within the kidney has been shown to stimulate sodium absorption within the thick ascending limb (157). Conversely, NO inhibits sodium reabsorption in rat thick ascending limb (126).

Lastly, superoxide is increased during nitric oxide deficiency and this may contribute toward the development of salt-sensitive hypertension (94)

Collectively, these data support our hypothesis that chronic ingestion of resveratrol would reduce fructose-induced salt sensitive increases in blood pressure through decreasing sodium reabsorption through a nitric oxide-dependent mechanism. In this study, we are the first group to use resveratrol treatment to measure sodium homeostasis using metabolic balance studies. Data from our study suggests: 1) 20% fructose in the presence of high salt intake is associated with positive sodium balance concomitant with significant increases in blood pressure. Strikingly, changes in blood pressure occurred within only two weeks of initiating 20% fructose treatment. 2) Resveratrol treatment was ineffective in ameliorating fructose-induced salt retention, nor did it blunt the salt-sensitive increases in blood pressure.

Resveratrol research crosses many disciplines, for purposes in this paper, we limit the scope of this discussion exclusively to the effects of resveratrol on the cardiovascular and renal systems. Within the literature there is currently a large gap of knowledge of the effect of resveratrol on normal renal physiology. However, there are an abundance of studies showing resveratrol lowering blood pressure in Ang II models of hypertension and spontaneously hypertensive rat (SHR). Thandapilly et al (169) treated SHR with grape powder (600mg/day) for ten weeks. At the end of 10 weeks, the grape powder-treated SHR had significantly lower blood pressure. Dolinsky et al (42) treated 10 week old SHR with resveratrol (4g resveratrol/kg rat chow) for 5 weeks and then removed resveratrol treatment in the final 2 weeks. Blood pressure was monitored via implanted telemetry. The SHR-resveratrol group initially had lower blood pressure, but with the removal of resveratrol from the diet, blood pressure steadily

increased back to the level of the non-treated SHR (42). These experiments are difficult to make direct comparisons with our study because they used SHR, the varying lengths of their experiments eclipse our protocol.

We wanted to study the effect of resveratrol in a fructose-induced salt sensitive model. Hwang et al. (73) made the original observation that fructose intake can induce hypertension and insulin resistance. They demonstrated 66% fructose over 2 weeks could result in a 22 mmHg increase in blood pressure. A review of the literature indicates the development of hypertension due to fructose is both time and concentration-dependent (19). Thus, dietary intake of 40-60% fructose can induce hypertension in rats more rapidly than 10-20% fructose (29, 39, 73). Further, lower percentages of enhanced dietary fructose intake (10-20%) can still induce hypertension, but the onset of hypertension requires a longer fructose treatment time (2). The prevailing literature suggests 20% fructose has little to no effect on blood pressure within 1 week and more often requires 4-12 weeks to increase blood pressure (19, 29). Within our study, in agreement with the literature, we find that 20% enhanced fructose intake over 2 weeks has no effect on blood pressure. However, blood pressure did increase with the addition of high salt in rats pretreated over 1 week with 20% fructose, with or without the presence of resveratrol. Overall, these data support salt-sensitive increases in blood pressure within our fructose model, and the time required for the onset of increased blood pressure is shortened with the presence of high salt in 20% fructose-treated rats.

The few reports of resveratrol supplementation in fructose-treated models in the existing literature are not equivalent counterparts to our study. We specifically use a mild level of added dietary fructose (20%), and we have a limited protocol using only 2

weeks of enhanced fructose-feeding. Extended fructose treatment can result in increases in blood pressure that are comorbid with the onset of diabetes and metabolic syndrome. These studies (discussed below) use significantly higher fructose intake (up to 3 times our dosage) or the length of their protocols were 2-5 times the length of our study. Leibowitz et al (98) treated rats with 60% fructose over 5 weeks with resveratrol of varying doses (200, 400 and 800 mg/kg/day) in the final 2 weeks (98). 60% fructose increased systolic blood pressure approximately 25 mmHg in all groups after 3 weeks. In the resveratrol-supplemented groups (by week 5) blood pressures were lower than fructose alone group. However, even with resveratrol treatment, blood pressures were still significantly elevated in the presence of 60% fructose (≈150 mmHg). These blood pressure values are significantly higher than the increases in blood pressure in our study.

In experiments with fructose levels more analogous to our fructose dosage, Akar et al. (3) treated rats with 20% fructose over ten weeks in the presence or absence of resveratrol (50 mg/L) and found 20% fructose induced significant increases in systolic blood pressure and resveratrol treatment decreased elevated blood pressure. The increases in blood pressure they report due to 20% fructose seen over 10 weeks are unusually high with systolic values in the 160 mmHg range, 40 mmHg above control group blood pressure readings. Miatello et al (115) treated rats with 10% fructose over 6 weeks with and without resveratrol (10 mg/kg/day). After 2 weeks, the 10% fructose group had very mild increases in systolic blood pressure and by the end of 6 weeks blood pressure was increased by approximately 16 mmHg in the 10% fructose alone group. By the end of 6 weeks, blood pressure in the resveratrol-treated group was significantly lower as compared to fructose alone.

Cheng et al (33) fed 10% fructose to rats for 4 weeks with the introduction of resveratrol treatment (10 mg/kg/day) at week 2. They show resveratrol reduced fructose-induced increases in blood pressure. However, their results are highly atypical when compared with the current literature. The increases in blood pressure with 10% fructose after 1 week were exceptionally high, inconsistent with all the literature using 10 or 20% fructose supplementation. At 4 weeks their blood pressure data in the 10% fructose group were also much higher than expected. This is in distinct contrast to prevailing literature which shows 20% fructose having little or no effect on blood pressure during 1 week. 10% fructose has been shown to require 4-12 weeks to induce increases blood pressure (1, 39). Overall, it is difficult to make direct comparisons to our study with the existing resveratrol-fructose studies because fructose intake is higher and varying time courses which may carry the rat into a stage of insulin resistance-associated hypertension and metabolic syndrome.

It was our aim to investigate resveratrol-stimulated changes in sodium excretion and balance during fructose-induced salt-sensitive changes in blood pressure prior to the development of diabetes and metabolic syndrome seen with longer durations or higher levels of fructose supplementation. At the conclusion of our study we measured plasma glucose levels in fasted animals. We found 20% fructose in all fructose-treated groups, regardless of high salt or resveratrol treatment mildly elevated plasma glucose levels. However, plasma glucose levels were still within normal glycemic range for a non-diabetic rat. The mild elevation of plasma glucose suggests that there is a nearly sufficient pancreatic compensation. Plasma insulin levels were also elevated in the fructose-fed rats, but still within a normal range, and further resveratrol had no effect insulin levels.

We also performed glucose tolerance tests in three groups of rats: control, 20% fructose treated (2 weeks), and 20% fructose and high salt. The glucose tolerance tests in the fructose-fed groups had higher baseline plasma glucose levels, but in response to a glucose bolus, had similar curve profiles as compared to the control group. Collectively, these data suggest some metabolic changes could be in the initial stages, but our rats are not diabetic.

Oudot et al (129) showed that insulin resistance associated with fructose intake is linked to increases in proximal tubular and glomerular area also known as renal hypertrophy. In our study we found that there were no differences in kidney to body weight ratios, suggesting within our 2 week time course there was no renal hypertrophy associated with fructose consumption. With all of these factors taken together, it appears the elevations in blood pressure within our model are occurring prior to the development of diabetes and metabolic syndrome.

Blood pressure in all four groups did not increase during week 1 while receiving a normal rat chow diet, nor did blood pressure increase with the addition of 20% fructose in the following week. In our study, blood pressure in response to 20% enhanced fructose-feeding was similar to Abdulla et al (1). In the next week, blood pressure significantly increased in groups 3 and 4 (20% fructose), only with the addition of the high salt diet with and without resveratrol. Resveratrol treatment was unable to prevent the salt-sensitive increases in blood pressure. It is unknown if resveratrol-induced reductions in blood pressure require extended treatment. However, it was the succinct aim of this study to measure possible resveratrol-stimulated changes in sodium balance during the salt-driven initial increase in blood pressure.

The literature suggests resveratrol may increase nitric oxide generation and

reduce oxidative stress (58, 177, 178, 185). Balancing sodium reabsorption is important in regulation of long term blood pressure, and explains why thiazide-type diuretics are often a first-line antihypertensive therapy (103). Increased nitric oxide or decreased superoxide in the renal thick ascending limbs is shown to inhibit sodium reabsorption (126, 157). If this is the case, resveratrol acting within the nephron may reduce the propensity to develop salt-sensitive hypertension as found in our fructose model.

Metabolic studies show that increased positive sodium balance is associated with the onset of hypertension in spontaneously hypertensive rats (12). Moreover, defects in sodium reabsorption are linked with salt-sensitive hypertension (13, 118). We found resveratrol had no effect in reducing cumulative sodium balance even with the addition of high salt to our fructose-induced salt-sensitive model. Interestingly, independent of resveratrol, the increases in blood pressure occurred during the increases in cumulative sodium balance in the presence of fructose when high salt was added. The data gained from our metabolic studies is the first to report a positive sodium balance linking increases in blood pressure to salt in the presence of 20% fructose.

With the introduction of fructose (Group 2, 3, and 4) we observed significant decreases in urinary sodium excretion; changes absent in the control group. The decreases in urinary sodium excretion also coincided with decreases in food consumption due to (sodium-free) fructose replacing calories within the diet. The decreases in sodium excretion suggest fructose may increase sodium reabsorption. When high salt was added to the diet, as expected we saw significant increases in sodium excretion. Divergent from our hypothesis, we saw no differences in urinary sodium excretion with or without resveratrol treatment in our high salt groups (Groups 3 and 4). We measured fecal sodium excretion in a third of the rats to assess whether the

high salt diet would disproportionately be excreted within the stool versus the kidney. We found that during normal diet with or without 20% fructose, fecal sodium excretion was relatively low and consistent across groups (3-5%) and when high salt was added to the diet the majority of sodium was excreted through urine and not the stool. Overall, we found resveratrol treatment, independent of fructose or high salt, had no effect on increasing sodium excretion.

There is substantial evidence that oxidative stress and nitric oxide deficiency contributes towards hypertension (182). A sodium-restricted diet in fructose fed rats has been shown to reduce oxidative stress and inflammation independent of blood pressure (129). Urinary excretion of nitric oxide has been shown to increase in a fructose fed rats (129). Additionally, high salt diets have been shown to increase urinary nitric oxide excretion (151, 181). This may be a compensatory response to facilitate increased sodium excretion to help maintain normal blood pressure. To what degree both fructose and high salt contribute towards increased nitric oxide excretion in the same *in vivo* model is unknown.

To test if resveratrol reduced sodium reabsorption through a nitric oxide or superoxide dependent mechanism, we measured urinary excretion of nitric oxide metabolites (NO_2/NO_3) and 8-isoprostane. In our study, we observed NO_2/NO_3 excretion was significantly increased in our high salt groups (Groups 3 and 4). Contrary to our hypothesis, our dose of resveratrol treatment had no effect on nitric oxide excretion. The explanation remains elusive, but it may be in part that since both fructose and high salt increased nitric oxide production, it exists at such a sufficiently high level that resveratrol would have little to no effect.

Pharmacokinetic studies in humans treated with oral 14C-resveratrol shows

resveratrol is well absorbed (70%+), but low circulating levels of non-degraded resveratrol suggest rapid degradation and low availability (176). Juan et al (84) demonstrated high concentrations of resveratrol in at kidneys following infusion. Resveratrol treatment in isolated thick ascending has been shown to increase nitric oxide bioavailability (50). Majid et al (108) have shown, when nitric oxide is inhibited in dogs, sodium excretion is decreased suggesting NO contributes towards diuresis and natriuresis *in vivo.* Bertelli et al (17), in a model of acute kidney failure induced by ischemia, showed rats pretreated with resveratrol raised urinary excretion of cGMP, a marker for renal nitric oxide synthase activity.

We also measured urinary excretion of 8-isoprostane to test if resveratrol would lower oxidative stress. Resveratrol, within multiple tissues, has been shown to decrease 8-isoprostane levels, and also decrease urinary isoprostane excretion in a mouse model of diabetes (34, 90, 116). Within the existing literature there are several reports of 60% fructose feeding from 6 to 12 weeks, as well as 10% fructose supplementation over 12 weeks, increasing 8-isoprostane excretion (43, 72, 129, 160). However, the fructose levels in these experiments are either really high or fed over 6-times the length of our experiment. In our study, we report significantly higher urinary 8-isoprostane excretion within all fructose-treated groups (Groups 2, 3, and 4), but resveratrol had no effect on 8-isoprostane excretion, presumably not decreasing oxidative stress. Notably, we observed that 20% fructose, even without high salt or increases in blood pressure, increased oxidative stress in as little as 2 weeks.

To evaluate the effect of 20% and high salt diet, with or without resveratrol on PRA, we measured PRA at the conclusion of our study. Angiotensin II type 1 receptor antagonists have been shown to reduce fructose-induced increases in systolic blood

pressure suggesting the renin-angiotensin system plays a role in the development of fructose-induced hypertension (71, 75, 87, 114). We found that PRA was suppressed in our fructose-treated high salt groups as compared to non-high salt groups. Increased sodium delivery within the nephron is known to have an inhibitory effect of plasma renin activity (7). Overall, resveratrol treatment had no effect on PRA in either normal salt or high salt diet rats.

In summary, our findings suggest 2 weeks of dietary resveratrol treatment at 146 mg/day is ineffective in preventing increases in blood pressure due to fructose-induced salt sensitivity. This may be a function of limited resveratrol exposure; either due to an insufficient dose or the lack of sufficient duration of the treatment. Alternatively, resveratrol may just not work as an antihypertensive in this model of hypertension. Notably, the increases in blood pressure with 20% fructose were only observed with the addition of high salt (15-16 mmHg increase in Groups 3 and 4) and this coincided with a large increase in sodium retention. The increase in blood pressure was produced within only 2 weeks preceding the onset of hypertension typically associated with 20% fructose alone (1, 29). The elevated blood pressure also preceded the onset of diabetes and metabolic syndrome seen with fructose-enhanced diets over longer durations or higher levels of fructose consumption. There were no indications of renal hypertrophy, hyperglycemia, shifts in the glucose tolerance tests, or overt hyperinsulinemia within our 2 week 20% fructose model. Digestion of resveratrol in pair-fed metabolic studies did not increase sodium excretion, nor did it decrease cumulative sodium balance. Resveratrol did not diminish oxidative stress due to fructose. Overall, our data suggests that high fructose consumption coupled with high salt taken can have deleterious effects, increasing blood pressure and oxidative stress.

Increases in urinary nitric oxide excretion, due to fructose and high salt were not able to compensate for increased sodium load, even with resveratrol added to the diet.

CHAPTER 5

CONCLUSION AND PERSPECTIVES

Conclusion

We systematically investigated the effects of resveratrol on renal physiology using the following approaches: 1) the acute effects of resveratrol on renal hemodynamics by administering a single bolus resveratrol into systemic circulation in anesthetized rats, 2) the sustained effects of resveratrol on renal hemodynamics and renal function during continuous intravenous resveratrol infusion, 3) the effects of chronically ingested resveratrol on blood pressure and sodium retention in a model of mild Ang II-induced increases in systemic blood pressure, and finally 4) the effects of chronically ingested resveratrol on blood pressure and sodium retention in a salt-sensitive fructose-induced model of hypertension.

An acute bolus of resveratrol delivered into systemic circulation, independent of changes in blood pressure, influenced changes in renal hemodynamics. Resveratrol increased renal blood flow and decreased renal vascular resistance. Renal vasodilation was contingent on the resveratrol dose administered. No effect was seen at lower doses. Renal vasodilation was mediated through resveratrol-stimulated nitric oxide and through a reduction in reactive oxygen species. Although resveratrol is reported to inhibit cyclooxygenase (78), vasodilation was not influenced by cyclooxygenase metabolism and vasodilatory prostanoids.

After our initial findings that resveratrol-induced renal vasodilation, we examined the effects of continuous intravenous resveratrol infusion. We found resveratrol infusion induced significant renal vasodilation while not altering either blood pressure or glomerular filtration rate. Unexpectedly, we found that resveratrol infusion also

produced significant natriuresis, independent of renal hemodynamic responses, suggesting it may have a direct effect on renal tubular sodium handling independent of perfusion pressure.

Data from the resveratrol-induced natriuresis experiment led us to test if resveratrol-induced natriuresis could blunt increases in blood pressure if resveratrol was consumed within the diet. The hypothesis was tested in two models: Ang II and fructose-induced salt sensitive hypertension. Resveratrol treatment was ineffective in preventing increases blood pressure in response to Ang II. Contrary to the original hypothesis, metabolic studies revealed that resveratrol had no measurable effect on net sodium handling within the nephron. Also, plasma renin activity was unaffected by resveratrol. Ultimately, we observed a reversal of the Ang II increase in blood pressure with chronic resveratrol treatment following 3 weeks of treatment, but this was due to an undetermined mechanism.

Further, we observed somewhat similar results with resveratrol in a model of fructose-induced salt sensitivity. Resveratrol, consumed through the diet, was ineffective in preventing increases in blood pressure due to fructose-induced salt sensitivity. The increase in blood pressure also preceded the onset of diabetes and metabolic syndrome seen with fructose-enhanced diets over longer durations or higher levels of fructose consumption. However, rats may have been considered pre-diabetic due to minor elevations in plasma glucose and insulin. Additionally, the increase in blood pressure was concomitant in the fructose treated groups only with the addition of high salt to the diet. Again, resveratrol ingestion did not measurably increase sodium excretion, nor did it decrease cumulative sodium balance. Fructose, independent of high salt or increases in blood pressure, increased oxidative stress and resveratrol had

no effect on decreasing oxidative stress as measured through urinary excretion of 8-isoprostane.

Taken all together the resveratrol data generated both from acute experiments and long term chronic studies reveal resveratrol stimulated biological effects are inherently dependent of its method of delivery. In our acute studies nitric oxide-dependent renal vasodilation following a resveratrol bolus was a transient event. With intravenous resveratrol infusion we observed preserved increased renal blood flow and decreased renal vascular resistance. Other agents can decrease renal vascular resistance in a more long action fashion. For instance, in two-kidney-one clip rats during nitric oxide inhibition, a losartan bolus (AT_1 receptors antagonist) has a long-lasting effect (past 40 minutes). In the non-clipped kidney, losartan can prevent large increases in renal vascular resistance during nitric oxide inhibition (153). Contrasting losartan to resveratrol suggests while both can reduce renal vascular resistance, the resveratrol effects are relatively short-lived.

The acute effects of resveratrol delivered by systemic injection juxtapose the chronic effects of resveratrol we observed in the Ang II and fructose-induced salt-sensitive models of elevated blood pressure. In the Ang II study, resveratrol did not blunt even mild Ang II-induced increases in blood pressure. It was only following 3 weeks of resveratrol consumption that we observed resveratrol reducing blood pressure. The effect of resveratrol lowering blood pressure was not due decreased sodium retention. A potential criticism of the Ang II study is that we did not perform metabolic studies prior to giving Ang II in osmotic minipumps. Also, it is possible that we could have missed changes in sodium handling and the rats could have adapted prior our metabolic measurements. However, blood pressure decreased in the final

weeks of the experiment in our resveratrol-treated group when we were measuring sodium balance. Conversely it could be argued that resveratrol-induced decreases in blood pressure require more extended periods of resveratrol treatment. Nevertheless, during the period we measured, resveratrol had no effect on sodium excretion or sodium balance.

Because of the possibility of missing changes in sodium retention during the Ang II experiment, we consciously decided to expand metabolic collections during the fructose-induced salt-sensitive model. We measured sodium excretion and cumulative sodium balance during the transition from a normal diet to experimental diets of fructose, high salt, and resveratrol. This would avoid the potential time-associated limitation of the previous study. Again, Resveratrol it did not blunt salt-sensitive increases it blood pressure. However, unlike the Ang II experiments in which we observed resveratrol eventually lowering elevated blood pressure, we saw no such effect in the fructose study. Our fructose study was two weeks shorter than the Ang II study. This raises the possibility that resveratrol may require extended treatment or resveratrol with its poor bioavailability simply has no effect in this model. Additionally, the chronic depressor effects could also have been due to a build-up of metabolites of resveratrol which might have additional biological activity especially over long durations.

The issue of bioavailability must enter into discussion. It might explain the seemingly diverging biological effects of resveratrol in our experiments: resveratrol entered directly into systemic circulation as opposed to dietary consumption. Poor bioavailability of unchanged resveratrol (4% or less; 4) within the blood stream may explain the striking contrast between the positive effects of renal vasodilation and natriuresis with resveratrol infusion while no natriuresis was observed when resveratrol

was taken through the diet. Resveratrol may reside in such a low concentration that no effect can be seen through normal dietary consumption.

A frequent criticism of resveratrol is that it is found in low concentration in wine and further upon consumption it has poor bioavailability. This criticism may carry weight. Despite total wine consumption increasing on per capita basis within the U.S. during the past two decades (U.S. Wine Institute), heart disease remains a leading cause of death for men and women within the United States (92, 143). Correlative studies do not seem to support resveratrol reducing cardiovascular disease risk. The results of the InCHIANTI Study (148), a longitudinal study of elderly individuals living in two Italian towns, were followed over a long term basis from 1998 to 2009 with follow up evaluations every three years. Participants were separated into quartiles based from total urinary resveratrol metabolite concentrations. The InCHIANTI study found no correlation between total urinary resveratrol metabolites and prevalence of cancer, cardiovascular disease, markers of inflammation, and cause of mortality. An editorial in Pharmacological Research by editor-in-chief, Francesco Visioli adeptly summarizes the failures of resveratrol research (175). Paraphrasing from “The resveratrol fiasco,” resveratrol has shown no proven human activity. He suggests that the use of non-physiological concentrations and cell culture experiments do not yield translatable clinical outcomes (175). In perhaps a definitive review of past resveratrol clinical trials, Tang et al (166), reports no resounding evidence that resveratrol lowers blood pressure in humans.

To that end, resveratrol supplementation may not act as an effective antihypertensive. The “resveratrol paradox” frequently mentioned in the literature may not be a paradox. The “resveratrol paradox” (a coined term designed to elicit intrigue)

describes an observation within French society. There are improved cardiovascular outcomes related to their wine consumption, despite French consumption of a high-fat diet. However, in the past twenty years wine consumption within the United States has increased and yet the U.S. is still plagued by high rates of hypertension and cardiovascular disease. Additionally, exogenous antioxidant supplements have not been proven to lower blood pressure in humans. The recent availability of resveratrol as dietary supplements at levels that are orders of magnitude higher than what wine provides may help end the resveratrol debate. Overall, the current literature suggests that resveratrol may exert a physiological effect in controlled animal models. It is clear from our data that resveratrol has distinct physiological actions on nitric oxide production, free radical formation, natriuresis, and sustained hypertension. However, the data does not yet suggest efficacy in prevention or diminishment of hypertension in humans.

My view of resveratrol is pragmatic. The lack of evidence of resveratrol having utility in humans is very forceful argument (166). While resveratrol consumption is not harmful to health, it is not a convincing antihypertensive treatment for humans. It is a fact that not all animal research may translate directly to humans. However, our observations showing consumption of fructose coupled salt intake abruptly increasing blood pressure is highly disturbing. In a broad sense and in retrospective, the fructose salt-sensitive model appears to offer greater translational opportunities for translational research in human hypertension over resveratrol.

Perspectives

It is rather trite, but the endeavor of research to answer questions has inevitably led to more unanswered questions. Although, resveratrol treatment in the Ang II and

fructose-induced salt sensitive models mostly generated negative data, the observation of continuous resveratrol infusion inducing natriuresis offers a prospect to further delve into the mechanisms of action of resveratrol.

Park et al. (7) have shown that resveratrol is a non-selective phosphodiesterase (PDE) inhibitor capable of inhibiting PDE-4. PDE-4 activity is found to be increased in Dahl salt-sensitive rats (8). Tanahashi et al demonstrated in anesthetized dogs that intrarenal arterial infusion of rolipram, a selective PDE4 inhibitor, increased renal blood flow, glomerular filtration rate, urine flow rate, and urinary Na^+ excretion. Their results of PDE4 inhibition are strikingly similar to the resveratrol data. In future studies, to further test the mechanism of resveratrol and its role in PDE4 inhibition *in vivo*, one could infuse with rolipram followed by Resveratrol.

To that end, resveratrol research and the scientific method to me is reminiscent of the Socratic Method, a form of inquiry between parties based on asking and answering questions. When experimental data is seemingly contradictory, experimental data forces the necessity of asking new questions and arriving at suitable conclusions. In this regard my training was not designed merely for the goal of performing technical work, but in preparation to learn how to be critical of my data and to ask questions.

APPENDICES

Appendix A – Wayne State University IACUC Approval Letter

WAYNE STATE UNIVERSITY

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
87 E. Canfield, Second Floor
Detroit, MI 48201-2011
Telephone: (313) 577-1629
Fax Number: (313) 577-1941

ANIMAL WELFARE ASSURANCE # A3310-01

PROTOCOL # A 12-04-13

Protocol Effective Period: February 17, 2014 – December 4, 2016

TO: Dr. Kevin Gordish
Physiology
5374 Scott Hall

FROM: Lisa Anne Polin, Ph.D.
Chairperson
Institutional Animal Care and Use Committee

SUBJECT: Approval of Protocol # A 12-04-13
"Resveratrol and Preservation of Renal Function (ADMIN-HFHS)"

DATE: February 17, 2014

Your animal research protocol has been reviewed by the Wayne State University Institutional Animal Care and Use Committee, and given final approval for the period effective **February 17, 2014** through **December 4, 2016**. Be advised that this protocol must be reviewed by the IACUC on an annual basis to remain active.

The work on the project will not involve the use of live animals conducted within Wayne State University or John D. Dingell VAMC facilities. The institution conducting this work requires an approved IACUC protocol for the live animal work conducted in that facility. However, be aware that the grantee always retains the primary responsibility for ensuring compliance with PHS Policy.

The Guide for the Care and Use of Laboratory Animals is the primary reference used for standards of animal care at Wayne State University. The University has submitted an appropriate assurance statement to the Office for Laboratory Animal Welfare (OLAW) of the National Institutes of Health. The animal care program at Wayne State University is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Appendix B – Henry Ford Health System IACUC Approval Letter

Research Administration
CFP-Basement 046
2799 West Grand Boulevard
Detroit, MI 48202-2689
(313) 916-2024 Office
(313) 916-2018 Fax

Date: April 7, 2014

To: William Bielrwaltes
Internal Medicine

Fm: Frederick Valeriote, Ph.D., Chair
Robert Knight, Ph.D., Vice Chair
Institutional Animal Care and Use Committee

Re: **"Effect of Resveratrol on Renal Hemodynamics and Modulation of Renin Secretion in a Model of Fructose-Induced Hypertension"** (IACUC No.1320)

Period of IACUC Approval: **April 4, 2014 through April 3, 2017**

Number of Animals Approved: 300 Rats (210 Sprague-Dawley and 90 SHR)

Dear Dr. Bielrwaltes:

Thank you for submitting an application for review by the Institutional Animal Care and Use Committee. The application was presented at a convened meeting on All concerns have been adequately addressed and full approval has been granted on April 4, 2014.

The expiration date for this study is April 3, 2017. In order to remain compliant with federal regulations, a new, complete IACUC Protocol must be submitted for full board review if you intend to proceed with this line of work beyond the above indicated expiration date. A Final Report must be submitted at the completion of the 3-year approval period or upon early termination of the project.

Any adverse effects on animals must be reported to the IACUC as soon as possible.

A copy of the signed and stamped protocol indicating approval of the Institutional Animal Care and Use Committee is enclosed for your files. Please feel free to contact Linda Murray at 916.2315 if you have any questions regarding this matter.

Enc.

cc: Bioresources

Appendix C – American Journal of Physiology Publishing Agreement

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Title: Resveratrol induces acute endothelium-dependent renal vasodilation mediated through nitric oxide and reactive oxygen species scavenging

Author: Kevin L. Gordish,William H. Beierwaltes

Publication: Am J Physiol-Renal Physiology

Publisher: The American Physiological Society

Date: Mar 1, 2014

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Appendix D – Physiological Reports Publishing Agreement

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Physiological Reports ISSN 2051-817X

ORIGINAL RESEARCH

Sustained resveratrol infusion increases natriuresis independent of renal vasodilation

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Keywords
Glomerular filtration, natriuresis, nitric oxide, renal blood flow.

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Physiol Rep, 2 (9), 2014, e12144, doi: 10.14814/phy2.12144

Abstract

Resveratrol is reported to exert cardio-renal protective effects in animal models of pathology, yet the mechanisms underlying these effects are poorly understood. Previously, we reported an *i.v.* bolus of resveratrol induces renal vasodilation by increasing nitric oxide bioavailability and inhibiting reactive oxygen species. Thus, we hypothesized a sustained infusion of resveratrol would also increase renal blood flow (RBF), and additionally glomerular filtration rate (GFR). We infused vehicle for 30 min followed by 30 min resveratrol at either: 0, 0.5, 1.0, 1.5 mg/min, and measured RBF, renal vascular resistance (RVR), GFR, and urinary sodium excretion. At all three doses, blood pressure and GFR remained unchanged. Control RBF was 7.69 ± 0.84 mL/min/gkw and remained unchanged by 0.5 mg/min resveratrol (7.88 ± 0.94 mL/min/gkw, $n = 9$), but urinary sodium excretion increased from 2.19 ± 1.1 to 5.07 ± 0.92 μmol/min/gkw ($n = 7$, $P < 0.01$). In separate experiments, 1.0 mg/min resveratrol increased RBF by 17%, from 7.16 ± 0.29 to 8.35 ± 0.42 mL/min/gkw ($P < 0.01$, $n = 10$), decreased RVR 16% from 13.63 ± 0.65 to 11.36 ± 0.75 ARU ($P < 0.003$) and increased sodium excretion from 1.57 ± 0.46 to 3.10 ± 0.80 μmol/min/gkw ($n = 7$, $P < 0.04$). At the 1.5 mg/min dose, resveratrol increased RBF 12% from 6.76 ± 0.57 to 7.58 ± 0.60 mL/min/gkw ($n = 8$, $P < 0.003$), decreased RVR 15% (15.58 ± 1.35 to 13.27 ± 1.14 ARU, $P < 0.003$) and increased sodium excretion (3.99 ± 1.71 to 7.80 ± 1.51 μmol/min/gkw, $n = 8$, $P < 0.04$). We conclude that a constant infusion of resveratrol can induce significant renal vasodilation while not altering GFR or blood pressure. Also, resveratrol infusion produced significant natriuresis at all doses, suggesting it may have a direct effect on renal tubular sodium handling independent of renal perfusion pressure or flow.

Introduction

Resveratrol (3,5,4′-trihydroxystilbene), a polyphenol found in red wine and other foods, is often attributed to providing cardioprotective effects through nitric oxide-mediated vascular relaxation and possible antioxidant properties (Baur and Sinclair 2006). However, little is known about its effects on the kidney. Recently, we reported an i.v. bolus of resveratrol induces renal vasodilation by increasing nitric oxide (NO) availability and inhibiting generation of reactive oxygen species (Gordish and Beierwaltes 2014).

NO has an important role in the control of renal function and long-term regulation of blood pressure (Persson 2002). NO has multiple effects within the kidney including increasing renal blood flow (RBF) (Navar et al. 1996), increasing glomerular filtration rate (GFR) (Gabbai and Blantz 1999), and inhibiting thick ascending limb (TAL) sodium reabsorption (Herrera et al. 2006). NO, a vasodilator, is tonically produced in the vascular endothelium by the enzyme endothelial nitric oxide synthase (eNOS) and synthesized from the amino acid substrate L-arginine (Palmer et al. 1988). Inhibition of NOS results in acute hypertension and renal vasoconstriction (Baylis et al.

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ABSTRACT

THE ACUTE AND CHRONIC EFFECTS OF RESVERATROL ON RENAL FUNCTION AND BLOOD PRESSURE

by

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Advisor: Dr. William Beierwaltes

Major: Physiology

Degree: Doctor of Philosophy

We investigated the acute and chronic effects of resveratrol on renal function and blood pressure. We hypothesized that resveratrol would act as a renal vasodilator through a nitric oxide-dependent mechanism. In our acute studies, we found an intravenous bolus of resveratrol influenced changes in renal hemodynamics by increasing renal blood flow and decreasing renal vascular resistance. The mechanism of renal vasodilation was nitric oxide dependent and through a reduction of endogenous reactive oxygen species. Resveratrol-induced renal vasodilation was not influenced by COX metabolism and vasodilatory prostanoids. We found with continuous intravenous resveratrol infusion induced significant renal vasodilation via a nitric-oxide dependent mechanism while not altering either glomerular filtration rate or blood pressure in normal rats. Resveratrol infusion produced significant natriuresis at all doses, independent of hemodynamic responses, suggesting it may have a direct effect on renal tubular sodium handling independent of perfusion pressure, renal blood flow, or changes in renal vascular resistance. We hypothesized chronic ingestion of resveratrol would reduce Ang II-induced and fructose-induced salt-sensitive increases in blood pressure by

decreasing sodium reabsorption through a NO-dependent mechanism. In both models of elevated blood pressure, we found resveratrol does not increase sodium excretion of decrease sodium retention. Resveratrol treatment did not blunt initial increases in blood pressure in either the Ang II or fructose-induced salt sensitive model. However, following 3 weeks of treatment the increases in blood pressure began to reverse on the Ang II model, but not the fructose-salt sensitive model (during 2 weeks of treatment). Resveratrol did not decrease oxidative stress, as measured by urinary excretion of 8-isoprostane, in either model. Notable, short term exposure to fructose (2 weeks), independent of blood pressure or salt, increased oxidative stress. It is well-defined from our data that resveratrol has distinct physiological actions on nitric oxide production, free radical formation, natriuresis, and sustained hypertension. However, the data does not yet show efficacy in prevention of hypertension.

AUTOBIOGRAPHICAL STATEMENT

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Education

2015 Ph.D. in Physiology, Wayne State University, School of Medicine

2003 B.A., Biology, Wayne State University

Peer-reviewed Publications:

1. Atchison DK, Westrick E, Szandzik DL, **Gordish KL**, Beierwaltes WH. Parathyroid hormone-related protein stimulates plasma renin activity via its anorexic effects on sodium chloride intake. Am J Physiol Endocrinol Metab. 303(4):E457-63, 2012.
2. **Gordish KL** and Beierwaltes, WH. Resveratrol induces acute endothelium-dependent renal vasodilation mediated through nitric oxide and reactive oxygen species scavenging. AJP Renal, 2014.
3. **Gordish KL** and Beierwaltes WH. Sustained Resveratrol Infusion increases natriuresis independent of renal vasodilation and without changing Glomerular Filtration Rate. Physiological Reports, 2014
4. Rossi, NF, **Gordish KL**, Beierwaltes WH, Ortiz PA. Dietary Fructose Increases Renal Sympathetic Nerve Activity in Response to High Salt Diet in Awake Freely Moving Rats. ASN, 2015
5. **Gordish KL**, Beierwaltes WH. Chronic Resveratrol reverses a mild angiotensin II-induced pressor effect in a rat model. Amer. J Physiology. 2015. (*in review*)

Invited Oral Presentations:

1. **Gordish, KL** and Beierwaltes, WH. Resveratrol induces acute endothelium- dependent renal vasodilation. Henry Ford Hospital Division of Hypertension and Vascular Research, 2013.
2. **Gordish, KL** and Beierwaltes WH. Sustained Resveratrol Infusion increases Renal Blood Flow without changing Glomerular Filtration Rate in Normal Rat. 2014.
3. **Gordish, KL** and Beierwaltes WH. Effects of Resveratrol on Sodium Balance and Blood Pressure in Angiotension II infused rats. September 2014.
4. **Gordish, KL** and Beierwaltes. Presented a progress update on ongoing work. 2015

Selected Poster Presentations:

1. **Gordish, KL** and Beierwaltes, WH. Resveratrol induces acute endothelium- dependent renal vasodilation. Experimental Biology, 2013.
2. **Gordish, KL** and Beierwaltes, WH. Resveratrol induces acute endothelium- dependent renal vasodilation. Wayne State University and University of Michigan Symposium, 2013.
3. **Gordish, KL** and Beierwaltes WH. Sustained Resveratrol Infusion increases Renal Blood Flow without changing Glomerular Filtration Rate in Normal Rat. Experimental Biology, 2014.
4. **Gordish, KL** and Beierwaltes WH. Sustained Resveratrol Infusion increases Renal Blood Flow without changing Glomerular Filtration Rate in Normal Rat. Michigan APS Annual Meeting, 2014.
5. **Gordish KL**, and Beierwaltes. Chronic Resveratrol reverses a mild angiotensin II-induced pressor effect in a rat model. Experimental Biology, 2015.
6. **Gordish KL**, and Beierwaltes. Resveratrol and Angiotensin work. Henry Ford Research Day, 2015.