The Effect of Lentils (*Lens culinaris*) on Hypertension and Hypertension–Associated Vascular Remodeling in the Spontaneously Hypertensive Rat

by

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Abstract

Consumption of pulse crops has been linked to improvements in blood vessel function, but which pulse variety/type is better and how they work remains unknown. Two distinct studies were conducted to address these questions.

In study 1, spontaneously hypertensive rats (SHR) were fed control diet, or diets containing 30% (w/w) beans, peas, lentils, chickpeas, or mixed pulses. Normotensive Wistar-Kyoto (WKY) rats were fed the control diet. The lentil–containing diet significantly attenuated the rise in BP (+8 and +31 mmHg, vs. control) and reduced large artery remodeling compared to control. In study 2, SHR were fed control, mixed lentil, green lentil, or red lentil diets for 8 weeks. WKY were fed control or mixed lentil diets. Green lentils decreased arterial remodeling and improved arterial compliance in the SHR.

A diet high in green lentils could be a valuable adjunct to allopathic medicine to mitigate effects of hypertension on the vasculature.

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"*Research is what I'm doing when I don't know what I'm doing.*"

–Wernher von Braun (1912-1977)

"*Remember, kids: the only difference between screwing around and science is writing it down"*

–Adam Savage (1967–)

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ABI	ankle-brachial index
ACE	angiotensin converting enzyme
ACEI	ACE inhibitor
ACh	acetylcholine
AKT	protein kinase B
AngII	angiotensin II
ANOVA	analysis of variance
AT_1R	angiotensin receptor type 1
AT_2R	angiotensin receptor type 2
B	bean
BH4	tetrahydrobiopterin
BP	blood pressure
BW	body weight
C	chickpea
Ctrl	control
CaMK	Ca^{2+}/calmodulin-dependent protein kinase
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CHD	coronary heart disease
CSA	cross sectional area
CVD	cardiovascular disease
DASH	Dietary Approaches to Stop Hypertension
DBP	diastolic blood pressure
***dd*H_2O**	double distilled H_2O
ECG	echocardiogram
ECM	extracellular matrix
eNOS	endothelial nitric oxide synthase
ERK1/2	extracellular signal-related kinase 1/2 (aka p42/44 MAPK)
ET1	endothelin 1
$G\alpha_i$	G_i alpha subunit
GFR	growth factor receptor
GL	green lentil
GPCR	G protein-coupled receptor
GRB2	GFR binding protein 2
H_2O_2	hydrogen peroxide
HDL	high-density lipoprotein
HDL-C	HDL-cholesterol
HW	heart weight
IGF-1R	insulin-like growth factor 1 receptor
IHD	ischemic heart disease
IMT	intima-media thickness
IP	intraperitoneal
JNK	c-Jun N-terminal kinase
L	lentil
LDL	low-density lipoprotein
LDL-C	LDL-cholesterol
LV	left ventricle
LVH	LV hypertrophy
LVW	LV weight
M/L	media:lumen
MAP	mean arterial pressure
MAPK	mitogen-activated protein kinase
MeFV	mean flow velocity
MFV	minimum flow velocity

MI	myocardial infarction
ML	mixed lentil
MLC	myosin light chain
MLCK	MLC kinase
MLCP	MLC phosphatase
Mx	mixed pulse
NA	noradrenalin
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
Nox	NADPH oxidase
O/N	overnight
$O_2\bullet^-$	superoxide anion
OCT	optimal cutting temperature compound
$ONOO^-$	peroxynitrite
P	pea
p	phosphorylated
p38 MAPK	p38 mitogen activated protein kinase
PAD	peripheral artery disease
PBS	phosphate-buffered saline
PDGF-R	platelet-derived growth factor receptor
PFN1	profilin 1
PI	pulsatility index
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PLC	phospholipase C
PP	pulse pressure
PV	peak velocity
PVDF	polyvinylidene fluoride
PWV	pulse wave velocity
QMR	quantitative magnetic resonance
RAAS	renin-angiotensin-aldosterone system
RhoA	Ras homolog gene family, member A
RI	resistance index
RL	red lentil
ROCK	rho-associated protein kinase
ROS	reactive oxygen species
SBP	systolic blood pressure
SDS-PAGE	sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SE	standard error
SHR	spontaneously hypertensive rat
SMC	smooth muscle cell
SNP	sodium nitroprusside
SOD	superoxide dismutase
T2D	type 2 diabetes
TC	total cholesterol
TG	triglycerides
TPR	total peripheral resistance
VSMC	vascular smooth muscle cell
WKY	Wistar Kyoto rat

1. Introduction

1.1 Hypertension

Cardiovascular disease (CVD) is the leading cause of death worldwide (Bazzano *et al.*, 2003; World Health Organization, 2011). In Canada, CVD accounted for 29% of all deaths in 2008 and is a major burden on the healthcare system, costing an estimated $21.2 and $457.4 billon *per annum* in Canada and the USA (2006), respectively (Tarride *et al.*, 2009; Heart & Stroke Foundation, 2012). CVD manifests in many forms including atherosclerosis, coronary heart disease (CHD), myocardial infarction (MI), and heart failure. Many of these disease manifestations are accompanied by underlying problems, with hypertension among the most common. One in five adults, or approximately 6 million Canadians, were diagnosed with hypertension as of 2007, with the prevalence of the disease expected to increase (Public Health Agency of Canada, 2010; American Heart Association, 2013). As many or more people are estimated to have prehypertension, thus increasing their risk for future hypertension development (Chobanian *et al.*, 2003; Wang & Wang, 2004). More than 90% of the population will develop hypertension in their lifetime (Vasan *et al.*, 2002).

Hypertension is clinically diagnosed as systolic blood pressure (SBP) ≥ 140 mmHg and/or diastolic blood pressure (DBP) ≥ 90 mmHg (Chobanian *et al.*, 2003; Appel *et al.*, 2006; Guyton & Hall, 2006; Public Health Agency of Canada, 2010). Hypertension can be further divided into stages – prehypertension, stage 1 hypertension, and stage 2 hypertension – as well as into categories such as essential (also known as primary), non–essential (also known as secondary) and isolated

systolic hypertension. Based on systolic pressure/diastolic pressure, the following are values used for diagnosis: prehypertension[Ψ] ≥ 120–139/80–89 mmHg, stage 1 ≥ 140–159/90–99 mmHg, and stage 2 ≥ 160/100 mmHg (Chobanian *et al.*, 2003; Giles *et al.*, 2005). Essential hypertension, or hypertension with no known cause, is the most commonly diagnosed form of hypertension in people and accounts for more than 90% of all diagnoses (Oparil *et al.*, 2003; Ivanov *et al.*, 2005; Guyton & Hall, 2006; Mohrman & Heller, 2006).

Raising blood pressure (BP) can be relevant in certain physiological conditions such as during a haemorrhagic incident or in the "fight–or–flight" response. The circulatory system is a closed system that relies on tightly regulated pressure to maintain proper perfusion of tissues to deliver nutrients and remove cellular waste. From the millisecond control mediated by the baroreceptor reflex and neuronal/humoral control of resistance arterioles to moderate total peripheral resistance (TPR), to the long–term blood osmolarity control regulated by the kidney, there are many physiologically important mechanisms to regulate BP (Heagerty *et al.*, 1993; Navar, 1997; Cushman, 2003). However, for most people, hypertension is not a response to an overt problem, and is likely the result of a dysfunction in any one or a combination of the BP regulatory mechanisms.

Chronic hypertension causes changes within the cardiovascular system to combat the undue stress it poses on the body. One notable change is an increase in the wall thickness of the left ventricle. High arterial pressure requires the heart to generate more force to eject blood against the higher than normal pressure present

[Ψ] It is important to note that prehypertension is not a disease; rather, it increases the probability of developing hypertension and CVD risk (Chobanian *et al.*, 2003).

in the arteries (Gardin & Lauer, 2004). The heart responds by increasing the musculature of the left ventricle to generate increased force and maintain cardiac output. Originally compensatory, this mechanism can continue to the point of decompensation and result in heart failure – a manifestation of CVD. It should be noted that left ventricle hypertrophy (LVH) is a short–term BP–independent risk factor for CVD, but is likely related to long–term hypertension (Chambers, 1995). LVH is also related to diabetes, obesity and past coronary events (Gardin & Lauer, 2004). Similar hypertrophic growth is seen in the smooth muscle cells of the vasculature in order to deal with the increased stretch and shear stress associated with hypertension. Vascular remodeling causes the arteries to stiffen and is a major risk factor for future CVD events.

1.2 Arterial Stiffness and Pulse Wave Velocity

In a chronic hypertensive state, the vasculature undergoes structural changes in order to deal with the stresses associated with the pathological condition. This results in arterial smooth muscle cells undergoing hypertrophy to deal with the tensile and shear stresses encountered, which in the long term results in decreased distensibility of the arteries and is known as arterial stiffness (Gündüz *et al.*, 2009). Initially, the stiffening is asymptomatic, but as it progresses it can affect ambulation and organ function, and is indicative of and a risk factor for cardiovascular events (Luft, 2012). In properly functioning large arteries, the vessel walls expand slightly in response to the rapid volume ejected from the heart during systole. Then, during diastole, the arteries recoil to their normal size and push the volume of blood

temporarily 'stored' by the expansion into the arterial tree. Termed the "Windkessel Effect," the arterial expansion and passive recoil act to ensure a constant flow of blood through the arterial tree and into the capillaries, which avoids pulsatile flow in the distal arterial/capillary beds (London & Guerin, 1999; Luft, 2012). In stiff arteries, the Windkessel Effect is either diminished or completely abolished, which has implications on the arteries to which the blood is flowing, by creating a fluctuating pressure environment and altering the stresses experienced by the vessel wall. This can further exacerbate the extent of vascular remodeling, especially in resistance arteries, and contributes to end–organ damage such as renal failure (Bataineh & Raij, 1998; Intengan & Schiffrin, 2001; Lin *et al.*, 2008).

Arterial stiffness and, in humans, atherosclerosis, are problematic diseases that are largely undiagnosed until they reach advanced stages (Loke *et al.*, 2010). Increased arterial stiffness, next to atherosclerosis, is the best predictor of future cardiovascular disease and mortality (Zhou *et al.*, 2011). Arterial stiffening occurs naturally with age, as the arteries become less distensible and lose some of their normal function. However, in the presence of chronic hypertension, this stiffening occurs earlier and to a greater extent, putting strain on the heart and vascular system. This hypertension–associated vascular remodeling is an attempt by the vasculature to normalize the increase in pressure and shear stress experienced under these conditions (Moustafa-Bayoumi *et al.*, 2007; Gündüz *et al.*, 2009; Nakano *et al.*, 2010; Zhang *et al.*, 2011a). Continuous instead of pulsatile blood delivery to the capillaries depends on the elasticity of the larger arteries and smaller arterioles. This makes the process of arterial remodeling an interesting area of research, with

restoration of proper vascular function and/or reversal of remodeling events potential targets for therapy.

Arterial stiffening can be assessed through measurement of the pulse wave velocity (PWV), in m/s, by use of a Doppler probe (Najjar *et al.*, 2008). There are other methods to determine arterial stiffness such as pulse waveform analysis, finger photoplethysmography, or relating vessel morphology to distending pressure (Laurent *et al.*, 2006; Sakuragi & Abhayaratna, 2010). PWV measurement is considered the best non–invasive test of vessel stiffness, with stiffer vessels having higher PWV values (Milan *et al*., 2011; Chirinos, 2012). PWV has been compared to *ex vivo* animal vascular elastic measurements and was shown to be closely related to, and a predictor of, cardiovascular mortality irrespective of BP status (Gribbin *et al.*, 1976; Sakuragi & Abhayaratna, 2010). PWV is higher in the spontaneously hypertensive rat (SHR) than in the Wistar–Kyoto (WKY) rat, even at a young age, and is indicative of early onset vascular remodeling, occurring even prior to manifestation of hypertension (Marque *et al.*, 1999; Zhang *et al.*, 2011a). It has been suggested that PWV measurements could be a tool to identify normotensive patients with a higher risk of developing hypertension as PWV is linked to future increases in SBP (London & Guerin, 1999; Najjar *et al.*, 2008).

In humans, peripheral artery stiffening is usually accompanied by atherosclerotic plaque development and is called peripheral artery disease (PAD) (Golomb *et al.*, 2006; Cooke & Wilson, 2010). Due to the expense of Doppler machines or invasive nature of histological methods, the accepted diagnosis of PAD is the ankle–brachial index (ABI), which is calculated from the systolic pressure

taken at the ankle and brachial arteries (Golomb *et al.*, 2006; Cooke & Wilson, 2010). An ABI < 0.90 is considered to be significantly associated with PAD and is used clinically for diagnosis (Selvin & Erlinger, 2004; Golomb *et al.*, 2006; Cooke & Wilson, 2010). However, this would be difficult in the rat model and instead PWV along the aorta measured through the use of a Doppler probe is considered an acceptable approach to show arterial stiffness.

1.3 Animal Models of Hypertension and Vascular Remodeling

The SHR is a model of essential hypertension (Lemmer *et al.*, 1993; Doggrell & Brown, 1998; Lerman *et al.*, 2005). Resulting from the selective inbreeding of WKY rats with high BP, the SHR is the mostly widely used animal model for hypertension and CVD research (Doggrell & Brown, 1998; Pinto *et al.*, 1998; Scoggan *et al.*, 2003). The BP of the SHR is prehypertensive for the initial 6 weeks of life, rising to 180–200+ mmHg systolic by around the 20th week (Eilam *et al.*, 1991; Doggrell & Brown, 1998). It is this dramatic increase in SBP and the resulting stiffening and remodeling of arteries that make the SHR rat model analogous to human hypertension, albeit an extreme example. The WKY rat makes an apt normotensive control, having stable BP and expected, age–associated changes in vessel morphology and function (Marque *et al.*, 1999). The SHR is an extreme model of hypertension, with pronounced vascular remodeling. At 12 weeks of age, the SHR already has significantly higher media:lumen (M/L) ratios in mesenteric arteries than WKY animals, which can be attributed to increased shear stress (Gao *et al.*, 2008). In addition, the SHR has been responsive in studies testing drugs, diets, and

bioactive compounds (Li *et al.*, 2010; Zhong *et al.*, 2011; Behbahani *et al.*, 2010; Duarte *et al.*, 2001; Mukai & Sato, 2011; Sánchez *et al.*, 2006). Attenuation of vascular remodeling, arterial stiffness and BP through dietary intervention is the ultimate goal of this study, and underscores why the SHR was chosen, instead of the many other available rat models of hypertension as outlined in Table 1.

1.4 Molecular Mechanisms of Vascular Dysfunction and Remodeling

1.4.1 Endothelial Dysfunction

Cyclic adenosine monophosphate (cAMP), produced by adenylyl cyclase (AC), is an important vasodilatory signaling molecule. cAMP activates protein kinase A (PKA), which in turn inhibits myosin light chain kinase (MLCK) activation and hyperpolarizes VSMCs through potassium channel activation. $G\alpha_{(i)}$ is able to inhibit AC and subsequent cAMP production from cellular ATP. It has been shown in hypertensive humans and the SHR that overexpression of $G\alpha_{(i)}$ effectively shuts down this vasodilatory pathway (Amer, 1973; Anand-Srivastava, 1992; Böhm *et al.*, 1992). The main pathway leading to vasodilation is elicited through nitric oxide (NO) signaling.

Endothelial dysfunction is characterized by decreased responsiveness to endogenous vasodilators such as NO. NO is released by vascular endothelial cells in response to acetylcholine (ACh) and mechanical stimuli such as shear stress (Awolesi *et al.*, 1995; Guyton & Hall, 2006). NO primarily exerts its action on

Table 1: Rat models of hypertension

Hypertension Model	Etiology	Pros	Cons
Spontaneously Hypertensive Rat (SHR)/ Stroke prone SHR (SHR-SP)	• Phenotypic	• Model of essential hypertension[1,2] and >90% of human hypertension is essential or 'primary' • Predictable disease progression[1] • Cardiovascular remodeling[1]	• Little genetic variation among colonies[1], though cosegregation of genes can occur[2]
Two-Kidney, One-Clip (2K1C) *Similar to one-kidney, one clip and two-kidney, two clip*	• Surgical (Renal Artery Ligation)	• RAAS§ dependent hypertension, allowing system targeted approach[1] • Cardiovascular remodeling[1,2] • Some sensitivity to certain diet components[2]	• Secondary hypertension model[1,2] • Primarily result of RAAS activation[1] • Rapid onset of hypertension (2-weeks)[1,2] • Not always successful at generating sustained hypertension[1,2] • Complications due to surgery
Deoxycorticosterone Acetate (DOCA) and Salt Loading (DOCA-salt)	• Drug & Diet	• Sodium retention and over-consumption common in hypertensive people • RAAS independent hypertension[1] • Cost effective, low mortality[1]	• Requires salt-loading, changing diet composition • Constant injections of DOCA may alter metabolism

Diabetic Hypertensive Rats	• Drug Induced (streptozotocin administration to SHR) • Genetic (Cohen-Rosenthal or Zucker)	• Hypertension and diabetes usually occur in the same populations	• Complications/confounding results due to diabetes (*e.g.* non-hypertension diabetes-related remodeling and organ damage) • Zucker has limited increases in BP or SHR is treated to induce diabetes
Nitric Oxide Synthase Inhibition	• Drug Induced ($_L$-NAME$^{\partial}$)	• Cost effective, low mortality[1] • Phenotype of essential hypertension[2]	• Inhibits nitric oxide synthase activity impairing endothelial function[1,2] • May generate a phenotype not responsive to diet • Alters endothelial function tests on a pressure myograph

Table based on information in the reviews by [1]Doggrell & Brown (1998) and [2]Lerhman et al. (2005)
[§]RAAS – rennin-angiotensin-aldosterone system
$^{\partial}{}_L$-NAME – N^{ω}-nitro-$_L$-arginine methyl ester

guanylyl cyclase in the underlying smooth muscle cells (SMCs). Guanylyl cyclase generates cyclic guanosine monophosphate (cGMP), causing the cells to relax through cGMP–dependent activation of protein kinase G (PKG) (Galleano *et al.*, 2010). NO is produced by nitric oxide synthase (NOS) and, more importantly in the vasculature, the endothelial isoform – eNOS (Safar & Laurent, 2003; Mukai & Sato, 2009; Mukai & Sato, 2011). Young SHR exhibit similar eNOS expression and activity to age–matched WKY (Linz *et al.*, 1999). With increasing age, SHRs experience less active eNOS and NO bioavailability, resulting in impaired endothelium–dependent relaxation (Pinto *et al.*,1998; Linz *et al.*, 1999).

During periods of elevated oxidative stress, the bioavailability of NO decreases for several reasons: decreased phosphorylation and activity of eNOS, increased reactions between NO and superoxide anions ($O_2\bullet^-$), and uncoupling of eNOS causing it to generate $O_2\bullet^-$ instead of NO. There are several causes of decreased eNOS activity in the cells, one of which is related to the increase in angiotensin II (AngII) seen in hypertension. AngII works through receptors on the cell surface, angiotensin receptor types 1/2 (AT_1R & AT_2R) (Otsuka *et al.*, 1998). mRNA levels of AT_1R, AT_2R, and angiotensin converting enzyme (ACE) have been shown to be elevated in aortae of the SHR (Otsuka *et al.*, 1998). Through AT_1**R**, AngII up–regulates the RhoA/Rho–kinase (ROCK) pathway. RhoA/ROCK are able to prevent phosphorylation and activation of eNOS by protein kinase B (AKT), as well as having the ability to inhibit myosin light chain phosphatase (MLCP) activity and directly phosphorylate myosin light chain (MLC), thereby increasing sensitivity to calcium and contraction in the vascular smooth muscle cells (VSMCs) (Nunes *et al.*,

2010; Wirth, 2010; Hassona *et al.*, 2011). This Rho/ROCK pathway has been shown to be up–regulated in hypertensive patients, as well as in the SHR (Nunes *et al.*, 2010).

The Rho/ROCK pathway, as well as p38 mitogen activated protein kinase (p38 MAPK) activation, are associated with increased activity of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) (Takeshima *et al.*, 2011). Nox activity is the driving force behind $O_2^{\bullet -}$ production in the endothelium, tunica media and adventitia of the vascular wall (Zafari *et al.*, 1998; Sánchez *et al.*, 2006; Takeshima *et al.*, 2011). Typically, the subunits that constitute Nox in the vasculature are $p22^{phox}$, $Rac/p20^{phox}$, $p47^{phox}$, $p67^{phox}$, and a catalytic NOX subunit (NOX1/2/4). However, these subunits differ between cell types (neutrophils vs. VSMCs, for example) (Griendling *et al.*, 2000; Lassègue *et al.*, 2001; Chabrashvili *et al.*, 2003; Loffredo *et al.*, 2012). Increased expression of one or many of the subunits, as a result of hypertension or in response to vascular injury, has been associated with increased Nox activity and levels of oxidative stress (Lassègue & Griendling, 2010). Two subunits in particular, $p47^{phox}$ and $p22^{phox}$, have been shown to be over–expressed in a variety of tissues in response to hypertension, including the kidney, heart, and VSMCs (Rajagopalan *et al.*, 1996; Zalba *et al.*, 2000; Chabrashvili *et al.*, 2002; Li *et al.*, 2002). $O_2^{\bullet -}$ reacts with NO to generate peroxynitrite ($ONOO^-$), also a reactive oxygen species (Bataineh & Raij, 1998; Bartuś *et al.*, 2005; Mukai & Sato, 2011; Schulz *et al.*, 2011; Takeshima *et al.*, 2011). The reaction between NO and $O_2^{\bullet -}$ occurs faster than the reaction of $O_2^{\bullet -}$ with superoxide dismutase (SOD), further

limiting the bioavailability of NO during times of increased oxidative stress (Rajagopalan *et al.*, 1996).

The generation of $O_2\bullet^-$ and subsequent reaction with NO has immediate and downstream implications. Immediately, the bioavailability of NO is greatly diminished, decreasing the ability of endothelial cells to mediate vascular relaxation (Schulz *et al.*, 2011). The downstream effect is caused by the reaction of $ONOO^-$ and other ROS with tetrahydrobiopterin (BH4) (Lu & Kassab, 2011; Zhang *et al.*, 2011b). BH4 is the coenzyme that couples the reaction of eNOS with L–arginine, generating L–citrulline and NO (Schulz *et al.*, 2008; Zhang *et al.*, 2011b). When BH4 is oxidized, eNOS no longer generates NO, instead producing harmful ROS (Linz *et al.*, 1999; Schulz *et al.*, 2008; Lu & Kassab, 2011; Zhang *et al.*, 2011b; Moreau *et al.*, 2012). Decreased endothelial mediated vasodilatation as a result of diminished NO production is used to diagnose endothelial dysfunction, which is present in SHR (Pinto *et al.*,1998; Bartuś *et al.*, 2005; Mukai & Sato, 2011; Sudano *et al.*, 2011). It has been demonstrated in humans that acute, high–dose therapy with ascorbic acid, an antioxidant compound, can restore arterial compliance, similar to BH4 supplementation on its own (Moreau *et al.*, 2012).

There is evidence that the ability of Rho/ROCK to inhibit the activity of eNOS can work the other way around and NO generation can inhibit the activity of Rho/ROCK (Wirth, 2010). NO has also, in addition to other molecules such as prostacyclins and statins, been shown to be anti–thrombotic and anti–atherogenic, as well as protecting against cardiac, vascular and renal hypertrophy (Linz *et al.*, 1999; Navar *et al.*, 1997; Sudano *et al.*, 2011). In fact, NO decreases expression of

both ACE and AT_1R, opposing the actions of AngII (Bataineh & Raij, 1998). One group showed that ACE inhibition was sufficient to restore eNOS expression and NO production (Linz *et al.*, 1999).

1.4.2 Vascular Remodeling

The vasculature undergoes remodeling to combat altered hemodynamic stresses encountered as a result of chronic hypertension. This can result in changes to the intima–media thickness (IMT), a measure of the thickness of the *tunica intima* and *tunica media* of the vascular wall. An elevated IMT is related to increased arterial stiffness and PWV, altered extracellular matrix composition, and VSMC hypertrophy (Owens & Schwartz, 1982; Marque *et al.*, 1999). Initially, vascular remodeling occurs to compensate for the altered tensile and shear stress experienced during hypertension (Gündüz *et al.*, 2009). Over time this remodeling may become deleterious and perpetuate the disease state. This relationship is best described by the Law of Laplace equation:

$$(1)\quad \sigma = P \times \frac{r}{w}$$

where σ is wall stress, P is intraluminal pressure, r is vessel radius and w is wall thickness. From this equation, it is evident that as the pressure increases – as seen in chronic hypertension – the radius of the vessel must decrease and/or the wall thickness must increase to normalize the wall stress. This results in an observably higher media:lumen (M/L) ratio and the vessel becomes less elastic and stiffer (Heagerty *et al.*, 1993; Thandapilly *et al.*, 2012). This remodeling increases total

peripheral resistance (TPR) and, as a consequence, maintains the hypertensive state (Eftekhari *et al.*, 2012). Remodeling is marked by an increased M/L ratio, and patients with diabetes and hypertension have a higher M/L ratio than those with either hypertension or diabetes alone (Rizzoni & Agabiti-Rosei, 2012). With age and progression of stiffness, this process of remodeling and growth becomes maladaptive and may act to increase or maintain increased BP, contribute to end organ damage, and cause endothelial dysfunction (Raij, 1998; Intengan & Schiffrin, 2001; Gündüz *et al.*, 2009).

Vascular remodeling occurs in two ways: through hypertrophic and/or eutrophic remodeling (Intengan & Schiffrin, 2001; Behbahani *et al.*, 2010). Hypertrophic remodeling results in an increased media cross sectional area (CSA), whereas eutrophic remodeling results in a smaller lumen (Intengan *et al.*, 1999; Intengan & Schiffrin, 2001). Cytoskeleton reorganization and cellular hypertrophy are, in part, mediated by the Rho/ROCK pathway ultimately resulting in stress fiber formation (Hassona *et al.*, 2010; Nunes *et al.*, 2010; Hassona *et al.*, 2011). AngII, through ERK1/2, is able to increase cell growth as mentioned above. ERK1/2 activation is also able to up–regulate synthesis of collagen I (Intengan & Schiffrin, 2001). Collagen I is produced at a higher level in the SHR (Otsuka *et al.*, 1998). Alterations in extracellular matrix (ECM) production and deposition result in arterial stiffening and eventually can result in end–organ damage (Lin *et al.*, 2008). The balance between the major ECM components – collagen I/III and elastin – is a major determinant of arterial stiffness (Briones *et al.*, 2003; Arribas *et al.*, 2010; Zhou *et al.*, 2011). Evidence suggests that deposition of excess levels of collagen in

combination with degradation of elastin results in stiffer arteries (Zhou *et al.*, 2011). As expected, treatment of arteries with elastase results in markedly stiffer arteries (Briones *et al.*, 2003).

Profilin–1 (PFN1), which is activated downstream of AngII and possibly ERK1/2, is a protein that binds to actin monomers and aids in the regulation of actin polymerization (Moustafa-Bayoumi *et al.*, 2007; Cheng *et al.*, 2011). Actin remodeling by over–expression of PFN1 has been linked to smooth muscle cell hypertrophy in vessels such as the aorta and mesenteric arteries (Hassona *et al.*, 2010). Over–expression of PFN1 is also directly related to hypertension as seen in a transgenic mouse model developed by Moustafa–Bayoumi *et al.* (2007), which constitutively expresses PFN1 in the blood vessel wall. It is also expressed in the aorta of SHR to a greater extent than in the WKY model (Cheng *et al.*, 2011). In humans, PFN1 has been shown to be over–expressed in atherosclerotic plaques and can be detected in serum samples, making it a possible marker of atherosclerotic plaque development and/or progression (Caglayan *et al.*, 2010). However, rats are not generally susceptible to development of atherosclerotic plaques, but expression of PFN1 in the VSMCs can be used as a possible marker of SMC hypertrophy.

Hypertrophy and vascular remodeling have also been postulated to be a result of the activation of growth factor receptors such as PDGF–R, IGF–1R and EGF–R, in both response to, and absence of, ligand activation. Activation by non–ligand forces is evident when VSMCs are exposed to mechanical stress or increased AngII, which is experienced in times of transient and chronic hypertension. Hu *et al.* (1998) showed that mechanical stress, through intracellular signaling, can activate

PDGF–Rα and its downstream signaling cascade in the absence of the receptor ligand PDGF. In fact, through endothelin–1 (ET1) signaling and the downstream Ca^{2+}/calmodulin–dependent protein kinase (CaMK), the AKT and MAPK signaling cascades are activated (Bouallegue *et al.*, 2007). Both AKT and MAPK signaling are activated by growth factor receptors (GFR), and are able to mediate VSMC hypertrophy and hyperplasia. Linseman *et al.* (1995) demonstrated that increased AngII signaling – primarily through AT_1R – was able to activate c–Src, Shc and growth factor receptor binding protein 2 (GRB2). c–Src is a potent activator of Shc and PDGF–Rβ and, as such, is a convergence point of AngII and GFR signaling (Linseman *et al.*, 1995; Sesiah *et al.*, 2002). Additionally, *via* c–Src, AngII trans–activates epidermal growth factor receptor (EGF–R), another member of the GFR family with tyrosine kinase activity, which also activates ERK and contributes to cardiac and vascular remodeling (Ushio-Fukai *et al.*, 2001; Kagiyama *et al.*, 2002; Sesiah *et al.*, 2002). GFR trans–activation may be redox–sensitive and ROS, through c–Src activation by hydrogen peroxide (H_2O_2), causes enhanced GFR phosphorylation and stimulate associated signaling cascades (Ushio-Fukai *et al.*, 2001).

1.5 Current Approaches in Hypertension Treatment and Prevention

Poor diet and a sedentary lifestyle are two of the many risk factors for developing hypertension and for increased CVD risk. Other CVD risk factors include smoking, obesity, genetics, and diabetes, which are usually accompanied by hypertension (Appel *et al.*, 2006). In fact, of the over 2.4 million Canadians

(2008/09) diagnosed with type 1 or type 2 diabetes (T2D), 63% also had diagnosed hypertension as of 2007 (Public Health Agency of Canada, 2010; Public Health Agency of Canada, 2011). People diagnosed with hypertension also had a 6–fold elevated diagnosis rate of diabetes when compared to people without hypertension, indicating a close link between these disease states (Public Health Agency of Canada, 2010). Further, >75% of Canadians with diabetes have a body mass index (BMI) in the overweight or obese range, and >32% of obese Canadians have diagnosed hypertension, which is twice the incidence of the non–obese population (Public Health Agency of Canada, 2011; Statistics Canada, 2012).

Current treatments for hypertension include drugs such as diuretics, ACE inhibitors (ACEIs), and β–blockers, as well as diet and lifestyle changes. BP control in more than 60% of patients requires two or more drugs (Chobanian *et al.*, 2003; Cushman, 2003; Williams *et al.*, 2004). However, since most hypertension has unknown causes, the drug treatments are aimed at alleviating symptoms as opposed to addressing the cause (Ivanov *et al.*, 2005; Ivey *et al.*, 2008). For example, treatment with the β–blocker atenolol can effectively reduce BP, but it does not affect arterial remodeling and structure (Thybo *et al.*, 1995; Schiffrin *et al.*, 2000). Stopping the progression of the pathologies associated with hypertension beyond symptoms should be the ultimate goal, making endothelial dysfunction and vascular remodeling attractive targets for therapeutic intervention. Furthermore, targeting the symptoms does not reverse the damage done, nor does it necessarily generate a state where hypertension will not return upon cessation of treatment.

Changing one's diet and lifestyle, while difficult, is an excellent approach to combating hypertension, as well as other diseases associated with CVD risk (Bazzano *et al.*, 2003; Pickering, 2006). The Dietary Approaches to Stop Hypertension (DASH) diet is one of the approaches to attenuate hypertension in the at risk/affected population. The DASH diet aims to limit sodium intake along with processed foods, substituting these foods for fruits, vegetables, low fat dairy, nuts and fish (Sacks *et al.*, 2001). The DASH diet also aims to increase calcium and potassium in the diet for their anti–hypertensive effects (Hummel *et al.*, 2012). A similar diet is recommended by the Public Health Agency of Canada and the American Heart Association to combat the risk of developing hypertension (Appel *et al.*, 2006; Public Health Agency of Canada, 2010).

Vegetables and legumes are especially valuable additions to the typical Western diet that is deficient with respect to daily intake of these food items. Canada's Food Guide for Healthy Eating recommends that teenagers and adults (ages 14+) consume 7 or more servings of fruits and vegetables per day (Health Canada, 2007). It is estimated that half of all adults and up to 70% of children do not meet their daily recommended servings of fruits and vegetables (Garriguet, 2004). Results from the World Health Survey indicated that fewer than 25% of respondents consumed 5 or more servings of fruits and vegetables a day (Hall *et al.*, 2009). Additionally, fewer than 1 in 7 Canadians consume pulses on a daily basis, with annual consumption below 3.5 kg/capita (Mudryj *et al.*, 2012). This falls well below the US Department of Agriculture Food Guide recommendation of 3 cups of pulses per week, equating to over 20 kg *per annum* (USDA, 2010).

Vegetarian diets, typically higher in pulses, have been linked to lower BP levels, and are associated with a 32% decreased risk of ischemic heart disease (IHD) according to a recent study (Sacks *et al.*, 1974; Crowe *et al.*, 2013). These foods are rich in vitamins, minerals, fibre and phytochemicals and are important to maintain over–all health and well–being (Bazzano *et al.*, 2003). Fibre is another nutrient lacking in the Canadian diet, with the average Canadian only consuming 14 g of the recommended 21–38 g/day (Heart & Stroke Foundation, 2011). High fibre intake, especially from cereal crops, has been associated with a decreased risk of CHD in both men and women (RR_{MEN} 0.81, 95% CI 0.70–0.93; RR_{WOMEN} 0.81, 95% CI 0.66–0.91; based on 10 g increments of fibre intake), as well as the potential to decrease the risk of colorectal cancer (Kromhout *et al.*, 1982; Rimm *et al.*, 1996; Wolk A. 1999; Park *et al.*, 2005). Fruits, vegetables, and legumes are excellent sources of dietary fibre.

Irrespective of the origin of the hypertension, increasing exercise and eating a healthy diet emphasizing non–processed, low fat foods would be beneficial in attenuating the progression of hypertension as well as decreasing the risk of CVD. The seventh report by the Joint National Committee (JNC; report referred to as JNC7) on the *Prevention, Detection, Evaluation and Treatment of High Blood Pressure* suggests that prehypertensive people control their BP with lifestyle changes (Chobanian *et al.*, 2003; Pickering, 2006). Improving diet and exercise patterns can also reduce required dosages for drugs, improve drug efficacy, and reduce CVD risk for those controlling their disease with pharmaceuticals (Chobanian *et al.*, 2003; Williams *et al.*, 2004). A salt–reduced DASH diet (1150 mg/2100 kcal) decreased

systolic (–17 mmHg, p=0.02), diastolic (–7 mmHg, p=0.02), and 24–h ambulatory systolic (–7 mmHg, p=0.02) and diastolic (–5 mmHg, p=0.02) BPs, as well as arterial stiffness (carotid–femoral PWV; –1.4 cm/s, p=0.03), in patients with treated hypertensive heart failure with preserved ejection fraction (Hummel *et al.*, 2012). Sacks *et al.* (2001) reported similar results in prehypertensive and stage 1 hypertensive patients.

1.6 Potential Vascular–Related Benefits of Pulse Crops

1.6.1 Pulse Crops Defined

One proposed method of reducing arterial stiffness and restoring endothelial function is increasing the amount of pulse crops in the diet. The Food and Agriculture Organization of the United Nations (FAO) defines pulse crops as the edible seeds of the legume family that are harvested for their dry grain (FAO, 2011). Peanuts and soybeans are generally not included under this definition as they are primarily grown for their oil (Thompson *et al.*, 2008; Patterson *et al.*, 2009; FAO, 2011; Singh & Basu, 2012). Additionally, those legumes harvested green and consumed non-dried (fresh or frozen), such as green beans and green peas, are not considered pulses (FAO, 2011). Pulse crops, specifically peas, lentils, and chickpeas, were among the first crops to be domesticated and farmed, originating in the Fertile Crescent around 6500 B.C. (Zohary, 1972; Lev-Yadun *et al.*, 2000; Brown *et al.*, 2008). Today, Canada is among the world's largest producers of pulse crops, with peas and lentils accounting for a significant percentage of Canadian–grown pulses (Roy *et al.*, 2010). The FAO estimated that Canada produced upwards of 37% of the

world's lentils, including 70% of the world's green lentil production (Agriculture and Agri-Food Canada, 2010; Roy *et al.*, 2010). However, pulse consumption in the typical Western diet is remarkably low at <3.5 kg/capita per year, compared to 3–10× that amount in countries such as India and Burundi where pulses are a staple of the diet (Schneider, 2002; Mudryj *et al.*, 2012). As such, most of the pulses grown in Canada are exported, accounting for an estimated 40% of the world pulse trade (Agriculture and Agri-Food Canada, 2010).

1.6.2 Pulse Crops and Health

Pulse crops are excellent sources of phytochemicals, such as flavonols, condensed tannins (proanthocyanidins), and catechins, as well as having high fibre, protein, vitamin (especially B vitamins) and mineral content, and a low glycemic index (Wang & Daun, 2004; Patterson *et al.*, 2009; Satya *et al.*, 2010; Faris *et al.*, 2012). Lentils, of all pulses, have among the highest content of flavonoids, condensed tannins and total phenolic compounds (Singh & Basu, 2012). Pulse crops also contain some "anti–nutritional compounds" such as protease inhibitors, saponins, and lectins (Patterson *et al.*, 2009; Roy *et al.*, 2010; Faris *et al.*, 2012). However, these "anti–nutritional compounds" may exert beneficial physiological effects in disease treatment or prevention, such as the hypocholesterolemic effect of saponins (Sidhu & Oakenfull, 1986; Roy *et al.*, 2010).

The interest in phytochemicals has been growing since the link between phytosterol consumption and LDL–C levels was established, allowing foods containing plant sterols and stanols to carry a health claim in several countries

including Canada and the U.S.A. (Health Canada, 2010). It has been well documented that dietary intake of 2 g/day of phytosterols can decrease LDL–C by up to 10% (Chen *et al.*, 2010; Hansel *et al.*, 2011). The ability of phytosterols to inhibit cholesterol uptake in the intestine due to structural similarities between the two is especially important as LDL–C oxidation is a source of oxidative stress as well as being one of the proposed initial steps in atherogenesis (Hansson, 2005; Health Canada, 2010). LDL–C oxidation is not as important in rats, which are generally resistant to atherogenesis, but phytochemicals have been shown to affect the progression of pathways associated with arterial stiffness and vascular remodeling *in vitro* as well as in rat and mouse models *in vivo*.

One study, based on food questionnaires from the Health Professionals Follow–up Study, has reported that with a serving of peas each day, the relative risk of a heart attack was 0.52 (95% CI 0.31–0.88) (Rimm *et al.*, 1996). Consumption of pulses 4 or more times a week – compared to 1 or fewer servings/week – was linked to a 22% and 11% reduction in the risks of CHD and CVD, respectively (Bazzano *et al.*, 2001). In unpublished data from our own lab, ½ cup of pulses consumed daily for 8 weeks decreased serum cholesterol and improved blood flow in people with PAD (Zahradka *et al.*, *in press*).

1.6.3 Antioxidant Capacity of Pulse Crops

Pulse crops have variable antioxidant capacities, with lentils having a greater capacity than beans, chickpeas or peas, based on *in vitro* assays (Amarowicz *et al.*, 2009; Amarowicz *et al.*, 2010; Yao *et al.*, 2010). The antioxidant ability of pulse crops

is largely associated with a group of compounds called phenolics, which includes quercetin, condensed tannins, and flavonols such as catechins (Figure 1A,B,C) (Amarowicz *et al.*, 2010; Galleano *et al.*, 2010; Zou *et al.*, 2011; Gharachorloo *et al.*, 2012; Singh & Basu, 2012). The antioxidant capacity is a result of the conjugated bonds, allowing absorption of free-radical electrons into a more stable structure. Quercetin, an antioxidant flavonol, can down–regulate $p47^{phox}$ and consequently prevent Nox activation in the SHR, which is the biggest cellular source of $O_2\bullet^-$ (Sánchez *et al.*, 2006). Quercetin also scavenges radicals such as $O_2\bullet^-$ and $ONOO^-$, exhibiting antioxidant effects (Duarte *et al.*, 2001). Duarte *et al.* (2001) have shown that chronic treatment with a quercetin isolate increases eNOS activity and decreases Nox activity in VSMCs of the SHR, significantly reducing blood pressure. This activity would increase endothelial function and decrease oxidative stress. Classic antioxidants such as ascorbic acid (Vitamin C; Figure 1D) and α–tocopherol (Vitamin E; Figure 1E) are present at varying amounts in pulses, and have been shown to increase SOD activity, decrease BP, $O_2\bullet^-$, and Nox activity in the SHR (Chen *et al.*, 2001).

Lentil phenolics have been shown to absorb oxygen radicals to a greater extent than many fruits including blackberries, cherries and apples

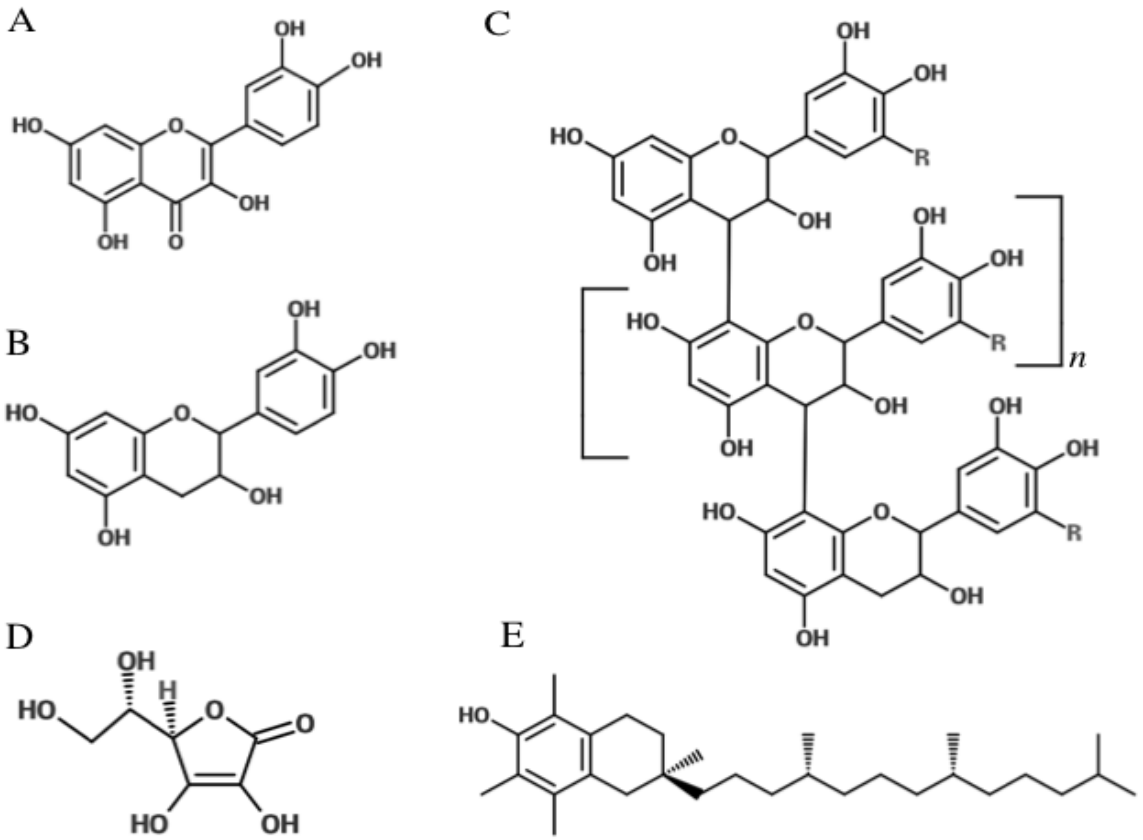


Figure 1: Polyphenols and Antioxidant Vitamins A) Quercetin, a flavonol, **B) Catechin**, a flavanol, **C) Condensed tannin** structure, **D) Vitamin C**, and **E) Vitamin E**. The antioxidant capacities are attributed to the conjugated bond systems present in all of the above molecules, allowing displacement of harmful free radical electrons over a large area. *A and B were adapted from (Manach et al., 2004), C was adapted from (Schofield et al., 2001).*

(Faris *et al.*, 2012). Of the known phenolics, most are present in the testa (seed coat) of lentils, and not in the much larger cotelydon; concentrations of phenolics vary depending on variety, year, and region of growth (Dueñas *et al.*, 2002; Xu & Chang, 2010). Resveratrol, a polyphenol abundant in grape skins and red wine and which is found in the *trans*–resveratrol–5–glucoside form in lentils (Dueñas *et al.*, 2002), has been shown to increase NO bioavailability without changing the expression of eNOS (Rush *et al.*, 2007). Polyphenols have also been shown to alleviate atherosclerosis and increase NO bioavailability in Apolipoprotein–E knockout mice (Loke *et al.*, 2010). People with PAD have lower circulating levels of Vitamin C, attributed to increased oxidative stress, indicating the importance of antioxidants in arresting disease progression (Langlois *et al.*, 2001). However, overconsumption of antioxidants can be dangerous and, at high doses, quercetin has been shown to become pro–oxidant instead of anti–oxidant, actually contributing to the production of reactive oxygen species (Perez-Vizcaino *et al.*, 2006).

1.6.4 Other Potential Effects of Phytochemicals and Pulses

Despite the touted benefits of antioxidants, basal levels of ROS are important signaling molecules in healthy individuals. In fact, a large, double-blind, placebo-controlled trial with over 18,000 smokers demonstrated that daily dosing with high levels of β-carotene (30 mg) and retinol (Vitamin A; 25 000 IU) increased the incidence of lung cancer (RR 1.46; 95% CI 1.07–2.00) and cardiovascular disease (RR 1.26; 95% CI 0.99–1.61) (Omenn *et al.*, 1996). This demonstrated that high doses of antioxidants are not protective as previously believed, but can actually be

deleterious. Coupled with the pro-oxidant effects of phenolics seen in *in vitro* assays, it has been postulated that polyphenols may elicit their beneficial effects through alternative mechanisms (Halliwell, 2007). Halliwell (2007) has promoted the concept that flavonoids have the potential to act as selective inhibitors of enzymes, including ACE and matrix metalloproteinases (responsible for ECM remodeling), alter cell signaling, including alterations to glucose uptake and increased eNOS activity, or mediate their effect in the gut where they are at the highest concentration. Furthermore, it cannot be ruled out that other aspects of the whole-food are contributing to health improvements. For example, fibre can be metabolized to short chain fatty acids by colonic microbiota, and in this way improve colonic health and function (Halliwell, 2007). Finally, the low consumption of pulses may be due to perceived negative effects such as flatulence, however, over time the gastrointestinal (GI) tract and microbiota can adapt to the high fibre.

1.6.5 Potential Anti–Hypertrophic Effect of Pulse Crops

Phytochemicals are promising with respect to attenuation of vascular remodeling and restoring proper vascular function. Quercetin was shown to decrease vascular remodeling in the SHR (Perez-Vizcaino *et al.*, 2006). The reduction of observed aortic hypertrophy has, by one group, been attributed to the inhibition of JNK and subsequent AP–1 activation downstream of AngII signaling (Yoshizumi *et al.*, 2002). In addition, a polyphenol extract from lentils was able to attenuate AngII–induced increases in cardiac and renal artery M/L ratio in Sprague Dawley rats (Yao *et al.*, 2012). The antioxidant capacity of certain phytochemicals

can also reduce the oxidative stress that appears to start, maintain, or enhance the cellular signaling that ultimately leads to aberrant growth and remodeling of the arteries. It has also been postulated and shown that restoration of function, as well as reduction of remodeling in the vasculature, is not dependent on the reduction of BP (Intengan & Schiffrin, 2001; Eftekhari *et al.*, 2012).

There is a growing body of evidence from *in vitro* studies that suggest pulse proteins and their metabolites are able to inhibit ACE and prevent some of the deleterious effects such as cardiomyocyte hypertrophy and reactive oxygen species generation (Boye *et al.*, 2010; Roy *et al.*, 2010; Yao *et al.*, 2010; Barbana *et al.* 2011; Faris *et al.*, 2012). The most active lentil protein hydrolysates in this regard were obtained through sequential degradation by pepsin, trypsin and α–chymotrypsin, an attempt to simulate digestion in the GI tract (Barbana *et al.* 2011). Much of this work needs to be verified *in vivo* to address their applicability to whole–body metabolism and effects, especially since multi–amino acid peptide fragments are not usually transported into the circulation.

1.7 Rationale

Due to the complexity of BP regulation in the body, there are many systems that can malfunction and ultimately result in hypertension. While drugs are able to control high BP for the most part, they are symptomatic treatments and do not necessarily correct the underlying problem. Additionally, drugs also have associated side effects, which can be as dangerous as the disease they are intended to treat. As a result, more people are looking to dietary options to control their diseases, or

reduce the reliance on pharmaceuticals. Pulse crops have many general health benefits associated with them due to their relatively high concentration of protein, fibre, and phytochemicals. There is also evidence that pulses may affect the systems associated with hypertension, and thus could provide an alternative or a complement to allopathic medicine.

These studies were designed to test the effect of pulse diets on a well–established model of hypertension and arterial stiffness, the SHR, and to provide insight into the mechanisms through which the pulses might operate. The first study was intended to determine if pulse crops could provide a benefit with respect to attenuation of hypertension and/or vascular remodeling and, if so, which pulse type provides the greatest benefit. The second study was designed to determine, of the most effective pulse type (lentil), if a particular variety (red or green) was more effective.

1.8 Hypotheses and Objectives

Hypothesis (Study 1)

If fed a diet high in pulse crops, the SHR will exhibit alleviation of hypertension and hypertension–associated pathologies. Specifically, there will be decreased vascular smooth muscle hypertrophy and vascular remodeling, ultimately resulting in decreased arterial stiffness.

Objectives (Study 1)

1. Determine the pulse type (bean, pea, lentil, chickpea) most effective at attenuating or lowering BP and PWV in a 4-week pilot study.
2. Investigate how these changes affect large artery and heart morphology, serum biochemistry, and body composition (gross anatomy).

Hypothesis (Study 2)

If fed a diet high in mixed lentils, the SHR will experience attenuation of BP and arterial remodeling, especially in resistance arteries, and over a longer study period these changes will translate into decreased arterial stiffness.

Objectives (Study 2)

1. Determine the lentil variety (red or green) most effective at attenuating resistance artery remodeling, BP, and PWV during an 8-week intervention period.
2. Determine the effects of lentils on small artery stiffness and arterial compliance.
3. Establish the effects of the lentil diets on body composition, serum biochemistry and heart morphology.
4. Investigate a potential mechanism of action for decreased arterial remodeling by probing the common cell hypertrophic stimuli, including ERK1/2 and p38 MAPK activation.

2. Materials and Methods

These experiments were carried out in accordance to proper animal care and experimentation as outlined by the Canadian Council on Animal Care and a protocol approved by the University of Manitoba Protocol Management and Review Committee.

For formulations of solutions described in Materials and Methods, see *Appendix A*.

2.1 Study 1: Determination of the Most Effective Pulse Type

2.1.1 Animals and Experimental Diets

Fifteen week old, male SHR and WKY rats were ordered from Charles River Laboratories (Charles River Laboratories, Saint-Constant, QC, CAN). The rats were weighed then housed individually in controlled laboratory conditions with a 12 hr light dark cycle (40–60% humidity, temperature controlled). The rats were acclimatized for a minimum of 12 days and were fed the American Institute of Nutrition 93–Growth (AIN–93G) control diet during this period; the formulation is described below and given in Table 2A.

After acclimatization, the rats were assigned to one of eight diet groups (n=8/group): WKY control (WKY), SHR bean (SHR–B), SHR pea (SHR–P), SHR lentil (SHR–L), SHR chickpea (SHR–C), SHR mixed pulse (SHR–Mx), SHR pair–weighed (SHR–PW), and a SHR control (SHR–Ctrl). Weekly body weight (BW) measurements were taken to ensure health and to alter the amount of diet provided to the PW control group if required. The PW group were SHR animals fed control diet in

Table 2: Diet formulations (Study 1)

	AIN–93G Control	Bean	Pea	Lentil	Chickpea	Mixed Pulse
A – Ingredients[1]			*g/kg*			
Casein	200	113	113	113	113	113
Cornstarch	397	229.5	229.5	229.5	229.5	229.5
Maltodextrin	132	132	132	132	132	132
Sucrose	100	100	100	100	100	100
Cellulose	50	5	5	5	5	5
L–cysteine	3	3	3	3	3	3
Choline Bitartrate	2.5	2.5	2.5	2.5	2.5	2.5
Mineral Mix[2]	35	35	35	35	35	35
Vitamin Mix[3]	10	10	10	10	10	10
Soybean Oil[4]	70	70	70	70	70	70
Pulse Powder[5]						
Black Bean	–	75	–	–	–	18.75
White Navy Bean	–	75	–	–	–	18.75
Red Kidney Bean	–	75	–	–	–	18.75
Pinto Bean	–	75	–	–	–	18.75
Green Pea	–	–	150	–	–	37.5
Yellow Pea	–	–	150	–	–	37.5
Red Lentil	–	–	–	150	–	37.5
Green Lentil	–	–	–	150	–	37.5
Chickpea	–	–	–	–	300	75
Total (g)	*1000*	*1000*	*1000*	*1000*	*1000*	*1000*
B – Proximate Analysis[6]			%			
Moisture	6.6	4.8	4.9	4.8	5.3	4.9
Dry Matter	93.4	95.2	95.1	95.2	94.3	95.1
Crude Protein	18.6	17.0	17.9	18.6	16.5	17.3
Crude Fibre	1.4	1.1	1.6	0.7	0.6	1.1
Fat	6.8	7.4	7.2	7.7	8.8	7.6
Ash	3.1	3.2	3.1	2.7	2.8	2.9

[1]Dyets Inc., Bethlehem, PA, USA, except pulse powders
[2]AIN–93G MX
[3]AIN–93 VX
[4]With 0.02% TBHQ – *tert*–butylhydroquinone
[5]Pulses sourced by Pulse Canada (Winnipeg, MB, CAN) and pulse powder prepared as described in *Methods*.
[6]Central Testing Labs Ltd., Winnipeg, MB, CAN

restricted amounts such that the BW of PW rats would be equivalent to the pulse group with the lowest BW; this group would eliminate the possible confounding effect of BW on BP. However, for the study duration there were no differences among SHR groups with respect to BW; the food intake of the PW animals was never restricted and this group was not included in the data analysis. Three rats, one from SHR–P, SHR–L and SHR–Ctrl, were excluded from the study due to a cage mix–up and possible diet change during the study; thus there was n=7 for aforementioned groups. Pulse–based diets were mixed in a similar manner as control diets with exact formulations given in Table 2A.

The AIN–93G control diet was prepared in 6 kg batches using the formula as outlined by Reeves *et al.* (1993) (Table 2A). All powdered diet components were ordered from Dyets Inc. (Bethlehem, PA, USA). Briefly, the separate diet components were weighed into a large plastic bag and stored overnight at 4°C. The powdered components were then mixed for 5 minutes at low speed to avoid heat generation and vitamin/component degradation (Reeves *et al.*, 1993). The soybean oil was measured separately and added slowly to the mixture. The whole diet was mixed for a further 10 minutes, stopping once to manually mix and ensure homogeneity. The diet was then weighed into plastic freezer bags and stored at 4°C/–20°C until used.

For each pulse type, diets were prepared with equal proportions of major pulse varieties grown in Canada and sourced by Pulse Canada for high quality seed. Pulses were: beans (black beans, red kidney beans, white navy beans, pinto beans), peas (green peas, yellow peas), lentils (green lentils, red lentils), and chickpeas. A

mixed pulse diet was added to the study design to determine if there was an additive or negative effect from consuming a mixture of pulse varieties, as is common in high-pulse diets. The pulses were soaked overnight[Ψ], cooked, and freeze–dried. The cooking process destroys some of the anti-nutritional compounds such as protease inhibitors, and is done before human consumption. The freeze–dried pulses were ground in a Retsch ZM 200 (Retsch, Haan, Germany) at 12,000 rpm until they passed through a 0.5 mm screen. The ground pulses were placed in separate freezer bags and stored at –20°C until included in the diets.

The diets were formulated to contain 30% pulses (w/w) and the weights were calculated based on measurements obtained after cooking, freeze-drying and grinding. The pulse–containing diets were formulated to be approximately isocaloric, isonitrogenous, and isofibrous when compared to the AIN–93G control diet. The calculations were based on the average nutrient composition of all of the pulses as obtained from the Canadian Nutrient File (Wang & Daun, 2004). A 30% pulse–containing diet was previously shown to have no adverse effects on animal growth (Thompson *et al.*, 2008). The diets were prepared in a similar manner to the control diets, with the appropriate adjustments to protein (casein), carbohydrate (cornstarch), and fibre (cellulose) (Table 2A). The calculation of protein was based on an analysis of the casein by Dyets, which showed it contained 87% protein (for calculation see Appendix B). Diet samples were sent to Central Testing Labs Ltd. (Winnipeg, MB, CAN) for proximate analysis and verification of diet composition (Table 2B).

[Ψ] Lentils do not require soaking prior to cooking and have shorter cooking times than other pulse varieties (Satya *et al.*, 2010).

The rats had access to food and water *ad libitum* for the duration of the study. At the end of the study period the rats were weighed, then euthanized by an overdose of pentobarbital (≥1.01 mg/kg BW) delivered via intraperitoneal (IP) injection. Death was ensured by decapitation. The aorta, heart, liver, kidneys, epididymal fat, mesenteric fat, and peri–renal fat pads were excised, weighed and frozen or sectioned as described in section 2.1.4.

2.1.2 Blood Pressure and Pulse Wave Velocity

BP was measured through tail–cuff plethysmography at the end of acclimatization (baseline) and within the week before termination (final). The animals were randomly assessed, 6 rats at a time with 5 acclimatization and 10 cycles, analyzed by the CODA™ system (Kent Scientific, Torrington, CT, USA). Measurements were repeated as required. The animals were acclimatized to the apparatus the day before both the baseline and final measurements. Measurements of SBP, DBP, and mean arterial pressure (MAP) were obtained. MAP was calculated as:

(2) $$MAP = DBP + \frac{1}{3}PP$$

where pulse pressure (PP) is:

(3) $$PP = (SBP - DBP)$$

PWV was measured once before the onset of experimental feeding, and once weekly for 4 weeks after the onset of experimental diets. The PWV was measured with an ECG–triggered 10–MHz Doppler probe (Indus Instruments, Weber, TX, USA). Blood velocity (v) was calculated based on the angle of the probe (θ),

frequency of the transmitted sound (f_t), frequency of the returned sound (f_r) and the speed of blood in tissue (c; ~1540 m/s) such that:

(4) $$v = \frac{f_d(c)}{2f_t(\cos\Theta)}$$

with f_d being the Doppler shift ($f_d = f_r - f_t$) (Oates, 2001). The rats were anesthetized using isoflurane. Rats were assessed in a random order, and 3 PWV measurements were taken per session. PWV analysis gives information on: heart rate (HR)(bpm), R–R interval (msec), peak velocity (PV)(cm/s), minimum flow velocity (MFV)(cm/s), mean flow velocity (MeFV)(cm/s), resistance index (RI), and pulsatility index (PI), where:

(5) $$RR\,interval = \frac{60}{HR}(1000)$$

(6) $$RI = \frac{(PV - MFV)}{(PV)}$$

(7) $$PI = \frac{(PV - MFV)}{(MeFV)}$$

(Oates, 2001)(Appendix C). RI is indicative of resistance of arterial beds distal to the site of measurements. An RI value of 0 indicates continuous flow, 1 indicates only systolic flow, and >1 indicates blood being reflected backwards during diastole. PI is representative of the variability of blood flow throughout the cardiac cycle (Oates, 2001).

2.1.3 Serum Biochemistry

Rats were placed in metabolic cages overnight for a 12-hour fast within the last week of the study. Fasting blood samples were taken from the saphenous vein after 12 hours in the metabolic cages. Blood was put on ice and allowed to clot for an hour before being spun in a centrifuge for 15 minutes at 1000 × *g*. Serum was then aliquoted in 50 μL aliquots and frozen at –80°C until analyzed.

Fasting serum samples were run on a Cobas C111 auto analyzer (Roche; Penzberg, Germany) to determine the blood lipid profile. Serum was analyzed for total cholesterol (TC), high–density lipoprotein (HDL–C), low–density lipoprotein (LDL–C), triglycerides (TG), glucose, creatinine, and urea.

2.1.4 Tissue Collection and Histology

To determine media thickness, M/L ratio and lumen diameter, the aorta was removed from the rat and a section of the middle of the descending aorta was embedded vertically in an embedding mould filled with optimal cutting temperature (OCT) compound (Sakura Fintek, Alphen aan den Rijn, NL). The sample was placed in an ethanol and dry ice bath to freeze, and then was kept at –80°C until sectioned. The aorta was further partitioned and placed into protein and mRNA foils, flash frozen in liquid nitrogen and then stored at –80°C until analyzed (Appendix D, Figure D.1 for section areas: *embedding, protein* and *mRNA*).

The OCT embedded segment of the aorta was sectioned into 5 μm sections, with 3 sections per slide, using a cryotome (Thermo Shandon, Thermo Fisher Scientific, Waltham, MA, USA). The sections were kept at –80°C until stained for

elastic proteins (Elastic Stain Kit, Reference #HT25A–1KT, lot #120M4344, Sigma Aldrich, St. Louis, MO, USA). The kit stained elastin black, muscle yellow and collagen red. For this procedure, the slides were fixed in 1% paraformaldehyde for 8 minutes. Slides were then washed in 1× phosphate–buffered saline (PBS; 1.4 M NaCl, 2.6×10^{-2} M KCl, 0.1 M Na_2HPO_4, 1.7×10^{-2} M KH_2PO_4 diluted 1:9 in *dd*H_2O) for 10 minutes, hydrated in *dd*H_2O for 15 minutes, and stained according to the manufacturer's protocol. Differentiation in the working ferric chloride solution took 3 minutes for the aorta sections, and Van Gieson staining showed the best contrast at 90 seconds. The stained slides were then fixed using VectaMount (Vector Laboratories, Burlingame, CA, USA), as Aqua Mount muted the stain colour due to some observed washing. The slides were then imaged with 4× and 20× objectives using an EVOS® microscope (AMG solutions, Seattle, WA, USA). An average of two non–consecutive sections were imaged per aorta. Image Pro Plus (version 4.5.1, Media Cybernetics, Rockville, MD, USA) was used to measure the lumen circumference and the outer *tunica media* (between *tunica media* and *adventitia*) circumference on the 4× images. Calculations were performed to determine lumen diameter, media thickness, media cross sectional area and media–to–lumen ratio (M/L ratio).

Lumen diameter was calculated as:

(8) $$\text{Lumen Diameter} = \text{Lumen Circumference}/\pi$$

Media thickness was calculated as:

(9) $$\text{Media Thickness} = \left(\text{Media Circumference}/2\pi\right) - \left(\text{Lumen Diameter}/2\right)$$

Media CSA was calculated as:

(10) $\text{media CSA} = \pi r_{\text{media}}^{2} - \pi r_{\text{lumen}}^{2}$

M/L ratio was calculated as:

(11) $\text{M/L ratio} = \text{media width} / \text{lumen diameter}$

The heart was excised and weighed to determine the heart weight to BW (HW/BW) ratio. The left ventricle (LV) was then isolated from the rest of the heart and weighed independently to determine left ventricle weight (LVW) and LVW to BW (LVW/BW). A section of left ventricle apex was then embedded in OCT. The sample was placed in an ethanol and dry ice bath to freeze, and then was kept at –80°C. The remainder of the left ventricle was placed in foil and frozen in liquid nitrogen, then placed at –80°C until analyzed. The liver, peri–renal adipose, mesenteric adipose, and epididymal adipose tissues were weighed individually.

2.2 Study 2: Determination of Most Effective Lentil Variety (Red/Green)

2.2.1 Animals and Experimental Diets

Fifteen-week-old SHR and WKY were ordered from Charles River Laboratories (Charles River Laboratories, Saint-Constant, QC, CAN). Animals for study 2 were housed individually in the same manner as for study 1, except study 2 had an 8–week dietary intervention period. There were 6 study groups (n=10/group): SHR control (SHR–Ctrl), SHR mixed lentil (SHR–ML), SHR green lentil (SHR–GL), SHR red lentil (SHR–RL), WKY control (WKY–Ctrl), and WKY mixed lentil (WKY–ML). Diets were formulated and prepared in the same manner as study 1, with ingredients listed in Table 3A and proximate analysis in Table 3B.

Table 3: Diet Formulations (Study 2).

	AIN–93G Control	Red Lentil	Green Lentil	Mixed Lentil
A – Ingredients[1]		*g/kg*		
Casein	200	113	113	113
Cornstarch	397	229.5	229.5	229.5
Maltodextrin	132	132	132	132
Sucrose	100	100	100	100
Cellulose	50	5	5	5
L–cysteine	3	3	3	3
Choline Bitartrate	2.5	2.5	2.5	2.5
Mineral Mix[2]	35	35	35	35
Vitamin Mix[3]	10	10	10	10
Soybean Oil[4]	70	70	70	70
Pulse Powder[5]				
Red Lentil	–	300	–	150
Green Lentil	–	–	300	150
Total (g)	*1000*	*1000*	*1000*	*1000*
B – Proximate Analysis[6]		%		
Moisture	7.1	7.5	4.6	4.6
Dry Matter	93.0	92.5	95.4	95.4
Crude Protein	19.2	17.3	17.3	17.2
Crude Fibre	1.4	1.3	1.4	1.4
Fat	7.2	7.1	7.3	7.5
Ash	2.3	2.8	2.8	3.0

[1]Dyets Inc., Bethlehem, PA, USA, except pulse powders
[2]AIN–93G MX
[3]AIN–93 VX
[4]With 0.02% *TBHQ* – *tert*–butylhydroquinone
[5]Pulses sourced by Pulse Canada (Winnipeg, MB, CAN) and pulse powder prepared as described in *Methods*.
[6]Central Testing Labs Ltd., Winnipeg, MB, CAN

The lentil varieties used were the same as those used in study 1. *For more on information on diet preparation and animal housing, see section 2.1.1.*

2.2.2 Blood Pressure and Pulse Wave Velocity

BP and PWV were measured in the same manner as study 1, however, in study 2 both PWV and BP were measured weekly for the duration of the study. Only animals giving five or more measurements per BP cycle were used in calculating SBP, DBP and MAP averages.

For study 2, the PWV analysis was done in a blinded manner. For more information on PWV measurements, *see section 2.1.2.* PWV analysis was done on the Doppler Signal Processing Workstation program (DSPW Version 1.624, Indus Instruments, Houston, TX, USA)(Appendix C). Briefly, the software is able to locate the baseline of the PWV trace. From there, it can outline the trace based on contrast readings, as well as determine the pulse waveforms on the EGC trace. The analyst then manually places markers at the flow velocity start (FVS), the peak flow velocity (PFV), and the minimum flow velocity (MFV) on the Doppler trace (Figure C.1BCD). The program then calculates HR (bpm), R–R interval (msec), PFV (cm/s), MFV (cm/s), MeFV (cm/s), PI, and RI. Approximately 17 peaks were analyzed per trace.

2.2.3 Body Composition

Body composition was assessed *in vivo* through use of an EchoMRI–700™ whole body Quantitative Magnetic Resonance (QMR) instrument (EchoMRI, Houston, TX, USA). The machine provided data on adipose mass, free water mass,

total water mass and lean mass. Body composition was measured at baseline (week 0), week 4, and week 7. Three measurements were performed on each animal each session, and averages of the 3 were used in calculations. Negative free water values were excluded from calculations.

2.2.4 Serum Biochemistry

Fasting serum samples were obtained at baseline (week 0), week 4, and week 7 in the same manner described in *section 2.1.3*. The serum samples were run on the Cobas C111 auto analyzer for TC, HDL–C, LDL–C, TG, glucose, creatinine and urea.

2.2.5 Tissue Collection

The tissue collection was done as described in *section 2.1.4*. Sections of the aorta saved for RNA were placed in micro–centrifuge tubes with 500 μL of RNAlater®. In study 2, the mesenteric fat was not weighed as the vessels contained within the fat were used for pressure myography.

2.2.6 Pressure Myography

The intestine was removed at the time of termination and immediately placed in cold 1× Kreb's buffer (2.5×10^{-2} M $NaHCO_3$, 5.5×10^{-3} M Glucose, 2.7×10^{-5} M NaEDTA, 400 mL 10× Kreb's). A third order mesenteric artery was then isolated from the first 10 cm of the mesenteric fat, proximal to the stomach. The selected artery was mounted on the pressure myograph (Living Systems Instrumentation,

Burlington, VT, USA), pressurized to 45 mmHg, and allowed to equilibrate for one hour in 1× Kreb's solution (37 ˚C, pH ~7.4). All solutions were bubbled with 5% CO_2 and 21% O_2 (~2 litres per minute). After equilibration, the artery was challenged with 125 mM KCl solution (in 1× Kreb's) to constrict and check artery viability. The artery diameter must constrict ≥ 50% to be considered viable. If viable, the artery was bathed for another hour in 1× Kreb's solution.

Arteries were then pre–constricted with 3×10^{-6} M norepinephrine (NE) (in 1× Kreb's), then treated with increasing concentrations of acetylcholine (ACh), an agonist causing endothelium–dependent vasorelaxation. The artery was then bathed for an hour in 1× Kreb's solution. Then the artery was again pretreated with 3×10^{-6} M NE, then challenged with increasing concentrations of sodium nitroprusside (SNP), an endothelium–independent donor of NO. Serial dilutions of ACh and SNP were used and the lumen diameter was measured at three places along the artery for each drug concentration. The dilutions were done at semi–log concentrations from 10^{-10} to 10^{-2} M.

Finally, the vessel was left to rest in 1× Ca^{2+}–free Kreb's solution containing 10 mM EGTA (2.5×10^{-2} M $NaHCO_3$, 5.5×10^{-3} M Glucose, 2.7×10^{-5} M Na EDTA, 10 mM EGTA, 200 mL 10× Ca^{2+} Kreb's) for 30 minutes. At the end of the 30 minutes, the pressure was increased to 140 mmHg three times, straightening the vessel each time. Finally, 3 separate measurements of the lumen diameter, left and right wall thickness were measured at 3, 10, 20, 30, 40, 60, 80, 100, 120, and 140 mmHg and these values were used to determine vascular compliance and stiffness. Measurements were also taken at 45 mmHg and used to determine the geometry of

the vessel but were not included in the stiffness or compliance calculations. For full procedure, see Appendix E.

Calculations for the stiffness of the artery were done as described previously (Park & Schiffrin, 2001; Behbahani *et al.*, 2010; Thandapilly *et al.*, 2012). Calculations were done to determine media stress, media strain, elastic modulus, media cross–sectional area (media CSA), remodeling index, and growth index. Calculations are as shown below:

Media stress:

$$(12)\ \sigma = (PD)/(2WT) = (P \times r)/(WT) = \text{equation}$$

where, P is intraluminal pressure, D is lumen diameter, and WT is media thickness.

Media Strain:

$$(13)\ \varepsilon = \frac{(D - D_0)}{D_0}$$

where, D is lumen diameter at a given pressure and D_0 is lumen diameter at 3 mmHg.

Elastic modulus:

$$(14)\ (y = a e^{bx}) : \sigma = \sigma_0 e^{\beta}$$

where, β is a constant and σ_0 is stress observed at the baseline diameter.

Media cross sectional area (CSA):

$$(15)\ \text{media CSA} = \pi(D_e^2 - D_i^2)/4$$

The conversion between mmHg and dynes/cm^2 is 1 mmHg = 1.334x10^3 dyn/cm^2.

Stress-strain data were log transformed, creating a linear relationship giving slopes that could be evaluated statistically. The equation for the curves was:

(16) $y(x) = e^{\ln a + x \ln b}$

and the log-transformed relationship was given as:

(17) $\ln y(x) = \ln a + x \ln b$

where:

(17a) $\ln b = \frac{n\sum x \ln y - \sum x \sum \ln y}{n\sum x^2 - \left(\sum x\right)^2}$

(17b) $\ln a = \frac{\sum \ln y}{n} - \bar{x} \ln b$

2.2.7 Western Immunoblotting

For protein extraction, tissue was weighed and then placed in a mortar. Liquid nitrogen was poured onto the tissue and the tissue was ground into a fine powder. Then 1200 μL (or 30 μL/mg of tissue, minimum of 500 μL) of 3× sample buffer was added to the ground tissue and allowed to sit for 15 minutes. The protein extract was then pipetted into 1.5 mL micro–centrifuge tubes and spun for 20 minutes at 13 000 rpm. This formed a pellet at the bottom, protein in sample buffer in the supernatant and a layer of fat on the top. The protein was carefully pipetted out into a new 1.5 mL micro–centrifuge tube and sonicated. The protein was then stored in aliquots at –80°C until analyzed.

Protein analysis was performed by a BCA protein assay (Pierce; Rockford, IL, USA). The standard curve was based on dilutions of bovine serum albumin (BSA) with concentrations of 2.0, 1.0, 0.8, 0.6, 0.4, 0.2 and 0 mg/mL; dilutions were made in *dd*H_2O. Samples and standards were pipetted in triplicate into a NUNC 96–well

plate, 10 μL/well. Aorta protein was diluted in 3 parts *dd*H_2O to 1 part protein isolate for analysis by the protein assay. Two hundred μL of a mixture of Pierce® BCA protein assay Reagents A and B (50 parts A to 1 part B) were added to each well (Cat. #23223 for Reagent A and #23224 for Reagent B; Pierce Thermo Scientific, Rockford, IL, USA). Plates were incubated for 30 minutes at 37°C and then read at 550 nm in a ThermoMax Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE) was used to separate proteins, which were then transferred to polyvinylidene fluoride (PVDF) membranes for analysis by Western blot. Blots were stained using Ponceau stain, then dried and imaged as a back-up protein–loading control. Membranes were blocked for 1 hour in 3% BSA–TBST, and washed for 3×5 minutes in 1× TBST. All antibodies were run in 3% BSA–TBST, with 1:10,000 rabbit secondary in 1% BSA–TBST unless otherwise specified. For antibody information, see Table 4. Membranes were dipped in Amersham™ ECL™ Prime Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, UK) and imaged on a FluorChem® Q Western Blot imager, and quantified using AlphaView SA software (Protein Simple, Santa Clara, CA, USA). In AlphaView, multiplex band analysis was selected, and in "Bkgrnd", local background was selected. Region tools were used to select bands of interest and BC average was exported into a Microsoft Excel file for analysis.

Table 4: Antibodies Used

Antibody[1]	Catalog/Lot #	Acrylamide (%)	1º Ab Dilution	Incubation Time	Exposure Time	μg Protein	Protein Source
$Galpha_i$	5290S/1	15	1:1000	O/N	20 sec	5	Aorta
p38 MAPK	9212/16	15	1:1000	O/N	3 min	5	Aorta
p–p38 $MAPK^{T180/Y182}$	9211/19	15	1:1000	O/N	15 min	5	Aorta
p42/44 MAPK	9102L/23	10	1:1000	1 hour	1 min	5	Aorta
p–p42/44 MAPK	9101/26	10	1:1000	1 hour	30 sec	5	Aorta
PFN1	3237S/1	10	1:1000	1 hour	10 sec	5	Aorta

O/N – overnight, p- – phosphorylated, 1° Ab – primary antibody
[1] All antibodies from Cell Signaling Technology, Danvers, MA, USA.

2.3 Statistics

Repeated measures ANOVA for *in vivo* measurements over time and one–way ANOVA for endpoint measurements were calculated using Statistical Analysis Software (SAS) (version 9.2, SAS, Cary, NC, USA). Outliers were calculated as those values outside the mean ± 2.5 standard deviations. A post–hoc Duncan's multiple range test was applied to determine significance at a $p<0.05$ level. When required, log–transformed or non–parametric statistics were used. Correlations were calculated using Pearson's test for normal data or Spearman's test for non–parametric data.

For pressure myography data, statistics were calculated using GraphPad Prism 5 (GraphPad Software, La Jolle, CA, USA). Linear regression one–way ANOVA was calculated, and a post–hoc Newman–Keuls multiple comparison test was performed to determine significance between columns. Log-transformed stress-strain relationships were calculated using a one-way ANOVA in SAS.

Data are expressed as Mean ± *Standard Error* (SE), unless otherwise indicated. A p–value ≤ 0.05 was considered to be significant.

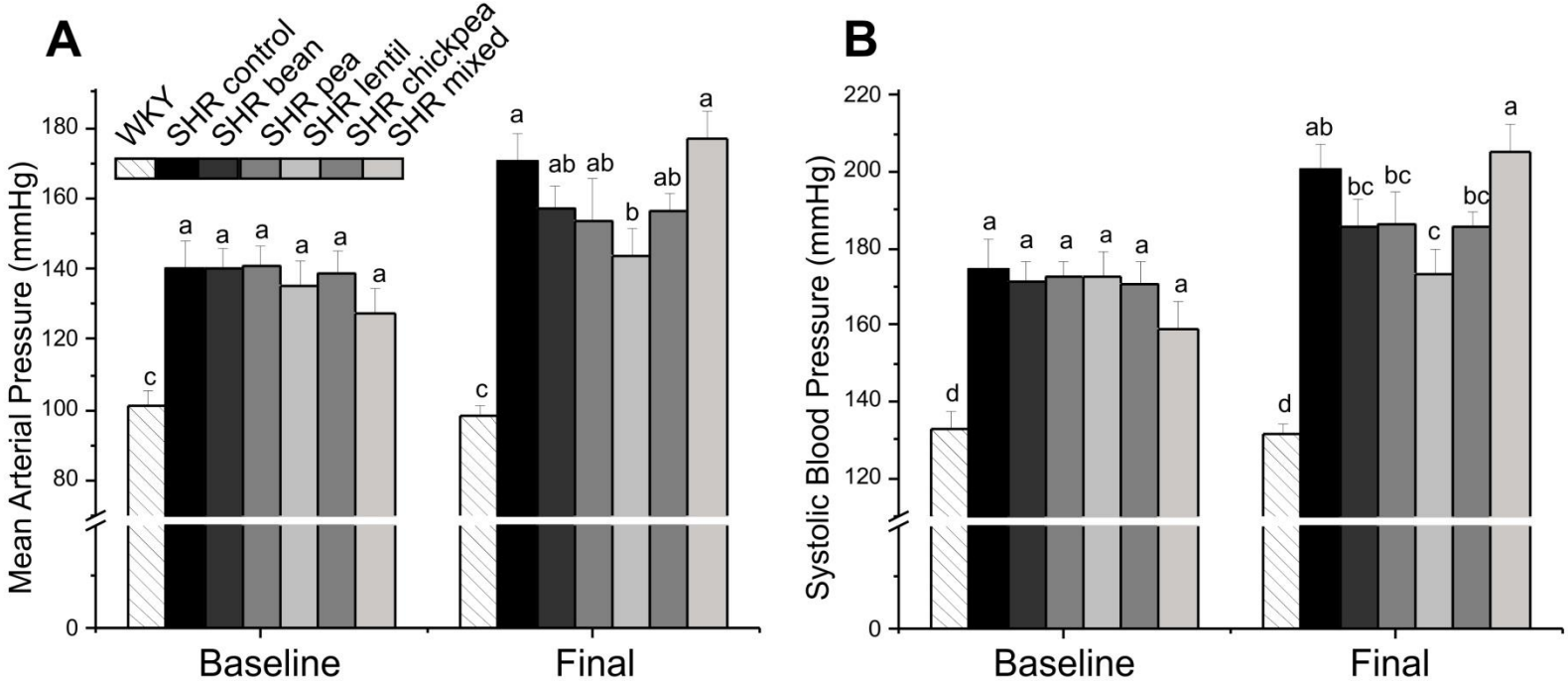
3. Results

3.1 Study 1: Determination of the Most Effective Pulse Type

3.1.1 Pulse Wave Velocity and Blood Pressure

At baseline, MAP in the SHR was higher than the WKY, however, there were no differences between SHR groups (Figure 2A). At week 4, the WKY MAP was still lower than in SHR, and the SHR–L had a smaller rise in MAP than SHR–Ctrl (+8 mmHg and +31 mmHg from baseline, respectively). The WKY had lower SBP and DBP at baseline compared to all SHR groups (Figure 2B,C). At week 4, SHR–L had lower SBP than SHR–Ctrl (+1 and +26 mmHg from baseline, respectively). The SHR–Mx group had higher SBP than all other SHR groups except SHR–Ctrl. At week 4, SHR–Mx had higher DBP than SHR–L, with all other SHR groups falling intermediate to these two groups.

WKY had lower PWV at baseline compared to the collective average for SHR (Figure 2D). For weeks 1–4, the WKY group had lower PWV compared to all SHR groups. There were no dietary–related changes in PWV observed between any of the SHR groups.


A
WKY
SHR control
SHR bean
SHR pea
SHR lentil
SHR chickpea
SHR mixed
Mean Arterial Pressure (mmHg)
180
160
140
120
100
80
0
a a a a a a
c
a ab ab b ab a
c
Baseline
Final
B
Systolic Blood Pressure (mmHg)
220
200
180
160
140
120
0
a a a a a a
d
ab bc bc c bc a
d
Baseline
Final

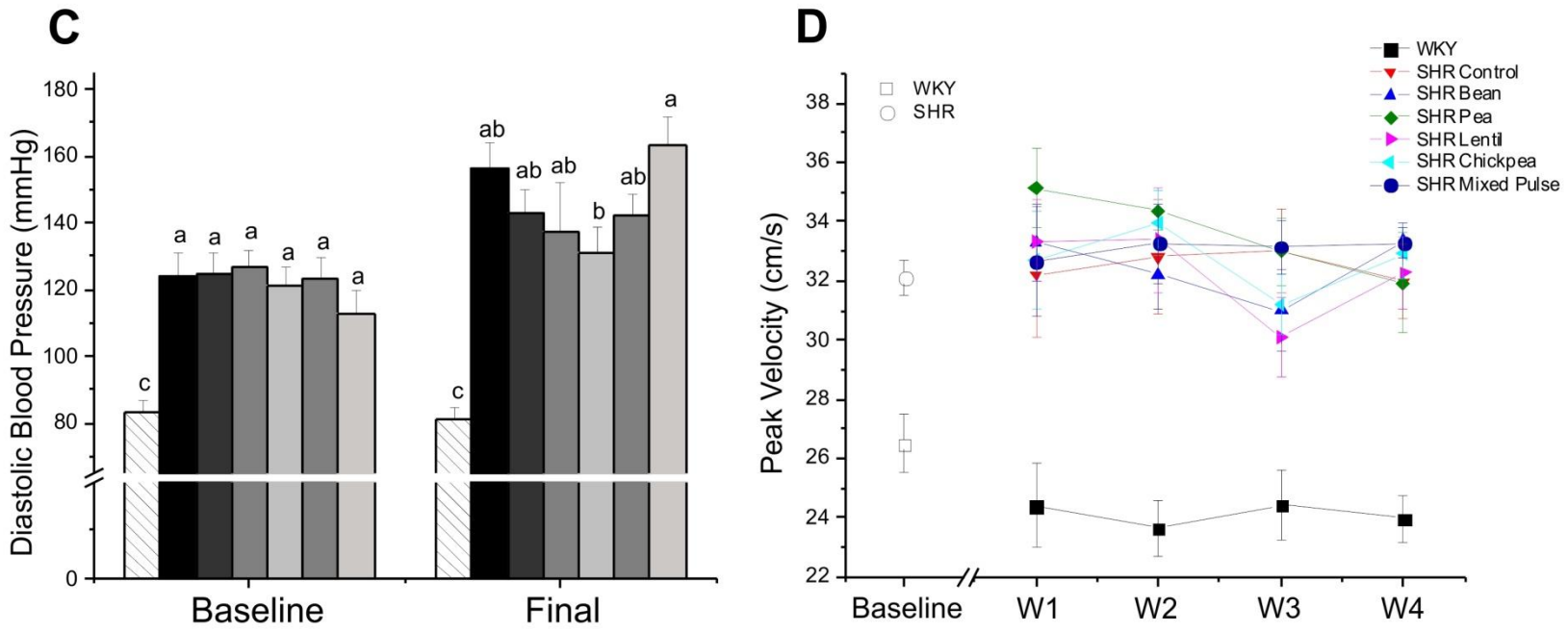


Figure 2: Blood Pressure and Pulse Wave Velocity (Study 1) A) Mean Arterial Pressure, **B) Systolic Blood Pressure**, **C) Diastolic Blood Pressure** as measured through tail-cuff plethysmography. Legend inset in A applies to B and C. Bars with different letters are significantly different ($p<0.05$). **D) Peak Velocity** as measured by an ECG-triggered Doppler probe, analyzed in Doppler Signal Processing Workstation. For all weeks, WKY had lower PWV values than all SHR groups ($p<0.0001$). Data are expressed as mean ± SE (n=7-8/group).

3.1.2 Serum Biochemistry

In study 1, SHR had lower TC, LDL–C, HDL–C and TG when compared to WKY (Figure 3A–D). All SHR groups had higher HDL–C as a percentage of total cholesterol compared to WKY, with the WKY having higher LDL–C to HDL–C ratios (data not shown). All pulse–fed SHR groups had lower LDL–C (–20.5%), HDL–C (–12.5%), and TC (–12.0%) when compared to SHR–Ctrl. All SHR groups had lower serum glucose and higher serum urea levels when compared to WKY (Figure 3E,F). There were no differences between any groups with respect to serum creatinine levels (data not shown).

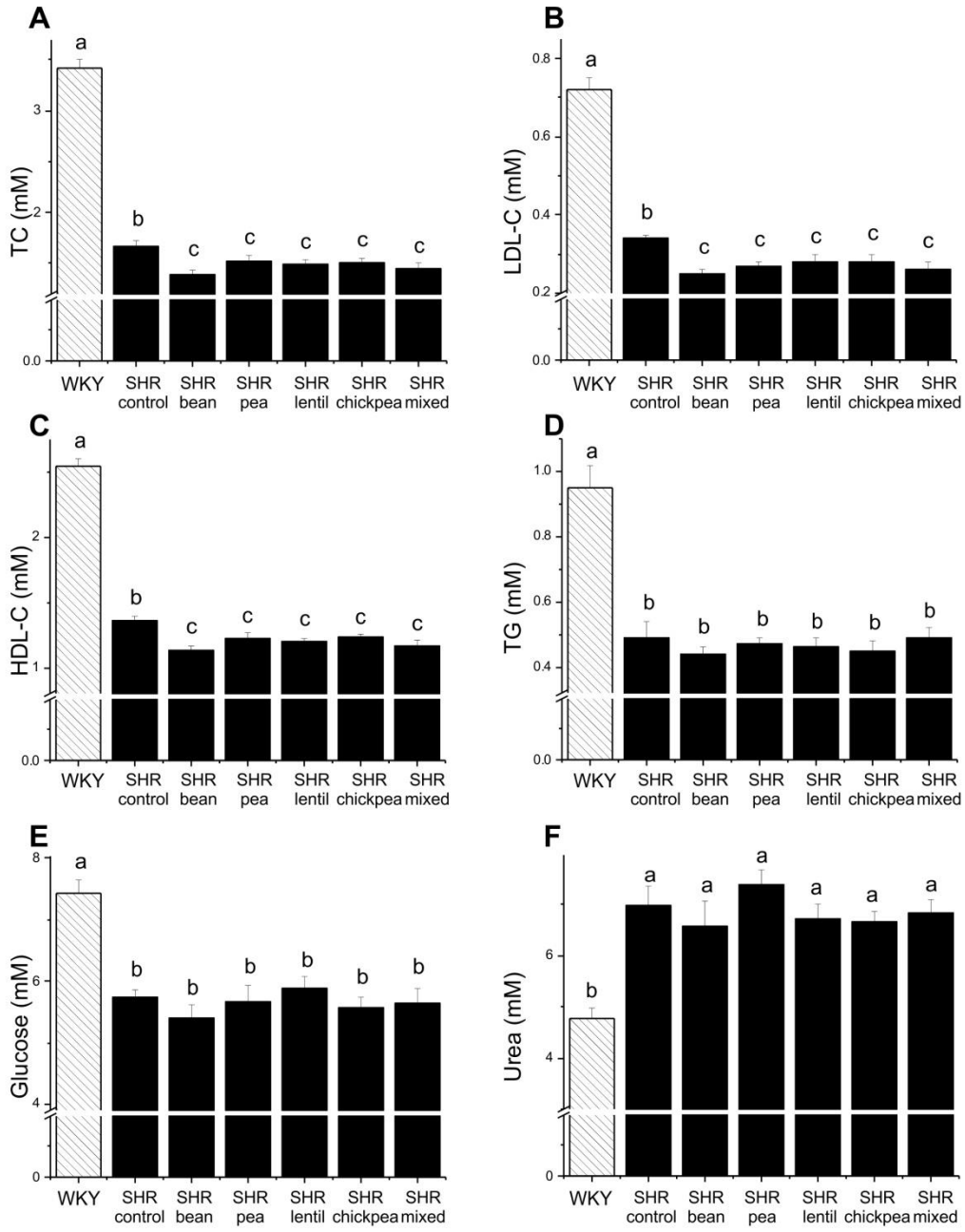


Figure 3: Serum Biochemistry (Study 1) A) Total Cholesterol, **B) LDL–C**, **C) HDL–C**, **D) TG**, **E) Glucose**, and **F) Urea** measured in serum samples obtained after a 12-hour fast on a Cobas C111 auto analyzer. Bars with different letters represent significant differences ($p<0.05$). Data are expressed as mean ± SE (n=7-8/group).

3.1.3 Tissue Weights and Vascular Measurements

At baseline, there were no differences among any of the groups with respect to BW. However, by week 2 until the end of the study, the WKY group weighed more than the SHR groups (data not shown), with no differences seen as a result of dietary intervention for final BW (Table 5).

WKY had higher peri–renal and mesenteric adipose to BW ratios compared to all SHR groups. SHR-B had significantly lower peri-renal adipose mass than SHR-Ctrl with all other SHR groups falling intermediate. WKY also had a higher epididymal fat to BW ratio compared to all SHR groups, and SHR–B and SHR–Ctrl were also different from one another. At the study end, all SHR groups had higher HW to BW ratios, as well as higher LVW weights and LVW to BW ratios compared to WKY. There were no differences between groups with respect to heart weight or liver weight to BW.

The SHR-Ctrl had higher M/L ratios than WKY, and the SHR–L diet was not different from SHR–Ctrl or WKY–Ctrl groups (Figure 4A). The WKY had lower media cross sectional area compared to all SHR groups (data not shown). There were no differences in lumen or external diameter among any of the groups (data not shown). WKY animals had smaller media widths than all SHR animals. SHR–L had smaller media width than SHR–C (–11%), with all other groups falling between the two (Figure 4B). Examples of aorta sections stained with the elastic stain kit are shown in Figure 4C.

Table 5: Tissue Weights (Study 1)

		WKY		SHR									
		Control		Control		Pea		Lentil		Chickpea		Mixed		Bean	
		Mean	*SE*	Mean	*SE*	Mean	*SE*	Mean	*SE*	Mean	*SE*	Mean	*SE*	Mean	*SE*
Body Weight (BW) (g)	*Baseline*	301	*5*	302	*2*	303	*2*	299	*2*	305	*2*	303	*4*	305	*3*
	Final	401	*7*^{a}	369	*4*^{b}	367	*4*^{b}	363	*6*^{b}	368	*5*^{b}	376	*7*^{b}	364	*5*^{b}
Peri–Renal Adipose (g/100 g BW)		1.96	*0.14*^{a}	1.33	*0.14*^{b}	1.09	*0.05*^{bc}	1.12	*0.09*^{bc}	1.19	*0.07*^{bc}	1.18	*0.13*^{bc}	1.00	*0.06*^{c}
Mesenteric Adipose (g/100 g BW)		1.01	*0.06*^{a}	0.81	*0.06*^{b}	0.78	*0.05*^{b}	0.67	*0.04*^{b}	0.72	*0.03*^{b}	0.76	*0.07*^{b}	0.67	*0.03*^{b}
Epididymal Adipose (g/100 g BW)		1.67	*0.05*^{a}	1.23	*0.07*^{b}	1.12	*0.05*^{bc}	1.12	*0.05*^{bc}	1.17	*0.06*^{bc}	1.12	*0.05*^{bc}	1.03	*0.06*^{c}
Liver (g/100 g BW)		3.23	*0.06*	3.19	*0.05*	3.37	*0.03*	3.27	*0.08*	3.38	*0.05*	3.34	*0.08*	3.35	*0.06*
Heart Weight (g)		1.42	*0.04*	1.50	*0.06*	1.53	*0.06*	1.48	*0.02*	1.53	*0.04*	1.50	*0.04*	1.50	*0.06*
(g/100 g BW)		0.35	*0.01*^{b}	0.40	*0.02*^{a}	0.42	*0.02*^{a}	0.41	*0.01*^{a}	0.42	*0.01*^{a}	0.40	*0.01*^{a}	0.41	*0.02*^{a}
LVW (g)		0.94	*0.02*^{b}	1.03	*0.02*^{a}	1.05	*0.02*^{a}	1.03	*0.02*^{a}	1.09	*0.03*^{a}	1.05	*0.03*^{a}	1.03	*0.03*^{a}
(g/100 g BW)		0.24	*0.00*^{b}	0.28	*0.01*^{a}	0.29	*0.01*^{a}	0.28	*0.01*^{a}	0.29	*0.01*^{a}	0.28	*0.01*^{a}	0.28	*0.01*^{a}

Within each row, means with different superscript letters are significantly different ($p<0.05$). The absence of superscript letters in rows indicates no significant differences.

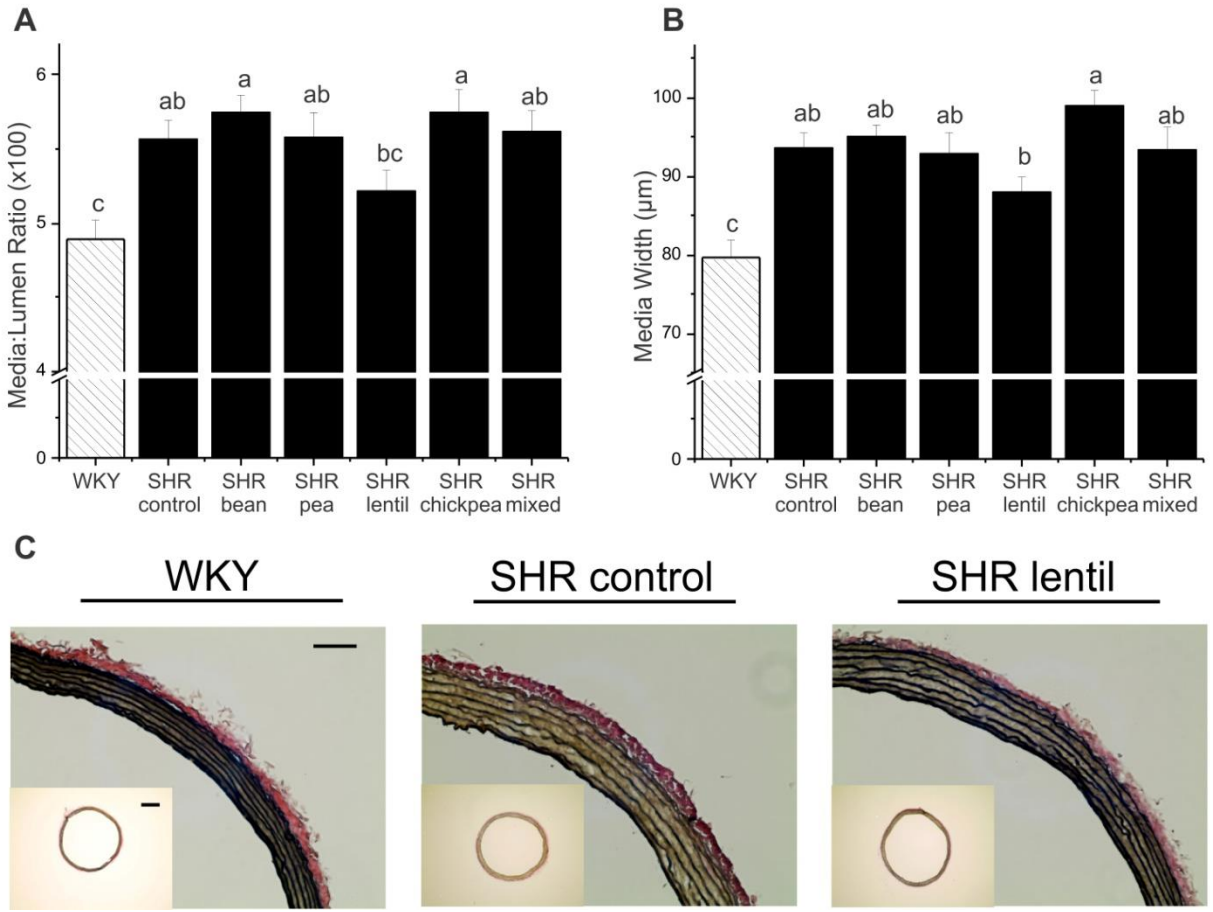
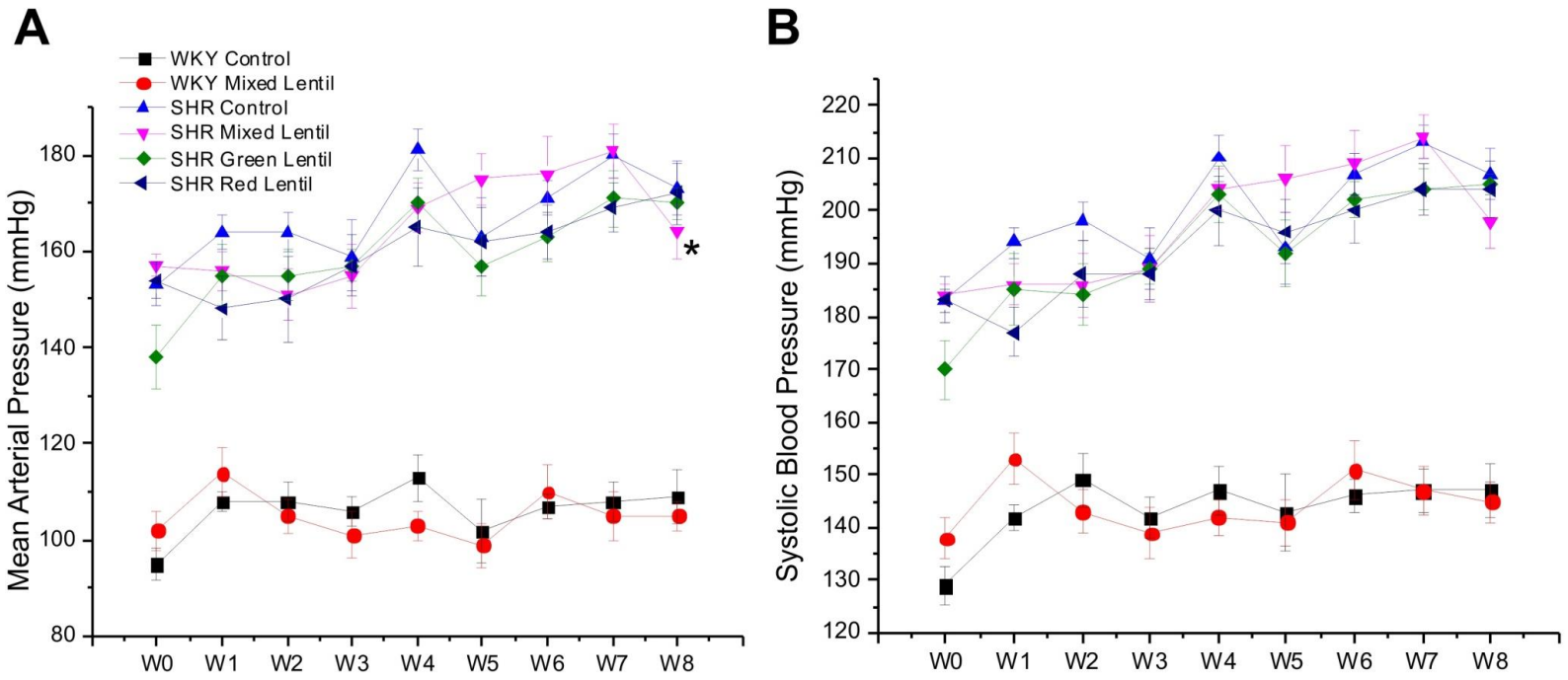


Figure 4: A) Media:lumen ratios of stained aorta sections **B) Media Width** of stained aorta sections using an Elastic Stain Kit, and measured on ImagePro Plus. **C) Representative Elastin Stained Sections** (bar = 0.1 mm, inset: bar = 0.5 mm). Bars with different letters are significantly different ($p<0.05$). Data are expressed as mean ± SE (n=7-8/group).

3.2 Study 2: Determination of the Most Effective Lentil Variety (Red/Green)

3.2.1 Pulse Wave Velocity and Blood Pressure

The SHR animals had higher MAP, SBP, and DBP than WKY animals at all time points (Figure 5A,B,C). There were no dietary-related differences in BP in WKY animals for the duration of the study. At week 8, the SHR-ML had lower MAP and DBP than all other SHR animals, but they were still higher than WKY animals. There were no diet-related SBP changes among SHR groups. Pulse wave velocity parameters PV (Figure 5D), MeFV and MFV (Table 6) were consistently higher in SHR animals than in the WKY. The RI and PI were higher in the WKY than in the SHR groups over the 8 weeks (Table 6). However, there were no significant dietary effects on any of these PWV parameters over the 8–week study.


A
WKY Control
WKY Mixed Lentil
SHR Control
SHR Mixed Lentil
SHR Green Lentil
SHR Red Lentil
Mean Arterial Pressure (mmHg)
80
100
120
140
160
180
W0
W1
W2
W3
W4
W5
W6
W7
W8
*
B
Systolic Blood Pressure (mmHg)
120
130
140
150
160
170
180
190
200
210
220
W0
W1
W2
W3
W4
W5
W6
W7
W8

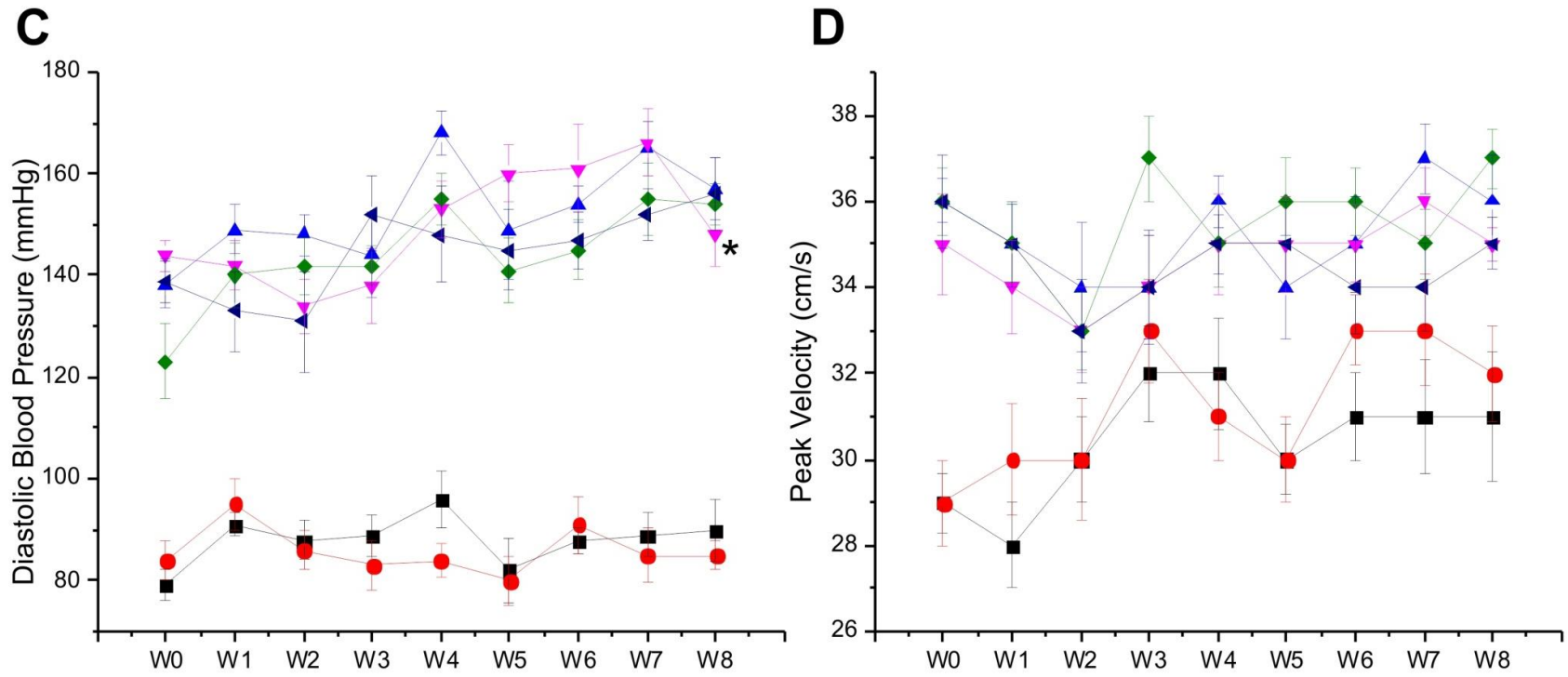


Figure 5: Blood Pressure and Pulse Wave Velocity (Study 2). **A) Mean Arterial Pressure, B) Systolic Blood Pressure. C) Diastolic Blood Pressure** measured through tail-cuff plethysmography. For all weeks, WKY-Ctrl and WKY-ML had lower BP values than SHR ($p < 0.0001$). **D) Peak Velocity** from PWV measurements using and ECG-triggered Doppler probe and analyzed on Doppler Signal Processing Workstation. For all weeks except W2 and W3, WKY-Ctrl had lower peak velocity values than SHR-Ctrl ($p < 0.0001$). *Represents significant difference compared to SHR-Ctrl. The legend inset in panel A applies to all graphs. Results are expressed as mean ± SE (n=10/group).

Table 6: Baseline and Final Values for BP and PWV Parameters[1]

		WKY				SHR							
		Control		Mixed Lentil		Control		Mixed Lentil		Green Lentil		Red Lentil	
		Mean	*SE*	Mean	*SE*	Mean	*SE*	Mean	*SE*	Mean	*SE*	Mean	*SE*
DBP	*Baseline*	79	*3*^{b}	84	*4*^{b}	138	*5*^{a}	144	*3*^{a}	123	*7*^{a}	139	*4*^{a}
(mmHg)	*Final*	90	*6*^{c}	85	*3*^{c}	157	*6*^{a}	148	*6*^{b}	154	*4*^{a}	156	*7*^{a}
SBP	*Baseline*	129	*3*‡	138	*4*‡	183	*4*	184	*2*	170	*6*	183	*2*
(mmHg)	*Final*	147	*5*‡	145	*4*‡	207	*5*	198	*5*	205	*2*	204	*5*
MAP	*Baseline*	95	*3*^{b}	102	*4*^{b}	153	*4*^{a}	157	*2*^{a}	138	*7*^{a}	154	*4*^{a}
(mmHg)	*Final*	109	*6*^{c}	105	*3*^{c}	174	*6*^{a}	164	*6*^{b}	170	*3*^{a}	172	*6*^{a}
PV	*Baseline*	29	*0.7*‡	29	*1.0*‡	36	*0.5*	35	*1.2*	36	*0.8*	36	*1.1*
(cm/s)	*Final*	31	*1.5*‡	32	*1.1*‡	36	*1.1*	35	*0.4*	37	*0.7*	35	*0.6*
MFV	*Baseline*	3.8	*0.4*‡	2.2	*0.2*‡	5.5	*0.7*	6.9	*0.7*	6.6	*0.7*	7.0	*0.7*
(cm/s)	*Final*	3.7	*0.4*‡	2.4	*0.2*‡	6.6	*0.7*	8.2	*0.6*	7.5	*0.6*	7.2	*0.6*
MeFV	*Baseline*	9.9	*0.6*‡	7.5	*0.4*‡	12.1	*0.6*	14.4	*0.6*	14.0	*0.6*	14.3	*0.7*
(cm/s)	*Final*	10	*0.5*‡	8.4	*0.4*‡	13.3	*0.6*	15.0	*0.5*	14.5	*0.5*	14.1	*0.6*
HR	*Baseline*	368	*5*	355	*5*	366	*8*	373	*8*	377	*9*	379	*6*
(BPM)	*Final*	373	*7*	360	*7*	374	*6*	377	*5*	377	*5*	373	*5*
PI	*Baseline*	3.1	*0.2*	3.7	*0.2*‡	2.6	*0.2*	2.0	*0.1*	2.1	*0.1*	2.1	*0.1*
	Final	3.0	*0.1*‡	3.6	*0.2*‡	2.4	*0.1*	1.9	*0.2*	2.0	*0.1*	2.1	*0.1*
RI	*Baseline*	0.92	*0.01*‡	0.93	*0.01*‡	0.80	*0.03*	0.81	*0.02*	0.82	*0.02*	0.81	*0.02*
	Final	0.92	*0.01*‡	0.92	*0.01*‡	0.77	*0.03*	0.78	*0.02*	0.79	*0.01*	0.81	*0.02*

Within each row, means with different superscript letters are significantly different ($p<0.05$). ‡Indicates significant difference compared to SHR-Ctrl at a given time point ($p<0.0001$).

[1]DBP – diastolic BP, SBP – systolic BP, MAP – mean arterial pressure, PV – peak velocity, MFV – minimum flow velocity, MeFV – mean flow velocity, HR – heart rate, PI – pulsatility index, RI – resistivity index

Correlation analysis of 486 measurements from week 0 to week 8 indicated a positive relationship between PV (cm/s) and MAP (mmHg) (W0–W8) ($r = 0.48$, $p<0.0001$). Similar relationships were observed in comparisons of PV with SBP and DBP ($r=0.47$, $p<0.0001$ and $r=0.50$, $p<0.0001$, respectively). However, when compared to pulse pressure there was a non–significant, negative relationship ($r = –0.09$). When analyzed on a week–by–week basis, there was an inconsistent relationship between PV and SBP, MAP or DBP (Table 7). The correlation between PV and MAP was significant for W0, W1, W2, W4 and W8. The correlation between PV and DBP was significant for W0, W1, W4, and W8. The correlation between PV and SBP was significant for W0, W1, W2, and W8. There was a consistent, non–significant, negative correlation between PV and PP for all 9 measurement weeks.

Table 7: Correlations Between Peak Flow Velocity (PV) and BP Parameters (Study 2)

	r–Value								
PV vs.	W0	W1	W2	W3	W4	W5	W6	W7	W8
DBP	0.60†	0.64†	0.51	0.26	0.57†	0.34	0.36	0.43	0.54†
SBP	0.62†	0.62†	0.55†	0.17	0.50	0.30	0.39	0.48	0.53†
MAP	0.67†	0.64†	0.53†	0.25	0.54†	0.33	0.36	0.44	0.55†
PP	–0.36	–0.37	–0.10	–0.24	–0.44	–0.29	+0.01	–0.25	–0.34

†p<0.0001 for correlation

3.2.2 Protein Analysis

Phosphorylated p38 MAPK$^{T180/Y182}$ was increased in the SHR control vs WKY, but returned to normotensive levels in lentil-fed animals when normalized to p38 MAPK (Figure 6). There were no differences among groups with respect to p-ERK1/2 when normalized to ERK1/2 (data not shown). SHR animals had higher PFN-1 levels than WKY, with no dietary differences observed (Figure 7). There were no differences between WKY and SHR animals with respect to $G\alpha_{(i)}$ levels, however, mixed-lentil fed WKY had a two-fold increase in levels compared to other groups (Figure 8).

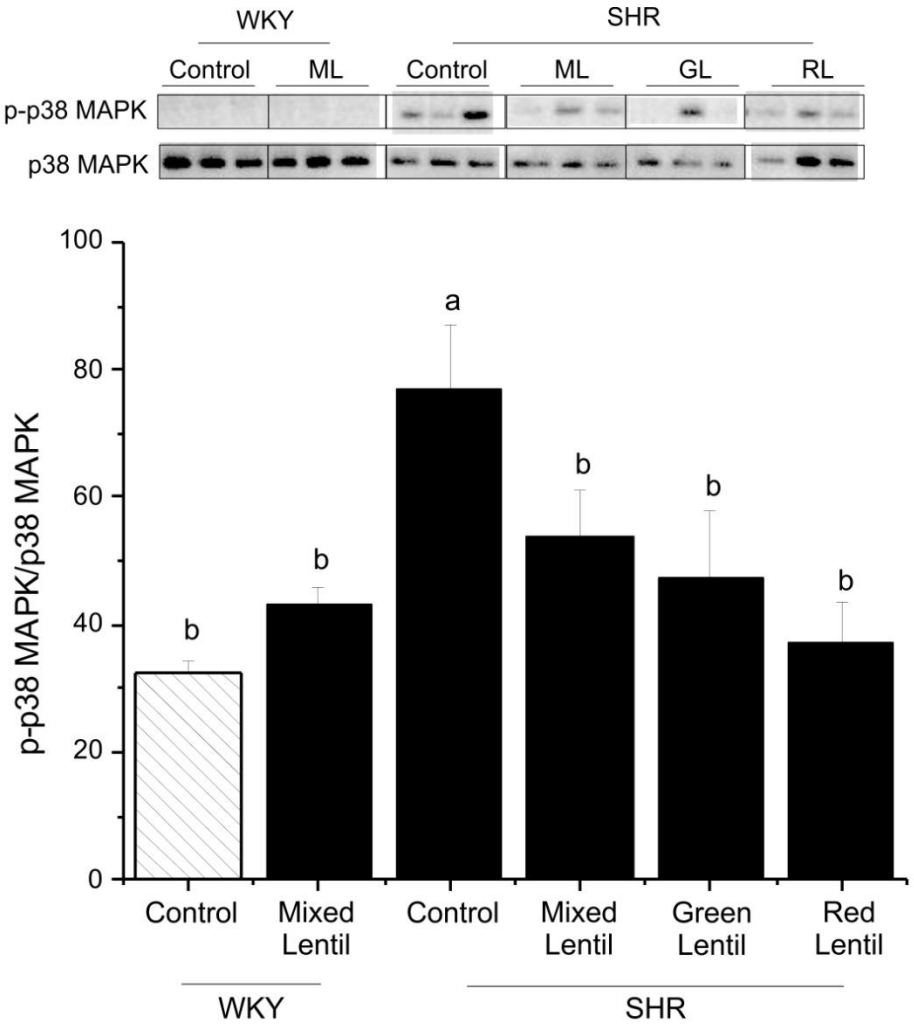


Figure 6: p-p38 MAPK$^{T180/Y182}$/p38 MAPK determined by Western Blotting from aorta protein samples (5 μg). p–p38 MAPK and p38 MAPK antibodies were applied at a concentration of 1:1000 and incubated overnight. Rabbit secondary was applied in a 1:10,000 concentration and incubated for 1 hour. Bars with different letters represent significant differences ($p<0.05$). Data are expressed as mean ± SE (n=9–10/group).

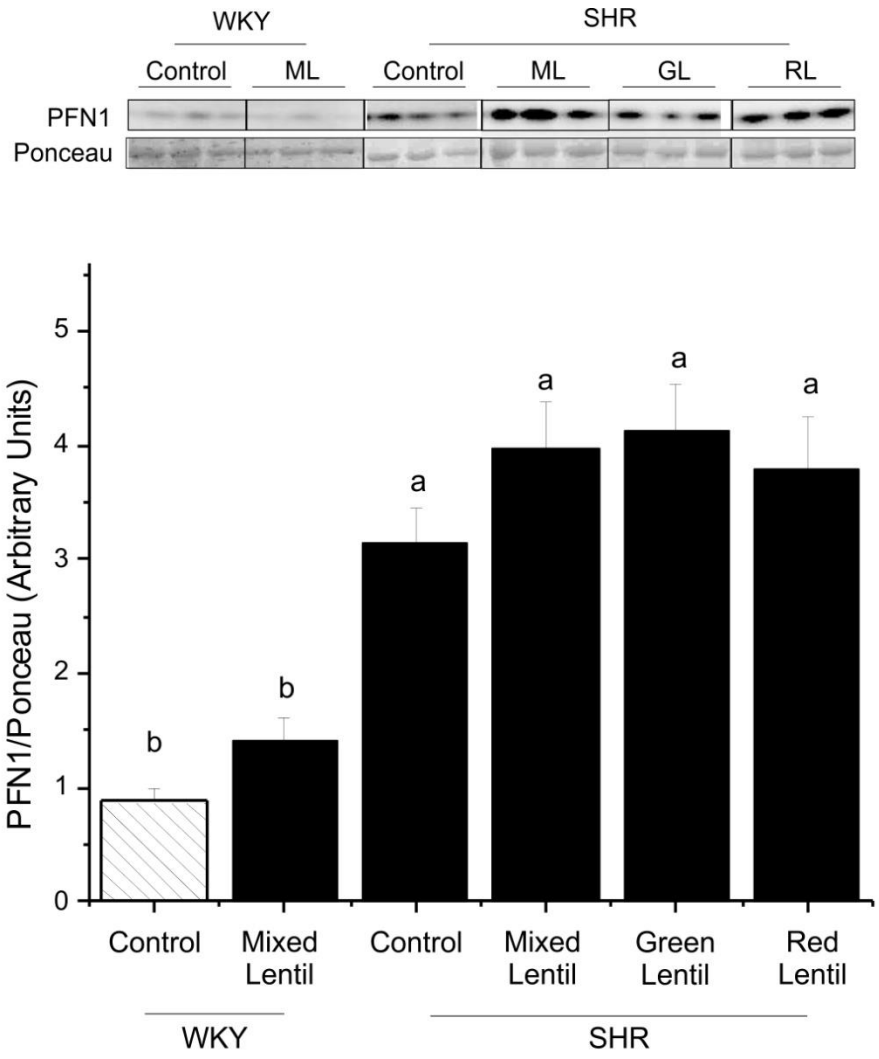


Figure 7: Profilin-1 (PFN1) levels as determined by Western Blotting on aorta protein samples (5 μg). PFN1 antibody was applied at a concentration of 1:1000 and incubated for 1 hour. Rabbit secondary was applied in a 1:10,000 concentration and incubated for 1 hour. Ponceau stained membranes were scanned then analyzed on AlphaView. "Blue background average" was selected. Bars with different letters represent significant differences ($p<0.05$). Data are expressed as mean ± SE (n=9–10/group).

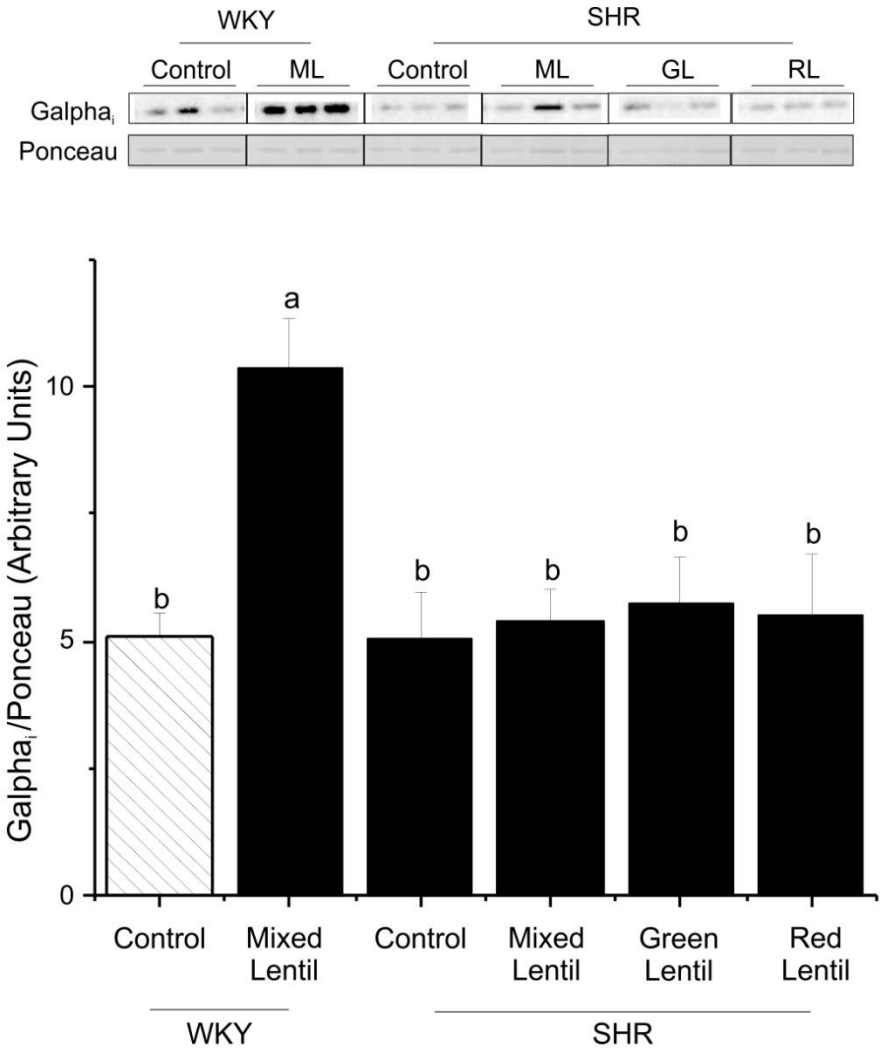
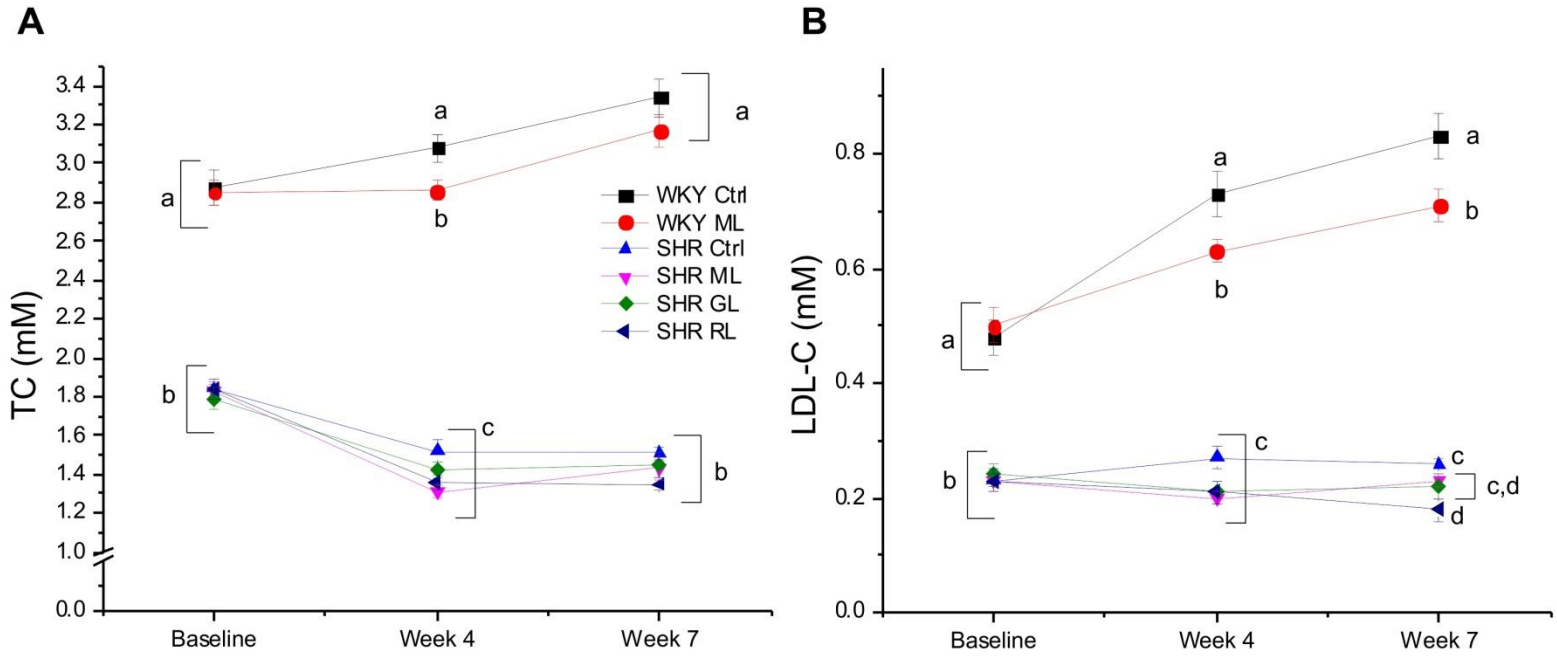


Figure 8: Galpha$_i$ (G$\alpha_{(i)}$) levels as determined by Western Blotting on aorta protein samples (5 μg). Galpha$_i$ antibody was applied at a concentration of 1:1000 and incubated overnight. Rabbit secondary antibody was applied at a concentration of 1:10,000 and incubated for 1 hour. Ponceau stained membranes were scanned then analyzed on AlphaView and "Blue background average" was selected. Bars with different letters represent significant differences ($p<0.05$). Data are expressed as mean ± SE (n=7–10/group).

3.2.3 Serum Biochemistry

At baseline, week 4, and week 8, WKY animals had higher TC, LDL-C, and HDL-C compared to all SHR groups (Figure 9A,B,C). WKY-ML had lower TC than WKY-Ctrl at week 4 (–7%). There were no differences among SHR groups with respect to TC at any time point. From week 4 until study end, WKY-ML had lower LDL-C and HDL-C compared to WKY-Ctrl (–14% and –7% at week 8, respectively). At 8 weeks, similar effects were observed in red lentil-fed SHR compared to SHR-Ctrl (–33% LDL-C and –15% HDL-C at week 8), with SHR-ML and SHR-GL falling intermediate to the two. At all time points, WKY animals had lower serum urea levels than all SHR with no dietary differences observed (Figure 9D). WKY animals had higher serum TG and glucose than SHR with no differences due to dietary intervention. There were no differences among groups with respect to serum creatinine. *For full serum profile, see Appendix G.*


A
TC (mM)
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.0
Baseline
Week 4
Week 7
WKY Ctrl
WKY ML
SHR Ctrl
SHR ML
SHR GL
SHR RL
a
b
c
B
LDL-C (mM)
0.8
0.6
0.4
0.2
0.0
c,d
d

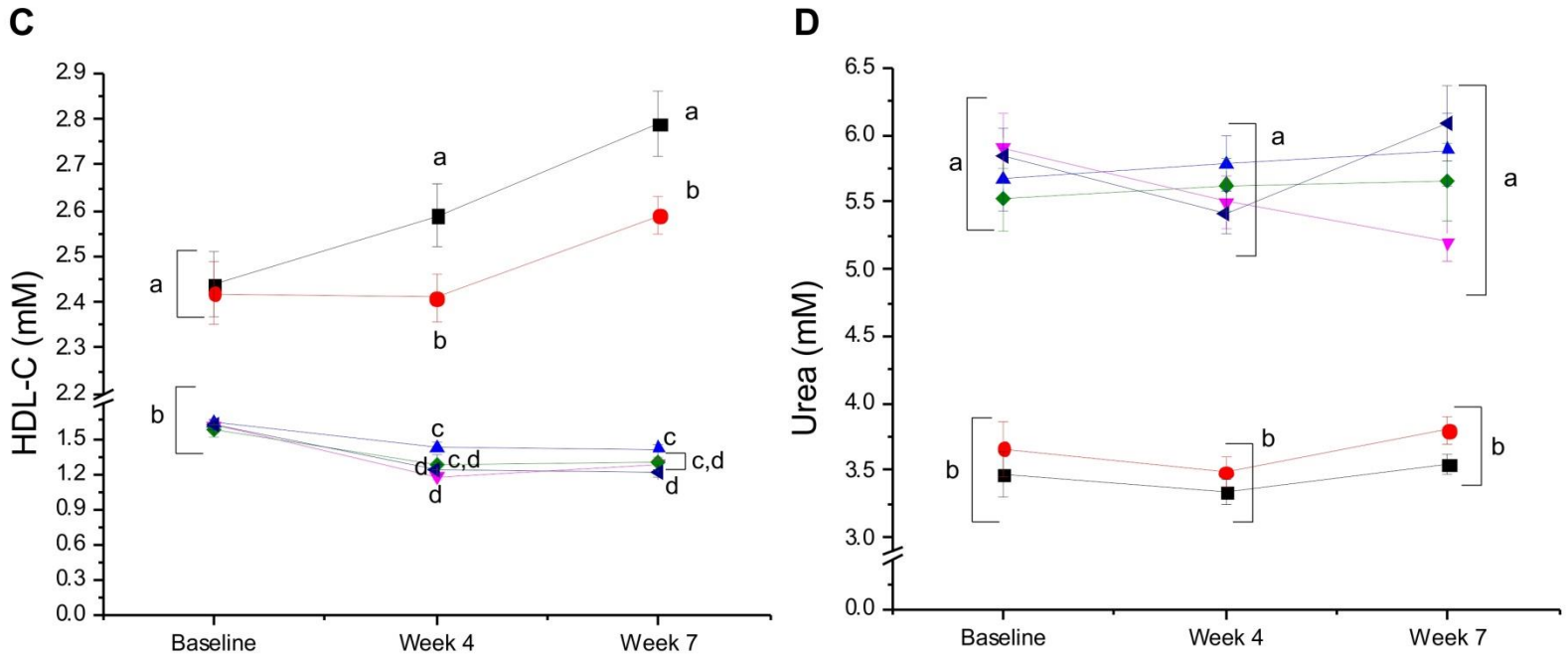


Figure 9: Serum Biochemistry (Study 2) A) Total Cholesterol, **B) LDL–C**, **C) HDL–C**, and **D) Urea** were measured in serum samples obtained after a 12-hour fast on a Cobas C111 auto analyzer. The legend inset in panel A applies to all graphs. Different letters represent significant differences ($p<0.05$). Data are expressed as mean ± SE (n=8–10/group).

3.2.4 Tissue Weights and Body Composition

At baseline and final (week 8), SHR animals weighed more than WKY control (Table 8). WKY-ML weighed less than SHR-Ctrl and SHR-GL at baseline, but were not different from SHR groups by week 8. At the end of the study, the WKY had higher peri-renal and epididymal adipose mass compared to all SHR groups (data not shown); the relationship was maintained when corrected for BW (Table 8). WKY rats had lower liver weights and liver/BW ratio compared to all SHR groups, with no dietary differences observed. There were no differences between any groups with respect to kidney weights (data not shown). The SHR groups had higher HW, HW/BW, LVW and LVW/BW when compared to both WKY groups, with no difference seen between diet groups.

When body composition was analyzed by QMR *in vivo*, there was no difference in lean mass, however, fat was higher in the SHR groups at baseline, compared to WKY (Table 9). By week 4, the WKY–Ctrl group had higher adipose mass than SHR–Ctrl, SHR–GL and SHR–RL but not SHR–ML or WKY–ML. By week 7, the WKY groups had higher adipose mass than all but the SHR–ML group, which fell intermediate to all other animals. When these data were corrected for lean mass, a weight also obtained from the QMR machine, differences were detected at weeks 4 and 7. In week 4, the WKY control group had a higher fat–to–lean–mass ratio than all SHR groups, with the WKY–ML falling intermediate. In week 7, WKY–ML was not different with respect to fat–to–lean–mass ratio compared to WKY–Ctrl and SHR–ML, and the SHR–ML was not different from all other SHR groups. The WKY–Ctrl group had a higher fat–to–lean–mass ratio than all SHR groups. WKY had higher FW

and TW than all SHR groups at baseline and week 7, with no significant changes over time.

Table 8: Tissue Weights (Study 2)

		WKY				SHR							
		Control		Mixed Lentil		Control		Mixed Lentil		Green Lentil		Red Lentil	
		Mean	*±SE*	Mean	*±SE*	Mean	*±SE*	Mean	*±SE*	Mean	*±SE*	Mean	*±SE*
Body Weight (BW) (g)	*Baseline*	322	*5^{c}*	327	*6^{bc}*	343	*3^{a}*	341	*5^{ab}*	343	*4^{a}*	337	*4^{ab}*
	Final	376	*7^{b}*	398	*8^{a}*	399	*5^{a}*	395	*8^{ab}*	399	*5^{a}*	384	*6^{ab}*
Peri–Renal Adipose (g/100g BW)		1.91	*0.14^{a}*	1.87	*0.09^{a}*	1.26	*0.06^{b}*	1.28	*0.12^{b}*	1.17	*0.07^{b}*	1.25	*0.07^{b}*
Epididymal Adipose (g/100 g BW)		1.65	*0.06^{a}*	1.63	*0.04^{a}*	1.23	*0.04^{b}*	1.26	*0.07^{b}*	1.19	*0.06^{b}*	1.22	*0.05^{b}*
Liver (g/100 g BW)		3.27	*0.06^{b}*	3.25	*0.06^{b}*	3.74	*0.06^{a}*	3.70	*0.11^{a}*	3.74	*0.09^{a}*	3.76	*0.06^{a}*
Heart Weight (g)		1.32	*0.04^{b}*	1.37	*0.03^{b}*	1.64	*0.01^{a}*	1.60	*0.04^{a}*	1.61	*0.02^{a}*	1.57	*0.04^{a}*
(g/100 g BW)		0.35	*0.01^{b}*	0.35	*0.01^{b}*	0.41	*0.01^{a}*	0.40	*0.01^{a}*	0.40	*0.00^{a}*	0.40	*0.01^{a}*
Left Ventricle Weight (g)		0.79	*0.02^{b}*	0.85	*0.02^{b}*	1.05	*0.04^{a}*	1.01	*0.05^{a}*	1.00	*0.03^{a}*	1.05	*0.03^{a}*
(g/100 g BW)		0.21	*0.00^{b}*	0.21	*0.00^{b}*	0.26	*0.01^{a}*	0.25	*0.01^{a}*	0.25	*0.01^{a}*	0.27	*0.01^{a}*

Within a row, means with different superscript letters are significantly different ($p<0.05$).

Table 9: Body Composition[1]

		WKY				SHR							
		Control		Mixed Lentil		Control		Mixed Lentil		Green Lentil		Red Lentil	
		Mean	*±SE*	Mean	*±SE*	Mean	*±SE*	Mean	*±SE*	Mean	*±SE*	Mean	*±SE*
Lean Mass (g)	*Baseline*	263	*4*	272	*3*	272	*2*	273	*2*	277	*2*	272	*2*
	Week 4	312	*9*	336	*7*	332	*4*	333	*5*	334	*5*	332	*5*
	Week 7	336	*8*	351	*8*	360	*4*	354	*5*	359	*5*	342	*5*
Fat Mass (g)	*Baseline*	25	*1*^{b}	25	*2*^{b}	35	*2*^{a}	36	*1*^{a}	32	*1*^{a}	34	*1*^{a}
	Week 4	28	*1*^{a}	27	*1*^{ab}	23	*1*^{bc}	24	*2*^{abc}	22	*1*^{c}	22	*1*^{bc}
	Week 7	33	*2*^{a}	32	*1*^{a}	22	*1*^{b}	27	*3*^{ab}	21	*2*^{b}	23	*2*^{a}
g/100 g lean mass	*Baseline*	9.2	*0.4*^{b}	9.2	*0.8*^{b}	13.1	*0.7*^{a}	13.3	*0.5*^{a}	11.7	*0.5*^{a}	12.6	*0.6*^{a}
	Week 4	8.9	*0.7*^{a}	8.0	*0.4*^{ab}	7.1	*0.5*^{b}	7.2	*0.7*^{b}	6.5	*0.5*^{b}	6.9	*0.5*^{b}
	Week 7	9.7	*0.7*^{a}	9.0	*0.4*^{ab}	6.2	*0.4*^{c}	7.2	*1.0*^{bc}	6.0	*0.6*^{c}	6.6	*0.7*^{c}
Total Water (TW)(g)	*Baseline*	220	*3*	229	*3*	229	*1*	230	*2*	234	*1*	227	*3*
	Week 4	262	*7*‡	283	*6*	280	*4*	281	*4*	282	*4*	280	*4*
	Week 7	279	*7*‡	295	*7*	299	*5*	298	*4*	302	*4*	287	*4*
g/100 g lean mass	*Baseline*	0.84	*0.01*	0.85	*0.01*	0.84	*0.01*	0.84	*0.01*	0.85	*0.01*	0.85	*0.01*
	Week 4	0.84	*0.01*	0.84	*0.01*	0.84	*0.01*	0.84	*0.01*	0.84	*0.01*	0.84	*0.01*
	Week 7	0.84	*0.01*	0.84	*0.01*	0.84	*0.01*	0.84	*0.01*	0.84	*0.01*	0.84	*0.01*
Free Water (g)	*Baseline*	0.91	*0.11*‡	0.90	*0.08*‡	0.63	*0.06*	0.82	*0.11*	0.79	*0.07*	0.65	*0.04*
	Week 4	0.91	*0.06*	1.09	*0.12*‡	0.76	*0.08*	0.92	*0.09*	0.91	*0.07*	0.92	*0.09*
	Week 7	1.25	*0.09*‡	1.39	*0.12*‡	0.89	*0.09*	0.94	*0.07*	1.04	*0.08*	0.84	*0.05*
g/100 g TW	*Baseline*	0.42	*0.06*‡	0.40	*0.03*‡	0.28	*0.03*	0.36	*0.05*	0.34	*0.03*	0.29	*0.02*
	Week 4	0.40	*0.05*‡	0.39	*0.04*‡	0.28	*0.03*	0.33	*0.03*	0.33	*0.03*	0.33	*0.03*
	Week 7	0.46	*0.03*‡	0.48	*0.04*‡	0.30	*0.03*	0.32	*0.02*	0.35	*0.02*	0.29	*0.02*

[1]Measurements presented were obtained *in vivo* with a QMR instrument. Results are expressed as mean ± SE (n=10/group). For means at the same time point, different superscript letters represent significant differences ($p<0.05$).
‡Indicates significant difference compared to SHR-Ctrl at a given time point ($p<0.0001$).

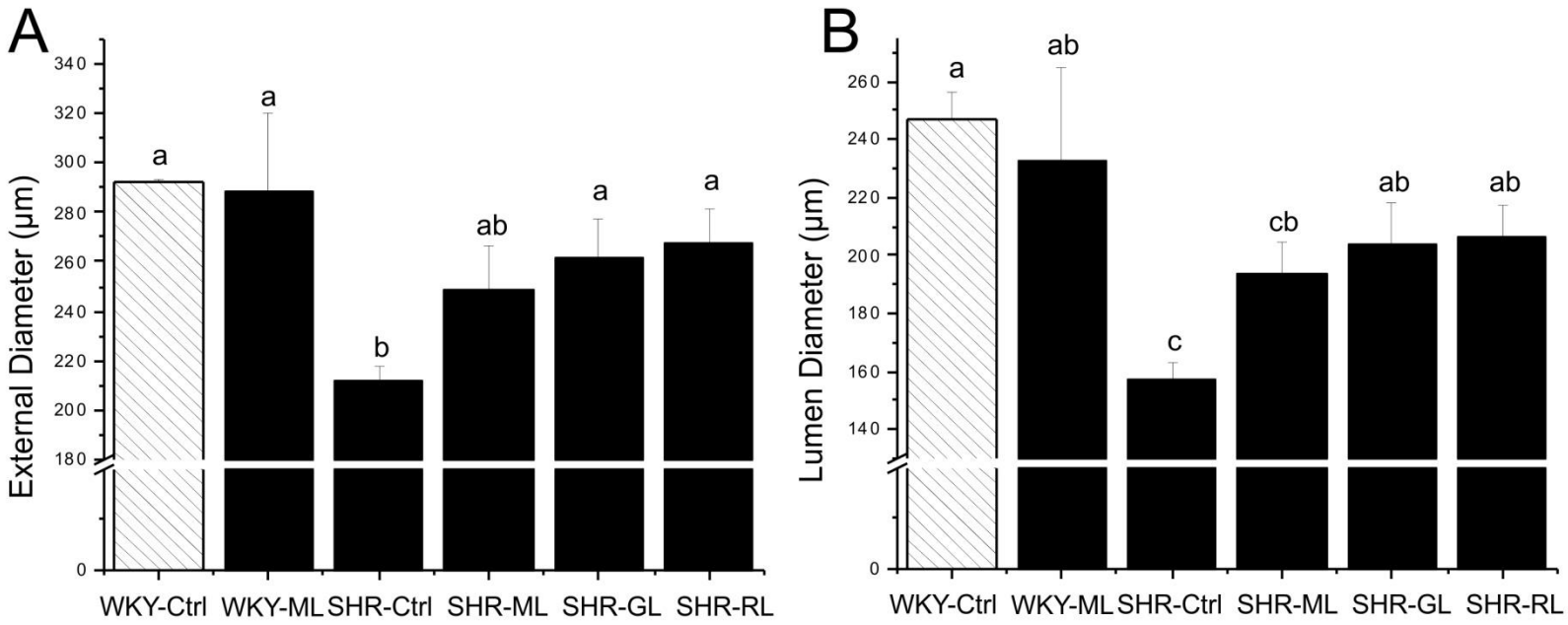
3.2.5 Pressure Myography

3.2.5a Endothelial Function

Due to large variability among measurements and small sample size, no meaningful data were obtained from endothelial function measurements.

3.2.5b Vascular Geometry (45 mmHg)

Wall thickness and lumen diameter readings taken at 45 mmHg indicated that the SHR–Ctrl had a smaller external diameter than all other groups except SHR–ML, which was intermediate to the other groups (Figure 10A). SHR–Ctrl had a smaller lumen diameter than WKY–Ctrl, WKY–ML, SHR–GL and SHR–RL (Figure 10B). There were no differences with respect to media width (Figure 10C) or media CSA (data not shown) between any of the animals. SHR–Ctrl had higher M/L ratios than the WKY–Ctrl (+93%) and WKY–ML (+34%) animals. WKY-ML had a higher M/L ratio (+45%) compared to WKY-Ctrl. SHR-ML decreased the M/L ratio (–22%) compared to SHR-Ctrl. SHR–GL and SHR–RL had similar values (–21% and –17%, respectively) compared to SHR-ML, but they were not statistically different from SHR-Ctrl (Figure 10D).


A
External Diameter (µm)
340
320
300
280
260
240
220
200
180
0
a
a
b
ab
a
a
WKY-Ctrl
WKY-ML
SHR-Ctrl
SHR-ML
SHR-GL
SHR-RL
B
Lumen Diameter (µm)
260
240
220
200
180
160
140
0
a
ab
c
cb
ab
ab
WKY-Ctrl
WKY-ML
SHR-Ctrl
SHR-ML
SHR-GL
SHR-RL

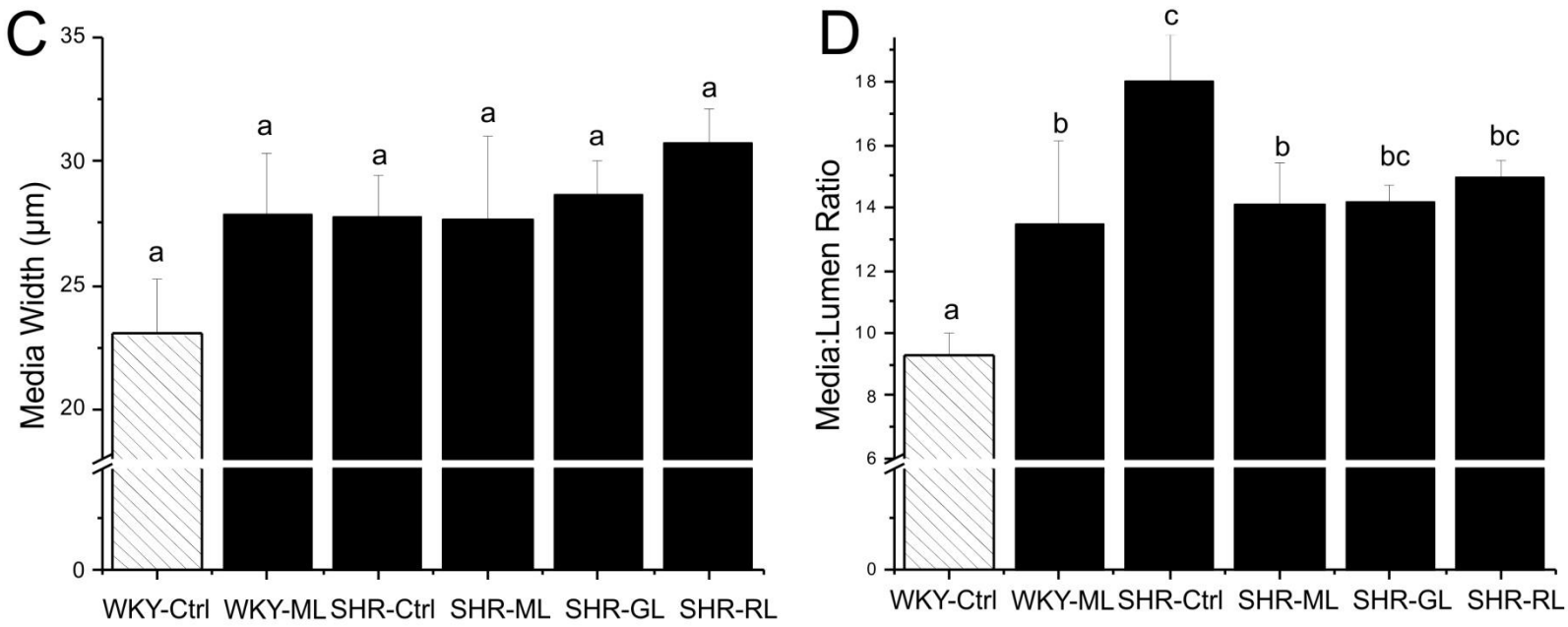
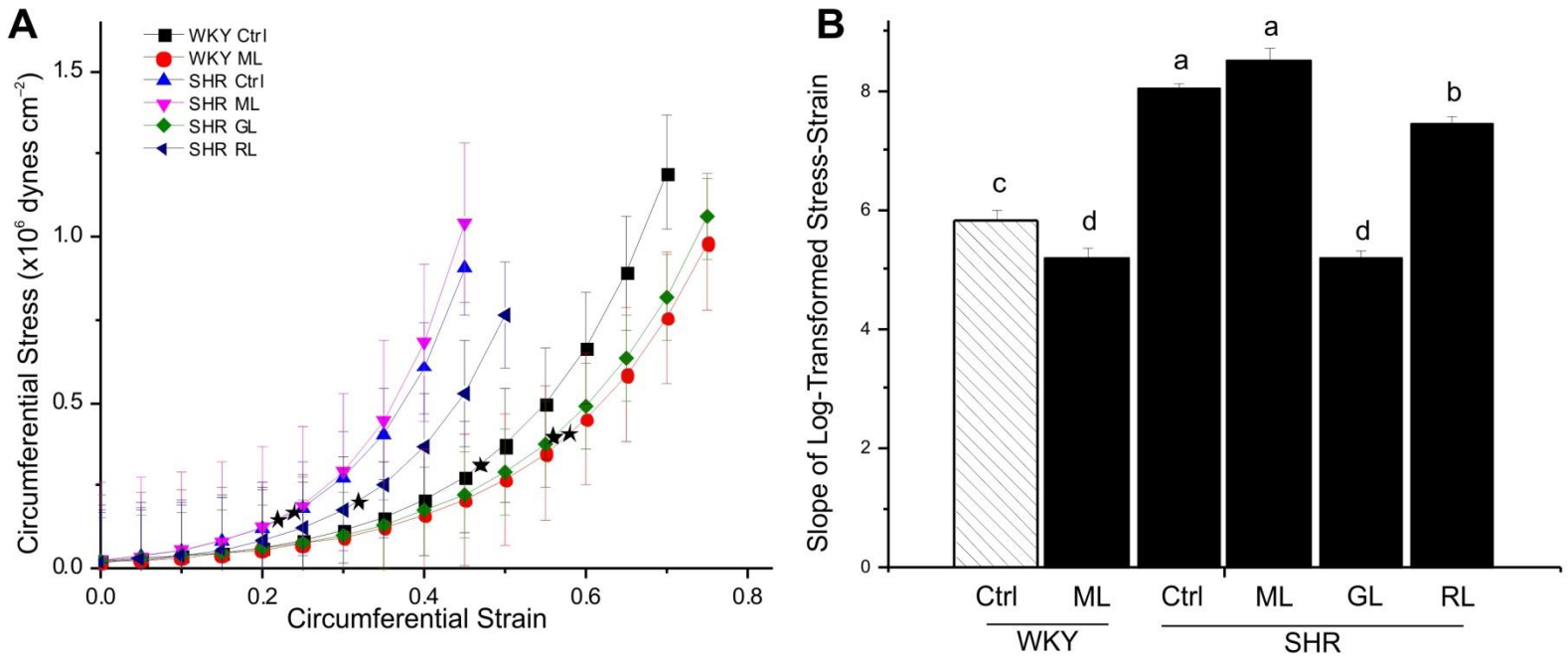


Figure 10: Vascular Geometry measurements of mesenteric arteries calculated from data obtained at 45 mmHg on the pressure myograph. **A) External Diameter** and **B) Lumen Diameter C) Media Width** and **D) Media:lumen Ratio**. Bars with different letters represent significant differences ($p<0.05$). Data are expressed as mean ± SE (n=6-7/group).

3.2.5c Isobaric Vascular Measurements

The stress–strain curve showed that the SHR–Ctrl animals had impaired ability to mitigate the tension caused by pressure compared to WKY–Ctrl, indicating decreased arterial compliance in these animals (Figure 11A). Again, the SHR–ML group showed no improvement compared to the SHR–Ctrl. The SHR–RL curve fell between the SHR–Ctrl and WKY–Ctrl. The SHR–GL curve fell to the right of the WKY–Ctrl curve indicating complete restoration of the vessel's ability to buffer pressure changes on the stress strain curve. The slopes of the log–transformed stress-strain relationship indicated that the SHR–GL and WKY–ML animals were not different, but both SHR-GL and WKY-ML were significantly lower than WKY-Ctrl indicating improved arterial compliance compared to normotensive control animals (Figure 11B). Every other group except SHR-Ctrl and SHR-ML had different slopes when compared to one another, indicating differences in vascular compliance.

The stiffness of the vessels based on the slope of the elastic modulus versus isobaric stress showed increased stiffness in the SHR–Ctrl (+46%) compared to the WKY-Ctrl animals (Figure 11C,D). The SHR-GL and SHR-RL had decreased stiffness (–38% and –24%, respectively) compared to SHR-Ctrl, with no significant difference between SHR-Ctrl and SHR-ML. WKY-ML had decreased arterial stiffness (–34%) compared to WKY-Ctrl, though this reduction was not significant. The changes seen in arterial stiffness assessed by the slope of the elastic modulus versus isobaric stress (Figure 11D) reflect similar changes seen in arterial stiffness assessed by the slope of the log–transformed stress–strain relationship (Figure 11B).


A
WKY Ctrl
WKY ML
SHR Ctrl
SHR ML
SHR GL
SHR RL
Circumferential Stress (x10^6 dynes cm^-2)
Circumferential Strain
0.0
0.5
1.0
1.5
0.0
0.2
0.4
0.6
0.8
B
Slope of Log-Transformed Stress-Strain
0
2
4
6
8
c
d
a
a
d
b
Ctrl
ML
Ctrl
ML
GL
RL
WKY
SHR

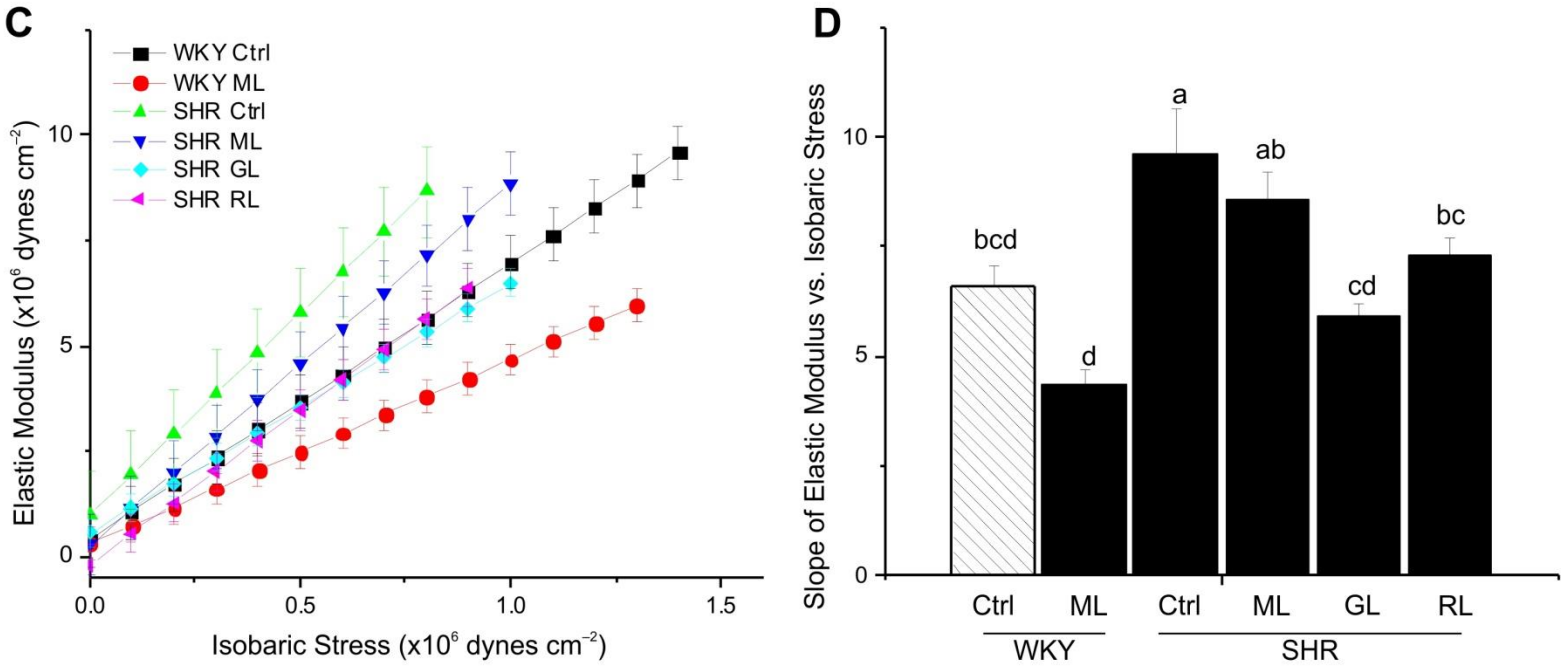


Figure 11: Isobaric Measurements obtained from the pressure myograph. **A) Stress–Strain Relationship**, **B) Slope of Log–transformed Stress–Strain Relationship** to determine vascular compliance, the ability of arteries to stretch in response to increased pressure. In **A)** the stars (★) on the graph represent the point when the change in slope becomes ≥100 000 dynes•cm^{-2}, an arbitrary 'break point' **C) Elastic Modulus vs. Isobaric Stress**, and **D) Slope of Elastic Modulus vs. Isobaric Stress**, a measurement of arterial stiffness. Bars with different letters represent significant differences ($p<0.05$). Data are expressed as mean ± SE (n=6-7/group).

4. Discussion

4.1 Arterial Stiffness, Blood Pressure, and Vascular Compliance

4.1.1 Arterial Stiffness and Blood Pressure

From the first study, it was evident that the SHR animals had higher PWV than the normotensive WKY. This indicates that a) they have stiffer arteries than WKY animals, which justifies the choice of animal model, and b) the Doppler machine was able to detect these differences. However, there was no dietary effect on the PWV in any of the animal groups over the four–week study period. On the other hand, the lentil diet was able to greatly attenuate the rise in BP experienced by the SHR control group (Study 1; $p<0.05$), but it did not cause it to decline. It is possible that much of the remodeling resulting in stiffness was established before the onset of experimental diet, and the subsequent decrease in BP; thus reduced hemodynamic stress was not enough to affect the stiffness causing remodeling in the short term. However, as evidenced from the aortic sections, there appeared to be changes in the remodeling of the aorta in those animals with decreased BP, namely the SHR–L group. Therefore, it could be that the intervention was not long enough to see these changes in vessel morphology translated into PWV as read by the Doppler probe. Alternatively, it has been reported that PWV is related closely to changes in BP, which is not indicative of changes in vessel morphology or function (Gribbin *et al.*, 1976; Kim *et al.*, 2007; McCall *et al.*, 2011).

It has been previously shown that a correlation exists between PWV and transmural pressure (linearly related to MAP), and this correlation was consistently and highly significant over a large range of pressures and ages ($r=0.9+$,

p<0.001)(Gribbin *et al.*, 1976). In another study, PP, of all other variables (including MAP, age, HP, SBP, DBP, waist–hip ratio, *etc.*), correlated to PWV to the greatest extent (Kim *et al.*, 2007). The study by Kim *et al.* (2007) was invasive, and likely had more accurate measurements of BP and PWV than would be obtained through the Doppler machine and tail–cuff plethysmography used in this study, which found a correlation of r=0.48 (p<0.0001) between MAP and PV. However, the loss of significance upon separation of WKY and SHR, and variability seen on a week–by–week basis indicates that the relationship between the values is not very strong and could be a number–biased result due to the large sample size. Then again, Najjar *et al.* (2008) showed that PWV predicts longitudinal increases in SBP. Therefore, a patient with a higher PWV will have a more rapid increase in SBP over time than an age matched patient with a lower PWV (London & Guerin, 1999). This could be indicative of positive feedback between the stiffening of the arteries and BP, causing increases in each parameter and resulting in escalating damage.

Another possible reason there was only a weak, positive correlation between PV and BP could be that the measurements were not taken at the same time. BP was measured on conscious animals, whereas PWV measurements were done on anesthetized rats, and the BP/PWV measurements were performed on separate days. If the BP measurements were made simultaneously with the PWV measurements, the fluctuations seen in BP may relate more closely to the variability in PV seen between consecutive Doppler measurements and would strengthen this relationship. Variability of PWV measurements could also be explained as a result of human error in the fact that the Doppler probe was held by hand, resulting in

variable angles of measurement. From equation (4), it is evident that changing the angle of the probe will affect the measured velocity and thus introduce greater variability between consecutive measurements.

Although the rise in BP was only attenuated by lentils in the first study, it has been estimated that a 3 mmHg drop in SBP could reduce CHD and stroke mortality by 5% and 8%, respectively (Stalmer, 1991). Alternatively, the mortality from stroke and IHD doubles for every 20 mmHg increase in SBP or 10 mmHg increase in DBP (Chobanian *et al.*, 2003). On average for study 1, the MAP in pulse-fed animals was 18 mmHg lower than the SHR control group (range 144 mmHg (lentil) to 156 mmHg (chickpea) vs. 171 mmHg), except the mixed pulse diet, which was not significantly different than SHR control (177 mmHg). In terms of SBP and DBP, the lentil–fed animals had average increases of 1 mmHg and 10 mmHg versus 26 mmHg and 33 mmHg increases in the control–fed SHR. The attenuation of both BP parameters seen in the lentil–fed SHR would, based on the information from Chobanian *et al.* (2003), result in more than a 4–fold decrease in CVD–related mortality.

In study 2 the average MAP in lentil–fed SHR was 5 mmHg lower when compared to SHR control (range 164 mmHg to 171 mmHg vs. 174 mmHg). The mixed–lentil fed WKY were also, on average, 4 mmHg lower when compared to WKY control. This shows that, while not significant, the lentils had a modest effect on BP over 8 weeks. Furthermore, the fact that a comparable difference in BP was seen in the lentil–fed normotensive animals is especially important as the JNC7 report suggested that prehypertensive patients should attempt to control their

hypertension with lifestyle modifications and not with pharmaceuticals (Chobanian *et al.*, 2003). Long-term dietary intervention with lentils appears to have a modest effect on BP regardless of BP status, without leading to a hypotensive state.

4.1.2 Arterial Compliance

As arteries become stiffer and less elastic, they lose their ability to temporarily stretch and store volume, known as compliance (Glasser, 2000). Arterial compliance is important in buffering the pulsatile blood flow imparted on the vasculature by the heart into the continuous flow received by the organs (McVeigh *et al.*, 1999; Luft, 2012). Mirroring stiffness, aging reduces arterial compliance (McVeigh *et al.*, 1999). Hypertension causes changes to the vasculature relating to stiffness and compliance (elastin and collagen remodeling, VSMC hypertrophy, *etc.*) to occur at an earlier age or a rate faster than would otherwise be normal. Endothelial dysfunction also decreases compliance, causing the basal vasculature tone to be increased (McVeigh *et al.*, 1999). According to the Bramwell–Hill equation:

$$c^2 = \frac{\Delta P}{\Delta v} \times \frac{V}{P} \quad (18)$$

it is evident that PWV (c) is inversely related to vascular compliance ($\Delta v/\Delta P$) (Bramwell & Hill, 1922; Gribbin *et al.*, 1976; London & Guerin, 1999). Therefore, as the compliance of the arteries decreases, the PWV will increase.

However, it is prudent to perform measurements of arterial stiffness other than PWV measurements. Due to the close relationship between BP and PWV, one

could reasonably expect a decrease in BP would result in a corresponding decrease in PWV, though this is not indicative of remodeling (McCall *et al.*, 2011). Isobaric measurements of vascular geometry through pressure myography in a Ca^{2+} free Krebs buffer offers an *ex vivo* method of arterial stiffness determination.

Pressure myography in study 2 indicated that there was a large increase in vascular compliance elicited by the green lentil diet in SHR. The stress–strain graph, indicative of vascular compliance, was shifted to the left in the SHR–Ctrl group, but restored to WKY levels in the SHR–GL group. The graph begins with a linear relationship, and this compliance is mainly due to the elastin content of the arteries, the elastic wall component. When the relationship becomes curved, this is indicative of the area when the elastin is no longer able to function with increasing pressure, and the stiffer collagen fibres prevent the arteries from over–expanding. The area where the linear part of the graph meets the curve in the curvilinear relationship is the break point, and is different between SHR–Ctrl and SHR–GL groups. This indicates that, for the green–lentil fed group, the arteries are able to effectively buffer higher arterial pressure better than those from the control–fed SHR. Additionally, slopes of log–transformed graphs indicate that green lentil-fed animals had better vascular compliance than the WKY–Ctrl animals, and not different than the WKY–ML group. Increased arterial compliance has been associated with reduced risk of CVD (Zieman *et al.*, 2005).

The improvement in vascular compliance seen in the arteries of WKY animals fed mixed lentils is also promising for arresting disease progression. Increased arterial stiffness and BP are both associated with increasing age, but are

preventable. Seeing improvements in normotensive animals suggests that the effects of lentils apply to all levels of BP, and improve arterial compliance regardless of BP status, though the effect was much greater in the hypertensive animals. Coupled with the fact there was a non–significant decrease in BP in lentil-fed WKY, these data indicate that lentils would be valuable additions to everyone's diet, whether healthy or with hypertension.

4.2 Cardiovascular Remodeling

4.2.1 Tissue Weights and Aortic Histology

Chronic hypertension has been associated with increased remodeling of the arteries as well as the heart, with chronic systemic hypertension noticeably affecting the left ventricle and resistance arteries. In studies 1 and 2, cardiac remodeling was indicated through higher HW/BW and LVW/BW ratios seen in the SHR groups compared to WKY ($p<0.05$; Tables 5&8). However, there was no dietary effect on these measurements, which could be due to the short duration of the intervention (4-8 weeks) or the onset of intervention. At the onset of experimental diet intervention, the animals were 17 weeks old, having experienced prehypertension and hypertension for many weeks prior, and thus the remodeling of the heart could have already be established past the point of reversal. However, there did appear to be a dietary effect on the vascular remodeling in study 1, with the lentil group having an aortic M/L ratio that was not significantly different, but between the SHR–Ctrl or WKY–Ctrl groups, which were significantly different. An increase in the M/L ratio indicates either vascular remodeling, VSMC growth, or a combination of the

two (Heagerty *et al.*, 1993). While there were no baseline measurements taken to determine if the lentils were able to arrest the remodeling or if the diet actually resulted in a reversal of the remodeling process, it is evident that the lentil diet had an effect on this cellular process. This relationship was strengthened through the use of pressure myography.

4.2.2 Arterial Remodeling

Typically, vascular remodeling is better observed in the smaller resistance arteries, or those arteries responsible for TPR – a major component of the BP regulatory system and contributor to BP. TPR is related to MAP through the equation

(19) $MAP = TPR \times CO$

where CO is cardiac output, the product of stroke volume and heart rate. Therefore, if TPR is decreased, MAP would decrease, thus highlighting the importance of properly functioning and morphologically–normal resistance arteries. Resistance arteries are vessels with a lumen diameter of 350 μm or less (Heagerty *et al.*, 1993; Schiffrin *et al.*, 2000; Briones *et al.*, 2003; Rhoades & Bell, 2009; Rizzoni & Agabiti-Rosei, 2012). The third–order mesenteric arteries (150–250 μm) selected for pressure myography are resistance arteries and were chosen due to their propensity to undergo remodeling during hypertension, as well as being relatively un–branched, which makes them easier to mount and pressurize on the myograph (Briones *et al.*, 2003; Thandapilly *et al.*, 2012). The remodeling of resistance arteries appears to occur at a similar rate throughout the body, including the brain, gut, and

heart, further justifying the choice to study the mesenteric arterial bed (Heagerty *et al.*, 1993; Eftekhari *et al.*, 2012). Additionally, resistance arteries may be the first organ damaged as a result of hypertension, and would therefore be an excellent end-point for treatment effectiveness, and could be a marker of early damage occurring before cardiac remodeling and renal damage (Park & Schiffrin, 2001; Rizzoni & Agabiti-Rosei, 2012).

Inward eutrophic remodeling is common in essential hypertension and is indicated by a smaller lumen and external diameters with little change in the media CSA (Eftekhari *et al.*, 2012; Rizzoni & Agabiti-Rosei, 2012)(Figure 12). This eutrophic remodeling may perpetuate the disease state and, through M/L ratio measurements, could be a useful marker for disease progression and, by extension, intervention effectiveness (Rizzoni & Agabiti-Rosei, 2012). In our studies, it is likely that the remodeling observed was eutrophic due to the alterations in lumen and external diameters, wall thickness, and M/L ratio measurements without significant changes to media CSA (Rizzoni & Agabiti-Rosei, 2012). This is a common remodeling process in response to vascular stresses as seen in hypertension and/or diabetes. In fact, people with both hypertension and diabetes – over one million Canadians – have greater M/L ratios of subcutaneous arteries than those with either hypertension or diabetes alone (Public Health Agency of Canada, 2010; Rizzoni & Agabiti-Rosei, 2012). The M/L ratio is also larger in hypertensive persons with previous cardiovascular events (*e.g.* MI) compared to hypertensive persons without (Rizzoni *et al.*, 2003). Arterial remodeling in beds responsible for peripheral resistance and BP regulation can lead to a state of dysregulated BP, eventually

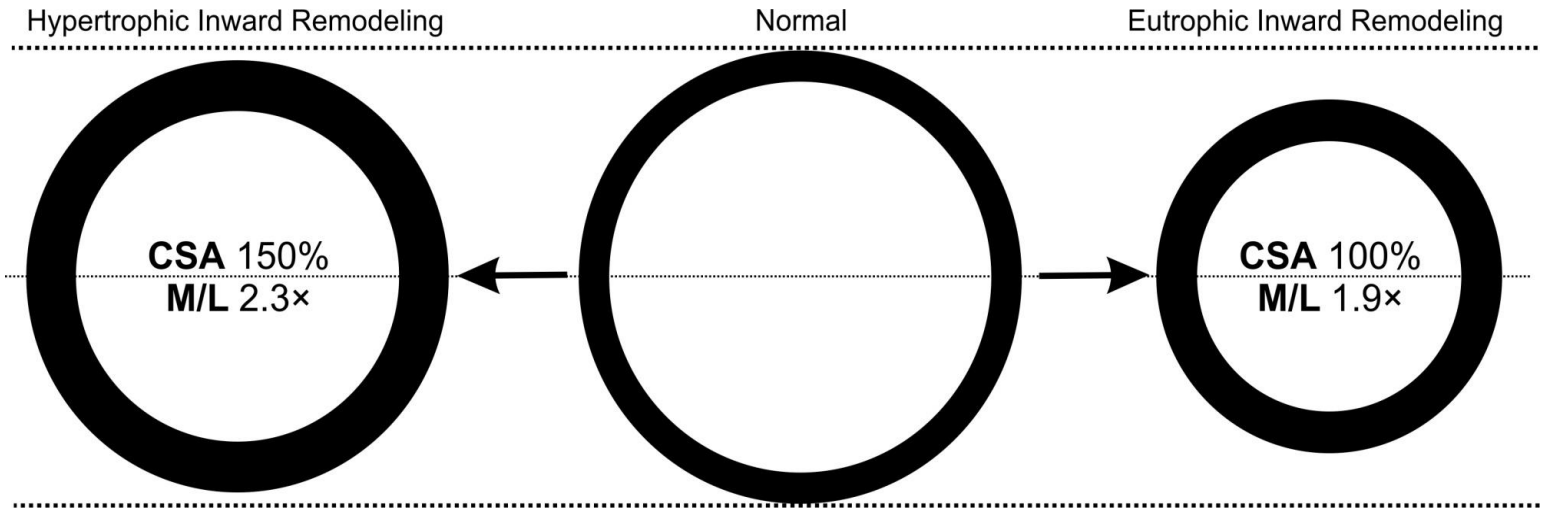


Figure 12: Vascular Remodeling. Hypertrophic inward remodeling (left) is marked by decreased lumen diameter and increases in media width, media cross sectional area (CSA), and media:lumen (M/L) ratio. Eutrophic inward remodeling (right) is marked by decreased lumen diameter; increased media width and M/L ratio; and unchanged media CSA. Adapted from New *et al.* (2004) & Heagerty *et al*. (1993).

resulting in organ damage including renal, cardiac, and cerebral events (Intengan & Schiffrin, 2001; Rizzoni *et al.*, 2003).

Symptomatic treatments of BP that are not able to alter this remodeled state and restore it towards normalcy are, in a way, ineffective. The ideal treatment state would be to both lower BP and, at the same time, stop and reverse vascular remodeling. In the eight–week lentil study, we were able to see the increased mesenteric artery remodeling in the control fed SHR versus WKY as evidenced by the decreased lumen diameter and external diameter. This resulted in the SHR–Ctrl animals having a higher M/L ratio than the WKY–Ctrl, WKY–ML, and SHR–ML groups, with the SHR–GL and SHR–RL falling intermediate to the two extremes ($p<0.05$; Figure 10). Additionally, SHR–GL and SHR–RL had significantly larger lumen diameters and external diameters than SHR–Ctrl with no appreciable change in media width, indicating decreased remodeling in the arteries of lentil–fed animals.

In the first study, the decreased aortic remodeling occurred in the lentil group, which also exhibited the greatest reduction in BP. However, no significant correlation existed between M/L ratio of the aorta and MAP in this study (data not shown). This lack of correlation was largely a result of the increased BP and reduced M/L ratio seen in the mixed pulse-fed group. In the second study, the mixed lentil group had the largest reduction of BP, unchanged vascular compliance, and remodeling similar to the green lentil–fed animals. The green lentil–fed animals showed a markedly improved vascular compliance, comparable remodeling and non–significantly higher BP than those fed the mixed lentil diet. This underscores

that changes in BP, vascular remodeling, and vascular compliance can occur independent of one another.

Despite the variability of BP values for animals on the lentil containing diet, there is consistent evidence that a diet high in lentils is able to affect the processes involved in arterial remodeling in both large conduit and smaller resistance arteries. Though the mechanism behind this is unclear, this would make lentils valuable adjuncts to pharmaceutical treatment, especially those treatments that effectively control BP but do not mediate an effect on the vasculature (*e.g.* β-blockers such as atenolol). A diet high in lentils may reduce the dose or number of drugs required to obtain the ideal treatment state of reduced remodeling and BP, by effectively restoring proper arterial morphology and compliance.

4.2.3 Protein Analysis

Hypertrophy in the vasculature is an outcome of the activation of many signaling pathways by growth factors, AngII, endothelin-1 (ET1), and oxidative stress to name a few. Of the downstream signaling mediators of hypertrophy, ERK1/2 and p38 MAPK have been widely studied. p38 MAPK showed increased phosphorylation in SHR compared to WKY, and lentil–fed SHR had lower p38 MAPK activation when compared to SHR-Ctrl. This indicates that p38 MAPK signaling, activated in signaling cascades of the aforementioned factors and associated with Nox activation (Touyz *et al.*, 2001; Takeshima *et al.*, 2011), is attenuated in lentil-fed SHR. Interestingly, ERK1/2 is also known to be activated downstream of hypertrophic stimuli, however, there were no differences in its phosphorylation in

this study. ERK1/2 activation, and not p38 MAPK activation, is dependent on PKC and MAPK-kinase (Viedt *et al.*, 2000). On the other hand, inhibition of Nox activity has been associated with decreased p38 MAPK and JNK activation, but it had no effect on ERK1/2 phosphorylation (Viedt *et al.*, 2000). This suggests that Nox activity is upregulated in the SHR leading to increased activation of p38 MAPK and not ERK1/2. Therefore, it can be concluded that p38 MAPK is redox sensitive, whereas ERK1/2 is not. It is possible that upon consumption of lentils, Nox activity or associated ROS levels are decreased in the SHR aorta, explaining the reduced activation of p38 MAPK with no effect on ERK1/2, though further investigation would have to be performed to test this hypothesis. The possible reduction in oxidative stress can be caused by decreased activation of Nox, decreased ROS signaling, or a combination of the two. Reduced ROS signaling would likely be a result of increased antioxidant compounds in the diet, which include the phenolic compounds and vitamins provided by pulses (Figure 1).

PFN1 has been shown to be over-expressed in VSMCs of SHR animals compared to WKY and is associated with actin structure reorganization and arterial remodeling (Hassona *et al.*, 2010; Cheng *et al.*, 2011). PFN1 was shown to be higher in the SHR in this study, but its levels were not decreased in aortae of lentil-fed rats with markedly reduced arterial remodeling. This could be explained by the fact remodeling was analyzed on mesenteric arteries, and protein was extracted from aortae.

Finally, $G\alpha_{(i)}$ is an inhibitor of cAMP generation and has been shown to overexpressed in aortae of SHR animals (Anand-Srivastava, 1992). Low levels of

cAMP are associated with hypertension (Amer, 1973). In our study, there was no difference between WKY control and SHR animals with respect to $G\alpha_{(i)}$ levels, however, it was increased 2–fold in mixed lentil-fed WKY animals compared to all other animals. This is interesting because the WKY-ML group had the same or non-significantly lower BP than control-fed WKY. This observation indicates that this pathway is not as important with respect to BP in normotensive animals, at least in the short-term.

4.3 Body Fat and Circulating Lipids

4.3.1 Body Composition and Tissue Weights

There was no dietary effect of any of the pulse–containing diets on BW throughout either of the studies. This was used as a surrogate measurement for feed intake, which is hard to determine when using a powdered diet. The similarities in BW indicates that a 30% (w/w) pulse–containing diet had no adverse effects on animal growth, as has been previously reported (Thompson *et al.*, 2008). However, there were significant differences between the bean and control diets with respect to the peri–renal and epididymal adipose tissue at the time of termination. This shows a diet–related effect on gross fat pads, but they account for only a fraction of the fat located in the body that includes other visceral and subcutaneous adipose tissue.

In the second study, through use of the QMR instrument, we were able to determine whether the diets had an effect on total body fat, and not just those that can be dissected and weighed. The QMR instrument provided information on the

total fat, lean mass, free water and total water. These data gave evidence that there were differences in body composition evident between the WKY and SHR animals with respect to fat mass. However, there did not appear to be significant differences as a result of the dietary treatments. Thus, all changes in BP and arterial remodeling and compliance occurred in the absence of changes to BW and adiposity.

4.3.2 Serum Lipid Analysis

There were significant changes to circulating lipid profiles of animals on pulse diets compared to control in study 1. The decrease in serum cholesterols (TC, LDL-C, and HDL-C) observed in animals eating pulse–containing diets is likely due to the high fibre content of the pulses (Sihag & Kawatra, 2003). However, the data from proximate analysis shows that all but the pea diet actually had lower total fibre content than the AIN–93G control diet (Table 4). The fibre content of the control diet is primarily derived from cellulose, an insoluble fibre. The pulse-containing experimental diets had some fibre from cellulose, as well as varying amounts of pulse–derived insoluble and soluble fibres, which have been shown to reduce total and LDL–C cholesterol in people (Tosh & Yada, 2010; Abeysekara *et al.*, 2012). Soluble fibre, and to a lesser extent insoluble fibre, are able to sequester and force the excretion of bile salts in the intestine. This prevents reabsorption and increases production by the liver, resulting in lower serum levels of cholesterol (Patterson *et al.*, 2009; Abeysekara *et al.*, 2012). Red and green lentil flours have been shown to exhibit high levels of bile salt binding *in vitro*, attributed to the fibre and protein content (Barbana *et al.* 2011). Pulses also contain saponins, which have the ability to

reduce cholesterol (Cheeke, 1971; Sidhu & Oakenfull, 1986). Lowering cholesterol may be beneficial from a clinical standpoint as increased circulating LDL–C levels has been considered a risk factor for CVD as well as being related to endothelial dysfunction (Jakala *et al.*, 2009).

Similar results were observed in the second study, especially in the red-lentil fed animals. Again there were no differences in dietary fibre so these results must be a result of a different component of pulse crops, which appears to be more abundant and/or effective in red lentils. Furthermore, the mixed lentil diet also had an effect on the normotensive WKY animals, lowering both LDL-C and HDL-C. WKY animals have higher serum cholesterol levels, indicating lentil-based diets are able to lower serum cholesterol regardless of basal level.

The fact that all pulse varieties decreased serum HDL–C, LDL–C and total cholesterol, but only the lentil diet had a significant effect on aortic remodeling, indicates that it was not LDL–C levels that were the driving force behind the vascular hypertrophy in study 1. Reduction of HDL-C is not usually considered beneficial, but decreases in TC (–0.35 mM), LDL-C (–0.28 mM), and HDL-C (–0.09 mM) were observed in analysis of the DASH diet study (Obarzanek *et al.*, 2001). The second study reaffirmed these results as the SHR-RL group had the lowest serum cholesterol and a modest effect on resistance artery function and remodeling compared to SHR-GL. Furthermore, SHR-ML and SHR-GL had similar serum cholesterol values, while SHR-GL had arterial compliance comparable to WKY-ML whereas SHR-ML showed no improvement. While LDL–C levels have for a long time been used as an indicator for CVD risk, this study would suggest that vascular

remodeling, arterial compliance, and attenuation of hypertension in the SHR is not dependent on cholesterol levels, but occurs through an entirely different mechanism.

5. Conclusions

Lentils, of all pulse types tested, showed the greatest effect on hypertension and vascular remodeling in the SHR. This was evident through a significant attenuation of BP increase as well as a decreased M/L ratio and media width of aortic sections. All pulse diets were able to lower circulating LDL–C, but only lentils had a significant effect on BP and aortic remodeling. This indicates that the changes in vascular remodeling and BP are independent of circulating cholesterol, and must be explained through another mechanism.

Of both lentil varieties tested, the green lentil–based diet was able to restore vascular compliance in SHR and to a level better than the normotensive control group. Mixed lentils showed a similar effect in WKY animals, while non-significantly lowering BP. Interestingly, the green lentil–fed animals did not have the lowest BP or greatest vascular remodeling of the lentil–fed animals. They did, however, have the greatest restoration of compliance, had less remodeling than red lentil–fed animals, and were similar to those on a mixed lentil diet. The remodeling can in part be explained by decreased phosphorylation of p38 MAPK, possibly resulting from decreased oxidative stress. This underscores the fact that changes in BP, vascular remodeling, and vascular compliance are not inextricably linked, nor are they dependent on LDL-C levels. These changes likely resulted from a combination of

phytochemicals contained within the lentils, which were able to decrease oxidative stress, thereby decreasing arterial remodeling and stiffness and improving arterial compliance.

5.1 Implications

Pulse crops, and specifically green lentils, are able to affect some of the pathways associated with vascular remodeling in the SHR, an animal model of essential hypertension. There was evidence from both studies that the vascular remodeling was decreased regardless of BP status or LDL-C levels. Despite morphological differences between rat and human arteries, they share many similarities in remodeling pathways. However, translation to human health is not possible until clinical trials are undertaken and the mechanism is better understood. At this point, a single trial of mixed pulses has been reported, and no change in BP was obtained, although there were improvements in blood flow in regions with atherosclerotic lesions (Zahradka *et al*., *in press*)

If lentils are able to abrogate vascular remodeling regardless of BP status, this could make them valuable adjuncts to pharmaceutical therapy by decreasing dosage or multiple drug therapy for control. Lentils could also be a component of prevention in prehypertensive patients. Pulses provide an inexpensive, nutrient dense food source that would be a valuable addition to the typical Western diet.

5.2 Strengths

Remodeling of arteries was measured on both large conduit arteries (aorta) and smaller, resistance arteries (mesenteric) through classic histology and pressure myography, respectively. Stiffness was determined *ex vivo* through isobaric measurements of mesenteric arteries on the pressure myograph and *in vivo* through PWV measurements. This gives a variety of measurements, and the myograph measures parameters in a manner that would be difficult to perform on human samples in a large clinical trial.

The results were obtained through diet alone, not dosing of purified compounds – the pulses were cooked and handled in a similar manner as one would at home. This increases the applicability to human populations with respect to diet preparation and consumption, as a clinical trial could be designed using lentil–based (or pulse–based) foods prepared in the same way as was done for these studies.

5.3 Limitations

The pulse diets in the present study contained 30% w/w cooked, freeze-dried pulses and provided ~26% of energy from the pulses. According to Mitchell *et al*. (2009), the highest quartile of pulse consumers in the USA consumed ~16% of their daily calories from dry beans, peas, and lentils; thus, the amount of pulses in the experimental diets was about 1.6-fold higher on a caloric basis. While a diet containing 30% pulses is not unattainable, it is not likely realistic for an everyday diet for the general population. This level of pulses provides phytochemicals and other pulse–associated nutrients to a greater extent than would be likely be

achieved by a human population. Although lentils showed the most improvement in the initial study, it could be that a component of the bean group was overlooked due to the fact it contained 4 bean varieties: navy, pinto, black and kidney. If one of these bean varieties were able to mediate an effect, it would have only been present in ½ the amount (w/w) of the corresponding lentil variety that also caused the effect. This could result in an oversight of a bean variety, something that may need to be looked at in the future.

BP in both studies was measured through tail–cuff plethysmography, and is therefore not as reliable as other options available such as telemetric measurements from an implant.

There were no baseline myography measurements taken for the second study. While one can assume that, at baseline, all SHR animals would be relatively similar, the full extent of variation is unknown at this time. Further, although we can see that there were diet–related changes over the 8–week study, the change from baseline, and degree of change cannot be calculated. Analyzing more vessels on the apparatus could have strengthened these results, but due to some vessels not functioning properly and the limited number of days based on initial study design, the sample size was not as high as necessary for statistical power in all areas. Had the endothelial-function measurements garnered useful data, this would have provided insight as to whether improved endothelial function and NO signaling contributed to the reduced arterial remodeling observed. Further, obtaining baseline measurements would have strengthened the results and given a stronger indication of the lentils effect on vascular remodeling, and whether the effect was an

attenuation and/or reversal of remodeling. These studies set the stage for further animal work to determine mechanistically what is happening, as well as the possible identification of bioactives for purification and possible human trials.

5.4 Future Directions

To further elucidate the mechanism(s) through which the lentils mediate their effect is an important step in advancing the research presented here. The exact mechanism of action, and the role of lentils in activating or deactivating associated pathway(s) is important in understanding the role of the bioactive in the body. For example, there is a substantial amount of evidence supporting oxidative stress as one of the driving factors behind vascular remodeling. A more in depth look at that system now that the effect of lentils on vascular remodeling has been demonstrated would be prudent.

Discovering the identity and location (*i.e.* testa vs. cotyledon) of the bioactive, if it is readily isolatable, would be the logical next step for these experiments. That being said, it is equally likely that several nutrients and/or bioactives contained in the lentils, and not a single one, elicited the beneficial effects seen throughout the study. It is also equally likely that there were a variety of effects contributing to the beneficial outcome, and not a singular change.

To see if these results are repeatable in other animal models of hypertension, and in humans would be an appropriate progression of the studies presented here. Due to the vast differences between rodent and human vessels, a human trial would have to be conducted to validate the results with respect to human health.

Additionally, a dose study would be appropriate to see the effects at lower doses to make diets that would be at a level more reasonably incorporated into an everyday diet.

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Appendix A – Solutions

10× Phosphate Buffered Saline

In 800 mL *dd*H_2O, add:

80 g NaCl

2 g KCl

14.4 g Na_2HPO_4

2.4 g KH_2PO_4

pH to 7.4, bring up to 1 L with *dd*H_2O. Store at 4°C.

To make 1× PBS add 100 mL of 10× PBS to 900 mL ddH_2O and mix. Make fresh before use.

1% Paraformaldehyde

Heat 100 mL 1× PBS (microwave for ~2 minutes).

Add 1 g paraformaldehyde.

Stir to dissolve in fume hood.

Allow to cool. pH to 7.4. Store at 4°C (1 week).

5× TBST

400 mL 1 M Tris–HCl pH 7.4

600 mL 5 M NaCl

10 mL Tween

Bring up to 4 L with *dd*H_2O. Store at 4°C.

To make 1× TBST, add 100 mL TBST into 400 mL ddH_2O.

3× Sample Buffer

67.5 mL 0.5M Tris–HCl pH 6.8

54 mL 10% SDS

54 mL glycerol

Bring up to 200 mL with *dd*H_2O. Store at RT.

10× Krebs (Stock)

In 3 L *dd*H_2O, add:

278 g NaCl (58.44 g/mol)

14 g KCl (74.55 g/mol)

11.64 g $MgSO_4 \bullet 7H_2O$ (246.48 g/mol)

6.4 g KH_2PO_4 (136.09 g/mol)

14.8 g $CaCl_2 \bullet 2H_2O$ (147.01 g/mol)

Bring up to 4 L with *dd*H_2O. Store at 4°C.

1× Krebs (Prepared Fresh Daily)

In 3 L *dd*H_2O, add:

8.4 g $NaHCO_3$ (84.01 g/mol)

4.00 g D–Glucose (180.16 g/mol)

0.04 g Na EDTA (372.24 g/mol)

400 mL of 10× Krebs

pH to 7.4 and bring up to 4 L with *dd*H_2O. Keep at RT.

10× Ca^{2+} Free Krebs (Stock)

In 3 L *dd*H_2O, add:

278 g NaCl (58.44 g/mol)

14 g KCl (74.55 g/mol)

11.64 g $MgSO_4 \bullet 7H_2O$ (246.48 g/mol)

6.4 g KH_2PO_4 (136.09 g/mol)

Bring up to 4 L with *dd*H_2O. Store at 4°C.

0.25 M EGTA

In 800 mL *dd*H_2O add

95 g EGTA

20 g NaOH (pellets, or until pH=8)

Dissolve. Bring up to 1L. Store at 4°C.

1× Ca^{2+} Free Krebs + 10 mM EGTA (Prepared Fresh Daily)

In 1 L *dd*H_2O, add:

4.20 g $NaHCO_3$ (84.01 g/mol)

2.00 g D–Glucose (180.16 g/mol)

0.02 g Na EDTA (372.24 g/mol)

200 mL Ca^{2+} Free Krebs

80 mL 0.25 M EGTA stock

pH to 7.4 and bring up to 2 L with *dd*H_2O. Keep at RT.

125 mM KCl Solution (Prepared Fresh Daily)

Add 932 mg KCl (74.55 g/mol) to 100 mL 1× Krebs.

Norepinephrine Stock Solution (10^{-2} M)

Dissolve 33.8 mg (337 g/mol) norepinephrine in 10 mL *dd*H_2O.

Aliquot (350 μL) and store at –20°C.

Norepinephrine Working Solution (3×10^{-6} M) (Prepared Fresh Daily)

300 μL 10^{-2} M NE stock

1000 mL 1× Krebs

Mix and pH to 7.4. Keep at RT.

Acetylcholine Stock Solution (10^{-1} M)

Dissolve 1.362 g (181.66 g/mol) of acetylcholine in 75 mL 1× Krebs solution. Aliquot in 2.5 mL and freeze at –20°C.

Acetylcholine Working Solutions

To make 15 ml of 10^{-2} M ACh, aliquot 13.5 ml of 1× Krebs. Add 4.5 µl of 10^{-2} M NE stock. Mix in 1.5 ml of 10^{-1}M ACh stock.

To make serial dilutions of ACh (10^{-2} to 10^{-10} M):

1.5 ml of 10^{-2} M ACh + 13.5 ml 3 x 10^{-6} M NE working solution = 10^{-3} M ACh

1.5 ml of 10^{-3} M ACh + 13.5 ml 3 x 10^{-6} M NE working solution = 10^{-4} M ACh

1.5 ml of 10^{-4} M ACh + 13.5 ml 3 x 10^{-6} M NE working solution = 10^{-5} M ACh

1.5 ml of 10^{-5} M ACh + 13.5 ml 3 x 10^{-6} M NE working solution = 10^{-6} M ACh

1.5 ml of 10^{-6} M ACh + 13.5 ml 3 x 10^{-6} M NE working solution = 10^{-7} M ACh

1.5 ml of 10^{-7} M ACh + 13.5 ml 3 x 10^{-6} M NE working solution = 10^{-8} M ACh

1.5 ml of 10^{-8} M ACh + 13.5 ml 3 x 10^{-6} M NE working solution = 10^{-9} M ACh

1.5 ml of 10^{-9} M ACh + 13.5 ml 3 x 10^{-6} M NE working solution = 10^{-10} M ACh

To make 15 ml of $10^{-2.5}$ M ACh, aliquot 13.5 ml of 1× Krebs. Add 4.5 µl of 10^{-2} M NE stock. Mix in 450 µl of 10^{-1}M ACh stock.

To make serial dilutions of ACh ($10^{-2.5}$ to $10^{-9.5}$ M):

1.5 ml of $10^{-2.5}$ M ACh + 13.5 ml 3 x 10^{-6} M NE working solution = $10^{-3.5}$ M ACh

1.5 ml of $10^{-3.5}$ M ACh + 13.5 ml 3 x 10^{-6} M NE working solution = $10^{-4.5}$ M ACh

1.5 ml of $10^{-4.5}$ M ACh + 13.5 ml 3 x 10^{-6} M NE working solution = $10^{-5.5}$ M ACh

1.5 ml of $10^{-5.5}$ M ACh + 13.5 ml 3 x 10^{-6} M NE working solution = $10^{-6.5}$ M ACh

1.5 ml of $10^{-6.5}$ M ACh + 13.5 ml 3 x 10^{-6} M NE working solution = $10^{-7.5}$ M ACh

1.5 ml of $10^{-7.5}$ M ACh + 13.5 ml 3 x 10^{-6} M NE working solution = $10^{-8.5}$ M ACh

1.5 ml of $10^{-8.5}$ M ACh + 13.5 ml 3 x 10^{-6} M NE working solution = $10^{-9.5}$ M ACh

Sodium Nitroprusside Stock Solution (10^{-1} M)

Dissolve 2.235 g (298.0 g/mol) SNP in 75 mL 1× Krebs solution. Aliquot in 2.5 mL and freeze at –20°C.

Sodium Nitroprusside Working Solutions

To make 15 ml of 10^{-2} M SNP, aliquot 13.5 ml of 1× Krebs. Add 4.5 µl of 10^{-2} M NE stock. Mix in 1.5 ml of 10^{-1} M SNP stock.

To make serial dilutions of SNP (10^{-2} to 10^{-10} M):

1.5 ml of 10^{-2} M SNP + 13.5 ml 3 x 10^{-6} M NE working solution = 10^{-3} M SNP

1.5 ml of 10^{-3} M SNP + 13.5 ml 3 x 10^{-6} M NE working solution = 10^{-4} M SNP

1.5 ml of 10^{-4} M SNP + 13.5 ml 3 x 10^{-6} M NE working solution = 10^{-5} M SNP

1.5 ml of 10^{-5} M SNP + 13.5 ml 3 x 10^{-6} M NE working solution = 10^{-6} M SNP

1.5 ml of 10^{-6} M SNP + 13.5 ml 3 x 10^{-6} M NE working solution = 10^{-7} M SNP

1.5 ml of 10^{-7} M SNP + 13.5 ml 3 x 10^{-6} M NE working solution = 10^{-8} M SNP

1.5 ml of 10^{-8} M SNP + 13.5 ml 3 x 10^{-6} M NE working solution = 10^{-9} M SNP

1.5 ml of 10^{-9} M SNP + 13.5 ml 3 x 10^{-6} M NE working solution = 10^{-10} M SNP

To make 15 ml of $10^{-2.5}$ M SNP, aliquot 13.5 ml of 1× Krebs. Add 4.5 µl of 10^{-2} M NE stock. Mix in 450 µl of 10^{-1}M SNP stock.

To make serial dilutions of SNP ($10^{-2.5}$ to $10^{-9.5}$ M):

1.5 ml of $10^{-2.5}$ M SNP + 13.5 ml 3 x 10^{-6} M NE working solution = $10^{-3.5}$ M SNP

1.5 ml of $10^{-3.5}$ M SNP + 13.5 ml 3 x 10^{-6} M NE working solution = $10^{-4.5}$ M SNP

1.5 ml of $10^{-4.5}$ M SNP + 13.5 ml 3 x 10^{-6} M NE working solution = $10^{-5.5}$ M SNP

1.5 ml of $10^{-5.5}$ M SNP + 13.5 ml 3 x 10^{-6} M NE working solution = $10^{-6.5}$ M SNP

1.5 ml of $10^{-6.5}$ M SNP + 13.5 ml 3 x 10^{-6} M NE working solution = $10^{-7.5}$ M SNP

1.5 ml of $10^{-7.5}$ M SNP + 13.5 ml 3 x 10^{-6} M NE working solution = $10^{-8.5}$ M SNP

1.5 ml of $10^{-8.5}$ M SNP + 13.5 ml 3 x 10^{-6} M NE working solution = $10^{-9.5}$ M SNP

Appendix B – Dietary Protein Calculation

The casein obtained from Dyets Inc. contained 87% protein. The 76 g of protein per 300 g of pulse powder was based on average protein content from analysis by Wang & Daun (2004). For the calculation of the pulse–containing experimental diets, the adjustment made was as follows:

$$200\ \text{g casein} \times 0.87 \frac{\text{g protein}}{\text{g casein}} = 174\ \text{g protein (control}$$

$$174\,\text{g protein} - 76\,\text{g protein per } 300\,\text{g pulse powder} = 98\,\text{g protein (still required)}$$

$$98\ \text{g protein} \times \frac{1}{0.87 \frac{\text{g protein}}{\text{g casein}}} = 112.6\ \text{g casein (required in pul}$$

Therefore, ~113 grams of casein was required in pulse–based diets to make them approximately isonitrogenous to the AIN-93G control diet.

This method of adjustment resulted in a total mass of 1044.5 g instead of 1000. The 'extra' 44.5 g were taken out of the cornstarch amount to maintain proper ratios of other ingredients (*e.g.* vitamins and minerals) and a 30% (w/w) pulse diet.

Appendix C – Doppler Waveform Analysis

***Procedure*.** Doppler Signal Processing Workstation (DSPW, v. 1.624, Indus Instruments, Webster, TX, USA) was used to analyze Doppler traces.

1. A trace is opened in the DSPW window. From the analysis menu in the control bar, 'open analysis window' is selected.
2. ***Step 1: File Info*.** Enter your Name.
3. ***Step 2: General Setup***. In the drop down menus under 'Type'
    a. Data select "Doppler"
    b. Signal select "Peripheral Flow"
    c. Animal select "Rat"
    d. Select all measurements
4. ***Step 3: FFT and Filter Control***. Leave default settings.
5. ***Step 4: Envelopes Editor***. Press the "Auto adjust display" button, followed by the "Auto Calculate" button (Figure C.1 Yellow Line).

Troubleshooting: If the yellow trace appears to truncate the peaks or not follow them correctly, it may be necessary to manually adjust the contrast on the right hand side of the trace, the re-press the "Auto Calculate" button.

6. ***Step 5: R-Peak Editor***. Press the "Auto Calculate" button. Check to make sure that the hearts generated by the program line up with the R-peaks of the ECG trace before moving on to the next step (Figure C.1A). If they don't hearts can be manually added, deleted, or moved.
7. ***Step 6: Beat Editor***. Press "Select all beat(s)".
8. ***Step 7: Marker Editor***. Place markers for "flow velocity start" (FVS), "minimum flow velocity" (MFV), and "peak flow velocity" (PFV) for each peak on the Doppler trace (Figure C.1A,B,C, respectively). Marker names are selected by choosing the appropriate title in the drop-down menu "Type" under the "Current Marker Selected" area.
9. ***Step 8: Measurement Results***. Choose display average, and then press "Copy Results to Clipboard". Results can then be pasted into a Microsoft Excel file for further analysis.

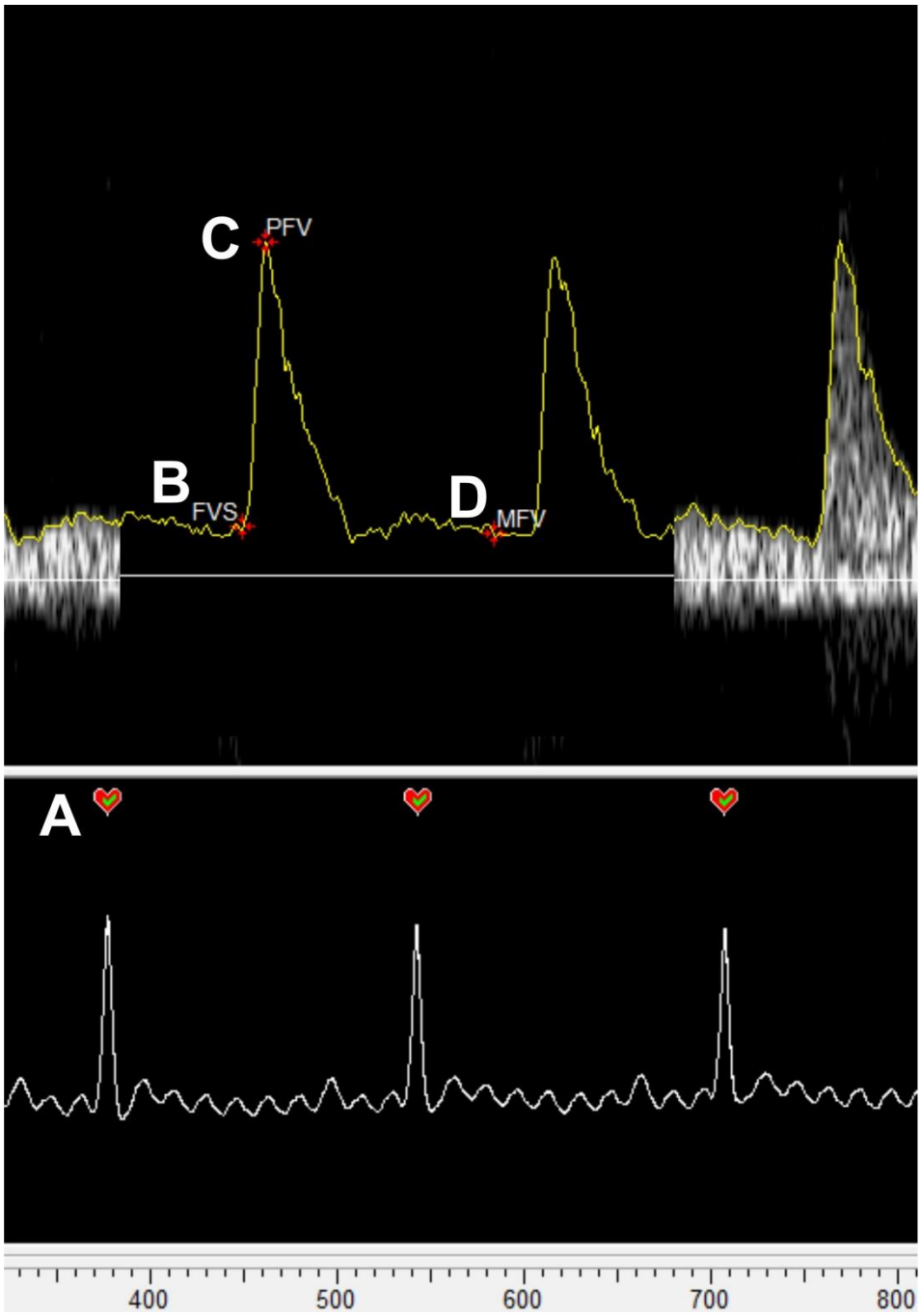

Figure C.1 – Sample Doppler Readout as viewed in Doppler Signal Processing Workstation. **A) R-peak editor** marked by hearts lined up with corresponding R-peak, **B) Flow Velocity Start (FVS) marker**, **C) Peak Flow Velocity (PFV) marker**, and **D) Minimum Flow Velocity (MFV) marker.**

Appendix D – Staining and Measurements of Aorta Sections

Upon termination a section of the descending aorta (Figure D.1) was removed and placed in Optimal Cutting Temperature (OCT, Sakura Fintek, Alphen aan den Rijn, NL) compound and frozen in an ethanol and dry ice bath. Using a cryotome, the aorta was sectioned into 5 μm sections, 3 sections per glass slide. Non-consecutive sections were collected. Slides were kept at –80°C until the time of staining.

D.1 Staining. An Accustain® Elastic Stain kit (Reference #HT25A–1KT, lot #120M4344; Sigma Aldrich; St. Louis, MO, USA) was used to stain elastin black, muscle yellow and collagen red (Figure D.2). The staining procedure, as follows, is described in the manufacturer's protocol:

1. Slides were thawed to room temperature and then placed in 1% paraformaldehyde for 8 minutes.
2. Washed in 1× phosphate–buffered saline (PBS; 1.4 M NaCl, 2.6×10^{-2} M KCl, 0.1 M Na_2HPO_4, 1.7×10^{-2} M KH_2PO_4 diluted 1:9 in *dd*H_2O) for 10 minutes.
3. Hydrated in *dd*H_2O for 15 minutes.
4. Placed into a working elastic stain solution (20 mL hematoxylin solution (alcoholic), 3 mL ferric chloride solution, 8 mL Weigert's iodine solution, and 5 mL *dd*H_2O) for 10 minutes.
5. Rinsed in *dd*H_2O.
6. Differentiated in working ferric chloride solution (3 mL ferric chloride solution and 37 mL *dd*H_2O). Differentiation occurred best at 3 minutes.
7. Rinsed in 95% ethanol (remove excess iodine).
8. Rinsed in *dd*H_2O.
9. Stained with Van Gieson Stain for 90 seconds.
10. Rinsed in 95% ethanol.
11. Placed in 95% ethanol for 5 minutes.
12. Placed in 100% ethanol for 5 minutes.
13. Placed in 90% xylene for 5 minutes
14. Placed in 100% xylene for 5 minutes

15. Mounted using VectaMount (Vector Laboratories, Burlingame, CA, USA) and left in the fume hood overnight.

D.2 Measurements. Stained and mounted sections were imaged using an EVOS® microscope (AMG solutions, Seattle, WA, USA). Only sections that were complete and not folded or collapsed were used.

1. Slides were imaged at 4× and 20× objectives.
2. Images were opened on Image Pro Plus (version 4.5.1, Media Cybernetics, Rockville, MD, USA)
3. The measurement tool was selected.
4. Measurements were taken along the inner surface of the *tunica media* (1)(Figure D.2C) and the outer surface of the *tunica media* between it and the *tunica adventitia* (2)(Figure D.2E).
5. Measurements were exported to Microsoft Excel.
6. The following calculations are done under the assumption that the aortas are circles.
7. Measurement (1) was used to calculated lumen diameter such that (1)/π is equal to the lumen diameter (3).
8. Measurement (2) was used to calculate external diameter such that (2)/π is equal to the external diameter (4).
9. Media width (5) was calculated as [(4)–(3)]/2.
10. Media:lumen diameter (6) was calculated as (5)/(3).
11. Media cross sectional (7) area was calculated as $\pi[(4)/2]^2 - \pi[(3)/2]^2$
12. An average of 2 non-consecutive aorta sections were measured per animal.
13. Measurements 1–7 were analyzed using SAS (version 9.2, SAS Institute Inc., Cary, NC, USA).

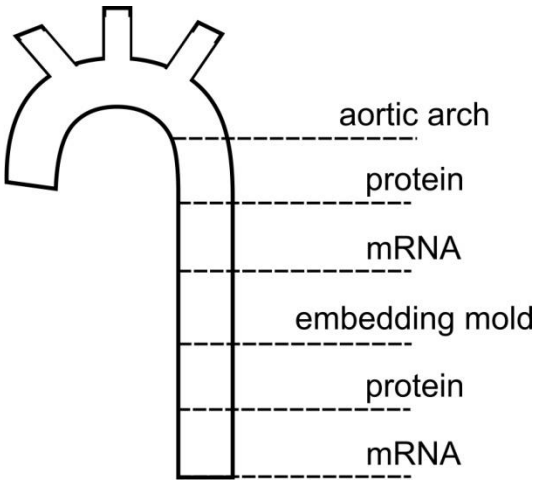

Figure D.1 – Aorta Sampling Sections used in both study 1 and study 2. Embedding mold samples were placed in Optimal Cutting Temperature (OCT) compound for sectioning and staining. mRNA sections were put into RNAlater for study 2 only.

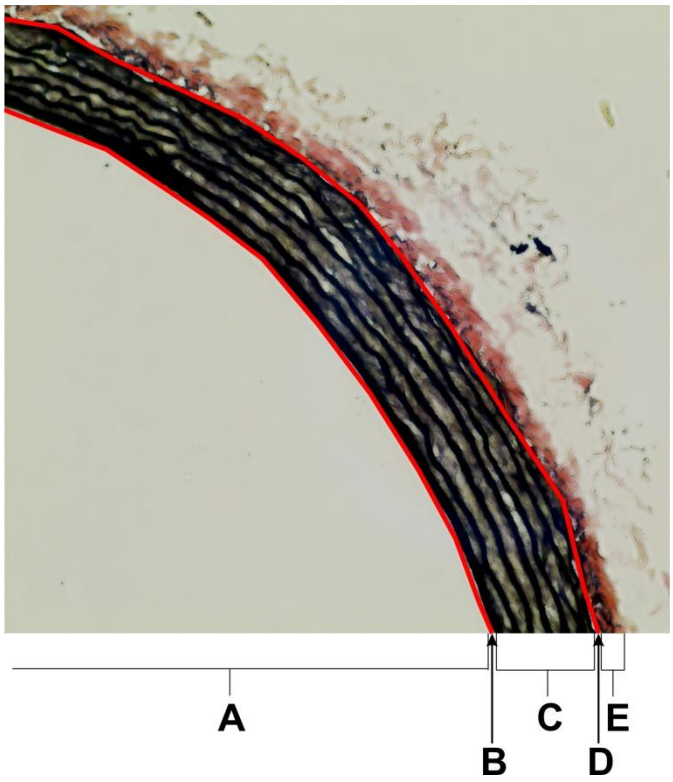

Figure D.2 Aorta Morphology and Measurements. **A) Vessel Lumen**, **B) Measurement** location for (1)**, C)** ***Tunica media*****, D) Measurement** location for (2), and **E)** ***Tunica adventitia***

Appendix E – Western Immunoblotting

E.1 Protein Extraction.

1. Weigh tissue samples and then place in a mortar.
2. Add liquid nitrogen to the mortar and grind the tissue to a fine powder.
3. Add 30 μL/mg tissue of 3× Sample Buffer to the powder. Tissue and sample buffer were left at room temperature for 15 minutes.
4. Pipette the tissue from the mortar into a micro-centrifuge tube.
5. Centrifuge the tube at 13000 rpm for 20 minutes at room temperature.
6. Pipette the supernatant into a new micro-centrifuge tube (the layer of fat on top of the supernatant was not pipetted this into the new tube). This was the protein sample.
7. Sonicate the protein sample for 8 seconds.
8. Prepare aliquots of the protein sample (2×50 μL, 1×10 μL, and the rest as stock).
9. Store aliquots at –80°C until used.

E.2 Protein Assay

1. Make serial dilutions (40 μL each) of bovine serum albumin (BSA); 2.0, 1.0, 0.8, 0.6, 0.4, 0.2, and 0 mg/mL. Vortex and keep on ice.
2. Dilute 10 μL protein samples with 30 μL *dd*H_2O. Vortex and keep on ice.
3. Pipette, in triplicate, 10 μL of each standard into a 96 well NUNC plate (Thermo Scientific, Waltham, MA, USA).
4. Mix Pierce® BCA protein assay Reagents A and B (50 parts A to 1 part B) (Cat. #23223 (Reagent A) and #23224 (Reagent B); Pierce Thermo Scientific, Rockford, IL, USA). Add 200 μL to each well.
5. Incubate the plate at 37°C for 30 minutes.
6. Read the plate at 550 nm in a ThermoMax Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Be sure to correct for dilution in step 2.
    a. Use the microplate reader to briefly agitate the plate prior to reading.
    b. The standard curve should have an r-value ≥0.99.

7. Use wells with coefficient of variation less than 10. Outlying wells can be masked at this point.
8. Print the readout, and use to calculate amount of protein required for gels.

a. *E.g.* to load 5 μg of protein/well on a gel and the readout says a particular sample contains 1.25 μg/μL, then 4 μL of that sample would need to be loaded.

9. From 50 μL protein sample aliquots, re-aliquot into amounts required for loading on a gel.

E.3 SDS–PAGE

1. Pour a separating gel at the desired acrylamide percentage. Add tert-butanol to the top and allow it to set for 30-60 minutes.
2. Make protein dye (1:1 dye to β-mercaptoethanol).
3. Add dye to each of your protein samples (to 10% of the volume; a 10 μL sample would require 1 μL of dye).
4. Boil water in the microwave for 4 minutes.
5. Place the protein samples and dye into a micro-centrifuge rack into the water bath. Boil in the microwave for 90-120 seconds.
6. Pour off the tert-butanol from the top of the gel. Wash with *dd*H_2O and dry.
7. Make and pour a 5% stacking gel and add the desired comb (appropriate number of wells and desired width).
8. Allow stacking gel to set for 15-30 minutes.
9. Clamp plates to the apparatus and add running buffer (1 part 5× Running Buffer to 4 parts *dd*H_2O).
10. Clean out and flush wells with loading syringe.
11. Load samples into each well.
12. Run the gel at 70 milliamps in the cold room until the dye–front reaches the bottom of the gel. Do not let the dye front run off of the gel.

13. Cut a membrane big enough for the gel. Label with pencil and wet in pure methanol before transferring to Running Buffer (200 mL methanol, 160 mL running buffer, 640 mL cold *dd*H_2O).
14. Remove the glass plates from the apparatus. Carefully pry one plate off of the gel.
15. Using blotting paper, transfer the gel from the remaining plate to the blotting paper.
16. Place the membrane over the gel. Roll out any bubbles.
17. Assemble the transfer cassette: black side, pad, blotting paper, gel, membrane, blotting paper, pad, red side.
18. Place the cassette in the running chamber and fill the chamber with Running Buffer.
19. Add a stir bar. In the cold room, turn on the stirrer and run the transfer apparatus at 100 volts for 45 minutes.
20. Carefully remove the membrane and store in TBST until used.

E.4 Western Immunoblotting.

1. Block the membrane in 3% BSA–TBST for 1 hour.
2. Pour off BSA–TBST from membrane.
3. Add primary antibody in desired concentration (*e.g.* a 1:1000 concentration would require 30 μL of antibody in 30 mL 3% BSA–TBST).
4. Place membrane back on shaker for 1 hour at room temperature or overnight in the cold room.
5. Pour off primary antibody.
6. Wash 3×5 minutes in 1× TBST.
7. Add appropriate secondary antibody: 1:10000 in 1% BSA-TBST. Place on the shaker for 1 hour.
8. Pour off secondary antibody.
9. Wash 3×5 minutes in 1× TBST.
10. Add chemiluminescent agent (ECL prime, Crescendo, etc.) to membrane.

11. Immediately take for imaging.

E.5 Imaging & Quantification.

1. Place the membrane (in blotting box) into the FluorChem® Q Western Blot imager (Protein Simple, Santa Clara, CA, USA).
2. Select the "Acquire" button and choose CHEMIULTRA from the drop down menu.
3. Select "Movie Mode".
4. Choose the amount of time for capture, the number of frames, check "stack frames" and press "copy to end".
5. Save images with good band resolution.
6. Invert images, can reduce band intensity by adjusting black, not gamma.
7. Use the noise reduction to decrease background noise.
8. Under the "Multiplex Band Analysis" tab, go to background and check "local background".
9. Use the band selection tools to select bands, and in the readout select "background count average" and export it to an Excel file for further analysis.

Appendix F – Pressure Myography

***F.1 Procedure*.** Pressure myography was performed on third order mesenteric arteries using a myograph from Living Systems Instrumentation (Burlington, VT, USA). Two vessels were analyzed per day.

1. Immediately after termination, the intestine and mesenteric fat were carefully removed and placed in ice cold 1× Krebs solution.

   NOTE: The end of the intestine proximal to the stomach must be indicated or remembered so the arteries can be correctly and consistently isolated.
2. The intestine was pinned to a dissecting dish exposing the first 10 cm of intestine and mesenteric fat.
3. Using tweezers, the fat was carefully removed from a third-order artery and vein. The artery will have thicker walls than the vein travelling along side of it.

   NOTE: Do not pinch the artery with tweezers, this will damage the vessel.
4. Fill the reservoir of the pressure myograph with 1× Krebs. Using a syringe filled with 1× Krebs, ensure there is no air in the cannulae.
5. Carefully cut out the artery and transfer it to the pressure myograph. As with step 3, do not pinch the artery anywhere but the end.
6. Put two ties on each cannula.
7. Mount the vessel on the myograph, careful to not damage the ends.
8. Using two ties for each end, secure the vessel to the cannula.
9. Attach the pressure myograph to the solution and pressure pumps. With the pressure pump on automatic, pressurize the vessel to 45 mmHg.
   a. Switch the pump to manual. If the pressure drops, there is a leak that needs to be fixed before continuing.
   b. If there is no leak, turn the pump back to automatic and continue with experiment.
10. Leave the artery to bathe in 1× Krebs for one hour.

NOTE: all solutions from this point forward are bubbled with 21% O_2, 5% CO_2, balance N_2. Solution heater is also on at 41.3°C heating solutions to ~37°C.

11. At the end of the hour, measure the vessel diameter (this measurement is "Baseline").
12. Treat the artery with 125 mM KCl solution. The artery diameter must constrict >50% for the artery to be viable. Record diameter measurements.
    a. If the artery is not viable, it must be replaced.
13. Leave the artery to bathe in 1× Krebs for an hour.
14. Prepare 3×10^{-6} M NE and then serial dilutions of ACh.
15. After the hour, pre-treat the vessel with 10^{-6} M NE. Record measurements.
16. Treat the vessel with increasing concentrations of ACh taking 3 diameter measurements at each concentration.
17. Bathe the artery in 1× Krebs for an hour.
18. Pre-treat with 3×10^{-6} M NE, then with increasing concentrations of SNP. Take 3 diameter measurements at each concentration.
19. Leave the artery to bathe in 1× Ca^{2+}-free Krebs for 30 minutes.
20. After the 30 minutes, increase the pressure to 140 mmHg three times, straightening the vessel each time.
21. Take 3 wall thickness and lumen diameter measurements at each of the following pressures: 3, 10, 20, 30, 40, *45**, 60, 80, 100, 120, and 140 mmHg.
    a. The measurement at 45 mmHg is used for vascular geometry determination. All other measurements are for arterial stiffness and compliance calculations.

F.2 Clean Up.

1. Flow 3 L *dd*H_2O through each myograph apparatus.
2. Flush cannulae with *dd*H_2O.
3. Turn off heater and pumps. Water can be left in myograph reservoir if it is being used the next day. Otherwise dry and pack it away.

Appendix G – Serum Biochemistry

Table G.1 – Serum Biochemistry (Study 2)

		WKY-Ctrl		WKY-ML		SHR-Ctrl		SHR-ML		SHR-GL		SHR-RL	
TC (mM)	*Baseline*	2.9	*0.09*^{a}	2.8	*0.07*^{a}	1.8	*0.03*^{b}	1.8	*0.05*^{b}	1.8	*0.06*^{b}	1.8	*0.02*^{b}
	Week 4	3.1	*0.07*^{a}	2.9	*0.05*^{b}	1.5	*0.06*^{c}	1.3	*0.03*^{c}	1.4	*0.04*^{c}	1.4	*0.04*^{c}
	Week 7	3.3	*0.10*^{a}	3.2	*0.08*^{a}	1.5	*0.03*^{b}	1.4	*0.06*^{b}	1.5	*0.07*^{b}	1.3	*0.03*^{b}
LDL-C (mM)	*Baseline*	0.48	*0.02*^{a}	0.50	*0.03*^{a}	0.23	*0.01*^{b}	0.23	*0.02*^{b}	0.24	*0.02*^{b}	0.23	*0.02*^{b}
	Week 4	0.73	*0.04*^{a}	0.63	*0.02*^{b}	0.27	*0.02*^{c}	0.20	*0.01*^{c}	0.22	*0.02*^{c}	0.21	*0.02*^{c}
	Week 7	0.83	*0.04*^{a}	0.71	*0.03*^{b}	0.26	*0.01*^{c}	0.23	*0.01*^{cd}	0.22	*0.02*^{cd}	0.18	*0.02*^{d}
HDL-C (mM)	*Baseline*	2.4	*0.07*^{a}	2.4	*0.07*^{a}	1.6	*0.03*^{b}	1.6	*0.04*^{b}	1.6	*0.05*^{b}	1.6	*0.04*^{b}
	Week 4	2.6	*0.07*^{a}	2.4	*0.05*^{b}	1.4	*0.05*^{c}	1.2	*0.02*^{d}	1.3	*0.03*^{cd}	1.2	*0.04*^{d}
	Week 7	2.8	*0.07*^{a}	2.6	*0.04*^{b}	1.4	*0.03*^{c}	1.3	*0.05*^{cd}	1.3	*0.05*^{cd}	1.2	*0.03*^{d}
TG (mM)	*Baseline*	1.1	*0.08*‡	1.0	*0.07*‡	0.8	*0.04*	1.0	*0.06*	0.8	*0.04*	0.9	*0.07*
	Week 4	0.7	*0.04*‡	0.7	*0.05*‡	0.4	*0.02*	0.5	*0.03*	0.5	*0.02*	0.5	*0.02*
	Week 7	0.8	*0.05*‡	0.9	*0.08*‡	0.4	*0.02*	0.5	*0.05*	0.5	*0.03*	0.4	*0.02*
Urea (mM)	*Baseline*	3.5	*0.17*‡	3.7	*0.20*‡	5.7	*0.23*	5.9	*0.25*	5.5	*0.23*	5.9	*0.20*
	Week 4	3.3	*0.10*‡	3.5	*0.12*‡	5.8	*0.21*	5.5	*0.19*	5.6	*0.19*	5.4	*0.16*
	Week 7	3.6	*0.08*‡	3.8	*0.10*‡	5.9	*0.27*	5.2	*0.15*	5.6	*0.29*	6.1	*0.28*
Glucose (mM)	*Baseline*	7.4	*0.31*	7.6	*0.23*	6.6	*0.24*	6.5	*0.24*	6.4	*0.26*	6.6	*0.18*
	Week 4	7.2	*0.15*‡	7.1	*0.11*‡	6.1	*0.13*	6.1	*0.18*	6.3	*0.24*	5.9	*0.12*
	Week 7	7.3	*0.14*‡	7.4	*0.30*‡	6.5	*0.20*	6.2	*0.16*	6.0	*0.11*	5.9	*0.11*
Creatinine (μM)	*Baseline*	28.7	*1.13*	27.5	*0.82*	29.0	*0.76*	27.6	*0.82*	27.8	*0.90*	29.0	*0.84*
	Week 4	28.4	*1.05*	27.1	*0.69*	29.6	*0.96*	29.9	*1.95*	29.4	*1.40*	32.0	*1.12*
	Week 7	29.4	*0.65*	30.1	*1.93*	29.4	*2.91*	29.4	*1.80*	33.6	*1.55*	36.3	*3.32*

Serum biochemistry of fasting serum samples run on a Cobas C111 auto analyzer. Data are presented as mean ± SE. Within each row, means with different superscript letters are significantly different ($p<0.05$). ‡Indicates significant difference compared to SHR-Ctrl at a given time point ($p<0.0001$).