THERAPEUTIC POTENTIAL OF A DIETARY PHASE 2 PROTEIN INDUCER IN STROKE-PRONE SPONTANEOUSLY HYPERTENSIVE (SHRsp) RATS

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ABSTRACT

Persistent hypertension is associated with a greater incidence of organ damage and conditions such as stroke, heart failure and end–stage kidney disease, which results in increased cardiovascular (CV) morbidity and mortality. Among the patients receiving antihypertensive treatment, the level of adequate blood pressure (BP) control (<140/90 mm Hg) is only 30%-50%. These antihypertensive drugs reduce the risk of CV events only by 20% and stroke by 40%, besides causing adverse effects.

The spontaneously hypertensive stroke-prone rat (SHRsp) is a good model of essential hypertension. It starts developing hypertension at 5-6 weeks of age which becomes established hypertension at 12-16 weeks. The SHRsp also develops oxidative stress (characterized by low glutathione levels) and inflammation.

We have previously shown that consumption of broccoli sprouts (BSp) decreased oxidative stress, inflammation and blood pressure in both male and female SHRsp. BSp contain sulforaphane glucosinolate (SGS), the precursor of the phase 2 protein inducer sulforaphane. BSp low in SGS did not have these effects. Furthermore, BSp did not have any measurable effects on the normal physiology of Sprague Dawley (SD) rats.

The objectives of this study were to determine: (1) the importance of a food matrix in causing these beneficial effects, i.e., can one get the same effects by administration of sulforaphane alone?, (2) whether dietary sulforaphane decreases the level of nitrosylated proteins in the kidneys of SHRsp, (3) whether dietary sulforaphane increases the phase 2 enzyme γ-glutamyl-L-cysteine ligase expression (γ-GCS), (4) whether dietary sulforaphane increases the phase 2 enzyme glutathione reductase (GRed) expression, (5) whether dietary sulforaphane increases the phase 2 enzyme thioredoxin reductase (TrxR1) expression and (6) whether dietary sulforaphane protects renal artery structure.

After 1 week of adaptation, the 4 week old female SHRsp and SD rats were divided into four groups and administered daily by gavage: (i) Corn oil (vehicle) alone (Control); (ii) sulforaphane (5 μmol/kg body weight) in corn oil; (iii) sulforaphane (10 μmol/kg body weight)

in corn oil; and (iv) sulforaphane (20 μmol/kg body weight) in corn oil. Systolic BP was determined weekly using a standard tail cuff noninvasive BP measurement system (model 29-SSP; Harvard Apparatus, St. Laurent, QC, Canada). The treatment lasted for 15 weeks. At the end of the treatment period, the animals were anesthetized with isoflurane (3%) and the BP was measured by the intra-arterial catheter method using a BP monitor (MK-2000 instrument; Muromachi Kikai Co., Ltd, Tokyo, Japan). Later, the animals were euthanized and perfused with normal saline, and tissues collected for histology, western blot, gene expression study or measurement of reduced glutathione (GSH).

The results of the study showed that chronic administration of sulforaphane in SHRsp significantly increased phase 2 proteins (i.e., significantly increased kidney γ-GCS [0.93. ± 0.07 arbitrary unit (AU)] when compared with SHRsp control [0.36 ± 0.05(AU)] , decreased kidney nitrotyrosine (significantly lowered the levels of nitrotyrosine [0.917± 0.16 AU] when compared with SHRsp control [1.37± 0.2 AU], protected the arterial structure of small resistance vessels in kidneys, and significantly attenuated the increase in blood pressure by 22-43 mm Hg by the end of the study.

In conclusion, the results of this thesis demonstrate that: (i) A minimal change in our diet may have a major impact on our health, (ii) The beneficial health effects previously seen with consumption of BSp are due to the conversion of SGS to sulforaphane and (iii) Long term administration of sulforaphane in SHRsp attenuates the increase in BP and vascular alterations

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DEDICATION

To my Mom, Jafala Heidub and my wife, Amal Abdelsalam, thank you for your invaluable support throughout all these years.To my dear friend, Abdulla Ahmad El-Alowanee, may Allah forgive his sins and increase his virtue.

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LIST OF ABBREVATIONS USED

ANOVA	analysis of variance	DTNB	dithio-bis(2-nitrobenzoic) acid
ATP	adenosine-5´-triphosphate	DEPC	diethylpyrocarbonate
AMP	adenosine monophosphate	EDTA	ethylenediaminetetraacetic acid
ANGII	angiotensin II	GS	GSH synthetase
ACE	angiotensin converting enzyme	GSH	glutathione
ARB	angiotensin II receptor blocker	GRed	glutathione reductase
ANP	atrial natriuretic peptide	GPx	glutathione peroxidase
ARE	Antioxidant(or electrophile) response element	GSTs	glutathione-S-transferases
BCA	bicinchoninic acid	γ -GC	γ-glutamyl-L-cysteine ligase (gamma-glutamyl-L-cysteine synthetase)
BMI	body mass index	g	gram
BSp	broccoli sprouts	HPLC	high pressure liquid chromotography
BP	blood pressure	H_2O_2	hydrogen peroxide
CCBs	calcium channel blockers	HF	heart failure
CV	Cardiovascular	OH	hydroxyl radical

CVD	cardiovascular disease	DOCA	deoxycortisone acetate salt
DNA	deoxyribonucleic acid	Nrf2	nuclear factor erythroid2-related fator2)
JNK	C-Jun N-terminal kinases	NOS	nitric oxide synthase
kg	kilogram	NADPH oxidase	nicotinamide adenine dinucleotide phosphate-oxidase
LV	left ventricle	$ONOO^-$	peroxynitrite radical
LVESP	left ventricular end systolic pressure	1K, 1C	1 kidney-1 clip
LDL	low-density lipoproteins	PVDF	polyvinylidene fluoride
MAP	mean arterial blood pressure	PP	peak pressure
µmol	micromole	PFA	paraformaldehyde
µl	microliter	PBS	phosphate buffered saline
µm	micrometer	PGs	prostaglandins
mg	milligram	RIPA	radioimmuno precipitation assay
ml	milliliter	Q.PCR	Quantitative polymerase chain reaction
nm	nanomole	RNS	reactive nitrogen species
MAPK	mitogen-activated protein kinase	ROS	reactive oxygen species
NO	nitric oxide	RAAS	renin-angiotensin-aldosterone system
NFkB	nuclear factor kappa	SMCs	smooth muscle cells
SHRsp	Spontaneously hypertensive stroke prone	SD	Sprague Dawley rats

	rats		
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis	UV	ultraviole
SGS	sulforaphane glucosinolate	4-HE	4-hydroxynonenal
SNS	sympathetic nervous system	Ub	ubiquitin
2K, 1C	two-kidney-one clip	WKY	Wistar Kyoto
TrxR1	thioredoxin reductase	U	unit

1. INTRODUCTION

1.1 Hypertension

Hypertension in humans is defined as persistent high arterial systemic blood pressure (BP); the systolic BP is ≥ 140 mm Hg and the diastolic BP is ≥ 90 mm Hg (Table 1) (Whelton et al. 2004). Persistent hypertension is associated with a greater incidence of organ damage (Hossain et al. 2007), causing conditions such as stroke, end–stage kidney disease (Khan et al. 2007) and hypertrophic changes in heart (Preston 2007), partly through endothelin-1 signaling pathway. Endothelin-1 stimulates signal regulated kinases (p38 MAPKS and JNK) (Preston 2007), and in the walls of large and small kidney arteries (Khan et al. 2007).

1.2 Epidemiology of hypertension

Persistent hypertension is linked to an increased risk of morbidity and mortality (Preston 2007). The health problem of hypertension is global and it is escalating at an alarming rate. Worldwide approximately one billion individuals are hypertensive (Preston 2007); by 2025, this number is projected to increase to 1.56 billion, constituting 29% of the entire population (Hossain et al. 2007; Kaplan and Opie 2006). More than 50% of Canadians aged between 55 to 74 years are hypertensive (Wexler and Aukerman 2006; Boulanger JM. Hill MD. CHEP (Canadian Hypertension Educational Program) 2005).

1.3 Types of hypertension

There are two types of hypertension: primary (essential) hypertension and secondary hypertension. 80-95% of cases are classified as essential hypertension, in which the cause of the hypertension is poorly understood. While in about 5-15% of the cases the hypertension is due to a specific disease or abnormality, such as renal parenchyma disease (1%), renovascular hypertension (1%) and primary hyperthyroidism (1%) (Preston 2007).

1.4 Mechanisms of hypertension

A widely held misconception is that hypertension is a single disease that can be treated with a single antihypertensive agent. In fact hypertension is a heterogeneous disorder. The homeostatic regulation of blood pressure depends on the balance between vasoconstrictive mechanisms, such as the renin-angiotensin-aldosterone system (RAAS), the sympathetic nervous system (SNS), the endothelin system, the vasopressin system and the reactive oxygen species (ROS) and the vasodilatory mechanisms such as nitric oxide (NO), atrial natriuretic peptide (ANP), bradykyinin and some prostaglandins (Whelton et al. 2004). Dysfunction in one of these mechanisms could lead to hypertension. For instance, oxidative stress could contribute to the vasoconstrictive mechanisms and attenuates the vasodilatory mechanisms. In order to establish BP reduction and organ protection, it is imperative to understand some mechanisms implicated in hypertension.

1.4.1 Activation of the sympathetic nervous system (SNS)

SNS activation plays an essential role in the pathophysiology of BP through its effects on brain, cardiac, renal function and vessel tone (Weir 2009). In the brain, SNS activation is accompanied by the release of norepinephrine (induces peripheral sympatho-stimulation) (Amagase 2006). Stimulation of β1 adrenergic receptors in the heart increases heart rate and contractility, and in the kidney leads to an increase in renin secretion (Dietz et al. 2008). Stimulation of α1 adrenergic stimulation in vascular smooth muscle leads to an increase in intracellular calcium and vasoconstriction (Weir 2009). Therefore, any suppression of SNS either with clonidine (brainstem α2-receptor agonist), carvedilol (β1 adrenergic receptor blocker), or doxazosin (α1 adrenergic receptor blocker) (Ohta et al. 2007), lowers BP.

1.4.2 Activation of the renin-angiotensin-aldosterone system (RAAS)

The RAAS sytem plays an essential role in the pathophysiology of hypertension (Amagase et al. 2001). Renin, produced in juxtaglomerular (JG) cells in the kidney, is the rate–limiting enzyme in the synthesis of the potent vasoactive peptide angiotensin II (ANGII) (Bader

and Ganten 2000). Renin secretion is regulated by the macula densa, which functions as a sensory structure in JG apparatus in the kidney. If macula densa cell senses any drop in salt concentration or a change in blood flow rate, it triggers renin release from the JG cells. And it does this by releasing prostaglandins (PGs). PGs activate renin secretion in JG by upregulating cylic AMP (Bader and Ganten 2000). To minimize pressure drop and blood loss, renin induces vasoconstriction through angiotensin II {cleaved from angiotensin I by the angiotensin converting enzyme (ACE)} and renal sodium and fluid retention through aldosterone production (Del Vecchio et al. 2007; Maisel 2009). Therefore, suppression of RAAS by aliskiren (renin inhibitor) (Solomon et al. 2009), captopril (ACE inhibitor) (Reboldi et al. 2009), candesartan {an angiotensin II receptor blocker (ARB)}(Stoukides et al. 1999) or eplerenone (aldosterone receptor antagonist; in resistant hypertension and hyperaldosteronism) (Karagiannis et al. 2009) attenuates hypertension.

1.4.3 Salt sensitivity

Salt sensitivity is considered as a BP determinant in both individuals and animals (Franco and Oparil 2006). It is defined as the tendency for BP reduction during salt depletion and BP rise during salt supplementation. The individuals who experience a large change in BP with acute or chronic salt depletion or repletion are termed “salt sensitive” (Katori and Majima 2008). Mechanisms that appear to be involved in salt sensitivity: deficiency in RAAS system, ANP expression and arterial baroreflex activity (Franco and Oparil 2006). Even though sodium intake activates RAAS, sodium intake reductions have similar renin and aldosterone responses in both hypertensive and normotensive individuals (Franco and Oparil 2006). This supports the weak effect of sodium reduction on BP. Secondly, in black and obese persons, deficiency in ANP expression or polymorphism of ANP genes may contribute to salt sensitive hypertension. Furthermore, in animal study, baroreceptors buffer the effects of any increase in dietary sodium intake on BP (Franco and Oparil 2006). This evidence confirms the baroreceptor dysfunction role in salt sensitive hypertension. In short, salt sensitive hypertensives experience an increase in BP during salt intake; this could be due to either activation of RAAS, or baroreceptor dysfunction or polymorphism of some genes, such as ANP.

1.4.4 Oxidative stress

Oxidative stress refers to an imbalance between producing ROS and the anti-oxidant defense system; oxidative stress occurs when the amount of ROS exceeds the anti-oxidant capacity. Thus, an excessive increase in ROS, a decrease in anti-oxidant capacity, or both result in oxidative stress (Roberts and Sindhu 2009; Juurlink 2001). These reactive species include ROS such as the superoxide radical ($O_2 \cdot^-$), the hydroxyl radical (OH.), and hydrogen peroxide (H_2O_2), and reactive nitrogen species (RNS) such as NO and the peroxynitrite radical ($ONOO^-$) (Juurlink 2001). Reactive species are produced by enzymes, such as nitric oxide synthase (NOS), isoforms of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, or as by-products from mitochondrial electron-transport chain (Whelton et al. 2004). At low physiological levels, reactive species are indispensible in several signaling processes, for example, in cellular differentiation, growth arrestment, apoptosis, immunity and defense against microorganisms. At high pathological levels, reactive species - anti-oxidant imbalance occurs and the amounts of reactive species exceed the anti-oxidants. Therefore, there is an excessive increase in reactive species and a depression in anti-oxidant capacity (Juurlink 2001).

1.4.4.1 Oxidative stress and cell dysfunction

The consequences of imbalance between ROS production and the antioxidant defense include lipid, protein and DNA modifications (Vaziri et al. 2000). Strong oxidants such as hydroxyl radicals or peroxynitrous acid initiate lipid peroxidation (Christman et al. 2000) causing alteration in membrane fluidity, increase in membrane permeability and decrease in membrane ATPase activity (Subramaniam et al. 1997; McGrath et al. 1995). This results in depletion of cellular ATP stores and cellular calcium overload. Consequently, it may contribute to hypertension as suggested by the efficiency of long acting calcium channel blockers, such as benidipine, that decrease systolic and diastolic blood pressure in essential hypertensive patients *via* reducing oxidative stress. Although the mechanism of the long acting calcium channel blockers in reducing oxidative stress is not well known, it could be due to inhibition of NADPH oxidase thereby causing less hydrogen peroxide formation and less tissue lipid peroxidation

(Subramaniam et al. 1997; Yasunari et al. 2005). Lipid peroxidation products such as isoprostanes, a reliable marker of oxidative stress, play a role in the vasoconstriction of renal glomerular arterioles, the retinal vessels, and the brain microcirculature (Comporti et al. 2008). In addition, superoxide produced by NAD(P)H oxidase rapidly inactivates the most important endothelium-derived relaxation factor, NO (Rodrigo et al. 2007). The peroxynitrous acid formed by the reaction of NO with superoxide causes oxidative damage in cell including protein nitration, protein oxidation and lipid peroxidation. Another major source of superoxide and thereby lipid peroxidation is mitochondrial dysfunction (Xiong and Hall 2009). If not adequately detoxified, these ROS result in a variety of pathologies, e.g., hypertension. Oxidative damage to DNA, as assessed by 8-hydroxydeoxyguanosine formation, is correlated with several pathologies, such as experimental diabetes (Hoeldtke et al. 2009). In summary, in oxidative stress, modification of lipid, protein and DNA affect normal cell function and trigger several pathological conditions, e.g., hypertension.

1.4.5 Vascular changes during hypertension

The common basic structure of a blood vessel are: (1) an inner single lining layer of epithelial cells called **endothelium** supported by a basement membrane called the **tunica intima**, (2) intermediate muscular layer, **the tunica media** and outer layer called **the tunica adventia** (Di Wang et al. 2010)**.**

Current research has indicated the role of these layers in vascular pathologies in hypertension. For instance, in vascular oxidative stress, in vitro and in vivo, Ang II-induced NADPH oxidase-derived ROS in the adventia and intima was associated with medial hypertrophy through activating growth–related signaling pathways (i.e., MAPK) and growth factors (Ushio-Fukai et al. 1998). In addition, media thickness was associated with essential hypertension. For example, the basis of the thicker media of mesenteric resistance vessels from SHR was hyperplasia (Mulvany et al. 1985). In rat thoracic aorta, the superoxide anion inactivates nitric oxide and inhibits endothelium-dependent relaxation (Wang et al. 1998).

In short, some vascular changes, such as hypertrophy, hyperplasia and endothelial dysfunction are associated with hypertension.

1.5. Rat models of hypertension

Hypertension is a multifactorial disease that involves an interaction between genetic and environmental factors. Several animal models, such as cat, dog, mouse and rat, are used to investigate human hypertension. But there is no ideal animal model of hypertension that develops all of the human disease characteristics and complications. What dictates the choice of animal models of hypertension are the experimental questions to be addressed. The most commonly used animal model is the rat model of hypertension (Pinto *et al*. 1998). This model of hypertension exhibits features of hypertension, such as end-organ damage, cardiac hypertrophy and renal dysfunction (Pinto *et al*. 1998). Lerman classifies rat models of hypertension into nongenetic and genetic animal models of hypertension (Lerman et al. 2005). In this section, we will outline how to produce some models of hypertension in rats, what are the features (tissue damage) and the efficacy of antihypertensive agents in experimental rodent models of hypertension.

1.5.1 Nongenetic models

1.5.1.1 Surgically induced hypertension

In 1934, Goldblatt *et al*. introduced the first model of renal hypertension in dogs by a unilateral constriction of the renal artery by a silver clip. This model is called the 2 kidneys-1 clip (2K, 1C), whereas the 1 kidney-1 clip (1K, 1C) is produced with a unilateral constriction and a contralateral nephrectomy (Monassier et al. 2006). In rats (Leenen and de Jong 1971)(Lerman et al. 2005), 2K, 1C leads to a gradual and chronic increase in blood pressure (Lerman et al. 2005). The pathophysiological mechanism in 2K, 1C model, is a reduction in renal perfusion that results in the juxtaglomerular apparatus to secrete renin that causes increased formation of ANGII (Lerman et al. 2005; Monassier et al. 2006; Al-Qattan et al. 1999). This, in turn, results in activation of NADPH oxidase that increases oxidative stress. Oxidative stress induces a number of changes in several cellular systems such as endothelial dysfunction, cardiac hypertrophy (Lerman et al. 2005; Monassier et al. 2006), hypertrophy of the contralateral kidney,

vascular remodeling (Lerman et al. 2005), and inflammation. ANGII also induces increased reabsorption of Na^+ that also promotes hypertension. The 2K, 1C Goldblatt model, with a cautious approach, may be extrapolated to study human renovascular hypertension (Lerman et al. 2005).

1.5.1.2 Deoxycortisone acetate (DOCA)-salt hypertension

Introduced over 60 years ago, DOCA-salt hypertension method is a common hypertension model in rats (Lerman et al. 2005; Monassier et al. 2006; Pinto et al. 1998). Administration of DOCA, in combination with a high salt diet and unilateral nephrectomy induces a low renin form of hypertension (Lerman et al. 2005; Monassier et al. 2006; Pinto et al. 1998). In the body (adrenal cortex), DOCA is hydroxylated by 11 β hydroxylase (CYP 11 B1) and produces corticosterone which is converted by aldosterone synthase (CYP 11 B2) into aldosterone (mineralocorticoid). Acting on renal tubules, aldosterone regulates sodium and potassium homeostasis. By binding to aldosterone receptors in renal tubules, aldosterone promotes reabsorption of sodium through sodium channels on distal renal tubules and on collecting ducts (amiloride-sensitive epithelial sodium channels) and induces sodium retention (Brown 2005). Thus, DOCA administration induces increased Na^+ retention (Lerman et al. 2005; Pinto et al. 1998; Brown 2005), leading to low renin type of hypertension; seen in hypertensives of African ancestry.

Regarding the efficacy of antihypertensive agents in DOCA-salt hypertension, it is the only model in which renin angiotensin inhibitors do not decrease blood pressure (Lerman et al. 2005; Pinto et al. 1998) while diuretics, calcium (Pinto et al. 1998) and endothelin antagonists are very effective not only in decreasing blood pressure but also in attenuating end-organ changes (Pinto et al. 1998).

In addition to aldosterone, the adrenal cortex produces cortisol that competes with aldosterone at mineralocorticoid receptors. In kidney and vasculature, cortisol is converted into inactive metabolite cortisone by 11 β-hydroxysteriod dehydrogenase. This permits aldosterone to compete effectively with mineralocorticoid receptors.

1.5.1.3 Fructose-induced hypertensive rats

Hwang, in 1987, fed normal Sprague-Dawley (SD) rats a high fructose-enriched (a simple monosaccharide, 60% of total calories) diet for 2 weeks; at the end of the 2 weeks, high systolic blood pressure (145 mm Hg) with hyperinsulinemia and hypertriglyceridemia were observed in fructose-fed rats (Hwang et al. 1987). In correlation with Hwang's study, Dai suggested that treating male Wistar rats with 10% fructose (equivalent to a diet containing 48-57% fructose) in drinking water at least for two weeks induces mild hypertension (Dai and McNeill 1995). This increase in SBP is much lower than that observed in the spontaneously hypertensive rat (SHR), renovascular and DOCA-treated rats (Dai and McNeill 1995). Even though the mechanism is not fully understood, a diet high in carbohydrates increases superoxide, hydrogen peroxide, isoprostanes (Song et al. 2005) and methylglyoxal, a reactive dicarbonyl intermediate (Wang et al. 2008). This leads to an increase in SNS activity and RAAS stimulation (vasoconstrictor mechanisms, e.g., isoprostanes) while it decreases the vasodilators, such as NO (Whelton et al. 2004; Song et al. 2005; de Champlain et al. 2004). As indicated in Fig. 1, features of fructose-fed rats are: hypertriglyceridemia (Johnson et al. 2009), hyperuricemia (Johnson et al. 2009), and hyperinsulinemia (Johnson et al. 2009) associated with down regulation of insulin receptors (Catena et al. 2003). Thus, this is a nongenetic rat model of hypertension. It is a useful model to study the diet induced hypertension in rats and the end organ damage in these species.

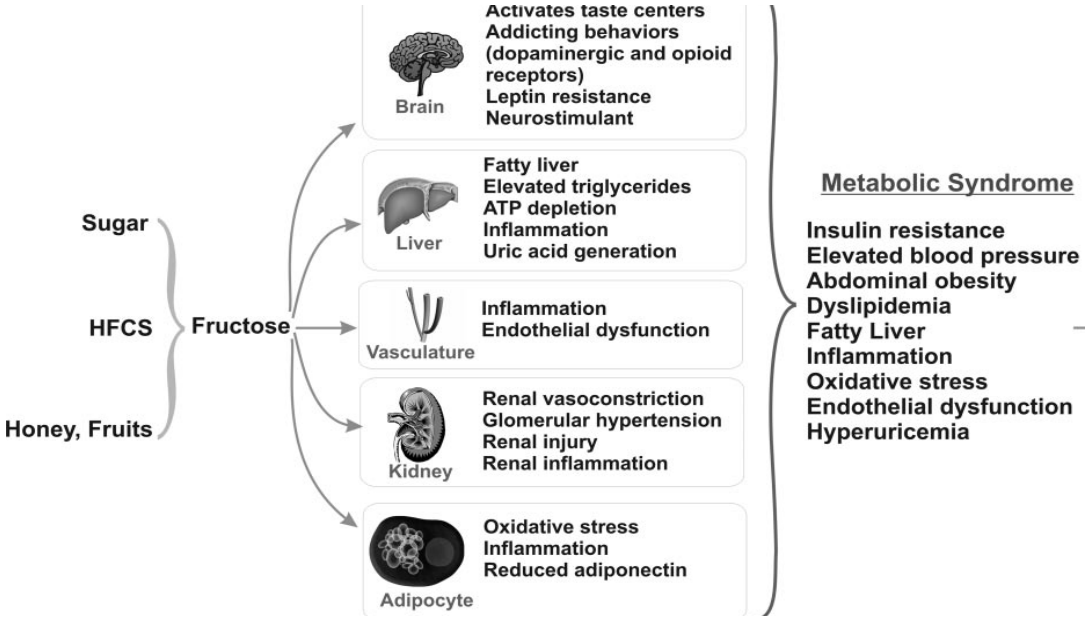


Figure 1 Effect of fructose in various rat organs. Table sugar, high fructose corn syrup (HFCS), and honey and fruits provide the source of fructose; triggering the metabolic syndrome(Johnson et al. 2009).

1.5.2 Genetic models

1.5.2.1 Spontaneously hypertensive rats (SHR) and stroke prone spontaneously hypertensive rats rats (SHRsp)

SHR is the most widely used rat model of hypertension as indicated by the number of research papers published (Fig. 2) (Lerman et al. 2005; Monassier et al. 2006; Pinto et al. 1998; Yamori 1999). The hypertensive rat strains such as SHR or SHRsp were developed by selective breeding of Wistar Kyoto males with marked elevation of blood pressure and mating to females with slightly elevated blood pressure. Brother x sister mating with continued selection for spontaneous hypertension was maintained for about 20 generations to develop hypertensive models such as SHR or SHRsp (Lerman et al. 2005; Monassier et al. 2006). In SHR, the systolic blood pressure, measured by tail cuff, rises around 5-6 weeks of age and steadily increases to reach systolic pressure of 180-200 mm Hg (Pinto et al. 1998). The features of hypertension in these genetic models are: oxidative stress (Vaziri et al. 2000), impaired glutathione (GSH)

system (Wu and Juurlink 2001), activation of the SNS (Yamori 1999), vascular smooth muscle remodeling (growth rate increase) (Lerman et al. 2005; Yamori 1999), impaired endothelium dependent relaxation (i.e., at 16 weeks of age) (Pinto et al. 1998), artery necrosis and sclerosis (Yamori 1999), cardiac hypertrophy (Lerman et al. 2005), (Pinto et al. 1998) and renal functional impairment (proteinurea, decreased creatinine clearance) (Lerman et al. 2005; Pinto et al. 1998). Due to these features such as impaired glutathione (GSH) system and endothelium dependent relaxation, SHRsp was used in our experiment.

The genetics of hypertension, recent studies were based on genetics mice and human to identify a novel candidate gene in hypertension (Broeckel et al. 2011). There are more than three major genes that are involved in the development of severe hypertension (Monassier et al. 2006), such as phenylethanol-N-methyltransferase, responsible for epinephrine biosynthesis (Nguyen et al. 2009), angiotensinogen, renin and ACE genes (Milsted et al. 2009) and 11beta-hydroxysteroid dehydrogenase type 2 (11beta-HSDII) which selectively metabolizes glucocorticoids to inactive metabolites, thus allowing for mineralocorticoid receptor activation by aldosterone (Takeda 2003).

With unknown genetic and environmental factors triggering hypertension, the most widely used hypertensive models are SHR (Fig. 1) and SHRsp. These hypertensive models mimic a subtype of human hypertension, i.e., primary hypertension that is inherited in a Mendelian fashion (Lerman et al. 2005). Therefore, these models may be used to understand the development of essential hypertension in human.

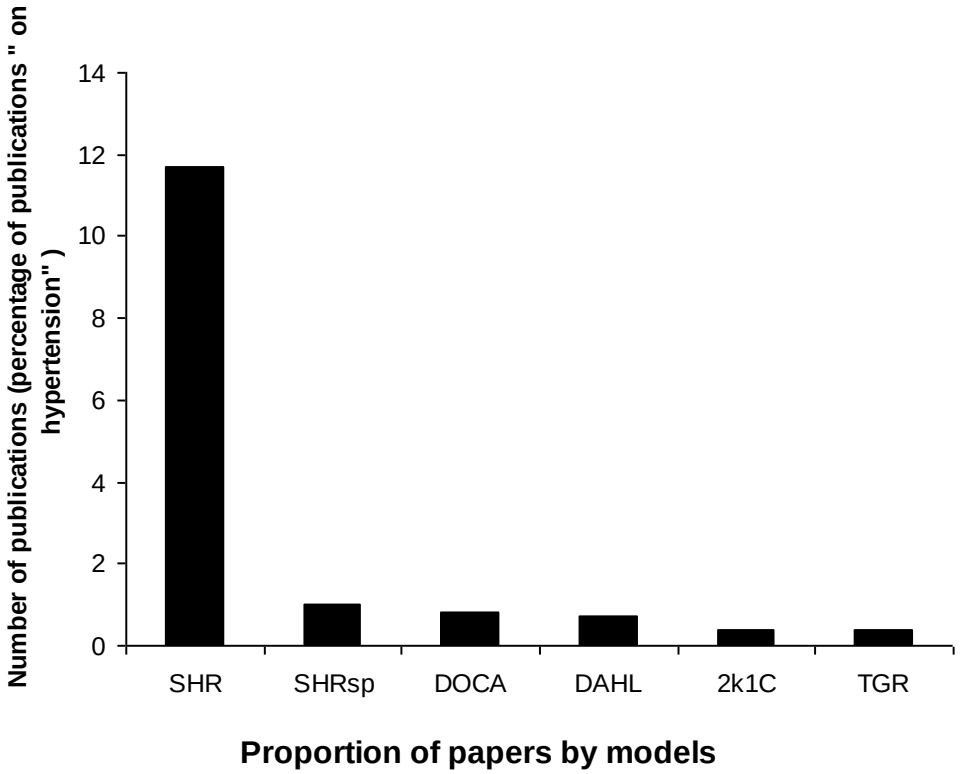


Figure 2 Number of publications on particular rat model of hypertension, as divided by the total number of papers on rat hypertension (Pinto et al. 1998).

To compare SHRsp to the stroke-resistant SHR strain, although little has been made in identifying the localized susceptible gene(s), Fornage,M. 2003 study has reported that gene and protein expression patterns in SHRSP are altered when compared with SHR. For instance, MAPKs regulate gene expression and protein synthesis and play a pivotal role in neuronal survival and apoptosis (Fornage et al. 2003). The MAPKs extracellular signal-regulated kinase (ERK) and JNK are associated with left ventricular hypertrophy (Kim et al. 2000). Compared with SHR, the JNK gene expression and activated ERK and JNK protein abundance is increased in SHRsp (Fornage et al. 2003). The increase in activity of ERK and JNK in SHRsp may suggest altered MAPK pathways and may contribute to the development of stroke and hypertension. Both SHRsp and SHR develop hypertension at early age. For example, by 10 week of age SHRsp BP levels are higher than SHR. In short, prior to the onset of stroke, multiple kinases

pathways are altered. This may enhance the SHRsp vulnerability to stroke and may play important role in the pathophysiology of hypertension.

1.5.2.2 Dahl salt-sensitive rats

Dahl salt-sensitive rat model is another genetic hypertensive rat model developed by Dr. Lewis K. Dahl (Dahl et al. 1962). Dahl introduced the Dahl salt-sensitive rat model by breeding 3 week-old SD male and female rats, administering 50 µg triiodothyronine intraperitoneally on each of four successive days and then at about 2-day intervals 5 µg doses administered for the next 21 days. All animals were fed a chow which contains salt (Dahl et al. 1962). In the first generation of animals some were resistant to triiodothyronine-high salt regimen (low BP) while others were triiodothyronine-high salt sensitive (high BP). The animals of the third generation were weaned at 3-4 weeks and immediately put on triiodothyronine-high salt regimen. In comparison with the original breeding stock, there was significant increase in the fraction of salt sensitive animals that responded with high blood pressure while a decrease in the fraction of salt resistant animals that responded with normal blood pressure. The salt sensitive Dahl rats develop severe and fatal hypertension when fed a high salt diet while the salt resistant rats do not develop such severe hypertension upon salt loading (Pinto et al. 1998). The salt sensitive rats need to consume the salt just for 2-6 weeks to maintain the systolic blood pressure between 180-206 mm Hg (Dahl et al. 1968). Dahl, L.K. in 1968 observed that younger animals are very sensitive to salt loading and develop severe hypertension in a short time and die after 8 months on high salt intake (Dahl et al. 1968).

This model of genetic hypertension may be linked with loci close to ACE receptors (Pinto et al. 1998) or to the deficiency of 11β hyroxylase which is encoded by gene CY11 B1 in the region of chromosome 8 (Imrie et al. 2006). The genetic influences of this gene CY11 B1 is highly inheritable. Major genetic effects on this gene CY11 B1 and other genes (Husted et al. 1998) may cause salt/water retention and consequently hypertension (Imrie et al. 2006).

The features of hypertension in this model are: salt sensitivity (Pinto et al. 1998; Dahl et al. 1962; Dahl et al. 1968), cardiac hypertrophy (Lerman et al. 2005; Pinto et al. 1998), endothelial dysfunction (Lerman et al. 2005; Pinto et al. 1998) and renal impairment (proteinuria, decreased creatinine clearance) (Lerman et al. 2005; Pinto et al. 1998).

Furthermore, the Dahl-sensitive rat exhibits coronary artery disease and over expresses the hepatic human cholesteryl ester transfer protein; this enzyme is implicated in lipoprotein metabolism (Lerman et al. 2005).

In this model, calcium antagonists and beta blockers are less effective and the effect on end organ damage may dissociate from the effect on blood pressure (Pinto et al. 1998). For example, diuretics decrease blood pressure but are not effective in attenuating end organ damage (Pinto et al. 1998). Not only in animals, but the salt variations are also present in humans; it has been documented that BP responses to high sodium interventions are greater in women than men, particularly at older ages (He et al. 2009).

The Dahl salt model of hypertension reflects the type of hypertension in black hypertensives because of low renin (Monassier et al. 2006). Furthermore, it mimics the essential hypertension because of the inherited salt sensitivity implications (Lerman et al. 2005).

1.6 Gender differences in blood pressure regulation before and after puberty

I will highlight the sex differences in blood pressure regulation in humans and animals and the possible mechanisms for gender differences.

1.6.1 Gender differences in blood pressure regulation in humans

Although the prevalence of cardiovascular diseases is higher in men (Reckelhoff 2001; Montague et al. 2006), women (after menopause) experience higher rates than men of stable angina, high blood pressure, congestive heart failure and stroke (Montague et al. 2006). Until the age of 60 to 70 years, men have higher BP levels than women. After the menopause, the women tend to have higher BP than men. For example, the blood pressures in women and men with type 2 diabetes is about 145/84 mmHg and 140/80 mmHg, respectively (Manjoo et al. 2010). Lower levels of vasodilating female sex hormones, such as estrogen, after the menopause (Hart et al. 2009) may partially explain why women have higher BP than men. In addition to BP regulation, estrogen inhibits collagen production and smooth muscle proliferation, aortic stiffness and endothelial dysfunction.

1.6.2 Gender differences in blood pressure regulation in animals

The gender differences in blood pressure observed in humans have also been documented in various animals, such as SHR (Reckelhoff et al. 1999), Dahl salt-sensitive rats (Rowland and Fregly 1992) and DOCA-salt rats (Ouchi et al. 1987). For example male SHR have higher blood pressure (187 mm Hg) than do females (155 mm Hg) of similar ages (Reckelhoff 2001; Sartori-Valinotti et al. 2007). The systolic blood pressure in male DOCA-salt rats (13 week old SD) is significantly higher than that in female DOCA-salt rats (Ouchi et al. 1987). Similarly, in normal rat strains, the systolic blood pressure in male and female SD (24 week old) rats is 120 ±3 mm Hg and 116 ±2 (Wang et al. 2006). In short, estrogen is a phase 2 protein inducer. This partially explains why females generally have less oxidative stress until menopause than males.

1.6.3 Mechanisms for gender differences in blood pressure control

Although the mechanisms responsible for the gender differences in blood pressure in humans and animals are not clear, there is significant evidence that oxidative stress and sex hormones, such as testosterone and estrogen play an important role in gender- associated differences in blood pressure (Comporti et al. 2008; Reckelhoff 2001; Sartori-Valinotti et al. 2007). Previous data suggest that testosterone contributes significantly to hypertension development (Comporti et al. 2008; Reckelhoff 2001). Castrated male SHR develop a blood pressure pattern similar to the female pattern of hypertension. Another evidence is that administration of testosterone to male SHR increases blood pressure (Comporti et al. 2008; Reckelhoff 2001). However, the mechanism by which testosterone increases blood pressure is not known. But it is known that testosterone is correlated with BP (Reckelhoff 2001) and that testosterone promotes renal angiotensinogen mRNA expression (Reckelhoff 2001; Kienitz and Quinkler 2008). Before menopause, females have lower blood pressure than males (Wang et al. 2006); it is possible that female sex hormones, such as estrogen partially play a role in protecting the female from developing higher blood pressure. Before menopause, the blood pressure does not increase; however the blood pressure increases after menopause (Reckelhoff 2001). This may suggest that female hormones, such as estrogen, contribute to the blood pressure control in females. The mechanism of estrogen in controlling blood pressure may be through stimulating

NO (Reckelhoff 2001). In addition to sex hormones, male hypertensive rats experience higher renal tissue oxidative stress than females (Sartori-Valinotti et al. 2007). Kidney tissue F_2-isoprostane (oxidative stress marker) and glutathione peroxidase (antioxidant enzyme) levels are slightly higher in male than that in female SHR (Sartori-Valinotti et al. 2007). Therefore, the increase in expression levels of the antioxidant enzymes in male SHR suggests that the male SHR experience more oxidative stress in their kidneys (Vina et al. 2007) than the females. To sum up, the sex hormones and oxidative stress may partially contribute to the gender differences in blood pressure control.

1.7. Management of hypertension

1.7.1 Life style

Most hypertensive patients would benefit from a healthy lifestyle. The majority of Canadians exhibit at least one hypertension risk factor, such as obesity, high dietary salt intake, high alcohol consumption (Appel et al. 2009) and physical inactivity (Buttar et al. 2005). Therefore, healthy body weight, salt reduction, regular exercise and moderate consumption of alcohol not only reduce blood pressure but can delay the incidence of hypertension, and enhance the antihypertensive drug therapy. In the past, clinicians focused on diastolic blood pressure (DBP) as predictor of cardiovascular diseases (CVD), now systolic blood pressure (SBP) appears to be a better predictor of CVD. With lifestyle modifications, a reduction in SBP may be associated with reductions in mortality caused by stroke and heart disease. For example, it has been conjectured that a 3 mm Hg reduction in SBP could lead to a reduction of 8% in mortality caused by stroke and a reduction of 5% in mortality caused by coronary heart disease (Appel et al. 2006).

1.7.2 Weight management

Overweight and obesity have become a major health issue. Adults have a body mass index (BMI) of $\geq$ 25 kg/ m^2 are classified as either overweight or obese (Khan et al. 2007). In the U.S.A., approximately 67 % of the adults are either overweight or obese. Studies have shown that adult men or women with a BMI of 30 kg/m^2 or more have a prevalence of hypertension of 38.4% and 32.2% , respectively while those with a BMI of 25 kg/m^2 have a hypertension prevalence of 18.2% and 16.5%, respectively (Lenz and Monaghan 2008). A weight loss of 10 lb (~4.5 kg) can reduce or prevent hypertension in a large portion of overweight / obese individuals (Wexler and Aukerman 2006).

1.7.3 Salt intake

Research studies have shown that sodium salt has a considerable effect on blood pressure (Appel et al. 2009). Sodium salts, particularly in some food components, have shown to increase blood pressure. Salt intake of 100 mmol/d corresponds to 2.3 g of salt intake/day (Appel et al. 2006). The BP correlates with salt intake. In normotensive subjects, for a 1 mm Hg decrease in SBP and 0.1 mm Hg decrease in DP, a 100 mmol decrease in daily sodium intake is required. This effect is very pronounced in hypertensive people over 44 years old. For every 100 mmol decrease in daily sodium intake, there is 6.3 mm Hg in SBP and 2.2 mm Hg in DPB (Fodor et al. 1999). A high salt diet stimulates NADPH oxidase (e.g. in kidney) and reduces antioxidant enzymes, such as superoxide dismutase, thus increasing oxidative stress (Titze and Ritz 2009). This is also accompanied by activation of SNS and RAAS system (Titze and Ritz 2009). Although these studies show that salt restriction leads to small reduction in BP and it may have small effect in mortality, excessive salt intake should be avoided, particularly in salt sensitive hypertensive patients, because high salt diet results in higher salt retention and thereby increasing blood pressure. Restriction of dietary salt intake may be an alternative or a supplement to antihypertensive medications.

1.7.4 Alcohol consumption

The effect of alcohol consumption varies depending on the amount consumed and on individual patient characteristics. Consuming alcohol has been linked to some pathological conditions, such as stroke and high blood pressure. By limiting alcohol consumption,

hypertensive individuals can decrease SBP (Lenz and Monaghan 2008). Furthermore, clinical trials have shown that 67% reduction in heavy alcohol consumption (3-6 drinks/day) leads to a 3.31 mmHg decrease in systolic blood pressure and 2.04 mmHg in diastolic blood pressure (Xin et al. 2001). Even though light to moderate amounts of alcohol consumption can have positive health benefits, e.g., decreasing coronary heart disease risk by lowering high density lipoprotein, it is not recommended for anyone to begin drinking on the basis of this positive effect (Lenz and Monaghan 2008) because there is no clear evidence that there is a very large protective effect of alcohol consumption and this protective effect is still disputed (Gronbaek 2004) .

1.7.5 Physical activity

As a non-pharmacological intervention physical exercise such as mild to moderate not vigorous, has been reported to decrease blood pressure. It has been shown that mild to moderate exercise produces 10-18 mmHg average reduction in systolic and 10 mmHg average reduction in diastolic pressure (Schulze 2009; Zaros et al. 2009). Although the antihypertensive mechanisms of the moderate exercise are unclear, in addition to the sympathetic slow down (Jennings et al. 1989), moderate exercise attenuates cardiac and vascular remodeling (Schulze 2009), may be, through decreasing P38 mitogen activated protein kinase (p38 MAPK) and extracellular regulated kinase (ERK) and improves the NO availability (Zaros et al. 2009). To sum up, for people at high risk of hypertension or who already have hypertension and on antihypertensive medications, maintaining healthy body weight, moderating consumption of alcohol, moderate exercise and reducing sodium salt intake may not only reduce hypertension but it may also delay or prevent the complications of hypertension, such as, left ventricular hypertrophy and vascular wall thickening.

1.7.6 Drug treatment of essential hypertension

In this section, I will highlight why some of the therapeutic agents, as monotherapy, are used in essential hypertension and what adverse effects might be produced in hypertensive patients.

1.7.6.1 Beta blockers

Beta blockers, such as atenolol and metoprolol (β_1, cardioselective), propranolol ($\beta_2 + \beta_1$, nonselective) and carvedilol and labetalol ($\alpha 1 + \beta_1$, nonselective) are used in younger and elderly hyperptensive patients (Pollack et al. 2009). As single drugs, beta blockers are effective, with low cost and cardio protective (Goose and Dallocchio 1989). However, they are not recommended as first-line therapy in hypertension because they worsen diabetes, bronchospasm, depression, sexual activity and their effect on cardiovascular morbidity and mortality remains controversial (Stafylas and Sarafidis 2008). Propranolol and atenolol, in comparison to other antihypertensive drug classes, increase risk of mortality and stroke (Stafylas and Sarafidis 2008).

1.7.6.2 Sympathoplegic agents

These agents lower blood pressure by inhibiting the SNS function. For example methyldopa is centrally acting antihypertensive agent that inhibits the sympathetic activity and lowers BP. Although it is still used in developing countries because of low cost, its use has been replaced by other antihypertensive agents. This is because methyldopa may cause serious adverse effects (Mah et al. 2009) such as hepatitis, which could be fatal, (Ali et al. 2009) in 2.5-10% of the patients (Thomas and Cardwell 1997). And this hepatitis is thought to be due to immune reaction to the resultant metabolite (Thomas and Cardwell 1997).

1.7.6.3 Diuretics

Diuretics, for example, thiazides (i.e., hydrochlorothiazide) and loop diuretics (i.e., furosemide) are widely prescribed as a first or a second line therapy in essential hypertension (Chen et al. 2009) and particularly, low-dose diuretics (i.e., 12.5 to 25 mg per day of hydrochlorothiazide) are very effective not only in lowering BP but also in preventing the occurrence of cardiovascular disease morbidity and mortality (Psaty et al. 2003). Even though both thiazides and loop diuretics produce a similar modest SBP/DBP lowering effect (-8/-4 mmHg), their current use may be limited because the antihypertensive effect is accompanied by

several adverse effects. The adverse effects include impaired pancreatic insulin release and tissue glucose utilization triggering hyperglycemia. Increased total serum cholesterol and low-density lipoproteins (LDL) result in lipidemia. An enhancement of uric acid reabsorption in the proximal tubule may precipitate attacks of gout. Inhibition of salt reabsorption in the thick ascending limb results in increased secretion of potassium and hydrogen in collecting duct and triggers hypokalemia (Musini et al. 2009).

1.7.6.4 Vasodilators

Vasodilators such as hydralazine, are used for short treatment as a second line therapy in severe hypertension, one of the antihypertensive mechanisms may be through rapidly triggering NO release (relaxing factor) (Katsumi et al. 2007). Even though they reduce mean BP by an average of 10 mm Hg (i.e., hydralazine) (Carlsen et al. 1985), the severe cardiovascular effects of tachycardia, cardiac contractility limit their antihypertensive effectiveness (Carlsen et al. 1985).

1.7.6.5 Calcium channel blockers

Calcium channel blockers (CCBs) are widely prescribed in the treatment of hypertension either alone or in combination with other antihypertensive agents. It is important to recognize why CCBs are used and what adverse effects they might produce. L-type calcium channel blockers (CCBs), particularly dihydropyridines like nifedipine or amlodipine are prescribed to patients with moderate and severe hypertension (Major et al. 2008), particularly in African Americans (Ferdinand and Armani 2007). CCBs appear to be effective regardless of the age or the ethnic background of the patients. As monotherapy or in combination with diuretics, CCBs preferentially bind to L-type channels and produce potent vasodilatory effects. Common adverse effects are induced vasodilatory edema in the skeletal muscle vasculature (Major et al. 2008), possibly due to selective precapillary arterioles or small arteries vasodilatation (Arroyo and Kao 2009), induced hyperglycemia through affecting L-type calcium channels in the pancreas, preventing the release of insulin (Arroyo and Kao 2009). Although dihydropyridines have slight direct effect in L-type calcium channels located at the cardiac smooth muscles (Arroyo and Kao

2009), dihydropyridines might trigger slight cardiovascular instability such as bradycardia and conduction abnormalities (Arroyo and Kao 2009). Although CCBs have common adverse effects such as edema and bradycardia which may decrease the compliance, CCBs should be used either as monotherapy or in combination to lower BP.

1.7.6.6 Inhibitors of angiotensin system

Inhibition of renin angiotensin aldosterone system (RAAS) may not only lower BP but also it may reduce end organ damage. Inhibition of RAAS *via*, renin inhibitor, angiotensin–converting enzyme inhibitors (ACEI) such as captopril or *via* angiotensin receptor blockers (ARBs) such as valsartan or aldosterone antagonists (such as spironolactone) may be effective in lowering BP (Weir 2009) in most hypertensive patients, with the exception of African Americans (Locatelli et al. 2009). The exception of the African Americans is due to the genetic abnormality in RAAS in those patients. Heart failure (HF), myocardial infarction (MI), and kidney disease (end organ damage) among patients with stable coronary artery disease are reduced with ACEI. Regardless of cough, abnormal creatinine rise (Germino 2009; Fox and EURopean trial On reduction of cardiac events with Perindopril in stable coronary Artery disease Investigators 2003), perindopril (ACEI) reduces BP (mean 5/2 mm Hg) and this reduction is accompanied by reduction in cardiovascular events (Fox and EURopean trial On reduction of cardiac events with Perindopril in stable coronary Artery disease Investigators 2003; Ram 2009). Despite the cardiovascular events reduction with ACEIs, the cardiovascular death remains the main cause of death. For example in the United States, more than 147,000 Americans died of CVD in 2004 (Rosamond et al. 2007). And the CV mortality and morbidity remains with the RAAS inhibition (Ram 2009). In short, RAAS inhibition, i.e., perindopril (ACEI), reduces BP, with specific adverse effects, such as cough, or abnormal creatinine rise, varied organ protection and CV morbidity and mortality.

In short, even though, in agreement, all antihypertensive drugs reduce BP, there are some differences between antihypertensive drug classes and some within class differences in organ protection (Hermann et al. 2006) and maybe in reducing or preventing CV morbidity and mortality. For instance, ACE inhibitors, ARBs, CCBs (Hermann et al. 2006) and low dose

hydrochlorothiazide (Psaty et al. 2003) improve organ protection may reduce or prevent CV morbidity and mortality. However, among the patients receiving antihypertensive treatment, the level of adequate BP control (<140/90 mm Hg) is only 30%-50% (Hermann et al. 2006) and in addition to the adverse effects, these antihypertensive drugs reduce the risk of CV events only by 20% and stroke by 40% (Hermann et al. 2006). In different models of hypertension, the balance between NO (potent vasodilator) and superoxide (oxidant, implicated in vasoconstriction) is altered (Zalba et al. 2001). So a mono-functional phase 2 protein inducer, which induces GSH and its dependent enzymes with or without life style modification, may attenuate hypertension, protect organ damage and reduce or prevent CV disease morbidity and mortality.

1.8. Anti-oxidant defense systems

1.8.1 Glutathione (GSH) –dependent scavenging mechanisms

1.8.1.1 Glutathione

Glutathione (L-γ-glutamyl-cysteinyl glycine, GSH) is a low molecular weight tripeptide containing glutamic acid, cysteine and glycine (Griffith 1999) (Fig.3). GSH is ubiquitous in mammalian and other living cells (Anderson et al. 1997). Depending on cell type and metabolic factors, growth, nutritional state and hormonal effect (Boyland and Chasseaud 1969) the intracellular GSH concentration is generally in the 1-8 mM range (Griffith 1999). Greater than 90% of the naturally occurring nonprotein thiol present in cells (i.e., the large pool in cytosol) is in the form of GSH (Pei et al. 2006). The reactive group in GSH is the thiol group. GSH was first identified in crude form by J. De Rey-Pailhade while F.G. Hopkins isolated GSH in a crystalline from yeast and showed that this molecule is a tripeptide (Boyland and Chasseaud 1969). Understanding GSH synthesis, biosynthesis, degradation and biological function provides the basis to upregulate the critical components of the biological anti-oxidant defense systems.

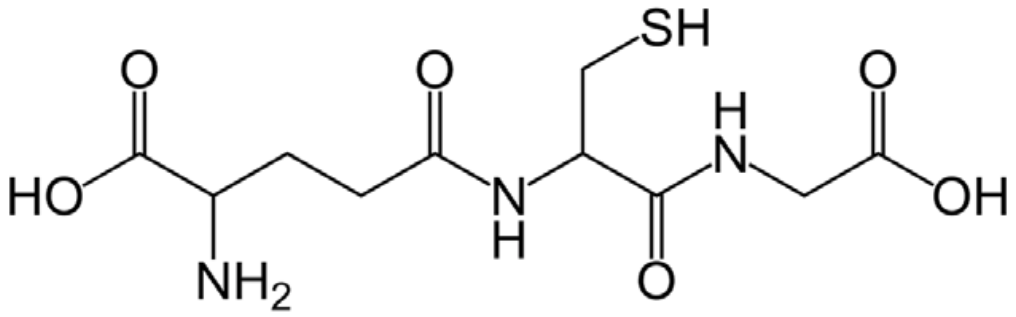


Figure 3 The structure of glutathione (L-γ-glutamyl-cysteinyl glycine, GSH)

(Griffith 1999).

1.8.1.2 Biosynthesis and Degradation

GSH is synthesized from its constituent amino acids by the following sequential action of gamma-glutamylcysteine synthetase (γ-GCS), also called glutamate cysteine ligase (GCL), (reaction 1) and GSH synthetase (GS, reaction 2) (Griffith 1999) :

[1] L-glutamic acid + L-cysteine + ATP ↔ L- γ –glutamyl –L-cysteine + ADP + Pi

[2] L- γ –glutamyl –L-cysteine + ATP + glycine ↔ glutathione + ADP + Pi

Both enzymes are cytosolic (Griffith 1999) with cysteine being the rate-limiting amino acid while γ-GCS in reaction 1 is the rate limiting enzyme (Juurlink 2001). In a physiological feedback phenomenon, GSH regulates its own synthesis by binding to the glutamate (Glu) and cysteine (Cys) sites of the enzyme (Richman and Meister 1975) (Fig.4). However, when GSH is oxidized, feedback inhibition is lost and the availability of L-cysteine as a precursor and γ-GCS can become the rate-limiting factor. The net loss of intracellular GSH is attributed to spontaneous or catalyzed addition of GSH to various electrophiles, transport of GSH across plasma membrane and out of the cell (Griffith 1999) while the rapid and irreversible extracellular degradation of GSH (few μM) is initiated by γ-glutamyltranspeptidase (Griffith 1999) localized at the plasma membrane of mammalian cells (Kumar et al. 2003). γ-Glutamyltranspeptidase breaks down extracellular GSH and yields cysteine, the rate limiting amino acid for intracellular GSH synthesis (Zhang et al. 2005).

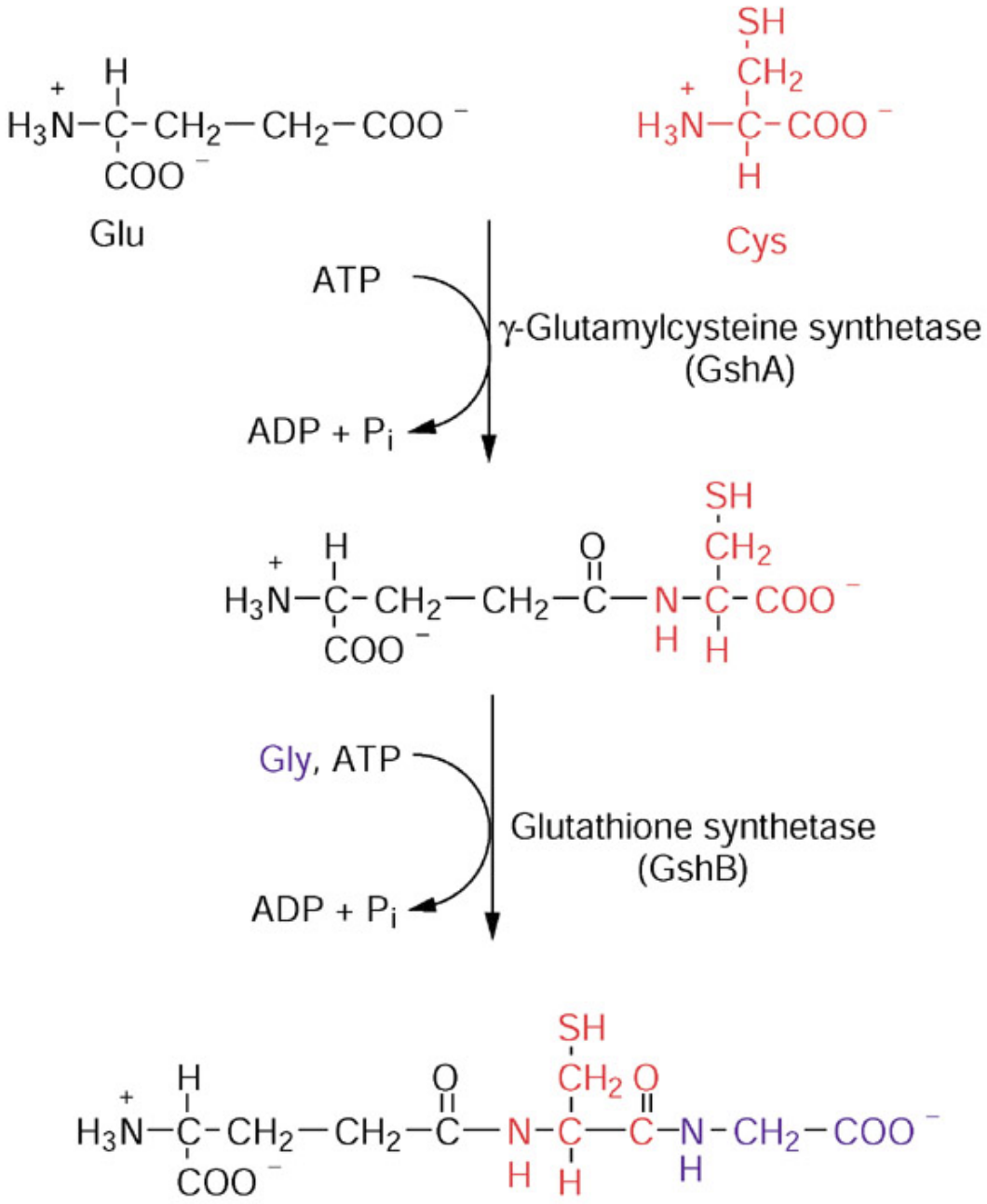

Glu
Cys
ATP
ADP + Pi
γ-Glutamylcysteine synthetase
(GshA)
Gly, ATP
ADP + Pi
Glutathione synthetase
(GshB)

Figure 4 Glutathione synthesis, GSH is synthesized from its constituent amino acids by the following sequential action of gamma-glutamylcysteine synthetase (γ-GCS), also called glutamate cysteine ligase (GCL), and GSH synthetase (GS) (Richman and Meister 1975) .

1.8.1.3 Functions of glutathione

In most cells, GSH is the major intracellular redox buffer (Lopez-Mirabal and Winther 2008). The balance between the GSH synthesis and catabolism maintains the cellular level of GSH between 2-10 mM range (Juurlink et al. 1998)., Glutathione participates in many cellular reactions: (1) using GSH as the electron donor, the selenium-containing protein (glutathione peroxidase, GPx) scavenges H_2O_2 and many other hydroperoxides such as phospholipid hydroperoxides, fatty acid hydroperoxides, cumene hydroperoxide, tert-butyl hydroperoxide, and cholesterol hydroperoxides (Liu et al. 2008), (2) GSH (GSH dependent dehydroascorbate reductase) initiates ascorbic acid (vitamin C) regeneration from its oxidation product, dehydroascorbate (Fornai et al. 1999). Ascorbic acid, in turn, regenerates Vitamin E from its innocuous vitamin E radicals (Aydin et al. 2004) where vitamin E quenches lipid peroxyl radicals, (3) using GSH as a substrate (cofactor), the glyoxalase pathway scavenges the strong oxidant dicarbonyl methylglyoxal (Wu et al. 2002). This methylglyoxal increases in many pathological conditions such as hyperglycemia (Wu et al. 2002) and hypertension (Wu and Juurlink 2002), (4) catalyzed by various glutathione-S-transferases (GSTs; a family of phase 2 enzymes), GSH mediates detoxification of xenobiotics (e.g., acetaminophen) and endogenously generated electrophiles such as strong oxidants (4-hydroxynonenal, 4-HE) (Goon et al. 1993). (5) GSH reacts with NO; forming nitrosothiol which can serve as a vehicle to transport NO in plasma; increasing the biological half life of NO (Fang et al. 2002). GSH reacts with peroxynitrite ($ONOO^-$) to form nitrosoglutathione which acts as a NO donor causing oxidant scavenging promotion and vasodilatation. In addition, tyrosine residues of proteins can be nitrosylated by NO or its derivative peroxynitrite. GSH can scavenge peroxynitrite (Fang et al. 2002) and (6) GSH is necessary for the proliferation of cells, including lymphocytes and intestinal epithelial cells (Wu et al. 2004).

1.8.2 Phase 2 proteins (cytoprotective proteins)

Phase 2 proteins (cytoprotective) are inducible proteins. The transcription of phase 2 proteins is under the control of the antioxidant (or electrophile) response element (ARE) (Juurlink 2001; Christman et al. 2000; Dinkova-Kostova and Talalay 2008). Induction of phase 2 genes is a highly effective way to counteract many chronic pathological conditions which have oxidative stress and inflammatory components, such as hypertension, diabetes and cancer (Dinkova-Kostova and Talalay 2008). Understanding phase 2 enzymes; particularly those proteins associated with the GSH-dependent pathways, may provide the basis for designing dietary phase 2 enzyme inducers. The term of phase 2 enzymes comes from the perspective that all xenobiotics undergo sequential two–step metabolism (Juurlink 2001; Dinkova-Kostova and Talalay 2008): (1) phase 1 enzymes, catalyze the initial metabolism of xenobiotics and produce highly reactive products (e.g., electrophilic). An example of phase 1 enzyme are mono-oxygenases such as cytochrome P450 enzymes (Juurlink 2001; Dinkova-Kostova and Talalay 2008); those reactive electrophiles are damaging to biological macromolecules and (2) phase 2 enzymes play an essential role, either directly or indirectly, inactivating xenobiotics (reactive electrophiles) (Juurlink 2001; Dinkova-Kostova and Talalay 2008) through increasing xenobiotics solubility and facilitating their excretion (Dinkova-Kostova and Talalay 2008). Therefore selective induction (by monofucntional enhancing transcription) of phase 2 genes may reduce the risk of chronic diseases and cancer. Phase 2 proteins include proteins associated with the GSH-dependent pathways: l-gamma-glutamyl-L-cysteine ligase (the rate limiting enzyme in GSH synthesis), glutathione reductase (GRed), thioredoxin reductases (TrxR1) (Juurlink 2003), glutathione-S-transferases (GST), quinone reductase (Juurlink 2001) and selenoprotein glutathione peroxidase (GPx) (Christman et al. 2000). Therefore, if phase 2 enzymes, e.g., GRed, TrxR1 and GC, are induced, they will ensure that GSH and reduced-thioredoxin are available to scavenge strong oxidants. The current definition of phase 2 proteins (cytoprotective proteins) are those proteins whose genes have ARE's in their promoter regions.

1.8.2.1 How are intrinsic cytoprotective phase 2 proteins regulated?

The mechanism of regulation of intrinsic cytoprotective phase 2 genes involves Kelch-like ECH-associated protein1 (Keap1), nuclear factor erythroid2-related fator2 (Nrf2) and antioxidant response elements (ARE) (Dinkova-Kostova and Talalay 2008) (as shown in fig.5): (1) AREs are present in the regulatory regions of the cytoprotective phase 2 genes (in nucleus), (ii) Nrf2 is a transcription factor responsible for basal and inducible expression of phase 2 cytoprotective genes. Nrf2 heterodimerizes with members of small Maf family transcription factors, binds to ARE and triggers the transcriptional machinery for expression of ARE regulated genes and (iii) Keap1 is a cytosolic repressor protein that binds to Nrf2 in the cytoplasm (Dinkova-Kostova and Talalay 2008; Kensler et al. 2007), equipped with highly reactive cysteine thiols. This transcriptional machine (the three cellular components) regulates the basal and inducible expression of cytoprotective phase 2 genes. For instance, environmental stress or inducers react with (or oxidize) a single thiol group of Keap1 (intracellular sensor) resulting in reversible inactivation (or modification) of Keap1 with Nrf2 release, triggering the transcriptional machinery of cytoprotective genes (Dinkova-Kostova and Talalay 2008; Kensler et al. 2007).

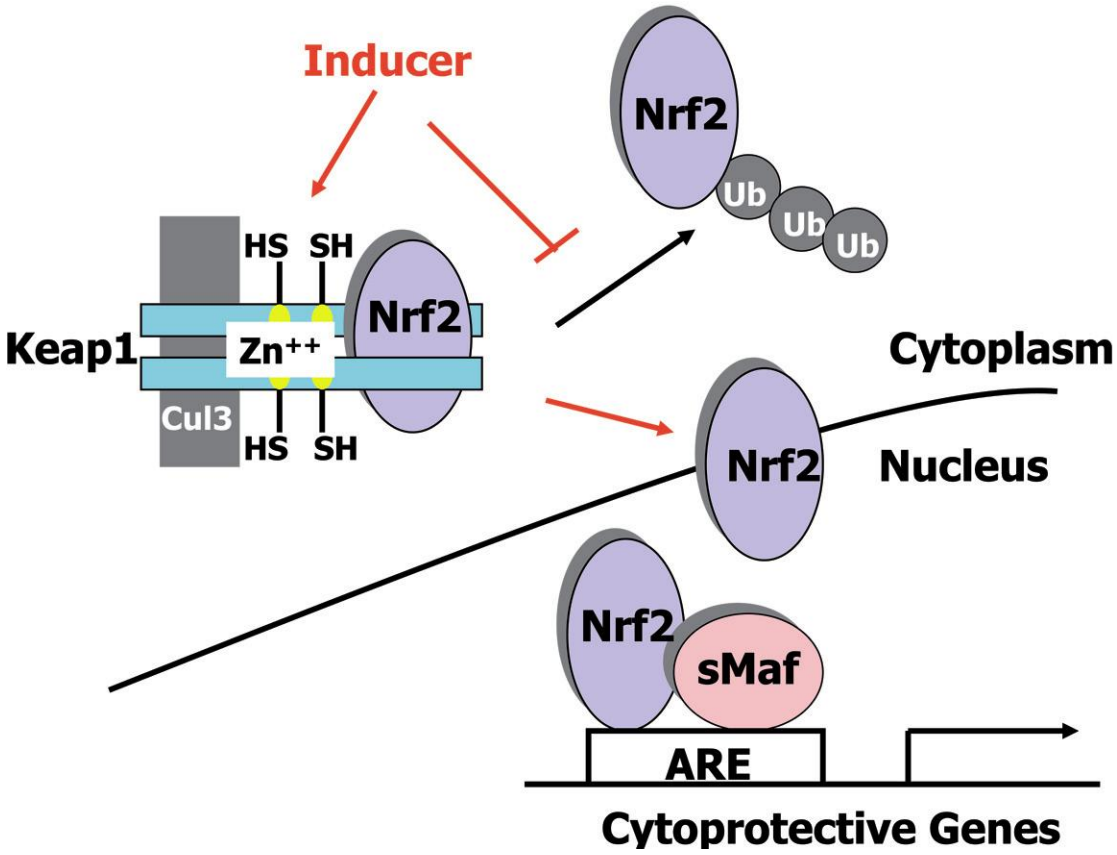


Figure 5 Mechanism of induction of cytoprotective genes, with permission from (Dinkova-Kostova and Talalay 2008). In the presence of inducer, Inducer interacts with highly reactive cysteine residues of Keap1 resulting in conformational changes (chemical modification), inhibiting the ubiquitination (Ub) of Nrf2, inducing nuclear translocation and binding of Nrf2 with small MAF (transcription factor). NRF2-small MAF complex binds with ARE and activates the transcription of cytoprotective genes. On the other hand, resynthesis of Keap1 inactivates the inducing activity of the inducers.

1.8.3 Dietary Phase 2 protein inducers

In our diets, we encounter phase 2 protein inducers including: diallyl sulfide found in garlic (Munday and Munday 2001b), the phytoestrogen genistein present in soy flour, lignan phytoestrogens present in flax seed (Pan et al. 2009), Kaempferol, a flavonoid present in kale

(Juurlink 2003), ellagic acid, polyphenols present in pomegranate (Haidari et al. 2009), strawberries and raspberries and / or blackberries (Bomser et al. 1996; Stoner et al. 2006), green tea polyphenolics (Yang and Wang 1993), curcumin [a polyphenolic of turmeric (Purkayastha et al. 2009)], quercetin (phytoestrogen isoflavone) (Bandera et al. 2009) present in onions and in a variety of berries (Arteaga et al. 2004) and sulforaphane, a metabolite of the glucosinolate glucoraphanin found in crucifers, particularly in the sprouts of certain broccoli cultivars (Fahey et al. 1997).

1.8.3.1 Dietary Phase 2 protein inducers and therapeutic effect

In many pathological conditions, diabetes, atherosclerosis and hypertension, phase 2 protein inducers, such as ellagic acid, increase phase 2 proteins (cytoprotective proteins), ameliorating conditions associated with oxidative stress (Dinkova-Kostova and Talalay 2008; Juurlink 2003). Using animal model of diabetes (alloxan-induced model), Sheweita (Sheweita et al. 2002) demonstrated that when diabetic male rats are maintained on Lupinus albus (Termis) (hypoglycemic herb) daily for 28 days, Lupinus albus decreases the insulin-dependent diabetes mellitus, called "Alloxan Diabetes". These hypoglycemic herbs have been shown to increase GST and GSH (Sheweita et al. 2002) (phase 2 protein induction). Thus, the hypoglycemic effect may be due to attenuation of oxidative stress and enhancement of insulin secretion and glucose uptake (Pi et al. 2009). In a chemically-induced cancer, the principal lignin of flax, SDG inhibits the chemically induced tumors in rats (Bommareddy et al. 2009). These effects may possibly be due to the inducing capability of phase 2 proteins. In two-kidney-one-clip (2K-1C) Goldblatt model (type of unilateral renovascular stenosis hypertension), garlic (aqueous not ethanolic extract) fed female SD rats experience lower systolic BP when compared with water vehicle-fed animals (Al-Qattan et al. 1999). The therapeutic effect of garlic has been attributed to the organosulfur compounds (diallyl sulfide, diallyl disulfide, and diallyl trisulfide). These substances act through induction of phase 2 detoxification enzymes such as quinone reductase and glutathione transferase (Munday and Munday 2001a). Moreover, in obese Zucker rats, a daily dose of quercetin (2 or 10 mg/kg of body weight) or vehicle for 10 weeks has been shown to attenuate systolic BP (~135 mmHg vs. 150 mmHg in control) and inflammation (Rivera et al. 2008). Although the mechanisms of the beneficial effects of quercetin have not yet been fully

explained, the antioxidant properties of flavonol seem to be involved in these effects. Male SHRsp rats fed 200 mg/day (daily for 14 weeks) of dried broccoli sprouts that contained glucoraphanin, which is a precursor of phase 2 protein-inducer sulforaphane. After 14 weeks, the treated rats experienced low oxidative stress as shown by increased GSH and GR and significantly lower (20 mm Hg) BP. In addition, if 5 week old pregnant and lactating female SHRsp rats consumed daily dried broccoli sprouts containing 5.5 μmol sulforaphane inducing equivalents (glucoraphanin) for 14 week, their adult offspring showed lower SBP and less inflammation than the control animals (Noyan-Ashraf et al. 2006). Moreover, in cell culture studies, sulforaphane (0.05–1 μmol/l) upregulated the impaired GSH system (GSH, GR and GPx) in aortic SMCs from SHR and this upregulated correlated with a decrease in oxidative stress (Wu and Juurlink 2001).

1.8.3.2 The beneficial health effects of cruciferous vegetables like cabbage, brussel sprouts and broccoli

In order to understand the greatly stimulated interest in using sulforaphane as an indirect dietary antioxidant in preventing or attenuating diseases including hypertension in animal model of hypertension, the therapeutic effect of cruciferous vegetables in cancer (cell culture) and hypertension (animal models) studies will be highlighted.

Previous studies

In cancer, the cancer preventive capacity of phytochemicals in brussel sprouts was clear in healthy male non-smoking volunteers. (Verhagen et al. 1995). 300 g of cooked brussel sprouts was consumed by healthy male, non-smoker volunteers daily for 3 weeks. At the end of the 3 weeks, the levels of oxidative DNA marker (8-oxo-7, 8 dihdro-2-deoxyguanosine) in sprouts group, in 24 h urine was decreased by 28% when compared with sprouts free group. Thus a reduction in oxidative DNA marker may indicate a decrease risk of cancer (Verhagen et al. 1997) or any diseases with oxidative components.

In hypertension, our interest in dietary phase 2 protein inducers present in broccoli sprouts was evident. In 2004, (Wu et al. 2004) 5-week-old male SHRsp rats were fed 200 mg of air-dried broccoli sprouts for 5 days/week for 14 week. Our data indicate that SHRsp rats experience significantly decreased oxidative stress as demonstrated by increased glutathione

(GSH) content and decreased oxidized GSH, decreased protein nitrosylation, as well as increased GSH reductase and GSH peroxidase activities in cardiovascular tissues. The decreased oxidative stress correlates with lower inflammation, better endothelial function in aorta and lower BP (20 mm Hg) as compared with age match SD rats (as the normotensive controls). In 2005, Noyan-Ahsraf *et al*., observed that using normotensive Wistar Kyoto (WKY) rats as control, dried broccoli sprouts significantly decreased degenerative changes in SHRsp CNS (Noyan-Ashraf et al. 2006). In 2006, Noyan-Ahsraf and Juurlink 2006, have shown that when five–week-old female SHRsp rats maintained either on diet containing no synthetic anti-oxidants or on 200 mg of four day old air dried broccoli sprouts (have 27.3 μmol sulforaphane per gram of dried sprouts) daily for 14 weeks, broccoli not only decreases the oxidative stress and inflammation but also attenuates hypertension in the females and her offspring (Noyan-Ashraf et al. 2006).

1.8.4 Sulforaphane: the isothiocyanate sulforaphane (1-isothiocyanato-(4R)-(methylsulfinyl) butane (fig 6)

Previous studies

In a rat model of mammary carcinogenesis (female SD rats), sulforaphane, at a dose of 75, 100, or 150 μmol/day for 4 days before and up to 1 day after the last dose of carcinogen, inhibited the tumor incidence and burden (Zhang et al. 1994). In a parallel study, using a mouse model of stomach carcinogenesis, sulforaphane, at a dose of 7.5 μmol/day from 7 days before and up to 2 days after the last dose of carcinogen, inhibited the stomach carcinogenesis (Fahey et al. 2002). In a mouse model of lung cancer, sulforaphane (1.5 or 3 mmol/kg diet), sulforaphane-N- acetylcysteine (8 mmol/kg diet), daily for 21 weeks after carcinogen (chemical carcinogen), inhibited the malignant progression of lung adenomas (Conaway et al. 2005). Importantly, sulforaphane is not only effective in chemically induced carcinogenesis. In intestinal adenomas in mice (in which apc tumor suppressor gene is altered), feeding sulforaphane at a dose of 6 μmol per mouse for 10 weeks (Myzak et al. 2004). The mechanism(s) of the anticarcinogenic effect of sulforaphane are not clear. Induction of cytoprotective proteins (phase 2 proteins), or inhibition of phase 1 enzymes (Barcelo et al. 1996), or changes in DNA methylation (Tost 2009), or modulation of the mitogen-activated protein kinase (MAPK) (Keum et al. 2006), or induction

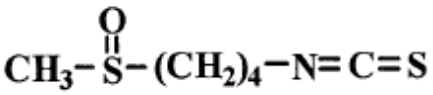
of cell cycle arrest and apoptosis, or inhibition of angiogenesis may partially contribute to the anticarcinogenic effect of sulforaphane. In addition, sulforaphane had no effect on tumor development in Nrf2-knockout mice (Dinkova-Kostova and Talalay 2008). This strongly indicates that the cytoprotective effects of sulforaphane are *via* Nrf2-dependent mechanism.

In hypertension, in addition to the protective effects of sulforaphane in neoplastic condition, aortic smooth muscle cells (SMCs) from SHR rats, l-sulforaphane (0.05-1 micromol/l) increases GSH in dose dependent manner (Wu and Juurlink 2001). The upregulation of phase2 proteins in smooth muscle cells is correlated with oxidative stress decrease.

To sum up, evidence from our *in vitro* study (Wu and Juurlink 2001) and *in vivo* studies (Wu et al. 2004; Noyan-Ashraf et al. 2006) indicate that dried broccoli sprouts, contain high amounts of glucuraphanin that gives rise to potent phase 2 inducer isothiocyanate sulforaphane, attenuates oxidative stress, inflammation and hypertension in hypertensive animals. These observations lead us to hypothesize that in SHRsp, early and long term oral administration of pure sulforaphane will have cardiovascular protective effect in these hypertensive animals.

1.8.4.1 Chemical structures

Sulforaphane (fig 6), (-)-1-isothiocyanato-(4R)-(methylsulfinyl)butane [CH3-SO-(CH2)4-NCS, is a major and very potent monofunctional phase 2 enzyme inducer isolated and identified in broccoli (Zhang et al. 1992).



Sulforaphane (SF)

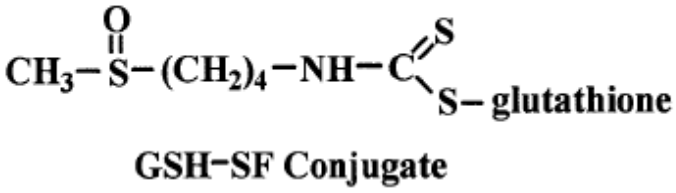


Figure 6 Structure of sulforaphane, (-)-1-isothiocyanato-(4R)-(methylsulfinyl) butane and GSH-SF (GSH-sulforaphane) conjugate (Zhang et al. 1992).

1.8.4.2 Pharmacokinetic characteristics of sulforaphane

Oral sulforaphane, in mice, is rapidly absorbed from intestinal mucosa (Yanaka et al. 2005) attains peak plasma concentration in an hour (Hanlon et al. 2008) may be due to its lipophilicity and the small molecular size of sulforaphane (Hanlon et al. 2008). Nevertheless, sulforaphane absorption does not increase proportionally with the dose; for instance, at higher doses (5 mg/kg) the absorption rate decreases in rat (Hanlon et al. 2008). This may imply that sulforaphane is absorbed *via* carrier-mediated transport mechanism that is saturated at higher doses or sulforaphane is susceptible either to first pass metabolism (enzymes in blood, intestine or in liver that interact with sulforaphane) or to hepatic first pass metabolism when the bioavailability is less than 1.0 (100%). Not only the absorption decreases at higher doses, but also the oral bioavailability was 80% at lower doses (0.5 mg/kg, in rat) while only 20% at higher doses (5 mg/kg) (Hanlon et al. 2008). The oral bioavailable sulforaphane displays high plasma protein binding affinity (Hanlon et al. 2008). This may explain why sulforaphane has long duration of action (an elimination half-life of 2.23 h (Hu et al. 2004b) and low volume of distribution. The remaining sulforaphane, free drug, is rapidly and reversibly conjugated with GSH (Zhang et al. 1995), either enzymatically catalyzed by GST (glutathione transferase) (Agrawal et al. 2006) or non enzymatically. This is strongly supported by the rapid decline in human plasma concentration of sulforaphane observed at 1 h after dosing (Ye et al. 2002). Sulforaphane and its metabolites (i.e., Sulf-GSH) accumulated in tissues and dissociated in to sulforaphane and GSH (Zhang 2000). Sulforaphane cleared within 72 h of dosing from the body

(Clarke et al. 2008; Hu et al. 2004a). In closing, sulforaphane, a portion of it, conjugates with thiol compounds, such as GSH, catalyzed by the phase II enzyme glutathione_S_transferase (GST). In addition, sulforaphane induces its own conjugation by inducing GST) (Zhu et al. 2008a; Zhu et al. 2008b) and sulforaphane accumulates in tissues and produces multiple biological effects.

1.8.4.3 Multiple biological effects of sulforaphane

In vitro studies

In normal cells, a balance exists between histone acetyl transferase and histone deacetylase. A disturbance of this balance can trigger cancer development. *In vitro* sulforaphane and its GSH conjugate (sulforaphane-GSH) have little effect on inhibition of histone deacetylase while the other metabolites, sulforaphane-cysteine and sulforaphane-N-acetycysteine have concentration-dependent inhibition of histone deacetylase (Myzak et al. 2004). In aortic smooth muscle cells (SMCs) from SHR rats, l-sulforaphane (0.05-1 micromol/l) increases GSH in dose-dependent manner (Wu and Juurlink 2001). Furthermore, in rat aortic smooth muscle cells, not only L-sulforaphane but also DL-sulforphane (0.25-5 μM) resulted in dose-dependent induction of phase 2 enzymes and important cellular antioxidants such as glutathione reductase (GRed) and glutathione S-transferase (GST) (Zhu et al. 2008a). Consistent with this in human hyperglycemic culture of endothelial cells, sulforaphane (4 μM) reversed the increase in ROS formation by 73%, while upregulating GSH and Nrf2 (Wu and Juurlink 2001; Zhu et al. 2008a). This effect was prevented by knockdown of Nrf2 expression (Xue et al. 2008). This strongly suggests that sulforaphane suppress endothelial dysfunction and the development of vascular diseases *via* activation of ARE in the promoter regions of phase 2 cytoprotective genes. At high concentration sulforaphane is cytotoxic (i.e., in mouse hepatocytes), for instance, at 50 μM, it causes 33% loss of cell viability as indicated by lactate dehydrogenase release (Barcelo et al. 1996).

Animal studies

Sulforaphane competitively inhibits the phase I cytochrome P450 isoenzyme 2E1 (CYP2E1), responsible for the activation of several carcinogens, of rat liver microsomes (Barcelo et al. 1996). Since there is high catalytic and regulatory specificity between rodent and

human forms, CYP2E1 is likely to be relevant to humans (Barcelo et al. 1996). In lung adenoma induced by tobacco in mice, sulforaphane (1.5 and 3 μmol/g) and sulforaphane-N-acetylcysteine (4 and 8 μmol/g) inhibit cell proliferation as indicated by inhibition of proliferating cell nuclear antigen and trigger apoptosis (programmed cell death) as demonstrated by activating caspase3 (Conaway et al. 2005) resulting in inhibition of the progression of lung adenoma. In female mice, *RS* sulforaphane (*RS* means racemic mixture of of dextro and levo isomers) (in daily doses of 15 μmol for 5 days) induces phase 2 enzymes such as quinone reductase (QR) and GST activities 1.6 to 3.1 in several organs (Zhang et al. 1992).

In short, sulforaphane, which is lipid soluble, is rapidly absorbed from intestinal mucosa. Since sulforaphane has high binding affinity to plasma protein, it has a low volume of distribution. Sulforaphane stimulates GST, which catalyzes the reversible conjugation with thiol compounds, such as GSH. As an anticarcinogenic, sulforaphane is extensively studied, but the protective mechanism is unclear. Presently, particular attention has been focused on activation of phase2 cytoprotective genes as a novel strategy to suppress the development of vascular diseases, such as hypertension.

2. Overall hypothesis

The following hypothesis was tested:

Independent of the food matrix, long term administration of a dietary phase 2 inducer (sulforaphane) would attenuate hypertension in female SHRsp. If our findings supported that hypothesis and sulforaphane treatment did attenuate hypertension in female SHRsp, we would preceed with testing our second hypothesis. Therefore our second hypothesis was that sulforaphane treatment will result in upregulation of phase 2 proteins such as γ-glutamyl-L-cysteine ligase (GCL: the rate limiting enzyme in GSH synthesis), glutathione reductase (GRed) and thioredoxin reductase-1 (TrxR1).

3. Specific objectives

Objective 1. To determine what level of sulforaphane incorporated into the diet would ameliorate development of hypertension in SHRsp? The dose that gave rise to maximal reduction in the increase in BP was used for future studies.

Objective 2. Determine whether dietary sulforaphane would markedly increase cellular GSH in SHRsp rats.

Objective 3. Determine whether dietary sulforaphane would decrease the level of nitrosylated proteins in the kidneys of SHRsp.

Objective 4. Determine whether dietary sulforaphane would increase the expression of the phase 2 enzyme γ-glutamyl-L-cysteine ligase.

Objective 5. Determine whether dietary sulforaphane would increase the phase 2 enzyme GRed expression.

Objective 6. Determine whether dietary sulforaphane would increase the phase 2 enzyme TrxR1 expression.

Objective 7. Determine whether dietary sulforaphane would protect renal artery structure.

4. Materials and methods

4.1 Animal model

A total of 42 4-week-old female rats, including 21 SHRsp and 21 age matched SD rats (end of the 4th week postnatal) –were purchased from Charles River Laboratories (St. Constant, Quebec Canada). The rats were treated in accordance with the guidelines of the Canadian Council on Animal Care, and the experimental protocols were approved by the Animal Care Committee at the University of Saskatchewan. Each two rats were housed together, received standard rat chow and water and kept in a 12 hour light/dark cycle at 25 ºC with controlled humidity. Animals were randomly assigned to experimental groups.

4.2 Animal groups

After 1 week of adaptation, the 4 week old female SHRsp and SD rats were divided into four groups and administered daily by gavage: (i) Corn oil (vehicle) alone (Control, *n*=5); (ii) sulforaphane (5 µmol/kg body weight, *n*=5) in corn oil; (iii) sulforaphane (10 µmol/kg body weight, *n*=5) in corn oil; and (iv) sulforaphane (20 µmol/kg body weight, *n*=6) in corn oil. Systolic BP was determined weekly using a standard tail cuff noninvasive BP measurement system (model 29-SSP; Harvard Apparatus, St. Laurent, QC, Canada). The gavage treatment lasted for 15 weeks. At the end of the treatment period, the animals were anesthetized with isoflurane (3%) and the BP was measured by the external catheter (intra-arterial) method using a BP monitor (MK-2000 instrument; Muromachi Kikai Co., Ltd, Tokyo, Japan). Later, the animals were euthanized and perfused with normal saline, and tissues collected for histology, western blot, gene expression study or GSH measurements.

4.3 BP measurements

4.3.1 Tail cuff BP measurement (non-invasive)

Prior to obtaining a stable baseline BP, the animals were acclimatized to the restraining device and to the sensation of the cuff inflation-deflation cycles for 5 days. To induce vasodilatation, the animals were slightly warmed at 37º C (external air temperature) for 5 min then an average of nine BP readings were obtained from each animal. The cuff-detector unit consisted of an inflatable cuff, light source, and photoelectric pulse detector (IITC Life Science Inc. CA, USA) of the BP monitoring system. On occlusion of the caudal artery, the photoelectric signals that are responsive to changes in blood density disappeared and the occlusion pressure applied to the inflatable cuff was recorded. By recording the disappearance and reappearance of pulse signals in conjunction with measurements of cuff pressure, Systolic BP was determined directly from the recordings (Fig 7).

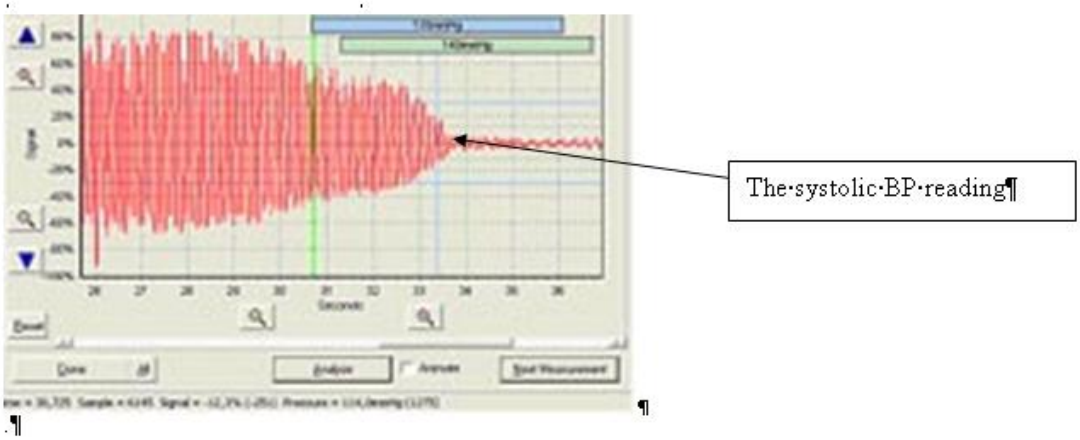


Figure 7 Blood pressure measured using tail cuffs on conscious rats. By optical detection, the pulse flow (pulse signal) will disappear during inflation of the cuff and reappear during deflation of the cuff. The point at which the pulse signal reappears is the systolic BP reading.

4.3.2 External catheter (intra-arterial) BP measurement (invasive):

Rats were anesthetized with isoflurane and prepared for surgery under aseptic conditions. An incision was made in the ventral region of the neck. The left carotid artery was isolated by blunt dissection and ligated at the proximal end. A catheter that had a pressure transducer (Transducer Control Unit, Model TC-510, Texas, and U.S.A) embedded at its tip, was introduced into the blood vessel and tied in place. Pressure fluctuations were measured through the pressure transducer, converted into a varying electrical signal that is amplified and converted into a digital signal by the data acquisition system, Analog Output (BIOPAC System, Inc, CA, U.S.A.). Finally, the cardiovascular parameters from left ventricular pressure signals were recorded and analyzed using Acqknowledge software (Version 3.8.1, BIOPAC Systems, Inc., CA, USA).

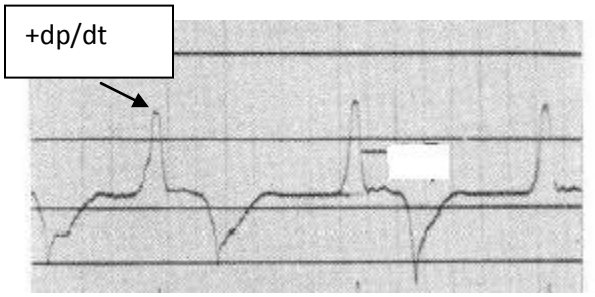
4.3.2.1 Measurement of left ventricular pressure and arterial pressure using the intra-arterial catheter method of BP measurement

The left ventricular pressure parameters were measured through a catheter inserted into the left ventricular chamber and the pressure wave signals of varying frequency were recorded.

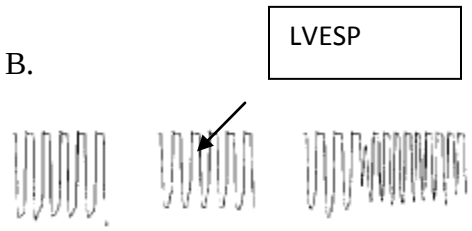
Then the same catheter was slowly withdrawn from the left ventricle chamber and positioned in the aorta and the pressure wave signals of varying frequency were recorded. The slope of ventricular pressure development (+dp/dt) (mmHg/sec) and the left ventricular end systolic pressure (LVESP, mmHg) reflects the signal with high ventricular pressure were shown below.

The arterial pressure parameters such as the systolic blood pressure (SBP, mmHg) and the diastolic blood pressure (DBP, mmHg) were recorded directly from the incoming signals (Fig 8). The pulse pressure (PP, mmHg) was obtained by subtracting the SBP reading from DBP. Mean arterial blood pressure (MAP) was obtained from this equation: DBP+1/3PP, while the heart rate (HR, beat per minute) was obtained from this equation: number of peak/deltaT*60.

A



B.



C.

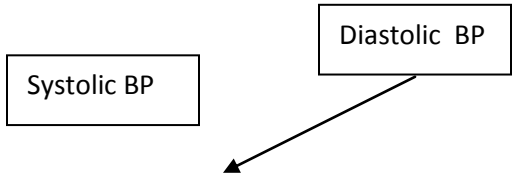


Figure 8 An example of left ventricle pressure and arterial pressure recordings using the intra-arterial catheter method of blood pressure measurement. (A) +dp/dt (mmHg/sec) and –dp/dt (mmHg/sec). (B) LVESP (mmHg), LVEDP (mmHg) and LVDP (mmHg). (C) Systolic and diastolic BP (mmHg).

4.4 Tissue harvesting

Animals were anesthetized with isoflurane, perfused with normal saline and the tissues were collected. The tissues collected were brain, heart, liver, kidney, and intestine, abdominal, thoracic, renal and mesenteric artery. The tissues were rinsed in normal saline, weighed and their volumes (measuring the difference of volumes of water before and after immersing the tissues in water) were taken. Each tissue was sectioned into three segments. After the tissues was rinsed in normal saline, some of the tissues were snap frozen in liquid nitrogen and stored at -80º C until further processing for Western blot, polymerase chain reaction (PCR) and GSH analyses. The remaining tissues were processed for histology as outlined below.

4.4.1 Histology

After the tissues were rinsed in normal saline, the tissues were kept in a solution containing 4 % paraformaldehyde (fixative) in PBS overnight at 4° C. Next day the samples were immersed in 10% formalin and stored at 4°C until further processing using a Tissue-Tek® VIP (Vacuum Infilteration Processor) machine from Miles Scientific.

4.4.1.1 Hematoxylin & eosin (H&E) staining of tissues

This includes tissue fixing, paraffin embedding, and tissue sectioning and staining. (i) After fixation, the tissues were removed from 10% formalin and washed in tap water, to remove excess fixative. (ii) The specimens were embedded in paraffin using a Tissue-Tek® VIP machine from Miles Scientific – this involved the passage of fixed tissues through a series of solutions for specified times: the dehydration steps include the passage of specimen in serial dilutions of alcohol (70%, 80%, 90% and absolute alcohol), clearing the alcohol with xylene (miscible with alcohol and paraffin) and then the specimen was infiltrated with paraffin and prepared for sectioning. (iii) The embedded samples were sectioned at 9 μm thickness (using "820" Microtome, American Optical Company). (iv) The sections were stained using Hematoxylin and Eosin (Ehrlich's H&E) (Clark 1981). Hematoxylin is a base and stains acidic cellular components (mainly DNA and RNA) dark blue. Eosin is an acid and stains basic components, which include most of the cytoplasmic components of the cell, pink. The protocol used in Ehrlich's H&E staining involved the passage of paraffin sections (9 μm thick) through a series of solutions for specified periods of time: these solutions included xylene, to deparaffinize the specimen, absolute alcohol, 95% ethanol, 70% ethanol, and tap water respectively before rinsing in distilled water; this was done to hydrate the sections. The slides were then immersed in Ehrlich's hematoxylin for 3 min and rinsed in tap water, to remove the excess of the stain. They were then dipped 2 times (5-10 seconds) in acid alcohol (0.5% HCl in 95% ethanol), this was a very critical step to remove the excess of hematoxylin where it was not firmly bound, such as in the cytoplasm while it left the hematoxylin in the nucleus where it was resistant to decolorization because it was firmly bound. Too much of acid alcohol will remove the stain from the nucleus. The specimens were washed in tap water (basic) to stop the action of acid alcohol and to turn the hematoxylin blue. Subsequently, they were rinsed in distilled water before being immersed in eosin Y solution (stains the cytoplasm pink) for 1 min. To avoid masking the hematoxylin in the nucleus or overstaining the cytoplasm, they were quickly removed, rinsed again in water and examined under the microscope to make sure that appropriate amount of staining was achieved. When the staining was adequate the slides were dehydrated in graded ethanols and cleared in xylene because the Etellan mounting medium is not water soluble. The slides were cover-slipped with Etellan mounting medium. The slides were then allowed to dry and were then ready for histological examination.

4.4.1.2 Morphometry

Morphometric measurements were done in a blind fashion. For renal artery measurements (wall thickness and smooth muscle cell number), MacBiophotonics version (McMaster University, Canada) of Image J software (NIH, Bethesda) was used. Renal arteries were photographed under x40 lens resolution and captured as TIFF images. For the wall thickness, an average of 3-5 readings from different areas of the renal artery per animal was possible. The renal artery smooth muscle cell number and the average number of cells in five different known areas of the renal arteries per animal were automatically calculated.

4.5 HPLC

High performance liquid chromatography (HPLC) was performed to quantify GSH in tissues using ultraviolet spectrophotometric detection. This method is based on the complete reaction of sulphydryl containing compounds (GSH, cysteine, homocysteine) with 5,5-dithio-bis-nitrobenzoic acid (DTNB), at pH 8.0, to yield DTNB derivatives of cysteine, GSH and homocysteine in tissues (Katrusiak et al. 2001). The sulphydryl-DTNB derivatives were detected by ultraviolet absorbance at 330 nm.

4.5.1 Sample preparation

For protein precipitation, samples were homogenized in 5% sulfosalicylic acid with 0.1 M EDTA. The homogenates were then sonicated on ice and centrifuged at 12000 rpm (15294 g) (Eppendorf Fixed-angle Rotors F-45-30-11(radius=9.5cm) for Centrifuge 5430 R, Eppendorf North America, NY,USA for 20 min at 4°C and the supernatant collected.

4.5.2 Derivatization

The acid supernatant was used for suflhydryl derivatization reaction mixture. The reaction mixture consisted of 0.5 ml Tris buffer (0.5 M, pH 8.9), 130 µl acid supernatant, 20 µl internal standard (penicillamine), 20 µl double distilled water and 350 µl DTNB (10 mM pH 7.2). The mixture was vortexed and allowed to sit for 5 min; to allow DTNB to react with the sulphydry compounds. The reaction mixture was re-acidified by addition of H_3PO_4 (20 µl 7M) then 130 µl from this reaction mixture was injected into the column.

4.5.3 Chromatography

The chromatography of the sulfhydryl-DTNB derivatives was accomplished using isocratic elution (the composition of the mobile phase is unchanged); on a Supelco LC-18 column (3µm particle size, L x I.D. 15 cm x 4.6 mm) at 37º C. The mobile phase consisted of 12.5% methanol, KH_2PO_4 (0.5 M pH 3.89); at a flow rate of 0.9 ml/min. To elute the excess of DTNB reagent, after 10 min of isocratic elution, the methanol concentration was increased to 40% for 7 min. The sulphydryl-DTNB derivatives were detected by ultraviolet absorbance at 330 nanometers (nm) (Hitachi, Ltd. Tokyo Japan).

4.6 SDS-PAGE and Western blotting

Western blot analysis was performed to determine the abundance of the following proteins: gamma-glutamylcysteine synthetase (γ-GCS) (1:1000 - using a rabbit polyclonal antibody, Santa Cruz Biotechnology, CA, USA), thioredoxin1 (TrxR1) (1:500 – using a rabbit polyclonal antibody, Santa Cruz Biotechnology), glutathione reductase (GR) (1:500 - using a rabbit polyclonal antibody, Santa Cruz Biotechnology). The secondary antibody was goat anti-rabbit IgG-Horse Radish Peroxidase (HRP)-conjugate. (1:5000, Santa Cruz Bbiotechnology). β-Actin (1:1000, mouse monoclonal, Santa Cruz Biotechnology) was used as a reference for sample loading. The secondary antibody was goat anti-mouse IgG-HRP-conjugate (1:5000, Santa Cruz Biotechnology).

4.6.1 Sample preparation

A radioimmuno precipitation assay (RIPA) buffer was prepared: this is a lysis buffer used to lyse cells and tissue, for the radio immunopreciptation assay and can be used for Western blotting. This buffer is more denaturing than NP-40 or Triton X-100 lysis buffer because it contains the ionic detergents SDS and sodium deoxycholate as active constituents and is particularly useful for nuclear membrane disruption for nuclear extracts. A protease (enzyme breaks down the proteins) inhibitor cocktail (this cocktail contains a protease inhibitors, such as cysteine protease inhibitors) (1% of the protease inhibitor: Bio Vision, Catalog #K268-50 -4) was added to RIPA buffer. Then the tissues were homogenized in a RIPA buffer containing: Tris

50 mM, NaCl 150 mM, SDS 0.1%, Na deoxycholate 0.5%, and Triton X-100. Homogenates were then sonicated on ice and centrifuged at 12000 rpm for 15 min at 4º C. The protein concentration was determined using the Bicinchoninic acid (BCA) protein assay. This is a very sensitive, simplified, low variability, and one–step analysis for total proteins. The principle of this assay is that the peptide bonds in proteins reduce Cu^{+2} ions (in the stock BCA solution) from cupric sulfate to Cu^{+1} (a temperature dependent reaction), then BCA chelates with Cu^{+1}, forming BCA Cu^{+1} complex (purple in color) that absorbs energy at wavelength of 562 nm. The purple color is directly proportional to the protein concentration (Smith et al. 1985).

4.6.2 SDS-Polyacrylamide Gel Electrophoresis

(i) For each SDS-PAGE gel sandwich one small and one large glass plate were used (Table 1 and2).

(ii) Using a large volume pipetor, 7.5 ml of the resolving (running) gel was slowly poured between the glass plates:

Table 1 SDS-Page Running Gel Recipe for Mini-Protean II System, for 2 gels

	Percentage of acrylamide
Ingredients	7.5%
DD-H_2O(sterile)	9.6 ml
1.5 M Tris-HCL (pH8.8)	5.0 ml
30% acrylamide mix	5.0 ml
10% SDS	200 µl

10% Ammonium persulfate	200 µl
Tetra-methyl-ethylenediamine	10 µl
Total volume	20.0 ml

(iii) 1 ml of tert-amyl alcohol, Reagent Plus, 99% (Sigma-Aldrich, Inc, Steinheim, Germany) was added to the top of the gel and the gel was allowed to polymerize (this takes 25 min.).

(iv) Tert-amyl alcohol was removed by inverting the gel while the residual liquid was wicked off with a Kimwipe®.

(v) 3.5 ml of the stacking gel was slowly poured between the glass plates:

Table 2 SDS-Page Stacking Gel Recipe for Mini-Protean II System (4% acrylamide stacking gel), for 2 gels.

	Percentage of acrylamide
Ingredients	4.0%
DD-H_2O(sterile)	6.1 ml
1.5 M Tris-HCL (pH8.8)	2.5ml
30% acrylamide mix	1.3 ml
10% SDS	100 µl
10% Ammonium persulfate	80 µl
Tetra-methyl-	10 µl

ethylenediamine	
Total volume	10.0 ml

(vi) The comb was inserted into the stacking gel, leaving at least 1 cm distance between the bottom of the comb and the running gel and the gel was allowed to polymerize (this takes about 25 min.).

(vii) Once the stacking gel had finished polymerizing, the comb was removed and the system was ready for loading the samples.

4.6.3 Loading, electrophoresis and transferring of proteins

(i) Prior to resolving the sample by SDS-PAGE, before the protein is denatured by heating (at 95° C for 5 min) and in the presence of the detergent (50 µl β-mercaptoethanol to 950 µl Laemmli Sample Buffer (Tris HCL 62.5 mM, 25% glycerol, 2% SDS and 0.01% bromophenol blue) {BIORAD}, the protein samples are diluted 1:4 in sample buffer, to ensure optimal band resolution.

(ii) The samples (50 µg) were loaded and electrophoresed on a 7.5% reducing SDS-polyacrylamide gel at 100 V for 90 min; the running buffer used:

Ingredients	The quantity
Tris Base (Tris[hydroxymethyl]aminomethane	15 gm
Glycine	72 gm
Sodium sulphate(sodium dodecylsulfate)	5 gm
Double distilled(DD)-H_2O (sterile)	1000 ml

(iii) After electrophoresis, proteins were transferred (at 90 V for 90 min) onto polyvinylidene fluoride (PVDF) membrane (Life Sciences BioTrace, Lawrence, Kansas, USA). The transfer buffer used was made up as follows:

Ingredients	Quantity
Tris Base (Tris[hydroxymethyl]aminomethane	5.85 gm
Glycine	2.93 gm
Sodium sulphate(sodium dodecylsulfate)	0.375 gm
Methanol	200 ml
DD-H_2O (sterile)	800 ml

4.6.4 Immunoblotting

(i) To prevent nonspecific binding, the membranes were blocked in 5% milk (Canada Safeway Limited, Alberta, Canada) (in PBS), overnight at 4° C.

(ii) The membranes were then hybridized in 5% milk, containing γ-GCS, GRed, TrxR1 or β-Actin antibodies overnight at 4°C.

(iii) To remove any unbound primary antibodies, the membranes were then washed for 3 h (in PBS), with changes every 30 min.

(iv) The membranes were then incubated for 2 h in 5% milk, containing either goat anti-rabbit or goat anti-mouse IgG HRP (Santa Cruz biotechnology), at room temperature.

(v) To remove any unbound secondary antibodies, membranes were then washed for 3 h, with changes every 30 min and then proceed immediately for detection.

4.6.5 Chemiluminescent Detection

(i) Using enhanced chemiluminescence, ECL kit (PerkinElmer Life Science, Inc), 2 ml of enhanced luminol reagent and 2 ml of oxidizing reagent were gently swirled for 1 min.

(ii) The membranes were completely covered with the detection reagent, incubated for 1 min and the excess was drained.

(iii) The air bubbles were gently smoothed out; the membranes were placed in a film cassette (Spectronics Corporation Medical Products Division, Western, New York, USA).

(iv) In the dark a sheet of autoradiography film (Kodak BioMax Film; N.Y., USA) was placed on top of the membrane, the cassette was closed and the film was exposed

4.6.6 Reblotting

The Western blot is carried out as usual and after the film exposures are complete the membrane is subjected to the stripping procedure. To do this, the membranes were washed for 4x20 min in PBS followed by incubation for 30 min at 50° C in the stripping buffer. The Tris HCL 0.5 M and membrane stripping buffer used:

Tris HCL 0.5 M, pH 6.8	
Ingredients	Quantity
Tris Base(Tris[hydroxymethyl]aminomethane)	6.0 gm
DD-H_2O(sterile)	200 ml

Membrane stripping buffer	
Ingredients	Quantity
Tris HCL 0.5 M, pH 6.8	31.25 ml

Sodium dodecylsulfate (SDS)	5.0 gm
Beta-mercaptoethanol	1.75 ml
DD-H_2O (sterile), made up to final volume	250 ml

The membranes were then washed for 4x15 min in PBS and new Western blotting started at the blocking step.

4.6.7 Densitometric analysis

The NIH image Software (ImageJ Processing and Analysis in Java, version 1.43) was used to determine the optical densities of the Western blot bands. To do this, films were scanned and opened in NIH image and a dashed box was selected from "Tool" option. To analyze the optical density of each band, it should be surrounded with this box. Then go to "Analyze" and choose "Measure". The optical density of the band will appear on the menu.

4.7 Quantitative polymerase chain reaction (Q.PCR)

4.7.1 RNA isolation

RNA isolation included the following steps: (i) homogenization using phenol and guanidinium thiocyanate in a monophase solution (1 ml per 50 mg tissue), (ii) RNA extraction by chloroform (1 vol. of homogenate + 0.2 vol. of chloroform), (iii) RNA precipitation (0.5 vol. of isopropanol) and(vi) RNA wash with 1 ml of 75% ethanol dissolved in diethylpyrocarbonate (DEPC, 0.1%).

4.7.1.1 Homogenization

Tissue samples (~50 mg) were homogenized in 1 ml of phenol and guanidinium thiocyanate in a monophase solution using a Mikro-Dismembrator S tissue homogenizer. The homogenized suspension was then sonicated (3x10 seconds). Following homogenization, the homogenate was stored for 5 min, to permit the complete dissociation of nucleoprotein complexes.

4.7.1.2 RNA extraction by chloroform

Next, to 1 ml of the phenol and guanidinium thiocyanate in a monophase solution, 0.2 ml of chloroform was added, shaken vigorously for 15 seconds and allowed to stay at room temperature for 2-3 min. To remove cellular debris and large tissue fragments, the homogenate was centrifuged for 15 min at 14,000 rpm at 4°C. The resulting supernatant (the colorless upper aqueous phase contains RNA) was removed, transferred to a new sterile polypropylene centrifuge tube (1.5 ml).

4.7.1.3 RNA precipitation

0.5 ml of isopropanol was added to precipitate total nucleic acids. After centrifugation at 14,000 rpm (20817 g) for 10 min at 4°C, RNA precipitates formed a white pellet at the bottom of the tube.

4.7.1.4 RNA wash

The supernatant was removed, the RNA pellet washed twice with 1 ml of 75% ethanol dissolved in diethylpyrocarbonate, vortexed and subsequently centrifuged at 10,000 rpm (10621g) for 5 min at 4° C. The supernatant was removed and the pellet air-dried in fume hood (~20 min); the pellet was not allowed to dry completely. If the pellet dries completely, it will have low solubility and it becomes more difficult to resuspend properly. The RNA sample was stored at -20ºC.

4.7.2 Determination of RNA Concentration and purity

a. 1 µl RNA sample from the original stock was diluted with 99 µl RNase free water treated with diethylcarbonate (DEPC water) in a 1.5 ml microcentrifuge tube. This gave 100 time dilution of the RNA sample.

b. 100 µl DEPC water was pipetted in a clean cuvette and the absorbance was read at 260 and 280 nm to give blank values.

c. 100 µl of the diluted RNA sample was pipetted into a clean cuvette and the absorbance was read at 260 nm and 280 nm.

d. The formula below was used to determine RNA concentration of the original sample:

[RNA µg/µl]= A260 x 40 x dilution factor / 1000

e. To determine the purity of the RNA sample, the ratio of A260/A280 was calculated. Ratios between 1.7 to 2 represented good RNA preparations.

4.7.3 PCR Reaction

The relative expression of GC, GRed and TrX1) was determined by quantitative real-time reverse transcription-polymerase chain reaction (QRT-PCR) on a Bio-Rad iCycler™ platform (Hercules, CA, USA) with the use of one-step Quantitect SYBR Green RT-PCR kits and reagents. Reactions were performed in a final volume of 25 µl as per Table 3.

Table 3 QRT-PCR master mix reaction components for use on Bio-Rad iCycler

QRT-PCR master mix reaction components for use on Bio-Rad iCycler

Component	Volume/Reaction	Final Concentration
SYBR Green	12.5µl	1x
Left Primer (Primer1)	0.25 µl	50 µm
Right Primer (Primer2)	0.25 µl	50 µm
Template RNA	1 µl	1 µg

(cDNA)		
Double Distilled Water	11 µl	-
Total Volume	25 µl	-

The reactions were quantified following determination of the threshold cycle (C_T; the amplification cycle when PCR products are first detected above baseline fluorescence) and fluorescence was measured from the intercalation of SYBR green dye into the double stranded product after the primer elongation phase . A non template negative control was incorporated into all analysis runs. The fold change of the target was calculated using the $2^{-\Delta C}{}_T$ method, where $\Delta\Delta C_T = (C_{T.\ target} - C_{.Actin})_{TimeX} - (C_{T.\ target} - C_{.Actin})_{Time0.}$ The fold change in gene expression normalized to an endogenous reference gene (β-actin) and relative to the untreated control.

PCR cyclic condition

95°C ..... 30 sec (cDNA strands separate)

55°C ..... 1 min (primers bind to template cDNA) strands)

72°C ..... 30 sec (Taq polymerase extends the strands)

95°C-55°C..... 1 min

(a melt curve analysis)

15°C ..... hold

4.8 Statistical Analysis

All data are expressed as means ± SEM. Statistical significance was tested using Students t test or one –way ANOVA followed by a post hoc analysis: check test of homogeneity of variances was performed. If equal variances were determined, Bonferroni was used while if equal variances were not determined, Tamhanes's T2 was used (using SPSS 14.0 for window). Significance level was set at $P<0.05$.

5. Results

5.1 Effect of sulforaphane on body weight:

The data for the final body weight of SD and SHRsp rats are presented in Fig. 9. In rats on control diet, the initial body weights (g) were significantly higher in SD rats (184.34 ± 3.94) than SHRsp (120.14 ± 3.84) and the final body weight were also significantly higher in SD rats (320.08 ± 11.3) than SHRsp (216.2 ± 1.84). Sulforaphane had no effect on body weights of SD or SHRsp rats.

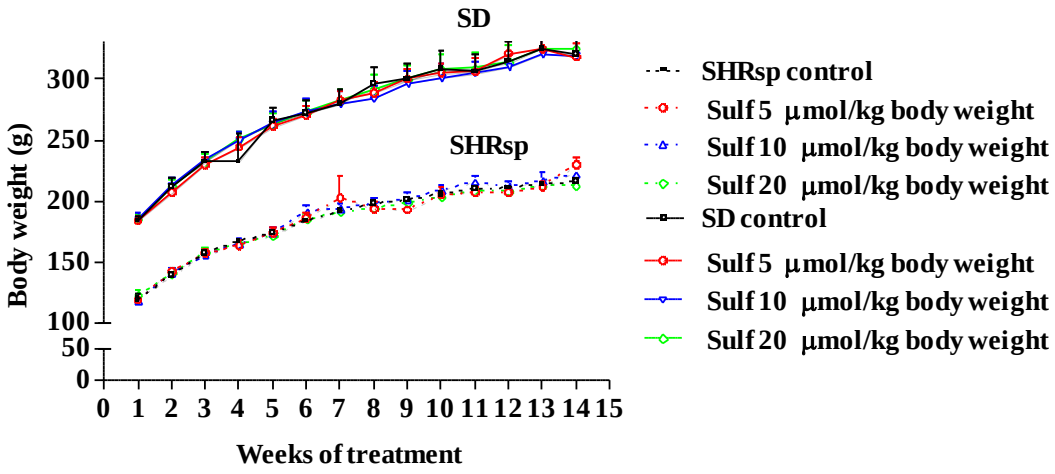


Figure 9 Effect of sulforaphane on body weight. Four-week old female SD and SHRsp ratswere treated daily by gavage corn oil (control) or sulforaphane (5, 10 and 20 µmol/kg body weight) for 15 weeks. During the treatment period, the animals were weighed, *n* = 5-6 rats per group, mean ± SEM.

5.2 Effect of sulforaphane on heart volume and weight

The data for the final heart weight and volume of SD and SHRsp rats are presented in Fig. 10 and 11. In rats on control diet, the weights (g) and the volume (ml) of the hearts in SD rats (0.96 ± 0.04 and 0.91 ± 0.04 respectively) were not significantly higher than that of SHRsp (1.05 ± 0.05 and 1.00 ± 0.03). In addition, sulforaphane had no effect either on heart weights or heart volume of SD or SHRsp rats.

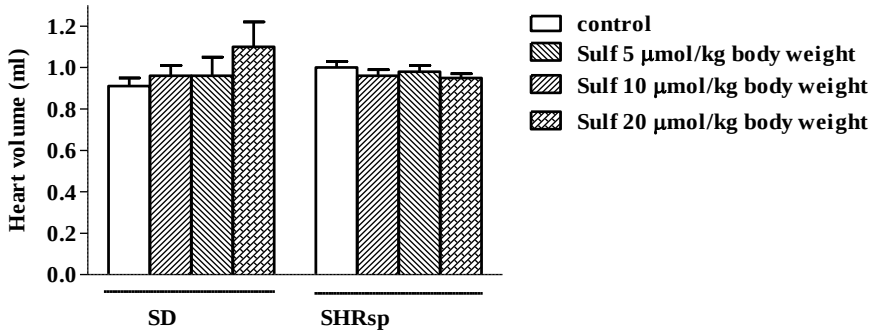


Figure 10 Effect of sulforaphane on heart volume of SHRsp and SD rats.

Four-week old female SD and SHRsp rats were treated daily by gavage corn oil (control) or sulforaphane (5, 10 and 20 μmol/kg body weight) for 15 weeks. At the end of the treatment period, the heart volume of SD or SHRsp rats were measured, *n* = 5-6 rats per group, mean ± SEM.

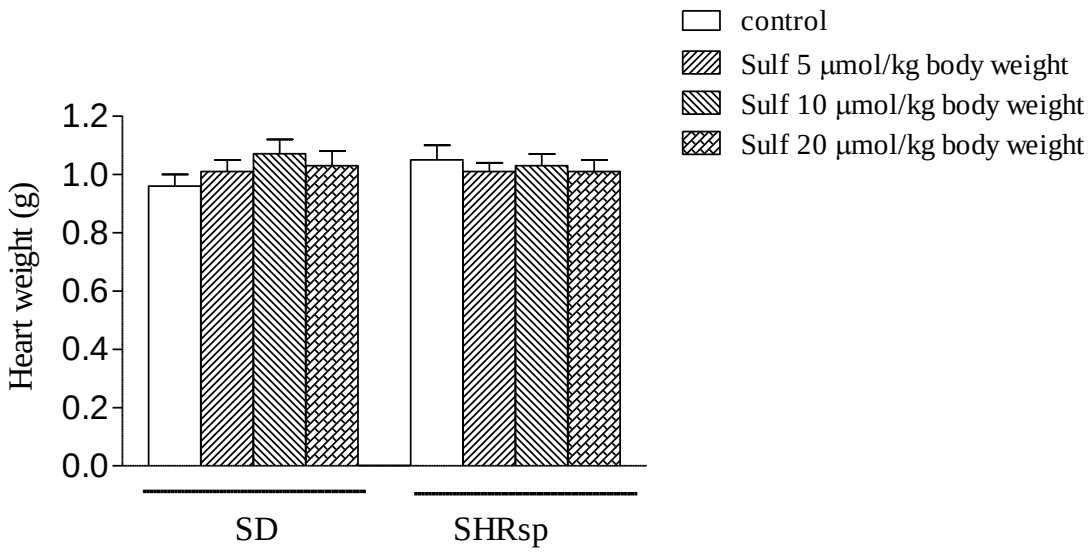


Figure 11 Effect of sulforaphane on heart weight of SHRsp and SD rats.

Four-week old female SD and SHRsp rats were treated daily by gavage corn oil (control) or sulforaphane (5, 10 and 20 μmol/kg body weight) for 15 weeks. At the end of the treatment period, the heart of SHRsp and SD rats were weighed, *n* = 5-6 rats per group, mean ± SEM.

5.3 Effect of sulforaphane on kidneys weight and volume

The data for the final kidney weights and volumes of SD and SHRsp rats are presented in Fig. 12. In rats on control diet, the weights (g) and the volume of the kidneys in SD rats (1 ± 0.06 and 0.94 ± 0.06 respectively) were not significantly higher than that of SHRsp (0.97 ± 0.03 and 0.94 ± 0.02 respectively). In addition, sulforaphane had no effect either on kidneys weights or volume of SD or SHRsp rats.

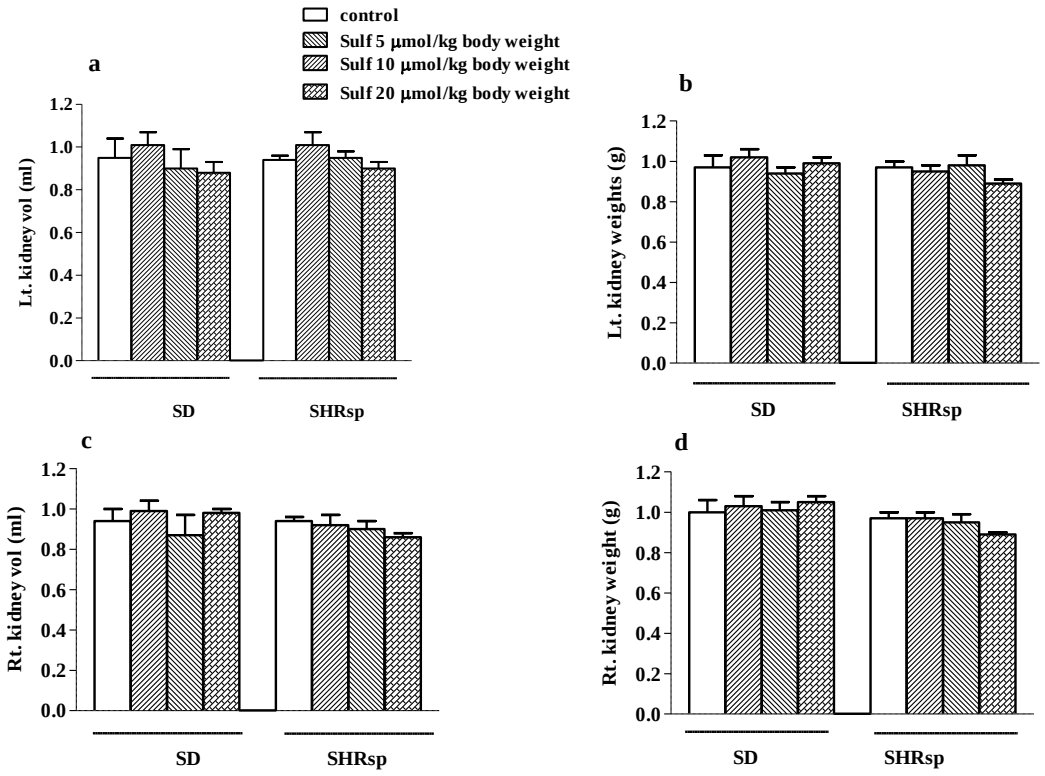


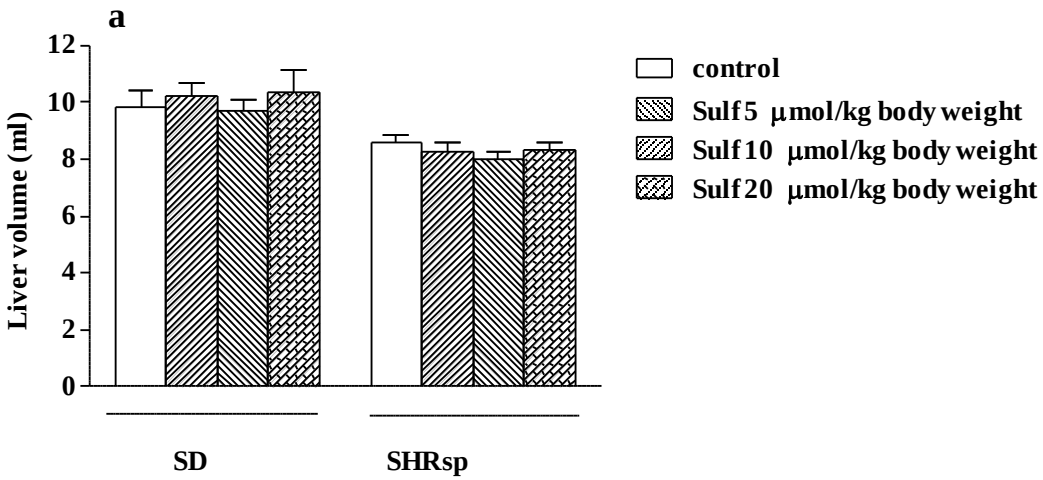
Figure 12 Effect of sulforaphane on kidneys weight and volume of SHRsp and SD rats,

(a) left kidney volume, (b) left kidney weight, (c) right kidney volume and (d) right kidney weight. Four-week old female SD and SHRsp rats were treated daily by gavage corn oil (control) or sulforaphane (5, 10 and 20 µmol/kg body weight) for 15 weeks. At the end of the treatment period, the kidneys weight and volume of SHRsp and SD rats were measured, *n* = 5-6 rats per group, mean ± SEM.

5.4 Effect of sulforaphane on liver weight and volume

The data for the final liver weights and volumes of SD and SHRsp rats are presented in Fig. 13. In rats on control diet, the weights (g) and the volume of the kidneys in SD rats were

10.46 ± 0.53 and 9.82 ± 0.57, respectively, while in SHRsp, the weight and volume were 9.02 ± 0.25 and 8.6 ± 0.24, respectively. The liver weights and volume of SD and SHRsp rats are not significantly different (*P*=0.059). In addition, sulforaphane had no effect either on liver weights or volume of SD or SHRsp rats.



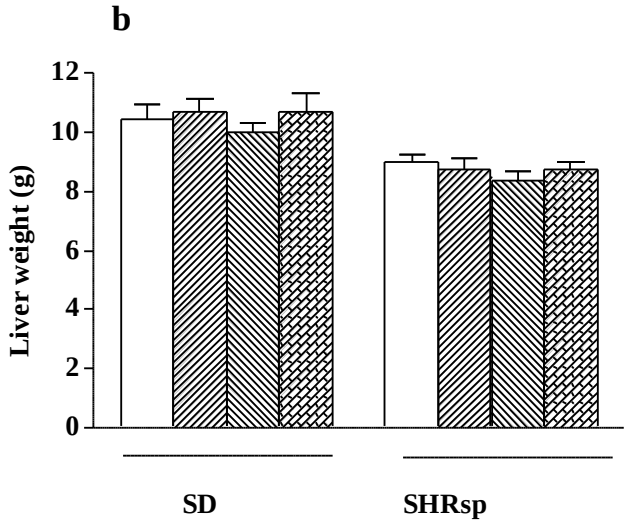


Figure 13 Effect of sulforaphane on liver weight and volume of SHRsp and SD rats, (a) liver volume, (b) liver weight, (c). Four-week old female SD and SHRsp rats were treated daily by gavage corn oil (control) or sulforaphane (5, 10 and 20 μmol/kg body weight) for 15 weeks. At the end of the treatment period, the liver weight and volume of SHRsp and SD rats were measured, n = 5-6 rats per group, mean ± SEM.

5.5 Effect of sulforaphane on brain weight

The data for the final brain weight of SD and SHRsp rats are presented in Fig. 14. In rats on control diet, the average weight (g) of the brains in SHRsp was 1.29 ± 0.03) while in SD rats, the average weight was 1.47 ± 0.03. The final brain weight of SD and SHRsp rats are significantly different (P = 0.0457). In addition, sulforaphane had no effect either on brain weights of SD or SHRsp rats.

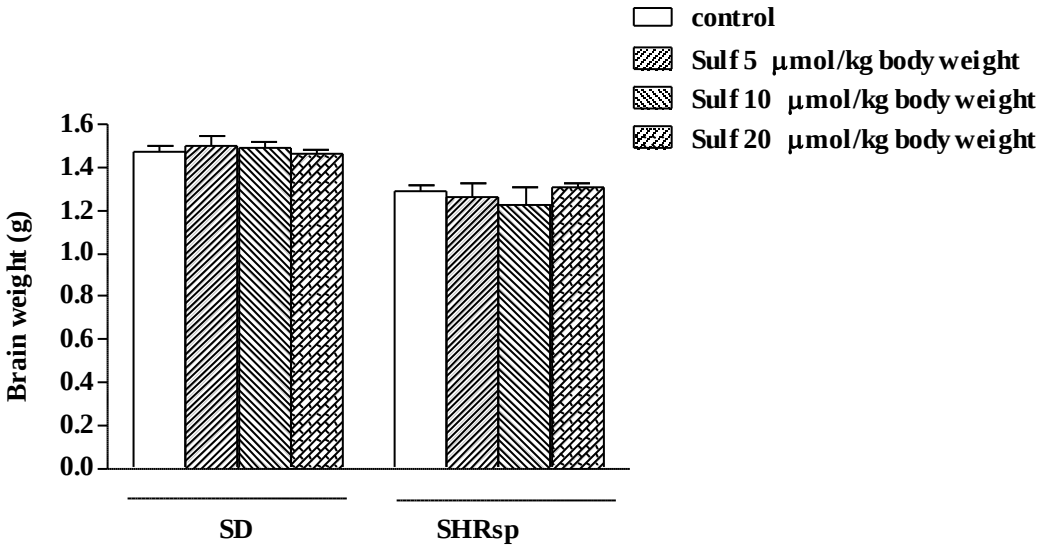


Figure 14 Effect of sulforaphane on brain weight of SHRsp and SD rats.Four-week old female SD and SHRsp rats were treated daily by gavage corn oil (control) or sulforaphane (5, 10 and 20

μmol/kg body weight) for 15 weeks. At the end of the treatment period, the brains of SHRsp and SD rats were weighed, *n* = 5-6 rats per group, mean ± SEM.

5.6 Effect of sulforaphane on systolic BP (measured by tail-cuff)

At the beginning of the experiment, for the SD controls, the systolic blood pressure was over 100 mm Hg. SD fed sulforaphane and the controls have an increased systolic blood pressure at the beginning of the experiment. This increase in systolic blood pressure seen in controls may be because the animals are still in restrain, cuff inflation, cuff deflation and heating training (i.e., using animals heating unit) for the noninvasive systolic blood pressure measurements. Over the course of the experiment, the systolic blood pressure was significantly ($P<0.05$) lower in SD rats (83.98 ± 4.3 mmHg compared with SHRsp (179.9 ± 4.3 mm Hg) (Fig. 16). In SHRsp, the systolic blood pressure was 117.67 ± 2.6 mm Hg at the beginning of the intervention (Fig. 15). Then the systolic blood pressure increased to 179.91 ± 4.3 mm Hg at the end of the study. In contrast, blood pressures of sulforaphane treated SHRsp rats were significantly lower by 22-43 mm Hg by the end of the study.

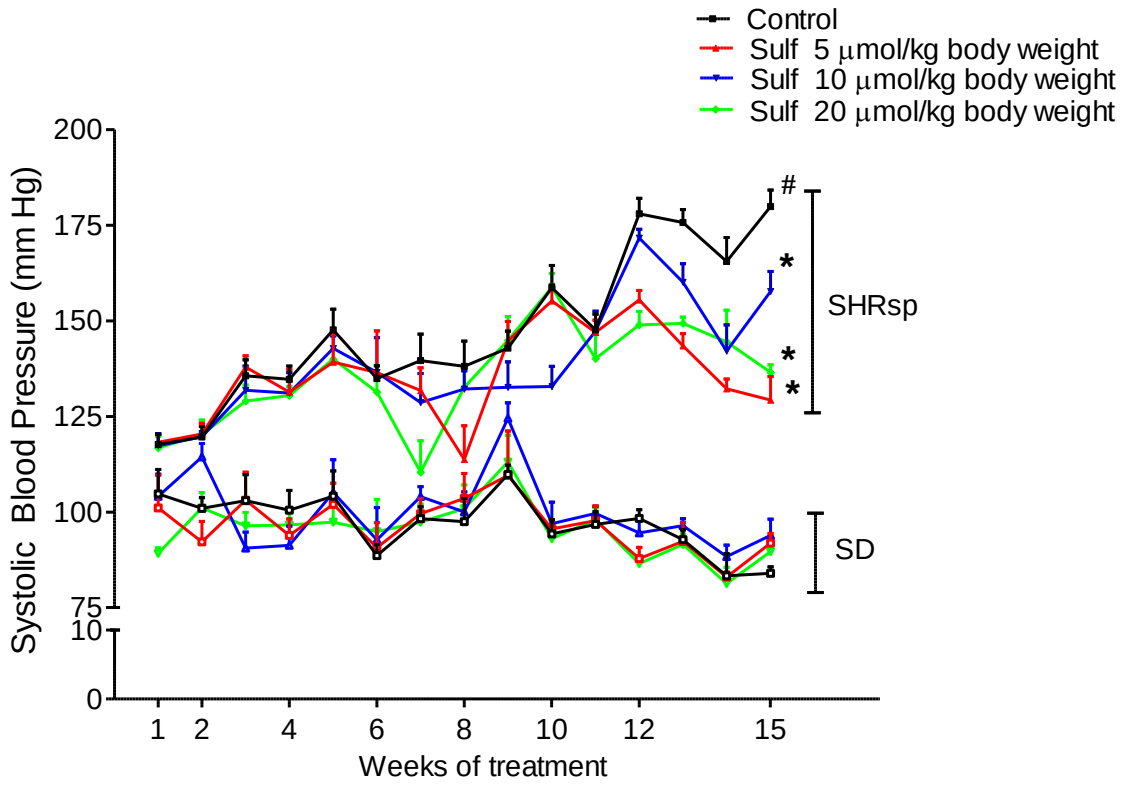


Figure 15 Effect of sulforaphane on systolic blood pressure (measured by tail cuff) in SHRsp and SD rats. Four-week old female SD and SHRsp rats were treated daily by gavage corn oil (control) or sulforaphane (5, 10 and 20 μmol/kg body weight) for 15 weeks. At the end of the treatment period, the systolic blood pressure in SHRsp and SD rats was measured, $^{\#}P<0.05$ *vs*. the control of the SD rats, $^{*}P<0.05$ *vs*. the control of the SHRsp rats, n = 5-6 rats per group, mean ± SEM.

5.7 Effect of sulforaphane on diastolic BP (DP) (measured by intra-arterial catheter method)

The data for the DP of SD and SHRsp rats are presented in Fig. 16. Rats on control diet, the DP in SD rats was 82.9 ± 5.9 mm Hg while in SHRsp the DP was 106.48 ± 2.44 mm Hg. In SD rats, DP was significantly lower compared with SHRsp. In addition, sulforaphane significantly lowered DP (87.64 - 91.7 mm Hg) in SHRsp rats compared with SD rats (82.9 ± 5.9 mm Hg).

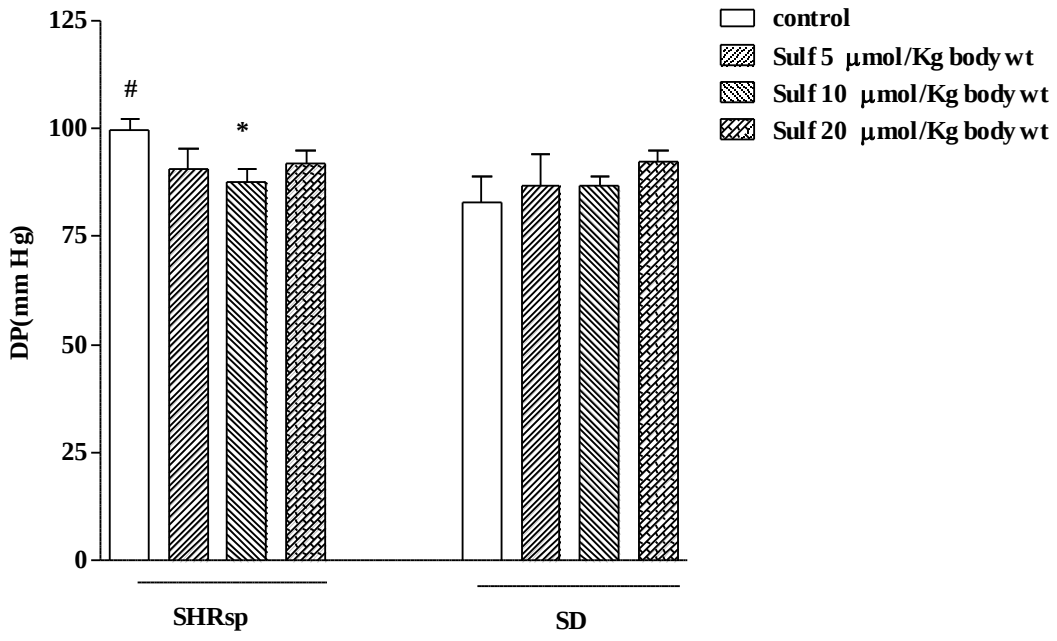


Figure 16 Effect of sulforaphane diastolic blood pressure (DP) (mm Hg) of SHRsp and SD rats. Four-week old female SD and SHRsp rats were treated daily by gavage corn oil (control) or sulforaphane (5, 10 and 20 μmol/kg body weight) for 15 weeks. At the end of the treatment period, the DP in SHRsp and SD rats was measured, $^{*}P<0.05$ *vs*. control of SHRsp rats, $^{\#}P<0.05$ *vs*. control of SD rats, n = 5-6 rats per group, mean ± SEM.

5.8 Effect of sulforaphane on mean arterial BP (MAP) (measured by intra-arterial catheter method)

The data for the MAP (MAP=DP+1/3(SP-DP) of SD and SHRsp rats are presented in Fig. 17. Rats on control diet, the MAP in SD rats were 95.1 ± 5.6 mm Hg while in SHRsp; the MAP was 116.89 ± 2.28 mm Hg. In SD rats, MAP was significantly lower ($P<0.05$) compared with SHRsp. In addition, sulforaphane significantly ($P<0.05$) lowered MAP (101 - 105 mm Hg) in SHRsp rats compared with SHRsp on control diet.

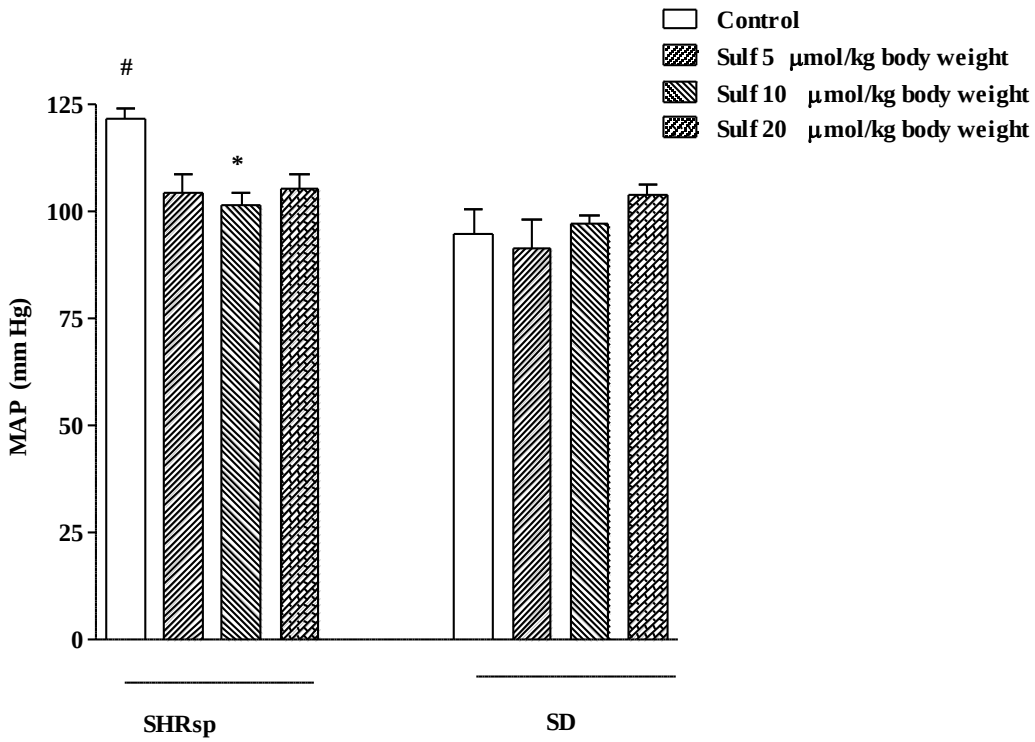


Figure 17 Effect of sulforaphane in mean arterial blood pressure (mm Hg) (measured by intra-arterial catheter method) of SHRsp and SD rats. Four-week old female SD and SHRsp rats were treated daily by gavage corn oil (control) or sulforaphane (5, 10 and 20 μmol/kg body weight) for 15 weeks. At the end of the treatment period, the mean arterial blood pressure (mm Hg) in SHRsp and SD rats was measured, $^{*}P<0.05$ vs. control of SHRsp rats, $^{\#}P<0.05$ *vs*. control of SD rats, n = 5-6 rats per group, mean ± SEM.

5.9 Effect of sulforaphane on Systolic BP (SBP) (measured by intra-arterial catheter method)

The data for the SBP of SD and SHRsp rats are presented in Fig. 18. The SBP in SD rats on control diet was 119.5 ± 5.7 mm Hg while in SHRsp, the SBP was 144.27 ± 2.58 mm Hg. In SD rats, SBP was significantly lower compared with SHRsp. In addition, sulforaphane treatment resulted in a lower SBP (128 - 132 mm Hg) in SHRsp rats compared with SHRsp on control diet.

Unlike the SBP in SHRsp, the SBP in normotensive SD rats remained constant during the experimental period.

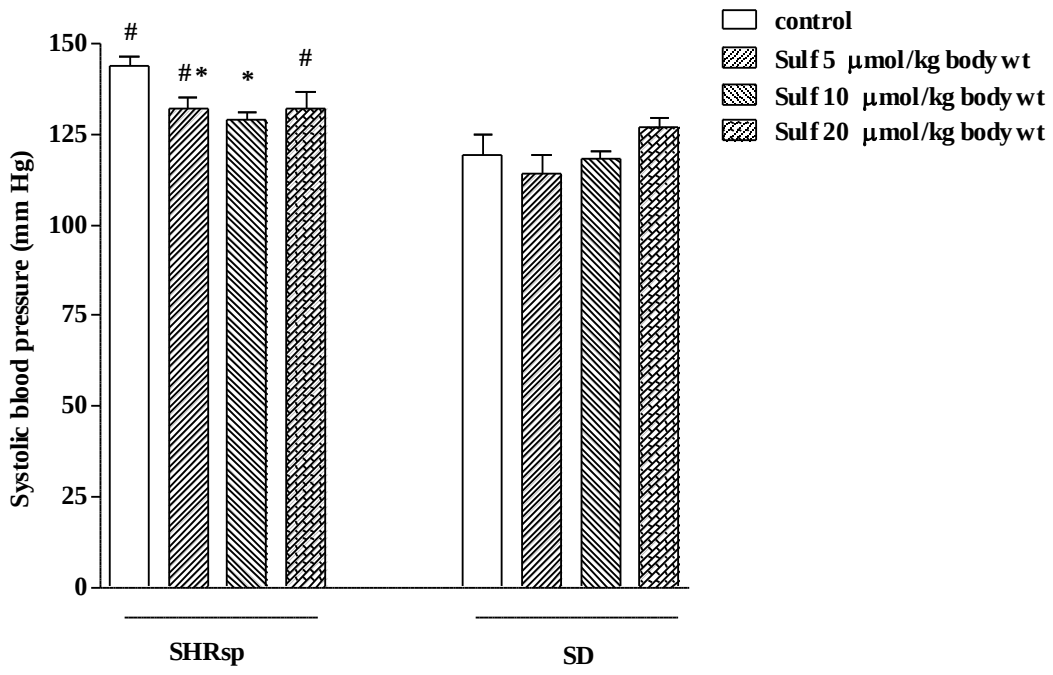


Figure 18 Effect of sulforaphane in systolic blood pressure (mm Hg) of SHRsp and SD rats. Four-week old female SD and SHRsp rats were treated daily by gavage corn oil (control) or sulforaphane (5, 10 and 20 µmol/kg body weight) for 15 weeks. At the end of the treatment period, the systolic blood pressure (mm Hg) of SHRsp and SD rats was meaured, **P*<0.05 *vs*. control of SHRsp rats, $^{\#}P$<0.05 *vs*. control of SD rats, *n* = 5-6 rats per group, mean ± SEM.

5.10 Effect of sulforaphane on pulse pressure (PP)

The pulse pressure is defined as the difference between the systolic and diastolic arterial pressures. The data for the PP of SD and SHRsp rats are presented in Fig. 19. For rats on control diet, the PP in SD rats was 36.7 ± 3.6 mm Hg while in SHRsp; the PP was 41.38 ± 1.95 mm Hg.

In SD rats, PP was lower compared with SHRsp. In contrast, sulforaphane did not affect PP (41.5±2.0 mm Hg) in SHRsp rats compared with SHRsp on control diet (41.38 ± 1.95 mm Hg).Similarly, compared with SD rats on control diet (36.7±3.6 mm Hg), the PP of the SD rats fed sulforaphane was 33.9±2.7, 31.7 ±1.4 and 34.9 ±0.4 on the 5,10 20 μmol/kg animal groups.

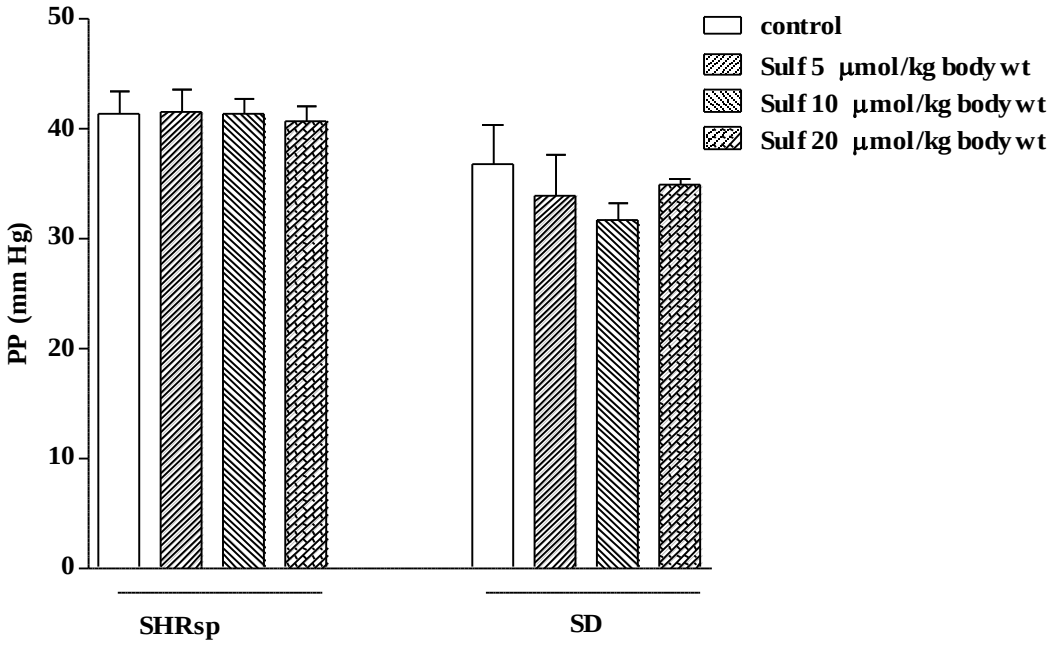


Figure 19 Effect of sulforaphane in PP (mm Hg) (PP = SBP-DBP) of SHRsp and SD rats. Four-week old female SD and SHRsp rats were treated daily by gavage corn oil (control) or sulforaphane (5, 10 and 20 μmol/kg body weight) for 15 weeks. At the end of the treatment period, the PP (mm Hg) of SHRsp and SD rats was meaured, *n* = 5-6 rats per group, mean ± SEM.

5.11 Effect of sulforaphane on heart rate (beats/min; bpm) in anesthetized rats

The data for the heart rate (HR) of SD and SHRsp female rats are presented in Fig. 20. In rats on control diet, the HR in SD female rats is significantly ($P < 0.05$) higher than those of

SHRsp female rats. The HR of SD and SHRsp female rats was not affected by sulforaphane administration.

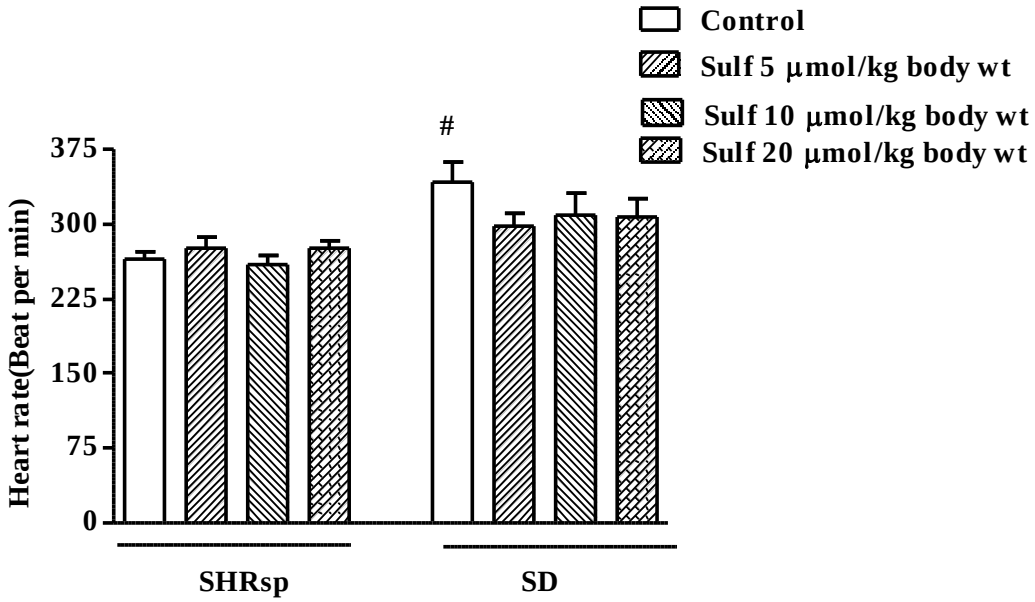


Figure 20 Effect of sulforaphane on the heart rate (beats/min; bpm) of female SD and SHRsp. Four-week old female SD and SHRsp rats were treated daily by gavage corn oil (control) or sulforaphane (5, 10 and 20 μmol/kg body weight) for 15 weeks. At the end of the treatment period, the heart rate of SHRsp and SD rats were measured, $^{\#}P<0.05$ *vs*. control of SHRsp rats, *n* = 5-6 rats per group, mean ± SEM.

5.12 Effect of sulforaphane on left ventricular (LV) end systolic pressure (LVESP) in anesthetized rats

One of the left ventricle function parameters is LVESP. In normal conditions such as in SD rats on control diet, the left ventricle pumps the oxygenated blood to the body after the ventricle contraction with no increase in LVESP. However, in pathological conditions, for instance in SHRsp rats on control diet, the increase in contractility may increase the LVESP (i.e., higher than SP). Moreover, the left ventricles of these hypertensive rats pump the oxygenated blood to the body but with more resistance than in SD. The data for the LVESP of SD and SHRsp female rats are presented in Fig. 21. In rats on control diet, the LVESP in SD female rats were significantly ($P < 0.05$) lower than those of SHRsp female rats. In contrast, while sulforaphane did not affect LVESP of SD, it significantly ($^{\#}P<0.05$) lowered LVESP of SHRsp female rats.

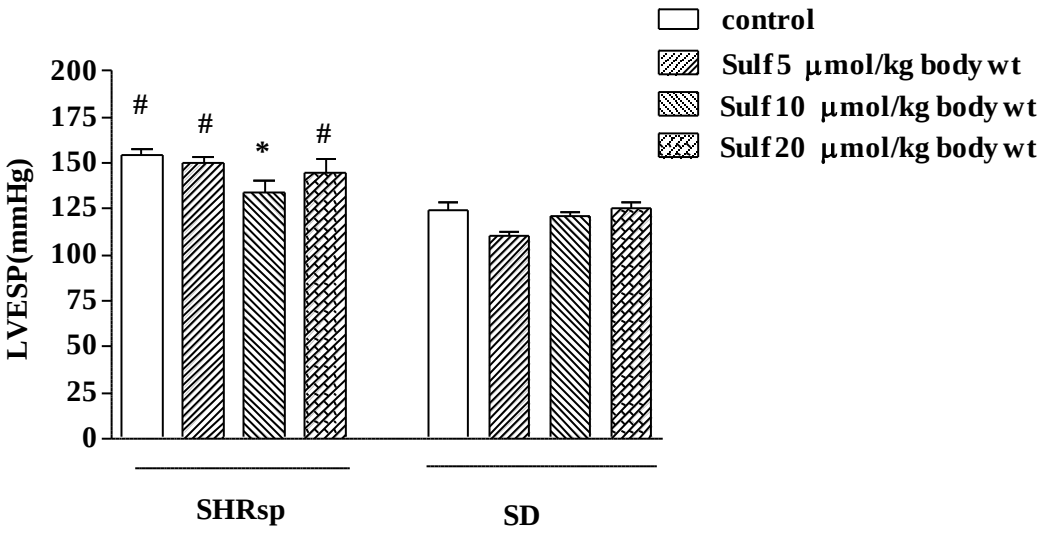


Figure 21 Effect of sulforaphane on the LVESP of female SD and SHRsp rats. Four-week old female SD and SHRsp rats were treated daily by gavage corn oil (control) or sulforaphane (5, 10 and 20 μmol/kg body weight) for 15 weeks. At the end of the treatment period, the LVESP of SHRsp and SD rats was measured, $^{*}P<0.05$ *vs*. control of SHRsp rats, $^{\#}P<0.05$ *vs*. control of SD rats. Each group contained 6 rats, mean ± SEM.

5.13 Effect of sulforaphane on left ventricular contraction (+dp/dt, mmHg/min) in anesthetized SD and SHRsp rats

Fig. 22 includes data concerning the effect of chronic sulforaphane administration on SD and SHRsp female rats. Consistent with the rate of left ventricular relaxation, the rate of left ventricular contraction may be used as an index or an estimate of left ventricular performance- how slow or fast the cardiac muscle contracts. In SD rats +dp/dt was not different from those in control SHRsp rats. In a normally functioning SD rats on control diet, the rate of cardiac muscle contraction was 8603.06 ± 1017 mmHg/min while in hypertensive rats maintained on control diet for 15 weeks, the rate of cardiac muscle contraction of the left ventricular cardiac muscle was 91569 ± 389.7 mmHg/min. Chronic sulforaphane administration did not affect the ventricular contraction in either SD or SHRsp rats with one exception, i.e., sulforaphane at the 10 μmol/kg dose in SHRsp rats decreased the rate of ventricular relaxation (8220.64 ± 578.72).

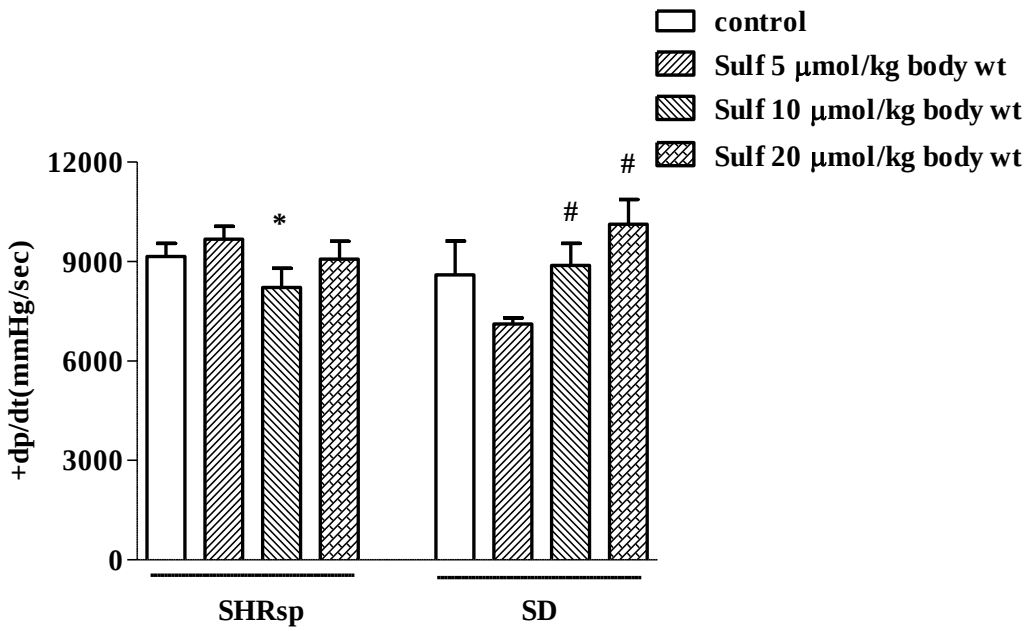


Figure 22 Effect of sulforaphane in rate of contraction of left ventricle (+dp/dt) (mm Hg/ sec) of SHRsp and SD rats. Four-week old female SD and SHRsp rats were treated daily by gavage corn oil (control) or sulforaphane (5, 10 and 20 μmol/kg body weight) for 15 weeks. At the end of the treatment period, rate of contraction of left ventricle (+dp/dt) (mm Hg/ sec) of SHRsp and SD rats was measured, **P*<0.05 vs. control of SHRsp rats, $^{\#}P$<0.05 vs. control of SD rats, *n* = 5-6 rats per group, mean ± SEM.

5.14 Effect of chronic administration of sulforaphane on glutathione levels (nmol/mg protein) in different tissues of SD and SHRsp rats

5.14.1 GSH levels in liver

In rats on control diet, the basal levels of GSH in livers were lower in SHRsp (261.7 ± 20.4) than in SD (315.1 ± 21.3 nmol/mg protein) rats (Fig. 23). In SHR, long term sulforaphane administration, in 5 and 10 μmol/kg increased GSH levels while the highest dose (20 μmol/kg) decreased GSH levels. The lower GSH in 20 μmol dose in SHRsp was not significantly different from that of 10 μmol dose. In SD rats, with the exception to the 10 μmol/kg group, the GSH level constant. The GSH level decreased observed with this group may be due to feeding pattern or diurnal rhythmic effect.

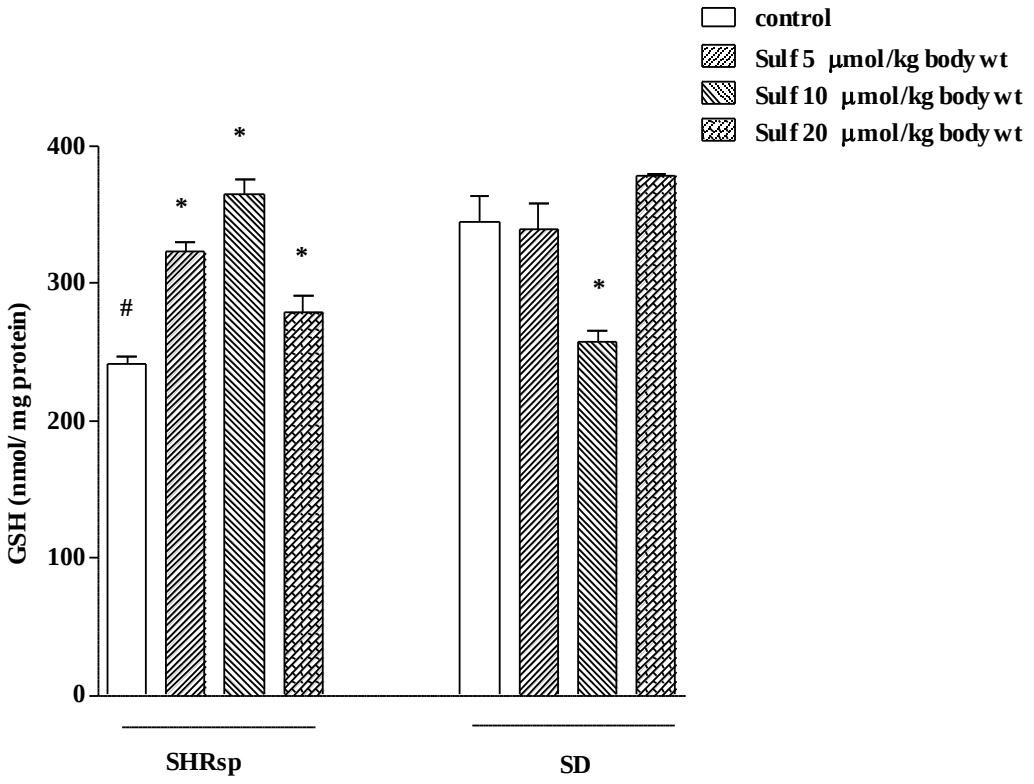


Figure 23 Effect of sulforaphane on glutathione levels (nmol/mg protein) in the liver of SHRsp and SD rats. Four-week old female SD and SHRsp rats were treated daily by gavage corn oil (control) or sulforaphane (5, 10 and 20 μmol/kg body weight) for 15 weeks. At the end of the treatment period, the livers were analyzed for GSH levels.*P<0.05 *vs*. control of same age and strain. Each group contained 3 rats, except with 20 μmol/kg dose in SD rats (n = 2). $^{\#}P$<0.05 *vs*. control of SD rats, mean ± SEM.

5.14.2 Cysteine levels (nmol/mg protein) in liver

In rats on control diet, the basal levels of cysteine in the liver were not significantly lower in SHRsp (8.52 ± 0.71) than in SD (10.53 ± 1.95) rats (Fig. 24). In SHR, long term sulforaphane administration increased cysteine levels significantly. In normotensive SD rats, with one exception, i.e., sulforaphane at the 20 μmol/kg dose in SD rats (n = 2), long term administration of sulforaphane did not affect the liver cysteine level in SD rats.

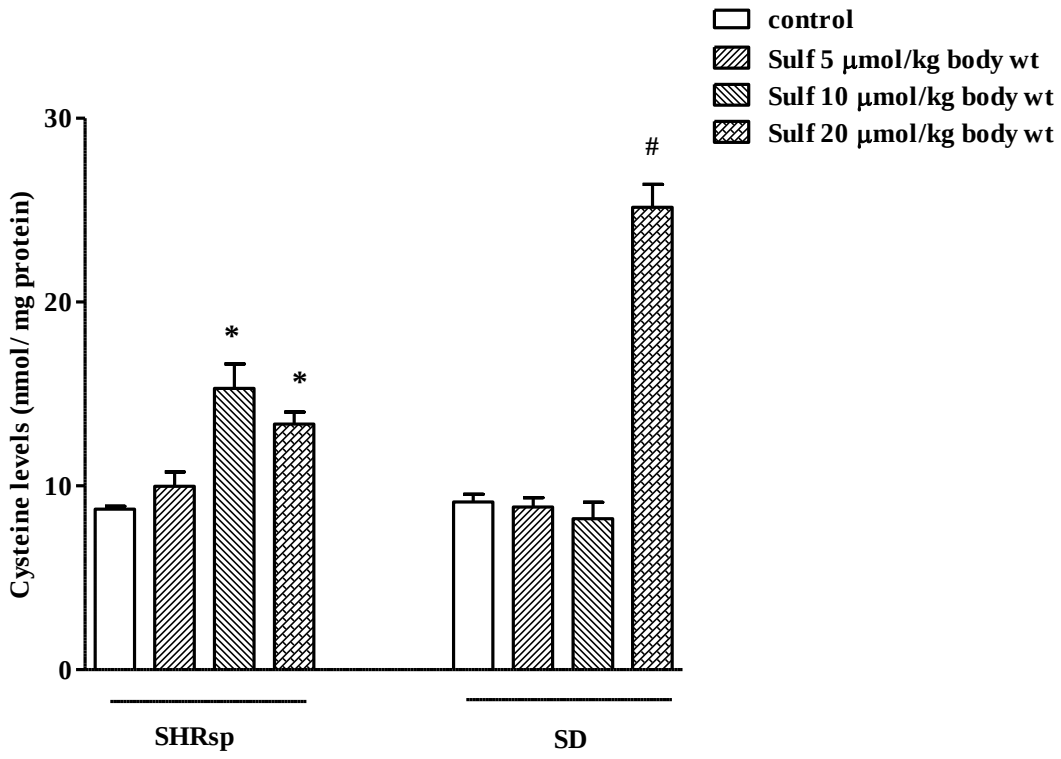


Figure 24 Effect of sulforaphane on cysteine levels (n moles/ mg protein) in liver of SHRsp and SD rats. Four-week old female SD and SHRsp rats were treated daily by gavage corn oil (control) or sulforaphane (5, 10 and 20 μmol/kg body weight) for 15 weeks. At the end of the treatment period, the livers were analyzed for cysteine levels. Each group contained 3 rats, except with 20 μmol/kg dose in SD rats (n = 2). *P<0.05 *vs*. control of SHRsp rats, $^{\#}P$<0.05 *vs*. control of SD rats, mean ± SEM.

5.14.3 GSH levels (nmol/mg protein) in heart

In rats on control diet, the basal levels of GSH in heart were not significantly lower in SHRsp (53.1 ± 11.4) than in SD (66.56 ± 6.04) rats (Fig. 25). In SHRsp, long-term sulforaphane administration not significantly upregulated GSH levels, in these analyses a sulforaphane dose of 10 μmol/kg was used.

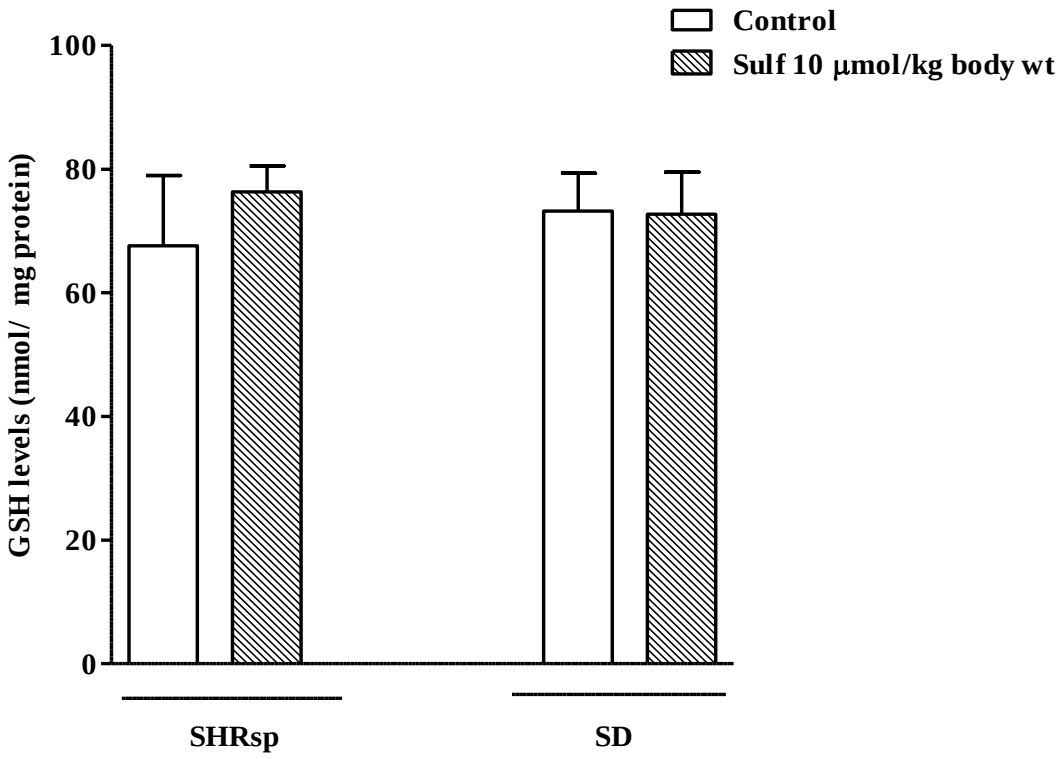


Figure 25 Effect of sulforaphane on glutathione levels (nmol/mg protein) in heart of SHRsp and SD rats. Four-week old female SD and SHRsp rats were treated daily by gavage corn oil (control) or sulforaphane (10 μmol/kg body weight) for 15 weeks. At the end of the treatment period, the heart of SHRsp and SD rats were analyzed for GSH levels, *n* = 5-6 rats per group, mean ± SEM.

5.14.4 GSH levels (nmol/mg protein) in the abdominal aorta

In rats on control diet, the basal levels of GSH in the abdominal aorta were not significantly lower in SHRsp (34.82 ± 3.17) than in SD (42.34 ± 8.18) rats (Fig. 26). In SHRsp, long term sulforaphane administration increased GSH levels, but it was not significant.

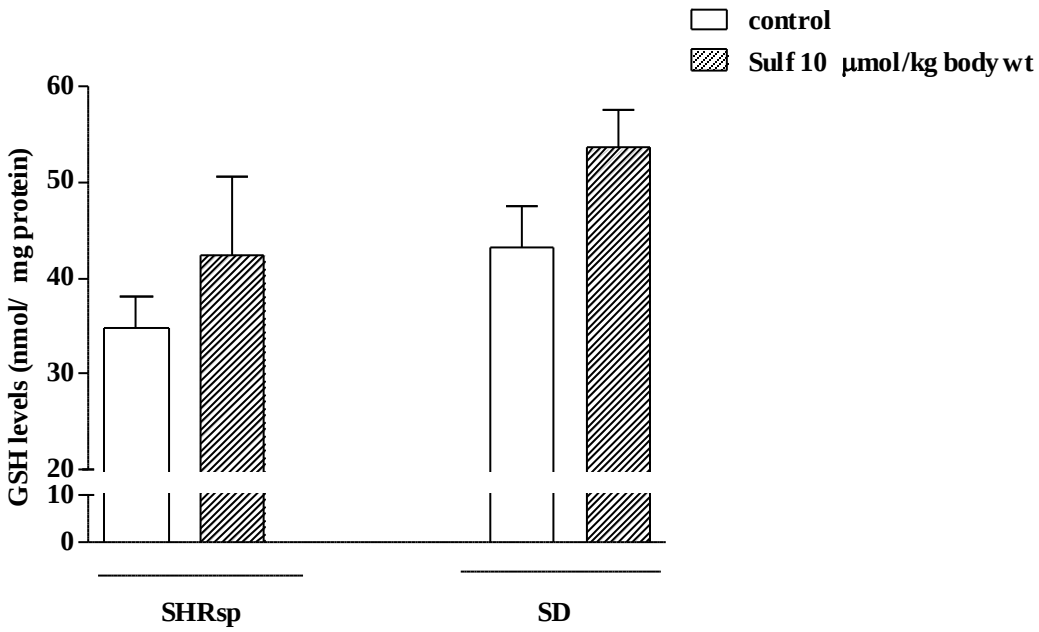


Figure 26 Effect of sulforaphane on glutathione levels (nmol/mg protein) in abdominal aorta of SHRsp and SD rats. Four-week old female SD and SHRsp rats were treated daily by gavage corn oil (control) or sulforaphane (10 μmol/kg body weight) for 15 weeks. At the end of the treatment period, the abdominal aorta of SHRsp and SD rats were analyzed for GSH levels. Each group contained 5 rats, mean ± SEM.

5.14.5 GSH levels in kidney

In rats on control diet, the basal levels of GSH in kidneys were significantly lower in SHRsp (11.83 ± 0.79) than in SD (15.25 ± 2.45) rats (Fig. 27). In SHR, long term sulforaphane administration non-significantly increased GSH levels.

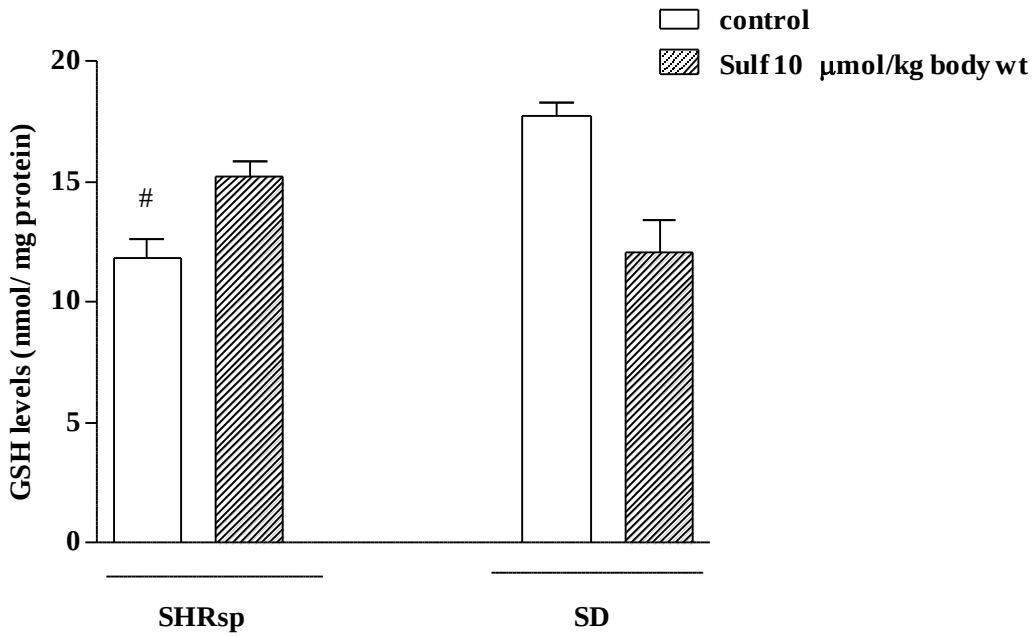


Figure 27 Effect of sulforaphane on glutathione levels (nmol/ mg protein) in kidneys of SHRsp and SD rats. Four-week old female SD and SHRsp rats were treated daily by gavage corn oil (control) or sulforaphane (10 μmol/kg body weight) for 15 weeks. At the end of the treatment period, the kidneys of SHRsp and SD rats were analyzed for GSH levels, $^{\#}P<0.05$ *vs.* control of SD rats. Each group contained 5 rats, mean ± SEM.

5.15 Effect of chronic administration of sulforaphane on γ-GCS (or GCL) protein in liver of SD and SHRsp rats, the dose of 10 μmol/kg was used: Immunoblotting of protein

5.15.1 γ -GCS in liver

In rats on control diet, the basal levels of γ-GCS in the liver were not significantly lower in SHRsp [3.12 ± 1.29 arbitrary unit (AU)] than in SD [8.15± 0.51 (AU)] rats (Fig. 28). In SHR and in SD rats, long term sulforaphane administration significantly increased γ-GCS in SD (P<0.05) while not significantly increased in SHRsp when compared with the same strain control.

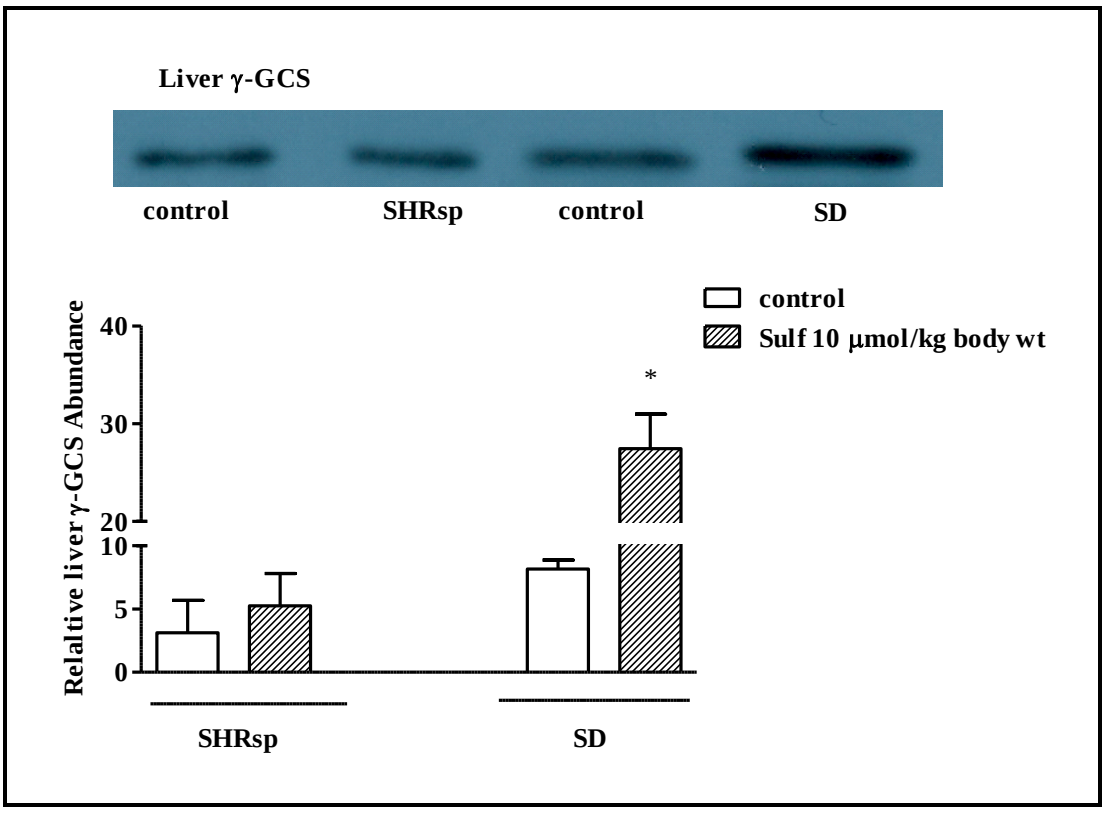


Figure 28 Effect of sulforaphane on γ-GCS in livers of SHRsp and SD rats. Four-week old female SD and SHRsp rats were treated daily by gavage corn oil (control) or sulforaphane (10 μmol/kg body weight) for 15 weeks. At the end of the treatment period, the livers of SHRsp and SD rats were analyzed for γ-GCS levels, **P*<0.05 *vs*. control of SD rats, mean ± SEM. Each group contained 4 rats.

5.16 Effect of chronic administration of sulforaphane on γ-GCS (or GCL) protein in kidney of SD and SHRsp rats

5.16.1 γ-GCS in Kidney

In rats on control diet, the basal levels of γ-GCS in kidneys were significantly lower ($^{\#}P$<0.05) in SHRsp [0.36 ± 0.05(AU)] than that in SD [0.78± 0.13(AU)] rats (Fig. 29). In SHRsp rats, long term sulforaphane administration significantly increased γ-GCS [0.93. ± 0.07 (AU)] when compared with SHRsp control while it did not produce any significant effect in SD rats.

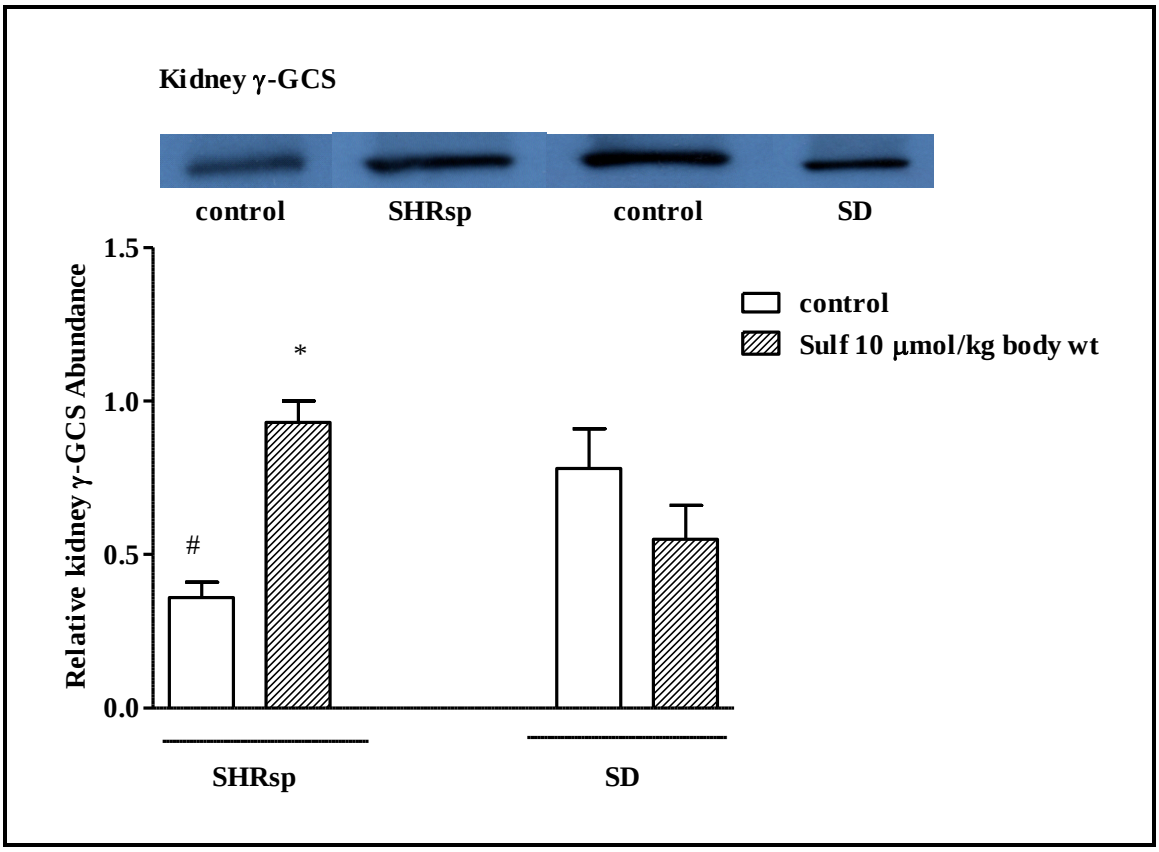


Figure 29 Effect of sulforaphane on γ-GCS in kidneys of SHRsp and SD rats. Four-week old female SD and SHRsp rats were treated daily by gavage corn oil (control) or sulforaphane (10 μmol/kg body weight) for 15 weeks. At the end of the treatment period, the kidneys of SHRsp and SD rats were analyzed for γ-GCS levels, $^{*}P<0.05$ *vs*. control of SHRsp rats, $^{\#}P<0.05$ *vs*. control of SD rats, mean ± SEM. Each group contained 3 rats.

5.17 Effect of chronic administration of sulforaphane on GRed protein in kidney of SD and SHRsp rats. The dose of 10 μmol/kg was used: Immunoblotting of protein

5.17.1 GRed in kidney

In rats on control diet, the basal levels of GRed in kidneys were significantly lower in SHRsp (0.87 ± 0.24 AU) than in SD (2.1± 0.05 AU) rats (Fig. 30). Long term sulforaphane administration significantly decreased GRed in normotensive sulforaphane treated SD rats while sulforaphane nonsignificantly increased GRed protein in hypertensive SHRsp rats.

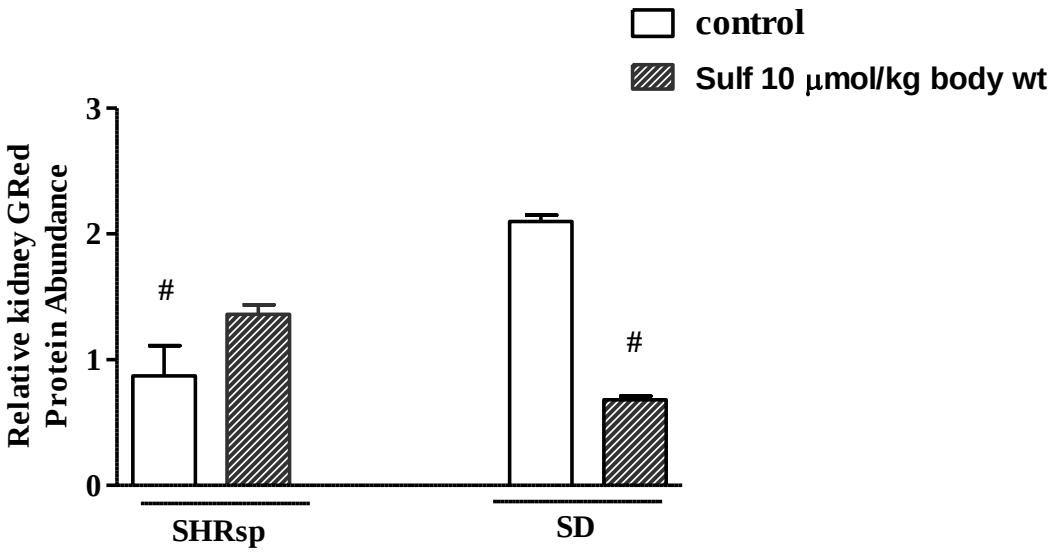


Figure 30 Effect of sulforaphane on GRed in kidney of SHRsp and SD rats. Four-week old female SD and SHRsp rats were treated daily by gavage corn oil (control) or sulforaphane (10 μmol/kg body weight) for 15 weeks. At the end of the treatment period, the kidneys of SHRsp and SD rats were analyzed for GRed levels, $^{\#}P<0.05$ when compared with control of SD rats. Each group contained 3 rats, mean ± SEM.

5.18 Effect of chronic administration of sulforaphane on nitrotyrosine (nitrosative stress marker) in kidney of SHRsp rats, Western blotting

In rats on control diet, the levels of nitrotyrosine in untreated SHRsp was 1.37± 0.2 AU while in treated SHRsp it was significantly lower at 0.917± 0.16 AU (Fig. 31).

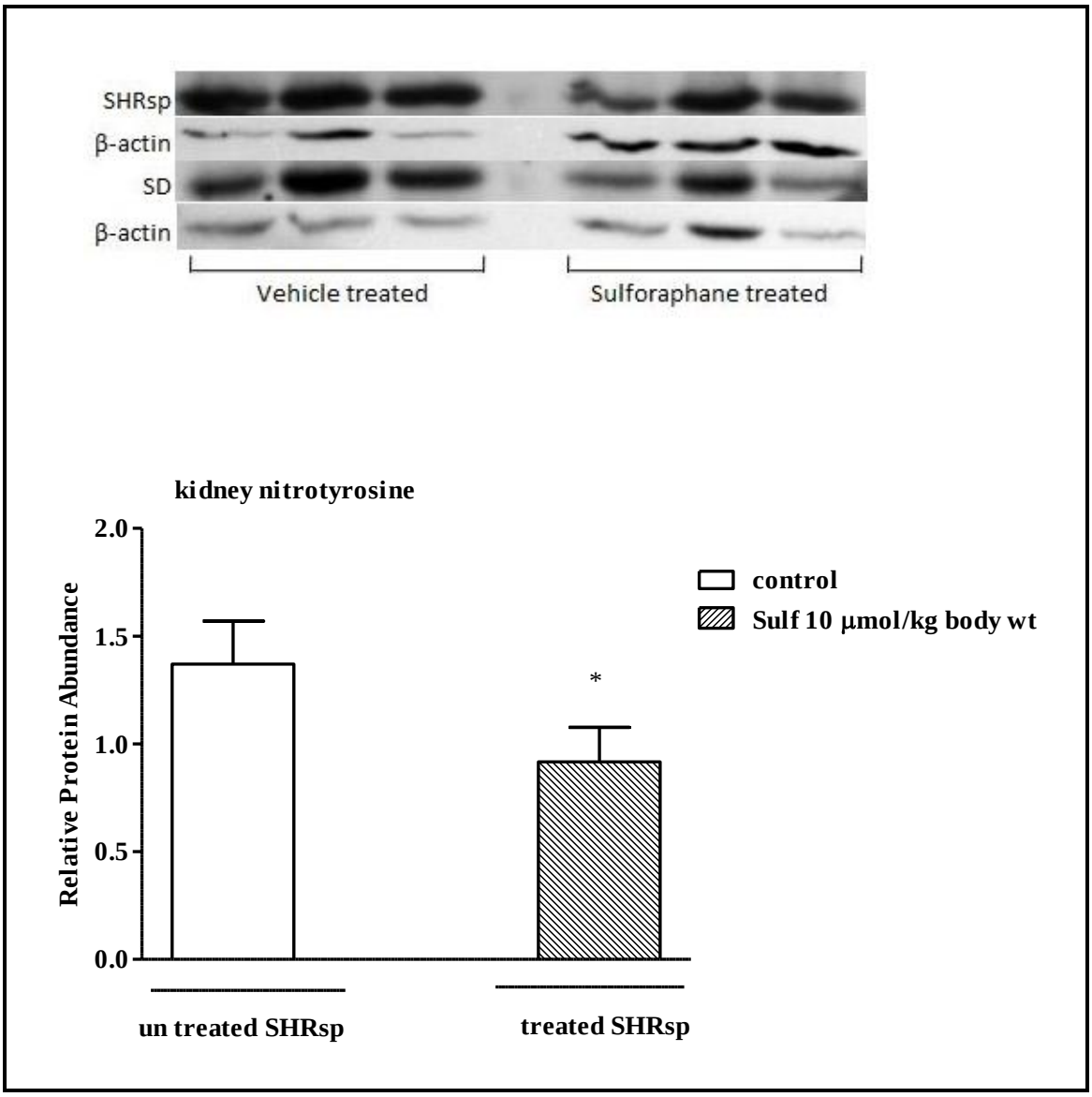


Figure 31 Immunoblot analyses of nitrosylated proteins (nitrosative stress marker) in kidney homogenates of treated and untreated SHRsp rats. Four-week old female SD and SHRsp rats were treated daily by gavage corn oil (control) or sulforaphane (10 µmol/kg body weight) for 15 weeks. At the end of the treatment period, the kidneys of SHRsp rats were analyzed for nitrosylated protein levels. The values were normalized to β-actin as loading control, **P*<0.05 *vs*. control of untreated SHRsp rats. Each group contained 3 rats, mean ± SEM.

5.19 Effect of chronic administration of sulforaphane on γ-GCS (or GCL) mRNA in liver and kidney of SD and SHRsp rats, the

dose of 10 µmol/kg was used: mRNA levels were determined by Q-PCR

5.19.1 γ -GCS mRNA in liver by Q-PCR

In rats on control diet, the basal levels of γ-GCS mRNA in livers were not significantly lower in SHRsp (70.63 ± 21.7) than in SD (100 ± 17.19) rats (Fig. 32). In SHRsp rats, long term sulforaphane administration no significantly increased γ-GCS mRNA while it no significantly decreased it in SD rats.

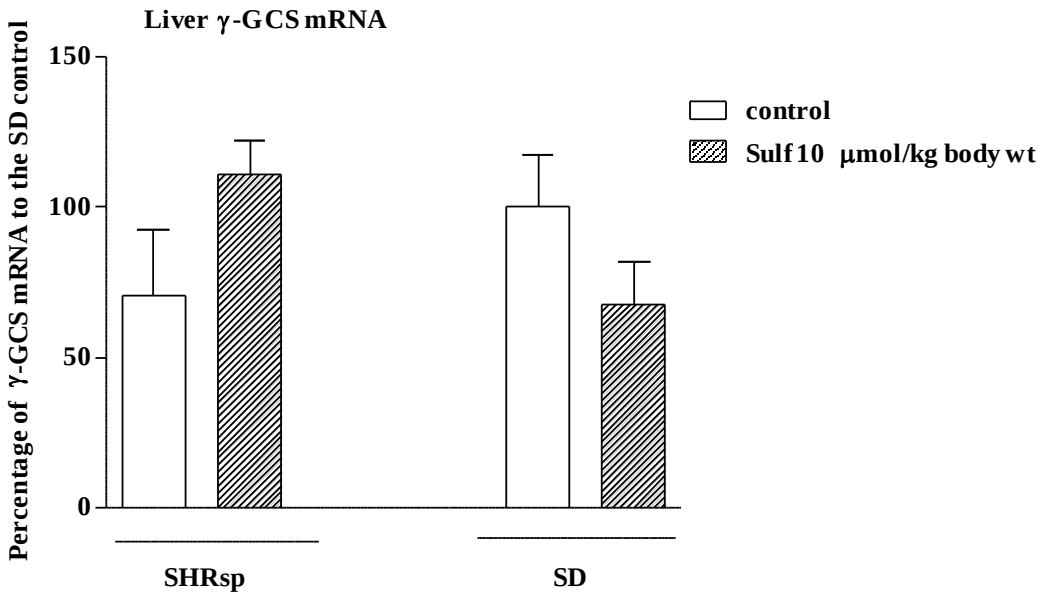


Figure 32 Effect of sulforaphane on γ-GCS mRNA levels in livers of SHRsp and SD rats. Four-week old female SD and SHRsp rats were treated daily by gavage corn oil (control) or sulforaphane (10 µmol/kg body weight) for 15 weeks. At the end of the treatment period, the livers of SHRsp rats were analyzed for γ-GCS mRNA levels. Each group contained 4 rats, mean ± SEM.

5.19.2 γ -GCS mRNA in kidney by Q-PCR

In rats on control diet, the basal levels of γ-GCS mRNA in kidneys were not significantly lower in SHRsp (97.1 ± 5.36) compared to SD (100 ± 23.21) rats (Fig. 33). In SHRsp rats, long term sulforaphane administration significantly (P<0.05) increased γ-GCS mRNA levels when compared with SHRsp control rats.

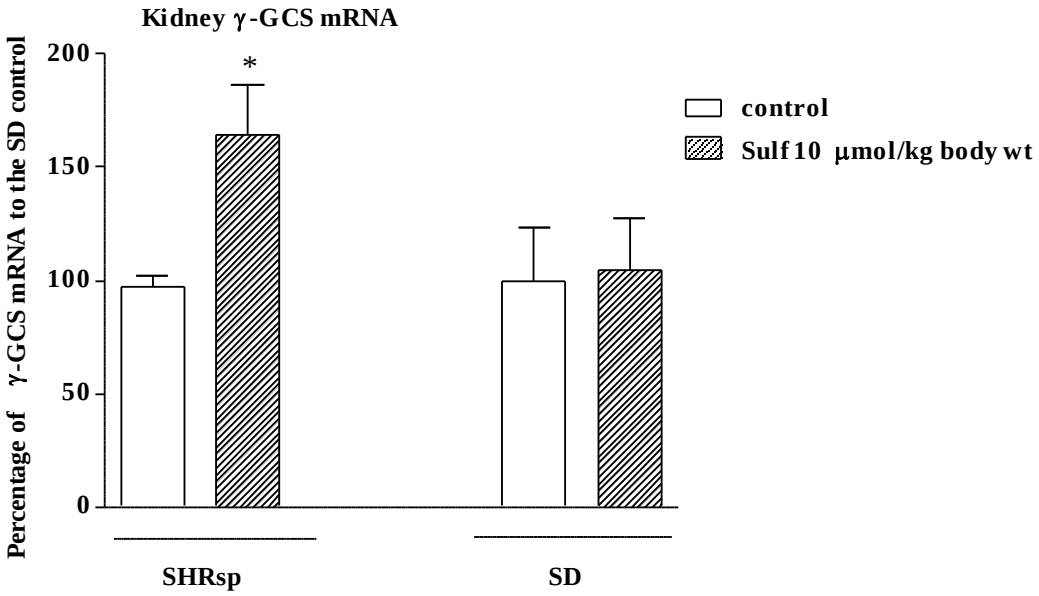


Figure 33 Effect of sulforaphane on γ-GCS mRNA levels in kidneys of SHRsp and SD rats. Four-week old female SD and SHRsp rats were treated daily by gavage corn oil (control) or sulforaphane (10 µmol/kg body weight) for 15 weeks. At the end of the treatment period, the kidneys of SHRsp rats were analyzed for γ-GCS mRNA levels. *P<0.05 when compared with control of the same strain. Each group contained 3-4 rats, mean ± SEM.

5.20 Effect of chronic administration of sulforaphane on TrxR1 mRNA in liver and kidney of SD and SHRsp rats, the dose of 10 µmol/kg was used: mRNA of the protein by Q-PCR

5.20.1 TrxR1 in liver by Q-PCR

In rats on control diet, the basal levels of $TrxR_1$ mRNA in livers in SHRsp (77.84 ± 12.23) and in SD (100 ± 19.18) rats (Fig. 34) were not significantly different. In SHRsp rats, long term sulforaphane administration had no effect on TrxR1 mRNA levels (84.06 ± 10.12) when compared with the SHRsp control. However, in SD rats, long term sulforaphane administration no significantly decreased TrxR1 (81.53 ± 15.1) when compared with the SD control.

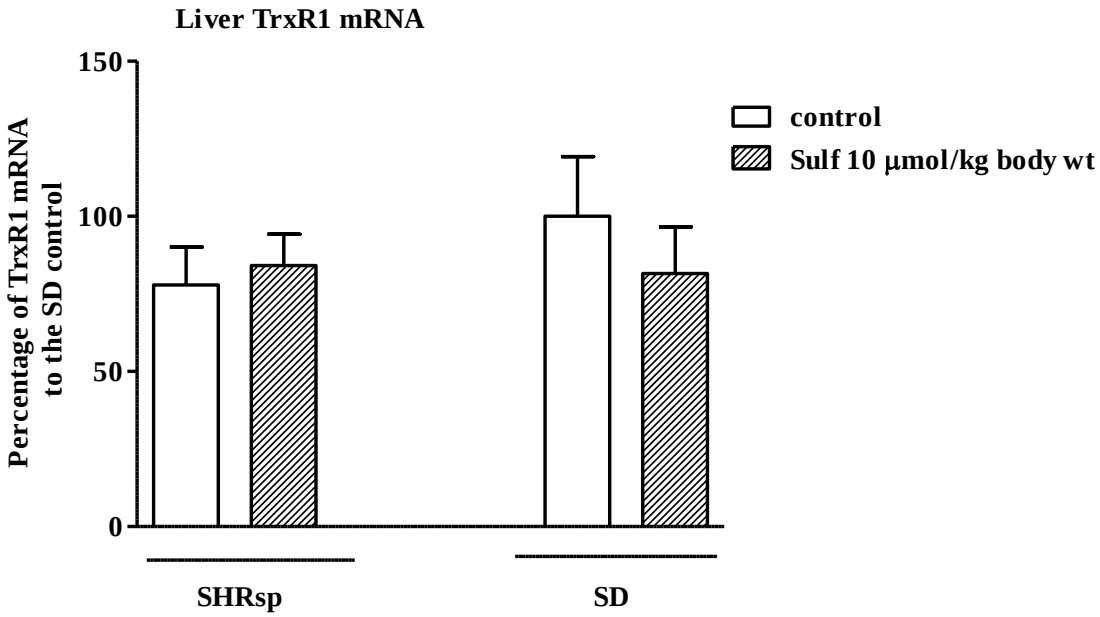


Figure 34 Effect of sulforaphane on TrxR1 mRNA in the liver of SHRsp and SD rats. Four-week old female SD and SHRsp rats were treated daily by gavage corn oil (control) or sulforaphane (10 μmol/kg body weight) for 15 weeks. At the end of the treatment period, the livers of SHRsp rats were analyzed for TrxR1 mRNA levels. Each group contained 4 rats, mean ± SEM.

5.20.2 TrxR1 mRNA in kidney by Q-PCR

In rats on control diet, the basal levels of $TrxR_1$ mRNA in kidney in SHRsp (98.22 ± 7.91) and in SD (100 ± 14.08) rats (Fig. 35) were not significantly different. In SHRsp rats, long term sulforaphane administration significantly increased (**P*<0.05 *vs*. control of SHRsp rats) $TrxR_1$. However, in SD rats, long term sulforaphane administration no significantly decreased TrxR1 (79.0 ± 11.59) when compared with the SD control

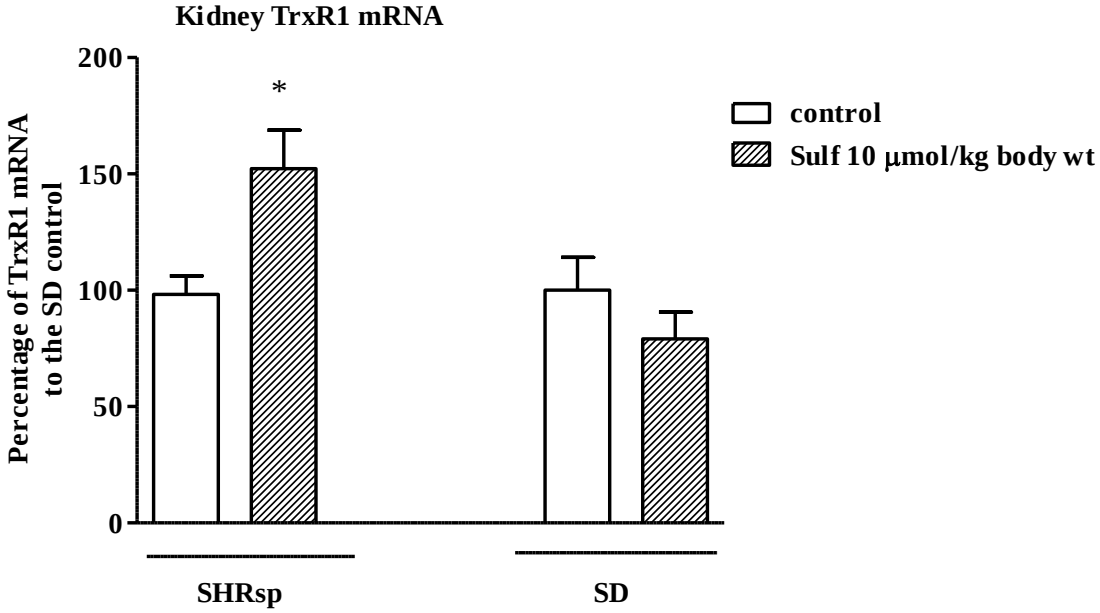


Figure 35 Effect of sulforaphane on TrxR1 mRNA in kidneys of SHRsp and SD rats. Four-week old female SD and SHRsp rats were treated daily by gavage corn oil (control) or sulforaphane (10 μmol/kg body weight) for 15 weeks. At the end of the treatment period, the kidneys of SHRsp rats were analyzed for TrxR1 mRNA levels, **P*<0.05 vs. control of SHRsp rats. All sulforaphane treated SHRsp and SD rats group contained 4 rats while the control animal groups contained 3 rats, mean ± SEM.

5.21 Effect of chronic administration of sulforaphane on renal artery structure of SD and SHRsp rats

5.21.1 Renal artery wall thickness in SHRsp and SD rats in H/E stained tissues

In rats on control diet, the renal wall thickness in SHRsp was significantly thicker than in SD rats (Fig. 36). In SHRsp rats, long term sulforaphane administration significantly decreased (**P*<0.05 *vs*. control of SHRsp rats) arterial wall thickness to that seen in control SD rats. In SD rats, long term sulforaphane administration had no effect on the renal wall thickness in SD rats.

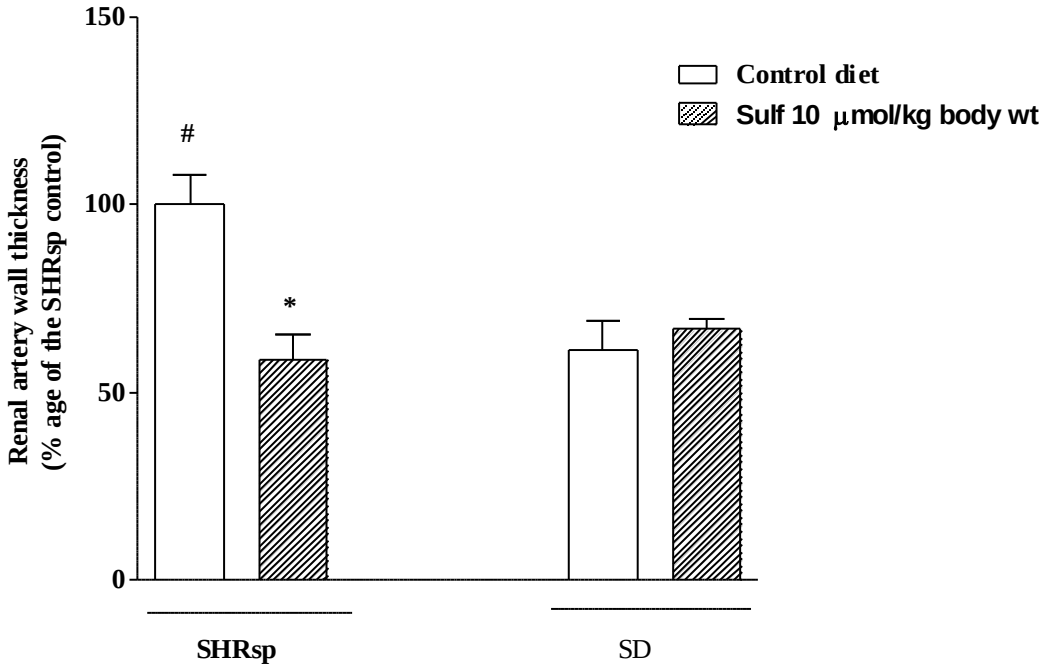


Figure 36 The structure of renal artery of treated and untreated SHRsp and SD rats. Four-week old female SD and SHRsp rats were treated daily by gavage corn oil (control) or sulforaphane (10 μmol/kg body weight) for 15 weeks. At the end of the treatment period, for the wall thickness, an average of 3-5 readings from different areas of the renal artery per animal was possible, **P*<0.05 *vs*. control of SHRsp rats, $^{\#}P$<0.05 when compared with control of SD rats. Animal groups contained 3 rats, mean ± SEM.

5.21.2 Renal artery smooth muscle cell number of SHRsp and SD rats in H/E stained tissues

In rats on control diet, the renal smooth muscle number in artery in SHRsp was significantly higher than in SD rats (Fig. 37). In SHRsp rats, long term sulforaphane administration significantly decreased (**P*<0.05 *vs*. control of SHRsp rats) the renal smooth muscle number in the renal artery to that seen in control SD rats. In SD rats, long term sulforaphane administration did not alter the renal arterial smooth muscle numbers.

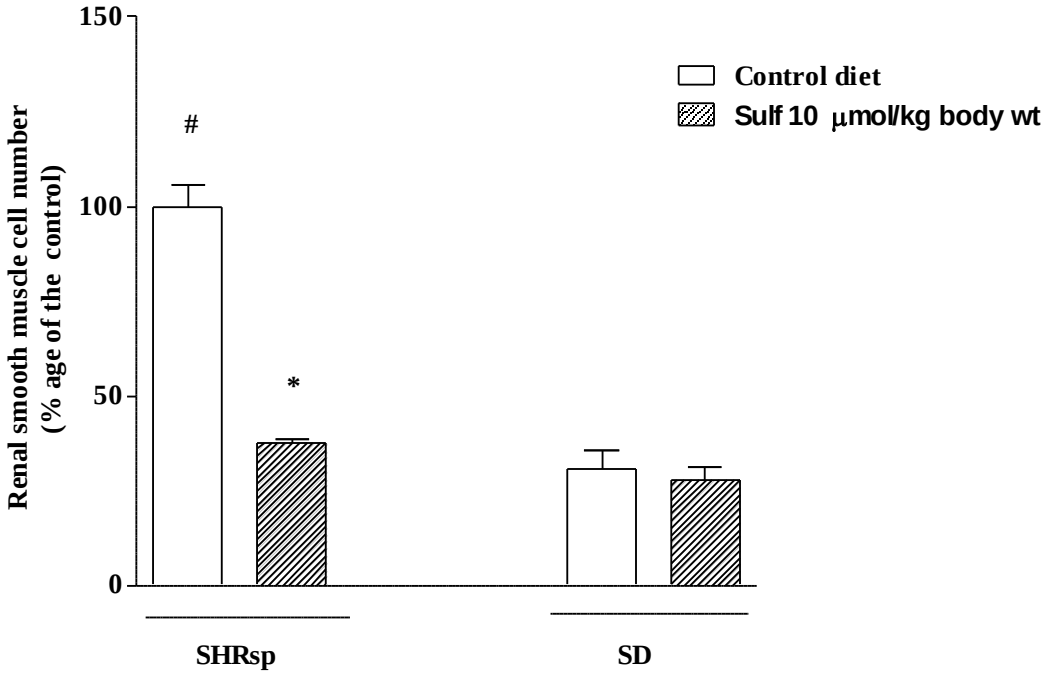


Figure 37 Renal smooth muscle number in artery of treated and untreated SHRsp and SD rats. Four-week old female SD and SHRsp rats were treated daily by gavage corn oil (control) or sulforaphane (10 μmol/kg body weight) for 15 weeks. At the end of the treatment period, the renal artery smooth muscle cell number and the average number of cells in five different known areas of the renal arteries per animal were automatically calculated, $^{*}P<0.05$. vs. control of untreated SHRsp rats, $^{\#}P<0.05$ *vs*. control of untreated SD rats. Animal groups contained 3 rats, mean ± SEM.

6. Discussion

Globally, the escalating health problem, hypertension, is increasing at an alarming rate. Worldwide approximately one billion individuals are hypertensive (Preston 2007; Kaplan and Opie 2006) by 2025, this number is projected to increase to 1.56 billion, 29% of the total population (Kaplan and Opie 2006). The great majority of hypertension cases (95%), where the cause is not clear, is essential hypertension. The consequences of sustained increase in BP are end organ damage, e.g., kidney damage, and structural and functional abnormalities in resistance vessels (Escobales and Crespo 2005). An imbalance between the production of ROS and the antioxidant defense mechanisms is believed to be one of the factors that lead to hypertension and its complications. An increase in ROS production leads to inactivation of NO, a potent vasodilator and reduces its availability (Garcia-Redondo et al. 2009). SHRsp is an experimental animal model with elevated BP together with structural and functional abnormalities (Aleixandre de Artinano and Miguel Castro 2009).

In this study, using an animal model of hypertension such as SHRsp, we evaluated the antihypertensive effects of pure sulforaphane. The results show that chronic oral administration of sulforaphane, a phase 2 protein inducer, obtained from extracts of edible plants: (a) had comparable effects on body weights amongst all sulforaphane treated groups throughout the experiment, (b) reduced BP, wall thickness and cell number of the small resistance arteries such as renal artery and (c) did not have effects in animals of normal redox physiology such as SD.

6.1. Effect of sulforaphane on body and organ weight

Numerous studies have documented that obesity contributes to cardiovascular diseases including hypertension, heart failure and congestive heart failure (Lavie et al. 2009). The present experiments have shown that chronic daily administration of sulforaphane has no significant effect on body weight of female SD or SHRsp rats. In contrast, in chronic pathologies, such as diabetes and hypertension, plant food containing flavonoids (the flavonol quercetin) significantly reduces final body weight and body weight gain of obese and lean Zucker rats compared with the controls. In addition, green tea catechins (polyphenols) induce weight reduction possibly *via*

inhibiting catechol-O-methyltransferase (the enzyme that degrades norepinephrine). This results in an increase in or more prolonged effects of norepinephrine on thermogenesis (Shixian et al. 2006) and fat metabolism. Reduction of energy intake or increasing energy expenditure is one way to treat obesity (Dulloo et al. 1999). Similarly, no significant effects on organ weights of female SD or SHRsp rats have been observed with chronic daily administration of sulforaphane. This study suggests that any beneficial effects of chronic administration of sulforaphane in SHRsp would be independent of body weight effects.

The comparable body and organ weights among the sulphoraphane treated and untreated groups suggest that there were no toxicities associated with the chronic oral administration of sulforaphane. These findings are in agreements with those of Conaway (Conaway et al. 2005). In A/J mice (lung cancer animal model), sulforaphane, phenyl isothiocyanate and their N-acetylcysteine conjugates treated mice had comparable body weights as compared with untreated groups.

6.2 Effect on BP

6.2.1. Comparing and contrasting of tail cuff plethysmography and intra-arterial catheters in SHRsp

SHRsp is an adequate experimental model of elevated BP together with structural and functional abnormalities (Aleixandre de Artinano and Miguel Castro 2009). Several methods have been described to record BP in animal model of hypertension. Among the most widely used methods are: tail cuff plethysmography (noninvasive) and intra-arterial catheters (invasive). Tail cuff plethysmography is noninvasive, relatively simple and suitable for large number of animals (20 animal/day), but imprecise (high variability) and restraining; furthermore, it requires warming the animal and thus induces stress. The intra-arterial catheter is precise, suitable for large number of animals (10 animal/day), but it requires surgery (invasive method) and anesthesia (Plehm et al. 2006). The necessity of anesthesia brings into question how do volatile

anesthetics such as isoflurane affect BP and other hemodynamics? To verify the monitoring of BP by tail cuff method, the intra-arterial catheter was used. This is an invasive method. In order to introduce the intra-arterial catheter surgically, anesthesia (i.e., isoflurane as volatile anesthetic) must be induced. We use intra-arterial catheters prior to euthanizing the rats.

In the present study, using the tail cuff method**,** the systolic blood pressure was 179.9 ± 4.3 mm Hg in SHRsp at the end of the experiment. When the anesthetic agent isoflurane was used in intra-arterial catheter method the SBP dropped to 144.27 ± 2.58 mm Hg SHRsp. As reported previously in animal (Conzen et al. 1992) and human (Doi and Ikeda 1993) studies, isoflurane (with increasing concentration and inhalation time) reduces the venous return to the heart, lowers filling pressure and may induce tachycardia. In addition, Conzen *et al*., 1992 reported that isoflurane decreases systemic vascular resistance, maintains cardiac output, reduces heart rate and left ventricular pressure. In short, in agreement with the previous studies, isoflurane attenuates BP - this may be through decreasing vascular resistance. It should be noted that the isoflurane effect on heart rate varies from study to another (Conzen et al. 1992; Doi and Ikeda 1993). This variation could be due to the different concentration or different inhalation time of the anesthetic.

6.2.3. Tail cuff plethysmography BP findings

In parallel with the previous studies (Wu et al. 2004; Noyan-Ashraf et al. 2006), the results of our study confirmed that chronic administration of pure phase 2 protein inducer sulforaphane significantly prevented the blood pressure rise (22 - 43 mm Hg by the end of the study) in SHRsp rats (Fig. 15). This decrease in blood pressure was independent of animal body weight (Fig. 9). In contrast, age-matched normotensive SD rats sulforaphane administration had no effect on blood pressure during the experimental period.

A growing body of evidence supports the protective effects of broccoli in several chronic diseases that have oxidative stress and inflammatory underlying components. For example, both *in vitro* and in a diabetic rat model, broccoli offers protective effect by reducing the oxidative stress load (Cho et al. 2006). In the hypertensive rat model, 14 week administration to rats of 200 mg/day of dried broccoli sprouts that contained glucoraphanin (5.5 μmol sulforaphane equivalents) attenuated blood pressure rise (Wu et al. 2004). Cruciferous vegetables (i.e.,

broccoli) contain a number of compounds with similar chemistry, metabolism, and protective effects as sulforaphane {e.g. glucobrassicin, gluconasturtiin–phenethylisothiocyanate, glucoerucin–erucin (sulfide analog of sulforaphane), and glucoiberin-iberin} (Clarke et al. 2008).

In addition, broccoli contains multiple other bioactive components, such as indole-3-carbinol and flavonoids (i.e., quercetin) (Keck et al. 2003). My study shows that independent of these bioactive components found in broccoli sprouts, long-term oral administration of sulforaphane alone reduces BP in hypertensive rats. The previous findings on the effect of broccoli sprouts on blood pressure Wu *et al*. 2004; Noyan-Ashraf *et al*. 2006) is likely due to sulforaphane and not other bioactive components present in broccoli sprouts: in other words the effect of sulforaphane appears to be independent of food matrix. Like broccoli sprouts, sulforaphane only prevents the final rise in blood pressure. Since hypertension is polyfactorial disease, in SHRsp, the final rise in BP in these animals is likely driven by oxidative stress.

6.2.4. Intra-arterial catheter findings

6.2.4.1 BP findings

The results show that chronic oral administration of sulforaphane, a phase 2 protein inducer, extracts from edible plants, decreased rises of SBP by 12 - 16 mmHg, DP by 15 - 19 mmHg and MAP by 12 – 16 mmHg when compared with untreated animals. On the contrary, sulforaphane did not exert hypotensive effects, or modify BP in control normotensive SD animals. The antihypertensive effects of sulforaphane are correlated with the reduced wall thickness and cell proliferation of the small resistance arteries such as renal artery.

Similar to sulforaphane, the polyphenols quercetin (a flavonoid) and resveratrol (3, 4', 5-trihydroxytrans-stilbene) present in our diet (Yu et al. 2010) are phase 2 enzyme inducers (Zhuang et al. 2003)(Myhrstad et al. 2002). Consistent with these findings, chronic administration of flavonol quercetin attenuated hypertension in obese male Zucker rats (Rivera et

al. 2008). In DOCA-salt hypertensive rats, resveratrol has been shown to reduce BP (Chan et al. 2010).

These phase 2 enzyme inducers attenuate hypertension, partly by enhancing GSH synthesis in endothelium. Flavonoids also increase the intracellular glutathione level by transactivation of the gamma-glutamylcysteine synthetase catalytical subunit promoter. For instance, in cell line (COS-1 cells), flavonoids in onion extract, i.e., quercetin, were able to increase the intracellular concentration of glutathione by approximately 50% *via* enhancing gamma-glutamylcysteine synthetase (the rate limiting enzyme of glutathione synthesis) (Myhrstad et al. 2002). Enhancement of the intracellular GSH level reverses endothelial dysfunction and increases NO (vasodilator) availability (Prasad et al. 1999).The physiological functions are controlled by a fine balance between prooxidants such as superoxide and hydrogen peroxide and antioxidants such as GSH and thioredoxin and their dependants enzymes (GR and TrxR1) (Arner and Holmgren 2000). In the vessel wall, superoxide dismutases dismute converts the superoxide free radicals into hydrogen peroxide and molecular oxygen (Faraci and Didion 2004). Catalyzed by catalase, or GSH peroxidase or thioredoxin peroxidase, hydrogen peroxide will be converted into water and oxygen (Arner and Holmgren 2000). Under pathological conditions, for instance, hypertension, may be correlated with increased production of superoxide free radicals or a decrease in thioredoxin and GSH dependent system. Increased superoxide free radicals react with NO and results in peroxynitrite formation (Faraci and Didion 2004). Peroxynitrite is potent oxidant with the potential to produce cytotoxicity (Faraci and Didion 2004). How do GSH and/ or thioredoxin inactivate the free radicals such as superoxide and hydrogen peroxide? Firstly, In presence of GSH or thioredoxin (reduced form), the peroxidases of GSH or thioredoxin convert hydrogen peroxide or lipid peroxide into water and molecular oxygen or alcohol, respectively (Yamawaki and Berk 2005). Secondly, the redox state of thiol groups is ideally suited for protein function. Oxidation of a critical SH group in cytosolic proteins will generally lead to an inactivation of protein biological functions (Arner and Holmgren 2000). The GSH-dependent glutaredoxin and the thiroredoxin-dependent thioredoxin reductase-1 (TrxR1) help to maintain proteins in their reduced functional state (Arner and Holmgren 2000). Decrease in GSH levels leads to proteins being maintained in their oxidized inactive states resulting in oxidative stress and hypertension (Vaziri et al. 2000). As explained

above, a decrease in GSH disturbs the balance between the prooxidants and antioxidants and correlates with hypertension in animals.

In agreement with the correlation of decreased blood pressure and increased GSH, Galisteo (Galisteo et al. 2004) showed that in DOCA-salt rats, chronic administration of an oral dose of the dietary flavonoid quercetin reduces SBP (Galisteo et al. 2004; Garcia-Saura et al. 2005): the antihypertensive effects are attributed partly to increasing cardiovascular GSH and improving kidney GST activity. In agreement with this is that in DOCA-salt hypertensive rats, prolonged administration (28 days) of antioxidants lowered the rise in blood pressure by inhibiting superoxide accumulation when compared with sham rats (Beswick et al. 2001).

6.2.4.2 Cardiac hemodynamic effects:

(i) The basic hemodynamic principles

Hemodynamic refers to the mechanical and physiological properties of controlling blood pressure and the flow through the body. The following cardiac hemodynamic parameters: heart rate (HR), left ventricular (LV) end systolic pressure (LVESP), left ventricular developed pressure (LVDP) and left ventricular contraction (contractility) (+dp/dt) were used to express the left ventricular functions.

(a) Left ventricular functions:

The major three factors in regulation of left ventricular functions are preload, afterload and contractility (Biondi et al. 2002). Preload defined as the tension exerted on ventricular wall before the myocardial contraction. The Frank-Starling mechanism helps the heart to match volume of the blood pumped by the ventricle per minute (cardiac output) to the volume of the blood available to the heart (venous return) (Truijen et al. 2010). For instance, any increase in end-diastolic volume (i.e., before the left ventricular contraction) leads to increase in ventricular pressure and ventricular contractility (+dp/dt). Afterload is generated by the resistance (i.e., two thirds of the resistance is determined by the arterioles) against which the ventricle is ejecting (Kenner 1987). So any increase in afterload (i.e., peripheral arteriolar resistance) may decrease the cardiac output.

(b) Normal hemodynamic:

The cardiac cycle is often divided into systole (ventricular contraction) and diastole (ventricular filling). In ventricular contraction, the ventricular pressure increases (the atrial pressure declines), the aortic valve opens and blood is ejected (Stehle and Iorga 2010). In diastolic period, it is the period during the cardiac cycle between the aortic valve closure and the mitral valve closure. When the ventricular pressure falls below atrial pressure, the AV (atrioventricular valve) opens, the rapid ventricular filling occurs (the ventricle is still relaxing) (Stehle and Iorga 2010). When the ventricular pressure increases (the atrial pressure declines), the aortic valve opens and another cardiac cycle starts.

(ii) Abnormal cardiac hemodynamic and protective role of sulforaphane

Clinically, a decrease in relaxation of the resistance artery results in a rise of systolic blood pressure. The resulting increase in afterload may induce cardiac hypertrophy, increase in rigidity and period lengthening in left ventricle relaxation (Tukkie et al. 1997). In hypertensive patients, the heart develops compensatory processes in order to overcome afterload. To set the contraction and relaxation at high rate, the left myocardial mass (LVM) and wall thickness increase (Salles et al. 2006). After a while, the hypertrophied heart exhibits impaired contractility and relaxation leading to heart failure and myocardial infarction (Koren et al. 1991).

In early stages (6-8 week old), SHRsp begins to develop a prominent left ventricular hypertrophy, maybe through enhancing hypertrophic signal regulated kinases in the myocardium) (Preston 2007).

Consistent with previous studies from our laboratory (Wu et al. 2004), at 6 weeks of age, SHRsp shows mild hypertension. At 19 weeks of age SHRsp rats exhibit severe hypertension. As a consequence of elevated blood pressure (afterload; reflecting peripheral arteriolar tone), a secondary change occurs in the heart in the form of elevated LV systolic blood pressure, rate of LV contraction and relaxation when compared with the control. Dahl (Dahlof et al. 1992) reported that in hypertensive patients, ACE inhibitors, beta blockers, and calcium antagonists all reduce LV mass by reversing wall hypertrophy when inducing BP reduction.

The data presented herein clearly demonstrate that 20 week old female SHRsp treated with sulforaphane exhibit a reduction in LV systolic blood pressure, LV developing pressure,

and with 10 μmol/kg dose, reduction of the rate of ventricle contraction and relaxation. This may be due, in part, to the decreased BP that decreases the afterload and improves cardiac function.

The possible mechanism of sulforaphane in improving the cardiac function could be *via* decreasing inflammation, oxidative stress, and hypertension (Noyan-Ashraf et al. 2006) of the cardiovascular tissues in hypertensive animals.

In accordance with our data, in angiotensin II dependent model of hypertension, administering green tea extract [GTE] (containing epigallocatechin-3-gallate and epicatechin) daily for 15 days decreases the systolic and diastolic BP and reduces the cardiac ERK1/2 (extracellular signal regulated kinase) (Li et al. 2006; Papparella et al. 2008). In this model, GTE attenuates the hypertension and inhibits development of cardiac hypertrophy *via* scavenging of ROS (Li et al. 2006; Papparella et al. 2008).

Previously, we have observed in our laboratory that a diet containing broccoli sprouts high in glucoraphanin, a sulforaphane precursor, in SHRsp protects the heart from oxidative stress *via* upregulating phase 2 enzymes such as glutathione peroxidase and increasing GSH (Wu et al. 2004). In this study long-term administration improved the rate of LV contraction and relaxation. In SHRsp, whether the attenuation of systolic blood pressure correlated with increased expression of cardiac thioredoxin reductase, glutathione reductase or γ-GCS [γ-glutamyl-cysteine synthase (ligase)) is unknown – see Discussion Section 6.3. Further experiments might answer this question.

Finally, in this study chronic administration (15 weeks) of sulforaphane (phase 2 protein inducer), in SHRsp, attenuates hypertension and improves the cardiac function in term of reducing LV systolic pressure and rate of LV contraction when compared with the control.

6.3 The renal vascular effects of sulforaphane

Small resistance arteries are a key element in controlling BP. Structural changes of small resistance arteries are associated with an increased cardiovascular risk in hypertensive patients (Rizzoni and Rosei 2009). It is widely accepted that in both, genetic and experimental hypertension animal models, as well as in hypertensive human beings thickened artery wall,

reduced lumen and hypertrophy (Schiffrin 2002) or hyperplasia (Yu et al. 1996) are associated with chronic hypertension (Rizzoni and Rosei 2009). While only some hypertensive patients exhibit endothelial dysfunction, almost all hypertensive patients exhibit vascular remodeling (Schiffrin 2002). In this study, we showed that long-term administration of sulforaphane protects against or corrects these alterations of small resistance vessels in SHRsp.

Consistent with our findings, Schiffrin (Schiffrin 2002) showed that, in hypertensive patients, a 1-year treatment with angiotensin AT1 receptor antagonists (losartan) or long acting calcium channel blockers (e.g. nifedipine) do not only lower BP but also correct the vascular remodeling. In contrast, β-blockers (e.g. atenolol) lower BP but have no effect on structure or function of the small arteries. In 2K, 1C Goldblatt animal model of hypertension (reproduces human renovascular hypertension), quercetin reduces SBP and cardiac hypertrophy (Garcia-Saura et al. 2005). The mechanism of this protection is partly due to antioxidant defenses improvement (Garcia-Saura et al. 2005). These finding are important because it does not only indicate that sulforaphane lowers BP but also suggest that sulforaphane protects the arterial structure of small resistance vessels.

In conclusion, this study shows that long-term administration of sulforaphane in SHRsp attenuates BP and improves vascular alterations. These findings show that this molecule (sulforaphane) is responsible for the beneficial effect seen previously with broccoli sprouts. In order to decrease the morbidity and mortality associated with essential hypertension, antihypertensive agents may not only lower BP but also may correct the vascular remodeling of small resistance arteries. This result may give a strong indication that the beneficial effects seen with sulforaphane here may be extrapolated to human essential hypertension leading to morbidity and mortality reduction

6.4 Effects of sulforaphane on the anti-oxidant defense system in cardiovascular and renal tissues (heart, kidney and aorta) and how improved anti-oxidant defense systems can improve blood pressures.

As in many diseases, in hypertension, in various cardiovascular tissues, abnormalities in oxygen metabolism could trigger oxidative and/or nitrosative stress.

Oxidative stress (by reactive oxygen species): GSH is one of the oxidative biomarkers. GSH levels are inversely related to oxidative stress, i.e., the lower the GSH level, the more oxidative stress. Sulforaphane administration resulted in increased GSH levels (i.e., reduced oxidative stress). GSH levels in normotensive SD rats was 345.55 ± 18.42 nmol/mg proteins in liver, 66.56 ± 6.04 nmol/mg proteins in heart, 17.68 ± 0.62 nmol/mg proteins in the kidney, and 43.07 ± 4.44 nmol/mg proteins in aorta. In contrast, the control hypertensive animals had 241.66 ± 5.6 nmol/mg proteins in liver, 53.09 ± 11.42 nmol/mg proteins in the heart, 11.83 ± 0.79 nmol/mg proteins in kidney, and 34.82 ± 3.17 nmol/mg proteins in the aorta. Oral administration of sulforaphane for 4 months resulted in a decrease in oxidative stress in hypertensive animals. This decrease was supported by increased intracellular GSH level in hypertensive animals on sulforaphane. Unlike the control hypertensive animals, sulforaphane increased GSH levels in liver of SHRsp by 133.69 ± 2.69 %, 151.05 ± 4.72 and 115.76 ± 4.8 (with 5, 10 and 20 μmol/kg body weight respectively). Being a net synthesizer of GSH and exporter of large quantities of GSH into the blood, the liver is a very important source of GSH (Deneke and Fanburg 1989); dietary components that enhance intracellular GSH in the liver may result in higher GSH levels in other tissues because of the interorgan flow of GSH. In accordance with higher GSH level in the liver, SHRsp fed sulforaphane (10 μmol/kg) had the following increases: 128.7 ± 4.82 % in the kidney, 139.36 ± 7.12 % in the heart and 121.59 ± 23.49 in the aorta. Thus, after 4 month of sulforaphane administration, GSH level increased in the liver, kidney, heart and the aorta in SHRsp. This consequential increase in the GSH level was in agreement with our previous findings with broccoli sprouts that contained glucoraphanin which is metabolized to the phase 2 protein inducer sulforaphane. This provides evidence that the beneficial effect seen with broccoli was due to the antioxidant-inducing properties of sulforaphane.

Nitrosative stress (by reactive nitrogen species), the level of nitrotyrosine is an indicator of nitrosative stress. The increased levels of superoxide increases the probability of interaction with nitric oxide to form peroxynitrite (Faraci and Didion 2004), thereby decreasing NO bioavailability. Peroxynitrite induces nitration of proteins (i.e., nitrotyrosine, a specific marker of $ONOO^-$) (Liang et al. 2010). By nitration of proteins, proteins lose their biological activity

(Faraci and Didion 2004). Besides nitration of proteins, peroxynitrite gives rise to strong oxidants such as the hydroxyl radicals and the nitrogen dioxide radicals (Christman et al. 2000). Sulforaphane administration resulted in decreased nitrosylated protein tyrosines (decreased nitrosative stress) in hypertensive rats. In another words, it indicates that sulforaphane increases the NO bioavailability. In addition, sulforaphane may attenuate hypertension through inhibiting the nitration of tyrosines of some proteins such as muscle proteins (actin and myosin) (Tiago et al. 2006). Inhibiting the nitration of tyrosines may not only improve the muscle contraction, but it may also improve the biological activity of various enzymes and other proteins. One of the mechanisms of sulforaphane could be through induction of phase 2 enzymes such as γ-GCS, particularly in endothelial cells.

To emphasize the role of GSH, in oxidative stress and nitrosative stress, in endothelial injury, (Ozkul et al. 2010) reported that low GSH level is correlated with endothelial injury in patients. In hypertensive animals, there is age-related decline in GSH (Lee et al. 2010). Phase 2 enzyme inducers, such as quercetin attenuates hypertension, partly by increasing the intracellular glutathione level *via* increasing γ-GCS (Scharf et al. 2003). Enhancement of intracellular glutathione levels reverses endothelial dysfunction and increases nitric oxide (vasodilator) availability (Prasad et al. 1999). Inversely, glutathione inhibition may result in oxidative stress and hypertension in animals (Vaziri et al. 2000). In agreement with the correlation of decreased blood pressure and increased GSH, using DOCA-salt rats, Galisteo (Galisteo et al. 2004) showed that orally administered quercetin attenuates SBP, partly *via* GSH that indirectly inhibits superoxide accumulation (Beswick et al. 2001).

The hypothesis initiating this research was that SHRsp rats on sulforaphane would have a decreased oxidative stress. The decreased quantity of the 45 kDa nitrosylated protein in the kidney tissue of SHRsp administered sulforaphane is another piece of data that supports this hypothesis.

In conclusion, the results show that the chronic daily administration of potent phase 2 protein inducer, sulforaphane, reduced hypertension, increased intracellular GSH levels in cardiovascular tissues (decreases oxidative stress) and reduced nitrosative stress (nitrotyrosine) in the animal model of essential hypertension.

6.4.1 GRed, TrxR1 and γ-GCS (phase 2 enzymes) expression in kidney and liver: Western blot data and mRNA data

Our data suggest that long term administration of phase 2 protein inducers such as sulforaphane induce GR, GC and Trx1 gene and protein expression. Sulforaphane, dietary phase 2 inducers could have a promising therapeutic value not only in hypertension but also in other pathological conditions with oxidative stress and inflammation components.

Per SHRsp, is this decrease in blood pressure due to modification of GRed, TrxR1 and γ-GCS (phase 2 enzymes) expression? GSH-dependent enzymes (i.e., GRed and γ-GCS) and thioredoxin-dependent enzymes (i.e., TrxR1) are the major free-radical scavenging systems (Eftekharpour et al. 2000). GSH and GSH enzymes are essential in the antioxidant system (Juurlink et al. 1998). The phase 2 protein genes such as GRed and γ-GCS have antioxidant response elements (ARE_s) in their promoter regions. In normotensive rats, long-term administration of sulforaphane did not alter GRed and γ-GCS levels. Although the mechanisms are not yet defined, this may be due to the feedback inhibition mechanisms of GSH and thioredoxin on anti-oxidant gene expression. In hypertensive rats, sulforaphane administration slightly increased liver γ-GCS (the rate limiting enzyme for GSH) but significantly increased renal γ-GCS. In addition, compared with SHRsp control, sulforaphane intake increased the phase 2 enzyme GRed that reduces glutathione disulfide (GSSG) to the sulfydryl form GSH in kidneys of hypertensive animals. In short, the very modest increase of GRed and γ-GCS in kidney by long term-sulforaphane administration may indicate that both the *de novo* synthesis of GSH and reduction of GSSG are important under conditions of oxidative stress. This may also indicate that GRed and γ-GCS have similar ARE governing the transcription. In SHRsp rats, long term-sulforaphane administration significantly increased (*P<0.05 *vs.* control of SHRsp rats) $TrxR_1$ mRNA in kidneys of hypertensive animals. Little is known about $TrxR_1$. The most important role in developing redox buffer, it reduces the oxidized thioredoxin (Trx-S_2) to the reduced thioredoxin [Trx-$(SH)_2$] (Eftekharpour et al. 2000). Trx-$(SH)_2$ can attenuate oxidative damage *via* scavenging peroxides, peroxynitrite and reducing GSSG thus releasing GSH (Eftekharpour et al. 2000). Again, the very modest increase of $TrxR_1$ mRNA in kidneys and livers of hypertensive

animals by sulforaphane administration may indicate different tissues have similar antioxidant response element (ARE) governing $TrxR_1$ transcription. These finding collectively suggest that the control hypertensive rats have lower GRed, TrxR1 and γ-GCS expression thus lower GSH level when compared with SD rats. Increases in GRed, TrxR1 and γ-GCS in hypertensive rats when maintained for 15 weeks in sulforaphane indicates a decrease in tissue oxidative stress.

In agreement with the present study, in male SHRsp, phase 2 enzymes such as GRed and GPx (GSH peroxidase) are increased when male SHRsp are maintained on 200 mg broccoli sprouts (5.5 μmol sulforaphane equivalents per rat per day) daily for 14 weeks (Wu et al. 2004) while there was no effect when the animals were on 0.5 μmol sulforaphane-equivalents of broccoli sprouts. In a similar study, in human lung epithelial cells, reseveratrol, a phase 2 protein inducer, induces intracellular GSH levels through upregulation of many antioxidant genes, including glutamate-cysteine ligase (Kode et al. 2008).

Our findings are supportive of the idea that chronic administration (15 weeks) of sulforaphane increases GRed and γ-GCS (phase 2 enzymes) and attenuates hypertension in hypertensive animals.

Oxidative stress activates the transcription factor NFkB which then translocates into the nucleus and promotes expression of proinflammatory genes, i.e., inducible nitric oxide (iNOS) in cardiovascular tissues of SHRsp (Noyan-Ashraf et al. 2006). Feeding SHRsp (5 week old) for 14 weeks broccoli sprouts that contained glucoraphanin (sulforaphane precursor) decreases NF-kB activation, activation of macrophages (Wu et al. 2004), iNOS and ICAM-1 (Inter-Cellular Adhesion Molecule 1) protein levels (Noyan-Ashraf et al. 2006) in cardiovascular tissues when compared with the control. Therefore, further studies to investigate whether prolonged administration of pure sulforaphane to SHRsp decreases the major inflammatory pathways in cardiovascular tissues are necessary.

Finally, our results show that prolonged administration of a phase 2 protein inducer sulforaphane increases phase 2 proteins and attenuates the rise in blood pressure. However, it is not clear if the long term administration of sulforaphane attenuates renal inflammatory response in hypertensive animals.

6.5 A phase2 protein inducer that has similar effects to sulforaphane: Quercetin

Similar to sulforaphane, the flavonoid quercetin (a polyphenol, (3, 3′, 4′, 5, 7-pentahydroxyflavone) is a phase 2 enzyme inducer (Zhuang et al. 2003); (Myhrstad et al. 2002) and is present in our diet such as grape (Yu et al. 2010), onions (Yoo et al. 2010), apples (Barbosa et al. 2010) and black tea (Verma and Sangai 2009; Galleano et al. 2010) Chronic administration of the flavonol quercetin attenuates hypertension in obese male Zucker rats (Rivera et al. 2008). Quercetin attenuates hypertension (Galisteo et al. 2004; Garcia-Saura et al. 2005) partly by enhancing tissue GSH level *via* inducing the rate-limiting enzyme in GSH synthesis (γ-GCS) (Scharf et al. 2003). In addition, quercetin reduces the cardiac and renal hypertrophy, and functional vascular changes in hypertensive animals without effect on control animals (Duarte et al. 2001). To sum up, as a phase 2 protein inducer with actions similar to sulforaphane, quercetin attenuates hypertension and reduces cardiac and renal hypertrophy in hypertensive animals. The protective mechanism of quercetin is partly attributed to enhancing GSH through γ-GCS.

Finally, we conclude that long term oral administration of sulforaphane: (1) does not appear to alter the normal redox physiology of the control animals (i.e., SD), (2) does not alter the growth characteristics of the animals as evidenced by organ and body weights**,** (3) the beneficial health effects previously seen with consumption of broccoli sprout is due to conversion of the sulforaphane precursor, glucoraphanin to sulforaphane, a potent phase 2 protein inducer and (4) the health promoting effects of sulforaphane can be independent of a broccoli food matrix.

6.6 Future aims

6.6.1 Rat model of hypertension

The rat model of hypertension used in this study is SHRsp. This model is used because it has the genetic and oxidative stress component (Vaziri et al. 2000) such as impaired GSH system

(Wu and Juurlink 2001). Therefore, this model is used to understand the therapeutic potential of compounds in essential hypertension in human. For future studies, it is important to investigate the efficacy of sulforaphane in DOCA-salt hypertension because it is the only rat model in which renin-angiotensin inhibitors do not decrease blood pressure (Lerman et al. 2005; Pinto et al. 1998).

6.6.2 Gender differences in blood pressure regulation of animals

In our experiments, using female SHRsp, we demonstrated that chronic administration of pure sulforaphane decreases hypertension. In female rats, the oxidative stress may not be severe enough to decrease the antioxidant system as markedly as in males. The reason is that estrogens such as 17-beta-estradiol induces expression of phase 2 enzyme (cytoprotective) such as glutathione peroxidase (GPx) and γ-glutamyl-L-cysteine ligase (the rate limiting enzyme for the synthesis of GSH) (Kondo et al. 2009; Urata et al. 2006). On the other hand, in male SHRsp, sex hormones, such as testosterone, play an important role in gender-associated differences in blood pressure (Comporti et al. 2008; Reckelhoff 2001; Sartori-Valinotti et al. 2007). Previous data suggest that testosterone contributes significantly to hypertension development (Comporti et al. 2008; Reckelhoff 2001). Castrated male SHR develop a blood pressure pattern similar to the female pattern of hypertension. Another evidence is that administration of testosterone to male SHR increases blood pressure (Comporti et al. 2008; Reckelhoff 2001). The mechanism by which testosterone increases blood pressure is not known. But it is known that testosterone is correlated with BP (Reckelhoff 2001) and that testosterone promotes renal angiotensinogen mRNA expression (Reckelhoff 2001; Kienitz and Quinkler 2008). In our experiments, we used female SHRsp; previous findings suggest that sex hormones such as estrogens induce the expression of GRed, TrxR proteins in vascular endothelial cells (Ejima et al. 1999). Future research should include SHRsp male rats and monitor the estrogen levels in female SHRsp rats and the testosterone levels in male SHRsp rats.

6.6.3 Can phase 2 protein inducer, such as sulforaphane, affect age-related progression of the hypertension, vascular and cardiac function in SHRsp?

In our experiments, we were able to demonstrate the positive effect of sulforaphane at one point (20 week old SHRsp). Future research may be carried out to investigate the potential effect of phase 2 protein inducer (sulforaphane) at three time points: 6 week old, 20 week old and 40 week old SHRsp. SHRsp develops not only hypertension, but also develops prominent left ventricular (LV) hypertrophy and fibrosis (Jesmin et al. 2005). Thus, SHRsp is a good model to study the effect of sulforaphane in cardiac hypertrophy and remodeling and compare the effect with the control maintained in AIN93-defined diet (a synthetic diet).

6.6.4 In addition to anti-oxistress properties, can phase 2 protein inducers, such as sulforaphane, have anti-inflammatory properties in attenuating hypertension in SHRsp?

Aside from the anticarcinogenic effect (Gibbs et al. 2009); sulforaphane possesses cytoprotective effect in cardiovascular diseases that have the oxidative stress and inflammatory components. Sulforaphane acts through multiple mechanisms. One of these includes: Induction of phase 2 metabolizing enzymes and enhancing antioxidant function. In addition to antioxidative effects, can phase 2 protein inducer, such as sulforaphane, through decreasing oxistress have anti-inflammatory properties in attenuating hypertension in SHRsp? If yes, what are anti-inflammatory gene protective mechanisms?

In our experiments, we demonstrated that long term administration of sulforaphane attenuated hypertension, partly *via* increasing TrxR system and GSH and its enzyme dependent system [increases phase 2 proteins (γ-glutamyl-L-cysteine ligase and GRed)]. Aside from oxidative stress, an inflammation is implicated in hypertension (Wu et al. 2004; Noyan-Ashraf et

al. 2006). Inflammation is a defense response of a tissue to injury. For instance, in cardiovascular tissues in hypertensive animals, an inflammation is activated by oxistress as explained in discussion section 6.4. Inflammation sometimes is not resolved and leads to irreversible severe tissue injury {{437 Pae,H.O. 2009;}. For this reason, the inflammation process must be controlled *via* interfering either with pro-inflammatory signals such as the transcriptional factor complex NF-κB (Wu et al. 2004) and iNOS (Noyan-Ashraf et al. 2006) or with the anti-inflammatory signals such as the inducible form of heme oxygenase: heme oxygenase-1 (HO-1) (phase 2 protein) {{437 Pae,H.O. 2009;}. In SHRsp, broccoli sprouts (containing glucoraphanin, a precursor of sulforaphane) consumption decreased inflammatory markers such as the transcriptional factor complex NF-κB (Wu et al. 2004) and iNOS (Noyan-Ashraf et al. 2006) in cardiovascular tissues when compared with the control. Therefore, for future experiments, it will be important to investigate if sulforaphane and other naturally occurring phase 2 protein inducers, in addition to anti-oxidative stress effects, decreases inflammation in SHRsp rats.

7. Significance and conclusions

Hypertension is a multifactorial disease that is characterized by a sustained increase in arterial systemic BP (≥140/90 mm Hg). Some consequences of the persistent increase in BP are arterial, kidney (Khan et al. 2007) and heart (Preston 2007) damage.

Globally, increasing at an alarming rate, the escalating health problem, hypertension, approximately one billion individuals are hypertensive; by 2025, this number is projected to increase to 29%, 1.56 billion (Hossain et al. 2007; Kaplan and Opie 2006). And more than 50% of Canadians aged between 55 to 74 years are hypertensive (Wexler and Aukerman 2006).

With unknown genetic and environmental factors triggering hypertension, the most widely used hypertensive model is the SHRsp. This hypertensive animal model mimics a subtype of human primary hypertension that is inherited in a Mendelian fashion (Lerman et al. 2005). Another feature of SHRsp is increased oxidative stress (Vaziri et al. 2000) such as impaired glutathione (GSH) system (Wu and Juurlink 2001). Therefore, this model is used to understand the therapeutic potential of compounds in essential hypertension in human.

After identifying the hypertension and the animal model that will be used to tackle hypertension, now why do we need a compound that attenuates BP? Among the patients receiving the current antihypertensive treatment, the level of adequate BP control (<140/90 mm Hg) is only 30% - 50% (Hermann et al. 2006) and in addition to the adverse effects, these antihypertensive drugs reduce the risk of CV events only by 20% and stroke by 40% (Hermann et al. 2006).

For people at high risk of hypertension or who already have hypertension and are on antihypertensive medications, maintaining healthy body weight, moderating consumption of alcohol, moderate exercise and reducing sodium salt intake may not only reduce hypertension but it may also delay or prevent the complications of hypertension, such as, left ventricular hypertrophy and vascular wall thickening.

In addition to life style modification, phase 2 proteins (cytoprotective, associated with the GSH-dependent pathways) are inducible proteins. The transcription of phase 2 proteins is under the control of the ARE (Juurlink 2001; Christman et al. 2000; Dinkova-Kostova and Talalay 2008). Induction of phase 2 genes is a highly effective way to counteract many chronic pathological conditions which have oxidative stress and inflammatory components, such as hypertension, diabetes and cancer (Dinkova-Kostova and Talalay 2008).

Evidence from our *in vitro* study (Wu and Juurlink 2001) and *in vivo* studies (Wu et al. 2004; Noyan-Ashraf et al. 2006) indicate that dried broccoli sprouts, contain high amounts of glucoraphanin that gives rise to potent phase 2 inducer isothiocyanate sulforaphane, attenuate oxidative stress, inflammation and hypertension in hypertensive animals. And this indication leads us to hypothesize that in SHRsp, early and long-term oral administration of pure sulforaphane will have cardiovascular protective effect in these hypertensive animals.

In our experiments long term administration of pure sulforaphane (as summarized in Fig. 38) has demonstrated many effects, including: (1) a minimal changes in our diet may have a major impact on our health, (2) the beneficial health effects previously seen with consumption of broccoli sprouts is due to conversion of the sulforaphane precursor glucoraphanin to sulforaphane, a phase 2 protein inducer, (3) the health promoting effects of sulforaphane can be independent of a broccoli food matrix and (4) long term administration of sulforaphane in SHRsp attenuates BP and regresses vascular alterations, increases intracellular GSH levels in cardiovascular tissues (decreases oxidative stress), reduces nitrosative stress (nitrotyrosine),

increases phase 2 proteins (γ-glutamyl-L-cysteine ligase, GRed and TrxR) and improves the cardiac function in terms of reducing LV systolic pressure, rate of LV contraction and relaxation when compared with the control.

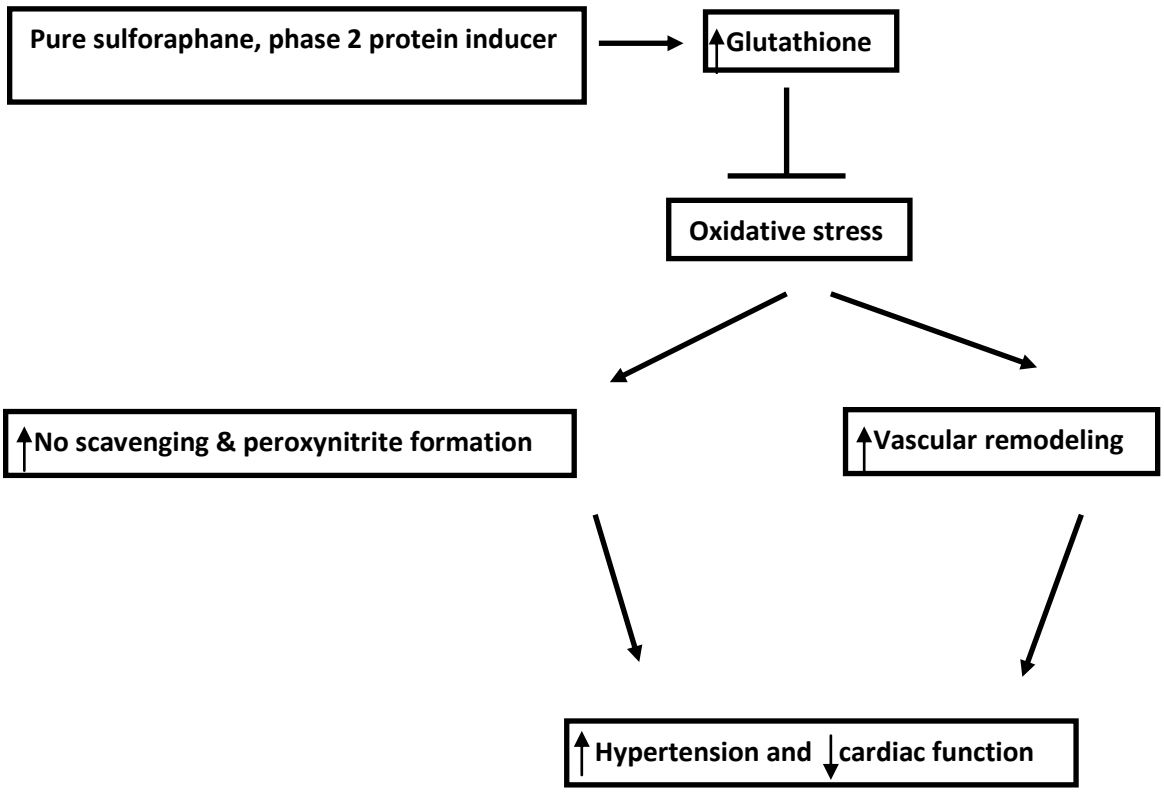


Figure 38 Summary of how sulforaphane attenuates hypertension and improves cardiac function. Decreasing oxidative stress decreases vascular remodeling of small resistance arteries and enhances nitric oxide availability**.**

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