THE EFFECT OF A DIETARY PHASE 2 PROTEIN INDUCER ON INFLAMMATORY PARAMETERS IN BLOOD AND LIVER OF SPONTANEOUSLY HYPERTENSIVE STROKE PRONE RATS

A Thesis Submitted to the College of Graduate Studies and Research in Partial Fulfillment of the Requirements for the Degree of Masters of Science in the Department of Anatomy and Cell Biology

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Abstract

Inflammatory diseases such as hypertension are associated with high levels of oxidative stress. Characteristic of oxidative stress is the inflammatory acute phase protein response. Oxidative stress and its accompanied inflammation can be reduced via phase 2 enzyme induction. Broccoli sprouts, a rich source of phase 2 enzyme inducers such as isothiocyanates, can be incorporated into the diet to increase phase 2 enzymes. The hypothesis of this study is that, the dietary intake of dried broccoli sprouts, by inducing liver phase 2 enzymes, will decrease oxidative stress and the acute phase response in the blood of spontaneously hypertensive stroke-prone rats.

Spontaneously hypertensive stroke-prone rats (SHRsp) and Sprague Dawley (SD) rats were placed either on a control diet of modified AIN-93G or an experimental diet of modified AIN-93G supplemented with dried broccoli sprouts. The following parameters were examined: 1. Isothiocyanate absorption (an increased level of dithiocarbamates is reflective of ITC absorption), 2. Oxidative stress (a reduction in oxidative stress is evidenced by an increase in plasma protein thiols and blood glutathione (GSH)), 3. Acute phase proteins (a decreased APR is reflected by an increase in plasma albumin and a decrease in ceruloplasmin), 4. Activity of phase 2 enzymes (increased phase 2 enzyme induction results in higher activities of liver quinone reductase (QR), glutathione-S-transferase (GST) and glutathione reductase (GR)).

My experimental results demonstrated that broccoli sprout feeding results in higher protein thiol levels in female SHRsp and higher blood GSH levels in males but no acute phase protein changes were observed in either male or female SHRsp. Broccoli sprout feeding caused higher QR and lower GST activities in female SHRsp but did not

affect the activities of phase 2 enzymes in male SHRsp. The activities of GST and QR were higher in SD rats than in SHRsp. Levels of dithiocarbamates were higher in the broccoli fed group than in the control fed group.

The results from this study do not present a clear pattern to support the hypothesis that dietary intake of broccoli sprouts by inducing phase 2 enzymes will decrease parameters of oxidative stress and the acute phase response. In conclusion, there is an interactive role played by animal gender and the induction of phase 2 enzymes by dried broccoli sprouts.

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List of Abbreviations

ACE	Angiotensin converting enzyme
AIN-93G	1993 Growth Diet of the American Institute of Nutrition
Ang I	Angiotensin I
Ang II	Angiotensin II
APP	Acute phase proteins
APR	Acute phase response
ARE	Antioxidant response element
Asc	Dehydroascorbate
AscH•	Ascorbate radical
$AscH_2$	Ascorbate
ASK1	Apoptosis signal-regulating kinase 1
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BHA	Butylated hydroxyanisole
BSA	Bovine serum albumin
CDNB	Chlorodinitrobenzene
CGMP	3'5' guanosine monophosphate
Cl^-	Chloride ion
CP	Ceruloplasmin
ddH_2O	Deionized distilled water
DNA	Deoxyribonucleic acid

DTC	Dithiocarbamates
DTNB	5,5′-Dithiobis(2-nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetate
ERK	Extracellular signal regulated kinase
FAD	Flavin adenine dinucleotide
Fe^{2+}	Ferrous cation
Fe^{3+}	Ferric cation
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Oxidized glutathione
GST	Glutathione-S- transferase
H^+	Hydrogen ion
H_2O	Water
H_2O_2	Hydrogen peroxide
HeLa	Human epithelial cancer cells
HepG2	Human hepatocyte carcinoma cell line
HX	Reduced xenobiotic
IL-1β	Interleukin-1β
IL-6	Interleukin-6
ITC	Isothiocyanate
IVR	Intervening region
JNK	c-Jun NH_2-terminal kinase

K_2HPO_4	Dipotassium hydrogen phosphate
Keap1	Kelch-like associated protein
KH_2PO_4	Potassium dihydrogen phosphate
$L^{\bullet}$	Carbon centred lipid radical
LH	Lipid
$LO^{\bullet}$	Lipid alkoxyl
$LOO^{\bullet}$	Lipid peroxyl radical
LOOH	Lipid peroxide
MAPK	Mitogen activated kinase
MAPKK	Mitogen activated kinase kinase
MAPKKK	Mitogen activated kinase kinase kinase
MEK	MAPK/extracellular signal-regulated kinase
mRNA	Messenger ribonucleic acid
MTT	3-(4, 5-dimethylthiazo-2-yl)-2, 5-diphenyltetrazolium bromide
Na^+	Sodium
Na_2HPO_4	Dibasic sodium phosphate
NAC	N-acetyl cysteine
NaCl	Sodium chloride
NAD^+	Nicotinamide adenine dinucleotide
NADP	Oxidized nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate

$NaH2PO_4$	Monobasic sodium phosphate
$NaHCO_3$	Sodium bicarbonate
NF-κB	Nuclear factor kappa B
NO•	Nitric oxide radical
Nrf2	Nuclear factor-erythroid 2p45-related transcription factor
O_2	Oxygen
$O_2^{\bullet -}$	Superoxide radical
OCl^-	Hypochlorite ion
OH^-	Hydroxyl anion
$OH^\bullet$	Hydroxyl radical
$ONOO^-$	Peroxynitrite anion
PBS	Phosphate buffered saline
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13 acetate
PP5	Protein phosphatase 5
QR	Quinone reductase
RAAS	Renin-angiotensin-aldosterone system
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
R-X	Electrophilic substrate

SD	Sprague Dawley
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SHR	Spontaneously hypertensive rats
SHRsp	Spontaneously hypertensive stroke prone rats
SOD	Superoxide dismutase
Sul	Sulforaphane
TAK1	Transforming growth factor-β activated kinase-1
tBHQ	tert-butyl-hydroquinone
TNB	5′-thionitrobenzoic acid
TNF-α	Tumour necrosis factor-α
TO•	Tocopheroxyl radical
TOH	Tocopherol
Trx	Thioredoxin
WKY	Wistar Kyoto rats

Chapter 1 Introduction

1.1. Rationale

During oxidative stress, reactive oxygen species (ROS) can activate transcription factors such as Nuclear Factor Kappa B (NF-κB) (Pinkus et al., 1996). Prolonged activation of NF-κB results in chronic inflammation (Makarov, 2000). Characteristic of NF-κB activation are a wide array of pro-inflammatory genes including Interleukin-6 (IL-6). IL-6 is a cytokine that modulates the acute phase response (APR). During the APR, acute phase proteins (APP) are produced (Shreiber, 1987). Some APP decrease during inflammation (negative APP) whilst others increase (positive APP). In the rat, albumin is an example of a negative APP and ceruloplasmin (CP) is an example of a positive APP. The APR is considered to be a marker of inflammation. Since the APR is a marker of inflammation, an APR should be present under conditions of oxidative stress.

ROS can act as signalling molecules under normal physiological conditions. However during oxidative stress, ROS can cause damage to deoxyribonucleic acid (DNA), lipids and proteins (Bourdon and Blache, 2001). It is desirable for cells to adequately scavenge ROS. Oxidant scavenging can occur via both enzymatic and non-enzymatic methods (Halliwell and Gutteridge, 1990). Of particular importance to this project are phase 2 enzymes that are involved in cellular detoxification processes and oxidant scavenging. Phase 2 enzymes are important in regulating the redox balance of the cell, and decreasing oxidative stress (Juurlink, 2001).

Phase 2 enzyme inducers can cause an upregulation in the synthesis of phase 2 enzymes. Phase 2 enzyme inducers, including tert-butyl-hydroquinone (tBHQ) and sulforaphane (Sul), are electrophilic compounds that are able to modify sulfhydryl

residues. Glucoraphanin found in broccoli sprouts, is converted into the isothiocyanate (ITC) Sul via the enzyme myrosinase (Fahey et al., 1997). Administration of phase 2 enzyme inducers should result in phase 2 enzyme induction and consequently a decrease in oxidative stress (see Figure 1).

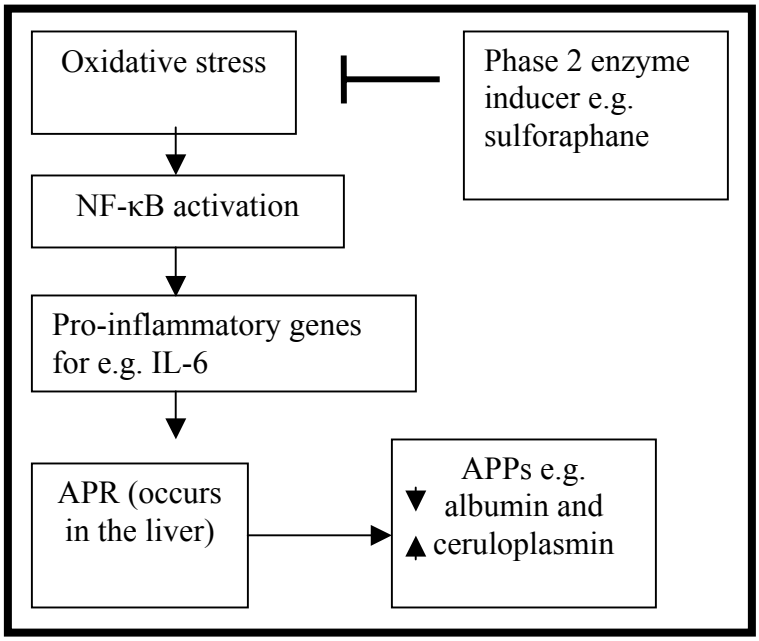

Figure 1. A proposed model by which feeding of a dietary phase 2 enzyme inducer decreases oxidative stress and the acute phase response. Oxidative stress results in NF-κB activation. This results in the transcription of pro-inflammatory products such as IL-6. IL-6 mediates the APR in the liver and gives rise to APP such as albumin and ceruloplasmin. Phase 2 enzyme inducers decrease levels of oxidative stress.

1.2. Hypothesis

Dietary intake of broccoli sprouts, by inducing phase 2 enzymes in the liver, will decrease oxidative stress and the acute phase protein response (APR) in the blood of spontaneously hypertensive stroke-prone rats (SHRsp).

1.3. Objectives

Blood analysis

1. To determine if ITCs from broccoli sprouts are detectable in the plasma of SHRsp.
2. To determine if the daily feeding of 200 mg of dried broccoli sprouts to male and female SHRsp reduces oxidative stress as reflected by an increase in levels of plasma protein thiols and blood glutathione (GSH).
3. To determine if the daily feeding of 200 mg of dried broccoli sprouts to male and female SHRsp reduces parameters of the APR as reflected by a decrease in levels of plasma ceruloplasmin and an increase in levels of albumin.

Liver Analysis

4. To determine if 200 mg of broccoli sprouts induces phase 2 enzymes in the livers of SHRsp as shown by an increase in glutathione reductase (GR), glutathione-S-transferase (GST), and quinone reductase (QR) activity.

Chapter 2 Literature Review

2.1 Oxidative stress

2.1.1 Background

Oxygen, which is central to our existence, plays a key role in oxidation and reduction reactions. Oxidizing agents take electrons from other compounds or add oxygen to them (Halliwell, 1994). Likewise, reducing agents donate electrons or remove oxygen (Figure 2.1).

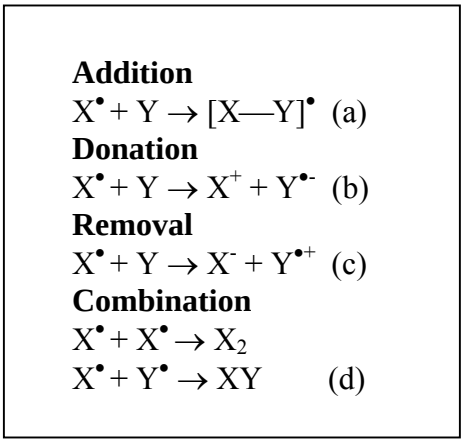

Figure 2.1 How radicals beget radicals: a fundamental principle of free radical chemistry. (Reprinted from J Lab Clin Med, 119 (6), Halliwell, B., Gutteridge, J. M., and Cross, C. E, Free radicals, antioxidants, and human disease: where are we now?, pages 598-620, 1992, with permission from Elsevier). $X^{\bullet}$ is a free radical and Y is non free radical. Equations (a-c) demonstrate the effects of combining a free radical with a non free radical or in equation (d) with another free radical ($Y^{\bullet}$)

Oxidizing or reducing reactions can generate free radicals. A free radical is "any species capable of independent existence (hence the term 'free') that contains one or more unpaired electrons" (as reviewed in Gutteridge, 1994). By this definition, oxygen which has 2 unpaired electrons is a free radical (as reviewed in Halliwell, 1994), whereas hydrogen peroxide (H_2O_2), which has no unpaired electrons is not a free radical. H_2O_2 is classified as a ROS. ROS are compounds that are derived from oxygen such as superoxide, the hydroxyl radical, hypochlorous acid and ozone. Superoxide and H_2O_2 are

not very reactive but still fall under the term ROS (as reviewed in Halliwell, 1994), as they can react with other free radicals to create species that are highly reactive. Likewise, reactive nitrogen species (RNS) describe molecules that are nitrogen derivatives. ROS and RNS are the most commonly encountered species during oxidative stress.

Under non-pathological conditions, the endogenous antioxidant system scavenges ROS and RNS ensuring that cellular damage is minimised. However during pathological conditions, ROS and RNS overwhelm the endogenous antioxidant system, giving rise to a condition known as oxidative stress. During conditions of oxidative stress, the generation of free radicals can have several consequences. They can regulate signalling pathways that activate transcription factors such as NF-κB (Kaul et al., 1998; Schmidt et al., 1996). Free radicals can react with and cause damage to DNA, lipids and proteins (as reviewed in Bourdon and Blache, 2001). They can play a role in the pathogenesis of diseases such as hypertension that have an underlying inflammatory component.

2.1.1.1 The generation of ROS and RNS

ROS and RNS are formed during normal physiological processes that occur when the cell is not under stress. For example, this occurs within the electron transport chain (see Figure 2.2). Of respired oxygen**,** 98% is utilized by mitochondria to generate adenosine triphosphate (ATP) (Duchen, 2004). The breakdown and oxidation of foods generates electrons that are passed onto electron carriers such as flavin adenine dinucleotide (FAD) or nicotinamide adenine dinucleotide (NAD^+) and then into the electron transport chain to generate ATP. Within the electron transport chain, oxygen receives a hydrogen ion and electrons and is reduced to water. During this process, premature leakage of an electron

to oxygen leads to the formation of superoxide ($O_2^{\bullet -}$) (Kamata and Hirata, 1999) (See reaction 1 in Table 2.1).

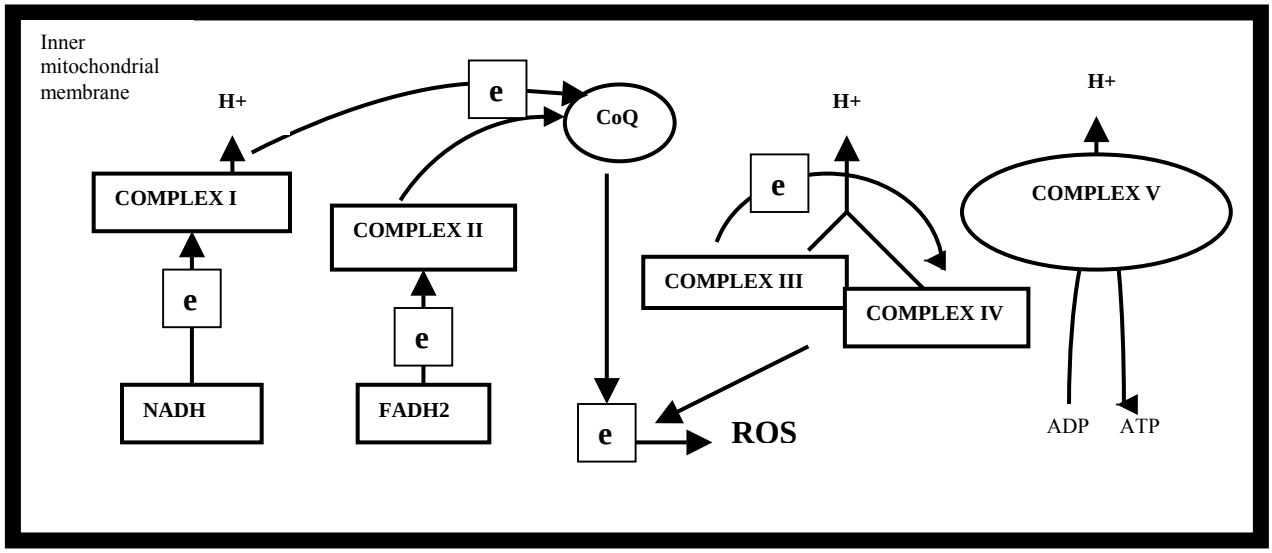

Figure 2.2 Electron transport chain. Electrons from NADH and FADH2 move through the various complexes of the electron transport chain. Leakage of these electrons can result in the generation of ROS.

In addition to the electron transport chain**,** superoxide is generated during arachidonate metabolism, cysteine oxidation, oxidation of catecholamines, peroxisome activity, and leukocytic respiratory bursts (as reviewed in Juurlink, 1999). Superoxide reacts to give rise to other oxygen-derived species such as H_2O_2 and peroxynitrous acid (Droge, 2002). Enzymatically, superoxide dismutase (SOD) generates H_2O_2 (See reaction 2 in Table 2.1). H_2O_2 is water and lipid soluble, and therefore crosses biological membranes readily. This is unlike superoxide, which diffuses at a slower rate across the membrane (as reviewed in Gutteridge and Halliwell, 1989). This allows H_2O_2 to participate in the Fenton reaction (See reaction 3 in Table 2.1) and generate the hydroxyl radical. In addition to giving rise to H_2O_2, superoxide reacts with nitric oxide (NO•) giving rise to peroxynitrous acid (OONOH) (See reaction 4 in Table 2.1). NO• is a reducing agent that is generated from the oxidation of a nitrogen atom in L-arginine. NO•

can give rise to other RNS (Droge, 2002). ROS and RNS that are generated during normal physiological conditions are scavenged by intracellular and extracellular antioxidant systems. During pathological states antioxidant systems may be overwhelmed by the excess production of oxidants. For example during hypertension excess production of superoxide acts as a sink for the vasodilator NO•. This decreases the bioavailability of NO• reducing vascular relaxation.

Table 2.1 Oxidative stress related reactions.

1)	$O_2 + e^- \rightarrow O_2^{\bullet-}$ (superoxide radical)
2)	Superoxide dismutase: $2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$
3)	$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^\bullet + OH^-$ (Fenton reaction)
4)	$O_2^{\bullet-} + NO^\bullet \rightarrow ONOO^-$
5)	$LOO^\bullet + TOH \rightarrow LOOH + TO^\bullet$
6)	$TO^\bullet + AscH_2 \rightarrow TOH + AscH^\bullet$
7)	$2AscH^\bullet \rightarrow AscH_2 + Asc$
8)	$2GSH + Asc \rightarrow GSSG + AscH_2$
9)	γ-glutamylcysteine ligase: L-glutamate + L-cysteine + MgATP→ L-γ-glutamyl-L-cysteine + MgADP +Pi
10)	Glutathione synthase: L-γ-glutamyl-L-cysteine + Glycine + MgATP → Glutathione + MgADP + Pi
11)	Glutathione-S-transferase: GSH + R-X → GSR + HX
12)	Glutathione Peroxidase: LOOH + 2GSH → HOH + GSSG
13)	Glutathione Reductase: GSSG + 2NADPH → 2GSH + $2NADP^+$
14)	Catalase: $2H_2O_2 \rightarrow 2H_2O + O_2$
15)	$LH + OH^\bullet \rightarrow L^\bullet + HOH$
16)	$L^\bullet + O_2 \rightarrow LOO^\bullet$
17)	$LH + LOO^\bullet \rightarrow L^\bullet + LOOH$
18)	$LOOH + Fe^{2+} \rightarrow Fe^{3+} + LO^\bullet + OH^-$
19)	$LOOH + Fe^{3+} \rightarrow Fe^{2+} + LOO^\bullet + H^+$
20)	Myeloperoxidase: $Cl^- + HOOH \rightarrow OCl^- + H2O$
21)	$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bullet-}$

2.1.2 Antioxidant defence systems

Antioxidant systems can be enzymatic or non-enzymatic and differ in importance depending on whether they are intracellular or extracellular. This is summarized in Table 2.2.

Table 2.2. Some important antioxidants of biological systems. (Reprinted from Best Practice and Research Clinical Haematology, 2 (2), Gutteridge, B. and Halliwell, J. M., Iron toxicity and oxygen radicals, pages 195-256, 1989, with permission from Elsevier)

Antioxidant	Mode of action
Intracellular	
Superoxide dismutases (Cu, Zn and Mn enzymes)	Removal of superoxide radical (O_2^-)
Catalase (heme protein)	Removal of hydrogen peroxide (H_2O_2)
Glutathione peroxidase (selenium enzyme)	Removal of hydrogen peroxide and probably organic hydroperoxides (LOOH) after cleavage from membranes
Membrane	
Vitamin E	Lipid-soluble, lipid radical , chain-breaking antioxidant ($LO^\bullet$ and $LO_2^\bullet$)
β-carotene	Singlet oxygen and $OH^\bullet$ radical scavenger
Extracellular fluids	
Transferrin	Binds Fe^{3+} and stops its participating in radical reactions
Lactoferrin	Binds Fe^{3+} at low pH values and stops its participation in radical reactions
Ceruloplasmin	Catalyses the oxidation of ferrous ions to the ferric state (ferroxidase activity). Can react stoichiometrically with O_2^-
Haptoglobins and haemopexin	Bind free hemoglobin/ heme and prevent lipid peroxide decomposition
Albumin	Binds copper tightly and iron weakly. Probably a 'site specific' sacrificial antioxidant
Extracellular superoxide dismutase (copper protein)	High-molecular-mass glycoprotein which removes O_2^-. May be located at endothelial cell surface. (Only trace amounts present)
Urate (uric acid)	Inhibitor of radical reactions can also bind iron and copper ions.
Glucose	High rate constant for reaction with $OH^\bullet$ radicals. Present at 3-5mM or even higher after a carbohydrate rich meal

2.1.2.1 Non-enzymatic defence system

Flavonoids, beta carotene and lycopenes constitute part of the non-enzymatic defence system. They play a role in iron chelation, inhibition of lipid peroxidation, decreasing cell adhesion molecules and scavenging free radicals such as peroxynitrite (as reviewed in Paterson, 1999). However, the most common non-enzymatic defence systems include vitamin C (which was omitted by Gutteridge et al in Table 2.2), vitamin E (tocopherol) and GSH. Vitamin E plays a major role in breaking the chain of lipid peroxidation that occurs in membranes, by binding peroxyl radicals (as reviewed in Halliwell and Cross, 1994; Juurlink, 1999) (See reaction 5 in Table 2.1). During this reaction, vitamin E becomes a free radical that is reduced by ascorbate (See reaction 6 in Table 2.1). This causes ascorbate to dismutate to oxidized ascorbate (See reaction 7 in Table 2.1) which is reduced by either nicotinamide adenine dinucleotide phosphate (NADPH) or GSH dependant systems (as reviewed in Halliwell, 1994) (See reaction 8 in Table 2.1).

GSH is the most abundant non-protein thiol that exists in millimolar concentrations in most cells (Dickinson and Forman, 2002). GSH plays an important role in cellular redox buffering. For example, an increase in the ratio of reduced to oxidized GSH, decreases the amount of NF-κB activation and consequently the amount of oxidative stress (as reviewed in Christman et al., 2000; Juurlink, 1999). Given that apoptosis is regulated by the redox state of the cell, GSH also plays a role in regulating cell viability (as reviewed in Filomeni et al., 2002).

GSH concentrations are regulated by phase 2 enzymes such as γ-glutamylcysteine ligase, GR and GST (as reviewed in Dickinson and Forman, 2002; Juurlink, 1999; Kirlin et al., 1999). GSH is made up of the amino acids glutamate, cysteine and glycine. The enzyme γ-glutamylcysteine ligase is the rate-limiting enzyme in the production of GSH. γ-glutamylcysteine ligase catalyses the formation of glutamate and cysteine giving rise to L-γ glutamyl-L cysteine (See reaction 9 in Table 2.1). Since cysteine is the limiting amino acid for GSH synthesis, pro-cysteine delivery systems such as L-2-oxothiozolidine-4-carboxylate or S-adenosyl methionine are used to increase intracellular GSH levels. Glutathione synthase catalyses the reaction of L-γ glutamyl-L cysteine with glycine, giving rise to GSH (See reaction 10 in Table 2.1). Once formed, enzymes such as GST or glutathione peroxidase (GPx) act on GSH. As an electron donor, GSH cycles between a reduced and oxidized state. The flavoprotein GR contains a NADPH and FAD site. GR catalyses the flow of electrons from NADPH to FAD and then to GSH (See reaction 13 in Table 2.1).

It has been suggested that plasma proteins, for example CP, hemoglobin and transferrin, constitute a major part of the non-enzymatic extracellular antioxidant defence system. They bind transition metal ions to prevent them from reacting to generate toxic species like the hydroxyl radical (as reviewed in Halliwell and Gutteridge, 1990). Some of these proteins in addition to being antioxidants are also APP. For example, the copper-binding protein CP, and the iron binding proteins hemoglobin and transferrin are examples of positive APP with antioxidant properties. CP has ferroxidase activity i.e. it is able to convert the ferrous cation (Fe^{2+}) into the ferric cation (Fe^{3+}) thus preventing the

Fenton reaction. CP is an APP that makes up part of both the enzymatic and non-enzymatic parts of the antioxidant defence system.

2.1.2.2 Enzymatic antioxidant defence system

The cell's main antioxidant defence systems are the enzyme systems of SOD, catalase, GPx, and GST (as reviewed in Halliwell and Gutteridge, 1990). They play a role in eliminating superoxides, peroxides, and xenobiotic compounds. SOD converts superoxide into H_2O_2. Catalase and GPx play a role in converting H_2O_2 to water and oxygen (See reaction 12 and 14 in Table 2.1). The advantage of GPx over the enzyme catalase is that the affinity of GPx for H_2O_2 is higher and GPx is not localized to peroxisomes as is catalase. GST catalyses the formation of glutathiol adducts (See reaction 11in Table 2.1) eliminating xenobiotic compounds.

In blood plasma, the enzyme systems of SOD, catalase, and GPx do not make up the main antioxidant systems, as their concentrations are too low (as reviewed in Halliwell and Gutteridge, 1990). Also, with respect to GPx, the amount of reduced GSH in rat plasma is in the range of about 20 μM, and GPx requires millimolar concentrations to function (as reviewed in Halliwell and Gutteridge, 1990). It has been found, however, that thioredoxin (Trx) and glutaredoxin may act as electron donors to human plasma GPx (Bjornstedt et al., 1994). This might explain the presence of GPx despite the low concentration of GSH in plasma. The next section deals with the consequences of oxidative stress that exist when antioxidant systems are overwhelmed by excess oxidant generation.

2.1.3 Consequences of oxidative stress

2.1.3.1 NF-κB

2.1.3.1.1 Overview

As part of a normal physiological defence mechanism, immune cells such as neutrophils, macrophages, and lymphocytes respond to infiltrating micro-organisms via NF-κB activation (as reviewed in Baeuerle, 1998; Makarov, 2000). NF-κB activation can also be mediated by ROS such as H_2O_2 (as reviewed in Li and Karin, 1999). In the absence of invading micro-organisms and in the presence of oxidative stress, oxidants can inappropriately activate NF-κB (Flohe et al., 1997; Janssen-Heininger et al., 2000; May and Ghosh, 1998; Pinkus et al., 1996). Antioxidants help to maintain the redox state of the cell, and are thought to counteract NF-κB activation. Interleukin-1 induced activation of NF-κB was enhanced when inhibitors of GPx, GR and catalase were administered (Renard et al., 1997). Maintaining the antioxidant defence system of a cell decreases oxidative stress and NF-κB activation, thereby avoiding NF-κB mediated pro-inflammatory pathways that result in cellular damage.

2.1.3.1.2 Mechanism of NF-κB activation

NF-κB in its inactive form resides in the cytoplasm where its nuclear localization site is masked by inhibitory proteins (as reviewed in Baeuerle, 1998; May and Ghosh, 1998). Prototypic NF-κB consists of a p50 and a p65 subunit (Brand et al., 1996). Upon activation, NF-κB translocates to the nucleus where it results in the transcription of over 60 pro-inflammatory genes, involved in cell adhesion, immune stimulation, apoptosis, chemoattraction, differentiation, extracellular matrix degradation, and redox metabolism (Baeuerle, 1998). The upregulation of these genes gives rise to the production of more oxidants (as reviewed in Juurlink, 2001). Prolonged activation of NF-κB and the upregulation of these pro-inflammatory genes perpetuates a cycle of inflammation, which

in the absence of invading organisms is not desirable (Lee and Burckart, 1998). Characteristic of chronic or recurring inflammation is the APR, which is regulated by IL-6.

2.1.3.2 Acute phase response

2.1.3.2.1 Overview

In response to inflammation, an APR occurs in the liver. The APR is considered to be a marker of acute inflammation, but components of it persist during chronic inflammation (as reviewed in Ceciliani et al., 2002). The APR is characterized by a change in production of a set of proteins known as APP (as reviewed in Shreiber, 1987). The definition of an APP is one "whose plasma concentration changes by 25% or more following inflammatory stimulus" (Mackiewicz, 1993). Positive APP increase in response to inflammation whereas negative APP decrease in response to inflammation (Mackiewicz, 1993). Examples of positive APP are CP, haptoglobin, fibrinogen and transferrin. Albumin is an example of a negative APP (see Table 2.3). It has been suggested that the APP pattern of expression occurs to decrease the production of certain proteins allowing for the production of others, as there is no need for a drastic increase in the overall amount of ATP or transfer ribonucleic acid (RNA) needed (Shreiber, 1987).

2.1.3.2.2 Function

Some of the metabolic changes that occur during the APP are listed in Table 2.4. The function of the APR in response to inflammation is not well understood. The APR appears to be a counterattack of the body against infection and an attempt to reach homeostasis (as reviewed in Ceciliani et al., 2002). A role for APP as part of an

antioxidant defence system is also proposed and as Ceciliani (2002) suggests, this may be to prevent some of the tissue damage that occurs during inflammation.

Table 2.3. Examples of acute phase proteins

Positive rat acute phase proteins	Negative rat acute phase proteins
α_1- Proteinase inhibitor	α_1-Inhibitor 3
α_1-acid glycoprotein	Albumin
α_2-macroglobin,	Prealbumin
Ceruloplasmin	
C-reactive protein	
Cysteine proteinase inhibitor	
Fibrinogen	
Haemopexin	
Haptoglobin	
Prekallikrein	
Transferrin	

Table 2.4. Metabolic effects of acute phase proteins. (Reprinted from Protein Pept Lett 9, Ceciliani, F., Giordano, A., and Spagnolo, V., The systemic reaction during inflammation: the acute phase proteins, pages 211-223, 2002, with permission from Bentham Science Publishers Ltd)

Neuroendocrine changes	Fever, Poor appetite and somnolence, Increased secretion of ACTH, Cortisol and Catecholamines.
Metabolic changes	Increased protein catabolism Hepatic production of APP Increased hepatic lipogenesis Increased adipose tissue lipolysis Decrease in bone mass Increased gluconeogenesis
Hematopoietic changes	Anemia (in chronic disease) Leukocytosis Thrombocytosis
Changes in non-protein plasma constituents	Hypozincemia, hypoferremia, and hypercupremia Increased plasma retinol and glutathione concentrations

2.1.3.2.3 Regulation

Glucocorticoids and growth factors regulate the activity of cytokines that in turn modulate the activity of the APR (as reviewed in Ceciliani et al., 2002). During the APR, the cytokine tumour necrosis factor-α (TNF-α) is released followed by interleukin-1β (IL-1β), and then IL-6 (van Deventer et al., 1990). The interplay between this network of cytokines is complex and not clear. It appears that IL-6 is induced by TNF-α and IL-1 but IL-6 also downregulates TNF-α (as reviewed in Ceciliani et al., 2002). The binding of IL-6 to its receptor causes the transcription factor nuclear factor IL-6 to translocate to the nucleus where it induces the transcription of APP and amplifies the APR. TNF-α activates NF-κB and this perpetuates an undesirable chain of inflammation.

2.1.3.3 Cellular damage

2.1.3.3.1 Lipid peroxidation

During conditions of oxidative stress, lipid peroxidation can disrupt cell membranes thereby altering membrane fluidity, decreasing cellular membrane potential, increasing permeability and ultimately leading to cell death (as reviewed in Halliwell and Chirico, 1993; Spatz, 1992). Lipid peroxidation can be mediated by free radicals (as reviewed in Halliwell and Gutteridge, 1990; Juurlink, 1999). A carbon centred lipid radical forms when a hydroxyl radical removes an electron from a polyunsaturated fatty acid (See reaction 15 in Table 2.1). This carbon centred lipid radical can react with oxygen to give rise to a lipid peroxyl radical (See reaction 16 in Table 2.1). When a lipid peroxyl radical reacts with a polyunsaturated fatty acid a lipid hydroperoxide is formed (See reaction 17 in Table 2.1). This lipid hydroperoxide can react with transition metals, generating more lipid peroxyl radicals (see reactions 18 and 19 in Table 2.1) and keeping

the chain of lipid peroxidation going. Lipid hydroperoxides are also formed via reactions involving myeloperoxidase. Activated monocytes/macrophages and neutrophils contain myeloperoxidase, which generates hypochlorous acid (See reaction 20 in Table 2.1). Hypochlorous acid can give rise to lipid hydroperoxides.

Transition metal ions such as iron affect the rate of lipid peroxidation (as reviewed in Aust et al., 1990). Fe^{2+} is autooxidized to Fe^{3+} by oxygen (See reaction 21 in Table 2.1), as well as peroxides including lipid peroxides and the hydroxyl radical. Lipid hydroperoxides can react with Fe^{3+} giving rise to lipid peroxyl radicals. When iron is chelated by iron binding proteins the rate of lipid peroxidation decreases (as reviewed in Gutteridge and Halliwell, 1989). Therefore, iron-binding proteins play an antioxidant role. During conditions of oxidative stress, damage to iron binding proteins increases the rate of lipid peroxidation.

2.1.3.3.2 Protein damage

Oxidative stress disables the normal functioning of many proteins, some of which include key antioxidant proteins, phosphatases, mitochondrial proteins and transcription factors. Given that free metal ions such as iron exacerbate conditions of oxidative stress, iron binding proteins such as ferritin and hemoglobin are key antioxidant proteins. Free radicals cause iron-binding proteins to release iron (as reviewed in Gutteridge and Halliwell, 1989). For example, in stimulated microglia superoxide caused ferritin to release iron (Yoshida et al., 1995). The antioxidant enzyme GR is also susceptible to oxidative stress. It is inactivated by advanced glycation products (Morgan et al., 2002), nitrosylation (Fujii et al., 2000), hydroxynonenal, an oxidative lipid breakdown product,

and sulfhydryl oxidation (Becker et al., 1998). Inactivation of GR disrupts GSH homeostasis.

In addition to antioxidant proteins, other proteins such as phosphatases are affected by oxidative stress. Phosphatases contain sulfhydryl groups that act like switches being activated and inactivated by reduction/oxidation reactions during normal physiological reactions. Certain ROS such as hypochlorous acid easily oxidize sulfur amino acids (Winterbourn, 2002). Therefore, theoretically excess hypochlorous acid can inactivate phosphatases disrupting cellular signalling pathways.

Several mitochondrial proteins, such as cytochrome oxidase and mitochondrial pore proteins are affected by oxidative stress. The enzyme, cytochrome oxidase that reduces oxygen in the electron transport chain, can be bound reversibly by NO• (as reviewed in Moncada and Erusalimsky, 2002). The ratio between NO• and $O_2•^-$ is suggested to act as an oxygen sensor for oxygen usage (as reviewed in Moncada and Erusalimsky, 2002). Moncada et al., (2002) suggest that inhibition of cytochrome oxidase by excess NO• results in more available oxygen, ATP depletion and possibly cell death (as reviewed in Moncada and Erusalimsky, 2002).

As electrons are moved through the electron transport chain in the inner mitochondrial membrane, protons are moved from the mitochondrial membrane into the intermembrane space, generating an electrochemical potential (Lehninger, 1995; as reviewed in Moncada and Erusalimsky, 2002). The proton motive force is used to generate ATP (as reviewed in Moncada and Erusalimsky, 2002) and is also associated with calcium accumulation in the cell (as reviewed in Duchen, 2004). Increases in extramitochondrial calcium result in an uptake of calcium into the mitochondria.

Intramitochondrial calcium exits via the Na^{+}/Ca^{2+} exchanger or the permeability transition pore. This pore appears to be regulated by sulfhydryls that during times of oxidative stress are oxidized and thereby opened. Opening of the pore is associated with cytochrome c release, which is pro-apoptotic. Irreversible opening of the pore leads to necrotic cell death (as reviewed in Duchen, 2004). Other than the mitochondrial transition pore**,** calcium release into the intracellular space occurs through other mechanisms. During oxidative stress, the oxidation of calcium binding proteins increases levels of intracellular calcium (as reviewed in Halliwell, 1994). Increased levels of calcium activate nucleases, proteases and other calcium dependant enzymes such as calpains and phospholipase C (as reviewed in Halliwell, 1994; Juurlink, 1999). The activation of calpains gives rise to superoxide whilst that of phospholipase C results in arachidonic acid release (as reviewed in Juurlink, 1999). Increased calcium is undesirable and leads to mitochondrial damage and consequently cell death (as reviewed in Juurlink, 1999).

The activity of proteins such as the transcription factor NF-κB is affected by oxidative stress. An oxidizing environment in the cytosol promotes NF-κB translocation. Prolonged conditions of oxidative stress can cause excess activation of NF-κB, perpetuating a cycle of chronic inflammation (Makarov, 2000).

2.1.3.3.3 DNA damage

Free radicals such as the hydroxyl radical, NO• and lipid breakdown products such as hydroxynonenal react with DNA to affect replication, signalling cascades and the cell cycle (Moncada and Erusalimsky, 2002). Consequently, DNA mutations can cause cancer, thereby increasing inflammation, oxidative stress and/or apoptosis. Mitochondrial DNA is particularly susceptible to oxidative attack. It does not contain histones and

therefore it is highly accessible to free radicals. Mitochondrial DNA is in close proximity to the electron transport chain, which is a major generator of superoxide. This increases the probability of superoxide reacting with mitochondrial DNA. Mitochondrial DNA mutations, by disrupting the normal activity of genes involved in energy production, may interrupt ATP synthesis ultimately leading to cell death.

2.1.4 SHRsp as a model of inflammation

2.1.4.1 Background

The animal model of inflammation used in this project is the SHRsp. This rat model was selectively bred from a strain of spontaneously hypertensive rats (SHR) in 1974 (Okamoto et al., 1975). The spontaneously hypertensive rats were derived from brother-sister breeding of Wistar Kyoto rats (WKY). SHRsp are well characterized as a model for studying stroke pathogenesis and hypertension. They are less characterized as a model for studying premature ageing, mental impairment and insulin resistance. Underlying many of these conditions is oxidative stress. Antioxidant therapy is effective at decreasing oxidative stress and consequent inflammation in SHRsp.

2.1.4.2 Stroke pathogenesis

Whilst the blood pressure of spontaneously hypertensive rats reaches 200 mm Hg, that of the SHRsp reaches up to 230 mm Hg. SHRsp develop hypertension over time that is linked to stroke incidence (Okamoto et al., 1975). At blood pressures under 200 mm Hg, stroke occurrence is not common (Okamoto et al., 1975). Lowering blood pressure decreases stroke incidence in the SHRsp (Okamoto et al., 1975). SHRsp experience both cerebral hemorrhage and infarcts and inflammation appears to underlie stroke pathogenesis.

APP are indicators of inflammation, and APP that correlate with stroke onset are detected in the serum of SHRsp (Sironi et al., 2001). Stroke onset was delayed by the administration of the statin, cervistatin (Kawashima et al., 2003). Since cervistatin decreased parameters of oxidative stress in the brain such as infiltrating inflammatory cells and excess superoxide production (Kawashima et al., 2003), a decrease in oxidative stress appears to be responsible for delaying stroke onset in SHRsp.

Diet plays an important role in the timing of stroke onset in SHRsp. The administration of a diet with a lower protein content causes strokes to occur quicker in SHRsp (Sarwar et al., 1999, Wexler, 1983). Also stroke occurs sooner in SHRsp consuming 1% sodium chloride (NaCl) than in SHRsp on tap water. Supplementation with NaCl and a diet low in protein accelerate blood pressure increases and decrease longevity from 10 months to approximately 3-4 months (Sarwar et al., 1999, Sironi et al., 2001). Therefore the diet of SHRsp can be manipulated to offset the timing of stroke occurrence. However the genetics of these animals still predisposes them to other diseases such as hypertension.

2.1.4.3 Hypertension

SHRsp are a useful model for studying hypertension. Blood pressure is largely affected by arteriolar radius and blood viscosity (Sherwood, 1997). Factors that contribute to arteriolar radius include properties of the smooth muscle cells' and blood volume regulation. The myogenic response (spontaneous contractile ability of smooth muscle cells) and signalling molecules such as NO· and endothelin regulate smooth muscle cell contractility. Compounds such as angiotensin, and the sympathetic and parasympathetic nervous system regulate blood volume. During hypertension**,** blood

viscosity or arteriolar radius can be increased resulting in higher blood pressure. Several causes of hypertension include renal dysfunction, angiotensin deregulation, NO· shortage, improper functioning of baroreceptors, or endocrine problems whereby the adrenal cortex may produce too much aldosterone (Sherwood, 1997). Many of the characteristics of primary hypertension such as renal dysfunction, problems with angiotensin, and endothelial dysfunction, have been observed in SHRsp (Griffin et al., 2001; Hamilton et al., 2002; Park et al., 2002) making them a good model of hypertension.

2.1.4.3.1 Renin-angiotensin aldosterone-system (RAAS) and renal dysfunction

RAAS plays a role in sodium (Na^+) reabsorption (Sherwood, 1997). The kidneys produce renin that converts angiotensinogen (produced in the liver) into Angiotensin I (Ang I). Ang I is converted into Angiotensin II (Ang II) via angiotensin converting enzyme (ACE) (produced in the lungs). Ang II increases vasopressin, which increases water retention in the kidney, causes vasoconstriction and acts on the adrenal cortex to release aldosterone. The overall effect of RAAS is Na^+ retention that results in an increased plasma volume.

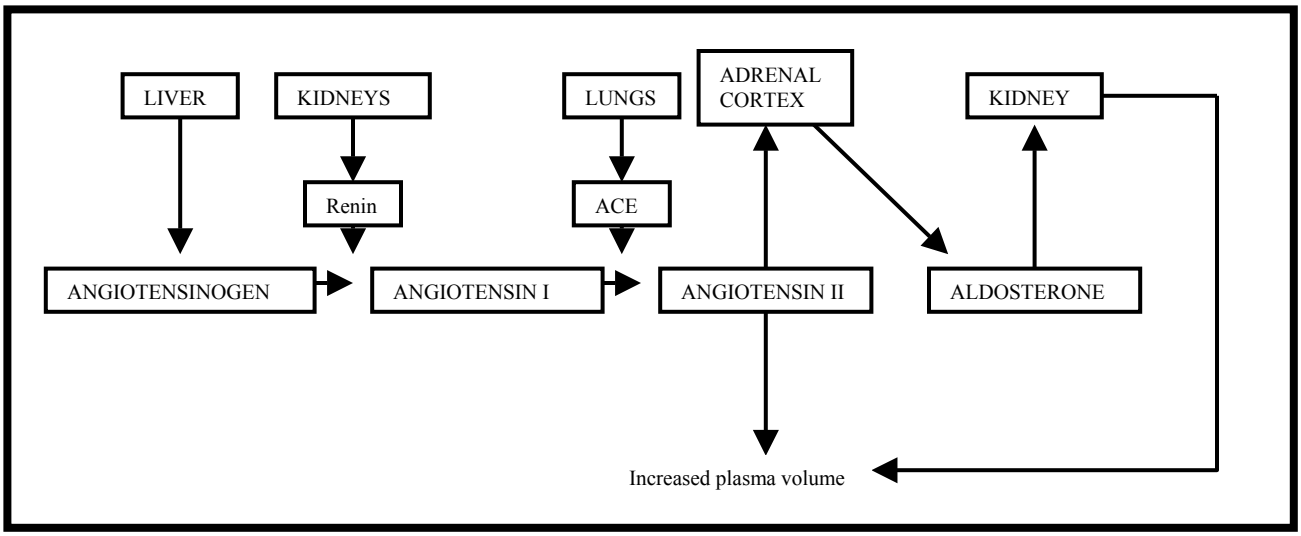

Figure 2.3. The renin-angiotenisin-aldosterone system

Ang II plays a role in cell proliferation/growth, blood pressure regulation, aldosterone release, sodium resorption, and serves as a pro-inflammatory mediator. Ang II is associated with endothelial dysfunction that is exhibited by an increased expression of adhesion molecules and activated monocytes. Ang II increases the pro-inflammatory cytokine IL-6, activates NADPH oxidase that generates superoxide, increases endothelin production, and activates NF-κB activity (as reviewed in Ruiz-Ortega et al., 2001). Monocytes in vascular lesions release ACE therefore increasing levels of Ang II (as reviewed in Ruiz-Ortega et al., 2001). Increased levels of Ang II result in higher levels of oxidative stress and have been implicated in the pathogenesis of hypertension in SHRsp. Treatments targeting the RAAS in SHRsp are effective at reducing hypertension (Romero and Reckelhoff, 1999) and also renal dysfunction. SHRsp are a good model of renal dysfunction. Proteinuria is indicative of kidney disease and in some cases of the overproduction of plasma proteins (as reviewed in Schrier, 2003). SHRsp fed a high salt/fat diet, which increases blood pressure, experience albuminuria, a marker of renal dysfunction (Ju et al., 2003). SHRsp are salt sensitive and this sensitivity is associated with their hypertension susceptibility and renal damage (Griffin et al., 2001).

Renal dysfunction in SHRsp is dependent on blood pressure, and therefore drugs that lower blood pressure are also renoprotective. For example Ang I receptor antagonists, ACE inhibitors, and aldosterone antagonists are renoprotective and lower blood pressure (Abrahamsen et al., 2002; Griffin et al., 2003; Griffin et al., 2001). Ang II increases levels of aldosterone. In SHRsp, the administration of the ACE inhibitor, captopril, reduced blood pressure but not when combined with aldosterone infusion (Rocha et al.,

1999). This study suggests that aldosterone is a key downstream player in RAAS regulating blood pressure in SHRsp.

2.1.4.3.2 NO· bioavailability

Endothelial cells produce vasoconstrictors, such as endothelin-I, ACE, superoxide anions and vasorelaxants such as NO· (also known as Endothelial derived relaxation factor) (as reviewed in Puddu et al., 2000). An imbalance between vasoconstrictors and vasodilators contributes to high blood pressure (as reviewed in Safar and Frohlich, 1995). Characteristic of hypertension is endothelial dysfunction whereby an increase in superoxide reduces NO• bioavailability (as reviewed in McIntyre et al., 1999; Zalba et al., 2001). NO· produces renal vasodilation that leads to diuresis and natriuresis that lowers blood pressure (as reviewed in McIntyre et al., 1999). Also, NO· acts on smooth muscle cells to activate guanylate cyclase, increasing 3'5' guanosine monophosphate (cGMP) that causes smooth muscle cell relaxation (as reviewed in Puddu et al., 2000). A decrease in NO· compromises smooth muscle cell relaxation and therefore contributes to hypertension. Causes for endothelial dysfunction may be mediated by the kinase, p38 mitogen activated kinase (MAPK).

NO· bioavailability is lower in SHRsp as compared to female WKY, which are a normotensive rat strain. In hypertension, NADPH oxidase is the main source of superoxide (as reviewed in Zalba et al., 2001). Immunocytochemical staining for p22phox (reflective of NADPH oxidase) was higher in SHRsp than WKY as was the concentration of superoxide generated (Hamilton et al., 2001). NADPH oxidase inhibitors prevent the translocation of the various NADPH subunits. By using apocynin, a

NADPH oxidase inhibitor, NO· bioavailability increased as was evidenced by more vasorelaxation (Hamilton et al., 2002). These studies demonstrate the important role played by NADPH oxidase in increasing NO· bioavailability. Levels of superoxide have been shown to be higher in SHRsp than in WKY (Kerr et al., 1999). In 16 week old male SHRsp fed a diet containing 4% NaCl, administration of the SOD mimetic tempol reduced blood pressure, increased the lumen diameter and decreased the width of the media, decreased superoxide production and increased total plasma antioxidant status (Park et al., 2002). This study by Park et al. validates the crucial role played by superoxide in hypertension. Overall a decrease in superoxide, either by inhibiting NADPH oxidase or by increasing SOD, can decrease high blood pressure.

The improper functioning of baroreceptors is associated with NO· bioavailability. Baroreceptors in the carotid sinus and aorta detect changes in blood pressure and act to regulate blood volume. In the brain stem, the nucleus tractus solitarii and the caudal ventrolateral medulla contain the baroreflex pathway. Transfection of endothelial nitric oxide synthase into the brainstem of male SHRsp resulted in an improved baroreceptor reflex (Kishi et al., 2003). Endothelial nitric oxide synthase is responsible for producing NO· in the endothelium.

2.1.4.3.3 Smooth muscle cell proliferation

ROS may act via Ang II activation of NF-κB giving rise to vascular smooth muscle cell hypertrophy (as reviewed in Zalba et al., 2001) (Irani, 2000). Vascular smooth muscle cell hypertrophy is characteristic of hypertension (as reviewed in Zalba et al., 2001) (Irani, 2000) and has been characterized in SHRsp and SHR. This increased

cellular proliferation has been attributed to a decrease in apoptosis (McCarthy and Bennett, 2000).

2.1.4.3.4 Role of p38 MAPK

Causes of endothelial dysfunction are proposed to act via p38 MAPK activation. In human umbilical vein endothelial cells, TNF-α activated p38 MAPK and this resulted in an increase in intracellular molecular adhesion molecule expression. Administration of p38 MAPK inhibitors blocked this effect (Ju et al., 2003). In SHRsp fed a high salt/fat diet, p38 MAPK inhibitors decreased blood pressure, increased vasorelaxation and decreased mortality (Ju et al., 2003). As evidenced by this study, p38 MAPK plays an important role in mediating blood pressure and vasorelaxation in the SHRsp. However given that oxidative stress can activate various signalling pathways and also that there is cross talk throughout the tier of kinase cascades**,** it is plausible that p38MAPK is acting in concert with other components of signalling pathways to give rise to the effects observed in Ju's study.

2.1.4.4 SHRsp as models of disease

2.1.4.4.1 Premature aging

SHRsp are described as a model of premature ageing. Parameters of oxidative stress such as superoxide or activated inflammatory cells increase with age. Levels of messenger ribonucleic acid (mRNA) levels for p22phox, a subunit of NADPH oxidase, were higher in older SHRsp than in the younger SHRsp (Hamilton et al., 2002). Levels of mRNA for p22 phox are also higher in SHRsp than in WKY (Hamilton et al., 2001). NO· bioavailability was also higher in younger SHRsp than in older SHRsp (Hamilton et al., 2001) When male 10 month old SHRsp were compared to age matched WKY rats fed

standard rat chow, parameters of central nervous system inflammation such as activated macrophages, nitrotyrosine positive cells and demyelination were increased in SHRsp (Ashraf et al., 2004 submitted).

2.1.4.4.2 Mental impairment

SHRsp are described as a model of attention deficit hyperactivity disorder and also of vascular dementia. Both male and female SHRsp who were 4 weeks old and fed standard rat chow were found to be more hyperactive, impulsive and memory impaired than age matched WKY (Ueno et al., 2002). Features of vascular dementia such as impaired learning-memory and decreased levels of brain acetylcholine and choline are observed in SHRsp (Kimura et al., 2000). However no senile plaques or tangles were detected. SHRsp are described as a "useful" model for studying vascular dementia (Kimura et al., 2000).

2.1.4.4.3 Insulin resistance

A comparison of 3 month old WKY to SHRsp demonstrates that in SHRsp glucose uptake was reduced in response to insulin (Collison et al., 2000). SHRsp may serve as a useful model for understanding mechanisms underlying diabetes. There is a strong link between oxidative stress and insulin resistance (Pessler et al., 2001; Tirosh et al., 1999).

2.1.4.5 SHRsp and oxidative stress

There are clear links between oxidative stress and the models represented by SHRsp. Increased levels of superoxide, advanced glycation products, APP, 8-hydroxy-2'-deoxyguanosine**,** activated immune cells, Ang II and decreased NO· bioavailability are present in SHRsp (Mizutani et al., 2000) (Morgan et al., 2002) (Romero and Reckelhoff,

1999) (Zalba et al., 2001) (Sironi et al., 2001). To what extent oxidative stress is a cause or a consequence of the pathologies seen in SHRsp is not clear. Regardless of this conundrum SHRsp are still a good model of oxidative stress and therefore inflammation.

2.1.4.6 Dietary intervention studies

Blood pressure, superoxide production and NADPH activity were all reduced in 16 week old SHRsp fed a diet containing 4% NaCl and supplemented with either vitamin E or C (Chen et al., 2001). Dietary administration of the polyphenolic compound, Resveratrol, a phase 2 enzyme inducer, was found to decrease levels of oxidative stress in SHRsp (Mizutani et al., 2000). Consumption of either black or green tea lowered blood pressure in SHRsp (Negishi et al., 2004). In cultured aortic smooth muscle cells form SHRsp, the isoflavones, genistein, daidzein and glycitein reduced smooth muscle cell proliferation (Pan et al., 2001). Dietary administration of antioxidant promoting agents decreases oxidative stress in SHRsp.

2.2 Dried Broccoli Sprouts

2.2.1 Profile of sprout metabolites

The genus brassica is one of approximately 350 genera in the cruciferae (also known as the brassicaceae) family of vegetables (as reviewed in Fahey, 2001). The cruciferae family, characterized by 4 petals or sepals, consists of subspecies such as broccoli, kale, brussel sprouts and cauliflower (as reviewed in Verhoeven et al., 1997). In our diets**,** the consumption of crucifers is encouraged for their anti-carcinogenic properties. Profiles of crucifers such as broccoli have been shown to contain vitamins, fibre, and phytochemicals such as flavonoids, glucosinolates**,** ITCs and phenols. Identifying the roles played by various compounds in crucifers has pointed to broccoli

sprouts having protective effects due to their high glucosinolate content (Zhang et al., 1992).

Cruciferous plants synthesize glucosinolates, which are β-thioglucoside *N*-hydroxysulfates that are known as N-hydroxyiminosulfate esters or S-gluopyranosyl thiohydroximates. They contain a side chain and a β-D-glucopyranose moiety attached to sulfur. Glucosinolates vary by having either straight or branched side chains, which may contain hydroxyl, or carbonyl, or sulfur groups that confer different properties upon them. Some brassica vegetables contain glucosinolates that are goitrogenic in certain animals, for example, rapeseed in swine and brussel sprouts in rats (Verhoeven et al., 1997). The effects of goitrogenicity are species and diet dependant. Plant injury, mastication or freeze- thaw cycles cause the conversion of glucosinolates to isothiocyanates via the enzyme myrosinase. Myrosinase, which is present in plants and the microflora of the digestive tract, breaks the *β*-thiogluoside bond of glucosinolates such as glucoraphanin giving rise to glucose, sulfate and aglucons such as the isothiocyanate Sul.

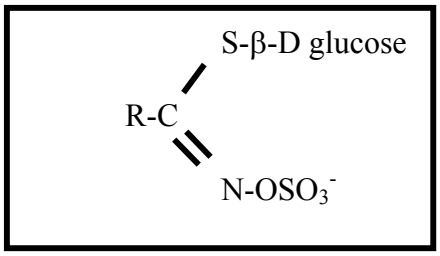

Figure 2.4. Diagram of a glucosinolate.

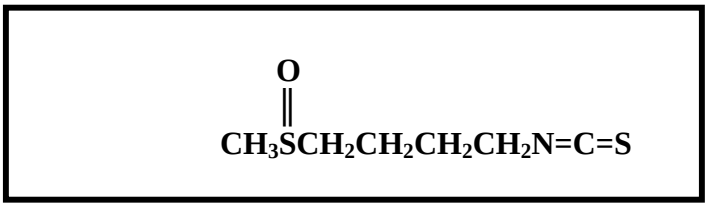

Figure 2.5. Structure of Sulforaphane

Environment, genetics and plant age affect the concentration of plant glucosinolates. For example, Jeffrey et al. (2003) suggests that plants that are more stressed due to handling and pests have higher levels of indole glucosinolates (e.g. gluconeobrassicin) as opposed to aliphatic glucosinolates such as glucoraphanin. Indole 3-carbinols are derived from trytophan whereas aliphatic glucosinolates are derived from methionine (as reviewed in Jeffery, 2003). Profiling of 2 day old broccoli sprouts revealed that less than 10% of the total sprout glucosinolates were indole glucosinolates (glucobrassicin and neoglucobrassicin) compared to 67% in the mature plant (Fahey et al., 1997). Levels of methylsulfinylalkyl glucosinolates (glucoraphanin and glucoerucin) were 20 times higher in the sprouts (Fahey et al., 1997).

Certain glucosinolates give rise to monofunctional inducers whilst others give rise to bifunctional inducers. For the most part, monofunctional inducers affect phase 2 enzymes whilst bifunctional inducers affect both phase 1 and phase 2 enzymes. Indole glucosinolates such as indole 3- carbinol and indole 3- nitrile when treated with myrosinase give rise to bifunctional inducers such as 3,3'-diidolylmethane and indole –3-carbazole (Bjeldanes et al., 1991). In contrast, the glucosinolate glucoraphanin gives rise to the monofunctional inducer, Sul. Monofunctional inducers such Sul are chemoprotective whereas bifunctional inducers have the potential to be pro-carcinogenic. Understanding the role of plant genetics, environment and age with regards to glucosinolate content is important for obtaining plants that are high in compounds such as Sul.

2.2.2 Sulforaphane

Sul, the major inducer of phase 2 protein (Zhang et al., 1992), is protective against inflammation and cancer causing agents. The protective effects of Sul have been attributed to its ability to induce phase 2 enzymes, inhibit phase 1 enzymes, and affect cell cycle arrest and apoptosis (See figure 2.6). Of particular interest to this study is Sul's role with respect to phase 2 enzyme induction.

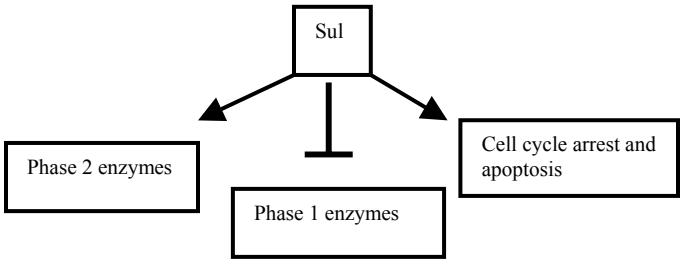

Figure 2.6. A proposed mechanism by which Sul provides protection.

2.2.2.1 Phase 2 enzymes

2.2.2.1.1 Overview of phase 2 enzymes

Xenobiotics or foreign compounds elicit various responses including homeostasis, proliferation, differentiation, apoptosis or necrosis (as reviewed in Rushmore and Kong, 2002). The body responds to xenobiotics by inducing phase 1 and/or phase 2 enzymes (as reviewed in Rushmore and Kong, 2002). Phase 1 enzymes (see section 2.2.2.2) are involved in oxidative reactions whereby xenobiotics can be rendered more electrophilic (as reviewed in West et al., 1997). Phase 2 enzymes then act on these toxic metabolites making them more readily excretable or less harmful to cells (Kirlin et al., 1999). Phase 2 enzymes also contribute to maintaining the redox balance of the cell. Members of the phase 2 enzyme family include QR, GST, epoxide hydrolase, thioredoxin reductase,

gamma glutamyl cysteine ligase, GR, and heme oxygenase-1 (Thimmulappa et al., 2002, Hintze et al., 2003).

Xenobiotics such as Sul that regulate levels of phase 2 enzyme activity are known as phase 2 enzyme inducers. Characteristic of most phase 2 enzyme inducers are the following: they are chemically reactive, electrophilic, GST substrates and they can alter sulfhydryl groups (Dinkova-Kostova et al., 2001). The following sections deals with Sul's induction of phase 2 enzymes and the possible involvement of nuclear factor-erythroid 2p45-related transcription factor (Nrf2) and NF-κB, mediated mechanisms of phase 2 enzyme induction.

2.2.2.2.2 Sulforaphane and phase 2 enzymes

Sul is protective against the administration of pro-carcinogenic compounds (Fahey et al., 2002; Zhang et al., 1994) and during conditions of oxidative stress (Wu et al., 2004; Wu and Juurlink, 2001). This protection may be due to Sul's ability to induce phase 2 enzymes. Several studies have shown that Sul directly induces phase 2 enzymes. For example, in a human hepatocyte carcinoma cell line (HepG2), in cells that were transfected with thioredoxin reductase luciferase reporter construct, the administration of 0.5, 1.0, or 2 μM of Sul for 24 hours resulted in a dose dependant increase of thioredoxin reductase activity (Hintze et al., 2003). In mouse intestine a dose of 3 μmol /gram Sul for 2 weeks induced QR activity and GST activity (McMahon et al., 2001). In female mice liver**,** GST and QR activities were increased by giving 15 μM of Sul for 5 days (Zhang et al., 1992). In smooth muscle cells from both spontaneously hypertensive and normotensive rats, Sul concentrations of 0.05-0.5 μM were shown to induce GR (Wu and Juurlink, 2001). In stroke prone spontaneously hypertensive rats**,** the administration of

dried broccoli sprouts induces GR in the aorta, carotid and kidney (Wu et al., 2004). These studies provide evidence that Sul induces phase 2 enzymes. A plausible mechanism for this phase 2 enzyme induction is via the transcription factor Nrf2.

2.2.2.2.2.1 Nrf2 overview

Phase 2 enzymes are induced via the antioxidant response element (ARE), which is responsive to various inducers (phenolic antioxidants and aromatic compounds) as well as H_2O_2 (Rushmore et al., 1991). The core sequence of the rat ARE is: 5' (G/A) TGA (G/C) NNNGC (G/A)-3' (Rushmore et al., 1991). A key player in the regulation of the ARE is Nrf2. The Nrf family is comprised of Nrf1, 2, and 3. Nrf1 is thought to play a role during biological development, whilst Nrf2 is implicated in regulating the basal (Huang et al., 2000) and inducible levels of phase 2-enzymes.

2.2.2.2.2.2 Nrf2 regulation

Nrf2 mRNA levels stay the same upon Nrf2 activation and translocation into the nucleus, therefore Nrf2 is regulated post transcriptionally (Nguyen et al., 2003). In the cytosol Nrf2 is regulated by Kelch-like associated protein (Keap1), a negative repressor of Nrf2 translocation. In the nucleus, Nrf2 heterodimerizes with Maf proteins, Fos-B, C-jun, JunD, and activating transcription factor 2 (as reviewed in Hayes and McMahon, 2001; Motohashi et al., 1997). These partners may regulate Nrf2's activity in the nucleus and consequently the transcription of phase 2 enzymes.

Nrf1 and 2 have been shown to heterodimerize with Maf G and Maf K**.** The latter are members of the Maf family of proteins which is made up of large proteins (c-Maf and MafB) and small proteins (Maf K, Maf F and Maf G). Recently it has been found that Maf K is a repressor of the ARE (Nguyen et al., 2000). Maf K and Nrf2 can bind to the

QR ARE with high affinity whereas they bind to the GST ARE with low affinity (Nguyen et al., 2000). The ability of this Nrf2/ MafK complex to bind with such high affinity seems to be dependant on the presence of a palindromic sequence in the QR ARE (Nguyen et al., 2000). Maf K repression reduces QR basal expression but not its inducible expression. The addition of inducers still leads to an increase in QR, despite there being an over expression of Maf K (Nguyen et al., 2000). Palindromic structures in the ARE of various phase 2 enzymes may determine the affinity with which Nrf2/Maf complexes bind. The regulation of phase 2 enzymes may depend on the type of Maf protein that Nrf binds to and the palindromic structures in the ARE of various genes.

Nrf2 has 6 domains namely, Neh1-6. Neh1 is a basic leucine zipper domain, Neh 4 and 5 are acidic domains and Neh6 is a serine rich domain (See figure 2.7) (Itoh et al., 1999) (Itoh et al., 1999). Neh4 and Neh5 are transactivation domains unlike Neh2, which contains a conserved carboxy hydrophilic region. Neh2 is the Keap 1 binding site. Keap1 is a negative repressor of Nrf2 translocation (Itoh et al., 1999). It regulates Nrf2 by keeping it bound in the cytosol until the appropriate signal is propagated. Deletion of Neh2 results in more potent transactivation of the ARE (Itoh et al., 1999). It is not known where Nrf2's nuclear translocation signal is located. It is possible that Keap1 masks this signal, thus maintaining Nrf2 in the cytosol. The location of Nrf2's nuclear translocation signal would help in understanding how Nrf2 translocates into the nucleus.

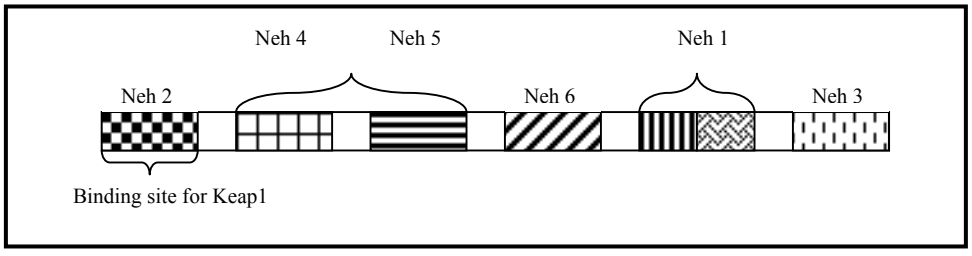

Figure 2.7. A schematic of Nrf2.

Keap 1 has 5 domains, which include a double glycine repeat motif, the BTB domain, the intervening region (IVR), which has 8 cysteines, an N-terminal repeat and a C-terminal region, (Dinkova-Kostova et al., 2002). The DGR motif is used to contact the cytoskeleton while the BTB interaction domain of Keap 1 is thought to play a role in the dimerization of Keap 1 and Nrf2 (Zipper and Mulcahy, 2002). Deletion of this domain in HepG2 cells hinders the ability of Keap 1 to sequester Nrf2 in the cytoplasm (Zipper and Mulcahy, 2002). Therefore the Neh2 domain of Nrf2 binds to the BTB domain of Keap 1. Sulfhydryl oxidation may release Nrf2 from Keap 1, in the cytosol. Murine Keap 1 has 25 cysteines and Nrf2 has 7 cysteines (Dinkova-Kostova et al., 2002). The cysteine residues in Keap1 are all reactive but there are 4 in particular in the IVR, which demonstrate the highest reactivity to compounds, such as sulforaphane (Dinkova-Kostova et al., 2002). The Neh2 domain of Nrf2 does not contain cysteine residues that are acted upon by inducers (Dinkova-Kostova et al., 2002). Inducers may react with the sulfhydryls in the IVR on Keap 1, changing the conformation of Keap 1, and releasing Nrf2. Cysteine reactivity is one of the mechanisms whereby Nrf2 is released. Kinases may also play a role in a phosphorylation event that results in Nrf2 release from Keap1.

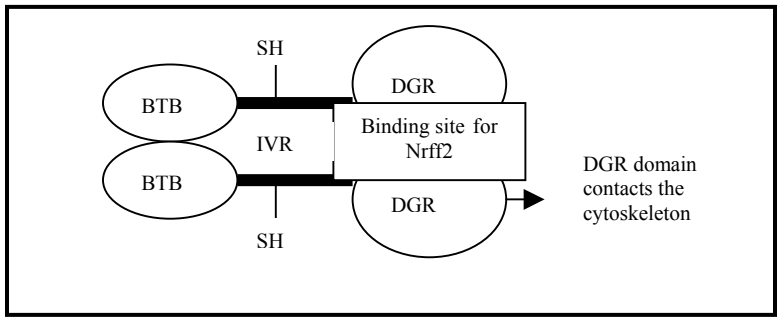

Figure 2.8. A schematic of Keap1.

Classical MAPK signalling involves phosphate additions through a 3 tiered cascade consisting of mitogen activated kinase kinase kinase (MAPKKK) (transforming growth factor-β activated kinase-1 (TAK1), apoptosis signal-regulating kinase 1(ASK1), Raf and MAPK/extracellular signal-regulated kinase kinase (MEKK1) to mitogen activated kinase kinase (MAPKK) (MAPKK 1-7 and MEK 1-5), and lastly to MAPK (p38, c-Jun NH_2-terminal kinase (JNK) and extracellular signal regulated kinase (ERK)), (See figure 2.9) (Kyriakis and Avruch, 2001). Examples of some other kinases that are not in this 3 tiered cascade include protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3K). It is important to keep in mind that MAPKs in different tiers do not always signal to the same downstream kinase. Instead, upstream kinases add phosphate groups onto an array of other downstream kinases. This introduces a tiered cascade that is not necessarily linear and therefore activation of a specific kinase will not always elicit the same response.

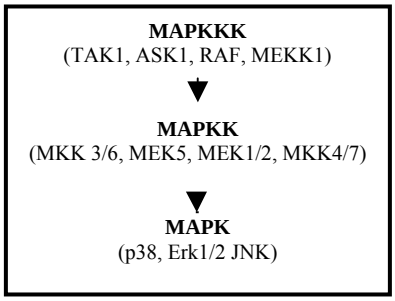

Figure 2.9. A schematic of the tiered MAPK families.

Some kinases, such as ASK1, are associated with phosphatases. ASK1 is bound to protein phosphatase 5 (PP5) and Trx in the cytosol (Morita et al., 2001). The redox sensor, Trx along with PP5, regulate ASK1 activity. Therefore kinase activation is dependant on other factors such as phosphatases. The regulation of kinases is complex and depends on the conditions at the time of activation. For example, ASK1 can be

activated to induce apoptosis (Morita et al., 2001) and/or phase 2 enzymes (Yu et al., 2000). This adds to the complexity of finding consistent themes in the literature that explain Nrf2 activation.

Several kinases have been implicated in Nrf2 activation, namely: MEKK1, TAK1, ASK1, JNK, ERK2, p38, phosphatidylinositol 3-kinase (PI3K), and protein kinase C (PKC). In HepG2 cells treated with tBHQ (100 μM), sodium arsenite and mercury chloride, MEKK1, TAK1 and ASK1, were activated (Yu et al., 2000). These kinases were thought to act via MKK4 and MKK6 to induce the ARE (Yu et al., 2000c). In human epithelial cancer cells (HeLa) and HepG2 cells the MEK inhibitor, PD98059, prevented Erk2 activation of the ARE but did not affect JNK activation induced by tBHQ (500 μM) and butylated hydroxyanisole (BHA) (250 μM) (Yu et al., 1997). In human and mouse hepatoma cells, tBHQ (50-100 μM) and Sul (12.5-50 μM) activates ARE reporter activity, via Erk2 and not JNK (Yu et al., 1999). Oxidative stress activates MEKK2 and this led to the activation of ERK2, but not JNK (Yu et al., 1997). These studies suggest that tBHQ may act via MEKK to activate Erk2 and induce phase 2 enzymes. The role of JNK is not clear.

p38 MAPK can either negatively regulate the ARE or have no effect on it. In HepG2 and in murine hepatoma Hepa1c1c7, inhibition of p38 resulted in greater QR activity, in the presence of tBHQ (Yu et al., 2000b). It appears that p38 negatively regulates the ARE (Yu et al., 2000b). Conversely, in HepG2 cells that were treated with phorbol 12-myristate 13 acetate (PMA), inhibition of MEK and p38 kinases had no effect on ARE reporter activity (Huang et al., 2000). In H4IIE cells, a rat hepatoma cell line, which was administered tBHQ, Erk negatively regulates the ARE whereas p38 MAP

kinase and JNK had no effect on phase 2 enzyme induction (Kang et al., 2001). This suggests that in the same cell type, different inducers may signal through different pathways; in different cell types, the same inducer may signal via different pathways.

PI3K may contribute to Nrf2 translocation possibly by acting through the cytoskeleton. PI3K has an implicated role in microfilament assembly, and the translocation of actin proteins (Kang et al., 2002). PI3K is thought to play a role in Nrf2 activation. H4IIE cells were examined to look at the effects of tBHQ on phase 2 enzyme induction, with an emphasis on the role of MAPK and PI3K (Kang et al., 2001). Activation of PI3K resulted in GST induction (Kang et al., 2001), Nrf2 translocation to the nucleus and increases in the amount of nuclear actin (Kang et al., 2002). Administration of cytochalasin B that disrupts actin resulted in Nrf2 translocation, whereas phalladoin, which prevents actin breakdown, prevented Nrf2 translocation into the nucleus (Kang et al., 2002). Administration of Wortmannin, which inhibits PI3K, prevented all of these changes from happening (Kang et al., 2002)(Kang et al., 2001). PI3K may contribute to Nrf2 translocation possibly by acting through the cytoskeleton.

Sites that can be phosphorylated by PKC have been found on both Keap 1 and in the Neh2 domain of Nrf2. PKC is thought to increase ARE activity. Huang and his colleagues have shown that if Ser 40 on the Neh2 domain is mutated to alanine, Keap 1 will still interact with Nrf2. However, in the presence of the PKC inhibitor staurosporine, there was a decrease in the amount of ARE activity (Huang et al., 2002). Huang et al conclude that Nrf2 is phosphorylated by PKC, released from Keap 1 and then shuttled to the nucleus. It is possible that this regulatory step is required for ARE activation. In HepG2 cells, PMA was able to activate the ARE (Huang et al., 2000). Staurosporine and

Ro-320432, inhibitors of PKC prevented PMA activation of the ARE (Huang et al., 2000). Therefore, PKC phosphorylation appears to increase ARE activity. The role played by various kinases in signalling is unclear (See figure 2.10). This is not surprising given that inducers may be working within a very complex communication network of kinases and phosphatases. The effect of inducers may depend on several factors such as the type of inducer, the cell type, and the state that the cell is in i.e. proliferating or not (Owuor and Kong, 2002) and perhaps the basal level of oxidative stress that is present.

2.2.2.2.2.3 Nrf2 and phase 2 enzymes

Several studies have demonstrated that Nrf2 deficient animals are not able to withstand certain xenobiotics. For example, Nrf2 deficient mice are more susceptible than wild type mice to gastric neoplasia after administration of the carcinogen, benzo [a] pyrene (Ramos-Gomez et al., 2001). Also, in Nrf2 wild type mice, BHT results in temporary lung damage, by destroying alveoli type 1 epithelial cells. Seven days later, the lungs appear normal again. Nrf2 deficient mice died from doses of BHT that were well tolerated in wild type mice (Chan et al., 2001). BHT metabolites are not lethal in Nrf2 wild type mice. It is proposed that BHT depletes GSH, thereby damaging the lungs. The lungs have lower levels of GSH and were more susceptible to BHT damage, compared to the liver, which contains higher levels of GSH (Mizutani et al., 1987). Given that phase 2 enzymes contribute to elevating GSH concentrations, then the inability of Nrf2 deficient mice to upregulate phase 2 enzymes may explain their inability to withstand xenobiotics. N-acetyl cysteine (NAC), a GSH promoting compound, decreased the mortality of Nrf2 deficient mice who were administered with acetaminophen (Chan et al., 2001). In Nrf2 deficient mice, constitutive levels of GST and QR were reduced by 50-80% (Ramos-

Gomez et al., 2001). Unlike in Nrf2 wild type mice, when the phase 2 enzyme inducer oltipraz was given to Nrf2 deficient mice, their incidence of benzo [a] pyrene induced gastric tumours was not decreased. These studies provide evidence that Nrf2 plays a role in phase 2 enzyme induction.

A direct link between Sul and an Nrf2 mediated mechanism of phase 2 enzyme induction is evidenced in studies by Thimmulappa (2002) and Morimitsu (2002). Thimmulappa (2002), shows that Sul acts via Nrf-2 to induce phase 2 enzymes such as GST mu, alpha 1,2,and 3, γ-glutamylcysteine ligase regulatory and catalytic subunits in rat liver. Morimitsu (2002) demonstrates that in the small intestine of mice the administration of Sul induces phase 2 enzymes via Nrf2.

2.2.2.2.3 Sulforaphane and NF-κB

Phase 2 enzymes are not solely under the regulation of Nrf2. A study by Hayes (2000), demonstrates that ethoxyquin, a bird food preservative, increases the levels of several GST isoforms in wild type mice (as reviewed in Hayes et al., 2000). In Nrf2 deficient mice, GST expression is still present, suggesting that GST expression is not solely dependent on Nrf2 (as referenced in Hayes et al., 2000). Some genes have an ARE and/or NF-κB elements in their promoter regions that are responsible for phase 2 enzyme induction. Ishii et al. (2000) examined peritoneal macrophages from Nrf2 deficient mice. In the wild type mice, Nrf2 was shown to regulate genes such as heme oxygenase-1 (Ishii et al., 2000) that play a role in decreasing levels of oxidative stress. Immunoblot analysis in this study demonstrated that different inducers failed to induce heme oxygenase-1 in the Nrf2 deficient mice (Ishii et al., 2000). The inducer menadione slightly increased heme oxygenase-1 and the cystine transporter, which was thought to occur via NF-κB

(Ishii et al., 2000). Heme oxygenase-1 and the cystine transporter have NF-κB elements in their promoter regions and menadione has the ability to activate NF-κB (Ishii et al., 2000). This study demonstrates that Nrf2 is required for the activation of the ARE whilst oxidative stress may be sufficient for NF-κB activation resulting in phase 2 enzyme induction (See figure 2.10).

In terms of a link between Sul and NF-κB, it has been shown that during lipopolysaccharide induced oxidative stress in cultured macrophages, Sul prevented DNA binding of NF-κB, reducing the amount of inducible nitric oxide synthase and cycloxygenase 2 protein (Heiss et al., 2001). During oxidative stress, Sul may increase phase 2 enzymes and by causing a more reduced environment, indirectly affect the binding of transcription factors such as NF-κB (See figure 2.10). Alternatively, Sul may directly affect NF-κB signalling pathways.

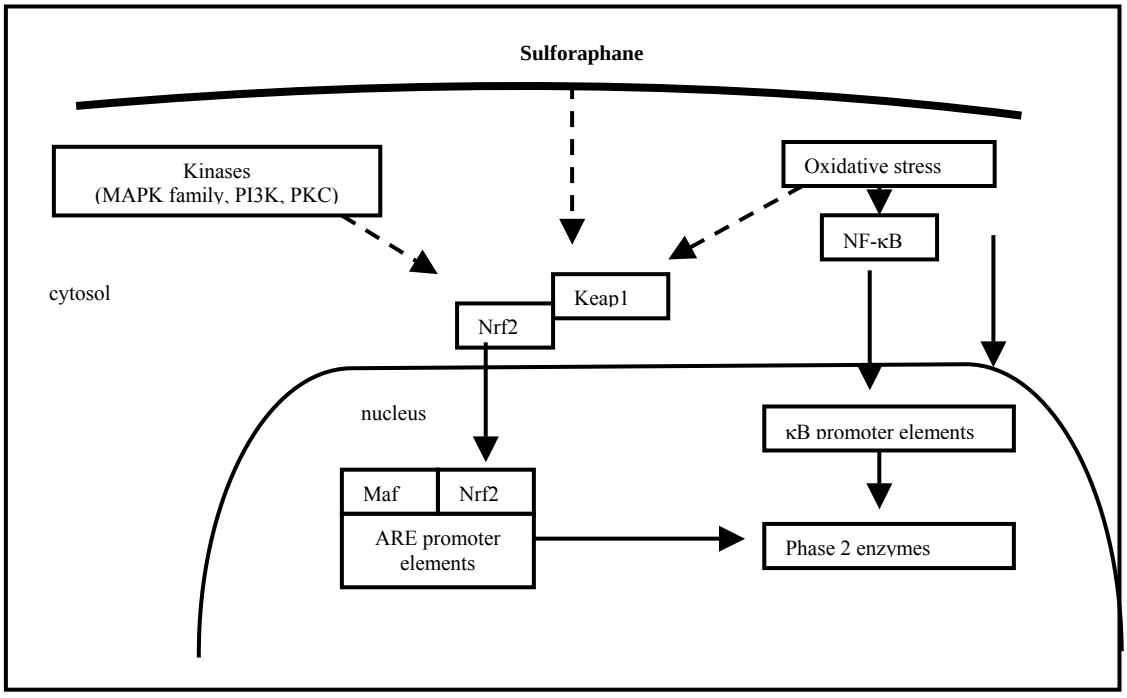

Figure 2.10. A proposed mechanism of phase 2 enzyme induction

2.2.2.2 Phase 1 enzymes

The protective effects of Sul may also be due to its inhibition of phase 1 enzymes. Amongst the family of enzymes that comprise phase 1 enzymes are the cytochrome P450 family which are found in the liver, gastrointestinal tract, lung and kidneys (as reviewed in Rushmore and Kong, 2002).

Polycyclic aromatic hydrocarbons, heterocyclic amines and nitrosamines are dietary carcinogens that when activated by phase 1 enzymes damage DNA (Steinkellner et al., 2001). Phase 1 enzymes are selectively inhibited and this inhibition is chemoprotective (Zhang and Talalay, 1998). In Salmonella typhimurium strain TA100 and T-antigen immortalized human liver epithelial cells, concentrations of Sul from 0.064-20 μmol/L inhibited N-nitrosodimethylamine induced unscheduled DNA synthesis via the inhibition of CYP2E1 (Barcelo et al., 1996; Barcelo et al., 1998) (Barcelo et al., 1998). Concentrations of Sul ranging from 0-40 μM were shown to inhibit several members of the human cytochrome family in E. coli membranes that express these human cytrochromes (Langouet et al., 2000).

2.2.2.3 Cell cycle arrest and Apoptosis

The protective actions of Sul may also be attributed to cell cycle arrest and or apoptosis. Concentrations of Sul ranging from 3-50 μM in human prostrate cells and in quiescent human colon carcinoma cells resulted in cell cycle arrest and apoptosis (Chiao et al., 2002; Gamet-Payrastre et al., 2000). Similar effects of Sul (concentration range of 3- 30 uM), were obtained in human T-cell leukemia cells by (Fimognari et al., 2002).

Inducers can be chemoprotective in that they promote apoptosis and/or detoxification (Kirlin et al., 1999). In human colon carcinoma cell line (HT29), administration of the phase 2 enzyme inducers, benzyl isothiocyanates, allyl sulfide, dimethyl fumarate, BHA, gave rise to an increase in phase 2 enzymes as well as to the activation of apoptosis (Kirlin et al., 1999). Kirlin points out that phase 2 enzyme induction and apoptosis may not be mechanistically linked together (Kirlin et al., 1999).

Mechanisms of protection by Sul are still not clear. Phase 2 enzyme induction, phase 1 enzyme inhibition, cell cycle arrest and apoptosis are all possible mechanisms of actions that may act in concert with each other or alone**,** depending on the context of administration. This study aims to explore Sul's effect on parameters of oxidative stress and inflammation in the blood and phase 2 enzyme induction in the liver of both male and female SHRsp.

Chapter 3 Materials and Methods

3.1 Source of Experimental Animals

SHRsp were initially obtained from the National Institute of Health and colonies were then bred by the College of Medicine Animal Facilities at the University of Saskatchewan. As it was not possible to acquire a sufficient number of rats for each experimental group at one time point, rats were collected as subsets over time, running from April 2002 to February 2003. Data were collected from a total of 5 subsets of animals. Tissue from the first and second subsets of animals was discarded as the timer that regulates the light switch to allow for 12 hour dark/12 hour light cycles was not functioning properly. The third and fourth subsets of animals were male SHRsp and the fifth subset was female SHRsp.

3.2 Source of diet and sprouts

The diet fed was purified 1993 Growth diet of the American Institute of Nutrition (AIN-93G) modified to contain no added antioxidant (tBHQ) (See Table 3.1) (Reeves et al., 1993).

Broccoli seeds (H6458) were obtained from Mumms Seeds and Sprouting (Shellbrook, Saskatoon, SK, Canada). These seeds were kept in a 10 % bleach solution for 10 minutes, washed and kept out of light for 3 days. The sprouted seeds were maintained in light and grown for 3 days after which time they were air dried for 7 days and were ready for usage. Analysis of these dried broccoli sprouts shows that they contain 27.3 μmols of sulforaphane per gram of sprouts (Wu et al., 2004).

Table 3.1 Composition of Experimental Diet[1]

Components	g/kg diet
Casein	200
Cornstarch	397.5
Dyetrose[2]	132
Sucrose	100
Cellulose	50
Soybean Oil	70
Salt Mix[3]	35
Sodium Bicarbonate	6.4
Vitamin Mix[4]	10
Choline Bitartrate	2.5
L-Cystine	3

[1]Diets were purchased from Dyets Inc. (Bethlehem, Pennsylvania, US).
[2]Dyetrose (dextrinized cornstarch)
[3]Salt mix supplied the following minerals (g/kg mix): calcium carbonate 357.0, monobasic potassium phosphate 196.0, potassium citrate·H_2O 70.78, sodium chloride 74.0, potassium sulfate 46.6, magnesium oxide 24.0, ferric citrate 6.06, zinc carbonate 1.65, manganous carbonate 0.63, cupric carbonate 0.3, potassium iodate 0.01, sodium selenate 0.01025, ammonium paramolybdate·$4H_2O$ 0.00795, sodium metasilicate·$9H_2O$ 1.45, chromium potassium sulfate·$12H_2O$ 0.275, lithium chloride 0.0174, boric acid 0.0815, sodium fluoride 0.0635, nickel carbonate 0.0318, ammonium vanadate 0.0066, finely powdered sucrose 221.026.
[4]Vitamin mix supplied the following vitamins (g/kg mix): niacin 3.0, calcium pantothenate 1.6, pyridoxine·HCl 0.7, thiamine·HCl 0.6, riboflavin 0.6, folic acid 0.2, biotin 0.02, vitamin E acetate (500 IU/g) 15.0, vitamin B_{12} (0.1%) 2.5, vitamin A palmitate (500,000 IU/g) 0.8, vitamin D_3 (400,000 IU/g) 0.25, Vitamin K_1/dextrose mix (10mg/g mix) 7.5, sucrose 967.23.

3.3 Experiment 1

Objective: To determine if ITCs from broccoli sprouts are detectable in the plasma of SHRsp.

3.3.1 Experimental Design

Male SHRsp were housed individually throughout the study in plastic shoebox cages in a room with a 12-hour light/dark cycle. Animals were handled daily and their weights were recorded on a weekly basis. Until 2 months of age SHRsp were fed

standard rat chow (Purina). At 2 months of age all SHRsp were changed to a modified AIN-93G diet (See Table 3.1). The rats were then also assigned to 1 of 2 groups.

One group of animals (n=5) was fed 200 mg of dried broccoli sprouts per day and the other group (control group) (n=7) did not receive broccoli sprouts. This feeding regime lasted for 3 months during which time SHRsp had free access to tap water.

At the end of the feeding trial, the rats were anaesthetized with halothane and blood was collected from the left ventricle in a lithium-heparinized vacutainer. Blood was centrifuged for 10 minutes at 1500 x g and stored at –70 °C until it was analyzed in Dr. Paul Talalay's laboratory at John Hopkins University. A cyclocondensation assay was performed followed by high performance liquid chromatography to determine levels of dithiocarbamates (DTC) as described by (Liebes et al., 2001). DTC are indicative of ITC absorption.

3.3.2 Principle of the cyclocondensation assay and high performance liquid chromatography

During the cyclocondensation assay ITC reacts with 1,2-benzene-dithiol giving rise to 1,3-benzenedithiol-2-thione. This product is then assayed by reverse phase high performance liquid chromatography and detected at 365 nm by an ultra-violet photodiode array detector.

3.3.3 Statistics

Data were analysed using independent 2 tailed t-tests on SPSS software version 11.0. Statistical significance was set at $p < 0.05$.

3.4 Experiment 2

Objective: To determine if the daily feeding of 200 mg of dried broccoli sprouts to male and female SHRsp reduces oxidative stress as reflected by an increase in concentrations of plasma protein thiols and blood GSH.

3.4.1 Experimental Design

Male (n=13 for the Control group and n=14 for the Broccoli group) and female (n=10 for the Control group and n=11 for the Broccoli group) SHRsp were used and treated as described in Experiment 1. Blood collection for the protein thiol assay was also the same as in Experiment 1 but differed for the blood GSH assay. For the latter, whole blood was added to an equal amount of 30% trichloroacetic acid containing 2% ethylenediaminetetraacetate (EDTA), centrifuged at 15000 x g for 5 minutes and the supernatant fluid snap frozen in liquid nitrogen and stored at –70 °C until assayed. Concentrations of blood GSH and plasma protein thiols are measures of oxidative stress (Asensi et al., 1999; Hu, 1994).

3.4.1.1 Blood GSH

3.4.1.1.1 Principle of the blood GSH assay

Blood GSH concentration was determined as per the method of Asensi et al. (1999), which uses chlorodinitrobenzene (CDNB). GST catalyses the formation of S-(2,4-dinitrophenyl) glutathione adduct from CDNB and GSH. This adduct can be detected spectrophotometrically (SPECTRA max³ 190, Sunyvale, CA) at 340 nm (Habig and Jakoby, 1981).

3.4.1.1.2 Preparation of reagents

A 0.5 M potassium phosphate buffer, with a pH of 7.00, containing 1 mM EDTA, was made up of 0.1746 mols potassium dihydrogen phosphate (KH_2PO_4) and 0.3253 mols of dipotassium hydrogen phosphate (K_2HPO_4) to 1 L of deionized distilled water (ddH_2O). The GST (500 U/ml) solution was made by adding 7 mg of stock GST (71 U/mg) in 1 ml of phosphate buffer. The CDNB solution (2 mg/ml) was made in ethanol and incubated at 37°C for 20 minutes to dissolve. The trichloroacetic acid solution (30%) was made by bringing 30 g of trichloroacetic acid (0.0744g of EDTA) to a volume of 100 ml of ddH_2O. The GSH stock solution (3.48 mM) was made by brining 10.7 mg of GSH to a final volume of 10 ml of 15% trichloroacetic acid containing 1 mM of EDTA.

3.4.1.1.3 GSH assay

A GSH standard curve was set up with a blank and concentrations of GSH including 6.25 µM, 12.5 µM, 25 µM, 50 µM and 100 µM that were diluted from the GSH stock (3.48 mM) in potassium phosphate buffer. To an eppendorf tube, 825 µl of potassium phosphate buffer, 25 µl of each respective standard, and 10 µl of CDNB were added. The contents were vortexed and 240 µl was pipetted into each microtitre well. To each microtitre well except for the one containing the blank, 10 µl of GST was then added. The blank was made up by adding 239.9 µl of buffer, 7.2 µl of 100 µM GSH, 2.9 µl of CDNB to an eppendorf tube and vortexing them and pipetting 240 µl of this mixture into a microtitre plate well. After 5 minutes, the optical density was read at 340 nm as an endpoint reaction. The concentration of each standard was derived according to the Beer-Lambert law using the extinction coefficient of CDNB ($9600 M^{-1}cm^{-1}$) (Lehninger, 1993). Blood samples were assayed as per the procedure described for the standards. Samples

were run in triplicate. The concentrations of the samples were obtained using linear regression and interpolation from the standard curve.

3.4.1.2. Plasma Protein Thiols

3.4.1.2.1 Principle of the plasma protein thiol assay

Levels of protein thiols were determined as described by Hu, (1994). In the presence of 5, 5'-dithiobis(2-nitrobenzoic acid) (DTNB), thiols give rise to the formation of 2, 5'-thionitrobenzoic acid (TNB). TNB can then be assayed spectrophotometrically at 412 nm.

3.4.1.2.2 Preparation of reagents

A DTNB (10 mM) solution was made up by bringing 4 mg of DTNB to a final volume of 1 ml of absolute ethanol. A Tris (0.25 M) buffer with EDTA (20 mM) was made up by bringing 30.275 g of Tris base to a final volume of 1 L of ddH_2O with 40 ml of an EDTA stock solution (0.5 M), pH 8.2. A GSH stock solution (.02 mM) for making up the standards was made up by bringing 0.06 g of GSH to a final volume of 10 ml of ddH_2O and stored at –20°C.

3.4.1.2.3 Plasma protein thiol assay

GSH standards ranged in concentration from 15.62 μM, 31.25 μM, 62.5 μM, 125 μM, 250 μM, 500 μM, and 1000 μM. Standards were made up from the GSH stock solution (1 mM) and diluted in Tris-EDTA buffer. To a microtitre plate well, 10 μl of GSH standard was added to 200 μl of Tris-EDTA. A reading (A1) was taken at 412 nm. Then, 4 μl of DTNB was added in and the plate was incubated at room temperature for 15 minutes. Another reading (A2) was taken at 412 nm. A DTNB blank (B) was used containing 210 μl of Tris-EDTA buffer and 4 μl of DTNB. The total sulfhydryl groups

were determined as described in Hu (1999). The molar absorption coefficient for TNB is 13600 $cm^{-1}M^{-1}$.

(A2-A1-B) X (0.2/0.01) 13.6 = (A2-A1-B) X 1.57 mM.

Samples were assayed in triplicate using the same procedure as the standards.

3.4.1.2.4 Protein concentration

To determine the amount of plasma protein present a bicinchoninic acid (BCA) assay was performed as described by Smith et al., (1985) using bovine serum albumin (BSA) as the reference protein. Standards were made up to concentrations of 0.0625 mg/ml, 0.125 mg/ml, 0.25 mg/ml, 0.5 mg/ml and 1 mg/ml using a Tris-EDTA buffer. The 1 mg/ml standard was stored at –70 °C. Working BCA solution was made up with BCA and copper sulfate (1:50). To each microtitre well 10 µl of standard or sample was added with 100 µl of working solution. Standards and samples were run in triplicate. The microtitre plate was incubated at 37°C for 30 minutes and then read at 562 nm by a microplate spectrophotometer (Spectra max 190) with SOFT max pro software version 4.0. Protein concentrations of the samples were calculated by linear regression from the standard curve.

3.4.2 Statistics

Data for blood GSH and plasma protein thiols were analyzed on SPSS software version 11.0. using an unpaired two tailed t-test. Statistical significance was set at $p < 0.05$.

3.5 Experiment 3

Objective: To determine if the daily feeding of 200 mg of dried broccoli sprouts to male and female SHRsp reduced parameters of the acute phase response as reflected by a decrease in plasma ceruloplasmin and an increase in plasma albumin concentration.

3.5.1 Experimental Design

Male (n=13 for Control group and n=14 for the Broccoli group) and female (n=10 for the Control group and n=11 for the Broccoli group) SHRsp were used and treated as described in Experiment 1.

3.5.1.1 Ceruloplasmin

3.5.1.1.1 Principles of Western blotting

Western blotting was used to determine levels of plasma CP in male and female SHRsp. A 12 % sodium dodecyl sulfate (SDS) gel was prepared as described by Sambrook et al. (1989), and 20 μg of proteins were electrophoresed. The protein was then transferred to a nitrocellulose membrane and detected with a mouse monoclonal CP antibody (1:750 dilution) (Transduction Laboratories). The secondary antibody used was peroxidase- labeled anti-mouse IgG (1:4000 dilution) (Sigma). Chemiluminescence detection of the proteins was performed using the NEN™ Renaissance Western Blot Chemilluminescence Reagent (NEN™ Life Sciences Products) followed by autoradiography.

3.5.1.1.2 Preparation of reagents

Loading buffer was made up by adding 1 ml of glycerol, 2 ml of 10% SDS, 0.25 mg of bromophenol blue, 1.25 ml of 0.5M Tris buffer (pH 6.8) and 4.25 ml of ddH_20. To 950 μL of loading buffer was added 20 μL of β-mercaptoethanol (1/20 v/v). Acrylamide

solution was made up by adding 30.0 g of acrylamide and 0.8 g of bisacrylamide to a final volume of 100 ml ddH_20. Running buffer (pH 8.5) was made up by adding 24.0 g of Tris, 115.2 g of glycine, 8.0 g of SDS to a final volume of 8.0 L ddH_20. Tris buffer (1M, pH 6.8) was made up of 31.5 g in 200 ml of ddH_20. Tris buffer (1.5M, pH 8.8) was made up of 36.3 g in a total volume of 200 ml of ddH_20. Stacking buffer was made up of 4.1 ml of ddH2O, 1.0 ml of 30% acrylamide, 0.75 ml of 1.0 M Tris (pH 6.8), 0.06 ml of 10 % SDS, 10% of ammonium persulfate (w/v) in ddH_2O, and 0.006 ml of TEMED. Separating buffer (12%) was made up of 6.6 ml of ddH2O, 8.0 ml of 30 % acrylamide, 5 ml of 1.5 M Tris (pH 8.8), 0.2 ml of 10 % SDS, 0.2 ml of 10 % ammonium persulfate, and 0.008 ml of TEMED. Transfer buffer was made up by adding 12.0 g of Tris, 56.4 g of glycine, 4.0 g of SDS, 800 ml of methanol (the methanol was added in after the contents was dissolved in ddH_2O), and brought to a final volume of 4.0 L. The transfer buffer was stored at 4°C. Phosphate buffered saline (PBS) (pH 7.2-7.5) was made up by adding 26.0 g of monobasic sodium phosphate (NaH_2PO_4)-H_2O, 115.0 g of dibasic sodium phosphate (Na_2HPO_4), and 85.0 g of NaCl to a final volume of 10.0 L of ddH_2O. PBST was made up by adding 0.5% of TWEEN 20 to PBS. Skim milk (5%) was made up by bringing 5 g of skim milk to a final volume of 100 ml of ddH_20 or for a 2.5 % solution, 2.5 g of skim milk was brought up to a final volume of 100 ml of ddH_20.

3.5.1.1.3 Protein concentration

Protein concentration was determined as described in section 3.4.1.2.4 except standards and samples were made up in 0.1 M potassium phosphate buffer.

3.5.1.1.4 Tissue preparation

The amount of protein was determined and 20 μg of each sample was pipetted into an eppendorf tube. To this 4 times the volume of acetone was added for 10 minutes at –20°C. Samples were then centrifuged at 16250 x g for 10 minutes after which time the acetone was removed and the pellets were allowed to air dry for about 20 minutes. Loading buffer at a concentration of 1 μL per μg of protein was added to the pellets, boiled for 5 minutes and then loaded onto the gel. A reference marker was also used and was boiled along with the samples, and 15 ul of it was loaded onto the gel.

3.5.1.1.5 Western blot

The resolving gel was poured between the glass plates of the western blotting apparatus. When it was set the stacking gel was poured in and the well comb was placed between the plates. When the gel was polymerized the samples were loaded and the gels were placed into the gel tank with running buffer. The gel was run for 30-34 minutes at 200V until the dye front approached the bottom of the gels. The gels along with some filter paper were placed in transfer buffer for 10 minutes. The gels were then sandwiched between filter paper and transferred at 13 V for 1 hour onto nitrocellulose membrane using the Trans-Blot SD Semi-dry Transfer Cell (Biorad). The membranes were then blocked in 2.5% skim milk for 1 hour. They were then incubated in primary antibody diluted in 2.5% milk for 1 hour at room temperature, followed by the secondary antibody (diluted in 5% milk) and incubated for 1 hour. The blots were then washed 3 times by 15 minutes in 2.5 % milk, followed by three 10 minute washes in PBST. The blots were then placed in chemiluminescence solution for 1 minute, placed into a cassette and exposed to a Kodak x-ray film for about 1 minute and the film developed. Band densities were then

scanned and blot densitometry was performed using Scion Image software. The band density for the control group was arbitrarily set at 100% and the band density for the broccoli fed-group was expressed relative to that of the control group.

3.5.1.2 Plasma albumin

3.5.1.2.1 Principle of the Albumin assay

Albumin levels were detected as described by Rodkey (1965). Bromcresol green has an absorbance maximum at 615 nm. Albumin binds to bromcresol green forming an albumin- bromcresol green complex. A decrease in optical density readings is reflective of the presence of albumin.

3.5.1.2.2 Preparation of reagents

Bromcresol green solution (0.6 mM) was made up by bringing 0.432 g of the sodium salt of bromcresol green to a final volume of 1 L of ddH_2O. A buffer indicator solution is made up by bringing 2.65 g of KH_2PO_4 and 4.33 g of Na_2HPO_4 to a volume of 500 ml of ddH_2O, then adding in 75 ml of bromcresol green stock solution and bringing the volume to 1L. Albumin standards were made up to concentrations of 6.25 mg/ml, 12.5 mg/ml, 25 mg/ml, 50mg/ml and 100 mg/ml.

3.5.1.2.3 Plasma albumin assay

A nonprotein standard (blank) was made up by adding 1.5 ml of indicator solution to 10 µl of ddH_2O. Standards were made by adding 10 µl of the respective standard to 1.5 ml of indicator solution. Eppendorf tubes were vortexed to mix, and 250 µl was placed in each microtitre well and run in triplicate. Readings were taken at 615 nm using the microtitre plate and reader and software as described above. Samples were assayed in

triplicate as per the procedure for the standards. Concentrations were obtained by using linear regression.

3.5.2 Statistics

Within the respective male and female SHRsp rat sets, samples were analyzed using unpaired 2 tailed t-tests on SPSS.

Experiment 4

Objective: To determine if 200 mg of broccoli sprouts induces phase 2 enzymes in the livers of SHRsp as shown by an increase in glutathione reductase (GR), glutathione-S-transferase (GST), and quinone reductase (QR) activity.

3.6.1 Experimental Design

Male (n=13 for Control group and n=14 for the Broccoli group) and female (n=10 for the Control group and n=11 for the Broccoli group) SHRsp were placed into either a control group or a broccoli group as described in Experiment 1 and kept on their feeding regime until they were 5 months of age. Male Sprague Dawley (SD) rats (n=3 per group) were also used and treated like the SHRsp with the exception that they were perfused at 6 months of age. SD were used to collect preliminary information as a control animal for the SHRsp. They were obtained from Charles River Laboratories, St Constant, Quebec, Canada. SHRsp and SD were anesthetized with halothane and perfused transcardially with 0.03 M PBS and 2500 U/L of heparin after blood was collected. Livers were collected and immediately snap frozen in liquid nitrogen and stored at –70 °C. The frozen tissue was ground with a homogeniser (Mickro-Dismembrator, B, Braun Biotech International GmBH) for 4 x 1 min at a frequency of 3000 oscillations per minute and

stored at –70 °C until assayed for GR, GST and QR activity. The activity of these enzymes is used as an indicator of phase 2 enzyme induction (Staack et al., 1998).

3.6.1.1 Glutathione Reductase

3.6.1.1.1 Principle of the Glutathione Reductase assay

GR was detected as described by (Carlberg and Mannervik, 1975). GR catalyses the reduction of oxidized GSH using NADPH as the electron donor. The disappearance of NADPH is detected spectrophotometrically at 340 nm.

3.6.1.1.2 Preparation of reagents

Incubation buffer consisting of 0.1 M sodium phosphate buffer (pH 7.6) contained 0.5 mM EDTA and was made up by adding 13 ml of 0.1 M NaH_2PO_4 buffer , 87 ml of 0.1 M of Na_2HPO_4 buffer and 0.01861 g of EDTA. A 0.1 M of $NaHPO_4$ buffer was made up by bringing13.8 g of NaH_2PO_4 buffer up to a final volume of 1 L of ddH_2O. A 0.1 M NaH_2PO_4 buffer was made up by bringing 14.2 g of Na_2HPO_4 buffer up to a final volume of 1 L of ddH_2O. A 1 mM NADPH solution was made by dissolving 0.8 mg of NADPH up to 1 ml of 0.1 % sodium bicarbonate ($NaHCO_3$)(pH 9). A 10 mM solution of oxidized GSH (GSSG) was made up by bringing 6.6 mg of GSSG to a final volume of 10 ml of incubation buffer.

3.6.1.1.3 Tissue preparation

Frozen liver tissue was ground with a homogeniser (Mickro-Dismembrator, B, Braun Biotech International GmBH) for 4 x 1 min at a frequency of 3000 oscillations per minute and stored at–80 C until assayed. Liver tissue was diluted in incubation buffer (1:100 dilution). Tissue was centrifuged for 10 minutes at 16250 x g. Samples were then

sonicated 3 times for 5 seconds on the Sonifier Cell Disruptor (Model W140 from Ultrasonics, Inc.).

3.6.1.1.4 NADPH standard curve

NADPH standards were made up by diluting the stock solution into concentrations of 1 nM, 5 nM, 10 nM and 15 nM. To a microtitre plate, 300 µl of standard was added in triplicate and read at 340 nm.

3.6.1.1.5 Kinetic assay

To each microtitre well, 140 µl of incubation buffer, 30 µl of 1mM NADPH and 100 µl of sample were added. The microtitre plate was incubated for 10 minutes at room temperature and 10 µl of GSSG was added. Readings were taken with the same plate reader and software as described above at 340 nm in 30 second intervals for 4 minutes. Data were collected using SOFTmax³PRO software version 4.0.

3.6.1.1.6 Protein concentration

Protein concentration was determined as described in section 3.4.1.2.4 except standards and samples were made up in incubation buffer.

3.6.1.2 Glutathione-S-transferase

3.6.1.2.1 Principle of the GST assay

GST was detected as described by (Habig and Jakoby, 1981). Glutathione transferase catalyses the formation of S-(2,4-dinitrophenyl) glutathione adduct from CDNB and GSH. This adduct can be detected spectrophotometrically at 340 nm.

3.6.1.2.2 Preparation of reagents

A 0.1 M potassium phosphate buffer (pH 6.5) was made by bringing 0.0659 mols of KH_2PO_4 and 0.034 mols of K_2HPO_4 to a final volume of 1 L with ddH_2O. A 25 mM

CDNB solution was made by bringing 0.05168 g of CDNB to a final volume of 10 ml of ethanol. A 10 mM GSH solution was made up by bringing 15.68 mg of GSH to a final volume of 5 ml of buffer.

3.6.1.2.3 Tissue preparation

Frozen liver tissue was ground with a homogeniser (Mickro-Dismembrator, B, Braun Biotech International GmBH) for 4 x 1 min at a frequency of 3,000 oscillations per minute and stored at–80 C until assayed. Liver tissue was diluted in 1.0 M potassium phosphate buffer (pH 6.5) (1:100 dilution). Samples were then sonicated 3 times for 5 seconds and then centrifuged for 10 minutes at 16250 x g.

3.6.1.2.4 Kinetic assay

To each well was added 10 µl of sample, 195 µl of phosphate buffer and 25 µl of 10 mM GSH. This mixture was kept at room temperature for 15 minutes and then 20 µl of 12.5 mM CDNB was added. Readings were taken kinetically at 340 nm every 30 seconds for 5 minutes.

3.6.1.2.5 Protein concentration

Protein concentration was determined as described in section 3.4.1.2.4 except standards and samples were made up in 0.1 M potassium phosphate buffer.

3.6.1.3 Quinone Reductase

3.6.1.3.1 Principle of the QR assay

QR was assayed as described by (Prochaska and Santamaria, 1988). The quinone menadione is reduced to menadiol using NADPH as the electron donor. Menadiol then is reduced into 3-(4, 5-dimethylthiazo-2-yl)-2, 5- diphenyltetrazolium bromide (MTT) that can be detected spectrophotometrically at 610 nm.

3.6.1.3.2 Preparation of reagents

A 50 mM Tris buffer (pH 7.4) was made and stored at room temperature by bringing 661 mg of Trizma –hydrogen chloride and 97 mg of Trizma base up to a final volume of 100 ml of ddH_2O. A second Tris buffer (25 mM, pH 7.4) was made by diluting the 50 mM Tris buffer 1:1. A 1.5% stock solution of TWEEN 20 was made by bringing 15 µl of TWEEN 20 to a volume of 850 µl of 25 mM Tris buffer and storing at room temperature for 2 weeks. A 10% BSA stock solution consisting of 1g in 10 ml of 25 mM Tris buffer, was made and stored at 4°C for 2 weeks. A stock FAD (7.5 mM) solution was made up by bringing 0.0328 g of FAD to a final volume of 5 ml of 25 mM Tris buffer, and stored at –20C. A 50 mM oxidized nicotinamide adenine dinucleotide (NADP) solution was made fresh by bringing 0.004 g of NADP to a volume of 100 µl of 25 mM Tris buffer. Glucose–6-phosphate (150 mM in 25 mM Tris buffer) was made up fresh by adding 0.0123 g in 300 µL of 25 mM Tris buffer. Glucose–6-phosphate dehydrogenase (372 U/mg of protein) was made fresh by adding 17.34 µl in 10 ml of working solution. Menadione sodium bisulfate was made fresh by adding 0.007 g in 1 ml of 25 mM Tris buffer. A 0.3 mM dicoumerol solution (Solution A) in 5 mM KPO_4 with 0.5 % DMSO was made up to a pH of 7.4 and stored at room temperature. A 1 M stock of KH_2PO_4 was made up by bringing 13.6 g to a final volume of 100ml of ddH_2O, and a 1M stock of K_2PO_4 was made up by bringing 17.2 g up to a volume of 100 ml of ddH_2O. To make a 0.1 M stock of potassium phosphate buffer, 7.22 ml of KH_2PO_4 and 2.78 ml of K_2PO_4 were brought to a final volume of 100 ml with ddH_2O. From the stock solution a 5 mM phosphate buffer was made up by adding 25 ml of the 0.1 M buffer and bringing it to a volume of 500 ml. To 0.02 g of dicoumerol 1 ml of dimethyl sulfoxide was added and

gently heated. To this 199 ml of the 5 mM phosphate buffer was added. An identical solution (Solution B) was made up but no dicoumerol was added to it. To make up a 30 ml working solution, 15 ml of 50 mM Tris buffer (25 mM final concentration), 1.5 ml of 10% BSA in 25 mM Tris (pH 7.4) (final concentration 0.005 %), 199.5 µl of 1.5 % TWEEN 20 (final concentration of 0.0001 %), 12.98 ml of ddH_2O, 20.01 ml of 7.5 mM FAD (final concentration of 5 µM), 199.5 µl of 150 mM glucose-6-phosphate (final concentration of 1 mM), 18 ml of 50 mM NADP (final concentration of 30 µM), 66.85 µl of glucose-6-phosphate dehydrogenase (final activity 2U/ml), 0.009 g of MTT (final concentration of 0.7 mM) and 230 ml of 5 mM menadione (final concentration of 50 µM) were added.

3.6.1.3.3 Tissue preparation

Frozen liver tissue was ground with a homogeniser (Mickro-Dismembrator, B, Braun Biotech International GmBH) for 4 x 1 min at a frequency of 3,000 oscillations per minute and stored at–80 C until assayed. Liver tissue was diluted (1:100 dilution) in 50 mM Tris buffer (pH 7.4) containing 1 mM EDTA. Samples were sonicated once for 3 seconds and centrifuged at 16250 x g for 3 minutes.

3.6.1.3.4 Kinetic assay

To each microtitre plate well 55 µl of sample was added two times in triplicate. To one set of each sample Solution A (contains dicoumerol) was added and to the other set 50 µl of Solution B (does not contain dicoumerol), was added. To all of the wells, 200 µl of working solution was added. Readings were taken with the pathcheck option on for intervals of 1 minute for a total of 5 minutes at a wavelength of 610 nm. The same spectrophotometer and software as described above were used.

3.6.1.3.5 Protein concentration

Protein concentration was determined as described in section 3.4.1.2.4 except standards and samples were made up in 25 mM Tris buffer.

3.6.2 Statistics

Within the respective male and female SHRsp rat sets, samples were analyzed using an unpaired two tailed t-test. As a consequence of small sample size a non-parametric Mann-Whitney U test was run for the male SD rats.

Chapter 4 Results

4.1. Male SHRsp Body Weights

Body weight in the male SHRsp control group ranged from 243.6 ± 4.6 g at the start of the feeding trial to 359.6 ±4.6 g at the end of the feeding trial. Male SHRsp in the broccoli group ranged from 253.1 ± 3.1 g at the beginning of the feeding trial to 375.9 ± 3.1 g at the end of the feeding trial. Significant differences were detected between the control and broccoli fed groups, with the broccoli group being heavier at weeks 2, 3, 6, 7, 8, 9, 10 and 11 with respective p values of 0.032, 0.013, 0.021, 0.011, 0.011, 0.051, 0.015, 0.006 (see Figure 4.1). Groups were not significantly different at weeks 1, 4, and 5 with respective p values of 0.095, 0.217, 0.504 (see Figure 4.1).

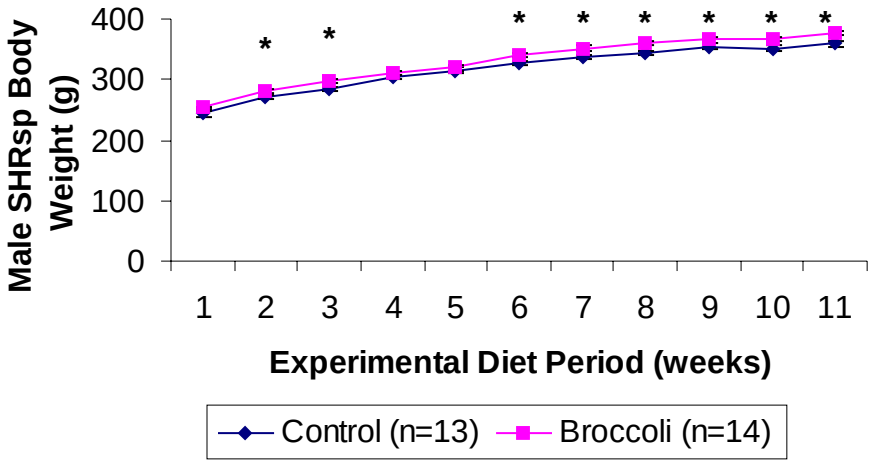

Figure 4.1. The effect of feeding 200 mg of dried broccoli sprouts on body weight in male SHRsp. Results are expressed as mean weight ± standard error of the mean (SEM). Groups were not significantly different by unpaired two tailed t-tests at weeks 1, 4, and 5 (respective p values are 0.095, 0.217, 0.504). Groups were significantly different by unpaired two tailed t-tests at weeks 2, 3, 6, 7, 8, 9, 10, and 11 (respective p values are 0.032, 0.013, 0.021, 0.011, 0.011, 0.051, 0.015, 0.006). Spontaneously hypertensive stroke prone rats (SHRsp) were fed a modified AIN-93G diet (Control) or a modified AIN-93G diet supplemented with 200 mg of dried broccoli sprouts (Broccoli) for 3 months.

4.2 Female SHRsp Body Weights

Body weight in the female SHRsp control group ranged from 182.1 ± 3.9 g at the start of the feeding trial to 245.6 ± 3.5 g at the end of the feeding trial. Male SHRsp in the broccoli group ranged from 177.4 ± 2.9 g at the beginning of the feeding trial to 239.7 ± 3.1 g at the end of the feeding trial. Groups were not significantly different throughout weeks 1 to 11 with respective p values of 0.349, 0.385, 0.379, 0.221, 0.192, 0.371, 0.194, 0.224, 0.818, 0.217 (see Figure 4.2). Data for body weights in week 5 were not collected.

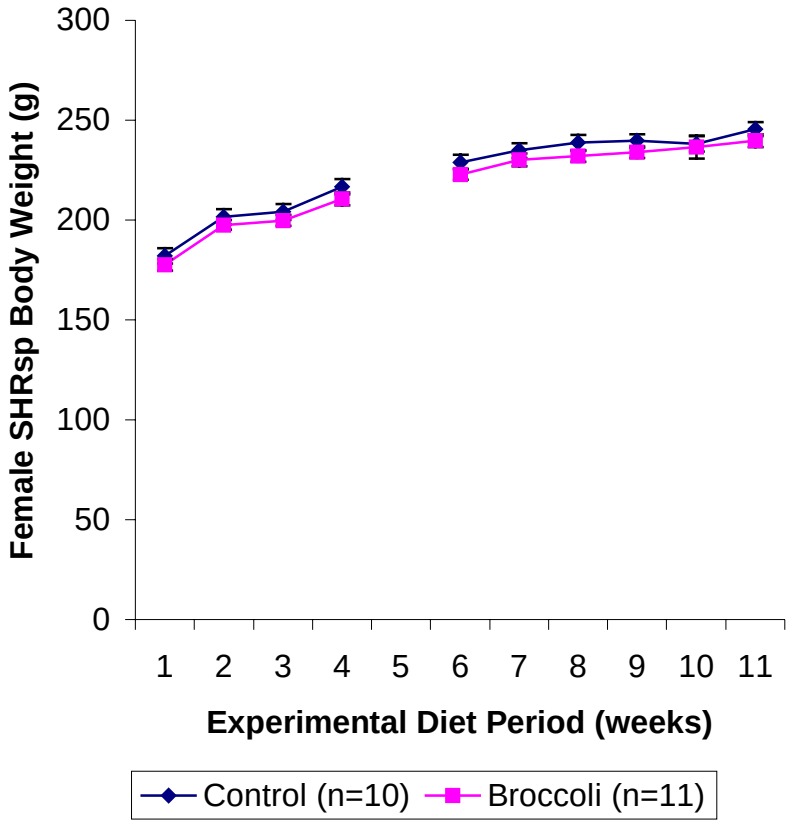

Figure 4.2. The effect of feeding 200 mg of dried broccoli sprouts on body weight in female SHRsp. Results are expressed as mean weight ±. SEM. Groups were not significantly different by unpaired two tailed t-tests (respective p values are 0.349, 0.385, 0.379, 0.221, 0.192, 0.371, 0.194, 0.224, 0.818, 0.217 for weeks 1 to11). Spontaneously hypertensive stroke prone rats (SHRsp) were fed a modified AIN-93G diet (Control) or a modified AIN-93G diet supplemented with 200 mg of dried broccoli sprouts (Broccoli) for 3 months.

4.3 Male SHRsp Food Intake

Food intake in the male SHRsp was not significantly different with values of 1562.9 ± 13.1 g in the control group and 1586.9 ± 11.2 g in the broccoli group (p = 0.174) (see Figure 4.3).

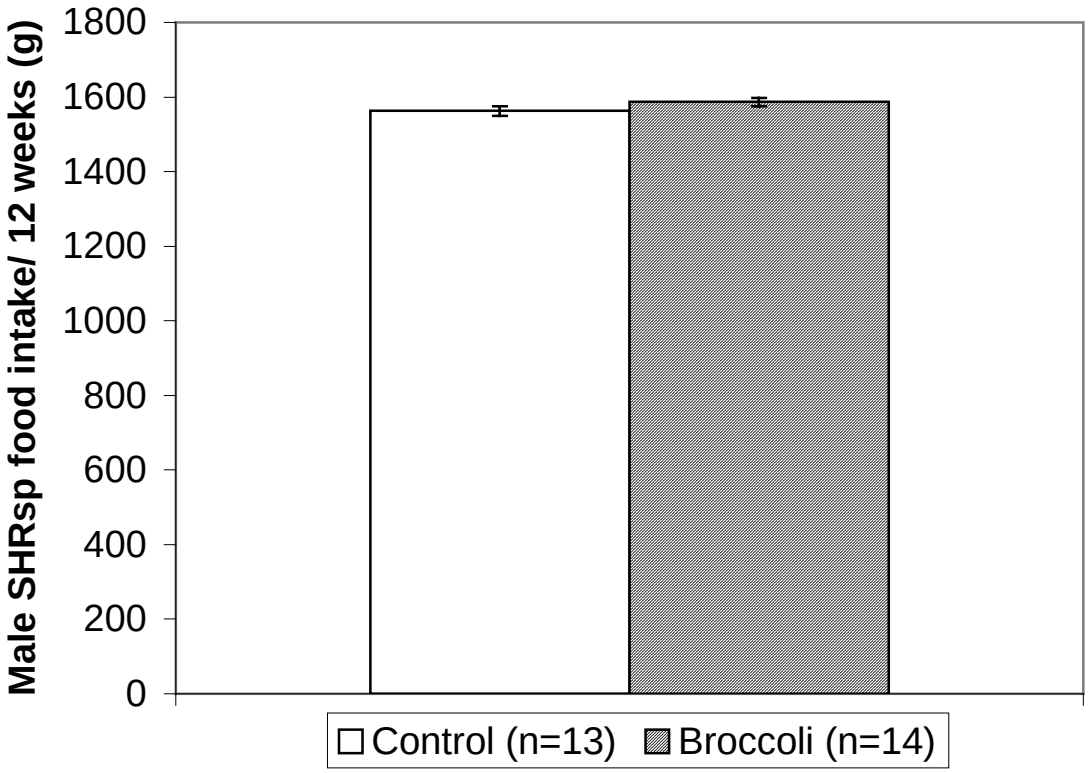

Figure 4.3. The effect of feeding 200 mg of dried broccoli sprouts on total food intake in male SHRsp. Results are expressed as mean total food intake ±. SEM. Groups were not significantly different by unpaired two tailed t-tests (p= 0.174). Spontaneously hypertensive stroke prone rats (SHRsp) were fed a modified AIN-93G diet (Control) or a modified AIN-93G diet supplemented with 200 mg of dried broccoli sprouts (Broccoli) for 3 months.

4.4 Female SHRsp Food Intake

There was a significant difference in food intake in the female SHRsp between the control and broccoli groups with respective values of 1206.6 ± 16.2 g and 1158.5 ± 14.0 g (p= 0.036) (see Figure 4.3).

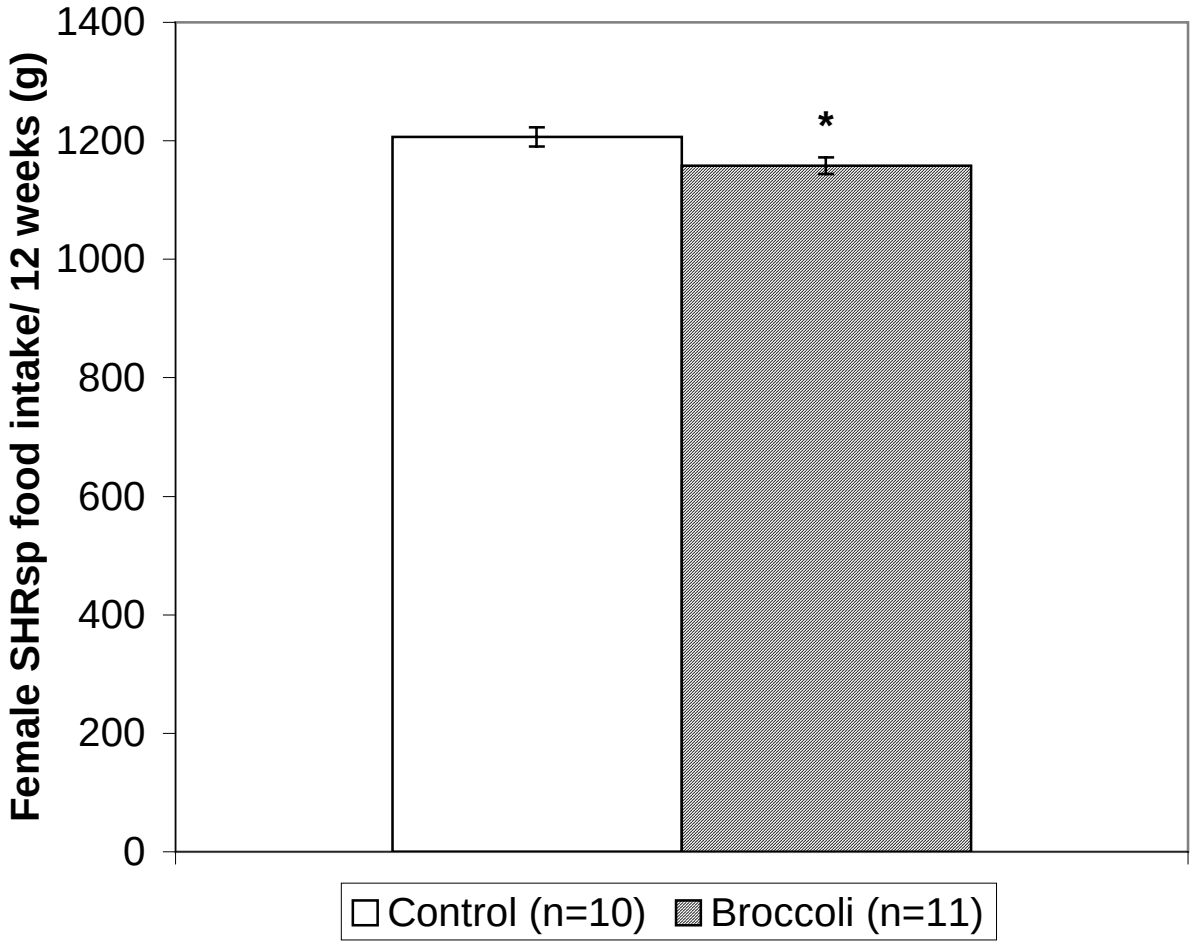

Figure 4.4. The effect of feeding 200 mg of dried broccoli sprouts on total food intake in female SHRsp. Results are expressed as mean total food intake ±. SEM. Groups were significantly different by unpaired two tailed t-tests (p= 0.036). Spontaneously hypertensive stroke prone rats (SHRsp) were fed a modified AIN-93G diet (Control) or a modified AIN-93G diet supplemented with 200 mg of dried broccoli sprouts (Broccoli) for 3 months.

4.5 Experiment 1

4.5.1 Plasma Dithiocarbamates

ITCs from broccoli sprouts are converted into DTC that can be detected to determine if the ITCs from the diet are absorbed. Levels of plasma DTC were 0.072 ± 0.015 μM in the control animals and 0.47 ± 0.1 μM in the broccoli fed animals. DTC levels were significantly higher in the broccoli fed group than in the control group, with a p-value of 0.007 (see Figure 4.3).

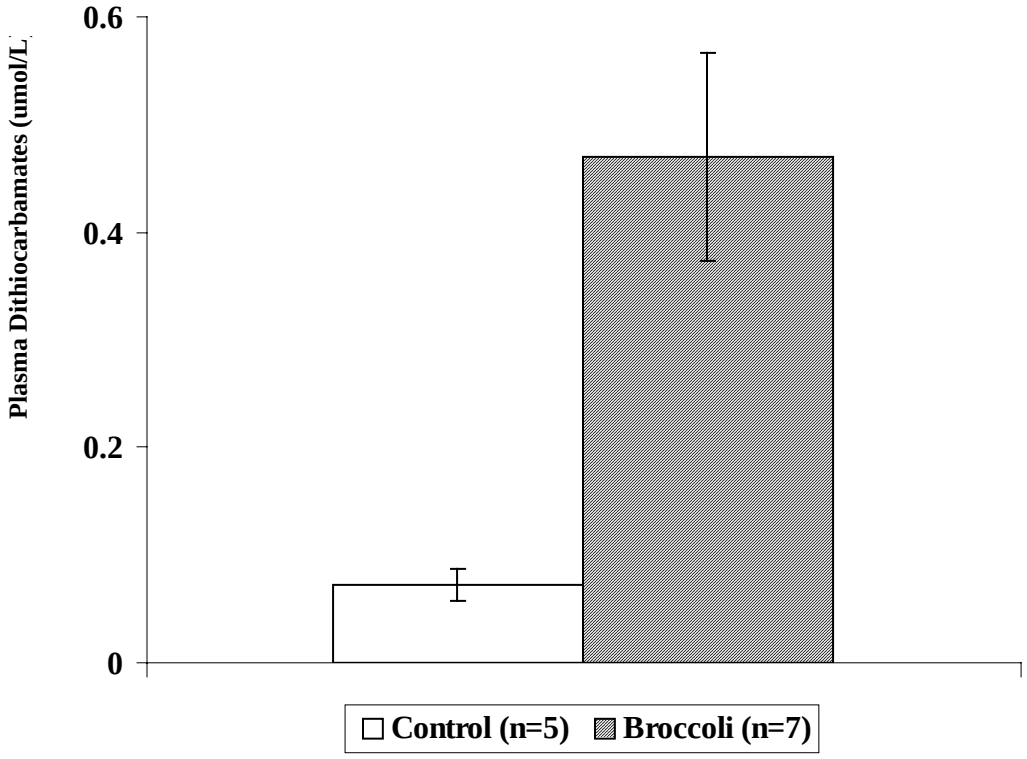

Figure 4.5. The effect of feeding 200 mg of dried broccoli sprouts on plasma dithiocarbamate concentrations in male SHRsp. Results are expressed as mean ± SEM. Groups were significantly different by an unpaired two tailed t-test (p = 0.007). Spontaneously hypertensive stroke prone rats (SHRsp) were fed a modified AIN-93G diet (Control) or a modified AIN-93G diet supplemented with 200 mg of dried broccoli sprouts (Broccoli) for 3 months.

4.6. Experiment 2

4.6.1 Blood GSH concentrations

An increase in blood GSH concentrations is indicative of a decrease in oxidative stress. Mean (± SEM) blood GSH concentrations in male SHRsp were 6.8 ± 0.6 μM for the control group and 8.6 ± 0.5 μM for the broccoli group (see Table 4.1). Blood GSH concentrations were statistically different with a p-value of 0.03 (see Table 4.1). In the female SHRsp, blood GSH concentrations were 9.0 ± 0.8 μM for the control group and 8.5 ± 0.9 μM for the broccoli group (see Table 4.1). No statistical difference was detected between the female broccoli and control groups (p-value = 0.669) (see Table 4.1).

4.6.2 Plasma protein thiol concentrations

An increase in plasma protein thiol concentrations can be used as a marker of reduced oxidative stress. In male SHRsp, mean (± SEM) plasma protein thiol concentrations were 163.9 ± 4.95 nmol/mg for the control group and 161.8 ± 5.4 nmol/mg for the broccoli group (see Table 4.1). Broccoli sprout feeding had no statistically significant effect on plasma protein thiol concentrations in male SHRsp (p-value = 0.771) (see Table 4.1). In the female SHRsp, plasma protein thiol concentrations were 188.4 ± 4.8 nmol/mg in the control group and 203.3 ± 4.4 nmol/mg in the broccoli group (see Table 4.1). Concentrations of plasma protein thiols were significantly affected by broccoli feeding in female SHRsp with a p-value of 0.033 (see Table 4.1).

Table 4.1. The effect of feeding 200 mg of dried broccoli sprouts on blood GSH and plasma protein thiol concentrations in male and female SHRsp.

Animal Gender	Groups	Blood GSH (μM)	Plasma Protein Thiols (nmol/mg protein)
Male	Control (n=13)	6.8 ± 0.6	163.9 ± 4.5
	Broccoli (n=14)	8.6 ± 0.5	161.8 ± 5.4
	p-value	0.030*	0.771
Female	Control (n=10)	9.0 ± 0.8	188.4 ± 4.8
	Broccoli (n=11)	8.5 ± 0.9	203.3 ± 4.4
	p-value	0.669	0.033*

Results are expressed as mean ± SEM. Male blood GSH and female plasma protein thiol concentrations were significantly different by unpaired two tailed t-tests (with respective values of p = 0.030 and 0.033) Spontaneously hypertensive stroke prone rats (SHRsp) were fed a modified AIN-93G diet (Control) or a modified AIN-93G diet supplemented with 200 mg of dried broccoli sprouts (Broccoli) for 3 months.

4.7 Experiment 3

4.7.1 Plasma albumin concentrations

During the APR albumin concentrations decrease, therefore a decreased APR would result in increased albumin concentration. Mean (± SEM) concentrations of plasma albumin in male SHRsp in the control group were 29.2 ± 2.2 mg/ml and 31.0 ± 2.0 mg/ml in broccoli fed animals, with no statistically significant difference found between the two groups (p-value = 0.561). In the female SHRsp, concentrations of plasma albumin were 28.3 ± 1.0 mg/ml in the control group and 27.6 ± 1.5 mg/ml in the broccoli group. No significant difference was detected between the control and broccoli group in the female SHRsp (p-value = 0.716).

Table 4.2. The effect of feeding 200 mg of dried broccoli sprouts on plasma albumin concentrations in male and female SHRsp.

	Groups	Plasma Albumin (mg/ml)
Male	Control (n=13)	29.2 ± 2.2
	Broccoli (n=14)	31.0 ± 2.0
	p-value	0.561
Female	Control (n=10)	28.3 ± 1.0
	Broccoli (n=11)	27.6 ± 1.5
	p-value	0.716

Results are expressed as mean ± SEM and analyzed by unpaired two tailed t-tests. Spontaneously hypertensive stroke prone rats (SHRsp) were fed a modified AIN-93G diet (Control) or a modified AIN-93G diet supplemented with 200 mg of dried broccoli sprouts (Broccoli) for 3 months.

4.7.2 Plasma ceruloplasmin levels

Ceruloplasmin is a positive acute phase protein that increases during the APR. Decreased levels of ceruloplasmin would be indicative of a dampened APR. In male SHRsp plasma CP concentrations as shown by blot densitometry were 100 % in the control group and 95% in the broccoli group, with no significant difference between the two groups. In the female SHRsp, plasma CP blot densitometry were 100% in the control group and 94.7 % in the broccoli group. Levels of plasma CP were not statistically affected by broccoli feeding in male or female SHRsp, with respective p-values of 0.442 and 0.571 (see Figures 4.7 and 4.9 with the respective blots in Figures 4.6 and 4.8).

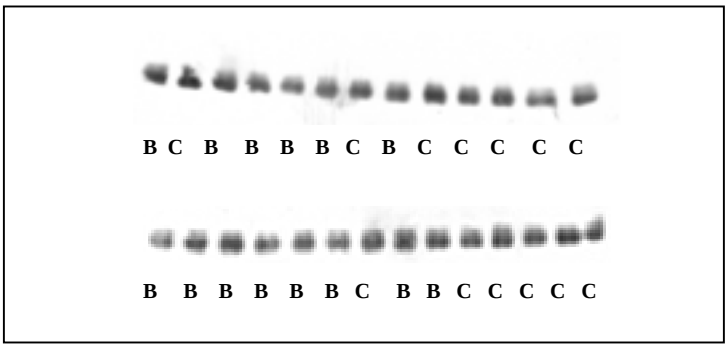

Figure 4.6. The effect of feeding 200 mg of dried broccoli sprouts on plasma ceruloplasmin levels in male SHRsp as shown by a western blot. Spontaneously hypertensive stroke prone rats (SHRsp) were fed a modified AIN-93G diet (C) or a modified AIN-93G supplemented with 200 mg of dried broccoli sprouts (B) for 3 months.

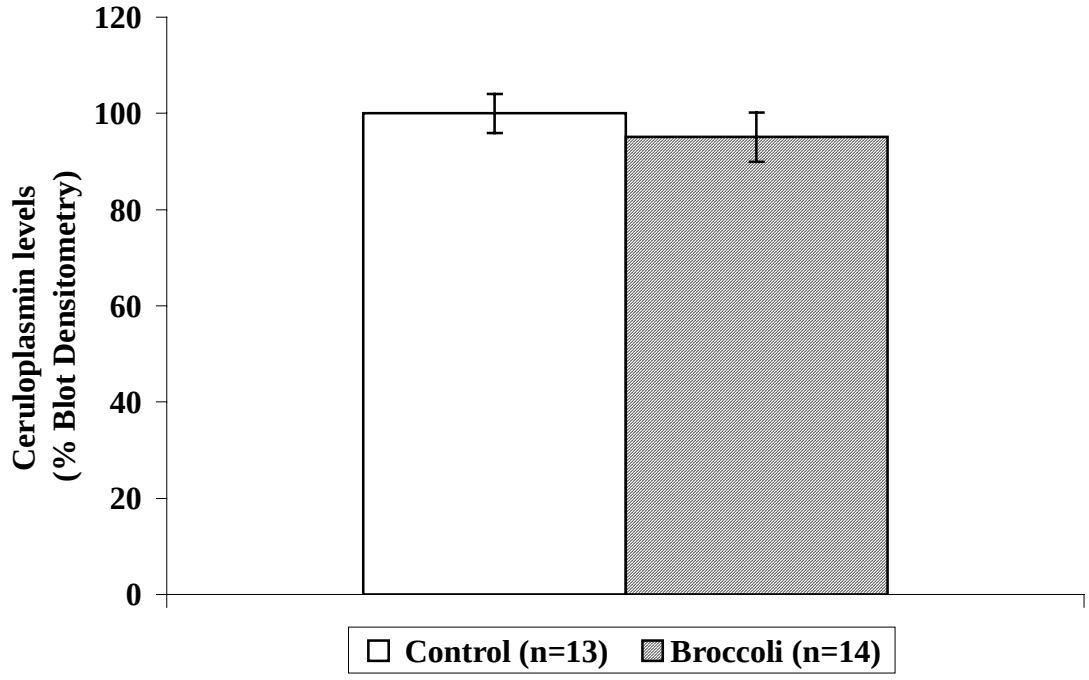

Figure 4.7. The effect of feeding 200 mg of dried broccoli sprouts on plasma ceruloplasmin levels in male SHRsp. Results are expressed as mean percentage ± SEM. Groups were not significantly different by an unpaired t-test with a p-value of 0.442. Spontaneously hypertensive stroke prone rats (SHRsp) were fed a modified AIN-93G diet (Control) or a modified AIN-93G supplemented with 200 mg of dried broccoli sprouts (Broccoli) for 3 months.

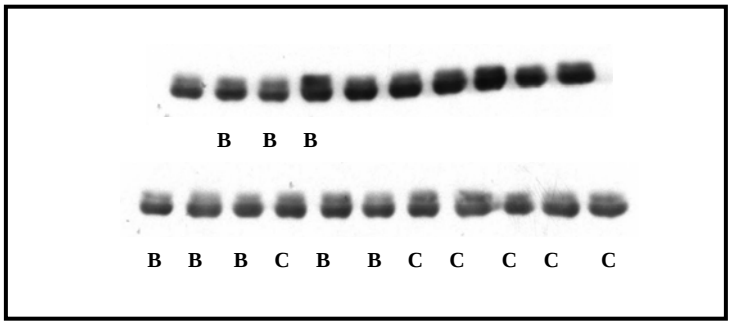

Figure 4.8. The effect of feeding 200 mg of dried broccoli sprouts on plasma ceruloplasmin levels in female SHRsp as shown by a western blot. Spontaneously hypertensive stroke prone rats (SHRsp) were fed a modified AIN-93G diet (C) or a modified AIN-93G supplemented with 200 mg of dried broccoli sprouts (B) for 3 months.

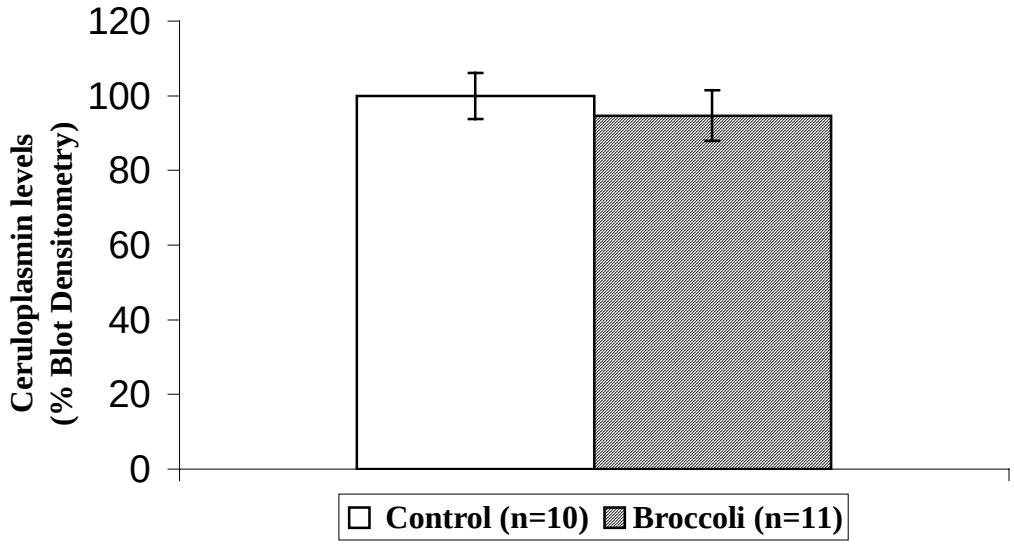

Figure 4.9 The effect of feeding 200 mg of dried broccoli sprouts on plasma ceruloplasmin levels in female SHRsp. Results are expressed as mean percentage ± SEM. Groups were not significantly different by an unpaired t-test with a p-value of 0.571. Spontaneously hypertensive stroke prone rats (SHRsp) were fed a modified AIN-93G diet (Control) or a modified AIN-93G supplemented with 200 mg of dried broccoli sprouts (Broccoli) for 3 months.

4.8 Experiment 4

4.8.1 Liver glutathione reductase activity

Increased GR activity is a marker of phase 2 enzyme induction. In male SHRsp, mean (± SEM) GR activity was 10.2 ± 0.6 nmol/min/mg in the control group and 11.2 ± 0.8 nmol/min/mg in the broccoli group (see Table 4.3). No statistically significant effect of broccoli feeding was detected in the male SHRsp (p-value = 0.268). In the female SHRsp, GR activity was 11.2 ± 0.6 nmols/min/mg in the control group and 10.4 ± 0.3 nmols/min/ng in the broccoli group with no significant difference detected between the control and broccoli fed group (p-value = 0.243). In the male SD rats, GR activity was 9.4 ± 1.2 nmols/min/mg in the control group and 9.9 ± 0.6 nmols/min/mg in the broccoli group. There was no significant effect of broccoli feeding in the male SD rats (p-value = 0.827).

Table 4.3 The effect of feeding 200 mg of dried broccoli sprouts in SHRsp and SD rats on liver phase 2 enzyme activity.

Animal gender and strain	Groups	Glutathione Reductase Activity (nmol/min/mg)	Glutathione –S-Transferase (nmol/min/mg)	Quinone Reductase (nmol/min/mg)
Male SHRsp	Control	10.2 ± 0.6	216.5 ± 17.5	10.2 ± 1.0
	N	13	13	13
	Broccoli	11.2 ± 0.8	213.5 ± 12.2	11.0 ± 0.8
	N	12	13	13
	p-value	0.268	0.887	0.539
Female SHRsp	Control	11.2 ± 0.6	283.6 ± 10.8	52.7 ± 2.4
	N	10	8	10
	Broccoli	10.4 ± 0.3	264.5 ± 6.4	64.6 ± 2.4
	N	11	11	11
	p-value	0.243	0.001*	0.002*
Male SD	Control	9.4 ± 1.2	363.9 ± 23.2	60.8 ± 14.0
	N	3	3	3
	Broccoli	9.9 ± 0.6	322.6 ± 7.9	96.6 ± 9.2
	N	3	3	3
	p-value	0.827	0.050*	0.127

Results are expressed as mean ± SEM and analyzed by unpaired two tailed t-tests for the SHRsp and by a Mann-Whitney U test for the SD rats. P-values are for the effect of diet within rat category. Spontaneously hypertensive stroke prone rats (SHRsp) and Sprague Dawley (SD) were fed a modified AIN-93G diet (Control) or a modified AIN-93G and 200 mg of dried broccoli sprouts (Broccoli) for 3 months.

4.8.2 Liver glutathione-S-transferase activity

During phase 2 enzyme induction the activity of GST is increased. Mean (± SEM) GST activity in the male SHRsp was 216.5 ± 17.5 nmols/min/mg in the control group and 213.5 ± 12.2 nmols/min/mg in the broccoli group (see Table 4.3). No significant effect of broccoli feeding was detected in the male SHRsp (p-value= 0.887). In the female SHRsp, GST activity was 283.6 ± 10.8 nmols/min/mg in the control group and 264.5 ± 6.4 nmols/min/mg in the broccoli group. For the female SHRsp, a boxplot was used to determine if there were any outliers. The values 232.81 and 212.75 nmols/min/mg were determined to be outliers. In the presence of the outliers, a p-value of 0.136 was obtained and removal of the outliers gave rise to a p-value of 0.001. In the male SD rats, GST activity was 363.9 ± 23.2 nmols/min/mg for the control group and 322.6 ± 7.9 nmol/min/mg for the broccoli group. A significance value of 0.050 was determined for the effects of broccoli feeding in male SD rats. Levels of GST activity were the highest in the SD rats followed by the female SHRsp and then the male SHRsp.

4.8.3 Liver quinone reductase activity

Increased quinone reductase activity is a marker of phase 2 enzyme induction. Mean (± SEM) liver QR activity in male SHRsp was 10.2 ± 1.0 nmol/min/mg for the control group and 11.0 ± 0.8 for the broccoli group. There was no significant effect of broccoli feeding in the male SHRsp (p-value = 0.539). In the female SHRsp, QR activity was 52.7 ± 2.4 nmol/min/mg for the control group and 64.6 ± 2.4 nmol/min/mg for the broccoli group. A significant effect of broccoli feeding was detected in the female SHRsp

with a p-value of 0.002. In the male SD, QR activity was 60.8 ± 14.0 nmol/min/mg in the control group and 96.6 ± 9.2 nmol/min/mg in the broccoli group. A significance value of 0.127 was detected for the effects of broccoli feeding on male SD rats. Levels of QR activity are the highest in the SD and female SHRsp category followed by the male SHRsp.

Chapter 5 Discussion

Does the daily feeding of dried broccoli sprouts affect animal weight?

Results from the body weight data show that there was a significant difference in male SHRsp body weights between the control and broccoli fed groups with the broccoli group being heavier for most of the time points during the feeding trial. The data on modified AIN-93G food intake shows that there was no significant difference between the control and the broccoli fed group, although the mean for the broccoli fed group is slightly higher than that of the control group. The increased body weight of the broccoli fed animals cannot be attributed to the broccoli fed group consuming more modified AIN-93G food than the control group.

There is no available expected energy intake for SHRsp. Male SHRsp were fed approximately 20 g of AIN-93G diet per day, and it was assumed that this was providing ad libitum energy intake based on the food consumption of adult male Fisher rats of 12-15 g per day. Therefore, a limitation of this study is that the male SHRsp were not strictly fed ad libitum.

A greater weight gain in the broccoli group may be due to extra kilocalories taken in from the broccoli sprouts. The number of kilocalories provided by the consumed dried broccoli sprouts is estimated to be in the range of 1 kilocalorie per day in addition to their energy intake of approximately 80 kilocalories per day from the modified AIN-93G diet. This value is taken from the United States Department of Agriculture nutritional database and is based on the kilocalories present in dried brussel sprouts as there were no reliable data for the energy content present in dried broccoli sprouts. Over 12 weeks the broccoli group would have consumed an extra 84 kilocalories compared to the control group. This

amount translates into an estimate of 10.9 g of body weight using the crude estimate that 0.454 kg of body weight equates to 3500 kilocalories. The difference in body weight between the control and the broccoli fed group is approximately 16 g. Therefore a portion of the 16 g of increased weight in the broccoli group may be attributed to the kilocalories present in the broccoli sprouts.

An alternate explanation to the increased weight gain in the broccoli fed SHRsp may be increased feed efficiency. This would be the case if the feeding of broccoli sprouts altered gastrointestinal function and increased nutrient absorption.

In other studies from our lab where male SHRsp were fed approximately 17 g of modified AIN-93G daily, the feeding of dried broccoli sprouts decreased inflammation in the cardiovascular system (Wu et al., 2004) and central nervous system (Ashraf et al., 2004 submitted). Therefore even if these animals are experiencing a mild caloric restriction, some of the results observed may be due to the actions of the dried broccoli sprouts. In addition, when the data are expressed as a total change of body weight from the start of the feeding trial until their time of perfusion, then there is no significant difference in weight gain between the control and broccoli fed groups. This suggests that even if the animals were fed slightly below their dietary requirement, both groups were restricted to roughly the same extent.

In the female SHRsp, there was no significant difference in body weight between the control and the broccoli fed animals, although the broccoli-fed animals significantly reduced their intake of AIN-93G diet. The female SHRsp consumed approximately 17 g out of the 20 g of modified AIN-93G that they were fed daily. There was usually food left over for all of the animals. Therefore it is safe to assume that the female SHRsp were fed

ad libitum and the results that are obtained are due to the effects of the dried broccoli sprouts. It is possible that the broccoli fed animals were compensating for the kilocalories obtained in their diet from the dried broccoli sprouts and therefore consumed less of the AIN-93G diet.

Can the ITCs in the dried broccoli sprouts be detected in the plasma of SHRsp?

The procedure used for examining ITC concentration in my study has been used to study the effects of glucosinolate consumption in humans (Liebes et al., 2001; Shapiro et al., 2001; Ye et al., 2002). When a dose of 200 μM of isothiocyanates was administered to humans, peak concentrations of ITCs were reached at 1 hour, and by 8 hours 58.3 ± 2.8% of the dose was detected (Ye et al., 2002). No pharmacokinetic studies examining the long-term administration of dried broccoli sprouts have been performed in rats. Therefore it is not possible to say what theoretical concentration of ITC concentration in the plasma of SHRsp should be present after 3 months of broccoli sprout administration.

Results from Experiment 1 demonstrate that concentrations of DTC, which are reflective of ITC concentration in the plasma of the broccoli fed animals, were significantly higher than those in the control group. Since glucosinolates which are converted into ITC and then into DTC are primarily found in the Cruciferae family of vegetables (as reviewed in Fahey, 2001), there should not have been glucosinolates in the diet of the control group. In the control group, the detection of low levels of DTC occurred due to incorrect sample preparation. Either rubber from the vacutainer lids or the latex gloves used is believed to have contaminated the samples. Within the broccoli group the variability in the DTC concentration was higher than expected. This variability can be accounted for by the time duration over which the samples were collected. In my

study, SHRsp were fed dried broccoli sprouts 24-31 hours before they were perfused. Ideally all of the animals would have had their blood collected at the same time from their last feeding.

Despite the issues raised concerning the way in which blood was collected, the difference observed in ITC concentration between the control and the broccoli group is thought to be reliable and indicative of the absorption of ITCs from the broccoli sprouts in the diets of the broccoli fed animals.

Does the daily feeding of dried broccoli sprouts decrease oxidative stress in blood?

The feeding of broccoli sprouts increased blood GSH concentration in male but not female SHRsp, whereas protein thiol levels were increased in female but not male animals. Overall, levels of protein thiols are higher in female SHRsp than in male SHRsp.

Blood GSH and protein thiols were used as markers of oxidative stress (Asensi et al., 1999; Hu, 1994), with an increase of either of these markers thought to be indicative of less oxidative stress. Respective concentrations of blood GSH and plasma protein thiols in rats is 20 μM and 300-500 μM (Hu, 1994). The amounts obtained in my study fall below this range. This may be due to the rat strain used in this study. This increase of blood GSH in males suggests that they were experiencing less oxidative stress and the increase in protein thiols in the females with broccoli sprout feeding suggests that they were experiencing less oxidative stress. However if both the male and female animals were experiencing less oxidative stress, then one would expect that both markers of oxidative stress would reflect this. A battery of tests is needed to reliably detect oxidative stress. Therefore, a limitation of this study is that only two markers of oxidative stress

were examined. Regardless of this limitation, the results that are observed in my study may be indicative of a gender effect in the SHRsp.

A number of studies provide evidence for a gender effect between male and female SHRsp. Female SHRsp experience lower blood pressure (Kato et al., 2003; Kerr et al., 1999), less kidney damage (Gigante et al., 2003), lower superoxide generation (Kerr et al., 1999) and smaller infarct size following stroke (Carswell et al., 1999) than male SHRsp. This gender effect appears to be dependent on diet and age. Blood pressure is higher in male SHRsp than in female SHRsp on a stroke permissive diet (5% fat; 0.4% sodium, and 0.75% potassium). However, if the same male and female SHRsp at the age of 20 weeks are then salt loaded for 1 month**,** then their blood pressure does not differ (Kato et al., 2003). Further evidence supporting an interplay between gender, age and diet is shown in a study by Kato et al., (2003). Screening of SHRsp male and female rats for quantitative blood pressure loci in the genome revealed the importance of sex, age and diet on blood pressure (Kato et al., 2003). Some blood pressure quantitative trait loci were present only in females, some were expressed only at certain ages, and some were more prominent on a salt loaded diet. Results from my study that support a gender effect are for blood GSH concentration, plasma protein thiols concentration, and activities of GST and QR. Results from this study that do not show a gender effect are those for albumin concentration, CP concentration and GR activity. It is possible that since there is an interplay between age and gender, a lack of gender effect in some of the parameters that I observed may be a consequence of animal age, even though the animals were age-matched.

A limitation of the experimental design in this project is that the estrous cycle of the female animals was not followed. It is possible that in the female SHRsp an increase in blood GSH concentrations induced by broccoli may occur at other times in the estrous cycle. Another possibility is that protein thiols concentration may decrease at different times of the estrous cycle. More studies are required whereby the effects of estrous cycling on parameters of oxidative stress are examined. During ischemia female SHRsp in metaestrus had larger brain infarcts than both females chosen at random and male SHRsp (Carswell et al., 1999). Carswell et al., (1999), therefore emphasizing the importance of gender and the stage of the estrous cycle on outcome in ischemia. It is possible that parameters other than infarct size may be influenced by estrous cycling.

What is evident from the examination of blood GSH and protein thiol concentrations is that there is an interaction between the effects of dried broccoli sprouts and animal gender. There are few studies that use both male and female SHRsp as a model. This study highlights the findings of a gender effect in SHRsp.

Does the daily feeding of dried broccoli sprouts decrease the APP?

The daily feeding of dried broccoli sprouts to SHRsp does not appear to have any effect on APPs as evidenced by no significant difference in albumin and ceruloplasmin levels between the controls and broccoli fed animals for both the male and female SHRsp. It is possible that the SHRsp are experiencing changes in APP that are not being detected due to the experimental design used in this study. Albumin and CP may not be sensitive enough markers for detecting a change in APP and other markers may be more sensitive. A proteomics approach such as that used by Sironi et al., (2001), which showed that SHRsp experience an APR, may be more suitable. The technique used to detect APP

in my study is western blotting. Western blotting is a semi-quantitative technique, which is more suitable for detecting large differences between groups. Western blotting may therefore have lacked the sensitivity to detect changes in APP in my study. Also, the SHRsp may experience changes in APP that are dependant on their age. Sironi et al used male SHRsp that were approximately 13 weeks old, whereas, the animals used in this study were perfused at 35 weeks of age. The animals in Sironi's study (2001), lost 10% of their body mass over time, whereas the animals in my study did not suffer from any weight loss. Therefore the experimental conditions namely, diet and age in Sironi's study may account for the increased presence of certain proteins in the serum and urine of SHRsp compared to SHR and WKY animals.

The other alternative is that in Sironi's study the SHRsp are not experiencing an APR. In the study by Sironi, the SHRsp are on a diet that is supplemented with 1 % NaCl. Given that SHRsp are salt sensitive and that this sensitivity is associated with renal damage as shown by proteinuria (Griffin et al., 2001), the SHRsp may be experiencing kidney damage whereby larger proteins pass through the kidneys and are released into the urine. Sironi detected more profound changes in urine proteins than in serum proteins and perhaps this is more indicative of kidney damage.

Drawbacks in experimental design of my study include the lack of a control strain of rat, no time and dose response curve for the dried broccoli sprouts, and the type of diet. In the absence of a control strain of rat, it is not possible to determine if both the broccoli and control groups were or were not experiencing an APR.

Another issue is the dose of broccoli sprouts that was used in my study. It is possible that a 200 mg dose of dried broccoli sprouts is not enough to dampen the APR.

A dose response curve would be useful in determining the effects of dose on APP. Also, the time at which the broccoli sprouts were administered may have been too late. At 2 months of age the SHRsp are already developing high blood pressure. Perhaps if the broccoli sprouts were administered earlier then an effect would be observed. Also at the time when the SHRsp were perfused they may have been too old for the broccoli to be effective. A time response curve for the broccoli sprouts would reveal if broccoli sprouts influence the APP in an age dependant manner. Lastly, the nature of the diet that the SHRsp were on may affect the APR. The SHRsp in my study were on a diet that is not supplemented with NaCl unlike that of Sironi et al. (2001), in which the animals received a diet that was NaCl supplemented. In the presence of a NaCl supplemented diet broccoli may have been effective at lowering an APR.

Does the daily feeding of dried broccoli sprouts induce phase 2 enzymes?

The activity of GR was not significantly different for either the male or female SHRsp nor for the SD rats. A gender effect was seen for GST and QR whereby the feeding of dried broccoli sprouts influenced values in the female SHRsp and not those of the male SHRsp. Overall activity levels were higher for the female SHRsp compared to the male SHRsp for GST and QR. The activity for QR was the highest in the SD rats compared to the female and male SHRsp.

Possible explanations for the results are that the regulation of phase 2 enzymes may be gender dependent, differentially controlled, and rat strain dependent. The gender differences seen in SHRsp are discussed above. The results obtained from GST and QR provide further examples of a gender difference between male and female SHRsp. Estrogen may play a role in the regulation of phase 2 enzymes. Other than via

electrophilic inducers, it is thought that phase 2 enzyme induction occurs when antiestrogens bind estrogen receptors that then bind to the electrophilic response element (Montano and Katzenellenbogen, 1997). Montano et al. have demonstrated that QR, GST-Pi and GCL are upregulated by an estrogen receptor mediated mechanism (Montano et al., 2004). Antiestrogens are compounds that compete with estrogen for the estrogen receptor and can act as either antagonists or agonists. There is some evidence that suggests that estrogen decreases the activity of phase 2 enzymes (Ansell et al., 2004). However compounds such as resveratrol that are phytoestrogens have been shown to induce phase 2 enzyme activity (Mizutani et al., 2000). This discrepancy between the roles played by agonistic versus antagonistic estrogenic effects may be accounted for by a study performed by Ansell et al., (2004). The administration of phytoestrogens and estrogenic and antiestrogenic compounds shows that estrogen receptor signalling to the ARE is dependent on cell type, ligand, and receptor (Ansell et al., 2004). From the results of my study**,** estrogen in the presence of broccoli sprouts may be increasing QR activity and downregulating GST activity. This suggests that the effect of estrogen in the presence of broccoli on phase 2 enzymes is differential in that it can vary from enzyme to enzyme.

Alternatively, different elements may be present in the promoter region of the respective phase 2 enzymes, explaining the differential effects of broccoli on GST and QR in the female SHRsp. For example, the phase 2 enzymes GCL and GST have a κB element in their promoter region and can therefore be activated by oxidative stress (as reviewed in Hayes and Pulford, 1995). It is possible that oxidative stress in the SHRsp control group upregulates GST activity.

Another explanation for the results obtained for GST is that broccoli is down regulating GST activity via either the ARE or other products that are in broccoli. This is plausible given that GST plays a role in Sul metabolism. Sul enters cells and forms DTC which are the products of ITCs reacting with thiols such as GSH (Zhang, 2000; Zhang, 2001; Zhang and Talalay, 1998). The accumulation of these DTC can result in phase 2 enzyme induction (Ye and Zhang, 2001). Given that GST catalyzes the formation of glutathiol adducts, the upregulation of phase 2 enzymes may be a consequence of requiring GST for Sul metabolism. Most studies that examine the effects of Sul on phase 2 enzyme induction are short term studies. It is possible that long term administration of Sul results in a negative feedback loop thereby decreasing GST activity.

Lastly**,** a limitation of the assay used to detect GST is that it is biased towards certain GST isoforms. The GST family of enzymes is comprised of about 5 related families namely, alpha, mu, pi, sigma and theta (as reviewed in Hayes and Pulford, 1995). CDNB has a high affinity for the Mu subfamilies M1 and M2), a moderate affinity for alpha and Pi subfamilies A1, A2 and P1, a low affinity for the Mu subfamily M3, and no affinity for the theta sub families (as reviewed in Eaton and Bammler, 1999). This suggests that there may be an upregulation of a specific GST subfamily in the broccoli fed group but this assay is not sensitive enough to all of the GST isoforms to detect it. The lack of sensitivity of the assay may also account for the lack of a significant difference in the male SHRsp. Males have higher levels of the M3 and M4 subfamilies of the GST Mu family than females (as reviewed in Tsuchida and Sato, 1992). Therefore**,** if in males the M3 subunit is upregulated**,** CDNB has a low affinity for M3 and will not maximally detect it.

The induction of GR and GST in SHRsp demonstrates tissue specificity. Work in our laboratory has shown that the administration of dried broccoli sprouts increases the activity of GR but not GST in the aorta of male SHRsp (Wu et al., 2004 and unpublished work). I have demonstrated that in the liver the activity of GR does not change where as that of GST decreases with administration of dried broccoli sprouts. Phase 2 enzymes have been shown to exhibit tissue specificity (as reviewed in Pickett and Lu, 1989).

Preliminary data from the male SD rats suggest that in the liver, the activity of GST is a little higher and QR is about six times higher than in male SHRsp. Activities of GST and QR in the female SHRsp are closer to those of the male SD rats than are the activities of GST and QR in the male SHRsp compared to the male SD rats. It is possible that the activities of GST and QR reflect better overall health in female SHRsp than male SHRsp since female SHRsp experience lower blood pressure (Kato et al., 2003; Kerr et al., 1999), less kidney damage (Gigante et al., 2003), lower superoxide generation (Kerr et al., 1999) and smaller infarct size (Carswell et al., 1999). Male SHRsp have been described to undergo premature central nervous system ageing (Ashraf et al., 2004 submitted) Given that the severity of the pathologies is worse in male SHRsp compared to female SHRsp, perhaps overall male SHRsp experience accelerated ageing. If GST is required for Sul metabolism, and the activity of GST is mirrored in female SHRsp by the control SD rat, then perhaps Sul metabolism is altered in the male SHRsp. Pharmacokinetic studies on the long term administration of broccoli sprouts would be required to test this hypothesis.

Concluding remarks

The findings for oxidative stress, APPs and phase 2 enzyme induction do not present a clear pattern to support the hypothesis that, the dietary intake of broccoli sprouts by inducing phase 2 enzymes in the liver will decrease oxidative stress and the acute phase response in the blood of SHRsp. In male SHRsp, broccoli feeding resulted in significantly higher levels of blood GSH relative to control diet but no differences were detected in protein thiol concentration, CP levels, albumin concentration, GR activity, GST activity or QR activity. In female SHRsp**,** broccoli feeding resulted in significantly higher levels of protein thiol concentration and QR activity, and lower levels of GST activity, whilst no difference was detected in blood GSH concentration, CP levels, albumin concentration or GR activity. For liver enzyme activity, broccoli feeding in male SD rats resulted in a significant difference in GST activity, but not in GR activity. A larger sample size may have rendered QR activity significant. There were some similarities between the male and female SHRsp with respect to albumin concentration, CP concentration and GR activity, which did not change significantly as result of diet.

The mechanism by which dietary intake of broccoli sprouts affect parameters of oxidative stress and phase 2 enzymes is unclear and appears to be further complicated by animal gender. An extension of this work would be to incorporate the ITC into the modified AIN93-G diet and then: 1) further examine the role of estrogen and Sul on phase 2 enzyme induction in various tissues of female SHRsp 2) perform a time response curve for the administration of ITC to examine if the protective effects exerted by Sul exhibit a temporal pattern 3) examine phase 2 enzyme protein levels of GR, GST and QR in both male and female SHRsp and SD rats 4) utilise 2-D protein gels to look at an increased number of APP such as transferrin, haemopexin, haptoglobin and 5) examine

other parameters of oxidative stress such as 4-hydroxynonenal, protein carbonyls, and 8-hydroxyguanosine (these are respective markers of lipid, protein, and DNA modification due to increased levels of oxidative stress).

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