Influence of acute and chronic glutathione manipulations on coronary vascular resistance and endothelium dependent dilation in isolated perfused rat hearts

by

Andrew Shawn Levy

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Glutathione (GSH), a 3-amino acid compound is ubiquitously expressed in eukaryotic cells and is the most abundant low molecular weight thiol. The importance of GSH is highlighted by its multitude of effects. Within the vascular wall GSH plays a crucial role as an intracellular antioxidant and it possess the ability to act as a signalling intermediate and store for nitric oxide (NO). The importance of NO and its role in vascular wall homeostasis is well recognized. Within the coronary circulation, NO is the primary dilator of many of the large arteries and the smaller arterioles. In addition to controlling coronary vascular tone, the importance of NO is highlighted by its antithrombotic, antihypertrophic, and antriproliferative effects. During instances of cardiovascular disease and normal aging, increases in the production of reactive oxygen species occur. A portion of the deleterious vascular effects of reactive oxygen species are believed to be due to reduction in NO bioavailability as a result of increased ROS-mediated destruction of NO. Altered GSH production in humans has been demonstrated to reduce endothelial function. Conversely, supplementation with GSH augments endothelium-dependent dilation. The mechanisms by which these alterations in GSH influence vasomotor function have not been resolved. The purpose of the studies within this thesis was to examine the impact of chronic and acute GSH modulations on coronary vascular resistance (CVR) and endothelium dependent dilation. In all experiments vascular reactivity was assessed in the isolated perfused rat heart. The advantage of this technique is that it allows the global coronary vasomotor functioning to be examined. Hearts were allowed to stabilize for 30 minutes to allow for the development of spontaneous coronary vascular resistance, followed by a bradykinin (BK) dose-response curve to assess endothelium-dependent dilation. The coronary circulation was then maximally dilated using an endothelium-independent agonist. In all cases BK-mediated dilation is expressed as a percentage of the endothelium-independent dilation.

Chapter 2 of this document examines the chronic nature of GSH depletion and examines whether GSH depletion augments the influence of natural aging. Animals (mean age 33 and 65 weeks) were randomized to receive L-Buthionine-(S,R)-sulphoximine (BSO) in the tap water in order to inhibit GSH synthesis, or regular tap water (normal controls). Following 10 days of BSO treatment, ventricular GSH content was reduced in the BSO group compared to the control (0.182±0.021 vs 2.022±0.084 nmol/mg wet weight, $p<0.05$) and there was increased ventricular H_2O_2 content (1.345±0.176 vs 0.877±0.123 pmol/μg PRO, $p<0.05$). Baseline CVR was significantly reduced in the older animals compared to the adult animals (3.92±0.34 vs 4.76±0.20 and 3.67±0.24 vs 5.12±0.37 mmHg/ml×min^{-1} in the control and BSO treated groups, $p<0.05$). Conversely, in the presence of LNAME there was a significant increase in CVR in the adult BSO group (14.15±0.99, $p<0.05$) compared to all other groups. In the absence of LNAME, maximal dilation (percent endothelium-independent response) was reduced in the older animals compared to the adult animals (77±10.3% vs 95.0±1.0% for older and adult control and 92.7±4.5% vs 98.6±0.6% for the older and adult BSO, main effect of age). In the presence of LNAME the adult BSO group had a significantly reduced sensitivity (EC_{50}) compared to all other groups (-7.39±0.09 Log M, $p<0.05$). Additionally, adult BSO treated animals had an increase in eNOS protein content. These results demonstrate that chronic thiol depletion resulted in an increased reliance on NO in the adult BSO group only.

In chapter 3 the beneficial effects of GSH supplementation on BK mediated dilation were examined. Acute GSH was administered in the perfusate at either 0 (control) or with 10 μM for 2 reasons, 1) this concentration does not reduce basal coronary vascular resistance, allowing for a similar baseline CVR across conditions and 2) the 10 μM concentration is a physiologically relevant concentration of plasma/extracellular fluid GSH. The sensitivity to the endothelial agonist bradykinin was enhanced in the presence of GSH (-8.70±0.16 vs -7.94±0.06 LogM, $p<0.01$). The GSH effect was not dependent on NO production or utilization by soluble guanylate cyclase (sGC) as the enhanced

dilation in the GSH group was maintained despite NOS (LNAME) and/or sGC inhibition. When the hearts were supplemented with a ROS scavenger TEMPOL, enhanced dilation was seen in the control group, but was not further enhanced in the GSH group. The requirement for ROS was best demonstrated when both the CON and GSH groups were supplemented with both TEMPOL and LNAME. This condition resulted in similar sensitivity (-7.76±0.19 vs -7.75±0.17 LogM, $p>0.05$) and area under the curve (182.33±12.70 vs 170±13.86, $p>0.05$) between GSH and CON. Thus, it was concluded that the effects of GSH administration requires the presence of ROS and exerts its effect in the microvasculature.

The study presented in chapter 4 examined the effects of acute thiol modulation (depletion) on CVR and endothelium-dependent dilation. Previous reports have suggested that a reduction in intracellular GSH causes impaired NO production, and functional data support this contention. However, a majority of the data regarding the effects of thiol manipulation are from endothelial-removed vessels. The following agents were used to reduce GSH: the glutathione reductase inhibitor, BCNU; the thiol oxidizing agent, diamide; the thiol conjugating agent, ethacrynic acid (EA); and a thioredoxin inhibitor (CDNB). Preliminary data revealed that only CDNB (11.46±0.71 mmHg/ml×min^{-1}) and EA (8.61±0.36 mmHg/ml×min^{-1}) caused an elevation in CVR compared to the control (6.73±0.24 mmHg/ml×min^{-1}). Conversely, Diamide and BCNU did not significantly affect baseline CVR, or the BK mediated responses. In the presence of EA, there was an overall blunting of the BK-response curve as observed by reduced EC_{50} (-7.85±0.07 Log M) and maximal dilation (90.8±1.8 %, percent endothelium-independent dilation) compared to the control group (-8.42±0.08 Log M and 97.7±1.6%). In the presence of CDNB the maximal dilation was 74.4±1.9% and the EC_{50} was -8.83±0.28 Log M. In addition to altering BK mediated responses, acute thiol depletion with all agents resulted in an increased minimal CVR with significant increases observed in the presence of CDNB and EA. There was a significant correlation with GSH:GSSG ratio and baseline (-0.547, $p<0.05$) and minimal CVR ($r=-0.581$,

$p<0.05$). This study demonstrates that modulation of the GSH:GSSG ratio using a variety of agents with diverse mechanisms elicits differential responses within the vasculature. Specifically conjugation of GSH and inhibition of thioredoxin significantly alters BK mediated response, where as BCNU and dimaide did not. These results suggest that a modulation in the GSH:GSSG ratio impairs endothelium-dependent dilation and alters total dilatory capacity (baseline-minimal CVR) and thus may have implications for adequate tissue perfusion.

Across all studies there were significant inverse correlations between GSH and GSSG with both baseline and minimal CVR. Therefore it is likely that changes in overall glutathione content plays a role in determining baseline and minimal coronary vascular resistance. These results demonstrate the complexity that manipulations of GSH have on both CVR and endothelium-dependent dilation, and provide mechanistic insight into how changes in GSH alter coronary vascular resistance and endothelium-dependent dilation.

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There are a number of people who need to be thanked here, for the sake of sanity and to save some trees I will try my best to keep it brief.

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Dedication

This thesis is dedicated to Dylan Matthew Levy.

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List of Abbreviations

±dP/dt	maximal rate of pressure increase or decrease
ACh	acetylcholine
ADO	adenosine
AUC	area under curve
BCNU	1,3-Bis(2-chloroethyl)-1-nitrosourea
BK	bradykinin
BSO	buthionine (S,R) sulphoximine, glutamate cysteine ligase inhibitor
CDNB	1-chloro-2,4-dinitrobenzene
COX	cyclooxygenase
CVR	coronary vascular resistance
EA	ethacrynic acid
EC	endothelial cell
EC_{50}	sensitivity, concentration required to elicit 50% of the dilatory response
EDCF	endothelial derived contracting factor
EDHF	endothelial derived hyperpolizing factor
EDRF	endothelial derived relaxing factor
eNOS	endothelial nitric oxide synthase
GCL	glutamate-cysteine ligase
GPx	glutathione peroxidase
GR	glutathione reductase
GRx	glutaredoxin
GS	glutathione synthetase
GSH	reduced glutathione
GSNO	nitrosoglutathione
GSSG	oxidized glutathione
INDO	indomethacin [non specific COX inhibitor]
iNOS	inducible nitric oxide synthase
KH	Kreb's Henseleit solution
LNAME	N_{ω}-Nitro-L-arginine methyl ester hydrochloride
LVdiaP	left ventricular diastolic pressure
LVDP	left ventricular developed pressure
LVsysP	left ventricular systolic pressure
ND	no drug
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
NS398	Selective COX-2 inhibitor, N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide
ODQ	1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one, sGC inhibitor

PPP	pentose phosphate pathway
ROS	reactive oxygen species
RSNO	nitrosothiols
SC560	Selective COX-1 inhibitor, 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole
SERCA	sarco(endo)plasmic reticulum ATPase
sGC	soluble gaunylate cyclase
SNP	sodium nitroprusside
SOD	superoxide dismutase
TDC	total dilatory capacity
TEMPOL	4 Hydroxy TEMPO (SOD mimetic)
TRx	thioredoxin
VEGF	vascular endothelial growth factor
VSM	vascular smooth muscle

CHAPTER 1

Introduction

1.1 Overview

Cardiovascular disease remains one of the leading causes of morbidity and mortality in the Western World, and is on the rise in many developing countries. Understanding the disease process and treatment strategies is essential in the prevention and treatment of disease. It has been 30 years since the discovery of endothelial derived relaxing factor (EDRF) which has subsequently been identified as nitric oxide (NO). Nitric oxide is generated by nitric oxide synthase (NOS), the most important isoform within the vascular wall being the endothelial specific isoform (eNOS). eNOS generates NO through a reduction/oxidation pathway converting L-arginine to NO. The importance of the identification of NO is highlighted by the pluripotent effects this molecule has at maintaining homeostasis within the vascular wall including vascular tone, as well as the inhibitory effects NO has on thrombosis, vascular smooth muscle proliferation and hypertrophy. The bioavailability of NO is reduced in disease states and is believed to be a contributing factor in the development of cardiovascular disease.

The ability of endothelium to respond appropriately to an agonist is a hallmark indicator of vascular health. However, NO is not the only dilatory agent released by the endothelium, as prostaglandins and non-NO-non-prostanoid derived vasodilatory agents have been identified. Furthermore, the identification of the release of endothelial derived contracting factors on endothelial stimulation has also been established. For the purpose of the current investigations, the primary focus is on NO given the prominent role it plays within the coronary circulation.

Free radicals or reactive oxygen species (ROS) are by-products of normal cellular respiration and immune responses. However in disease states, including cardiovascular disease, there is an

increased production of ROS, which results in pathological levels. These levels contribute to a reduction in NO bioavailability. For example, superoxide anion (O_2·-) rapidly combines with NO forming peroxynitrite (ONOO) which does not have the same antiproliferative or antithrombotic properties as NO. Within the vascular wall several key antioxidants play a role at mitigating the effects of free radicals including superoxide anion. A crucial intracellular antioxidant is glutathione (GSH). This ubiquitously expressed tripeptide is found within all eukaryotic cells. The role of GSH within the vascular wall extends beyond its antioxidant capabilities as GSH is also involved as a signalling agent used to alter protein function. The central focus of this thesis is examining the impact of modulating GSH levels chronically (chapter 2) and acutely (chapters 3 and 4) to determine how changes in GSH content or availability impact endothelium-mediated vasomotor function within the intact coronary circulation.

1.2 Background

1.2.1 Glutathione metabolism

Thiols are the product of sulphur metabolism and refer to compounds which contain a sulfhydryl group (-SH). Glutathione (reduced glutathione, GSH) is a 3 amino acid compound consisting of glycine, cysteine, the sulphur containing amino acid, and glutamate (1, 46, 65, 124, 160). Glutathione is ubiquitously expressed in all eukaryotic cells and is the most abundant of the low molecular weight thiols (46, 65, 160, 192). The concentration of GSH varies between 0.5-10 mmol/L depending on the specific tissues and cells being examined. The cytosolic compartment contains 85-90% of the total cell GSH, ~10% is located within the mitochondria and a small amount is located in the endoplasmic reticulum and the nucleus. In plasma, GSH can be found at concentrations ranging between 2 – 20 µmol/L (38, 65, 150, 192). GSH can be oxidized to oxidized glutathione (GSSG), but it is kept primarily (99%) as GSH within the cell (46, 65, 124, 150, 160) by the widely expressed and highly

active enzyme glutathione reductase (GR, (123, 124)). The GSH:GSSG ratio is important at maintaining cellular oxidation/reduction (redox) potential. Therefore the activities of both GR and glutathione peroxidase (GPx) are key enzymes in the maintenance of cellular redox balance and control (65, 117, 123, 150, 155, 160, 178).

The generation of GSH is the subject of many reviews (1, 46, 123, 124, 160). The purpose of this section is to provide an overview of glutathione synthesis, the γ-glutamyl cycle, and to highlight some of the key enzymes involved in the generation and transport of GSH. The synthesis of GSH (Figure 1.1) is controlled by 2 enzymes. γ-glutamylcysteine synthetase (GCS) catalyzes the reaction between L-glutamate and L-cysteine utilizing one molecule of ATP and Mg^{2+} as cofactors (1, 46, 77, 123, 124, 160). The activity of this enzyme is controlled by the availability of L-cysteine (1, 77, 124, 160, 192), but can be inhibited by negative feedback as high levels of GSH can attenuate GCS activity (1, 46, 77, 123, 124, 160). The activity of GCS can also be modulated by phosphorylation/dephosphorylation and oxidative/nitrostative stress. In the presence of oxidative stress there is both a compensatory increase in the transcription and activity of GCS (77). Furthermore, studies have demonstrated that concentrations of nitric oxide (NO) < 1 mM promote cystine (oxidized cysteine) uptake (111) and activation of GCS (127) in endothelial cells. This suggests that within the vasculature, signalling by NO may help promote GSH synthesis. The final step in GSH synthesis involves the combination of the newly formed L-glutamyl-L-cysteine molecule with glycine. This step is catalyzed by glutathione synthase and also utilizes ATP and Mg^{2+} (1, 46, 77, 124, 160). Most of the research regarding the control of GSH synthesis has focused on the GCS as it is the rate limiting step and the primary control point for GSH synthesis (77).

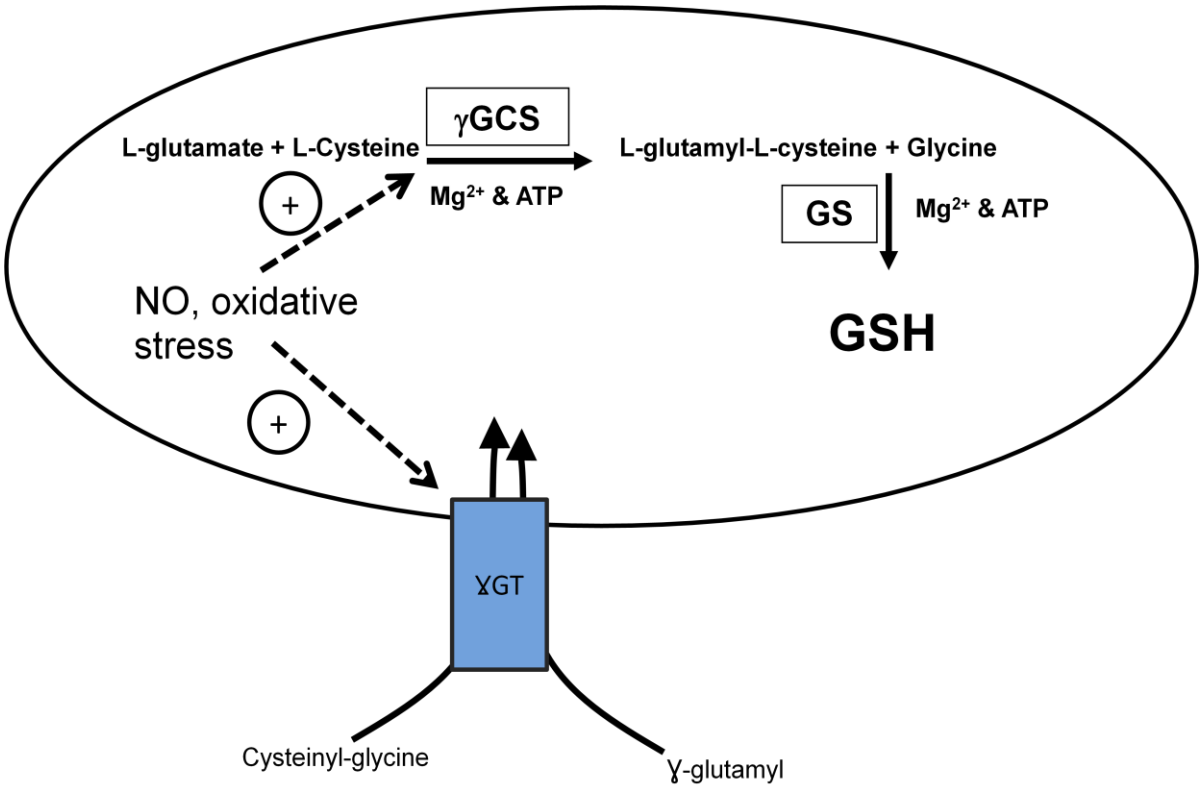

Figure 1.1 Pathway of Glutathione Synthesis
GSH is generated by 2 enzymes, each of which uses 1 molecule of ATP and Mg^{2+} as cofactors. The rate limiting step in this reaction is GCS, as it is controlled primarily be cysteine availability. ɣGT located on the outer cell membrane serves as an anchor for catabolic products of GSH and is used to secure and uptake the specific amino acids required for GSH synthesis.

GSH can also be generated through a salvage pathway. This pathway involves ɣ-glutamyl transpeptidase (ɣ-GT). The ɣ-GT is located on the outer cell membrane and aids in the transport of catabolised GSH and other glutathione complexes into the cell. Typically a cysteinylglycine component and a ɣ-glutamyl amino acid component are transferred to the cytosol where they can then be further processed to amino acids and reused for glutathione synthesis (46, 65, 123). NO-induced increases in

GSH in endothelial cells requires the activity of γ-GT, but it remains unknown if NO activates γ-GT (127). Most cells possess the ability to export both GSH and GSSG by a group of GSH transeferases (46, 65, 123). During instances of elevated GSSG, it is possible for GSSG to be exported from the cell, which can lead to a depletion of GSH (46, 65, 85). The function of the salvage pathway and γ-GT is to recoup the extruded GSSG, and this represents a second mechanism for GSH synthesis as there is no direct transport of GSH into the cell.

1.2.2 Control of coronary blood flow - the primary role of nitric oxide

Ensuring an adequate supply of oxygen and nutrients to the myocardium requires a near constant supply of blood. This would explain why much attention has been directed at matching myocardial perfusion to the metabolic demands of the heart, and the existence of multiple and redundant pathways that ensure this relationship is sustained regardless of the metabolic demands (59, 129, 176, 185). The primary mechanisms controlling coronary artery tone can be classified into 4 primary categories: metabolic, myogenic, neurohormonal, and endothelial and have been the subject of many reviews (59, 176, 185). A combination of these factors is used to determine myocardial perfusion and flow distribution with the relative importance of each mechanism varying as a function of the anatomical location of the vessel within the vascular network and depth within the myocardium itself. Large coronary arteries and arterioles rely heavily on endothelium-dependent mechanisms for maintenance of proper tone; conversely, the tone of smaller arterioles is more dependent on metabolic and myogenic mechanisms as they are often found deeper with the myocardial wall (27, 61, 93, 129). The capacity to change coronary vascular resistance varies within the vascular network of the coronary circulation. Approximately 75% of the resistance of the coronary circulation is determined by arteries between 75 – 200 μm in diameter (35, 93, 115, 129), albeit resistance also varies according to the location of the vasculature within myocardial wall (34, 93, 129). Generally, as the size of the

coronary vessel decreases the pressure drop observed across the same vessel is increased (27). Therefore, the ability of the coronary vasculature to regulate perfusion and tone is dependent on the distinct functioning of each segment of the coronary arterial network (27).

Nitric oxide is the primary dilator of large epicardial coronary arteries (61, 98, 176) as well as the mediator of flow-induced dilation in the coronary microcirculation (61, 93, 129). Furthermore, NO interacts with the α-adrenergic control of the coronary circulation by blunting the constriction induced by α-agonists (129). It is because of the multitude of effects of NO on the coronary circulation that make it arguably one of the most important vasoactive substances acting on the coronary circulation. Szekeres and colleagues (2001) demonstrated that arterioles dilate to a greater extent to the endothelium dependent dilators bradykinin (BK) and acetylcholine (ACh) than do the arteries. Additionally, arterioles demonstrate a greater amount of constriction in the presence of NOS inhibitors compared to large arteries (168). Interestingly, the eNOS protein content within the coronary vascular network is not reduced until reaching the smallest coronary arterioles (106), suggesting that NO production can occur to a similar extent throughout the vasculature of the heart. Therefore it is likely that NO can act as a dilator at all levels of the coronary vascular tree, despite the differences that exist as to what the primary dilatory agent is within a specific portion of the coronary vascular network. The general regulatory effects of NO and the role of reduced NO have been the subject of numerous reviews (26, 60, 64, 110, 145, 173) all of which highlight that the control of vasomotion is driven by NO and the bioavailability is controlled by production, destruction and sensitivity of the target tissue. The importance of prostacyclin and other endothelial derived vasodilators are not as important as NO with respect to coronary endothelial-mediated dilation, but may be important in pathological states (60, 71, 115, 176). The powerful influence of NO is demonstrated by the suppressive nature NO has on the dilation to other dilatory pathways in the coronary circulation (18).

This discussion demonstrates the important role NO plays as a dilatory agent within the coronary circulation. The importance of NO extends beyond its ability to maintain coronary perfusion as it plays an important role in the overall homeostasis of the vascular wall including antiplatelet capabilities, suppression of vascular smooth muscle growth and proliferation and the ability to suppress adhesion and atherosclerotic plaque development (26, 125). Therefore, alterations in the ability of the coronary vasculature to respond to endothelial stimulation are used as a surrogate of overall vascular health.

1.3 Rationale for examining the interaction between thiols and vascular function

1.3.1 The association between changes in GSH content and GSH:GSSG ratio and the risk of cardiovascular disease.

Recent studies have demonstrated that genetic polymorphisms in the enzymes of glutathione synthesis are associated with increased risk of negative cardiovascular outcomes. Koide and colleagues found a genetic variation in the *catalytic* subunit of glutamate-cysteine ligase (GCL or GCS). This mutation resulted in a -129C/T leading to 3 distinct genotypes CC, CT and TT. After controlling for traditional risk factors the individuals with the CT and TT polymorphism were at an increased risk of myocardial infarction (MI) and demonstrated robust endothelial dysfunction, as assessed by coronary angiography, compared to the CC. Furthermore, this mutation (CT or TT) resulted in reduced ability to modulate the GCL activity in response to an acute oxidative stress challenge. This suggests that the inability to increase GSH is an important predictor of MI and strongly associated with endothelial dysfunction (101). Similarly, a mutation on the *modifier* subunit of GCL (-588C/T allele) was also found to be associated with MI (133) and subsequently with impaired ACh response demonstrated by increased constriction and an attenuated increase in flow in patients undergoing angiography (134). The -588T polymorphism was also associated with a reduced plasma level of GSH (133) and basal NO

bioavailability as demonstrated by a blunted response to infusion of l-arginine analogue, L-NMMA (134). These findings indicate that reductions in GSH are associated with an impaired bioavailability of NO and increased risk of MI. Despite this evidence more information is required to identify the mechanisms responsible for the altered endothelium-dependent dilation. Thus, by examining both acute and chronic manipulations in GSH a better understanding of the role that GSH plays in maintaining vasomotor health will be established.

The reduction in GSH content may represent one aspect of increased risk of cardiovascular disease; however decreases in GSH will also alter cellular redox balance. The importance of the GSH:GSSG ratio and redox balance was highlighted by Ashfaq and colleagues who showed this ratio was significantly correlated with the Framingham risk score in an apparently healthy population ($r = 0.25$, $p<0.01$). Furthermore, there was an inverse relationship between GSH content and carotid artery intima-medial thickness ($r = -0.39$, $p<0.0001$) (12). In a subsequent study in a slightly healthier population (Framingham risk score ~ 0.2±6.4), no relationship was seen between the GSH, GSSG, or the GSH:GSSG ratio and flow mediated dilation. However, significant inverse correlations were observed between extracellular disulfide of cysteine and FMD and the mixed thiolated disulfide and FMD (11). Conversely, in patients with chronic renal failure, endothelial function was correlated with GSH ($r = 0.44$, $p<0.01$), inversely related to GSSG ($r = -0.4$, $p<0.05$) and the GSSG:GSH ratio ($r = -0.47$, $p<0.01$). These relationships remained significant after controlling for classical risk factors (9). These studies highlight that the relationship between endothelial function and GSH content may only become apparent in situations where endothelial function is reduced, as no relationship was apparent in healthy individuals but was apparent in those with renal failure. However, in healthy individuals a relationship was seen between oxidized cystine and FMD (11) suggesting that plasma levels of cystine (oxidized cysteine) may be a more important predictor of endothelial function than GSH in healthy individuals.

These studies lack a mechanistic insight into the relationship between GSH and coronary vasomotion, although they do suggest a strong independent relationship between GSH and cardiovascular events and endothelial dysfunction. This suggests that chronic impairment of GSH synthesis in humans is associated with increased risk of cardiac events and endothelial dysfunction. Despite this apparent limitation they do highlight that these polymorphisms and reduced GSH:GSSG ratio may result in impaired NO bioavailability. Therefore, in order to understand the relationship that exists between GSH or GSH:GSSG and endothelial function it is important to examine the mechanisms potentially responsible for this relationship utilizing alternative models.

1.3.2 The antioxidant effects of glutathione and its role in vascular aging and vasomotor function

GSH is a key component of the cellular antioxidant system, redox balance and cellular detoxification. GSH plays an integral role as an intracellular antioxidant (65, 155, 160, 178, 192). The primary antioxidant effect of GSH is its ability to be oxidized, thereby reducing H_2O_2 to water and O_2 (65, 178). This occurs via the enzymatic activity of GPx which serves to oxidize GSH to GSSG. GSSG is rapidly converted back to GSH by GR. The reduction of GSSG is accomplished through the oxidation of NADPH to $NADP^+$, which is recycled to NADPH + H^+ in the pentose phosphate pathway/cycle (1, 65, 77, 117, 124, 178, 192). This same process accounts for the detoxification of lipid peroxides to lipid alcohols and water (Figure 1.2). In instances of extreme oxidative stress, the GSSG concentration can become increased and extruded from the cell by transferases or via the concentration gradient (38, 77, 85, 117, 124). The loss of GSSG has the potential to decrease cellular GSH content. This may explain why during periods of increased oxidative stress the expression of GPx, GR, as well as GCS and ɣGS are increased (65, 77, 117, 189).

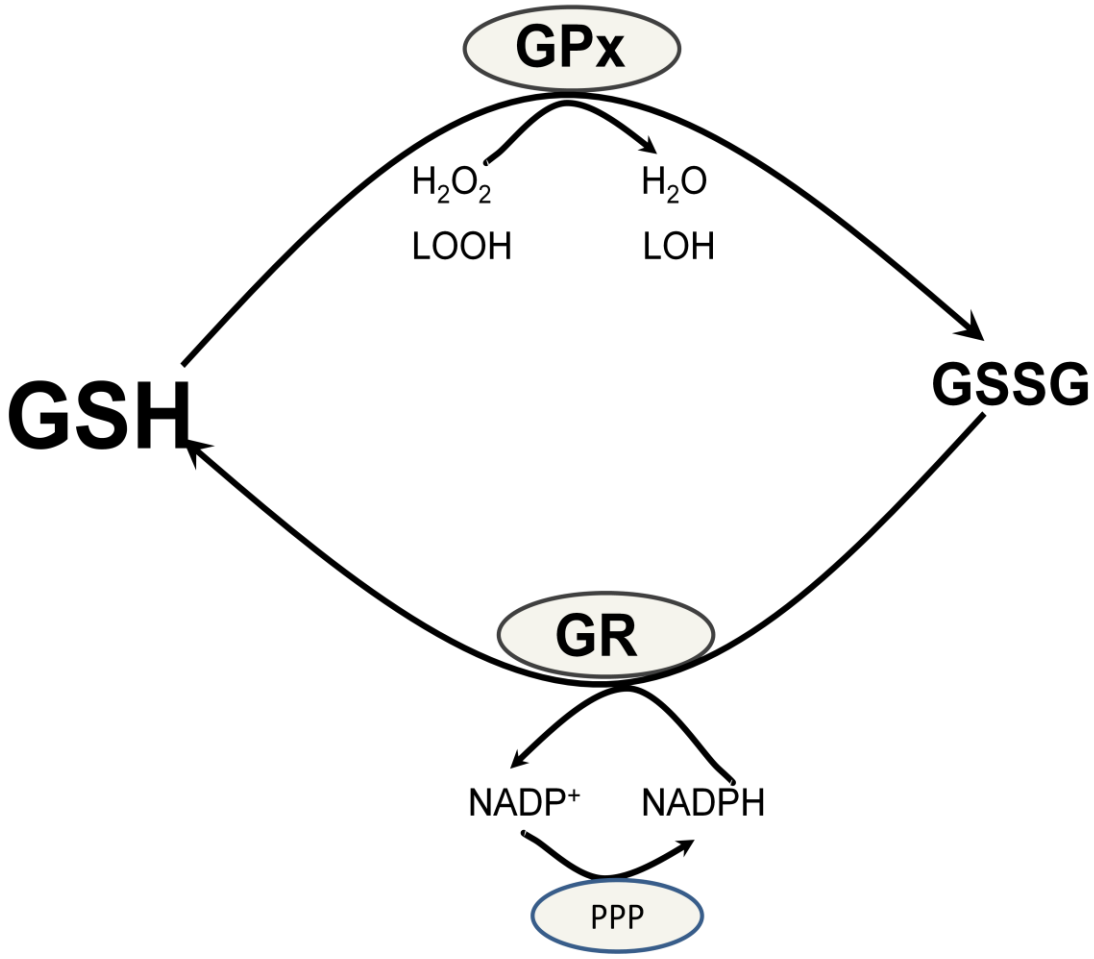

Figure 1.2. Primary detoxification of peroxides by GSH and its associated enzymes
Glutathione peroxidise (GPx) utilizes GSH as an oxidizing agent to reduce H_2O_2 and other peroxides. GSSG is rapidly reduced to GSH by glutathione reductase (GR) which utilizes NADPH as a cofactor. The pentose phosphate pathway (PPP) is the primary determinant of NADPH. GSH is presented as larger as it is found at higher concentrations within the cell (~10:1 GSH:GSSG).

Other radicals that can be quenched by GSH include O_2·-, ·OH, and reactive nitrogen species including NO. The reaction between O_2·- and GSH is independent of GPx, but a hydrogen ion is required. In this reaction, GSH is converted to a thiyl radical (GS·) and superoxide is converted to water. The thiol radical GS· will rapidly react with protein thiyl radicals or another GS· to yield thiolated proteins (RSSG) or GSSG (65, 85). It is believed that this mechanism helps protect proteins

against oxidative damage using a reversible thiolation process (38, 65). This clearly highlights that GSH possesses the ability to act as a general ROS scavenger and suggests a mechanism for preserving protein function (Figure 1.3).

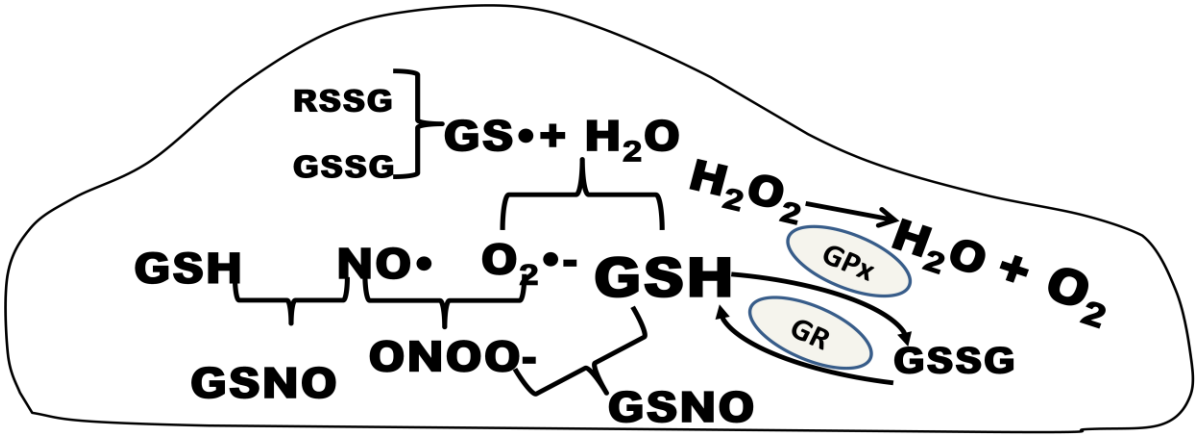

Figure 1.3. Summary of antioxidant effects of GSH

GSH possess several different antioxidant properties. While a majority of the antioxidant properties are focused on the enzymatic activities of glutathione peroxidase (GPx), glutathione reductase (GR), thioredoxin and glutaredoxin (omitted for clarity). GSH possess the ability to act as a scavenger for oxygen derived radicals such as $O_2^{\cdot -}$ or $OH^{\cdot}$, which requires the availability of an H^+ ion. These reactions yield a thiyl radical which is readily combined with a similar thiyl radical or sulphur residues on proteins forming thiolated proteins (RSSG).

Given the ability of GSH to mitigate the effects of free radicals it can be postulated that alterations in GSH influence vasomotor function by preventing the interaction between NO and O_2·- thereby improving NO bioavailability and preserving endothelial function. This effect is highlighted in the above discussion examining the vasomotor response in individuals with polymorphisms of the GCL (101, 133). In individuals with polymorphisms of GCL, NO bioavailability appeared to be reduced as demonstrated by the lack of constriction to l-arginine analogues and impaired dilation to endothelial agonists (101, 134). Furthermore, in animal preparations in which GSH had been chronically reduced, elevated levels of reactive oxygen species are a common observation (13, 19, 63, 67, 68, 107, 181, 196). In these animal preparations, reduction in endothelium-dependent dilation is also commonly observed (13, 63, 68, 107). These results suggest that in conditions where GSH is chronically reduced, there is an increase in the amount of reactive oxygen species and impaired endothelial mediated dilation. With the exception of the changes in humans with the GCL mutations, the effects of GSH depletion on coronary vascular function have not been examined.

Surprisingly there is limited data regarding changes that occur in GSH with aging. Total glutathione content of the vascular smooth muscle is reported to be increased with aging (112, 136), whereas in the endothelium GSH content has been reported to be reduced (157). In whole aortic homogenate, an age-dependent decrease in GSH, increase in GSSG and reduced GSH:GSSG ratio has been reported (62). These changes may explain why aging, like GSH depletion, is associated with increased ROS production (37, 95) and endothelial dysfunction (6, 37, 95). Several different mechanisms have been proposed for the vasomotor dysfunction with aging, albeit increased ROS production plays a pivotal role (for selected reviews see (24, 60, 125, 130, 190)). The importance of GSH at preventing and/or mitigating the effects of aging on vasomotor function has not been examined and warrants further investigation. This is the focus of chapter 2 in which the effects of GSH depletion and aging are examined in the coronary vascular bed.

1.3.3 GSH can modulate endothelium-dependent dilation and vascular tone

Given the apparent association between GSH and free radicals it is not surprising that GSH has been administered as an antioxidant to improve vasomotor functioning. Kugiyama and colleagues (1998) demonstrated that infusion of 1 mmol/L GSH into the coronary vascular bed was without effect on coronary vascular resistance. However, when GSH was co-infused with ACh, coronary dilation was observed. This dilation was in contrast to the coronary artery constriction observed in response to ACh alone (104). Subsequently, Prasad and colleagues demonstrated GSH could augment the dilatory responses in the peripheral circulation and suggested that NO bioavailability, was increased as demonstrated by increase in venous cGMP following co-infusion of GSH and ACh. In a subsequent study, Kugiyama and colleagues demonstrated that GSH reduced free radical production in the coronary sinus in those with and without vasomotor dysfunction, suggesting that GSH was acting as an antioxidant. One consensus that can be drawn from these studies is that while GSH possesses the ability to augment endothelial-mediated vasomotion, the effects appear to be limited to individuals with existing vasomotor dysfunction. The lack of effect of GSH at improving vasomotor functioning in isolated vascular ring preparations has similarly been observed in animal studies (5, 92).

Unlike the human studies, a unique dilatory effect of GSH itself has been observed in animal preparations. In humans, a large majority of GSH is found bound to proteins within blood such as haemoglobin (99). Thus when GSH is administered *in vivo*, GSH is likely broken down to individual components (ie cysteinyl-glycine see above) for uptake and GSH synthesis (4, 76). Conversely, the GSH reduction and breakdown is less likely to occur in a bicarbonate buffer which does not contain proteins (161). Akpaffiong and Taylor observed that GSH could dose-dependently dilate isolated aortic segments (5). In the isolated perfused heart, Cheung and Schulz (1996) demonstrated that GSH induced dilation required NO, sGC and free radicals (33). The requirement of free radicals warrants further investigation in light of the finding that GSH only augmented vasomotor function in the

presence of pre-existing vasomotor dysfunction. This is the focus of Chapter 3, which examines the mechanisms whereby acute GSH administration alters endothelial mediated vasomotor function.

1.3.4 Acute oxidation/depletion of GSH and vasomotor functioning

Given the complex nature and different pathways discussed in the following sections, Figure 1.4 and Figure 1.5 provide schematic overviews of several of the pathways already discussed. They illustrate the effects of the different pharmacological agents related to GSH concentration (Figure 1.4) and the influence of GSH within the vascular wall (Figure 1.5).

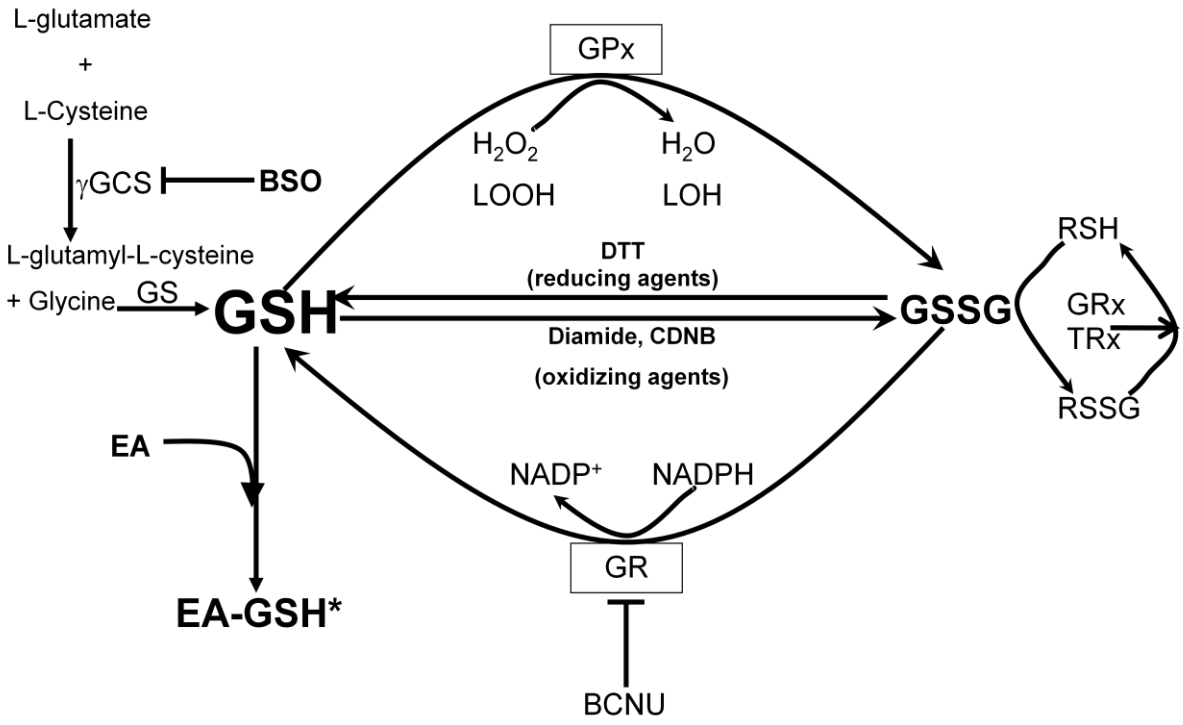

Figure 1.4. Influence of different thiol-altering pharmacological agents

Different pharmacological agents influence different components of glutathione availability. EA, ethacrynic acid forms a conjugate with GSH resulting in a loss of GSH content. BSO, L-buthionine-S,R-sulfoximine inhibits GSH synthesis through irreversible inhibition of glutathione cysteine synthetase. BCNU, 3-Bis(2-chloroethyl)-1-nitrosourea inhibits glutathione reductase, CDNB, 1-chloro-2, 4-dinitrobenzene is an inhibitor of thioredoxin. Diamide is a thiol oxidizing agent. Note both TRx, thioredoxin and GRx, glutaredoxin are involved in the dethiolation of proteins, which helps maintain GSH:GSSG ratio (21, 150).

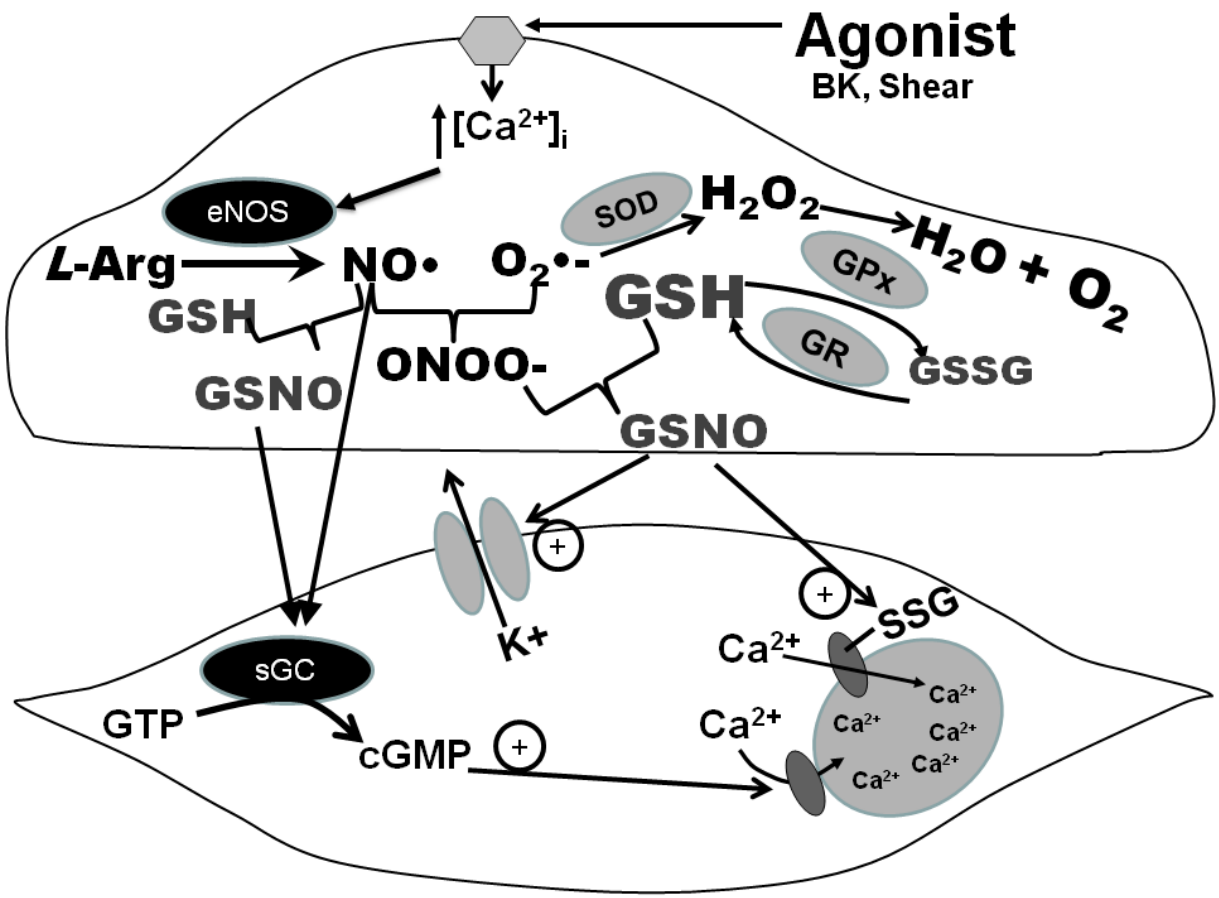

Figure 1.5. Interaction between GSH and vasomotor function

The following figure is a composite of several different pathways leading to vasodilation. The primary role of endothelial-derived NO, is to activate sGC (soluble guanylate cyclise) which in turn increases sarcoplasmic reticulum Ca^{2+} uptake. NO can be inactivated by O_2·- thereby generating peroxynitrite (ONOO-). ONOO- can react with GSH to from nitrosoglutathione which itself has several roles. GSNO can act as stored NO which may also be released by BK. GSNO possess the ability to activate sGC as well as open K^+ channels on both the endothelium and vascular smooth muscle to induce hyperpolarization. GSNO is also involved in the thiolation of SERCA by acting as a reactive intermediate. Additionally the antioxidant effects of GSH are shown with the exception of the direct interaction between GSH and O_2·- which was excluded for clarity. For additional details refer to text.

1.3.4.1 Thiols and eNOS activity

Several studies examining isolated endothelial cells have suggested that NO production may be impaired through depletion of intracellular thiols (72, 87, 132). More recently, it was determined that a portion of the reduced NO production may be mediated by a reduction in eNOS co-factors, and in particular NADPH (87) and tetrahydrobiopterin (164). This suggests that changes in overall thiol levels may result in an impaired redox state of the cell leading to the oxidation/reduction of eNOS cofactors. Interestingly, the phosphorylation of eNOS through a shear dependent mechanism required GR, as treatment with BCNU inhibited the phosphorylation of both Akt and eNOS (184). Using siRNA for glutaredoxin, it was speculated that glutathione reductase maintains Akt in a reduced state enabling the phosphorylation and activation (184). Taken together both the activity of eNOS and the availability of cofactors appear to be closely related to thiol status.

Nitrosothiols (RNSO) can modify a wide array (>100) of different proteins at their cysteine residues yielding altered function (159). Erwin and colleagues demonstrated that in response to the endothelial agonist VEGF, there was a rapid de-nitrosylation and an increase in phosphorylation of eNOS (56). Several different sites of nitrosylation of eNOS have been identified both in humans (175) and animals (57). However, the nitrosylation of eNOS only at Cys^{101} appears to regulate eNOS activity. It was subsequently identified that de-nitrosylation at this site was important for eNOS trafficking to the caveolae and subsequent increased NO production (57).

These studies highlight that NO production may not necessarily be dependent on the level of intracellular thiols but rather that eNOS activation through agonist or shear requires the appropriate redox environment. The removal of a thiol group from a thiolated protein (RSSG) or the removal of a nitroso group from RSNO may be dependent on the activity of a family of enzymes including thioredoxin, glutaredoxin and other reducing enzymes (38, 65, 116, 189). It may be postulated that

the appropriate reducing cofactors for these reducing enzymes including GSH and GSSG and NADP+ and NADPH may influence the overall activity and redox balance (21, 86, 86, 150).

In light of these findings, it is not surprising that endothelial-mediated dilation has been shown to be reduced in the presence of both BCNU and diamide (2). It was suggest that the impaired dilation was a result of an overall depletion of intracellular GSH, as replenishment of intracellular GSH with a GSH ester partially restored the endothelial-mediated dilation. It is also possible that in the presence of these reducing agents the activation of eNOS would be blunted, thus explaining the impaired dilation. An additional mechanism which may be attenuated in the presence of altered GSH content is altered Ca^{2+} uptake into the sarcoplasmic reticulum of the vascular smooth. A portion of NO-mediated dilation involves the nitrosylation and subsequent glutathiolation of the sarco(endo)plasmic reticulum Ca^{2+}-ATPase leading to enhanced Ca^{2+} uptake into the sarcoplasmic reticulum (3). Therefore, acutely depleting GSH may result in impaired dilation as a result of impaired Ca^{2+} uptake. In conditions of prolonged oxidative stress, the thiolation site on SERCA can undergo irreversible modification resulting in the thiol site being oxidized to either sulfinic ($R\text{-}SO_2H$) or sulfonic ($R\text{-}SO_3H$) acids which impair its overall function (36, 38). There is very limited data on how acute changes in intracellular thiols affect endothelium-intact vascular preparations. It is important to understand how acute changes in thiols influence endothelium-dependent dilation as thiols represent not only an important antioxidant within the vascular wall, but also clearly play in role in the activation of eNOS and the availability of eNOS cofactors. It is therefore paramount to determine the influence of acute alterations in GSH content on intact vasomotor function in order to understand and identify how early changes in thiols, such that could be caused by oxidative stress, may impair vasomotor function and vascular health in conditions of cardiovascular disease.

1.3.4.2 Interaction between intracellular thiol status and coronary vascular tone and the response to hypoxia

Despite the impairment in endothelium-dependent dilation seen in the presence of thiol oxidation, acute thiol oxidation induces dilation of isolated coronary arteries. In denuded coronary arteries diamide was shown to dose-dependently, dilate precontracted arteries (88). In this preparation, the addition BCNU to inhibit GR, significantly augmented the dilation to diamide. This dilation was shown to be independent of the activation of K^+ channels, sGC and oxygen derived radicals. It appeared to involved the closure of L-type Ca^{2+} channels thereby reducing Ca^{2+} entry into the vascular smooth muscle and inhibiting contraction (88). Similarly, inhibition of the pentose phosphate pathway (PPP), which is responsible for regenerating NADPH from NADP+, closely coupled to the reduction of GSSG to GSH has been shown to induce vascular smooth muscle relaxation. In the absence of the PPP, Gupte and colleagues demonstrated that the increase in NADP+ and subsequent reduced GSH:GSSG ratio resulted in a reduction in tone of endothelium removed coronary arteries (79). Similar to the previous finding using diamide, the dilation was partially mediated by an inhibition of L-type Ca^{2+} channels. However, unlike generalized oxidation-mediated relaxation (diamide), the inhibition of PPP also resulted in opening of K^+ channels and inhibition of sarco(endo)plasmic reticulum Ca^{2+} release (79). More recently, hypoxic coronary artery dilation was shown to be related to reduced NADPH:NADP ratio which is closely coupled to the GSH:GSSG, such that the oxidation promoted coronary vasodilation (80). Interestingly, in the presence of a general thiol reductant, the dilation to: diamide (88), PPP inhibition (79) and hypoxia (80) were all attenuated, suggesting that thiol oxidation leads to dilation in endothelium-removed coronary artery preparations. Thiol-mediated relaxation in endothelium removed coronary arteries appears to be controlled by increases in NADP:NADPH and GSSG:GSH through the opening of K^+ channels and the inhibition of Ca^{2+} entry into the vascular smooth muscle (190). These results suggest that in the absence of the modulatory effects

of the endothelium, overall thiol oxidation promotes a relaxed state of vasomotor tone in the coronary circulation.

1.3.4.3 Stored NO

The idea of RSNO as NO stores has gathered interest, as several studies demonstrated that the addition of NO scavengers to preparations containing NOS inhibitors, further reduced agonist-mediated dilation, suggesting that NO may be released from a separate site other than eNOS (32, 40, 70). Similarly, light-induced NO release is thought to be a result of the decomposition of nitrosothiols into NO and free thiol group (7, 8, 15, 120, 121). The ability of RSNO to induce relaxation appears complex and involves several different putative mechanisms including activation of endothelial K^+ channels resulting in hyperpolarization of the vascular smooth muscle and stimulation of sGC (15). The origin and storage location of the NO stores has been shown to be localized to both the endothelium and vascular smooth muscle and does not necessarily require eNOS-derived NO to generate RSNO (7).

Stored NO has been shown to mediate systemic decreases in mean arterial pressure. Davisson and colleagues demonstrated that *in-vivo* administration of ACh could repeatedly and consistently reduce mean arterial pressure (MAP); however, following the administration of LNAME, with each successive dose of ACh there was a progressive decline in the ability of ACh to lower MAP (41). Similar results were seen in the mesenteric vascular bed (41). These studies suggest that the reduction in blood pressure or mesenteric vascular resistance following NOS inhibition could be a result of release of NO from RSNO that are be progressively depleted in the presence of LNAME. In isolated porcine coronary arteries, repetitive BK curves did not alter maximal dilation of the arteries under control conditions; however, in the presence of LNAME, the BK dose-response curve was reduced and shifted ~10 fold to the right (40). Taken together these results suggest that repeated exposure to agonists can cause a reversible depletion of NO stores which may contribute to blunted dilation following repeated

agonist exposure. A similar response is observed in photorelaxation, a condition completely dependent on the replenishment of NO stores and independent of endothelial stimulation (15).

In the isolated perfused heart, BK mediated dilation was nearly abolished following prolonged incubation with LNAME; however, following a co-infusion of LNAME with BK, limiting only the *de novo* NO production, a similar dilatory response was seen compared to when BK was administered alone (39). It was concluded that BK mediated dilation occurred through NO and this NO was in part derived from preformed pools, as chronic but not acute eNOS inhibition resulted in a loss of vasodilation (39). Similarly, Batenburg and co-workers (2004) demonstrated that, *p*-hyroxymercurobenzoic acid, a thiol depleting agent, caused a significant reduction in BK-induced dilation which was not significantly further reduced with the addition of LNAME suggesting that depletion of GSH and therefore RSNO impaired dilation to BK (14). Interestingly, when the ability of nitrosothiols to be regenerated was inhibited with the thiol alkylating agent ethacrynic acid (EA), BK-induced dilation was not affected (14). This suggests that thiol depletion, and not replenishment, reduced BK mediated dilation in isolated coronary segments. In the absence of RSNO, NO release from preformed pools is abolished, yet when GSH is alkylated, NO can still induce relaxation. This may reflect the ability of BK to both activate eNOS and to release RSNO (15). That is why it is possible that at least part of the dilation mediated by NO is a result of stored NO, possibly GSNO releasing NO, which activates sGC as demonstrated in isolated rat hearts and purified NOS (119). Taken together these data suggest that in the isolated perfused heart, there appears to be a component of RSNO mobilization following BK stimulation, whereas the importance of RSNO in isolated vessels appears to be minimal depending on the mechanism of depletion. This suggests that the mobilization and activation of RSNO may be more important as either a stabilizer of NO or RSNO are more important in the microvasculature.

1.4 The utility of the Langendorff heart in assessing coronary vasomotor function

The primary outcome of the current work is coronary vascular resistance and endothelium-dependent dilation. In order to assess these variables in the presence of altered GSH levels the Langendorff heart preparation was used. The utility of this section is to provide a detailed description of the experimental methodology, procedure as well as a rationale for experimental protocols.

The isolated perfused heart system is based on the pioneering work of Oscar Langendorff circa 1895 (50, 156, 166). Its basic design has the heart being cannulated through the ascending aorta. Thus the heart is being perfused in a retrograde fashion, which closes the aortic valves and allows the perfusate to flow through the coronary ostea and hence through the coronary vascular network which is returned to the right atria from the coronary sinus and inevitably ejected from the heart (156). Similar to *in vivo*, during systole the intramural pressure is greater than the perfusion pressure; and there is minimal to no coronary flow (50). There are two different methods to provide flow to the isolated heart: the constant pressure system, and the constant flow system (50, 156). In the constant flow system, flow is delivered by a peristaltic pump, thus flow is adjusted and manipulated by the experimenter. Conversely in the constant pressure system, the height of the reservoir above the heart determines the perfusion pressure. Regardless of the methodology chosen, coronary vascular resistance can be described based on Ohm's law. The outflow pressure is essentially 0 mmHg (atmospheric pressure) therefore, in either the constant pressure or constant flow coronary vascular resistance (CVR) can be expressed as:

$$CVR = P_{inflow} - P_{outflow} \div flow$$

Given that the outflow pressure is essentially null, the inflow pressure, the perfusion pressure (PP) represents the total coronary vascular pressure. Poiseuille's equation which describes the relationship between resistance, and the radius of tube and viscosity of the fluid states:

$$\text{Resistance} = \frac{8 \times viscosity \times length}{\Pi \times radius^4}$$

or

$$\text{Resistance} \propto 1/\text{radius}^4$$

This simplification of Poiseuille's equation can be used in the Langendorff preparation as the length of the tube does not change (the perfusion tubing or the coronary vascular tree), nor does the viscosity of the perfusion solution. Therefore, changes in resistance of the system must reflect composite changes in vascular diameter (50, 52).

The constant flow is ideal for the study of vasoactive substances (50) and is reported to be more reproducible (166) and easier to use (50). The downside of this method is that coronary autoregulation cannot adjust flow to allow for changes in heart rate or contractility, which could lead to relative ischemia as coronary flow cannot be increased to account for increases in O_2 demand (156, 166). Both the constant pressure and constant flow preparations are currently utilized in the study of coronary artery vasomotion. A recent report using both techniques shows that similar experimental outcomes are seen regardless of whether constant flow or constant pressure is used (167). Given the primary outcome measure was vasomotor function, it was decided that a constant flow Langendorff would be most appropriate. Therefore, changes in vascular tone (CVR) would be result in increases or decreases in PP, as flow would be held constant. In order to assign flow rate and avoid ischemia, flow rate was assigned to mimic the *in vivo* flow rate by assigning the flow rate based on the estimated heart weight derived from body weight (45, 50). This can be done as there is a close relationship between heart weight and coronary flow (50). This equation yields flow rates of ~7 ml/min/g heart weight, which is higher than the typical *in vivo* flows of 2-3 ml/min/g heart weight (156, 166). However, the oxygen carrying capacity of Kreb's Henseleit solution is less than blood, therefore, the higher flow rates are used in this preparation to avoid ischemia and ensure adequate O_2 delivery (156,

166). In the absence of a ventricular balloon total cardiac work is zero. However, in the presence of the left ventricular balloon the heart is performing isovolumetric work (52). Therefore, in order to meet the demands of the a heart performing isovolumetric work, the flow rate is set slightly higher than *in vivo* to overcome the reduced O_2 carrying capacity and meet the metabolic demands of the perfused heart performing isovolumetric work which is less than the normal myocardial demands *in vivo* (52). The efficacy of this experimental choice is highlighted by the relative stability of this preparation over several hours and the ability of Langendorff hearts to increase contractility in response to increases in PP or in response to inotropic agents (52, 156, 166).

The constant flow rate allows for an easier delivery of any vasoactive agents. In a constant pressure system, when a dilatory agent is delivered there is an increase in coronary flow. Therefore, in order to appropriately deliver the subsequent concentration changes in drug infusion rate or concentration have to be adjusted accordingly. The use of a constant flow system allows for a constant rate of drug infusion meaning that each stock concentration can be delivered at the same rate allowing the transit time from the infusion adaptor to the coronary vasculature to remain constant.

The advantage of using the Langendorff apparatus to study vasomotor functioning is highlighted by the previous discussion describing the complex nature and regulation of coronary blood flow across different microvascular networks within the coronary circulation. Thus, unlike isolated perfused vascular rings or isolated vascular segments mounted in a myography unit, the Langendorff technique allows the global assessment of vascular function not limited to a single segment of the vascular tree. Therefore, if there are compensatory pathways activated during disease states they will be captured using the Langendorff. For example, it has been reported that spontaneously hypertensive rats have preserved coronary artery dilatory function when assessed using the

Langendorff (96, 97), whereas studies of isolated coronary artery segments often report coronary artery endothelial dysfunction (137, 182).

The constant flow Langendorff utilizes a perfusion reservoir and aerator, hydrostatic pump, compliance chamber, cannula and heart chamber. Flow from the reservoir is delivered via the pump to the compliance chamber through water-jacketed tubing. The compliance chamber is used to ensure a laminar flow in the absence of air bubbles (Figure 1.6). The perfusate, a Krebs Henseleit solution, is gassed with 95%O_2:5%CO_2, delivered through water-jacketed tubing designed to maintain a temperature of ~37^{o}C for delivery to the heart. Perfusion pressure is monitored using a pressure transducer, while an ultrasonic flow probe measures total flow. Both of these measures are done proximal to the aortic cannula thus representing total coronary perfusion pressure and flow.

Using the Langendorff apparatus, it is possible to measure cardiac hemodynamics; however, this requires the use of a left ventricular pressure balloon as the perfusate is not delivered into the left ventricle; therefore, the heart is not doing any external work (50, 52). The pressure balloon must be small enough to fit into the left ventricle but big enough to allow for expansion within the ventricle. Once inserted into the left ventricle the balloon needs to be filled to yield a diastolic pressure of between 5-10 mmHg to mimic end diastolic pressure (50, 156). The pressure balloon and transducer can be used to generate several indices of cardiac function including left ventricular developed pressure (LVDP, LVsystolic – LVdiastolic pressure), heart rate, and rate of pressure development (±dP/dt).

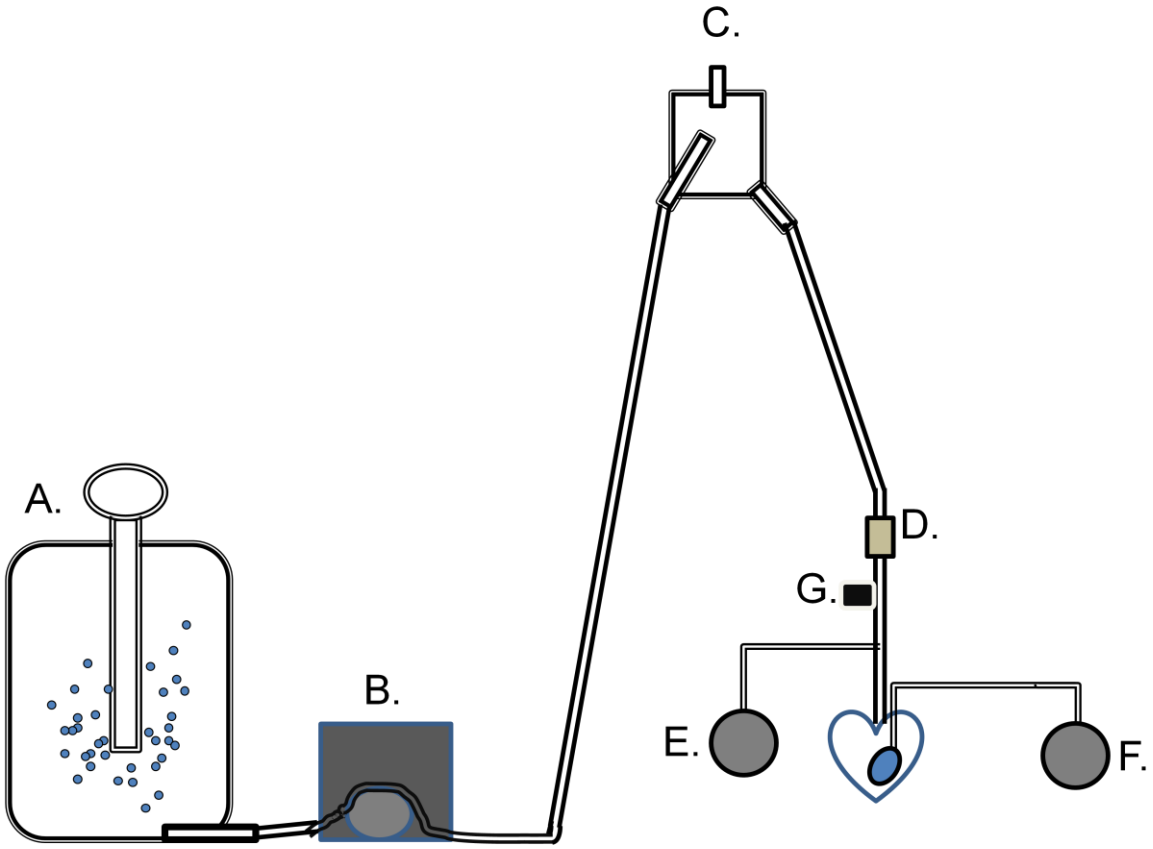

Figure 1.6. Constant flow Langendorff heart apparatus
All glass wear and tubing were water-jacketed by ~37^{o}C water. Aerated Kreb's Henseleit buffer is pumped from the reservoir (A.) by pump (B.) to the bubble trap (C.) which ensures laminar flow is delivered to the heart. Flow rate is measured by an ultrasonic paratubular flow meter (D.) While a fluid filled pressured transducer continuously monitors perfusion pressure (E.). Similarly a left ventricular pressure balloon and associated pressure transducer (F.) generates left ventricular pressure tracing. Vasoactive agents are delivered into the system by a syringe pump (not shown) through an in-line infusion adapter (G.). Also omitted for clarity is the heart chamber a water jacketed open bottom cylinder which is raised around the heart to maintain temperature and humidity.

In the experiments outlined within the current document, all hearts were allowed to develop spontaneous vascular tone and heart rate. Therefore, all hemodynamic variables represent the natural intrinsic characteristics of the hearts. Hearts which failed to achieve sufficient tone to allow for appropriate dilatory responses to endothelium-independent dilators were always excluded from the analyses. All hearts were allowed to equilibrate for at least 30 minutes at the *in vivo* flow. In most instances, this was sufficient to see a stabilization of the perfusion pressure and cardiac hemodynamics. If the PP was not stable following 30 minutes, additional time was given to allow for a stable PP. This ensured that dilatory responses were assessed from a stable tone to eliminate the confounding effects of potential increases in PP that could occur at the lower concentrations of the dilatory curves if the PP was not stable.

Once a stabilized perfusion pressure was achieved a dose-response curve to endothelial-dependent or independent agonists was generated (Figure 1.7). This involved the infusion of the dilatory agent through an infusion adapter located just proximal to the aortic cannula. The infusion rate was 1% of the total coronary flow as this infusion rate was without effect on total coronary flow. Following the final concentration of endothelial agonist, hearts were dilated with an endothelial independent agonist which was used as a reference point of maximal dilation.

Therefore, the Langendorff apparatus provides the ability to assess both vasomotor function and cardiac hemodynamics. In this regard compared to other techniques examining vascular function in which a single vascular segment is used, the vasomotor responses observed using the Langendorff provide a global assessment of the coronary vascular bed. Therefore, changes that occur in response to treatment or pharmacological agents are observed across the entire organ and do not reflect changes that occur at a given segment within the vascular tree.

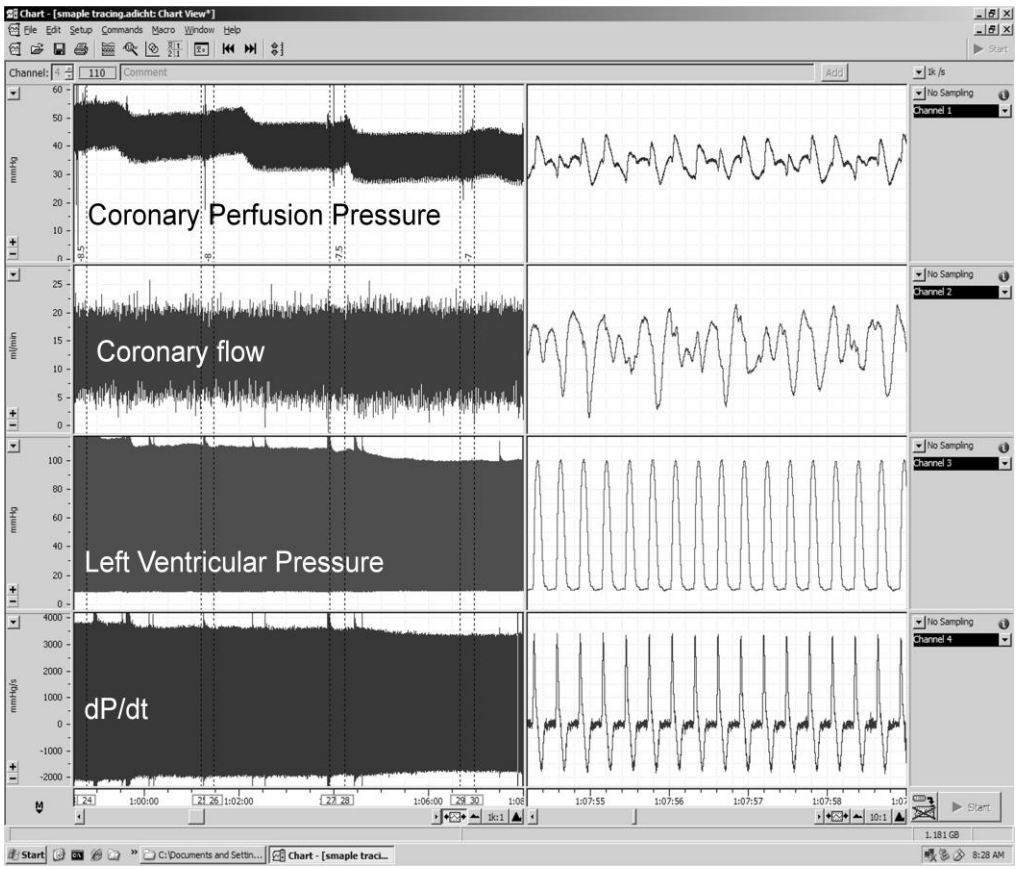

Figure 1.7. Sample tracing

This tracing was derived from a control rat in the middle of a bradykinin dose-response curve. Each channel is labelled for clarity. The left side of the tracing is a condensed view showing the infusion of bradykinin (dashed vertical lines). The right hand side is a continuation of the tracing demonstrating the beat-by-beat nature of the recoding.

1.5 Summary and Purpose

1.5.1 Global thesis purpose

The outline of the above literatures illustrates that within the vasculature, the control of GSH represents an important antioxidant and signalling molecule. As demonstrated, changes in GSH are associated with altered contractile state and impaired endothelium mediated dilation. Additionally changes in GSH and its associated enzymes were shown to impair NO production. Given the dominant role that NO plays in the endothelium-dependent control, interactions between GSH and NO warrant further investigation within the coronary circulation. The advantage of using the Langendorff to examine vascular function allows for a systemic evaluation of the coronary vascular bed. This is important as this vascular bed has several different mechanisms of control on baseline vascular tone and vasomotor function. Despite advances in the understanding of the interactions between thiols and nitric oxide, few studies have examined the role of altering thiol levels as a tool to understand how these specific changes influence endothelial derived vasomotor function.

Currently much of the information that has been generated regarding both acute and chronic thiol manipulations has typically focused on conduit vascular function (as is the case for chronic thiol manipulations), endothelium-removed coronary segments, or isolated cellular preparations (as is the case for acute thiol oxidation/depletion). Furthermore few studies have examined the interactions of acute thiol modulations on vasomotor functioning specifically examining if GSH is merely acting as an antioxidant to improve vasomotor functioning. The global purpose of this thesis is to examine and characterize the effects of thiol manipulations on the coronary vascular network. The goal is to characterize both the basal coronary vascular resistance and endothelium-mediated vasomotor responses with a primary focus on nitric oxide. The results of the current experiments will provide novel insight into how changes in thiols influence vasomotor functioning. By examining these changes in the isolated perfused heart, the entire vascular bed can be examined thus allowing for a more

systemic approach. This will provide further insight from studies utilizing isolated vascular rings or cellular preparations and will build on previous work from both human and animal studies regarding the complex relationship between thiols vascular function.

The global purpose of this thesis is to examine how changes in GSH influence coronary vascular resistance and coronary artery endothelium-dependent dilation. In order to address these complex relationships the following purposes and hypotheses were addressed.

1.5.2 Chronic thiol depletion results in an age-dependent increased reliance on nitric oxide in determining coronary vascular resistance and endothelium-dependent dilation in the isolated perfused heart (Chapter 2).

Purpose: To examine and characterize the effects of 10 days of thiol depletion on coronary vascular resistance in adult and older Sprague-Dawely rats.

Hypotheses:

i. Older rats will have an increase in basal coronary vascular resistance (CVR) compared to adult rats.
ii. Treatment with BSO will result in an increased CVR that will be further augmented with age.
iii. NOS inhibition will cause a increase in CVR in all groups which will be most pronounced in the adult and older controls, as the thiol depleted animals will have a reduced NO bioavailability.

Purpose: To examine the effects of aging and 10 days of thiol depletion on endothelial mediated vasomotor function.

Hypothesis:

i. Both aging and thiol depletion will result in impaired endothelium-dependent vasodilation.
ii. NOS inhibition (LNAME) will blunt the vasodilation primarily in the control groups as BSO will increase ROS production limiting NO bioavailability.

1.5.3 Glutathione enhances endothelium-mediated coronary vascular dilation in a reactive oxygen species-dependent manner and independent of effects on NO production and soluble guanylate cyclase. (Chapter 3)

Purpose: To examine the direct dilatory effects of GSH in isolated perfused rat hearts

Hypotheses:

i. GSH will dose-dependently induce vasodilation in the isolated perfused rat heart.

Purpose: To examine the effects of GSH infusion on bradykinin-mediated dilation in the isolated perfused rat heart in the absence of differences in baseline CVR.

Hypotheses:

i. GSH will augment the dilatory response to bradykinin, this response will be driven primarily through increased NO bioavailability.
ii. In the presence of NOS inhibition or sGC inhibition, no beneficial effect of GSH will be observed.
iii. TEMPOL will not further augment the vasodilatory response seen in the presence of GSH as both are antioxidants.

Purpose: To determine if the effects of GSH infusion enhances NO utilization by examining sodium nitroprusside-mediated vasodilation in the isolated perfused rat heart.

Hypothesis:

i. Sodium nitroprusside-mediated vasodilation will not be altered by GSH infusion.

1.5.4 Effect of thiol modulation on coronary vascular resistance and endothelium-dependent dilation in the isolated perfused heart (Chapter 4).

Purpose: To examine the influence of different thiol depleting agents on coronary vascular resistance.

Hypothesis:

i. Glutathione reductase inhibition (BCNU) and general thiol oxidation (Diamide) will result in a decrease in CVR.

ii. Thiol conjugation and thioredoxin inhibition will result in an increase in coronary vascular resistance as a result of impaired NO bioavailability.

Purpose: To examine the relationship between GSH:GSSG ratio and baseline CVR, minimal CVR, and change in CVR.

Hypothesis:

i. A reduction in the GSH:GSSG ratio will result in an increased minimal CVR.

ii. There will not be a relationship between GSH:GSSG and either baseline or total dilatory capacity as the baseline CVR will be reduced in some instances of thiol depletion but not others (see above).

Purpose: To examine the effects of thiol depletion on bradykinin mediated vasomotor functioning.

Hypotheses:

i. Treatment with all thiol oxidizing agents will impair endothelium-dependent dilation.

ii. Given the hypothesized impairment in developing tone (see above), it is hypothesized that a portion of the impaired dilatory ability will be related to a reduced dilatory capacity.

Purpose: To examine if the impairment in vasodilation is a result of altered smooth muscle sensitivity to nitric oxide:

Hypothesis:

i. There will not be any differences in vasodilation observed in the responsiveness to sodium nitroprusside.

CHAPTER 2

Chronic thiol depletion results in an age-dependent increased reliance on nitric oxide in determining coronary vascular resistance and endothelium-dependent dilation in the isolated perfused heart.

2.1 Authorship

This study was part of a larger study that involving several different experiments examining aging and thiol depletion. Given the contributions of Steven G. Denniss to study design, and animal care and treatment I would like to acknowledge his contributions. Steven G. Denniss will appear as a co-author on this manuscript.

2.2 Synopsis

Previous reports have documented that both thiol depletion and aging are independently associated with increased production of reactive oxygen species (ROS) and vasomotor dysfunction. The purpose of the current experiment was to examine the effects that thiol depletion and aging have on coronary vascular resistance (CVR) and endothelium-dependent dilation in the intact coronary vascular bed. Adult male Sprague-Dawley rats (33 weeks) or older rats (65 weeks) were randomized to receive 10 days of L-Buthionine-(S,R)-Sulphoximine (BSO). Hearts were isolated and perfused using the Langendorff technique. Endothelium-dependent dilation was assessed via administration of bradykinin (BK) in the presence or absence of LNAME. BSO treatment, but not aging, resulted in a reduction in glutathione content of the left ventricle, and was associated with an increased production of ROS. Aging resulted in reductions in baseline CVR and in maximal-endothelium dependent dilation, but no effect of BSO treatment on these parameters was observed. Intriguingly, LNAME resulted in a greater increase in CVR and a greater blunting of endothelium-dependent dilation in the adult BSO

animals compared to all other groups. BSO treatment resulted in an age-dependent increase in eNOS protein content in the adult BSO group. Compared to controls, BSO-treated animals were less responsive to exogenous H_2O_2 administration with respect to reduction in CVR, an effect that may be explained by a BSO-dependent increase in catalase expression. The results of the current study demonstrate that chronic thiol depletion results in an increased reliance on nitric oxide in the intact coronary circulation.

2.3 Introduction

Glutathione (GSH, reduced glutathione) is an intracellular antioxidant (155) important in maintaining vascular health (65, 146, 160). Changes in glutathione metabolism and reduction in tissue glutathione content have been shown to occur in humans with cardiovascular disease (28, 101, 133, 134, 152), and to be associated with coronary artery endothelium-dependent dysfunction (134).

Cellular glutathione deficiency can be experimentally induced in animals by administration of L-Buthionine-(S,R)-Sulphoximine (BSO); a selective inhibitor of the γ-glutamylcysteine synthetase (122, 123). Several investigations have demonstrated that chronic inhibition of GSH synthesis with BSO induces oxidative stress (13, 19, 63, 67, 68, 107, 181, 196). *In vivo* studies suggest that the vascular function of BSO-treated rats is impaired as demonstrated by a blunted reduction in mean arterial pressure to acetylcholine (107) and enhanced elevation in blood pressure in response to phenylephrine (68). While *in-vitro* studies using isolated conduit arteries demonstrate only modest effects of BSO-mediated GSH depletion on endothelium-dependent dilation in response to ACh (13, 63). This suggests that the larger effects observed *in vivo,* in intact vascular beds may be mostly dependent on the BSO-effects on the control of tissue vascular resistance, although this has not been specifically investigated.

Endothelial GSH and GSSG content have been reported to be lower in older compared to young animals (157), and it is plausible that alterations in GSH may be a contributing factor to the reduced endothelium-mediated vasomotor responses that accompany aging. Age-associated endothelial dysfunction has been reported in a variety of vascular beds under basal conditions, and in response to stimulation of the endothelium (6, 22, 29, 37, 95, 118, 131, 142, 157, 165, 179). Age-associated reductions in NO bioavailability contribute to this functional effect, and likely include a ROS-dependent component, as vascular wall ROS content is increased in aging (22, 37, 43, 81, 95, 130, 135, 142, 143, 165, 179). There is, however, limited data concerning the impact of manipulations of GSH on vascular function with age.

Although it is evident that both thiol depletion and aging are associated with increased ROS production, there is a lack of data regarding the effects of thiol depletion on the coronary circulation, as most isolated tissue studies have strictly examined individual conduit arteries. The purpose of the current study is to examine the effects of thiol depletion and aging on coronary vascular resistance and endothelium-dependent control of vascular function in the isolated perfused rat heart. The advantage of this model in this experimental context is that it allows the assessment of vascular function across the entire coronary vascular bed, and is thus not limited to the responses of a single segment of the vasculature. It is hypothesized that older animals, and thiol depleted animals, will both demonstrate an elevation in baseline coronary vascular resistance and impairment of vasomotor function, associated with increased levels of reactive oxygen species and impairment of NO bioavailability. It is further hypothesized that BSO-induced GSH depletion will exacerbate age-related impairment of vascular function.

2.4 Materials and Methods

2.4.1 Animals

A total of 50 adult (mean age 33 weeks) and 41 older animals (mean age 65 weeks) Sprague-Dawley rats were used in this study. All animals were obtained from a breeding colony at the University of Waterloo. Animals from both age categories were randomized to receive either L-Buthionine-(S-R)-sulphoximine (BSO; 30 mmol/L in tap water) or vehicle (tap water) *ad libitum* for 10 days. The dose, duration and method of delivery were based on a previous report from our laboratory (63). Animals were weighed prior to randomization, and individually-housed for the duration of treatment in order to monitor food and fluid consumption. Animals were kept on a reverse 12:12 light:dark cycle, and provided free access to food. Following the 10 day treatment, animals were weighed and sacrificed, and tissues were harvested as described below. All procedures were approved by the University of Waterloo Animal Care Committee.

2.4.2 Langendorff Heart

Animals were anaesthetized (60 mg/kg pentobarbital sodium; I.P.) and hearts were removed and prepared for a constant flow Langendorff protocol. Briefly, after removal the heart was immersed immediately in 4^{o}C Krebs Henseleit (KH) buffer (composition in mmol/L NaCl 118, KCl 4.7, $MgSO_4 \cdot 7H_2O$ 1.2, $NaHCO_3$ 24, KH_2PO_4 1.1, Glucose 10 and $CaCl_2 \cdot 2H_2O$ 1.25) to reduce myocardial contractility, oxygen consumption and ischemic injury (166). Hearts were then transferred to the aortic cannula which was dripping gassed (95%O_2-5%CO_2) 37^{o}C KH, cleaned of adherent tissue, and prepared for the insertion of a left ventricular pressure balloon.

A constant flow protocol was utilized as this is most suitable for the study of the effects of vasoactive agents (50, 166). The constant flow rate was assigned to mimic *in-vivo* coronary flow using the following equation: Flow = 7.43 × ((body weight×0.0027+0.6)$^{0.56}$) (45, 50). The pre-treatment body

weight was used in this calculation to avoid complications associated with BSO-induced changes in body weight. The flow probe and associated transducer (2PXL and TS410, Transonic Systems, Ithaca NY) and pressure transducer (MLT844, AD Instruments, Boulder Co) were positioned proximal to the aortic cannula for the measurement of total coronary flow and coronary perfusion pressure. A pressure balloon was inserted through the left atria, positioned in the left ventricle and adjusted to a diastolic pressure of 5 – 10 mmHg. Heart rate, left ventricular developed pressure (LVDP, left ventricular systolic pressure – left ventricular diastolic pressure), and ±dP/dt were derived from the left ventricular pressure tracing. The balloon was connected to a pressure transducer (MLT844, AD Instruments, Boulder Co). All data were collected using a Powerlab data acquisition board (4/sp, AD Instruments, Boulder Co) and stored digitally using Chart for Windows (5.5.1, AD Instruments, Boulder Co) for off-line analysis.

2.4.3 Assessment of endothelium dependent dilation

Hearts were allowed to stabilize for 30 minutes with or without the non-specific nitric oxide synthase inhibitor L-$_{\omega}$Nitro-L-arginine (LNAME, 0.1 mmol/L) to allow for the development of spontaneous coronary vascular tone and heart rate. Hearts that failed to establish a stable spontaneous tone to allow for dilation to SNP or adenosine were excluded from the study. A total of 9 hearts were excluded from the analysis (2 adult control, 1 adult BSO, 3 older control, 3 older BSO). Once the perfusion pressure stabilized, baseline hemodynamics were assessed by taking the average over 1 minute.

Following the baseline measurements, a dose-response curve to bradykinin (BK, 10^{-11} – 10^{-6} M) was generated. For each concentration a total volume of 250 µl was infused at a rate of 1% of the coronary flow using a syringe pump (Cole Parmer 74900, Montreal PQ). Following the final

concentration of BK, the vasculature was maximally dilated by sodium nitroprusside (SNP 10^{-5} M) in order to obtain the minimal CVR value.

2.4.4 Assessment of hemodynamics and endothelium-dependent dilation at an elevated flow

Baseline tone was re-established by washout of SNP. The flow rate was then raised to yield a standard perfusion pressure of 75mmHg (High Flow). This perfusion pressure was chosen as it represents a physiologically relevant perfusion pressure and has been employed by previous studies assessing vasomotor function in isolated perfused rat hearts (50, 126). New baseline measurements were obtained (as above) and the BK dose-response curve was repeated at this elevated perfusion pressure (High Flow) condition. LNAME alone results in an elevated perfusion pressure in the constant flow preparation as a result of the decrease in NO production. If we were to match the flow rate to achieve 75 mmHg in the presence of LNAME the flow would have been significantly lower and hearts would have become ischemic. Therefore, we duplicated the flow rate that resulted in 75mmHg in the corresponding no drug condition for each group (adult or older CON and BSO) in the experiments conducted in the presence of LNAME (High Flow LNAME). This was done to ensure that group differences in flow rates remained consistent in the presence of LNAME despite differences that may exist in perfusion pressure as a result of NOS inhibition. Therefore changes in CVR associated with LNAME would be a result of changes in perfusion pressure not changes in flow.

2.4.5 Does an acute oxidative challenge reveal impaired function in BSO-treated animals?

Given the accumulating evidence that H_2O_2 is a vasodilatory agent in this vascular bed (54, 149), and a recent report suggesting that BSO-treatment can enhance H_2O_2-mediated endothelium-dependent vasodilatory responses (90), in a subset of hearts from the adult groups, responses to H_2O_2 were assessed to determine if there was a differential response seen in the coronary circulation of CON vs BSO. Hearts were prepared and stabilized as described above, and endothelium-dependent

dilation was assessed by infusion of bradykinin (0.1, 10, and 1000 nM), followed by a washout and restabilization period. Once the perfusion pressure had stabilized, H_2O_2 was administered (10^{-5}-10^{-3} M). This protocol was chosen on the basis that when these H_2O_2 concentrations are applied exogenously to tissue preparations they result in intracellular concentrations 10-fold less, and produce a range of intracellular H_2O_2 concentrations that results in effects from reversible cell signalling to oxidative stress and possible damage (163). Following the final concentration of H_2O_2, hearts were allowed to re-equilibrate, and endothelium-dependent dilation to BK was reassessed, as was the response to SNP. This was done to determine if the acute H_2O_2 challenge protocol caused irreversible impairment of endothelium-dependent dilation.

2.4.6 Measurement of left ventricular glutathione, ROS, and H_2O_2

Left ventricular glutathione was assessed using sample preparation and HPLC techniques as previously described by our laboratory (63) according to the methods derived by Reed and colleagues (141).

ROS production was measured as previously described by our laboratory (147). Briefly, left ventricle was homogenized in phosphate buffered saline (PBS) and centrifuged at 1000g for 10 minutes, and the supernatant was extracted. The homogenate was diluted further in PBS and incubated with 5 µM of dicholorflouricine diacetate (H_2DCF-DA, Molecular Probes, Invitrogen, Carlsbad, CA) in the dark for 15 minutes. Fluorescence was detected every 15 minutes at excitation/emission wavelengths of 488/525 nm using a SPECTRAmax GEMINI-XS (Molecular Devices, Sunnyvale, CA). The fluorescent signal generated following 1 hour was normalized to protein content determined by a bicinchoninic acid (BCA) protein concentration assay.

H_2O_2 content in left ventricular homogenates was assessed using an Amplex Red Hydrogen Peroxide kit (Molecular Probes, Invitrogen, Carlsbad, CA). Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) reacts with H_2O_2 in a 1:1 stoichiometry in the presence of peroxidase to

produce the oxidation product resorufin that can be measured fluorescently. 50µl of diluted tissue homogenate was added to 50µl of Amplex Red reagent (10µM Amplex Red stock and 0.2U/ml horseradish peroxidase in PBS) (51) and incubated in the dark at room temperature for 30min. Fluorescence was detected every 15 minutes at 530/590nm excitation/emission wavelengths. H_2O_2 concentrations were calculated with reference to a contemporaneous H_2O_2 standard curve. Tissue concentrations are expressed relative to µg protein. Data reported represent H_2O_2 concentration at 1 hour and subsequently normalized to the adult control group.

2.4.7 Immunoblotting

Immunoblotting was preformed as previously described by our laboratory (63). A portion of the left ventricular wall was homogenized in 20 volumes of extraction buffer (10 mM NaH_2PO_4, 1% SDS, 6 M urea, pH 7.4). Samples were centrifuged at 4^{o}C for 2 minutes at 8000 rcf and the supernatants were collected. Total protein content was determined using BCA assay. Samples were then diluted to 1 µg protein/µl, and a sample containing 30 µg total protein was loaded for gel electrophoresis. Proteins were then transferred to polyvinylidene diflouride membranes (Roche Diagnostics, Mannheim Germany). Immunoblotting was preformed for eNOS (1:350, BD Bioscience, San Jose, CA), catalase (1:2500, Millipore (Chemicon), Billerica, MA) and superoxide dismutase 1 and 2 (SOD1 and SOD2, 1:1000, 1:5000, Stressgen/Assay Designs Ann Arbor, MI). Membranes were subsequently incubated using horseradish-peroxidase conjugated secondary antibodies. Signals were detected using enhanced chemiluminescence detecting reagents (GE Healthcare (Amersham) Little Chalfont, UK) and a Syngene gel detection system (Syngene, Cambridge, UK). Protein density was compared relative a standard loaded on each gel.

2.4.8 Chemicals

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) or Bioshop Canada (Burlington, ON, Canada) unless otherwise indicated. H_2DCF-DA and Amplex Red kits were purchased from Molecular Probes (Invitrogen, Carlsbad CA).

2.4.9 Statistics

All data are presented as mean ± standard error. Curves were fit to obtain EC_{50}, maximal response, and area under the curve (AUC) using Prism (4.03, GraphPad Software, San Diego CA). Differences between groups were assessed using 2 way ANOVA (treatment by age), and significant results were subsequently analyzed using least squares difference *post hoc* test. For comparisons made between adult CON and BSO only a 1-way ANOVA was utilized. A p value <0.05 was considered statistically significant. All statistics were performed using Statistica 6.0 (StatSoft Inc, Tulsa Ok).

2.4 Results

2.4.1 Animal Characteristics

Of the 50 adult animals used in this study, 24 were used as controls (AC), and 26 were BSO-treated (AB). Of the 41 older animals used, 20 were used as controls (OC), and 21 were treated with BSO (OB). The older animals had greater body, heart, and kidney weights compared to the younger animals (Table 2.1). Over the 10 day study period, treatment with BSO resulted in significant body weight loss in both the AB and OB groups. The loss in body weight may have been in part a result of reduced food consumption over the course of the treatment period (Table 2.1). BSO treatment was not associated with a change in fluid consumption.

Table 2.1 Animal Characteristics

	Adult Control	Adult BSO	Older Control	Older BSO	p values		
					Age	Treatment (TMT)	Age × TMT
Age (weeks)	33 ± 1	34 ± 1	65 ± 1	65 ± 1	<0.05	NS	NS
Body weight pre (g)	501.3 ± 8.9	508.1 ± 5.4	567.3 ± 11.6	600.5 ± 10.0	<0.05	<0.05	NS
Body weight post (g)	502.7 ± 9.3	487.0 ± 4.9	555.5 ± 11.8	549.8 ± 6.7	<0.01	NS	NS
Change in BW (g)	1.5 ± 1.8	-21.1 ± 2.9	-11.9 ± 2.4	-50.7 ± 6.7	<0.01	<0.01	NS
Heart weight (mg)	1923 ± 33	1974 ± 28	2166 ± 38	2211 ± 38	<0.01	NS	NS
Kidney weight (mg)	1439 ± 33	1442 ± 24	1754 ± 57	1816 ± 44	<0.01	NS	NS
Fluid intake (ml/day)	45.8 ± 1.8	54.9 ± 2.2	57.0 ± 1.2	48.7 ± 2.6	NS	NS	<0.01
Food intake (g/day)	23.4 ± 0.6	19.9 ± 0.5	23.9 ± 0.9	17.6 ± 0.9	NS	<0.01	NS

Data are presented as mean ± SE. Body weight pre refers to body weight prior to the initiation of BSO or vehicle, body weight post refers to body weight following 10 days of BSO or vehicle. *p<0.05 vs age matched control (n=20-26/group).

2.4.2 GSH content and ROS levels in the left ventricle

The GSH content in the left ventricle was reduced following 10 days of BSO treatment (2.022±0.084 vs 0.182±0.021 nmol/mg wet weight in the AC and AB, and 1.892±0.144 vs 0.175±0.025 nmol/mg wet weight in the OC and OB respectively, $p<0.05$ for treatment). Lower values were also observed for GSSG and for the GSH:GSSG ratio in both the BSO treated adult and older animals vs respective controls (Figure 2.1).

The DCF assay is a non-specific index of ROS production, while the Amplex Red assay more specifically provides an index of H_2O_2. Treatment with BSO resulted in an increase in both of these ROS indices in the left ventricle (Figure 2.2). There was no effect of aging observed on either index of ROS production.

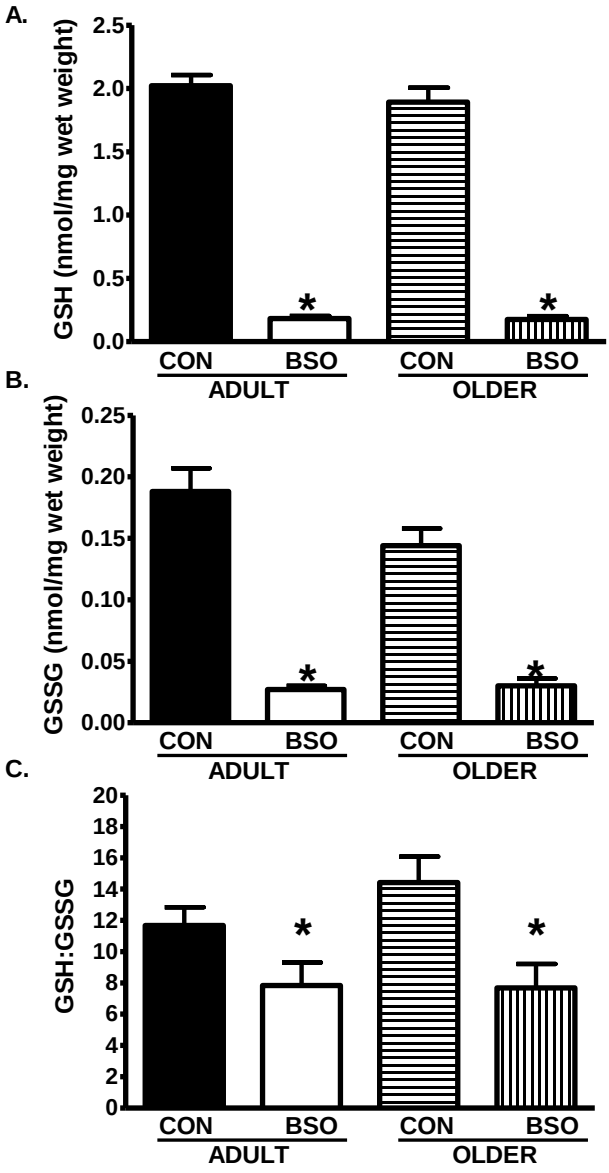

Figure 2.1. Ventricular thiol concentrations

Data are levels of reduced glutathione (A), oxidized glutathione (B) and GSH:GSSG ratio (C). Data are presented as mean±SE. * $p<0.01$ for treatment effect. No age dependent effects were observed (n=10/group).

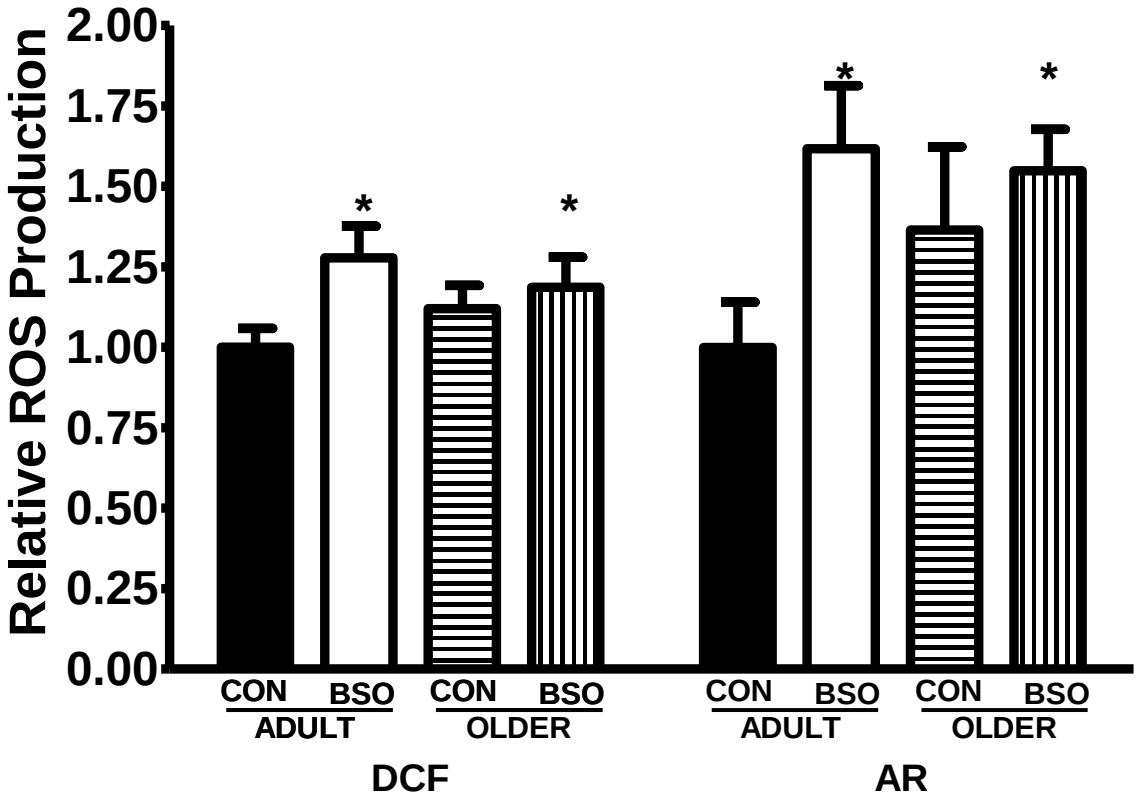

Figure 2.2. Levels of reactive oxygen species in the left ventricle

Left ventricular (A) levels of general ROS (DCF) and H_2O_2 production (AR). Data are presented as mean±SE from adult control (filled bars), older control (horizontal hatch), adult BSO (empty bars), and older BSO (vertical hatch). * $p<0.05$ main effect of BSO (n=8-10/group).

2.4.3 Hemodynamics under in vivo flow conditions

The hemodynamic data were obtained following the stabilization period both in the presence and absence of NOS inhibition, prior to the assessment of endothelium-dependent dilation. In the absence of NOS inhibition, the baseline CVR was reduced in the older rats compared to the adult rats (main effect for age $p<0.01$, Table 2.2). In the older group, the reduced CVR occurred despite a higher flow rate, which was assigned based on body weight (averaged across treatment groups: 11.53±0.09 vs 10.95±0.06 ml/min in older vs adult, respectively, $p<0.05$). No other differences were observed in any other hemodynamic variables in the absence of LNAME (Table 2.2). Treatment with LNAME resulted in a significant increase in CVR across all groups. The LNAME-induced increase in CVR in the AB group was significantly greater than in all other groups (Table 2.2), and the LNAME-induced increase in CVR seen in the AC group was significantly greater than that achieved in either OC or OB ($p<0.05$, Table 2.2).

In the presence of LNAME, BSO treatment resulted in a significant decrease in maximal rate of pressure development (+dP/dt), while no age differences were apparent for this effect (Table 2.2). No other significant cardiac contractility effects were noted.

2.4.4 Effects of endothelial stimulation on CVR

Bradykinin (BK) dose-dependently reduced CVR in all groups (expressed as a % of the maximal reduction in CVR in response to SNP; Figure 2.3, Table 2.3). We found a significant age-dependent reduction in minimal CVR in older vs adult animals, and this resulted in a reduction in total dilatory capacity (difference between baseline CVR and minimal CVR, Table 2.3). Despite this discrepancy, maximal BK dilation, expressed as % of TDC, was greater in hearts of the adult animals compared to the older animals. This was associated with a reduction in the AUC with age, while no age-dependent differences were observed in the sensitivity (EC_{50}) to BK in the adult compared to older animals in the absence of LNAME.

Table 2.2. Hemodynamic variables at *in vivo* flow rate

	Adult Control	Adult BSO	Older Control	Older BSO
	No Drug			
Perfusion Pressure (mmHg)	51.90 ± 2.17	56.37 ± 4.03	43.99 ± 3.64 *	42.95 ± 2.72 *
Flow (ml/min)	10.91 ± 0.09	11.03 ± 0.11†	11.25 ± 0.13 *	11.72 ± 0.08 *,†
CVR (mmHg/ml×min^{-1})	4.76 ± 0.20	5.12 ± 0.37	3.92 ± 0.34 *	3.67 ± 0.24 *
Heart Rate (beats/min)	229 ± 9	238 ± 12	213 ± 15	226 ± 7
LVDP (mmHg)	117 + 7	108 ± 6	103 ± 11	103 + 8
+dP/dt (mmHg/sec)	3959 ± 210	3661 ± 233	3277 ± 274	3564 ± 239
-dP/dt (mmHg/sec)	- 2105 ± 119	-1950 ± 118	-1726 ± 165	-1824 ± 136
	LNAME			
Perfusion Pressure (mmHg)	90.77 ± 7.50 §	149.39 ± 10.12	68.88 ± 8.39 §	70.01 ± 5.90 §
Flow (ml/min)	10.86 ± 0.15	10.57 ± 0.08	11.68 ± 0.15 *	11.81 ± 0.18 *
CVR (mmHg/ml×min^{-1})	8.40 ± 0.77 §	14.15 ± 0.99 ‡	5.94 ± 0.75 ‡,§	5.96 ± 0.51 ‡,§
Heart Rate (beats/min)	226 ± 13	241 ± 14	218 ± 10	223 ± 8
LVDP (mmHg)	122 ± 6	105 ± 8	112 ± 8	105 ± 6
+dP/dt (mmHg/sec)	4086 ± 259	3442 ± 83†	4046 ± 215	3657 ± 215†
-dP/dt (mmHg/sec)	-2114 ± 136	-1852 ± 87	-1982 ± 144	-1866 ± 99

Hemodynamic variables in the absence and presence of NOS inhibition (LNAME). *, $p<0.01$ main effect of age, †, $p<0.05$ main effect of BSO treatment; ‡, $p<0.05$ VS Adult con; §, $p<0.01$ vs Adult BSO. Data are presented as mean±SE (n=7-13/group).

Table 2.3 Curve characteristics from *in vivo* flow rate bradykinin dose-response curve

	Adult Control	Adult BSO	Older Control	Older BSO
			No Drug	
Max (%)	95.0 ± 1.0	98.6 ± 0.6	77.0 ± 10.3*	92.7 ± 4.5 *
EC_{50} (log M)	- 8.15 ± 0.04	- 8.21 ± 0.07	-8.48 ± 0.31	- 8.14 ± 0.09
AUC	231.9 ± 9.2	249.0 ± 9.3	201.9 ± 24.5*	209.3 ± 9.9 *
TDC	1.61 ± 0.23	2.06 ± 0.31	0.73 ± 0.27 *	0.89 ± 0.18 *
			LNAME	
Max (%)	94.5 ± 1.3	89.7 ± 2.2	91.4 ± 2.0	89.4 ± 1.7
EC_{50} (log M)	- 7.75 ± 0.07 †	- 7.39 ± 0.09	- 7.70 ± 0.07 †	- 7.75 ± 0.09 †
AUC	178.4 ± 8.5 †	137.2 ± 9.6	165.1 ± 11.3 †	171.8 ± 9.0 †
TDC	5.47 ± 0.68 †	10.72 ± 0.92 ‡	3.21 ± 0.72 †,‡	3.10 ± 0.45 †,‡

Curve characteristics in the absence (control) and presence of NOS inhibition (LNAME). Curves were fit as described in the methods section. TDC, total dilatory capacity (mmHg/ml×min^{-1}).*, $p<0.05$ main effect of age; †, $p<0.05$ vs Adult BSO; ‡, $p<0.05$ vs adult con. Data are presented as mean±SE (n=7-13/group).

Conversely, in the presence of LNAME, there was a marked decrease in the sensitivity to BK in the AB, as indicated by the rightward shift in the BK curve of this group compared to all other groups (Table 3). The AUC for the AB group was significantly reduced compared to all other groups (Figure 2.3, Table 2.3). These results suggest that in the AB group there is a greater relative reliance on NO to mediate the baseline CVR as well as endothelium-dependent responses to BK over several concentrations (1 nM – 0.03 μM) than the other treatment groups. Therefore within a physiologically relevant range of BK (98), the AB appear to be more reliant on NO than other endothelium-derived dilators (eg prostacyclin, EDHF).

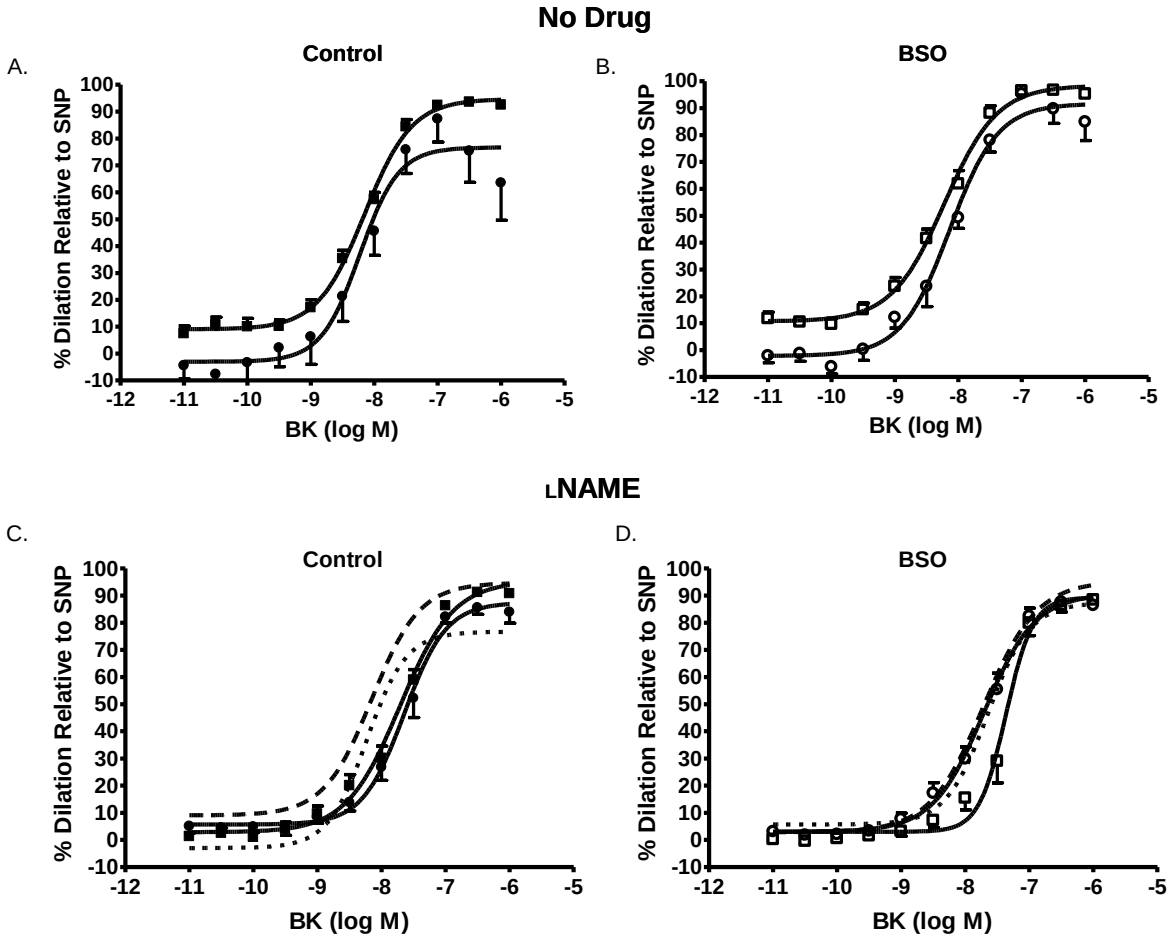

Figure 2.3. Bradykinin dose-response curves derived at *in vivo* flow rate

Data are from adult and older control (solid square and circle), and BSO adult and older animals (open square and circle) in the absence (A and B) and presence of NOS inhibition (C and D). The dashed line represents the adult CON no drug and the dotted line represents the older CON no drug (C). The dashed line in represents the adult CON LNAME and the dotted line represents the older Con LNAME (D). Data are presented as mean ± SE (n=7-13/group).

2.4.5 Hemodynamics at High Flow

Following the curves at the *in-vivo* flow rate, the flow rate was increased to yield a standardized perfusion pressure of ~75mmHg in the no LNAME groups to determine if this would result in altered CVR or BK-mediated responses. After a stabilization period, hemodynamics were re-assessed at the higher flow rate. Despite the fact that all groups had a perfusion pressure of ~75mmHg (p=NS, Table 2.4), the flow rate required to achieve the 75 mmHg perfusion pressure was significantly greater in the older animals compared to the adult (grouped by age: 18.30±1.31 vs 14.29±0.42 ml/min, $p<0.05$). Therefore, there was an age-dependent reduction in baseline CVR in the older groups (Table 2.4). Similar to the lower flow condition in the absence of LNAME, there were no group differences in any of the measures of cardiac function (Table 2.4). In the presence of LNAME there was also a main effect of age on CVR, such that adult animals had a greater CVR than the older animals (Table 2.4). There was a trend for an elevated +dP/dt in the presence of LNAME control groups compared to the BSO groups (p=0.070, main effect for treatment) and no differences were seen in any other indices of cardiac function (Table 2.4).

Table 2.4. Hemodynamic variables at High Flow

	Adult Control	Adult BSO	Older Control	Older BSO
	No Drug			
Perfusion Pressure (mmHg)	75.47 ± 0.85	83.66 ± 6.49	77.46 ± 3.99	73.06 ± 1.30
Flow (ml/min)	14.57 ± 0.61	14.00 ± 0.59	20.26 ± 2.52 *	16.84 ± 1.22 *
CVR (mmHg/ml×min^{-1})	5.27 ± 0.25	6.06 ± 0.49	4.30 ± 0.87 *	4.53 ± 0.38 *
Heart Rate (beats/min)	250 ± 12	228 ± 16	219 ± 20	222 ± 11
LVDP (mmHg)	92 ± 4	86 ± 5	97 ± 13	104 ± 10
+dP/dt (mmHg/sec)	3787 ± 187	3424 ± 222	3367 ± 389	3909 ± 358
-dP/dt (mmHg/sec)	-1657 ± 70	-1485 ± 75	-1709 ± 193	-1766 ± 153
	LNAME			
Perfusion Pressure (mmHg)	138.67 ± 8.85‡	168.49 ± 14.42†	107.40 ± 7.51†,‡	96.94 ± 4.98†,‡
Flow (ml/min)	14.16 ± 0.44	14.29 ± 0.10	18.57 ± 0.12 †,‡	16.48 ± 0.28 †,‡,§
CVR (mmHg/ml×min^{-1})	9.86 ± 0.68	11.80 ± 1.02	5.79 ± 0.41 *	5.93 ± 0.35 *
Heart Rate (beats/min)	248 ± 13	247 ± 17	226 ± 6	239 ± 10
LVDP (mmHg)	97 ± 14	90 ± 8	105 ± 6	95 ± 7
+dP/dt (mmHg/sec)	3636 ± 363	3169 ± 236 [a]	3997 ± 215	3445 ± 248 [a]
-dP/dt (mmHg/sec)	-1692 ± 249	-1514 ± 105	-1744 ± 73	-1695 ± 99

Hemodynamic variables in the absence (control) and presence (LNAME) of NOS inhibition at a standardized perfusion pressure.*, p<0.05 main effect for age, †, p<0.05 VS Adult con; ‡, p<0.05 vs Adult BSO, §, p<0.05 vs older control; [a], p=0.07 main effect of BSO treatment. Data are presented as mean±SE (n=7-13/group).

In the high flow condition, the maximal endothelium-dependent dilation in older animals remained blunted compared to the adult (105.4±1.4% vs 98.6±3.3, p=0.05, grouped by age). However, the older animals demonstrated a slight increased sensitivity to BK compared to the adult animals (EC_{50}; -8.18±0.11 vs -7.86±0.10 log M, p<0.05, grouped by age). There was no difference in the AUC between the groups (Figure 2.4, Table 2.5).

In the presence of LNAME, there was a marked reduction of the endothelium-dependent response to BK observed in the AB group; unlike the LNAME effect at lower flow this resulted in blunting of both the maximal response and AUC compared to all other groups (Figure 2.4, Table 2.5). Also unlike the LNAME effect at lower flow, there were no differences in the EC_{50} across the groups in the high flow conditions. Similar to the lower flow conditions, however, there was a reduction in TDC seen in the older animals compared to the adult animals in both the presence and absence of LNAME (Table 2.5).

Table 2.5 Curve characteristics from High Flow bradykinin dose-response curve.

	Adult Control	Adult BSO	Older Control	Older BSO
	No Drug			
Max (%)	104.8 ± 1.5	106.1 ± 2.5	99.0 ± 7.6 *	98.4 ± 2.1 *
EC_{50} (-logM)	7.80 ± 0.05	7.95 ± 0.15	8.23 ± 0.19 *	8.14 ± 0.19 *
AUC	218.1 ± 7.6	230.1 ± 15.2	244.7 ± 25.3	226.6 ± 21.7
TDC	1.94 ± 0.18	2.71 ± 0.33	1.15 ± 0.62 *	1.45 ± 0.87 *
	LNAME			
Max (%)	89.8 ± 4.7 †	66.8 ± 6.8	92.1 ± 3.9 †	94.3 ± 2.5 †
EC_{50} (-log M)	7.30 ± 0.13	7.13 ± 0.13	7.42 ± 0.09	7.37 ± 0.11
AUC	127.2 ± 15.9	83.6 ± 9.9	146.4 ± 12.0 *	147.1 ± 9.7 *
TDC	6.58 ± 0.60	7.84 ± 0.86	2.81 ± 0.34 *	2.83 ± 0.24 *

Curve characteristics in the absence (control) and presence of NOS inhibition (LNAME). Curves were fit as described in the methods section. TDC, total dilatory capacity ($mmHg/ml \times min^{-1}$). *, p<0.05 main effect of age, †, p<0.05 vs Adult BSO. Data are presented as mean±SE (n=7-13/group).

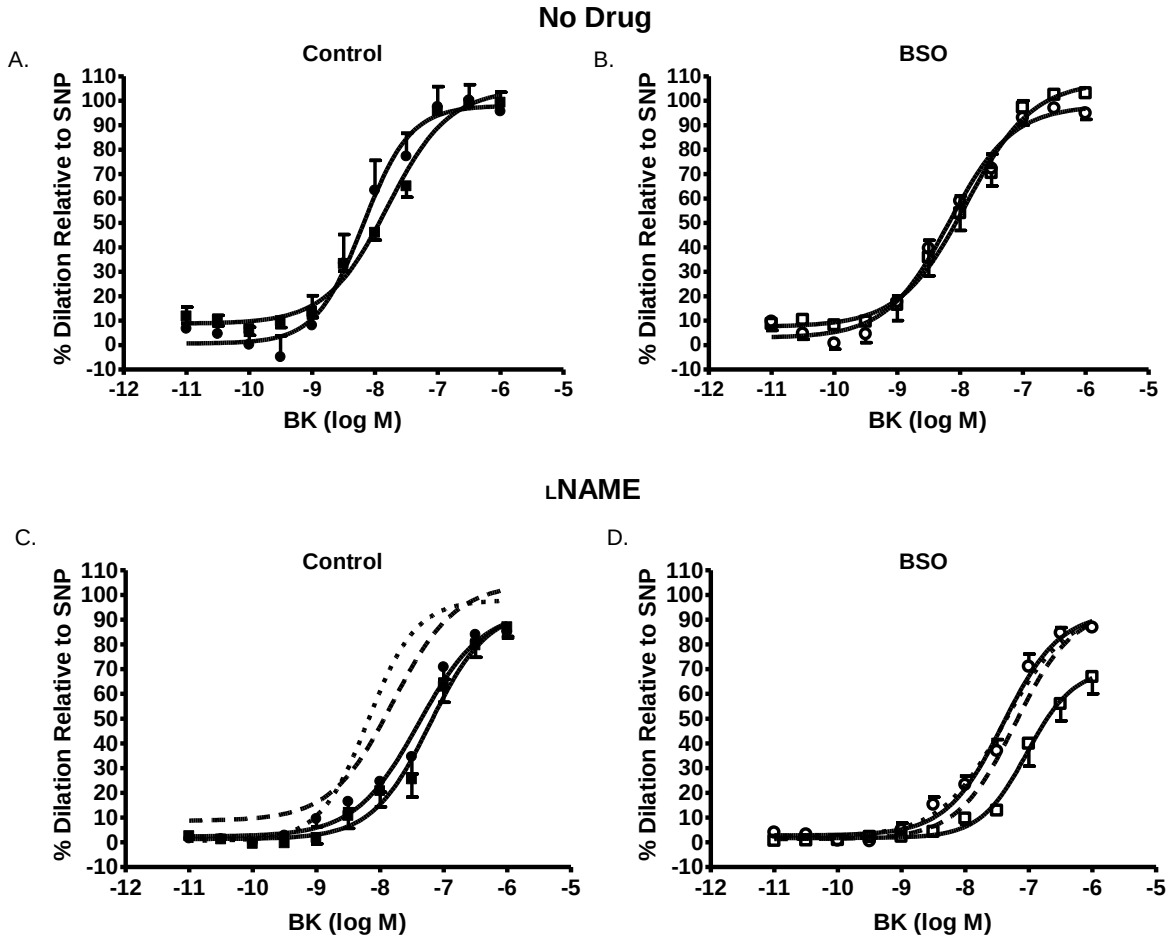

Figure 2.4. Bradykinin dose-response curves at High Flow

Data are from adult and older control (solid square and circle), and adult and older BSO animals (open square and circle) in the absence (A and B) and presence of NOS inhibition (C and D). The dashed line represents the adult CON no drug and the dotted line represents the older CON no drug (C). The dashed line in represents the adult CON LNAME and the dotted line represents the older CON LNAME (D). Data are presented as mean ± SE (n=7-13/group).

2.4.6 Effects of H_2O_2 on Coronary Vascular Resistance

Given that H_2O_2 is believed to act as a dilatory agent in the coronary circulation (148, 149), and that a previous study speculated that H_2O_2 was a mediator of endothelium-dependent effects observed in BSO treated adult animals (90), we wanted to assess if there was an effect of BSO treatment on the management of an acute exogenous H_2O_2 stimulus in the intact coronary vascular bed of AB animals. Prior to the assessment of H_2O_2-mediated effects, we confirmed that the responses to BK were similar between the CON and BSO animals. There were no differences in the dilation to 1 μM BK between the adult control and adult BSO groups (94.2±1.4% vs 91.9±1.6%; Figure 2.6). After washout of BK, H_2O_2 was administered at increasing concentrations (10^{-5} – 10^{-3} M) and a dose-dependent decrease in CVR was observed in CON, reaching 100% of the BK dilation. The response to H_2O_2 was blunted at several concentrations in BSO vs CON (Figure 2.5). Following the final concentration of H_2O_2, hearts were allowed to recover and redevelop a stable perfusion pressure. Once a stable perfusion pressure was achieved we assessed if there were alterations to the BK response, and found no differences between the adult CON and adult BSO animals following the H_2O_2 exposure (Figure 2.6). These results demonstrate that the BSO-treated animals were less sensitive to H_2O_2, and that a prior H_2O_2 exposure did not have a deleterious effect on subsequent endothelium-dependent dilation.

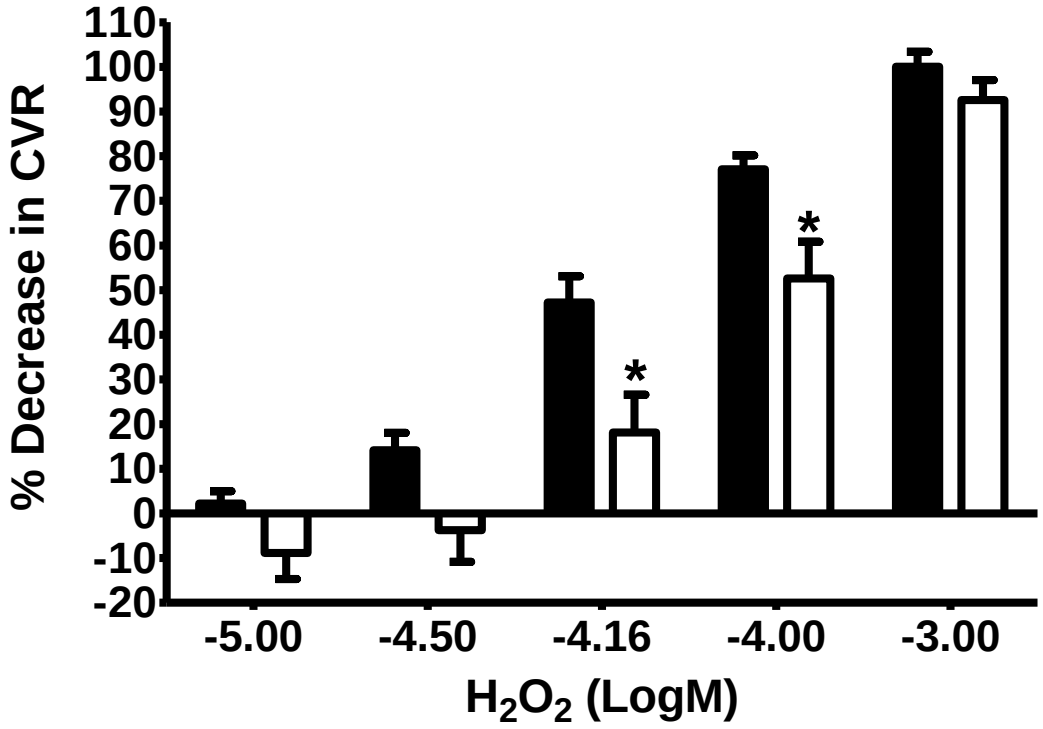

Figure 2.5. H_2O_2 induced dilation

Results are from adult control (■)and adult BSO (□). Decrease in CVR was expressed relative to maximal endothelium dependent dilation (1 μM BK). Data are presented as mean±SE (n=7-8/group).

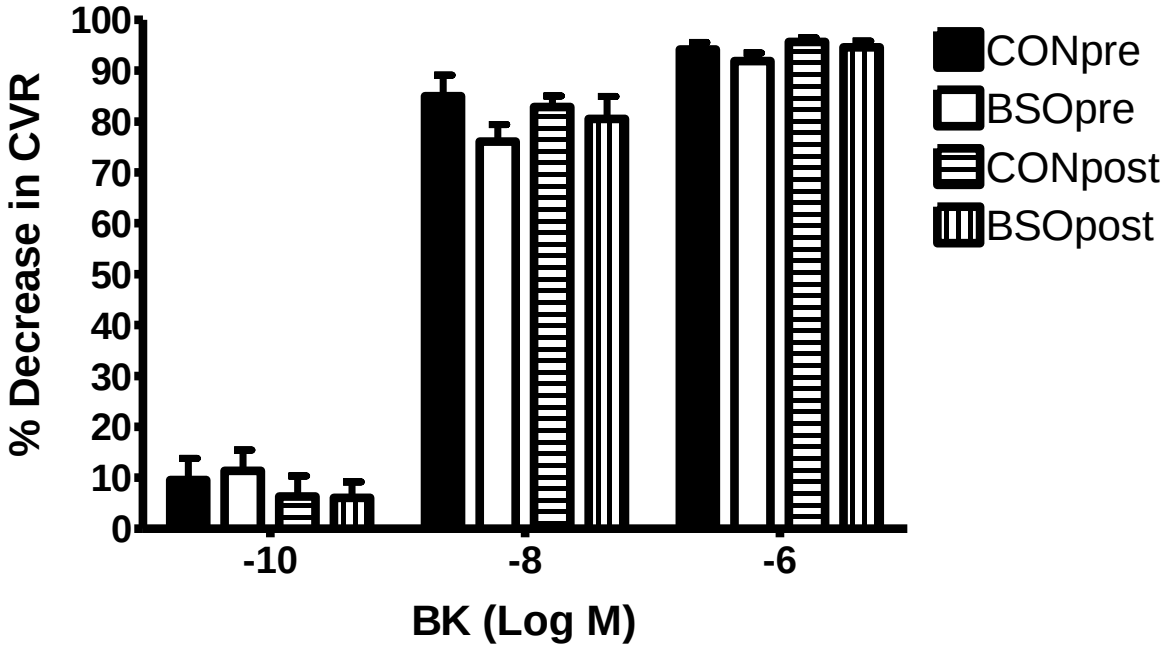

Figure 2.6. Bradykinin responses prior to and following H_2O_2 dose response curves
Bradykinin responses from adult control (filled bars) and adult BSO (empty bars) prior to, and following H_2O_2 exposure from adult CON (horizontal hatch), and adult BSO (vertical hatch). Data are presented as mean±SE (n=7-8/group).

2.4.7 Effect of BSO treatment on protein expression

An age dependent, BSO-induced increase in LV eNOS protein content was noted in the AB group compared to all other groups (Figure 2.7). Treatment with BSO induced a significant increase in left ventricular catalase protein expression in both adult and older animals (Figure 2.7). No differences in SOD 1 or 2 protein expression were observed (Appendix Figure A1).

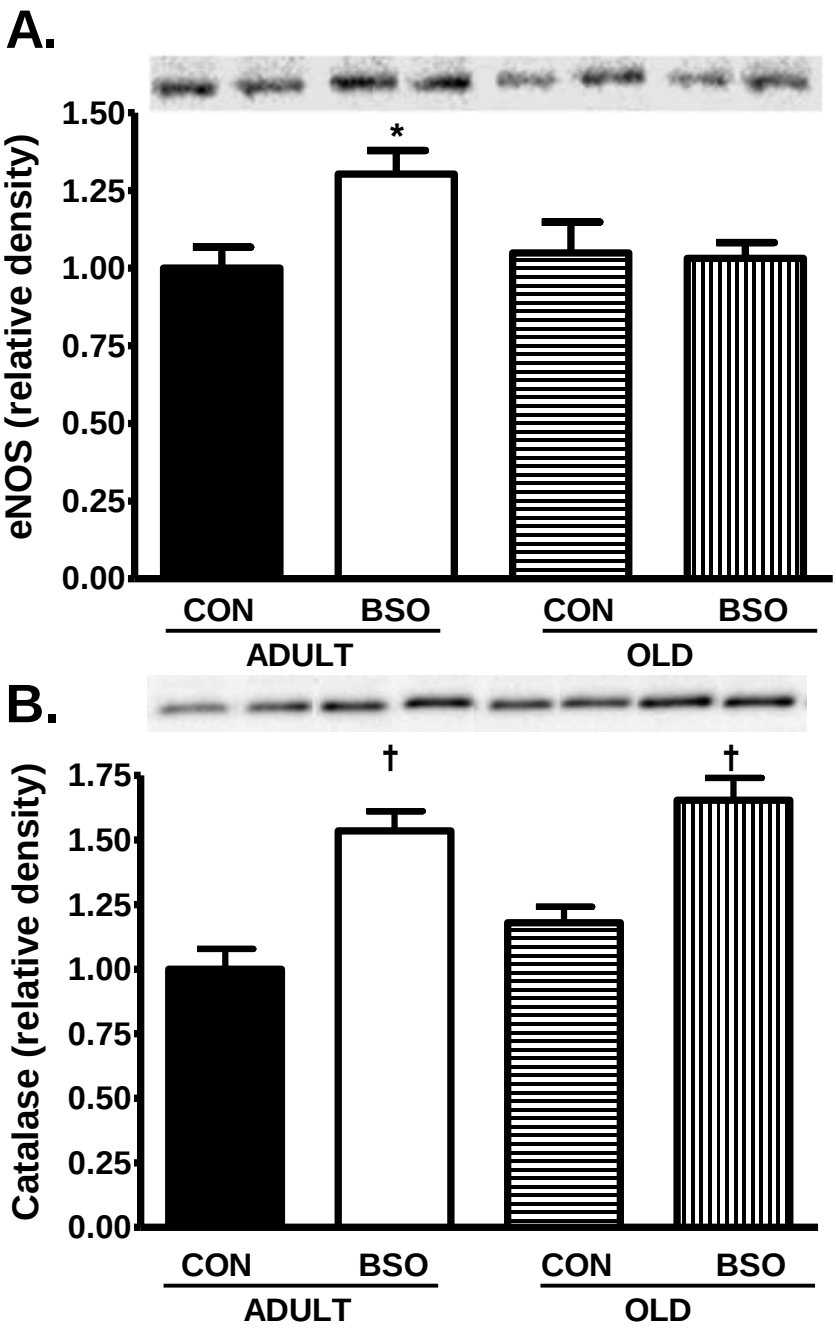

Figure 2.7. Relative change in protein expression following 10 days of BSO treatment
Data are expressed relative to adult control. eNOS protein expression (A) and catalase protein expression (B). Above each graph is a representative immunoblot of (n=2/group) in the order that they appear on the graph. *, $p<0.05$ vs all groups, †, $p<0.05$ main effect of BSO treatment. Data are presented as mean±SE (n=7-9/group).

2.5 Discussion

The primary finding of this study is that chronic thiol depletion in adult animals resulted in an increased reliance on NO for maintaining CVR and endothelium-dependent dilation, as supported by the observation that the increase in CVR and blunted endothelium-dependent responses in the presence of LNAME was greater in AB compared to AC. Furthermore, we demonstrated that this effect of BSO was age-specific, as no BSO-dependent increased reliance in NO was observed in the older animals, despite the fact that marked reduction in ventricular thiols and elevated ROS production still occurred in older BSO vs older control animals. In the older animals there was a reduction in the amount of spontaneous coronary vascular resistance and this appears to be unrelated to NO production, as it also was evident in the presence of LNAME. Intriguingly, coronary vascular sensitivity to H_2O_2 was lower in hearts from BSO treated animals compared to those from controls. This may be explained by the compensatory increase in catalase protein content seen with BSO treatment.

2.5.1 Effects of BSO treatment and aging on endothelium-dependent dilation

Studies which have examined the effects of BSO on systemic and conduit vascular function demonstrated that endothelium-mediated NO-induced dilation is reduced (13, 63, 68, 107), likely due to elevated oxidative stress associated with BSO treatment (13, 67, 181). In contrast, in the current study we observed an *increased* reliance on NO in the coronary circulation of adult BSO-treated animals. It appears that this effect is age-dependent as the adult BSO animals achieved a greater CVR and a greater blunting of the BK-mediated dilation in response to LNAME compared to the adult controls while no differences were observed between the older controls and older BSO animals, despite reduction in GSH content, and elevated ROS.

We expressed vasodilation responses relative to the degree of maximal SNP-stimulated reduction in CVR (minimal CVR). Expressing the responses in this manner does not account for

differences that may exist in capacity to dilate, as the change in CVR (from baseline to minimal CVR) is used as the total dilatory capacity. Importantly, we found that both baseline CVR and minimal CVR were reduced in the older animals, and overall this resulted in a reduced total dilatory capacity with age. Thus, unlike other studies from the coronary circulation (6, 37, 95, 172), we observed only a modest decrease in maximal BK-mediated dilation with aging at either flow condition. However, when we express the dilation relative to baseline vascular resistance, we also show that maximal BK-mediated dilation is blunted with age (31.4±1.5% vs 15.0±2.0% in adult vs older, $p<0.05$), similar to the effect reported in previous studies (6). It is also possible that our aging effect was smaller than previous reports as our older animals tend to be younger than old animals from other aging studies (eg (6, 37, 154)). Our older animals were chosen specifically to examine if thiol depletion would accelerate the aging-induced endothelial dysfunction. Although the older rats utilized may not be considered senescent, we demonstrated that the older animals had endothelial dysfunction as demonstrated by reduced maximal dilation and total dilatory capacity. Had BSO treatment accelerated these age-related changes as hypothesized, the current model would have been ideal to demonstrate a dynamic range of effects.

2.5.2 Effects of BSO treatment on eNOS protein expression

We have extended previous findings from other tissues (13, 63, 90, 195) by demonstrating that BSO treatment causes an increase in eNOS protein expression in the left ventricle of adult animals. It is possible that this is mediated by increased H_2O_2 as has been previously suggested (53). Further support for this as a potential mechanism is the intriguing finding that despite the effect of BSO treatment on catalase expression, H_2O_2 levels were still elevated in left ventricular homogenates from BSO treated vs control animals. It is also noteworthy, however, that although there was a general BSO effect at increasing H_2O_2 regardless of the age cohort, the increase in eNOS protein content was

limited to the adult BSO group. This suggests that the mechanisms are more complex and that aging modifies the signals causing eNOS adaptations to BSO, and/or the sensitivity of eNOS regulation to those signals. In support of this finding we found that catalase protein content was slightly, but non-significantly elevated with age (p=0.06). Thus if H_2O_2 is accounting for the increase in eNOS protein content, the ability of a small increase in catalase to reduce H_2O_2 production in the OC group could account for the age-dependent effect of BSO treatment to increase eNOS protein content observed. This is supported by the amplex red H_2O_2 production data, which demonstrated that relative increase in ROS production was greater between the AC and AB group compared to the OC and OB groups.

2.5.3 Effects of aging on coronary vascular resistance

The reduced amount of spontaneous tone that we observed as a result of aging is in agreement with previous reports from isolated perfused hearts (6, 89) and isolated arterioles (154). The myogenic response is blunted in isolated coronary arterioles from old animals compared to young animals, an effect that was also present in response to agonist induced constriction (154). It was further demonstrated that the impaired ability to constrict and develop spontaneous tone could be abolished either through LNAME or denudation, suggesting that increased endothelium-derived NO was involved in blunting the constriction (154). In contrast, in the current study, the effect of age on CVR persisted despite NOS inhibition, suggesting that the age-dependent reduction in CVR was not dependent on an increase in NO production. In response to LNAME, the percentage increase in CVR from baseline was similar in the adult controls (42%) compared to the older control (38%). Under the lower *in vivo* basal level flow conditions there are not differences in the relative contribution of NO between the adult and the older animals. Thus, our aging results are in contrast to both Amrani and co-workers (6) and Ishihata and colleagues (89) who found that NO production in isolated, perfused hearts was reduced in older animals. Under the higher flow conditions, however, we did find that the

LNAME effect was blunted in the old animals as the LNAME-induced increase in CVR was only ~18%, compared to 47% seen in the adult animals. It would therefore appear that under our lower flow conditions designed to mimic a basal *in vivo* flow rate, basal NO production is maintained; however when flow rate is increased, this reveals an age-dependent relative reduction in NO production. We found no differences in eNOS protein content in the LV with age, suggesting that the reduction in NO production seen under the High Flow is not related to differences in eNOS content and therefore likely reflects changes in either eNOS activation or NO destruction. Our data would suggest that enhanced NO destruction is not the case as ROS production in the LV was not significantly increased with age. It is therefore, likely that the attenuated increase in CVR in the older rats in the presence of LNAME is related to altered eNOS activation which was more apparent under conditions of higher shear stress (158).

As already suggested, alterations in the myogenic response could account for the reduction in CVR seen in the older animals (154). It is possible that other mechanisms could contribute to the observed age-dependent reduction in CVR including alterations in the expression of K^+ channels (94). The reduction in CVR seen with age likely relates to heterogeneous nature of the coronary circulation (93, 105) and may reflect a compensatory change to maintain cardiac perfusion and function.

2.5.4 Effects of BSO treatment on cardiac contractility

We found that chronic BSO treatment did not alter any measures of left ventricular function assessed in the Langendorff preparation. This is consistent with a previous report using acute administration of BSO (108). In the current study, however, NOS inhibition did reveal an interesting contractility effect of chronic BSO treatment, as the maximal rate of ventricular pressure development (+dP/dt) was reduced in BSO-treated adult rats in the presence of LNAME compared to controls in the presence of LNAME. In the older controls, we found that NOS inhibition resulted in an increased

+dP/dt, whereas no changes were seen in contractility in the other groups (Tables 2 and 4). It is plausible that in the older control animals that NOS inhibition removed excess NO which can cause a blunting of contractility (102, 139) whereas in both BSO groups this was not observed, possibly as a result of excess ROS reducing basal NO availability in the myocardium. Although this phenomenon was not directly assessed in the current study, we did observe increases ROS production in the BSO treated animals and, speculatively, the effect of BSO may be mediated in part by altered sarco(endo)plasmic reticulum function, as previously demonstrated in diaphragm (177).

2.5.5 Effects of exogenous H_2O_2 on coronary vascular resistance and endothelium dependent responses

We observed that BSO treatment impaired the ability of coronary vasculature to dilate to H_2O_2. This is inconsistent with the hypothesis put forward in a previous report suggesting that endothelial-mediated vasomotor function is enhanced with BSO treatment as a result of endothelium-dependent H_2O_2 mediated dilation (90). A possible explanation for the reduced dilation to H_2O_2 observed herein is that increased expression of catalase in the hearts of BSO-treated compared to control animals resulted in better management of the exogenous H_2O_2 stimulus. The increase in catalase may also explain why a larger increase in H_2O_2 production was not observed in heart homogenate. It is clear that the sensitivity of the coronary vascular bed to H_2O_2-mediated vasodilation is reduced in BSO treated vs control animals. Thus, the previous speculation regarding H_2O_2-mediated enhancement of endothelium-dependent dilation to be possible, it would require very large local increases in H_2O_2 in the vascular environment.

2.5.6 Summary

The current study reveals that BSO treatment age-dependently increases the reliance on NO in the intact coronary circulation of the adult BSO group. This is based on observations of greater CVR, blunted endothelium-dependent responses in the presence of NOS inhibition, and increased eNOS

expression in the adult BSO group (vs older) only. Furthermore, we have demonstrated that isolated perfused hearts from older animals fail to develop as much spontaneous coronary vascular resistance as the adult animals, and that BK-stimulated, endothelium-dependent coronary vasodilation is reduced with age. Our results indicated that the age-dependent reduction CVR is not dependent on increased NO as in the presence of LNAME, a similar relative increase in CVR was observed at the *in-vivo* flow rate compared to the adult animals. A portion of the impaired endothelium dependent vasodilation may be masked, as the capacity to dilate is smaller in the older animals with lower basal tone. Despite an increased H_2O_2 production seen in the left ventricle of BSO animals, the vasculature of these animals is less sensitive to exogenous H_2O_2 as demonstrated by the attenuated decrease in CVR compared to control animals. In summary, both thiol depletion and aging differently impact vasomotor function in the isolated perfused rat heart. These changes may reflect differential adaptations to different modes of chronic oxidative stress, and that the effects of age and BSO-dependent chronic glutathione depletion do not appear to be additive.

2.6 Acknowledgements

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CHAPTER 3

Glutathione enhances endothelium-mediated coronary vascular dilation in a reactive oxygen species-dependent manner and independent of effects on NO production and soluble guanylate cyclase

3.1 Synopsis

The purpose of this investigation was to determine the effects of acute physiological GSH administration on NO-mediated coronary artery dilation. Endothelial function was assessed in isolated, perfused hearts. A dose-response curve to GSH was conducted to determine a threshold concentration of GSH. We demonstrate that 30 μM GSH was sufficient to reduce coronary vascular resistance, and maximal dilation was achieved with 1 mM. Subsequently, GSH was administered at concentrations of 0 (CON), 1 μM or 10 μM (GSH_{10}) and dose-response curves to the endothelial agonist bradykinin (BK) were constructed. These GSH concentrations were chosen because of the physiological relevance, and because the effects of GSH on BK action could be assessed independent of baseline differences. The sensitivity (EC_{50}) to BK was enhanced in GSH_{10} vs CON ($p<0.05$). This enhancement remained in the presence of nitric oxide synthase inhibition (LNAME) and/or soluble guanylate cyclase inhibition. Treatment with TEMPOL enhanced the sensitivity to BK in CON similar to GSH_{10} and GSH_{10}+TEMPOL. Conversely, the GSH_{10}-dependent enhancement observed in the presence of LNAME did not occur in the presence of LNAME+ TEMPOL. Collectively, these results suggest that GSH enhances BK-mediated dilation through an antioxidant-dependent mechanism, unrelated to NO production or guanylate cyclase-dependent effects of NO. These results suggest a mechanism whereby the effects of endogenous reactive oxygen species are modulated by physiologically relevant

levels of GSH. This mechanism could help protect/enhance endothelium-dependent vascular function in health and in disease conditions of elevated vascular oxidative stress.

3.2 Introduction

Reduced glutathione (GSH) is an important antioxidant and contributes to the maintenance of vascular health via its role in mitigating the effects of free radicals (65, 146, 160). Several investigations have examined the effects of acute GSH administration on endothelial function in patients with suspected and known cardiovascular disease. Kugiyama and colleagues (1998) found that infusion of either GSH or ACh alone into the coronary arteries of patients undergoing angiography did not alter vessel diameter or blood flow. However, when GSH was co-infused with ACh, the normal healthy dilatory response to ACh was observed (104). Similarly, Prasad and colleagues demonstrated that GSH enhanced the ACh-induced reduction in femoral vascular resistance compared to ACh alone (138). In these studies, the effects of GSH administration were more pronounced in individuals with endothelial dysfunction than in those with normal vasomotor responses (103, 104, 138). It is plausible that the effects of acute GSH administration are most prominent in individuals with existing endothelial dysfunction, due to the action of GSH as an antioxidant protecting NO bioavailability in those with cardiovascular disease.

Direct vasodilatory effects of GSH were not observed in the human studies discussed above (103, 104, 138). However, animal studies utilizing pre-contracted arteries, and isolated perfused hearts have demonstrated that GSH alone is capable of inducing vasodilation (5, 33, 66, 92). In isolated perfused rat hearts, Cheung and Schultz demonstrated that GSH dose-dependently increased coronary flow in a constant pressure preparation. These vasodilatory effects of GSH were mediated through a pathway involving nitric oxide, sGC and free radicals (33). The Cheung and Schultz report establishes

that GSH can directly influence vascular tone, and therefore could potentially alter signalling pathways known to mediate endothelium-dependent vasorelaxation. However, the issues of whether there are significant effects of physiological levels of extracellular GSH on the action of endothelium-dependent vasorelaxation pathways, and the mechanisms involved have not been directly addressed. It is important to address these issues experimentally in order to understand both the physiological role of GSH in vascular function, and the clinical utility of GSH in cardiovascular disease states.

The purpose of the current study, therefore, was to investigate the hypothesis that acute, low dose GSH infusion co-administered with an endothelial agonist would augment endothelium-mediated reductions in coronary vascular resistance in the isolated perfused rat heart, and that this response would result from enhanced NO bioavailability.

3.3 Methods

3.3.1 Animals

Male Sprague-Dawely rats (n=105, mean age 40 weeks) were group housed (4/cage) in a temperature- and humidity-controlled environment, and kept on a reverse 12 hour light/dark cycle. Food and water were provided ad libitum. All procedures were approved by the University of Waterloo Animal Care Committee. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication NO. 85-23, revised 1996).

3.3.2 Langendorff Heart

Animals were anaesthetized (60 mg/kg pentobarbital sodium, i.p.), and the heart was removed and immersed in 4^{o}C Krebs Henseleit (KH) buffer (composition in mmol/L NaCl 118, KCl 4.7, $MgSO_4 \cdot 7H_2O$ 1.2, $NaHCO_3$ 24, KH_2PO_4 1.1, Glucose 10 and $CaCl_2 \cdot 2H_2O$ 1.25) to reduce myocardial

contractility and ischemic injury (166). Hearts were transferred to the aortic cannula which was dripping gassed (95%O_2-5%CO_2) 37^{o}C KH and secured with suture. The time from excision to the establishment of flow was less than 1 minute, and the heart was at the '*in-vivo'* flow in approximately 2.5 minutes.

We utilized a constant flow protocol as this is best suited for the study of vasoactive agents (10, 32). The constant flow rate was assigned to mimic *in-vivo* coronary flow where Flow=7.43×((body weight×0.0027+0.6)$^{0.56}$) (45, 50). A flow probe and associated transducer (2PXL and TS410, Transonic Systems, Ithaca NY) and pressure transducer (MLT844, AD Instruments, Boulder Co) were positioned proximal to the aortic cannula for the measurement of total coronary flow, and coronary perfusion pressure. A pressure balloon was inserted through the left atria into the left ventricle and adjusted to achieve a diastolic pressure of 5 – 10 mmHg. Heart rate, left ventricular developed pressure (LVDP, left ventricular systolic pressure–diastolic pressure) and +dP/dt were derived from the left ventricular pressure tracing. All data were collected using a Powerlab data acquisition board (4/sp, AD Instruments, Boulder Co) and stored digitally using Chart for Windows (5.5.1, AD Instruments, Boulder Co) for off-line analysis.

Hearts were stabilized for 30 minutes to allow for the development of spontaneous coronary vascular tone and heart rate. Preparations which failed to establish spontaneous tone were excluded from the study. Once the perfusion pressure had stabilized, baseline hemodynamic measurements were made by taking an average over 1 minute.

3.3.3 Series 1 GSH dose-response relationship in isolated perfused hearts

Following baseline measurements, increasing concentrations of GSH (1.0 µmol/L – 1.0 mmol/L) were administered. Each concentration was infused at 1% of total coronary flow for 10 minutes (33). A 10 second average around the peak response to each concentration was used to derive the dose-

response relationship for GSH-induced vasodilation. Following the maximal dose of GSH, sodium nitroprusside (SNP, 10 μM) was used to induce endothelium-independent dilation.

3.3.4 Series 2 Effect of GSH administration on BK-mediated dilation in isolated perfused hearts

Hearts were infused with either 0 (CON), 1 μM (GSH_1) or 10 μM (GSH_{10}) of GSH. These concentrations were chosen for two reasons: 1) Series 1 experiments demonstrated that these GSH concentrations do not cause a decrease in basal coronary vascular resistance, allowing for a similar baseline CVR across conditions, and 2) the 10 μM concentration is a physiologically relevant concentration of plasma/extracellular fluid GSH (65). Steady state baseline measures were made, followed by a dose response curve to bradykinin (BK, 10^{-11} – 10^{-6} M). After the final concentration of BK, the vasculature was dilated with adenosine (ADO 10^{-5}M), and endothelium-independent dilation to exogenous NO was also assessed using SNP (10^{-9} – 10^{-4} M). Each dilatory agent was infused at a rate of 1% of the coronary flow using a syringe pump (Cole Parmer 74900, Montreal PQ). L-$_{\omega}$Nitro-L-arginine (LNAME, 0.1 mmol/L) was used to inhibit nitric oxide synthase, 1H-[1,2,4]oxadiazolo[4,3]quinoxalin-1-one (ODQ, 3 μmol/L) was used to inhibit soluble guanylate cyclase, and the superoxide dismutase (SOD) mimetic, 4-Hydroxy TEMPO (TEMPOL, 0.1 mmol/L) was used as a non-thiol exogenous antioxidant. Therefore, 'no drug' refers to the condition in the presence or absence of GSH (CON no drug/GSH no drug) in the absence of other pharmacological agents. Only one drug protocol was performed per heart.

Changes in coronary vascular resistance were reported as a percentage of the maximal decrease in CVR induced by ADO, using the following equation: $[((CVR_{BL}–CVR_A)–(CVR_{BL}–CVR_C))÷(CVR_{BL}–CVR_A)]×100$. Where CVR_{BL} is baseline CVR, CVR_A is minimal CVR (adenosine) and CVR_C is the CVR at the concentration of interest. No differences in the absolute minimal CVR elicited by adenosine were

observed across groups ($p>0.05$). Curves were fit to obtain EC_{50}, maximal dilation, and area under the curve (AUC) using Prism (4.03, GraphPad Software, San Diego CA).

3.3.4 Series 3 Effect of GSH administration on ACh-mediated responses in isolated aortic rings

In a subset of animals (n=8/group), the thoracic aorta was isolated for wire myography as previously described (63, 74, 75). Rings (2mm axial length) were mounted on a myography unit (Radnoti, Morovia, CA), immersed in Kreb's bicarbonate buffer gassed with $95\%O_2/5\%CO_2$, stretched to a resting tension of 5g (12), and allowed to equilibrate for 30 minutes with or without 10 µM GSH. A cumulative dose-response protocol to ACh ($10^{-9} – 10^{-4}$ M) was performed in vessels contracted with phenylephrine (0.1 µM). Curves were fit as described above.

3.3.5 Measurement of left ventricular glutathione content

Following the experimental protocol, hearts were gently blotted and weighed. A portion of the left ventricle was snap frozen in liquid nitrogen. Glutathione was assessed using HPLC as previously described by our laboratory (63) according to the method derived by Reed and colleagues (141).

3.3.6 Chemicals

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO), Bioshop Canada (Burlington, ON, Canada) and ODQ which was purchased from Cayman Chemicals (Ann Arbor, MI). All drugs were diluted to their final concentrations in KH buffer.

3.3.7 Statistics

Data are presented as mean±standard error (SE). Differences between groups were assessed using ANOVA for planned comparisons for each drug condition between the CON and GSH. Significant results were subsequently analyzed using Least Squares Difference *post hoc* test. To assess the

changes that the pharmacological agents had within the specific groups data was normalized to the control no drug condition and compared using ANOVA to allow for within group comparisons. A p value <0.05 was considered statistically significant. All statistics were performed using Statistica 6.0 (StatSoft Inc, Tulsa OK).

3.4 Results

3.4.1 Series 1 GSH dose-response relationship in isolated perfused hearts

Glutathione caused a dose-dependent reduction in coronary vascular resistance (Figure 3.1). This reduction became significant at ~30 µM. At 1 mM GSH the reduction in CVR was similar to that observed in response to maximal SNP. Given that both 1 µM and 10 µM GSH failed to significantly reduce CVR, and that these levels are in the physiological range of extracellular fluid GSH values, these concentrations were chosen for subsequent studies to examine how GSH would influence endothelium-dependent, agonist-mediated vasodilation in Series 2 experiments.

3.4.2 Series 2 Effect of GSH administration on BK-mediated dilation in isolated perfused hearts

Baseline characteristics for the CON, GSH_1 and GSH_{10} groups are presented in Table 3.1. There were no differences in heart weight or body weight between any of the groups, and acute administration of GSH did not result in a change in left ventricular GSH content. Likewise, no group differences were noted in baseline CVR, or indices of cardiac function (Table 3.2).

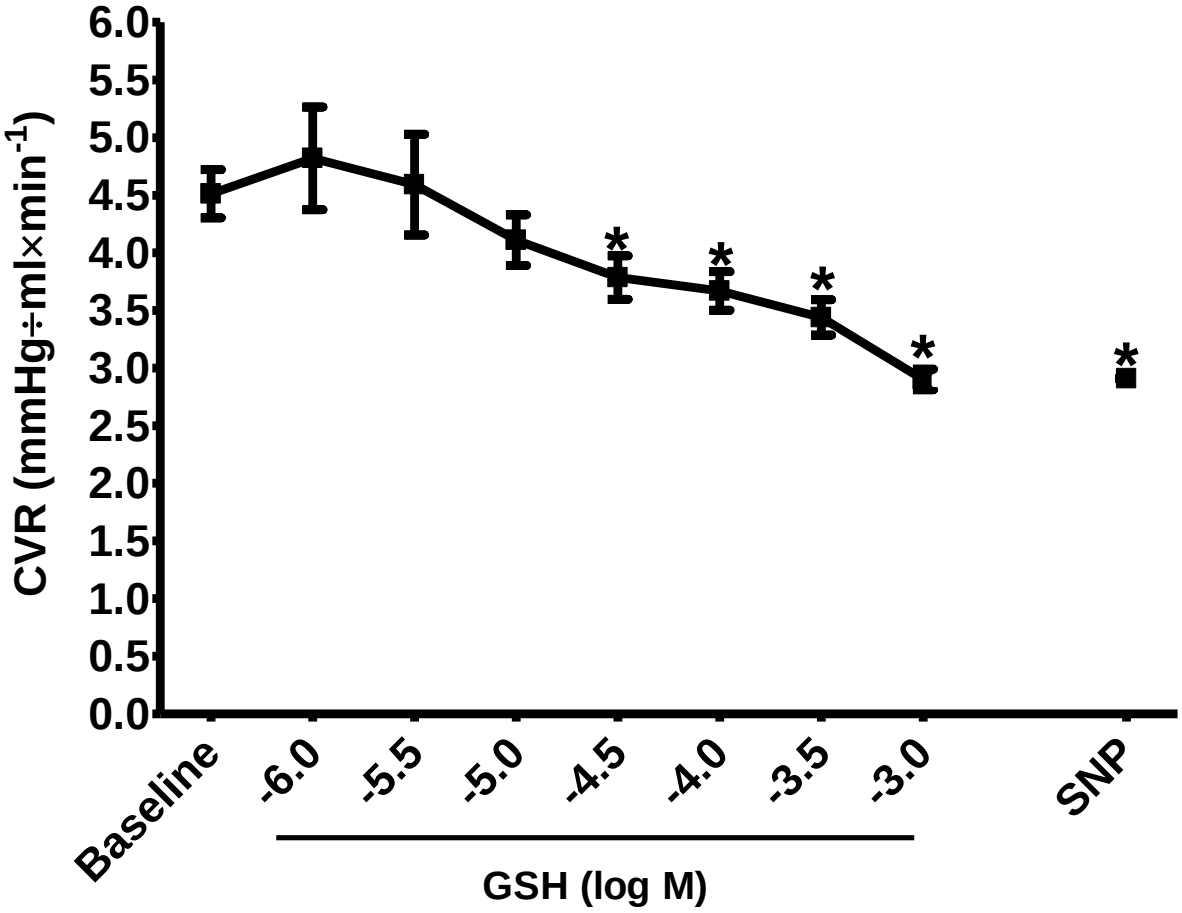

Figure 3.1 GSH mediated dilation

Acute administration of GSH led to a dose-dependent dilation of isolated perfused rat hearts. * $p<0.05$ vs baseline. Data are mean±SE (n=10).

Table 3.1. Baseline characteristics

	CON	GSH_1	GSH_{10}
Body weight (g)	511.7 ± 13.5	484.9 ± 12.5	514.4 ± 9.9
Heart weight (mg)	1836 ± 34	1828 ± 48	1907 ± 37
GSH (nmol×mg wet weight^{-1})	2.181 ± 0.109	1.936 ± 0.040	1.988 ± 0.173
GSSG (nmol×mg wet weight^{-1})	0.228 ± 0.010	0.197 ± 0.010	0.209 ± 0.018

No differences were noted between the CON and GSH_{10} groups. GSH content was determined using HLPC as described in the methods section. Data are presented as mean±SE (n=7-40/group for physical characteristics, n=7-14/group for thiol analyses).

Table 3.2. Hemodyamics values in the presence of no drug, NOS and sGC inhibition

	CON			GSH_{10}		
	No Drug	LNAME	ODQ	No Drug	LNAME	ODQ
CVR (mmHg÷(ml/min))	5.53 ± 0.35	10.74 ± 0.98*	5.32 ± 0.61	5.11 ± 0.47	7.97 ± 0.80*,†	5.40 ± 0.31
Heart rate (beats×min^{-1})	240 ± 9	226 ± 14	253 ± 17	218 ± 12	237 ± 14	240 ± 7
LVDP (mmHg)	116 ± 7	122 ± 14	117 ± 9	120 ± 7	117 ± 15	125 ± 6
+dP/dt (mmHg×sec^{-1})	4225 ± 262	4196 ± 565	4267 ± 525	4402 ± 243	3998 ± 518	4702 ±372

For details on specific comparisons refer to text. CVR; coronary vascular resistance, LVDP; left ventricular developed pressure, +dP/dt; maximal rate of pressure development. Data are presented as mean±SE (n=7-14/group). *, $p<0.05$ vs respective no drug,† $p<0.05$ vs CON+LNAME (planned comparison).

Acute GSH administration at 1 or 10 μM did not alter *maximal* BK-induced endothelial-mediated dilation compared to controls (Figure 3.2). However, acute administration of 10 μM GSH, which is similar to the concentration seen in the plasma/extracellular fluid (65), did enhance the *sensitivity* (lowered the EC_{50} value) to BK in the GSH_{10} group compared to CON (2.93±1.09 vs 12.19±1.37 nM), and this was associated with increased area under the curve (264.4±13.8 vs 191.1±10.2). Conversely, the effects of 1 μM of GSH did not reach significance for EC_{50} (7.12±1.90 nM) or AUC (229±17.3) compared to CON. Thus, the GSH_1 group was excluded from subsequent studies in this series designed to identify the mechanisms responsible for GSH effects on responses to BK (Appendix Figure A2).

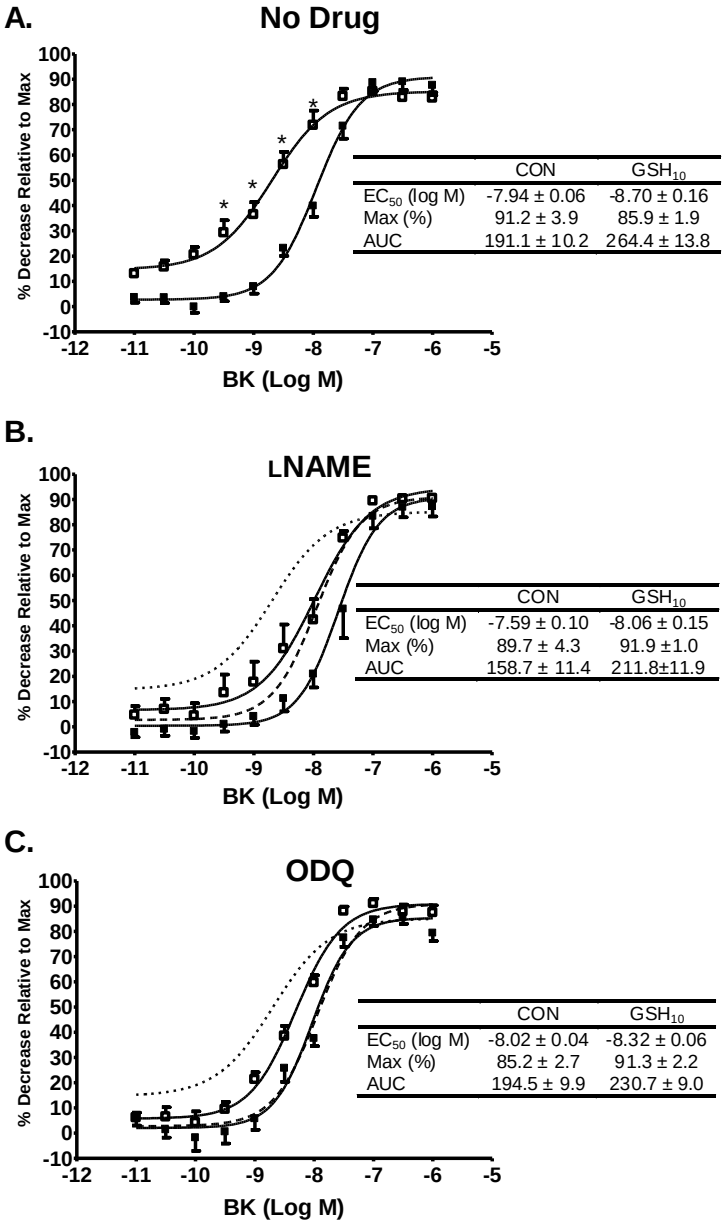

Figure 3.2 Bradykinin mediated dilation is augmented in the presence of GSH

Dilatory curves from the control (■)and GSH_{10} (□).The presence of GSH increases the sensitivity to BK in the 10 µM group but not the 1 µM group (A). In the presence of LNAME (0.1 mM), the GSH effect persisted (B). ODQ (3 µM) had minimal effect in both groups (C). Maximal dilation refers to minimal CVR elicited by adenosine (10 µM). The long dashed (control) and short dashed (GSH_{10}) are included for clarity. Data are presented as mean±SE (n=7-8/group) *p<0.05 vs respective control curve

3.4.3 Effects of NOS inhibition on BK-mediated responses in the presence of GSH

To assess the role of NO *production* in mediating the increased sensitivity to BK observed in the presence of 10 μM GSH, LNAME (0.1 mM) was employed (Figure 3.2). LNAME increased the baseline CVR in both the CON and GSH_{10} groups (Table 2), but GSH_{10} blunted the LNAME-associated increase in CVR (Table 3.2, Figure 3.3). No differences were noted between groups in any indices of cardiac function. LNAME reduced the sensitivity to BK (increased the EC_{50} value) in the CON group, yet failed to significantly reduce the sensitivity in the GSH_{10} group compared to GSH_{10} no drug condition (Figure 3.3). Thus, the effect of GSH_{10} to increase sensitivity to BK remained intact in the presence of NOS inhibition (Figure 2; EC_{50} = 6.94±3.33 vs 30.00±11.40 nM), as did the enhancement in the AUC (211.8±11.9 vs 158.7±11.4).

3.4.3 Effect of sGC inhibition on BK-mediated responses in the presence of GSH

Having established that GSH still enhanced vasodilation to BK even in the presence of NOS inhibition, we sought to determine if there was a sGC dependency of the GSH effect, independent of NO production. This would allow us to test the possible role of 'stored' NO, such as nirosothiols, acting through sGC in mediating the GSH effects (14, 16, 30, 174). In the presence of the sGC inhibitor ODQ, no differences were observed in the hemodynamic variables versus the no drug group (Table 3.2). In the control condition, ODQ (3 μM (33, 151)) did not alter the sensitivity, the AUC, nor maximal dilation to BK compared to the no drug control group (Figure 3.3). Increasing the concentration of ODQ to 10 μM (as some previous studies have employed (14)), also did not affect responses to BK (n=6, Appendix Figure A3). The lack of effect of ODQ on EC_{50} and CVR in the control group demonstrates that the sGC/cGMP pathway is not necessary to mediate the observed effects of GSH, thus suggesting that if there is any NO involvement in mediating the GSH effects it is due to sGC/cGMP-independent effects of NO (23, 128). Similarly, in the presence of GSH_{10}, ODQ had no significant effect on sensitivity (EC_{50},

5.16±0.80 vs 2.93±1.09 nM), yet resulted in a small decrease in the AUC compared to the GSH_{10} responses in the absence of ODQ (230.7±9.0 vs 265.4±13.8, Figure 1.3).

The sensitivity to BK remained enhanced in the presence of GSH_{10}+ODQ compared to CON+ODQ (EC_{50}, 5.16±0.080 vs 9.53±0.71 nM), as was the AUC (230.7±9.0 vs 194.5±9.9) (Figure 3.2). These results suggest that the observed effects of GSH do not require an obligatory role of sGC activation.

3.4.4 The effect of combined NOS and sGC blockade on the BK-mediated responses in the presence of GSH

In the presence of combined NOS and sGC inhibition, the GSH_{10} effect persisted, such that sensitivity to BK (EC_{50}, 4.28±0.46 vs 16.92±3.07 nM) and AUC (248.2±9.7 vs 183.1±6.2) remained enhanced (Figure 3.3, Appendix Figure A4). This suggests that the effects of GSH can be independent of both NO production and NO-dependent sGC activation, and that another non-NO dependent pathway contributes to the GSH effect.

3.4.5 Effect of GSH on vasodilatory responses to exogenous NO

In order to differentiate whether GSH affected the ability of vascular smooth muscle to respond to NO, the vasorelaxation effects of sodium nitroprusside were assessed. SNP dose-dependently decreased CVR in both groups. No differences were observed between the CON and GSH_{10} groups (Figure 3.4).Thus the GSH_{10} effects appear to be independent of changes in vascular smooth muscle NO-dependent vasodilatory capacity, as both the SNP- and ADO-mediated responses were not different than the control.

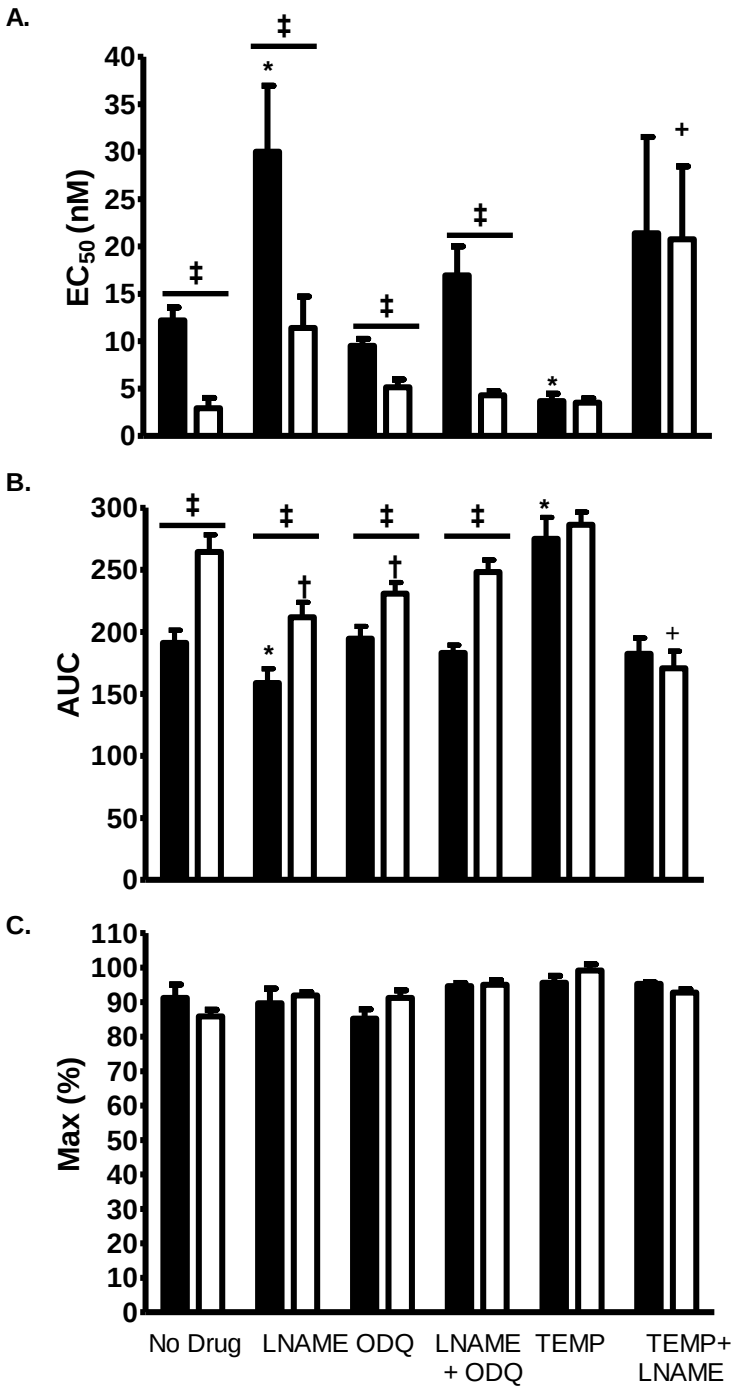

Figure 3.3 Summary of curve parameters

Effects of pharmacological agents on the sensitivity, EC_{50} (A), AUC (B) and maximal dilation (C) in the control (■)and GSH_{10} (□).*, p <0.05 vs control no drug; †, p<0.05 vs GSH_{10} no drug. ‡ p<0.05 vs CON a specific drug condition. Data are presented as mean±SE (n=3-9/group).

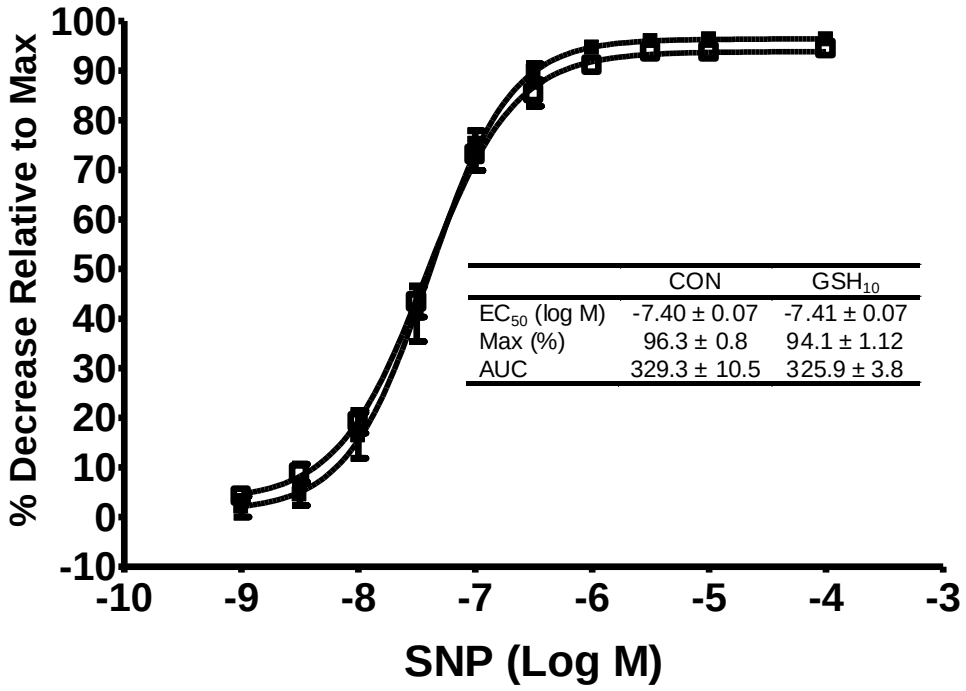

Figure 3.4. Sodium nitroprusside mediated dilation

Exogenous NO dose-dependently decreased CVR in the control (■) and GSH_{10} (□) groups. No differences were seen between groups. Dilation is expressed as a percentage of minimal CVR elicited by adenosine (10 μM). Data are presented as mean±SE (n=6/group).

3.4.6 Effect of GSH and exogenous non-thiol antioxidant on the dilatory responses to BK

The activity of GSH may be mediated in part by its antioxidant-dependent effects. We assessed whether the observed effects were thiol-specific, or were general antioxidant effects by determining whether addition of a non-thiol antioxidant would alter the effects of GSH_{10} on the BK-mediated responses. In the control group, the addition of excess SOD mimetic TEMPOL resulted in a leftward shift of the BK dose-response curve (Figure 3.5). The enhanced response seen in the CON group in the presence of TEMPOL was a result of an increase in sensitivity (EC_{50}, 3.68±0.78 vs

12.19±1.37 nM) and an increase in AUC (275.0±17.2 vs 191.1±10.2) compared to the no drug condition.

In the presence of TEMPOL, no changes were noted in any hemodynamic variables between the CON and GSH_{10} groups (Table 3). In addition, in the presence of TEMPOL there were no differences in the sensitivity to BK (EC_{50}, 3.68±0.78 vs 3.51±0.47 nM), the AUC (275.0±17.2 vs 286.3±10.4), or maximal dilation (95.6±2.0 vs 99.2±1.8%, Figures 3.3 and 3.5) between the CON and GSH_{10} groups. Furthermore, TEMPOL did not alter the response to SNP in either the CON or GSH_{10} group (Appendix Figure A5). The fact that TEMPOL had a pronounced effect in the CON group, yet failed to augment the response in the GSH_{10} suggests that there is likely a reactive oxygen species buffering component responsible for the GSH_{10} effect on BK mediated dilation that is superseded in the presence of excess TEMPOL. It is noteworthy that physiological GSH (10 μM) is quantitatively as effective as excess TEMPOL in augmenting the responses to BK.

The effects of NOS inhibition and ROS scavenging on the effects of GSH administration

Co-treatment with LNAME+TEMPOL resulted in an increase in CVR in CON which was not reduced by GSH_{10} under these conditions (Table 3.3). In the CON group, the presence of LNAME+TEMPOL resulted in reduced sensitivity (EC_{50}=21.38±10.17nM) compared to the no drug condition (12.19±1.37 nM), and slightly enhanced sensitivity compared to the LNAME only group (30.01±6.94 nM). The area under the curve and maximal dilation were not different from the control no drug condition (Figure 3.3). Taken together these results demonstrated that LNAME+TEMPOL abolished the increased sensitivity seen in the presence of TEMPOL alone, suggesting that the observed effect of TEMPOL is NO production-dependent.

In the GSH_{10} group, the presence of LNAME+TEMPOL resulted in a significant increase in the EC_{50} compared to the GSH_{10} no drug condition (20.77±7.69 vs 2.93±1.09 nM). This is in contrast to the

effect of GSH_{10}+LNAME (11.38±3.33 nM) which did not alter the EC_{50} compared to the GSH_{10} no drug. Thus in the presence of GSH_{10}, LNAME+TEMPOL was able to augment the sensitivity where as LNAME alone was not. There was a significant decrease in the AUC in the GSH_{10} group in the presence of LNAME+TEMPOL (170.57±13.86) compared to the GSH_{10} no drug (264.4±13.8) and GSH_{10} LNAME (211.8±11.9). No changes were observed in maximal dilation amongst these groups (Figure 3.3).

In the presence of LNAME+TEMPOL the curve characteristics were similar between the GSH_{10} and the CON; EC_{50} (20.77±7.69 vs 21.38±10.17 nM), AUC (170.0±13.9 vs 182.3±12.7) and maximal dilation (92.8±1.0 vs 95.2±0.5%).

Table 3.3. Hemodynamics in the presence of TEMPOL and LNAME and TEMPOL

	CON		GSH_{10}	
	TEMPOL	LNAME+TEMPOL	TEMPOL	LNAME+TEMPOL
CVR (mmHg÷(ml×min^{-1}))	5.25 ± 0.35	10.74 ± 1.88*	5.76 ± 0.44	10.68 ± 0.78*
Heart rate (beats×min^{-1})	228 ± 12	258 ± 13	238 ± 7	248 ± 9
LVDP (mmHg)	110 ± 5	130 ± 3	106 ± 5	102 ± 12
+dP/dt (mmHg×sec^{-1})	3793 ± 242	4677 ± 224	3576 ± 153	3709 ± 408

Data are presented as mean±SE (n=3-10/group). For details on specific comparisons refer to text. CVR; coronary vascular resistance, LVDP; left ventricular developed pressure, +dP/dt; maximal rate of pressure development. *, $p<0.05$ vs respective TEMPOL

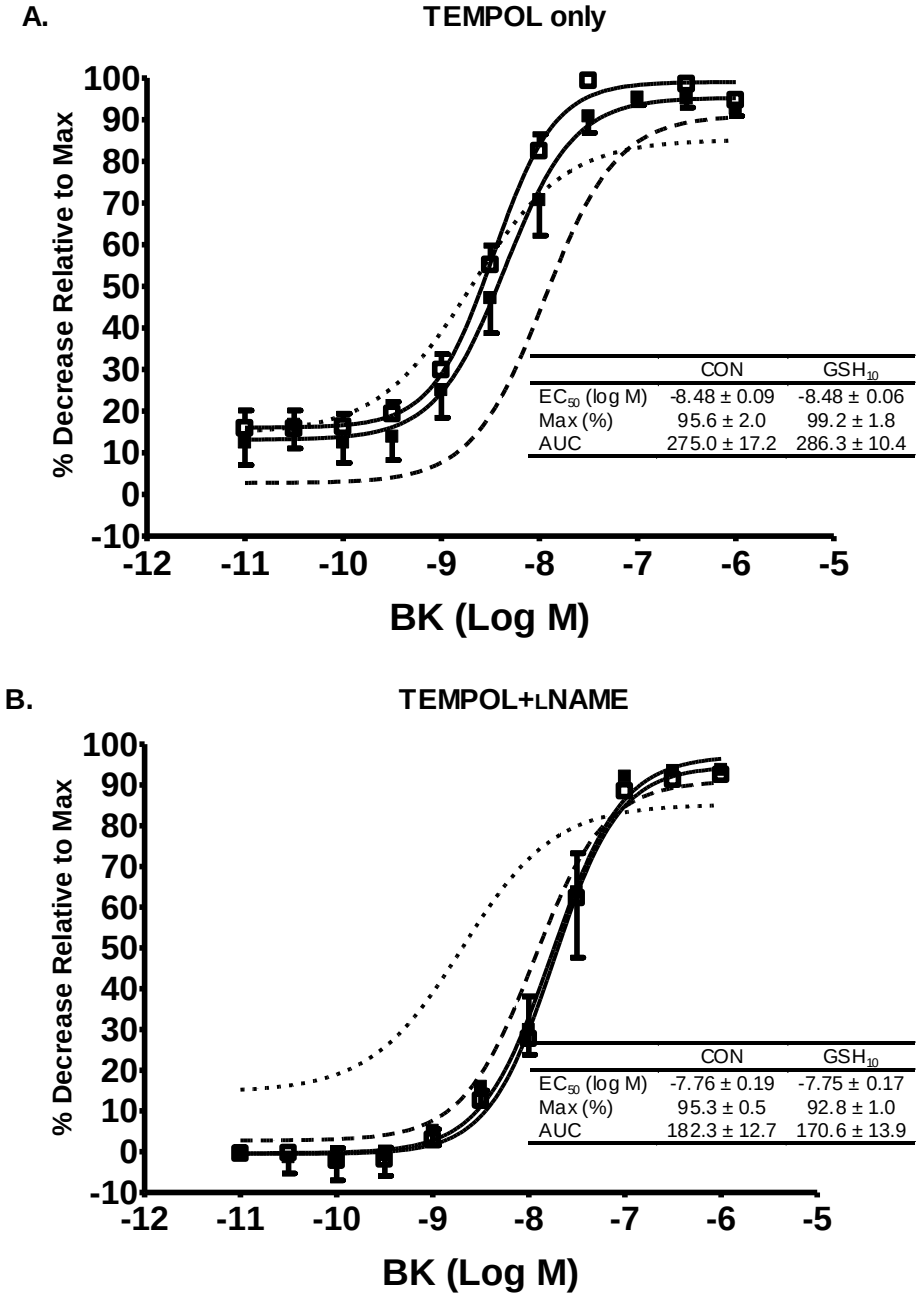

Figure 3.5. Endothelium dependent dilation in the presence of TEMPOL

Hearts were perfused with TEMPOL (0.1 mM) (A) or TEMPOL (0.1mM)+LNAME (0.1 mM) (B). TEMPOL increased the sensitivity to BK in the control group (■) compared to the respective no drug condition but not the GSH_{10} group (□). LNAME induced a rightward shift in equally in both groups. The no drug curves from the CON (long dash) and GSH_{10} (short dash) are included for clarity. Data are mean±SE (n=3-10/group).

3.4.7 Series 3 Effect of GSH administration on ACh-mediated responses in isolated aortic rings

There were no differences in precontraction to 0.1 µM phenylephrine between CON and GSH_{10} groups (69.1±2.7% vs 72.2±2.2%, percent of KCl contraction). Dose-response curves generated using the endothelial agonist ACh, revealed no differences between CON and GSH_{10} groups in sensitivity, AUC, or maximal dilation (Figure 3.6). This lack of GSH_{10} effect on isolated conduit artery suggests the effects of GSH on endothelium-dependent responses observed in the isolated perfused heart likely reflect changes that occur in the *resistance* vasculature of the heart.

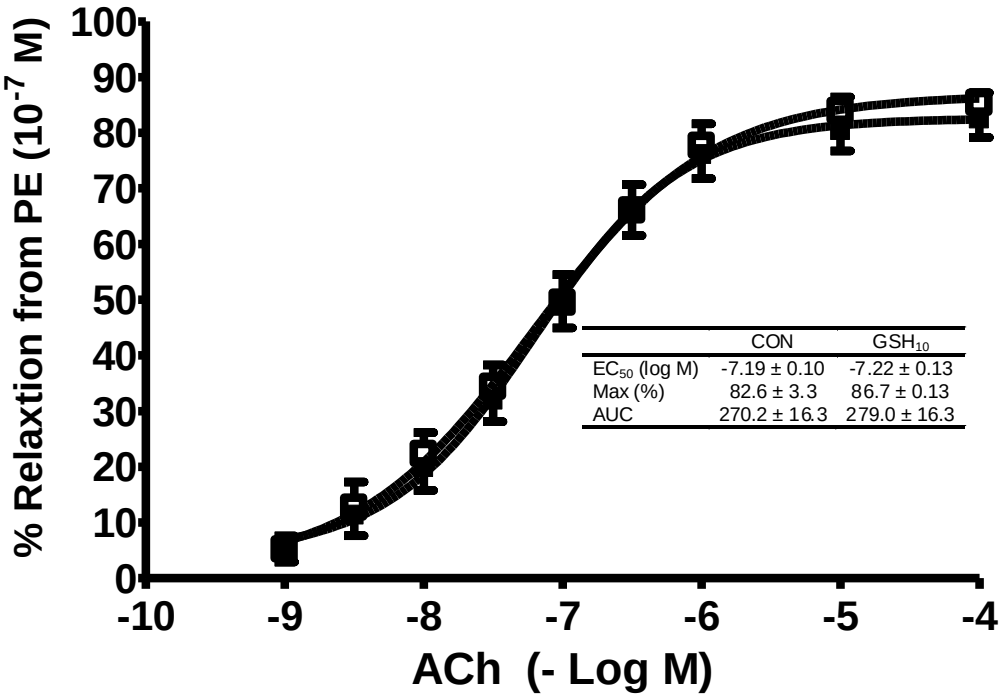

Figure 3.6. Effect of 10 µM GSH on endothelium dependent dilation in isolated aortic segments
Rings were precontracted with phenylephrine (10^{-7} M) and dose dependently dilated with ACh. No differences were seen in the control group (■) compared GSH_{10} group (□). Data are presented as mean±SE (n=7/group).

3.5 Discussion

The primary findings of this study are that exogenous glutathione, at a physiological concentration that does not alter basal CVR, enhances BK-mediated dilation of the intact coronary vascular bed in isolated perfused hearts in a manner that is dependent on reactive oxygen species. Furthermore, the effects of GSH on endothelium-mediated dilation appear to be independent of NO production or NO signalling mediated by sGC. The results we obtained in both the isolated perfused heart and conduit vasculature suggest that the mechanism of the ROS-dependent effects of physiologically relevant GSH infusion may occur in the coronary resistance vasculature.

Contrary to our hypothesis, the GSH_{10}-dependent improvement in BK-mediated dilation observed in the current investigation does not appear to be mediated through the ability of NO to be produced, or the activation of sGC, as demonstrated by the enhanced sensitivity and increased AUC in the GSH_{10} group that persists despite LNAME and/or ODQ. In the *control group*, the presence of the SOD mimetic TEMPOL was sufficient to augment the EC_{50} of the LNAME curve so that it was not different from the no drug condition. However, the *GSH_{10}* LNAME+TEMPOL and the CON LNAME+TEMPOL groups had similar baseline CVR and BK dose-response curve characteristics. The baseline CVR observed in the presence of LNAME+TEMPOL in the GSH_{10} group was greater than that observed in the presence of LNAME alone, and the GSH_{10} LNAME+TEMPOL group had an increased EC_{50} and reduced AUC of the BK dose-response curve compared to the GSH_{10} LNAME condition. These results suggest that the scavenging of ROS by TEMPOL, abolishes the GSH effects.

The effects we observed on CVR in the GSH_{10} group with LNAME vs LNAME+TEMPOL are intriguing as we did not observe any basal effects on CVR in response to GSH_{10} in the LNAME+TEMPOL group. The blunted LNAME-induced increase in CVR in the presence of GSH suggests that, unlike TEMPOL, GSH is not merely scavenging ROS and improving NO bioavailability. If this were the case then the CVR in the presence of LNAME would be similar in the GSH_{10} group compared to the control.

It is possible that in the presence of increased CVR, as a result of LNAME, the GSH itself could be inducing a direct vasodilatory effect (as occurs under normal CVR conditions at higher [GSH] as demonstrated in series 1 experiment). The fact that, in the presence of LNAME+TEMPOL, CVR in the GSH_{10} group was similar to that in the CON group suggests that when ROS are quenched (by TEMPOL), the protective/dilatory effects of GSH are abolished. This is supported by reported findings using a similar preparation in which the dilatory effects of GSH alone (no additional endothelium-dependent agents present) were abolished in the presence of exogenous SOD (33)). Thus, it is likely that the ROS management characteristics of GSH allow this molecule to enhance endothelium-mediated vasomotor function and therefore control changes in CVR observed in the current study, independent of NO bioavailability.

A reduction in NO-bioavailability as a result of increased reactive oxygen species is a hallmark of cardiovascular disease (26, 145). Given the importance of NO in mediating vasodilatation in the coronary circulation (98, 110), a reduction in oxidative stress should enhance NO bioavailability. Our study clearly demonstrated that the addition of either GSH or TEMPOL separately increased the sensitivity to the endothelial agonist BK. In the current study, we thus report a relevant and novel physiological role of GSH in mediating endothelium-dependent control of the coronary vasculature that was reactive oxygen species-dependent.

In previous human studies (103, 104, 138), GSH effects were most pronounced in individuals with pre-existing endothelial dysfunction. Here we demonstrate that the GSH is able to enhance endothelial function in normal animals, but that the enhanced function was not further augmented in the presence of ROS scavenging. Given that endothelial dysfunction is associated with increased levels of reactive oxygen species (26, 60, 145) and that the GSH effects were more apparent in the presence of reactive oxygen species and endothelial dysfunction, this suggests that the effects of GSH are likely mediated in part by its antioxidant-dependent effects. In the current study, had GSH been acting

solely to increase NO bioavailability through ROS scavenging, then in the presence of LNAME we would have demonstrated a similar blunting of the BK dose-response curve in our CON and GSH_{10} groups. However, this was not the case, as the BK curve remained enhanced in the GSH_{10} group despite NOS inhibition and/or sGC inhibition. This further suggests that the presence of GSH is not merely acting as an exogenous antioxidant protecting NO bioavailability with respect to the findings of the current study.

In agreement with our observations from aortic segments from healthy animals, Akapffiong and Taylor (5) and Jia and Fruchgott (92) failed to demonstrate an effect of GSH on ACh-mediated responses in thoracic aortic segments. Interestingly, in the SHR animals which were shown to have endothelial dysfunction, likely mediated in part by increased ROS, 1 μM GSH enhanced the ACh mediated response (5). These findings suggest the requirement of ROS for GSH to augment endothelium-dependent dilation.

In the isolated aorta preparation it is necessary to pre-contract the tissue (with PE) in order to then evaluate vasorelaxation effects, unlike in the isolated perfused heart in which spontaneous tone is present. The experiments in the isolated perfused heart demonstrated that GSH was without effect on spontaneous tone. Similarly, GSH_{10} did not alter the amount of contractile tone obtained with PE in the isolated vascular preparation. The effect of GSH on endothelium-dependent dilation as result of NO production was evaluated using BK in the heart preparation and ACh in the aortic preparation; both agents result in elevations in endothelial cell cytosolic calcium and the consequent activation of eNOS and other pathways (125, 180). Based on our observations of the effects of GSH observed in the intact coronary bed, but not in the isolated conduit artery preparation, we postulate that the effects of GSH administration are possibly more pronounced in the resistance portion of vascular beds, and in the presence of ROS.

During our protocol, no increase in left ventricular GSH occurred, and the GSH_{10} was without effect on cardiac function, similar to the results of Cheung and Schulz (33). This would suggest that the observed enhancement of BK mediated vasodilation/drop in CVR in the presence of GSH is a direct vascular effect. Furthermore, the endothelium-dependent effects we observed in the presence of GSH are likely not simply related to the interaction between GSH and NO, and do not reflect the ability of the vascular smooth muscle to either utilize NO, or dilate to adenosine.

While our results suggest that the GSH-dependent effects on BK-mediated dilation are mediated primarily through a ROS-dependent pathway, it is possible that GSH acts through a separate vasodilatory pathway, independently of ROS or NO. For instance, bradykinin and other endothelial agonists can cause dilation through cyclooxygenase (COX)-derived prostaglandins including prostacyclin (78). However, Cheung and Schulz (33) demonstrated that the vasodilatory effects of GSH alone (in the absence of endothelial agonists) are independent of COX. Furthermore, preliminary data from our laboratory demonstrate that inhibition of the COX pathway with indomethacin does not alter BK-mediated vasodilation/decline in CVR in isolated perfused rat hearts (Appendix Figure A6). Thus, in the current study it is unlikely that GSH effects are mediated by COX-derived vasodilators yet the lack of abolishment of dilation in the presence of LNAME and/or ODQ further suggests that a non-NO, non-prostanoid dilator is active in the isolated perfused heart. It is this pathway that may be altered by GSH infusion. It can be speculated that the GSH_{10} enhancement in BK-mediated dilation may relate to increased Ca^{2+} uptake in the vascular smooth muscle. This is consistent with the limited existing functional and biochemical evidence (3), demonstrating that this process is a ROS-dependent process, requiring the gluathiolation and increased the activity of vascular smooth muscle sarco(endo)plasmic reticulum Ca^{2+} ATP-ase (3). It is also possible that the presence of GSH was sufficient to alter the redox status through its antioxidant effects (103) and therefore increase open probability of smooth muscle K^+ channels (115) or increase the utilization of nitrosothiols which can act on K^+ channels and have

been suggested to be a source of endothelial derived hyperpolarizing factor in the coronary circulation (14-16). Further experiments specifically designed to test hypotheses related to the involvement of these or other mechanisms will greatly enhance the understanding of the importance of GSH to vascular function in health and disease.

In conclusion, our results demonstrate that physiologically relevant concentrations of extracellular GSH can significantly augment endothelium-mediated responses in the isolated perfused rat heart We identified, for the first time, that acute GSH enhanced BK-mediated endothelium-dependent dilation through a pathway requiring ROS, but not dependent on NO production or activation of sGC. By minimizing ROS through the addition of TEMPOL we were able to abolish the enhancement seen in the presence of NOS inhibition. These results extend previous findings from both animal and human studies by identifying a novel pathway whereby GSH enhances endothelium-dependent dilation. We speculate that acute GSH administration enhances endothelium-mediated vasomotor responses through a direct vascular effect, by interacting with endogenous ROS management in the microcirculation.

3.6 Acknowledgements

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CHAPTER 4

Effect of thiol modulation on coronary vascular resistance and endothelium-dependent dilation in the isolated perfused heart

4.1 Synopsis

Despite recent advances in the understanding of the role of thiols in vascular health, the effect that acute modulation of reduced glutathione (GSH): oxidized glutathione (GSSG) ratio (GSH:GSSG) has on endothelial intact preparations has not been studied extensively. The purpose of the current study was to examine how acute manipulations of GSH:GSSG ratio would impact coronary vascular resistance and endothelial-mediated dilation in the isolated perfused heart. Hearts were perfused with diamide, bis-chloronitrosourea (BCNU), ethacrynic acid (EA), or 1-chloro-2,4-dinitrobenzene (CDNB) in order to alter GSH:GSSG ratio. We found that there was a significant inverse correlation between GSH:GSSG and baseline coronary vascular resistance (CVR, $r=-0.55$, $p<0.05$) such that the lower the GSH:GSSG the higher the baseline vascular tone. Endothelium-dependent dilation was preserved in the presence of BCNU and dimaide but markedly attenuated in the presence of CDNB and EA. Despite this differential response we found a significant inverse correlation between GSH:GSSG and minimal CVR ($r=-0.58$, $p<0.05$) suggesting that the lower GSH:GSSG, the higher the minimal CVR. The BK mediated response seen in the presence of CDNB was bi-phasic and demonstrated early onset of dilation followed by a recontraction. EA resulted in an overall reduction in maximal dilation and sensitivity to BK. The results of the current studies demonstrate that alterations in the GSH:GSSG ratio affect baseline CVR and minimal CVR and therefore have implications for adequate tissue perfusion both dependent and independent on the ability of the endothelium to modulate tone.

4.2 Introduction

Regulation of intracellular thiols plays an integral role in the suppression of reactive oxygen species (ROS) and maintenance of vascular health (65, 146, 160). Population based studies have also demonstrated that changes in the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) glutathione, is a potential predictor of cardiovascular events (12). Clinical findings indicate that the GSH:GSSG is lower in patients with chronic kidney disease (9), diabetes mellitus (42), and aging (114). Collectively, these findings suggest that altered redox signalling is involved in several different disease states, and may be related to altered thiol antioxidant capacity. These relationships have been established based mostly on studies related to chronic alterations, but few studies have examined how an acute change in intracellular thiols impacts endothelium-intact vasomotor function.

Given the association between NO and cardiovascular disease (125), it could be postulated that alterations in GSH:GSSG would alter NO production and endothelium-dependent vascular function, and thus may be an important early determinant in cardiovascular disease development. There are several lines of evidence which suggest that acute alterations in the GSH:GSSG ratio have implications for the generation and production of NO. Isolated endothelial cells have reduced NO production (72, 132) and cGMP accumulation (87) following thiol depletion. Similarly, alterations in thiol metabolizing proteins including thioredoxin and glutathione reductase are also associated with impaired NO production (164). These studies indicate that in isolated cellular preparations, manipulation of intracellular thiols impairs NO production. The loss of NO production as a result of thiol depletion is a potential mechanism of altered endothelial dependent dilation in the presence of thiol oxidation (2). There is, however, a limited amount of data which has examined how acute reduction in the GSH:GSSG impacts endothelium-dependent dilation and whether or not the responses are related to NO production.

In the absence of the endothelium, thiol oxidization has been shown to promote a reduced contractile state in isolated coronary vessels (79, 80, 88). For example diamide resulted in a dose-dependent dilation, which was further enhanced through the inhibition of glutathione reductase (GR) and mediated by altered Ca^{2+} entry into the vascular smooth muscle through the closure of L-type Ca^{2+} channels (88). Furthermore, hypoxia promotes an oxidized state, and it appears that the hypoxic coronary dilation is mediated by increases in the NADP:NADPH which increases the GSSG:GSH ratio and promotes the opening of K^+ channels and altered Ca^{2+} handling by the smooth muscle (190). It is evident that thiol depletion alters coronary vascular tone in the absence of the endothelium; however the modulatory effects of the endothelium on vascular tone in response to thiol depletion has not been assessed.

There is growing evidence to suggest that alteration in GSH:GSSG, and acute changes in intracellular thiols can have an impact on NO bioavailability and vasomotor function. However, there are a limited number of studies which have examined how acute changes in intracellular thiols, in particular GSH, impact endothelium dependent vasomotion. Given the long term associations of altered GSH:GSSG seen in vasomotor dysfunction, it is important to examine the extent to which acute modulations of GSH:GSSG impact vasomotor tone and function to provide insight into the potential mechanisms underlying the relationship between GSH:GSSG and vasomotor function. The purpose of the current investigation was to examine the extent to which alterations in the GSH:GSSG ratio impact coronary vascular resistance and endothelium-dependent dilation in the isolated perfused heart. Several different agents designed to manipulate the GSH:GSSG via divergent mechanisms were employed to study the effects on endothelium-dependent dilation. Given that reductions in GSH:GSSG are known to impair NO production, it is likely that the extent to which GSH:GSSG is altered will be related to both basal coronary vascular tone and endothelium-dependent dilation.

4.3 Methods

4.3.1 Animals

Male Sprague-Dawely rats (n=172) weighing approximately 300g (mean age 10 weeks) were used in the current experiment. Animals were group housed (4-5/cage) in a temperature and humidity controlled environment, and kept on a reverse 12 hour light/dark cycle. Food and water were provided ad libitum. All procedures were approved by the University of Waterloo Animal Care Committee.

4.3.2 Langendorff Heart

Hearts were prepared for the Langendorff procedure. Briefly, animals were anaesthetized with 60 mg/kg of pentobarbital sodium (I.P.), the heart was removed and immersed immediately in 4^{o}C Krebs Henseleit solution (KHS, composition in mmol/L NaCl 118, KCl 4.7, $MgSO_4 \cdot 7H_2O$ 1.2, $NaHCO_3$ 24, KH_2PO_4 1.1, Glucose 10 and $CaCl_2 \cdot 2H_2O$ 1.25). Hearts were then transferred to the aortic cannula which was dripping gassed (95%O_2-5%CO_2) 37^{o}C KHS, secured with suture, and cleaned of adherent tissue. The time from excision to the establishment of flow was less than 1 minute, and the time until the heart was at the '*in-vivo'* flow was approximately 2.5 minutes.

We utilized a constant flow protocol as this is best suited for the study of vasoactive agents because the constant flow rate allows for a constant drug infusion rate (50, 166). The constant flow rate was assigned to mimic *in-vivo* coronary flow using the following equation: Flow = 7.43 × ((body weight×0.0027+0.6)$^{0.56}$) (45, 50). A flow probe and associated transducer (2PXL and TS410, Transonic Systems, Ithaca NY), and a pressure transducer (MLT844, AD Instruments, Boulder Co) were positioned proximal to the aortic cannula for the measurement of total coronary flow, and coronary perfusion pressure. A pressure balloon was inserted through the left atria and advanced into the left ventricle and adjusted to achieve a resting diastolic pressure of 5 – 10 mmHg. The balloon was connected to a pressure transducer (MLT844, AD Instruments, Boulder Co). Heart rate, left ventricular

developed pressure (LVDP, left ventricular systolic pressure – left ventricular diastolic pressure) and ±dP/dt were all derived from the left ventricular pressure tracing. All data were collected using a Powerlab data acquisition board (4/sp, AD Instruments, Boulder Co) and stored digitally using Chart for Windows (5.5.1, AD Instruments, Boulder Co) for off-line analysis.

A 30 minute stabilization period was included to allow for the development of spontaneous coronary vascular tone and heart rate. Hearts which failed to establish spontaneous tone were excluded from the study. Once the perfusion pressure had stabilized, baseline measurements including coronary vascular resistance (CVR, perfusion pressure ÷ flow), heart rate, and left ventricular developed pressure ±dP/dt) were made continuously and data points were extracted by taking an average over 1 minute.

4.3.4 Assessment of vasomotor function

Following the stabilization period, hearts were exposed to increasing concentrations of bradykinin BK (10^{-12} – 10^{-6} M). After the final concentration of BK, adenosine (ADO 10^{-5} M) was administered to assess minimal CVR. Endothelium-independent dilation to exogenous NO was assessed by SNP (10^{-10} – 10^{-4} M). For each agent a total volume of 250 µl was infused at a rate of 1% of the coronary flow using a syringe pump (Cole Parmer 74900, Montreal PQ) to yield the final desired concentration. Only 1 curve was generated per heart in order to limit potential time and order effects of the different agonists.

The following agents were used to alter thiol status: diamide (1 or 10 µM), a thiol oxidizing agent; bis-chloronitrosourea (BCNU 10 or 100 µM), a glutathione reductase inhibitor; ethacrynic acid (EA, 10 µM), a thiol alkylating agent; or 1-chloro-2,4-dinitrobenzene (CDNB, 20 µM), a thioredoxin inhibitor. No differences between 10 or 100 µM BCNU were observed for GSH:GSSG therefore the data obtained from the two concentrations was combined into one group (BCNU).

L-$_{\omega}$Nitro-L-arginine (LNAME, 10^{-4} M) was used to inhibit nitric oxide synthase, the superoxide dismutase (SOD) mimetic 4-Hydroxy TEMPO (TEMPOL, 10^{-4} M) was used as a non-thiol exogenous antioxidant, and the preferential COX-1 inhibitor (SC560 3×10^{-7} M) and the preferential COX-2 inhibitor (NS398, 10^{-6} M) were used to assess the potential role of cyclooxygenase-derived products in the responses. The group name 'no drug' refers to the condition in the presence or absence of an indicated thiol manipulating agent, but in the absence of any other pharmacological agents (LNAME, TEMPOL, SC560, and NS398).

Changes in coronary vascular resistance were reported as a percentage of maximal decrease in CVR induced by ADO, by using the following equation: $[((CVR_{BL} - CVR_A) - (CVR_{BL} - CVR_c)) \div (CVR_{BL}-CVR_A)]\times100$. Where CVR_{BL} refers to baseline CVR, CVR_A refers to minimal CVR elicited by ADO and CVR_C refers to the CVR at the concentration of interest. Curves were fit to obtain EC_{50}, maximal dilation and area under the curve (AUC) using Prism (4.03, GraphPad Software, San Diego CA). Total dilatory capacity (TDC) was determined by examining the total change in coronary vascular resistance ($CVR_{BL}-CVR_A$).

4.3.5 Measurement of left ventricular glutathione content and reactive oxygen species

Following the experimental protocol, hearts were gently blotted and weighed. A portion of the outer wall of the left ventricle was snap frozen in liquid nitrogen. Glutathione was assessed using HPLC as previously described by our laboratory (63) according to the method derived by Reed and colleagues (141).

4.3.6 Assessment of reactive oxygen species production

Hydrogen peroxide production was assessed using the Amplex Red Hydrogen Peroxide kit (Molecular Probes, Invitrogen, Carlsbad, CA) as previously described by us (147). Diluted left ventricular homogenate was added to 50µl of Amplex Red reagent (10µM Amplex Red stock and

0.2U/ml horseradish peroxidase in PBS) (51) and incubated in the dark at room temperature for 30min. Fluorescence was detected every 15 minutes using at 530/590nm excitation/emission wavelengths. H_2O_2 concentrations were calculated using a H_2O_2 standard curve ($R^2 \geq 0.998$). Tissue concentrations are expressed relative to μg protein as determined from a bicinchoninic acid (BCA) protein concentration assay.

General ROS production was measured as previously described by our laboratory (147). The left ventricular homogenate was diluted further in PBS and incubated for 15 minutes in the dark at 37^{o}C with 5 μM of dicholorflouricine diacetate (H_2DCF-DA, Molecular Probes, Invitrogen, Carlsbad, CA). Fluorescence was detected every 15 minutes at excitation/emission wavelengths of 488/525 nm and subsequently normalized to protein content.

For each measure of ROS production the total change in ROS was assessed by calculating the difference between the fluorescent signals at 1 hour from the baseline signal. Thus the values presented represent the increase from initial ROS content.

4.3.7 Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Bioshop Canada (Burlington, ON, Canada). SC560 and NS398 were purchased from Cayman Chemicals (Ann Arbor, MI). All drugs were diluted to their final concentrations in KHS.

4.3.8 Statistics

All data are presented as mean ± standard error (SE). Differences between groups were assessed using ANOVA. Significant results were subsequently analyzed using Least Squares Difference *post hoc* test. Correlational analysis was performed using Prism (4.03, GraphPad Software, San Diego CA). A p value <0.05 was considered statistically significant. All statistics were performed using Statistica 6.0 (StatSoft Inc, Tulsa Ok).

4.4 Results

4.4.1 Animal Characteristics

Baseline characteristics for the animals can be found in Table 4.1. There were no apparent differences in the rat body weights across treatment groups; however, hearts that were treated with CDNB, EA and $Diam_{10}$ had elevated heart weights compared to the control animals (Table 4.1). Acute treatment with all thiol manipulating agents (with the exception of $DIAM_1$) resulted in a lowering of the GSH:GSSG ratio (Table 4.1). Despite the effect that thiol manipulation had on GSH:GSSG, only modest effects were observed on ROS production (Table 4.1).

Table 4.1. Animal Characteristics

	CON	CDNB	EA	BCNU	$DIAM_1$	$DIAM_{10}$
Body Weight (g)	308.01±3.59	312.16±3.91	312.16±4.95	315.25±8.82	316.46±6.04	312.39±5.27
Heart Weight (mg)	1321±17	1568±22*	1378±20*	1333±32	1350±34	1471±26*
GSH (nmol/mg wet weight)	1.223±0.076	0.004±0.001*	0.650±0.124*	1.080±0.082	1.110±0.098	0.732±0.055*
GSSG (nmol/mg wet weight)	0.082±0.010	0.002±0.000*	0.065±0.011	0.093±0.010	0.088±0.013	0.074±0.008
GSH:GSSG	16.01±1.54	2.25±0.64*	10.29±1.80*	11.87±0.52*	12.98±1.04	10.31±0.83*
General ROS (RFU/µg PRO)	44.0±1.9	43.3±2.0	36.3±1.1 *	50.5±2.8 *	58.3±2.1 *	39.6±2.4
H_2O_2 (ρmol/µg PRO)	1.28±0.15	1.41±0.07	1.24±0.06	1.55±0.06 *	1.42±0.06	1.61±0.11 *

Data are presented as mean±SE (n=7-59/group for body and heart weights). Thiols were assessed using HPLC (n=5-10/group). General ROS was measured using DCF fluorescence, H_20_2 was measured using amplex red (n=5-10/group). *,$p<0.05$ vs CON

4.3.2 Effects of acute thiol depletion on cardiac hemodynamics

Acute treatment with the different thiol manipulating agents differentially altered the amount of spontaneous vascular tone developed. Treatment with CDNB, EA and $DIAM_1$ all resulted in greater baseline coronary vascular resistance compared to the control group, while BCNU and $DIAM_{10}$ were

without effect (Table 4.2). Across all treatment groups a significant inverse relationship existed between GSH:GSSG ratio and the baseline CVR ($r = -0.53$, $p<0.05$, Figure 4.1). A significant decrease in left ventricular systolic pressure was observed in all treatment groups compared to the control, and this was associated with a blunted LVDP in all groups (Table 4.2). Treatment with CDNB, and $DIAM_{10}$ resulted in significant blunting of +dP/dt and –dP/dt, whereas BCNU, EA and $DIAM_1$ were without effect (Table 4.2). Correlational analysis revealed significant relationships between GSH:GSSG and HR, LVsysP and +dP/dt (Figure 4.1).

Table 4.2 Baseline Hemodynamics

	Con	CDNB	EA	BCNU	$DIAM_1$	$DIAM_{10}$
CVR ($mmHg/ml\times min^{-1}$)	6.73±0.24	11.46±0.71*	8.61±0.36*	6.13±0.36	9.02±0.65*	6.61±0.27
HR (BPM)	265±11	298±7*	284±7	278±8	273±9	271±13
LVsysP (mmHg)	131±5	94±4*	114±4*	114±5*	114±9*	95±8*
LVDP (mmHg)	122±5	85±5*	106±4*	106±4‡	106±9‡	87±7*
+dP/dt (mmHg/sec)	4053±143	3066±140*	3736±139	3939±127	3681±298	2585±119*
-dP/dt (mmHg/sec)	2405±100	-1690±98 *	2163±67	2141±93	2102±179	1643±123*

Data represent the steady state hemodynamics following 30 minutes of stabilization. CVR coronary vascular resistance; LVsysP, left ventricular systolic pressure; LVDP, left ventricular developed pressure; ±dP/dt, maximal or minimal rate of pressure development; ‡p≤0.058 *p<0.05 vs CON Data are mean±SE (n=7-21/group).

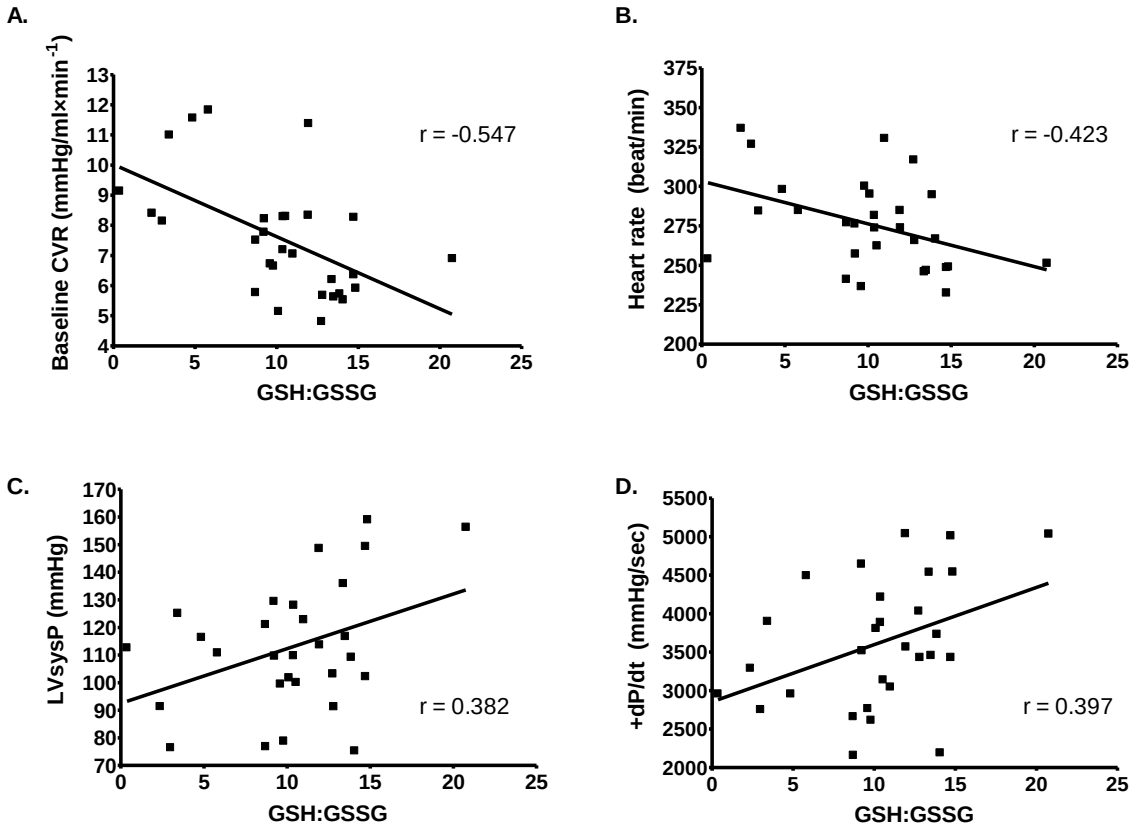

Figure 4.1. Correlational analyses between GSH:GSSG and selected hemodyamic variables
GSH:GSSG was inversely correlated with baseline CVR, and heart rate are significantly correlated with left ventricular systolic pressure and +dP/dt (n=29/graph). All Correlations were significant $p<0.05$.

4.4.3 The mechanism of thiol oxidation has varying affects on BK induced endothelium-dependent dilation.

Following the stabilization measurements, BK dose-response curves were generated for all conditions. BCNU, $DIAM_1$ and $DIAM_{10}$ were all without effect on BK mediated dilation (Table 4.3). Conversely, both CDNB, and EA significantly altered the BK dose-response curve compared to the control group (Figure 4.2). Treatment with CDNB resulted in a slightly earlier onset of dilation yet resulted in a significant redevelopment of vascular tone (reduced dilation) at the higher concentrations of BK. Conversely, EA resulted in a rightward shift and an overall reduction of the BK dose-response curve compared to the controls (Figure 4.2, Table 4.3).

Table 4.3. Curve Characteristics

	CON	CDNB	EA	BCNU	$DIAM_1$	$DIAM_{10}$
Maximal Dilation (%)	97.7±1.6	74.4±1.9 *	90.8±1.8*	96.0±3.4	97.6±1.1	101.7±1.6
EC_{50} (- Log M)	8.42±0.08	8.83±0.28 *	7.85±0.07 *	8.28±0.08	8.21±0.08	8.26±0.03
AUC	261.6±9.0	248.1±14.4	195.6±11.5 *	254.0±7.0	262.4±9.5	250.2±5.1

Data represent mean±SE (n=7-12/group). Curves were fit as described in the methods section.
*p<0.05 vs controls

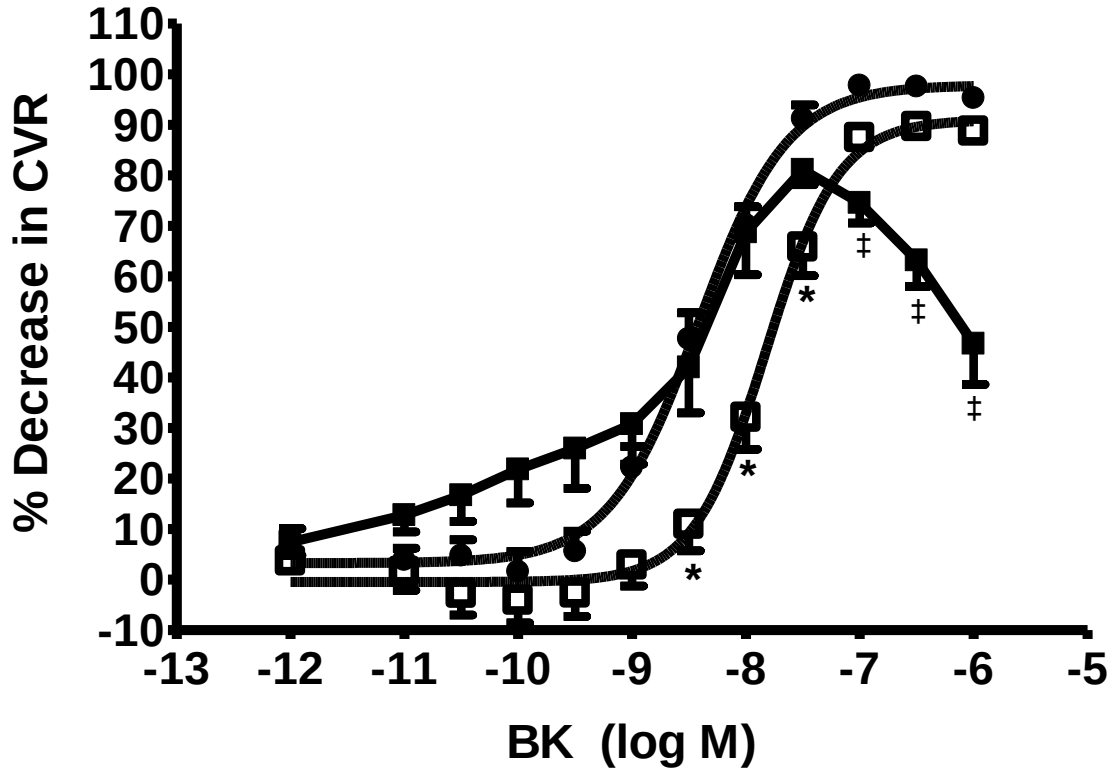

Figure 4.2 Bradykinin dose-response curves
Data are from CON (●), CDNB (■), and EA(□). CDNB elicits an earlier onset of dilation with significant blunting at the top end of the curve. EA results in a generalized blunting of the response. Dose-response curves for BCNU, $DIAM_1$ and $DIAM_{10}$ were excluded for clarity as they overlapped with the control group. Data are presented as mean±SE (n=9-12/group). *, p<0.05 EA vs CON ‡,p<0.05 CDNB vs CON

4.4.4 Thiol modulation alters minimal CVR and total dilatory capacity.

Following the BK dose-response curves all hearts were exposed to adenosine (10^{-5} M) to elicit maximal endothelium-independent dilation in the coronary vascular bed. Adenosine resulted in a significant reduction in CVR in all groups. However, the minimal CVR achieved in the presence of CDNB, EA and $DIAM_1$ was significantly greater than the minimal CVR achieved by the CON group (Figure 4.3a). Despite having greater minimal CVR the total dilatory capacity was also greater in the CDNB, EA and $DIAM_1$ compared to the control (Figure 4.3b) due to the fact that these groups also all had elevated baseline CVR (Table 4.2). There was a significant correlation between the GSH:GSSG ratio and minimal CVR such that the greater the ratio, the lower the minimal CVR ($r=-0.581$, $p<0.05$ Figure 4.4). There was no relationship between thiol status and TDC ($r=-0.305$, $p>0.05$).

4.4.5 Effect of exogenous NO on the dilatory responses in the presence of thiol depletion

Given that we found impaired BK-mediated dilation in the presence of both EA and CDNB we sought to determine if the altered dilation was as a result of altered ability of the smooth muscle to respond to NO. The NO-donor SNP was therefore employed in the presence of these two agents. SNP dose-dependently dilated the coronary vasculature. No differences were observed in maximal dilation, sensitivity or AUC between the groups (Figure 4.5). Thus, the impaired ability to dilate in the presence of CDNB or EA was not related to an altered sensitivity of the vascular smooth muscle to NO.

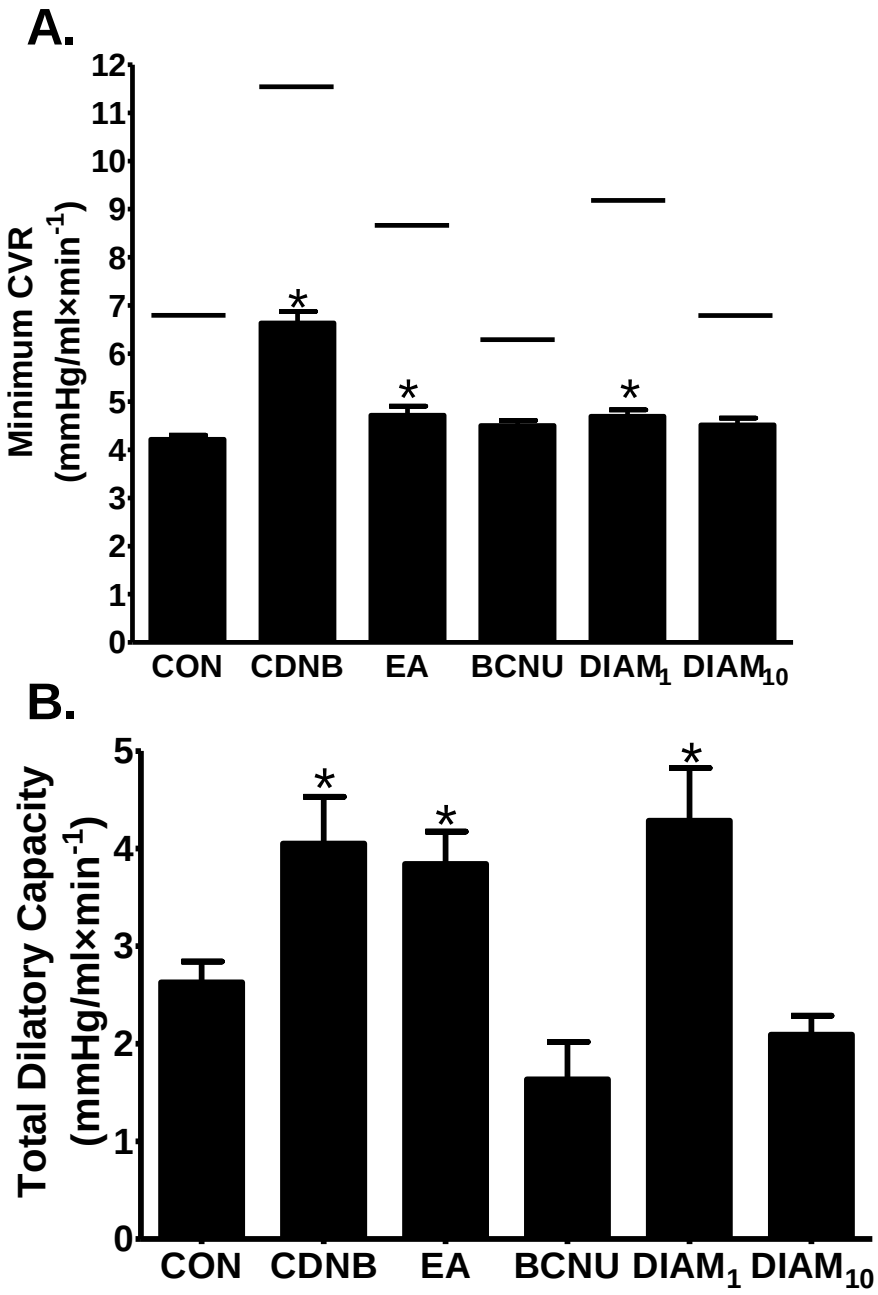

Figure 4.3. Effect of thiol manipulation on minimal CVR and total dilatory capacity

Absolute minimal CVR elicited following administration of adenosine 10 μM (A).Total capacity to dilate as assessed by the change from baseline CVR to absolute minimal CVR elicited by ADO (B). Included in A are the baseline CVR values for each condition demonstrated by the solid line above the minimal CVR. *$p<0.05$ vs CON Data are mean±SE (n=7-21/group).

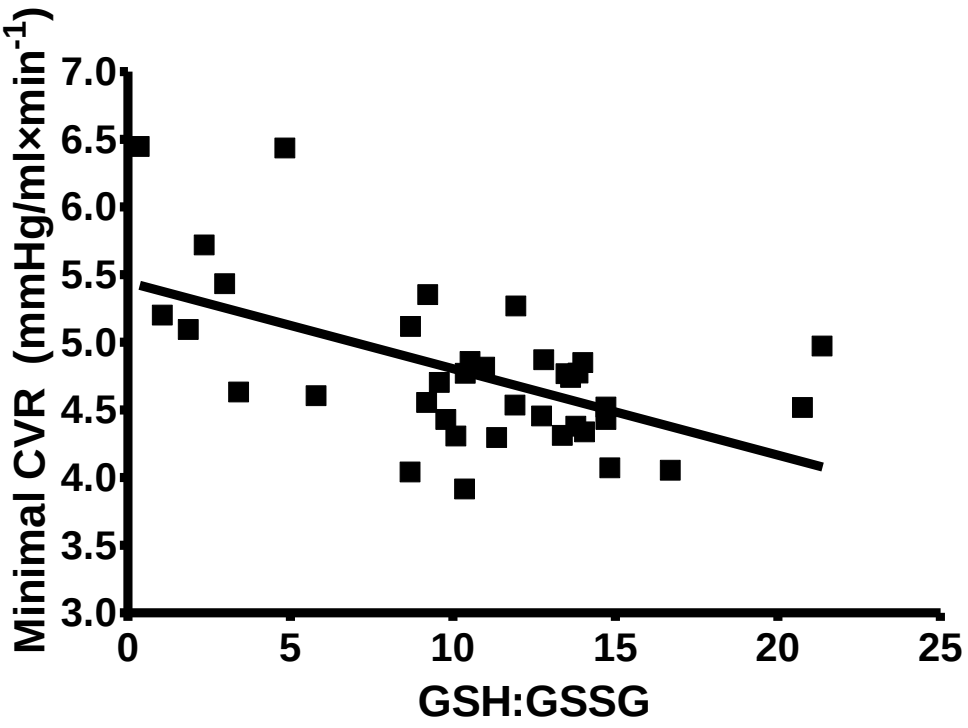

Figure 4.4. Relationship between GSH:GSSG ratio and minimal CVR

An elevated GSH:GSSG ratio is associated with a lower minimal CVR (n=38).

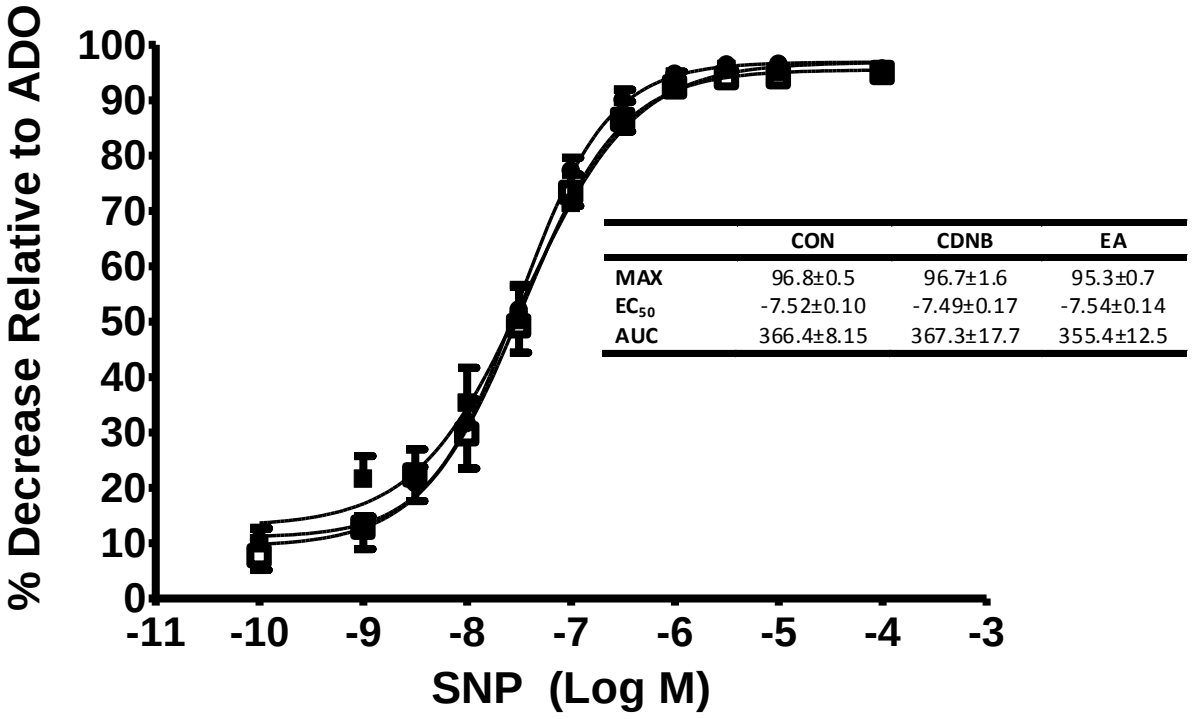

Figure 4.5. Endothelium independent dilation

SNP-induced dilation from the CON (●), CDNB (■), and EA(□). No differences were observed between the 3 groups. Data are presented as mean±SE (n=8-9/group).

4.4.6 Effect of NOS inhibition on BK mediated responses in response to CDNB and EA

In the presence of NOS inhibition, all hearts experienced increases in CVR; however, the CVR achieved by the control group in the presence of LNAME (14.00±0.76 mmHg/ml×min^{-1}) was significantly greater than that of EA+LNAME (10.45±0.95 mmHg/ml×min^{-1}, $p<0.01$), and no differences were seen between CON+LNAME and CDNB+LNAME (13.00±0.64 mmHg/ml×min^{-1}, Figure 4.6).

In the presence of NOS inhibition, all groups experienced a reduction in the endothelium dependent dilation in response to BK (Figure 4.7). Maximal dilation was significantly blunted in the CDNB+LNAME compared to CON+LNAME (17.5±5.6 vs 73.3±7.2%) whereas EA+LNAME (82.6±2.9%) was not. Despite the reduction in overall dilation, redevelopment of tone was still observed in the presence of CDNB+LNAME (Figure 4.7). The AUC was greater in the EA compared to the controls

(152.9±7.2 vs 113.8±10.4). LNAME had a profound effect on the sensitivity (EC_{50}) in the control group (-7.29±0.07 Log M) compared to CDNB (-8.54±0.52 Log M, $p<0.01$) but the EC_{50} in CON+LNAME was not different compared to EA+LNAME (-7.61±0.04 Log M, Figure4.6).

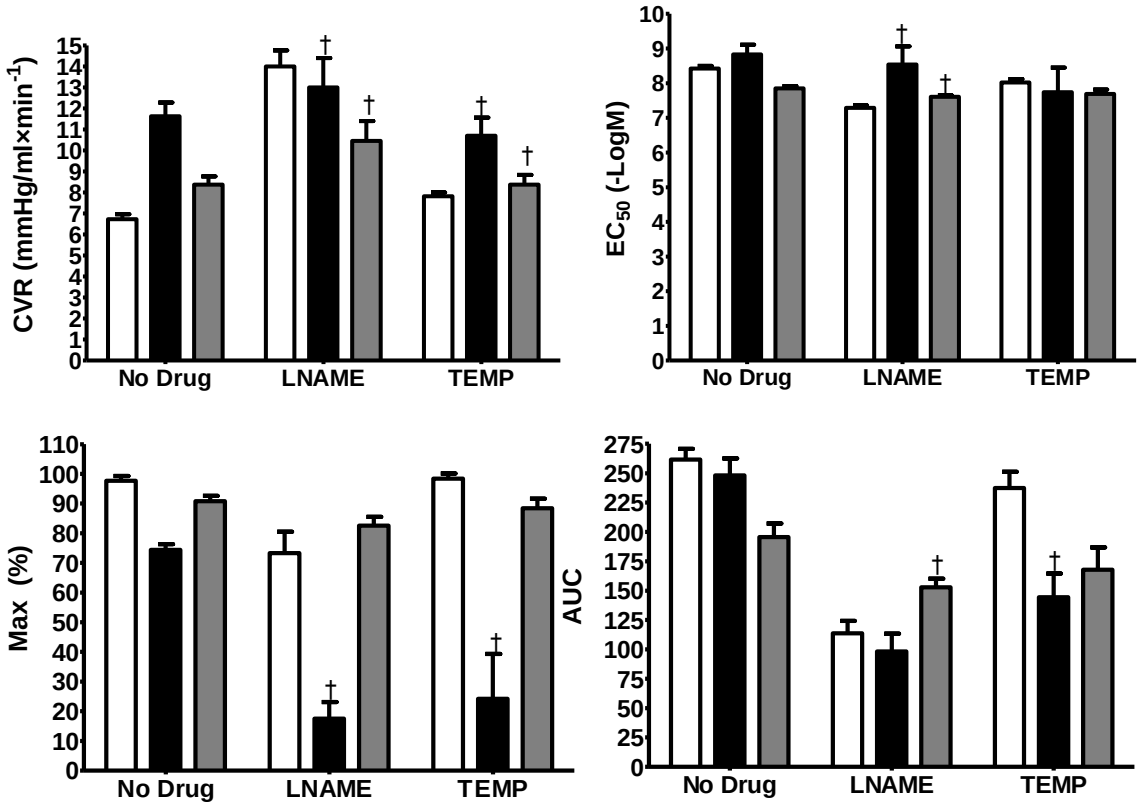

Figure 4.6 Comparison of baseline CVR and curve characteristics in the presence of NOS inhibition and ROS scavenging

Data highlight the effects that LNAME and TEMPOL had on control (white), CDNB (black) and EA (gray). For details on specific comparisons refer to text. †, $p<0.05$ for the relative effectiveness compared to the control. Data were normalized to the respective no drug condition for control (white), CDNB (black) and EA (gray) to allow for differences in the effectiveness of NOS inhibition or ROS scavenging to be compared to the respective control condition in the presence of the same drug. Data are presented as mean±SE (n=7-21/group).

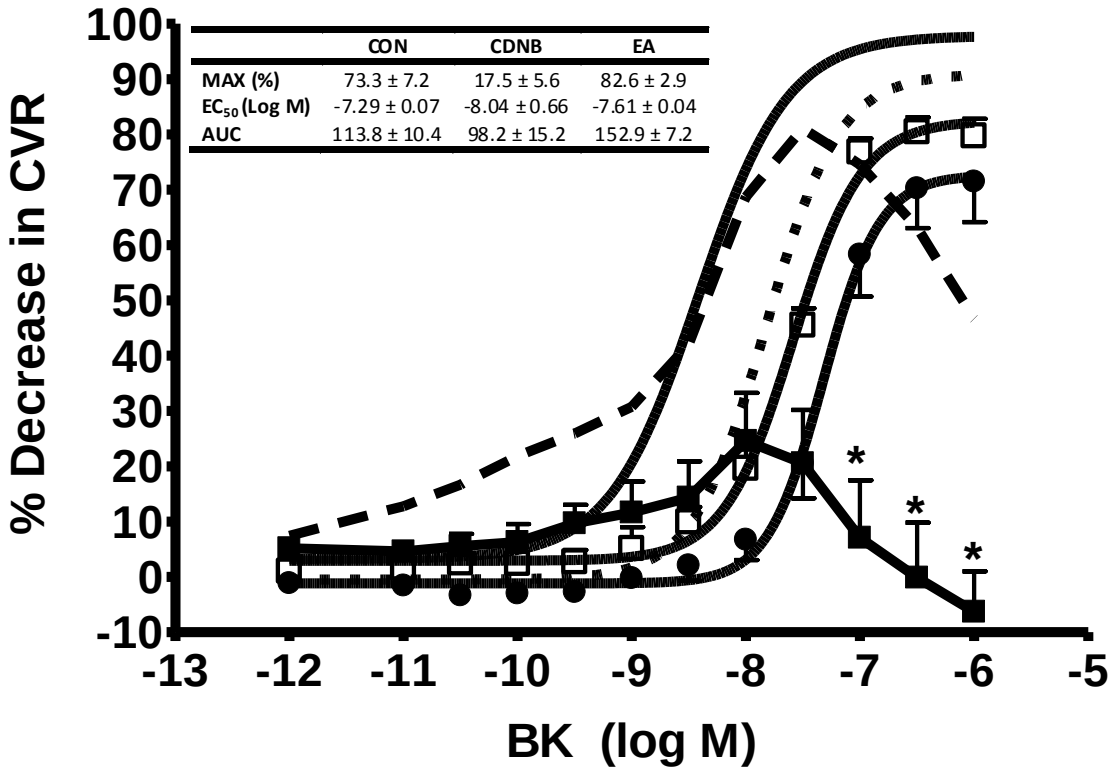

Figure 4.7 Effect of thiol depletion and NOS inhibition on bradykinin mediated dilation

Hearts were allowed to equilibrate for 30 minutes in the presence of LNAME (0.1 mM) followed by dose-response curve to BK. Data are from the CON (●), CDNB (■), and EA(□) in the presence of LNAME. The solid (CON) dashed (CDNB) and dotted (EA) represent the no drug BK mediated responses. Data are presented as mean±SE (n=9-11/group). *, $p<0.05$ vs CON

4.4.7Effect of TEMPOL on the baseline CVR and the BK-dose-response curve

Given that the changes in CVR and EC_{50} in response to LNAME were greatest in the CON we sought to determine if the acute thiol oxidation resulted in an increased ROS-mediated alteration in NO. Therefore, the SOD mimetic TEMPOL was added to the perfusate to scavenge superoxide, thereby improving NO bioavailability. The CVR achieved by CON+TEMPOL (7.82±0.18 mmHg/ml×min^{-1}) was not different than EA+TEMPOL (8.37±0.46 mmHg/ml×min^{-1}), but was less than CDNB+TEMPOL (10.70±0.86 mmHg/ml×min^{-1}, $p<0.01$, Figure 4.6)

The presence of TEMPOL resulted in a rightward shift of the dose-response curve to BK in all groups (Figure 4.8). As such there were no differences in the EC_{50} across all groups, yet the AUC was significantly greater in the CON (237.4±14.0) compared to either the CDNB (144.5±20.1) or EA (167.9±18.9). Furthermore, the maximal dilation of the CDNB (24.2±15.1%) was significantly blunted compared to the CON (98.4±1.7%). What is most intriguing is the enhanced (compared to CDNB no drug) redevelopment of vascular tone at the higher concentrations of BK observed in the presence of CDNB+TEMPOL.

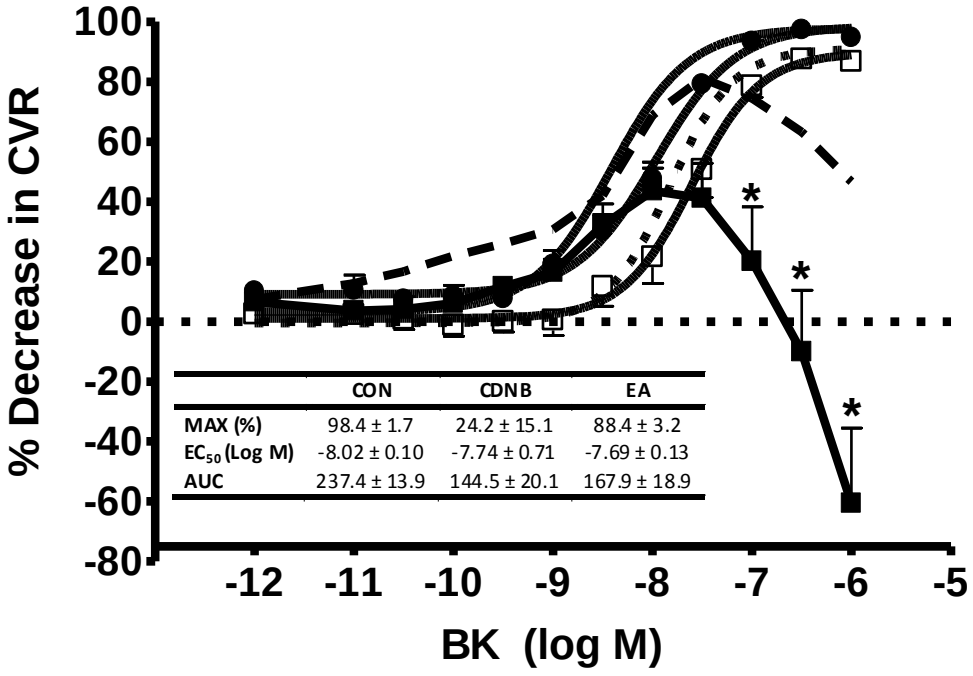

Figure 4.8. Effect of an SOD mimetic on the bradykinin dose-response curves

Hearts were allowed to equilibrate for 30 minutes in the presence of TEMPOL (0.1 mM) followed by dose-response curve to BK. Data are from the CON (●), CDNB (■), and EA(□).The solid (CON) dashed (CDNB) and dotted (EA) represent the no drug BK mediated responses. Data are presented as mean+SE (n=7-8/group). *p<0.05 vs CON.

4.4.8 Is the redevelopment of vascular tone in the CDNB-treated hearts dependent on COX derived prostanoids?

Previous reports from our laboratory and others (47, 48, 73, 75, 169) have demonstrated that COX derived products can mediate endothelium-dependent vasocontractions observed in cardiovascular disease states such as hypertension. The endothelium-dependent responses typically demonstrate enhanced vasodilation at lower agonist concentrations followed by recontraction at higher agonist concentrations (48, 75). Therefore, we wanted to assess whether the recontraction seen in the presence of thioredoxin inhibition could be accounted for by COX derived prostanoids acting as endothelial derived contracting factors (EDCF). In the initial series of experiments, we utilized the non-specific COX inhibitor indomethacin (INDO 10^{-5}M). In the presence of INDO, there was no significant alteration in the dilation seen in the control group to either BK or ADO vs the respective no drug condition. In the presence of both INDO and CDNB, the vasculature failed to respond to either the endothelium-dependent or -independent agonists (Appendix Figure A7, n=6/group). In light of this unexpected finding we chose to examine the effects of the selective COX inhibitors in the CON and CDNB groups.

In the presence of either COX-1 or COX-2 inhibition there remained a significant redevelopment of vascular tone such that the max in the presence of SC560 was blunted in the CDNB compared to the control (68.6±5.7 vs 100.9±1.7), as was the max in the presence of NS398 (40.5±10.7 vs 99.9±2.5). This was associated with an increased AUC in the controls in the presence of SC560 compared to the CDNB (290.0±9.7 vs 160.8±21.0) and NS398 (156.9±21.0). No differences were observed in the EC_{50} between the CON and CDNB in the presence of either SC560 or NS398 (Figure 4.9). These results suggest that COX derived prostanoids are not responsible for the redevelopment of vascular tone in the presence of CDNB. This finding was further highlighted by combining SC560 and

NS398 with CDNB; much like the control group in the presence of INDO, hearts treated with CDNB+SC560+NS398 responded similar to the CDNB no drug condition (Appendix Figure A6 n=6).

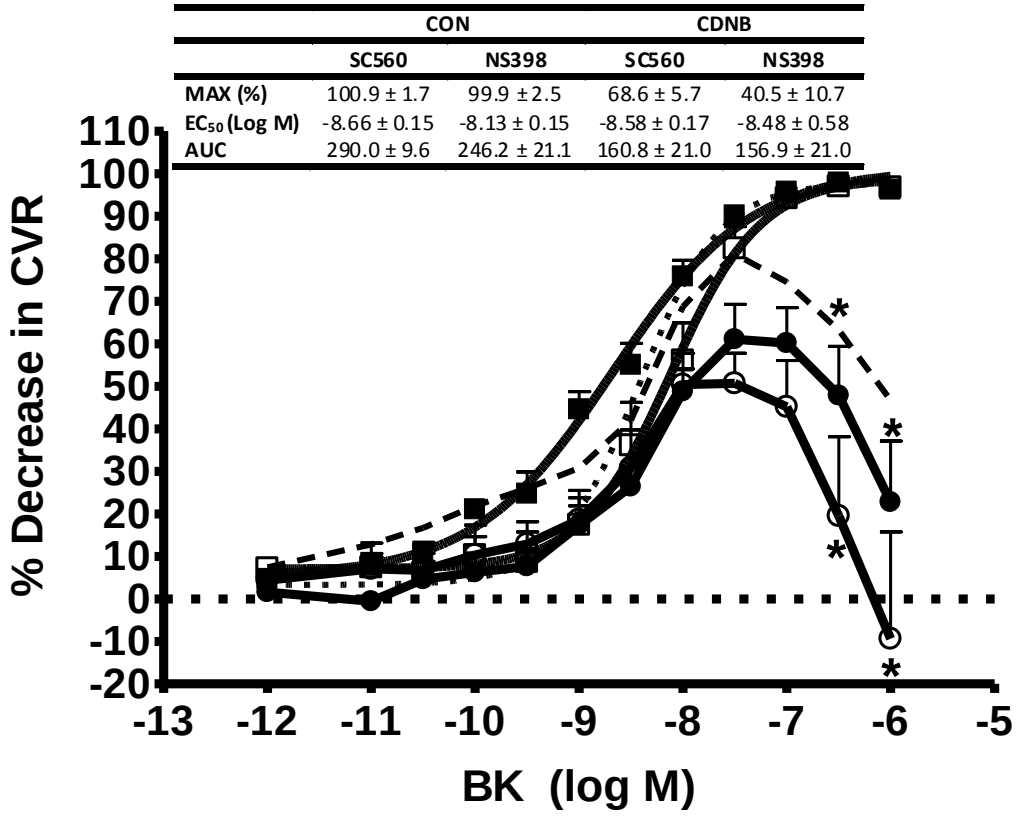

Figure 4.9. Effect of specific COX inhibitors on BK dose-response curve

Hearts were allowed to stabilize for 30 minutes with either the COX-1 inhibitor SC560 (3×10^{-7}) or the COX-2 inhibitor NS398 (10^{-6} M) followed by a dose-response curve to BK. Data are presented as mean±SE from CON+SC560 (■) CON+NS398(□) , CDNB+SC560 (●) and CDNB+NS398 (○).*$p<0.05$ vs respective CON. The respective NO drug curve for the CON (dotted) and CDNB (dashed) were included for clarity. Data are presented as mean+SE (n=4-8/group).

4.5 Discussion

It is evident that changes in thiol levels play an integral role in the control of vascular tone and function. We have described a relationship that exists between baseline vascular tone and the GSH:GSSG ratio in the isolated perfused heart. Given the acute nature of the current treatment we can make the following conclusions regarding endothelial-mediated vasomotor function in the intact coronary vascular bed. Depending on the nature of thiol depletion, BK-mediated dilation appears to be unchanged (BCNU, diamide) or is significantly altered as was the case in the presence of thioredoxin inhibition and GSH alkylation. In the presence of CDNB (thioredoxin inhibition) or EA (GSH alkylation), the impairment in vasomotor function does not appear to be mediated by an altered response of the vascular smooth muscle to endogenous NO. Therefore, these two thiol depleting agents affect signalling upstream of sGC. This indicates that the impairment in the response to BK is not indicative of altered target tissue sensitivity suggesting that these agents affect other aspects of NO signalling, production or destruction, or may be independent of NO altogether. More importantly we describe a significant relationship between GSH:GSSG ratio and minimal CVR such that the greater the GSH:GSSG ratio the lower the minimal CVR. This suggests that changes in intracellular thiol status may alter tissue perfusion.

4.5.1 Thiol modulation alters baseline CVR

The inverse relationship between GSH:GSSG and CVR is intriguing and suggests that in the intact coronary vascular bed acute oxidation and/or depletion of thiols results in an elevation of baseline vascular tone. In the current study both the thiol alkylating agent EA, and the thioredoxin inhibitor, CDNB resulted in a significant elevation of baseline coronary vascular resistance. CDNB had the most pronounced effect on the GSH:GSSG ratio and resulted in the greatest increase in CVR. In the presence of CDNB and EA it is possible that the increase in CVR observed is related to a reduction in NO bioavailability. In the current experiments, the presence of LNAME only slightly increased the

baseline CVR in the CDNB and EA treated hearts whereas in the control group LNAME caused a significant elevation in CVR. Recent reports have suggested that treatment with CDNB (72, 87) and altered thioredoxin expression (164) can attenuate NO production. In agreement with these studies the lack of effect of LNAME on CVR in the CDNB group suggests that NO production is likely reduced under conditions of CDNB exposure.

Previous reports utilizing EA, have demonstrated that thiol alkylation impairs the replenishment of NO stores (15, 120, 121). In the current study, it can be postulated that in the presence of EA, the altered CVR could be related to impaired NO transfer to GSH as a result of a loss of GSH through alkylation, leading to a reduction in the formation of nitrosoglutathione. As nitrosoglutathione and nitrosothiols have longer half lives than exogenous NO (58, 65), less nitrosothiol in the coronary microcirculation could contribute to the increase in basal CVR. Given that LNAME did not significantly influence CVR in the presence of EA, it is likely that the ability of RSNO to be regenerated plays an important role in determining baseline CVR. Therefore, RSNO are likely released and regenerated under basal conditions in response to physiological stimuli such as shear stress (174). In the presence of EA alone, *de novo* NO is able to contribute to baseline CVR, despite the impaired ability to regenerate RSNO. Yet when NO synthesis is inhibited, RSNO are being depleted and cannot be replenished. Given that the CVR was not greatly enhanced in the presence of EA+LNAME compared to EA alone, these results suggest that RSNO play an important role in determining baseline CVR.

Contrary to the effects of CDNB and EA are the effects of BCNU and Diamide (10 μM) which reduced the GSH:GSSG yet did not affect baseline CVR. Thus unlike studies from endothelium-removed rings where agonist mediated contraction was attenuated (2) and dilation in precontracted arteries was observed (79, 80, 88) the current study suggests that the presence of the endothelium may augment this overall dilatory response seen in either the presence of BCNU or Diamide.

In the current study, treatment with BCNU did not result in significant overall increase in intracellular GSSG compared to the controls, yet the GSSG concentration was significantly elevated compared to all other agents which resulted in altered GSH:GSSG ratio (Table 4.1). In light of this finding, we can assume that in the current experiment the excess GSSG was likely extruded from the cell given the overall reduction in GSH:GSSG. Compared to CDNB and EA which resulted in increases in CVR, the lack of elevated tone in the presence of BCNU compared to the CON may reflect the altered calcium handling and altered K^+ channel open probability seen in denuded coronary vasculature (69, 79, 88). These results suggest that in the presence of BCNU baseline CVR is not elevated as is the case for CDNB and EA. The lack of effect of BCNU on baseline CVR is in agreement with a previous report in the isolated perfused heart (33). Taken together, this suggests that treatment with BCNU in endothelium-intact preparations does not result in altered CVR.

Similarly the lack of increase in CVR in the presence of dimaide can be explained by the ability of diamide to dose-dependently activate sGC (193). A 10 µM concentration was demonstrated to cause a doubling of basal sGC activity from human platelets (193). This suggests that the reduced tone seen in the $DIAM_{10}$ vs $DIAM_1$ could represent either the activation of sGC (193) or the altered Ca^{2+} entry into the vascular smooth muscle preventing contraction (88). In the current preparation, higher concentrations of diamide resulted in a pronounced decrease in CVR to a similar extent as endothelium-independent dilation (Appendix Figure A8). This would have abolished TDC thus higher concentrations were not used.

In light of these finding, it likely that the different agents used had divergent effects on the development of spontaneous vascular tone. These divergent effects may be related to the mechanism of thiol depletion and the how the reduction in GSH:GSSG and altered redox balance affect NO production, RSNO replenishment and the NO independent effects including altering the ability of the vascular smooth muscle to generate tone (100, 173, 190). Importantly we demonstrate that the

presence of the endothelium alters the general dilatory effects observed with thiol oxidation seen in endothelium-removed preparations.

4.5.2 Effects of thiol depletion on minimal CVR

In the current study we demonstrated that minimal CVR was affected by alterations in the GSH:GSSG ratio. All hearts dilated to adenosine; albeit, there were differences in the absolute minimal CVR seen across groups. Treatment with CDNB, EA and $DIAM_1$ resulted in a significant increase in minimal CVR, whereas BCNU and $DIAM_{10}$ caused a small but non-significant increase in minimal CVR compared to the control. When we examined the extent to which the GSH:GSSG ratio was associated with minimal CVR, we determined that reductions in GSH:GSSG are related to elevated minimal CVR. This suggests that overall depletion of thiols attenuates the ability of the smooth muscle to elicit maximal dilation. Similar to the current study, Adachi and Cohen demonstrated a significant correlation between GSH content and ability to dilate to endothelium-derived NO, authentic NO and SNP derived NO (2). We extend these finding by demonstrating that the reduction in GSH:GSSG ratio is associated with an impaired ability to modulate minimal vascular resistance in the intact coronary circulation as demonstrated by the significant correlation between GSH:GSSG and minimal CVR. Therefore the GSH:GSSG ratio likely influences myocardial perfusion especially during instances of increased metabolic demand where dilation is enhanced through both metabolic and endothelial derived factors (176, 185).

4.5.3 Effects of thiol modulation on BK mediated dilation

In the presence of CDNB the BK dose-response curve demonstrated an earlier onset of dilation to BK (increased EC_{50}), followed by a redevelopment of tone at the higher concentrations of BK >0.1 μM. Recent work from our laboratory using hypertensive rats has demonstrated that endothelium-dependent dilation is slightly enhanced at low agonist concentrations; however, a recontraction at

higher agonist concentrations is observed (48, 75). It is possible that altered thioredoxin expression may play a role in the recontraction as thioredoxin expression has been reported to be reduced in the heart and aorta of hypertensive rats (170). In studies examining endothelial derived contracting factor (EDCF), this response is dependent on the endothelium and is mediated through alterations in COX metabolism, altered TP receptors, and downstream activation of the Rho-Kinase pathway (31, 47). In the current study we demonstrated that the redevelopment of CVR in response to BK, was independent of COX. However in the presence of TEMPOL, the redevelopment of tone is greatly augmented. In non-precontracted carotid artery rings, ACh mediated contraction was enhanced in the presence of TEMPOL (47). It is possible that the enhanced recontractions observed in the current investigation represent altered redox based signalling including direct activation of Rho-Kinase or through altered extracellular signal-related kinase (ERK) signalling (10, 100, 171). In the coronary circulation, activation of ERK by mitochondria-derived H_2O_2 attenuated the hypoxic induced dilation (69) which has been previously attributed to altered GSH:GSSG ratio (190). We speculate that the altered redox status associated with CDNB treatment shifts the dilatory effects of H_2O_2 to constrictor effects perhaps by increased ROS production from the mitochondria in part as a result of altered intracellular thiols (86, 100, 190).

One interesting finding regarding CDNB was marked blunting of the BK mediated dilation seen in presence of LNAME compared to the CDNB no drug condition at lower BK concentrations (≤0.1 μM). This result is surprising given that CDNB has been shown to impair NO production (87, 164). The enhanced dilation to the lower BK concentrations in the absence of LNAME (CDNB no drug) accounted for the maintained AUC compared to the control group, as the sensitivity in the CDNB no drug was not different than CDNB+LNAME. This suggests that at the lower agonist concentrations BK mediated dilation, even in the presence of CDNB, is mediated in part by NO. This is supported by the data which demonstrate that the BK mediated dilatory profile was similar between the CDNB+LNAME and

CON+LNAME until 0.1 μM where the recontraction began in the CDNB. Similar to the effects on CVR it appears as though in the presence of CDNB basal NO production was not completely abolished but it was likely reduced. It is possible therefore that a portion of the enhanced dilation seen in the absence of LNAME (CDNB no drug) could be a result of elevated levels of H_2O_2 as a result of uncoupled eNOS (25, 164), which has been demonstrated to mediate dilation in the coronary circulation (144, 148). Thus CDNB likely affects BK mediated dilation through both NO and a non-NO dependent process mediating both the enhanced dilation (lower BK concentrations) and recontraction at higher BK concentrations.

EA is a thiol conjugating agent (150) as such EA has been used to prevent the replenishment of NO stores thus acting as a nitrosothiol depleting agent (14, 15). We found that EA had no effect on the sensitivity of the smooth muscle to exogenous NO (SNP). It is likely that GSH alkylation and depletion associated with EA treatment impairs BK-mediated dilation through a NO/thiol-dependent pathway. A previous report utilizing the isolated perfused heart suggested that BK mediated relaxation is mediated primarily through the release of NO from NO stores (39). Danser and colleagues (1998) demonstrated that dilation to BK is not reduced when BK is administered with a single dose of LNAME (acute LNAME treatment) limiting *de novo* NO production; the dilatory response following prolonged LNAME treatment, which reduces NO production and limits the generation of stored NO, was attenuated (39). Given that the dilation was reduced following prolonged treatment with LNAME only, it was concluded that BK mediated dilation was mediated largely due to NO release from NO stores (39). In the current study, we extend these findings by demonstrating directly, through the use of EA to inhibit the regeneration of NO stores, that replenishment of RSNO is necessary for a portion of the BK mediated dilation in the isolated perfused heart.

In the presence of EA+LNAME there was only a small reduction in curve characteristics (EC_{50}, AUC and max) compared to EA alone whereas the control had greater relative decreases in the

response to BK. This suggests that at lower concentrations of BK, there is a greater reliance on RSNO. The dilation to GSNO is reduced to a greater extent in large coronary arteries compared to small coronary arteries in the presence of EA (153). However, the dilation to BK in isolated porcine coronary arteries was not shown to be blunted in the presence of EA, yet in the presence of a specific RSNO depleting agent, p-hydroxymercurobenzoic acid (PHMBA) dilation was attenuated (14). This suggests that in these arteries, NO production and the existing NO stores were sufficient to prevent a reduction in dilation in the presence of EA (14). In the current preparation both large and smaller coronary arteries/arterioles contribute to the BK-mediated responses. The current findings indicate that in the Langendorff preparation, the BK mediated dilation is mediated through both *de novo* NO synthesis and RSNO/GSNO replenishment and release. The inhibitory effects of EA on BK-mediated dilation are possibly being mediated both as a result of altered GSNO release in the larger coronary arteries (15, 153) coupled with altered GSNO/RSNO availability in the coronary microcirculation in which RSNO can act as EDHF (16). Thus, replenishment of NO stores and subsequent release by the endothelial agonist BK appears to be of importance for maintaining endothelium-dependent dilation in the isolated perfused heart.

Contrary to the findings with both CDNB and EA are the apparent preserved endothelium-dependent responses in the presence of both diamide and BCNU. Given the relationship we found between minimal CVR and GSH:GSSG ratio and the non-significant reduction in the change in CVR seen with both BCNU and $DIAM_{10}$, we postulate that the preserved function in the presence of these agents may in fact be related to a smaller dilatory capacity, not necessarily preserved endothelial mediated function. In the BCNU group maximal BK mediated dilation is 23.6±4.5% when expressed relative to the baseline coronary vascular resistance, whereas the control group maximal dilation was 34.5±1.7% ($p<0.05$). Therefore, when total dilatory capacity is reduced, even small changes in CVR may result in large percentage dilation when expressed as maximal dilation, as the capacity to

modulate tone is reduced. As such when the dilation to BK is expressed based on the baseline vascular resistance; maximal dilation is blunted in the BCNU group compared to the controls, whereas $DIAM_{10}$ is not, likely because the TDC is more preserved (Figure 4.3).

Previous studies have suggested that diamide treatment (87, 132) could induce a reduction in endothelial derived NO. Furthermore, Wang and colleagues (184) and Sugiyama and Michel (164) demonstrated that BCNU resulted in altered eNOS phosphorylation in response to flow or stimulation with an endothelial agonist respectively. Adachi and Cohen reported that diamide (0.2 mmol/L and 1 mmol/L) reduced ACh mediated dilation; and that both diamide and BCNU impaired endothelium independent dilation to NO (2).Therefore the apparent preserved endothelium dependent dilation is a result of smaller TDC. The reduction in TDC in the presence of $DIAM_{10}$ and BCNU explains why there is no correlation between GSH:GSSG and TDC. Both of these agents resulted in small but non-significant reductions in baseline CVR and small but non-significant elevations in minimal CVR which slightly attenuated TDC. Conversely, CDNB and EA resulted in greater TDC as a result of primarily an elevated baseline CVR.

4.5.4 Thiol modulation alters cardiac contractility

In agreement the data which suggest altered tissue perfusion following thiol manipulation we found that left ventricular systolic pressure and +dP/dt were significantly correlated with GSH:GSSG and heart rate was inversely correlated with this ratio. In the presence of all of the thiol manipulating agents we demonstrated that there are altered cardiac hemodynamics including a reduction in LVDP and altered ±dP/dt. The altered contractility noted in the current study is likely related to altered redox state of the myocytes and altered Ca^{2+} signalling related to alterations in the nitrosylation and modification of the sulphydryl groups of the ryanodine receptor (RyR), L-type Ca^{2+} channel and sarco(endo)plasmic reticulum Ca^{2+} ATPase (for reviews see (113, 197)). In conditions of oxidized thiols,

the RyR mediated Ca^{2+} release is increased transiently then reduced, whereas L-type Ca^{2+} channel activity and sarcoplasmic reticulum Ca^{2+} ATPase activities are reduced. This could result in a reduction in LVDP and altered Ca^{2+} cycling (197). Similarly, altered nitrosylation of these channels as well as the cardiac pacemaking cells contributes to impaired and attenuated cardiac contractility (113).

4.5.5 Conclusion

The primary findings of this study are that coronary vascular resistance and endothelium dependent dilation are altered in response to changes in the GSH:GSSG. Thioredoxin inhibition and glutathione conjugation increase CVR, whereas glutathione reductase inhibition and thiol oxidation do not result in increases in CVR. Similarly the effects of thiol modulation on endothelium-dependent dilation in the isolated perfused heart vary in response to the nature of reduced GSH:GSSG. In the presence of thioredoxin inhibition, BK-mediated dilation was enhanced at the lower agonist concentrations, yet resulted in a recontraction at the higher concentrations which were independent of COX derived prostanoids. Conversely, EA resulted in an overall blunting of the BK mediated response which was only modestly affected by NOS inhibition, suggesting this alteration is likely mediated by the ability to regenerate NO stores. Importantly, across all treatment groups there is an inverse relationship between GSH:GSSG and baseline and minimal CVR and suggests that alterations in GSH:GSSG may impair tissue perfusion because of an inability to properly modulate vascular tone. In summary, acute alterations in the GSH:GSSG ratio play an important role in determining both the baseline vascular tone and the ability of the vasculature to respond to endothelial-dependent and independent stimuli.

4.6 Acknowledgements

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CHAPTER 5

Discussion

5.1 General Discussion

The purpose of this collective work was to examine how changes in GSH either chronically or acutely alter CVR and endothelium-dependent dilation in the isolated perfused heart. The current literature suggests that changes in GSH levels have differential effects depending on the experimental model. This is highlighted by discrepancies that exist between endothelial-intact preparations compared to endothelial removed preparations as well as the discrepancies seen in cellular preparations compared to isolated vascular segments. The individual studies discussed above provide novel insight into the role that GSH has within the coronary circulation. Contrary to previous studies, chronic GSH depletion resulted in an age-dependent increased reliance on NO, such that only adult animals demonstrated an adaptive response, which included an increased eNOS protein content and reliance on NO. In the second study it was demonstrated that acutely administering GSH at physiological concentrations enhances bradykinin-mediated dilation. The enhancement was independent of NO production as GSH-mediated enhancement persisted despite NOS inhibition. The presence of LNAME and TEMPOL together abolished the GSH mediated effect suggesting a role for reactive oxygen species mediating the effects of GSH administration. Acutely modifying thiols demonstrated that endothelium-dependent dilation is altered to different degrees depending on the method through which GSH:GSSG was reduced. Additionally, inverse correlations exists between the GSH:GSSG ratio and baseline CVR and the GSH:GSSG ratio and minimal CVR.

These studies have used similar treatments, and endothelial agonists that have been previously used in isolated vascular segments in order to specifically examine the intact coronary circulation using the Langendorff heart. This has allowed the analysis of the influence of changes in

glutathione on vasomotor tone (CVR) and endothelium-dependent dilation. In all studies there was consistency between the changes that occurred in CVR and BK-mediated dilation. For example in the presence of NOS inhibition, there was both an increase in CVR and an increase in EC_{50}. The similarity between CVR and endothelium-dependent dilation is extremely important given the unique role NO plays within the coronary circulation (61, 64, 98, 115). The results of these studies demonstrate the complexity associated with changes in GSH and the role that GSH plays within the vasculature, as substantial differences were seen with the acute and chronic depletion of GSH. Furthermore, the ability of GSH to enhance BK-mediated dilation independent of changes in baseline CVR was determined to extend beyond the perceived antioxidant properties.

Within each chapter there is an extensive discussion section which is used to highlight the current state of the literature and integrate the unique findings of the chapter. The purpose of this overall discussion section is not to provide a repetition of these findings but rather to expand on the novel findings by providing additional integrative insight, while reconciling discrepancies that exist between the studies and expanding on certain findings where relevant. This discussion will include a section of the potential limitations of the current studies, and suggest future directions.

5.2 Contrasting effects of different modes of GSH modulation on coronary vascular resistance.

Based on the findings from the studies the following conclusions can be drawn with respect to the effect of thiol modulations on baseline coronary vascular resistance: acutely depleting GSH with EA or CNDB resulted in an elevated CVR whereas BCNU and diamide did not, chronic depletion of GSH did not alter baseline CVR, and acute GSH administration at concentrations greater than 30 μM was able to reduce baseline CVR.

In agreement with reports utilizing isolated endothelium-removed coronary segments (79, 88), and endothelium intact aortic ring preparations (2), acute thiol oxidation with BCNU and diamide can induce a reduction in CVR. This reduction appears to be dependent on increases in GSSG as demonstrated in the presence of BCNU but not diamide. Similarly, aging is also associated with a reduction in baseline CVR, which does not appear to be mediated by altered NO production or as the result of increased concentration of GSSG. Conversely, the effects observed with CDNB and EA suggest that reductions in the bioavailability of NO, likely mediated by altered production of NO or altered RSNO bioavailability, result in increases in CVR. This suggests that RSNO are an important determinant of baseline CVR. Given the apparent adaptive response seen in the presence of BSO treatment, no increases in baseline CVR were observed despite increased ROS production. In the presence of chronic GSH depletion it is apparent that a compensatory mechanism is in place to prevent reduced NO bioavailability. Conversely GSH at physiologically relevant concentrations does not itself influence baseline CVR, albeit it does blunt the effects of LNAME suggesting that GSH can itself induce a dilatory effect (also observed at concentrations >30 μM). Therefore, it can be concluded that the mechanism by which GSH, GSSG and GSH:GSSG ratio is altered must be identified in order to understand how changes will influence baseline coronary vascular resistance.

5.2.1 Relationship between GSH, GSSG and GSH:GSSG ratio on coronary vascular resistance – a cross study comparison.

Collectively, these studies demonstrate that acute thiol manipulation has a greater effect on baseline coronary vascular resistance than chronic alteration. This likely reflects the compensatory mechanisms associated with the chronic nature of the BSO treatment including increased ROS production and greater reliance on NO. The ability of acute changes in GSH to modulate baseline CVR is demonstrated by the ability of GSH at concentrations greater than 30 μM to reduce baseline CVR. When GSH is acutely depleted increases in CVR can be observed as demonstrated by the profound

increases seen with CDNB and EA; conversely BCNU and diamide moderately reduced baseline CVR. The ability to assess how acute and chronic changes in GSH and GSSG can influence CVR can be examined as the different agents designed to induce thiol depletion had a differential effect on baseline CVR. Similarly, as rats of different ages were used across the 3 studies, the natural effects of aging on GSH and GSSG can be examined. Not surprisingly, given the ability of changes in GSH and GSSG to augment CVR, we found that as rats age there was a progressive decline in CVR and this was associated with an increases in GSH and GSSG content (Figure 5.1A and B). By compiling the baseline CVR data across the three studies and including the data regarding the differences in age and GSH and GSSG content, there was a significant correlation between all thiol measures and baseline CVR (GSH vs CVR: $r = -0.71$, $p<0.01$, GSSG vs CVR: $r = -0.71$, $p<0.01$ and GSH:GSSG vs CVR $r=-0.29$, $p<0.05$, $n=58$, Figure 5.1 C and D). Interestingly, there was a gradual increase in the GSH and GSSG content, despite a reduction in GSH:GSSG ratio from 10 weeks to 35 weeks. This altered GSH:GSSG was recovered at 65 weeks of age likely as a result of the decrease in GSSG observed in this age group. The reduced association between GSH:GSSG and CVR likely reflects the age-dependent changes that occur in GSH and GSSG. The current data demonstrates that the youngest animals had lowest GSH and GSSG values, yet the GSH:GSSG ratio was the highest (Figure 5.1B). GSH and GSSG content and the ratio have been reported to be reduced in the left ventricle with age (82-84, 112). Within the aorta, Smith and colleagues demonstrated a marked reduction in GSH as well as GSSG in aged rats compared to young rats (157). In the heart, several reports have also demonstrated that aging results in a progressive decline in GSH content (82, 91, 183). However, a recent report also suggested that there are no changes in GSH content with aging in the heart, yet, there is a small increase in GSSG content and reduced GSH:GSSG ratio in the oldest group, 26 months compared to the youngest group 4 months (140). In agreement with the current data, Leichtweis and colleagues (2001) found that old rats (24 months) compared to young rats (6 months) had higher GSH, GSSG and similar GSH:GSSG values (109).

The current results demonstrate that myocardial GSH and GSSG increase with age, and that aging is associated with a reduction in the GSH:GSSG ratio, which is partially recovered at 65 weeks as a result of a reduction in the GSSG concentration. The rebound of GSH:GSSG may be in part related to the age associated increase in GPx and glutathione reductase activity (44, 109, 112, 142). The increased activity of GPx and GR may be a compensatory mechanism that is used in an attempt to maintain an adequate level of GSH and GSSG and to help scavenge free radicals and maintain redox balance (109, 112, 162).

Because of the chronic nature of BSO treatment these rats were excluded from the analysis as compensatory mechanisms likely prevented differences in CVR to be observed in both the adult and older BSO treated animals. In the presence of the BSO treated animals correlations between GSH and GSSG remained significant albeit they were blunted (GSH vs CVR: $r= -0.37$, $p<0.01$, GSSG vs CVR: $r=-0.43$, $p<0.01$ and GSH:GSSG vs CVR $r=-0.13$, $p>0.05$, $n=69$). Therefore, these findings demonstrate acute alterations in GSH and GSSG and alterations in GSH and GSSG associated with age are strongly inversely correlated with baseline CVR and suggest that the lower these values, the higher the baseline CVR.

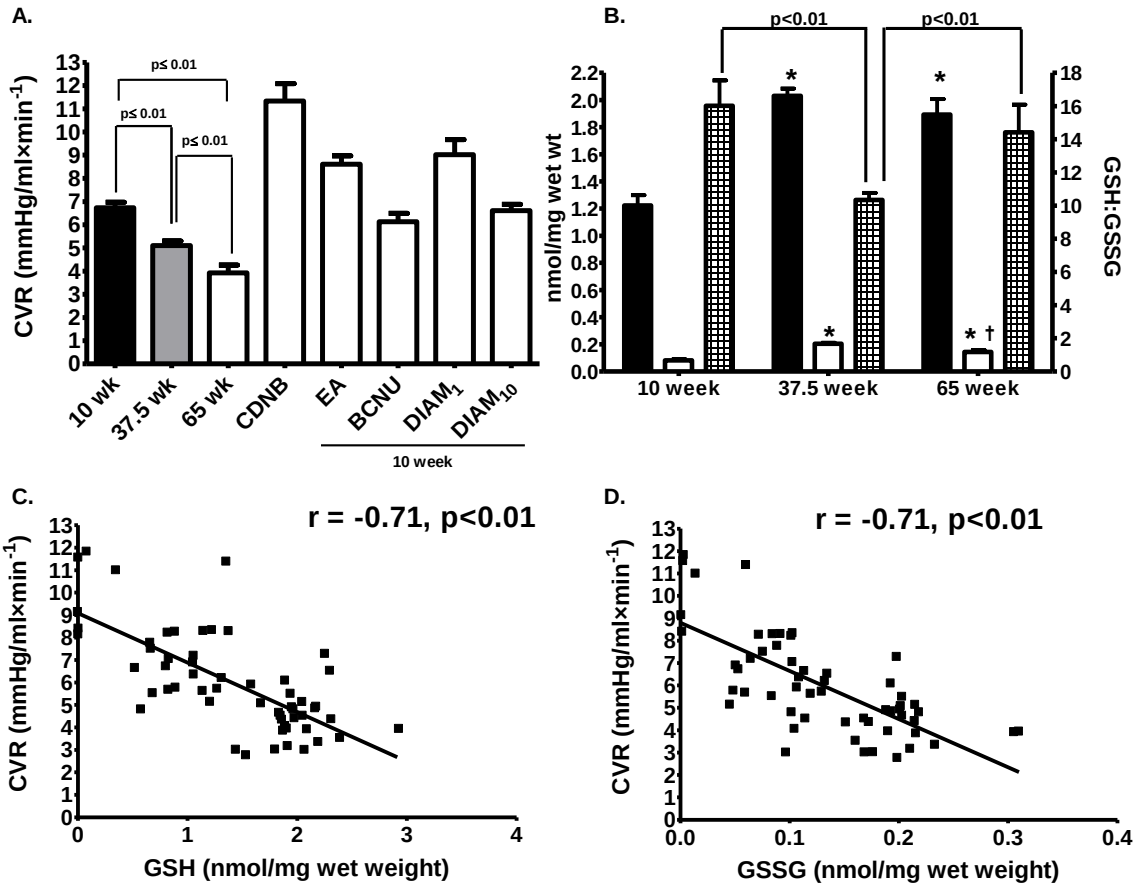

Figure 5.1. Relationship between glutathione levels and baseline coronary vascular resistance
Summary of CVR from all groups excluding BSO treated animals, for clarity the control animals from chapters 2 and 3 were collapsed (A). Summary of changes in left ventricle GSH, GSSG and GSH:GSSG ratio across study ages (B). There was a significant inverse correlation seen between GSH and CVR (C) and GSSG and CVR (D). Data was compiled from all experimental groups from each specific study, with the exception of BSO treated animals (both adult and older animals, for details refer to text). Thus this graph demonstrates primarily the effects of aging and acute thiol modulation on baseline coronary vascular resistance (n=58 for correlation analysis).

5.2.2 Potential mechanisms responsible for altered CVR with thiol manipulation.

Given the ability of GSH to reduce ROS it could be postulated that in conditions where GSH is reduced an increase in ROS mediated NO destruction could account for alterations in baseline CVR. A reduction in NO bioavailability as a result of increased ROS was originally postulated to account for the increase in CVR in the presence of the acute depletion elicited by thioredoxin inhibition (164, 194), glutathione conjugation (49) and BSO treatment (13, 63). Contrary to the findings of chronic thiol depletion, acute thiol modulation resulted in a modest increase in ROS in the presence of CDNB, and no difference was observed with EA. Further support for a lack of ROS mediated NO destruction is demonstrated by the lack of effect that the addition of TEMPOL to the perfusate had on baseline CVR in the presence of CDNB or EA (ie CDNB+TEMPOL and EA+TEMPOL compared to CON+TEMPOL, chapter 4). Additionally, the other agents utilized, BCNU and diamide (10 μM) also only moderately increased ROS, and did not affect baseline CVR. Conversely acute GSH administration was demonstrated to reduce basal CVR provided a sufficient extracellular GSH was provided (≥ 30 μM). These results indicate that alterations in GSH do result in an increased ROS, and therefore, altered NO bioavailability as a result of increased ROS cannot completely explain the alterations in CVR observed. Thus it appears that the baseline CVR in response to thiol depletion was not influenced by an overall ROS mediated NO destruction.

Changes in intracellular thiols have been reported to augment NO production (see above); therefore, in order to understand the role for NO and how the thiol manipulations influence CVR, LNAME was employed to inhibit NO production. NOS inhibition resulted in an increase in CVR in all conditions of GSH manipulation, but the increase in CVR varied depending on the experimental conditions. Unexpectedly, in the BSO treated animals (adult only) there was an increased reliance on NO as suggested by the effects of LNAME on baseline CVR. Concurrent with the effect on CVR was the increased eNOS protein content. It is therefore hypothesized that in this model which showed

substantial effects on ROS production that the preserved CVR is likely related to the increased NO production. This compensatory increase in NO likely accounts for the increased NO-mediated destruction by O_2·-. Indeed, in BSO treated animals despite the often reported increase in eNOS protein content (13, 63, 90, 195), there is often an increased amount of tissue nitrosylation, indicative of elevated levels of peroxynitrite (13, 181, 181, 196). Thus, it is postulated that the lack of effect on CVR in the BSO treated animals is a result of increased NO production, accounting for the increased NO loss as a result of ROS.

Contrary to this are the responses to CDNB and EA, which did not result in a large compensatory increase in NO production. The effects of LNAME on CVR that were observed in the presence of CDNB and EA were much less than the control group suggesting that overall these agents impaired basal NO bioavailability by a mechanism that appears to be unrelated to ROS. Thioredoxin function may be important for availability of eNOS cofactors and eNOS activation (87, 164). It is therefore, postulated that there is likely a loss in basal NO production in the presence of CDNB. This is likely the case as treatment with LNAME resulted in a small increase in CVR in the presence of CDNB and suggests that there is not a complete loss in basal NO production, yet it is substantially reduced.

The increase in CVR in the isolated perfused heart in the presence of EA is postulated to be a result of reduced GSNO and/or RSNO replenishment, suggesting that GSNO or RSNO is an important source of NO for maintaining baseline CVR. Support for the requirement of RSNO/GSNO is based on the data which demonstrate that while EA does not alter endothelium-dependent dilation (14); EA prevents the dilation to repeated light exposure (photorelaxation)(15), a condition solely mediated by RSNO/GSNO. Therefore, during the 30 minute stabilization period in the presence of EA, shear mediated NO production is available, yet the ability of NO stores to be replenished is reduced. Given that shear induced release of both RNSO and NO can occur (15, 174), the current results suggest that RSNO are constantly being depleted and replenished under normal conditions, and the increase in

CVR in the presence of EA represents the inability of RSNO to be regenerated and released. The increase in CVR in the EA condition in the presence of LNAME likely reflects a reduction in a portion of *de novo* NO synthesis, coupled with a gradual depletion of NO stores. In the presence of EA and LNAME, a mild increase in CVR is observed suggesting that eNOS-derived NO is still able to partially control baseline CVR, albeit not to the same extent as GSNO. It could be postulated that the lack of similarity achieved in the presence of EA+LNAME and the control+LNAME may represent a compensatory mechanism of dilation by the coronary microcirculation in the presence of EA or the fact that RSNO are more important in the responses of larger coronary vessels (153).

One final explanation of the altered baseline CVR is unrelated to ROS and nitric oxide and reflects the ability of changes in intracellular glutathione to impact vasomotor tone and coronary vasoconstriction through the ability to modulate Ca^{2+} release and K^{+} channels (190). Thus it is possible that alterations in GSH and GSSG have an endothelium-independent affects on baseline CVR and suggests that alterations in GSH concentration influence vascular smooth muscle contractile state. A surprising finding was the effect of aging on CVR. Contrary to the original hypothesis of an elevated CVR with age, a clear reduction was observed. As already discussed several potential mechanisms have been postulated including altered myogenic tone as a result of increased NO production (154), and altered K^{+} channel contribution (94). While these are likely mechanisms, another potential mechanism related to altered thiol status may explain the reduction in CVR seen with age and the small changes seen in the presence of BCNU and diamide as these agents typically promote oxidation. Thiol oxidation is reported to limit the amount of vascular constriction as demonstrated by a series of studies from Wolin's laboratory in denuded coronary vasculature (79, 80, 88). Similarly in endothelial intact aortic rings thiol oxidation resulted in an increased agonist concentration, required to elicit ~70% vasoconstriction (2). The results with BCNU, diamide and aging demonstrate that thiol oxidation resulted in either an impaired ability to generate tone, or sufficient oxidation which failed to affect

CVR (ie 1 vs 10 µM diamide). There were no age-dependent increases in the thiol content of LV in the older compared to the adult animals (Figure 5.1B). However, we cannot exclude this as a potential mechanism as the vascular tissue (whole aortic homogenate) did demonstrate an age-dependent increase in GSSG (adult and older controls only: 0.2430±0.0019 vs 0.0352±0.0035 nmol/mg wet weight, $p<0.05$). Similarly, Ferrini and colleagues demonstrated an age-dependent increase in GSSG in the aorta (62). Therefore, it could be postulated that with aging altered levels of GSSG may contribute to impairment in the development of CVR.

Unlike the age-dependent reduction in CVR which is postulated to be in part as a result of increased vascular GSSG, the two thiol modulating agents designed to shift the GSH:GSSG ratio towards a more oxidized state (increased GSSG) failed to significantly affect baseline CVR. Both BCNU and diamide (10 µM) were without effect on CVR, despite a decrease in GSH:GSSG ratio. Higher concentrations of diamide are typically used in studies examining the effects of this agent on vascular function (2, 88). However, in the current preparation increasing the concentration of diamide to 100 µM resulted in a substantial drop in CVR which would have significantly limited TDC (Appendix Figure A7). In the BCNU animals, there was a small but non-significant increase in GSSG compared to the control group. Yet, compared to all other thiol manipulating agents used, BCNU resulted in a significant increase in GSSG. This small increase in GSSG may explain the small but non-significant decrease seen in CVR. This suggests that relatively small increases in GSSG in endothelial-intact preparations also promote a dilatory state. In light of the significant correlation between GSSG and CVR it is not surprising that small increases in GSSG impact CVR in the isolated perfused heart.

The results of the current studies suggest alterations in GSH, GSSG and GSH:GSSG ratio may augment basal NO production as is the case for CDNB. Additionally it has been demonstrated that alterations in the ability and availability of GSNO and RSNO, which are reduced with EA, to be regenerated play a key role in determining baseline CVR and this appears to be relatively independent

of NO production. Conversely, general thiol oxidation (dimaide, BCNU and aging) may impact baseline CVR through mechanisms unrelated to NO production per se as the effects that these conditions/treatments have on baseline CVR may be related to altered vascular smooth muscle contractile properties (Figure 5.2).

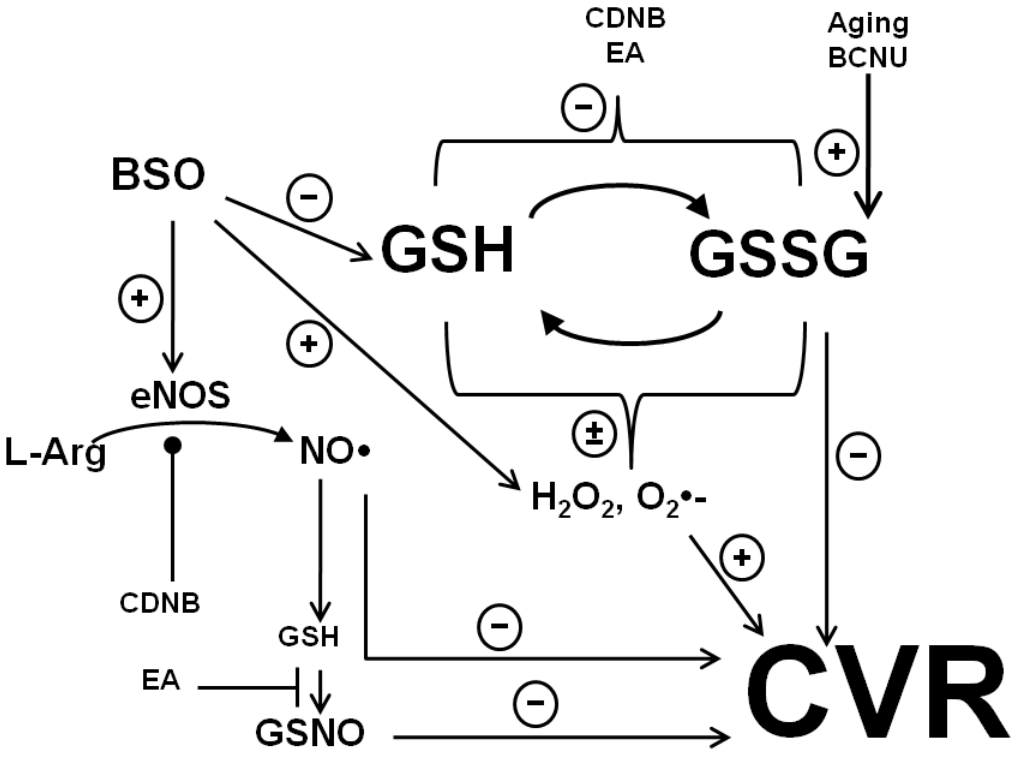

Figure 5.2. Contrasting effects of thiol modulating agents and aging on coronary vascular resistance
The primary determinants of CVR based on the current work are NO, RSNO, ROS and elevated GSSG. Each agent altered 1 or more pathways which resulted in augmented CVR. Notably, BCNU and aging resulted in an increase in GSSG which reduced CVR. Conversely, CDNB and EA reduced NO and RSNO respectively which resulted in an elevated CVR. BSO had a 2 different effects as it both increased ROS, which alone should cause an increase in CVR, however there was also increased NO production as a result of increased eNOS protein content. The affect of high concentration of GSH which are able to reduce CVR have been omitted from the diagram.

5.3 Contrasting effects of GSH modulation on endothelium-dependent dilation

Individually each of the studies adds to the literature by demonstrating: a compensatory age-dependent increased reliance on NO in the presence of chronic thiol depletion (chapter 2), mechanistic insights into the action of GSH administration on endothelium-dependent dilation (chapter 3), and that different thiol manipulating agents have a varying impact on endothelium-dependent dilation depending on the mechanism of action and there is a relationship between minimal CVR and GSH:GSSG status (chapter 4). Collectively this work highlights the complex nature of the interaction between GSH and vasomotor function. Given the recent evidence suggesting a link between GSH:GSSG and cardiovascular risk (12, 114), and the beneficial effects of GSH infusion on coronary artery dilation (103, 104) these studies provide insight into the potential mechanisms of altered GSH, GSSG and GSH:GSSG leading to impaired and improved vasomotor function.

Much of the preceding discussion regarding the mechanism of thiol depletion can be restated in this section as similar endothelium-dependent responses (typically EC_{50}) were observed compared to the effects the different agents had on CVR (Table 5.1). This is not surprising given the effects that the endothelial-derived NO has on coronary vascular resistance (61, 71, 110, 115). The following broad conclusions can be made regarding the effects of altered thiols on endothelium-dependent dilation: 1) administration of GSH at physiological levels is sufficient to enhance endothelium-dependent dilation; 2) acute thiol depletion through the inhibition of thioredoxin or glutathione conjugation appears to augment endothelium-dependent dilation as observed by altered BK-meditated dilation; 3) inhibition of glutathione reductase and aging affected BK mediated dilation through a process of altered minimal and baseline CVR and therefore altered dilatory capacity. Regardless of the mechanism of thiol depletion, previous studies using acute depletion in either isolated endothelial cells (87, 164, 184), isolated intact vascular rings (2) or chronic depletion (13, 107) results in augmented NO production

and/or impaired dilation. However, the results of the current study provide additional information regarding endothelium-dependent dilation and minimal CVR.

Table 5.1 Summary of changes in coronary vascular resistance and bradykinin mediated dilation across studies.

	Aging			GSH (10μM)	BSO		10 week			
	10 weeks	37.5 weeks	65 weeks		Adult	Older	CDNB	EA	BCNU	DIAM
CVR										
Baseline	↑	REF	↓	↔	↔	↓	↑↑	↑	↔/↓	↔/↓
Minimal	↑	REF	↓	↔	↔	↓	↑↑	↑	↔/↑	↔/↑
TDC	↑	REF	↓	↔	↔	↓↓	↑	↑	↓	↓
Dilation										
EC_{50}	↑	REF	↓	↑	↔	↔	↑	↓	↔	↔
AUC	↑	REF	↓	↑	↔	↔	↑	↓	↔	↔
Max	↔/↑	REF	↓	↔	↔	↔	↓↓	↓	↔	↔

Relative changes in curve characteristics, CVR and TDC (total dilatory capacity) in the absence of other pharmacological agents (no drug conditions). The 37.5 group was used as reference (REF) for all comparisons across age, GSH, and BSO. The 10 week controls were used as a reference for CDNB, EA BCNU and Diamide. ↑, increase; ↑↑, substantial increase; ↓, decrease; ↓↓, substantial decrease; ↔, no change; ↔/↑, small increase relative to 10 week group; ↔/↓, small decrease relative to 10 week group.

5.3.1 Relationship between GSH and GSSG on minimal coronary vascular resistance, total dilatory capacity and endothelium-dependent responses

Cross study analysis revealed a significant inverse correlations between: minimal CVR and GSH ($r = -0.81$, $p<0.01$), minimal CVR and GSSG ($r= -0.78$, $p<0.01$) but not minimal CVR and GSH:GSSG ($r = 0.20$, $p=0.10$, Figure 5.3). Additionally, no relationships between GSH, GSSG or GSH:GSSG and total dilatory capacity were observed. This is a result of enhanced TDC seen with CDNB, and EA despite lower thiols levels whereas BCNU and aging were associated with reduced TDC despite opposing effects on thiol content.

In order to assess if there were significant relationships between GSH, GSSG, or the GSH:GSSG and endothelium dependent responses across studies, both the 1 and 10 µM GSH groups as well as the adult and older BSO groups were excluded. There was a small but significant correlation between GSH and EC_{50} ($r = 0.36$, $p<0.05$, $n=43$) and GSH was inversely correlated with AUC ($r = -0.30$, $p=0.05$, $n=43$). Similarly the GSSG was significantly correlated with both EC_{50} ($r= 0.35$, $p<0.05$, $n=43$) and inversely correlated with AUC ($r = -0.38$, $p< 0.05$, $n=43$). These results must be interpreted with caution as the low GSH and GSSG values associated with CDNB and the enhanced EC_{50} and AUC associated with these results skew this data; without these values all the correlations become non-significant with the exception of the GSSG with AUC ($r = -0.34$, $p<0.05$). The lack of association between BK-mediated dilation and thiol content is in agreement with a recent report demonstrating no relationship between GSH:GSSG and flow mediated dilation in an apparent healthy population (11). Thus while GSH and GSSG are inversely correlated with minimal CVR, the results of the current series of experiments suggest that there is no correlation between either GSH or GSSG and the bradykinin curve parameters. Thus while, modulation of GSH content does affect endothelium-dependent dilation, there are no consistent effects observed.

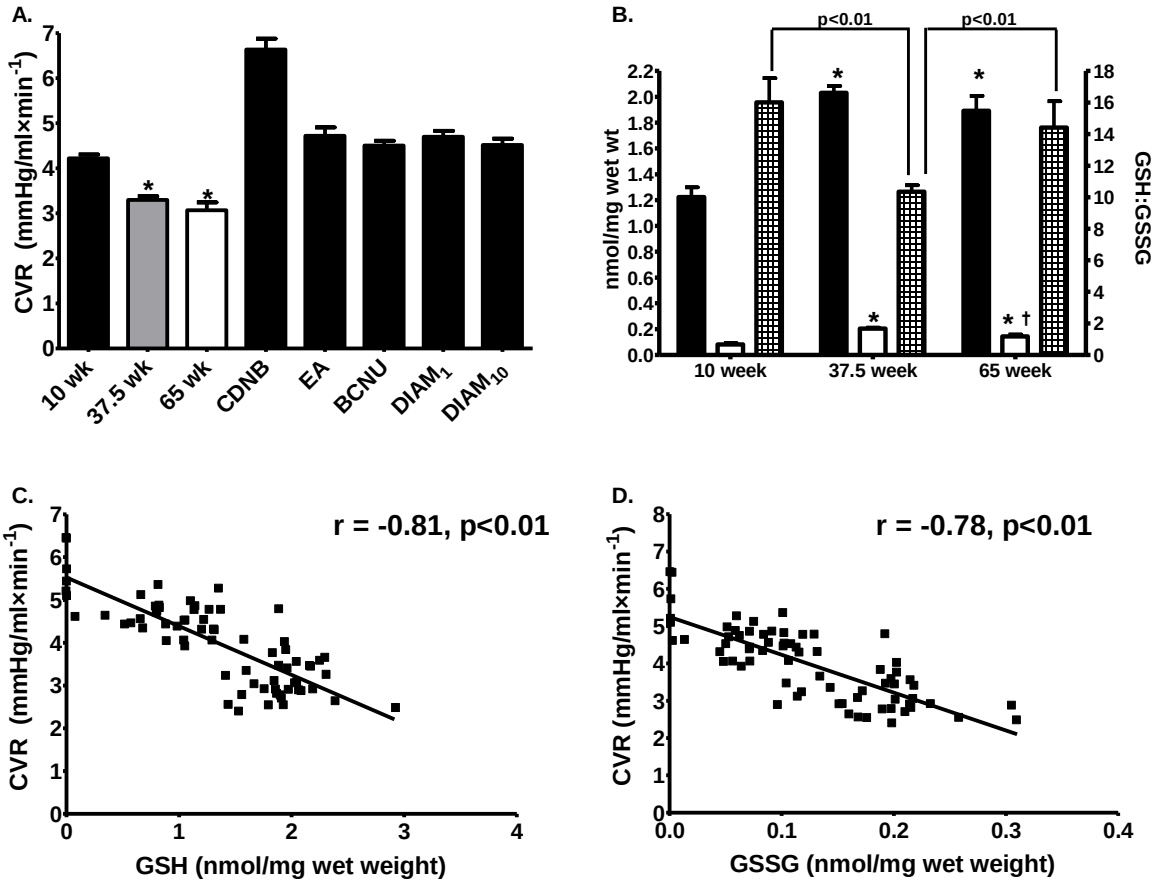

Figure 5.3. Relationship between glutathione levels and minimal coronary vascular resistance
Summary of minimal CVR from all groups excluding BSO treated animals, for clarity the control animals from chapters 2 and 3 were collapsed (A). Summary of changes in left ventricle GSH, GSSG and GSH:GSSG ratio across study ages (B). There was a significant inverse correlation seen between GSH and minimal CVR (C) and GSSG and CVR (D). Data was compiled from all experimental groups from each specific study, with the exception of BSO treated animals (both adult and older animals, for details refer to text). Thus this graph demonstrates primarily the effects of aging and acute thiol modulation on baseline coronary vascular resistance (n = 71 for correlation analysis). *, $p<0.05$ vs 10 week. †, $p<0.05$ vs 37.5 week.

In the current series of experiments, similar dilatory responses to endothelium-dependent dilation were observed in the old animals (chapter 2) and BCNU treated hearts (chapter 4). In both groups there was a reduction in TDC. This suggests that changes in GSSG concentration may not impair BK-mediated dilation when expressed as percentage of maximal endothelium-independent dilation as was the case with BCNU and aging. However, increases in GSSG such as those caused by aging (elevated thoracic aorta GSSG content) or BCNU (left ventricle) appear to blunt the total capacity to dilate. Therefore, when expressed as maximal dilation, potential differences are minimized as the total capacity to augment tone is reduced (Table 5.1, Figure 5.4). When dilation is expressed *relative to baseline CVR* both aging (chapter 2) and BCNU (chapter 4) were shown to have blunted BK mediated dilation.

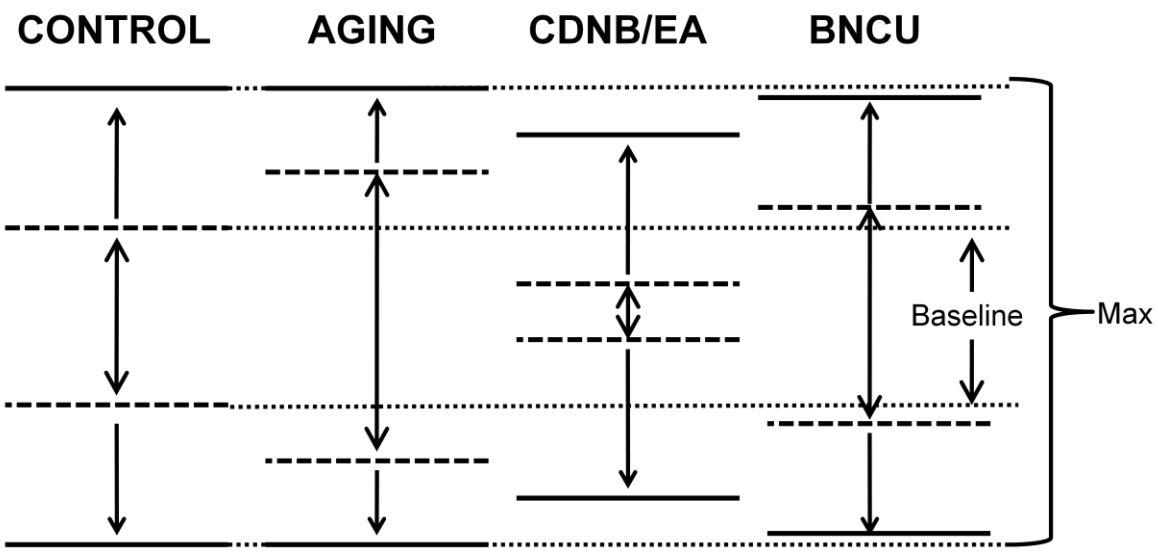

Figure 5.4. Effects of aging and thiol manipulation on baseline resistance and total dilatory capacity. For each condition, the dashed line represents the baseline vascular tone, the closer the lines the smaller the radius, the greater the baseline CVR. The solid lines represent the minimal CVR and the dotted lines are a continuation of the control condition. Aging results in a reduced TDC as a result of a reduction in baseline CVR, similarly BCNU results in a small reduction in baseline CVR and small increase in minimal CVR, net loss of TDC. Conversely, TDC is increased with CDNB and EA, yet there is a substantial increase in minimal CVR.

As discussed above, the compensatory increase in eNOS protein content observed in the adult BSO-treated animals was associated with an exaggerated increase in CVR in the presence of LNAME; a similar effect was observed in endothelium-dependent dilation. The sensitivity (baseline *in vivo* flow rate) and maximal dilation (high flow) were reduced in the adult BSO animals to a greater extent than all other groups. Unlike previous studies (eg. (13, 107)) in which endothelium-dependent dilation was reduced, there was no effect of BSO on endothelium-dependent responses in the absence of LNAME. That being said, they were not enhanced compared to the control group as recently reported (90). As with CVR, the compensatory increase in eNOS likely normalized total NO produced to account for the increased ROS mediated NO destruction. This explains the lack of effect on BK-dilation in the absence of LNAME.

Conversely, acute treatment with both CDNB and EA resulted in marked impairment of the BK-mediated response despite a greater minimal CVR and greater TDC. Overall, it appears that alterations in GSH:GSSG ratio impacts dilatory capacity and minimal CVR which may have implications for adequate perfusion of the myocardium (54, 176, 185). Indeed, both CDNB and EA resulted in increased minimal CVR in response to adenosine. Therefore, when the dilatory responses to these agents are examined, there is an *underestimate* of the impairment as the data was expressed relative to the adenosine, which is elevated compared to the control group. Interestingly, the ability of the coronary circulation to dilate to NO was not altered in the presence of either of these agents as demonstrated by the normal SNP responses, albeit this dilation was also normalized to the minimal CVR which was elevated. This is an important finding, and suggests that any impairment in BK mediated dilation is not a result of an impaired ability of the vascular smooth muscle to respond to NO. As discussed in chapter 4 alterations in NO production and the ability of GSNO to regenerate and release appear to account for the blunted BK-mediated responses in the presence of CDNB and EA respectively.

Acute GSH administration enhanced endothelium-dependent dilation in the isolated heart without altering GSH, GSSG or the GSH:GSSG ratio. Similar to *in vivo* studies where the effects were more pronounced in individuals with existing dysfunction (103, 104, 138) the results with TEMPOL and LNAME+TEMPOL suggest that GSH administration exerts effects aside from the antioxidant dependent effects (103). These beneficial effects were only apparent in the presence of ROS. Preliminary data examining the effects of GSH administration in isolated perfused hearts suggests that normotensive Wistar Kyoto (WKY) rats were more responsive than the spontaneously hypertensive rats (SHR) to GSH (Appendix Figure A9). This finding is unique as previous *in vivo* work using the same hypertensive model in which GSH infusion lowered the mean arterial pressure of the SHR not the WKY (5).

The current series of studies demonstrate that alterations in GSH, GSSG and GSH:GSSG ratio differentially effect endothelial-dependent dilation in the isolated perfused heart. Through the utilization of several different pharmacological agents specific conclusions can be drawn with respect to the interaction between GSH:GSSG and GSH itself (Figure 5.5). GSH possesses an antioxidant effect; as such when co-administered with an endothelial agonist, there is an increased sensitivity to endothelium-dependent dilation. The effects extend beyond a simple antioxidant effect as despite inhibition of NOS and/or sGC enhanced sensitivity to BK remained, yet there is an abolishment of the GSH effect which is apparent in the presence of TEMPOL and LNAME. Conversely, treatment with a GSH alkylating agent suggests that a portion of the BK-mediated response seen in the isolated perfused heart is through the release and replenishment of RSNO. This finding elaborates on previous work from the isolated perfused heart model (39) while suggesting that BK-mediated dilation in the isolated perfused heart may differ from isolated coronary vessels (14). It appears that CDNB resulted in biphasic response with dilation seen at concentrations ≤1 nM followed by recontraction at higher concentrations of BK (≥0.1 μM). Conversely, prolonged GSH depletion resulted in enhanced reliance on NO for endothelium dependent dilation; however this was restricted to adult rats. This likely

represents a compensatory mechanism to preserve vasomotor function within the coronary circulation. Conversely, in conditions where total dilatory capacity is reduced there is often a 'preserved' endothelium-dependent response, albeit this preserved function is not indicative of the ability to modulate vascular resistance over as wide a range (Figures 5.4 and Figure 5.5).

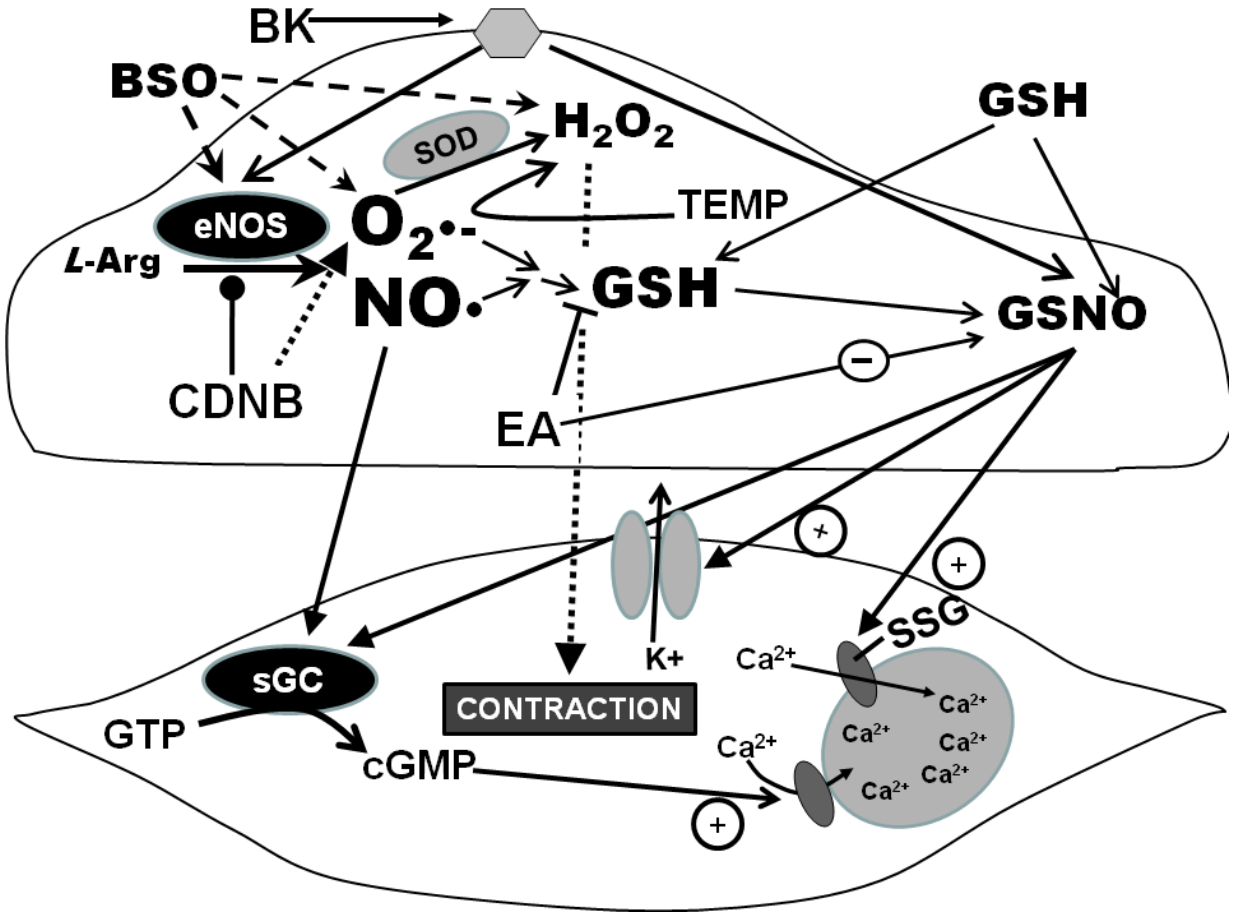

Figure 5.5. Complex interaction of thiol modulation on endothelium-dependent dilation

Depending on the nature of thiol depletion or acute GSH infusion, several potential pathways interact when determining the effects on BK mediated dilation. BSO results in a compensatory increased reliance on NO as a result of increased eNOS protein content as a compensatory mechanism for the increased ROS. CDNB likely results in eNOS uncoupling resulting in superoxide formation which is converted to H_2O_2 by either SOD or Tempol (TEMP) which causes vasoconstriction in the presence of the altered redox environment. EA prevents the replenishment of the NO stores by conjugating free GSH. These stores are released by BK stimulation through an unidentified pathway. Acute treatment with GSH enhanced BK mediated dilation through a pathway requiring ROS. It is possible that this may be due to increased GSNO stores which may require the interaction between NO and superoxide. For additional details refer to text.

5.4 Future directions and limitations

5.4.1 Limitations

The previous discussion and synthesis of results highlights the consistency of the data both within the individual studies and across the chapters. This is best demonstrated when the effects of LNAME on CVR and endothelium-dependent dilation are examined, as in the presence of LNAME larger increases in CVR were always accompanied by larger decreases in BK mediated dilation. The focus of this work was on vasomotor function; however, for all three studies, the left ventricle was used to measure GSH:GSSG as well as the different ROS measures. This was done as the yield of vascular tissue from a single heart would not have been sufficient to run the different assays. The LV was chosen to make these measures to avoid pooling of sample and provide adequate amount of tissue/sample. One caveat that may still exist is highlighted from the BSO study and suggests that the vasculature may be better able to preserve GSH content compared to the ventricle. Given that GSH, GSSG and GSH:GSSG were correlated with several measures of ventricular function it is likely that similar changes in vascular GSH occurred. The ability of the vascular wall to preserve GSH content may warrant further investigation.

Another potential discrepancy that should be noted is the differences in the age cohorts used across the current studies. Animals used in chapters 2 and 3 are representative of adult animals, whereas the animals used in chapter 4 were younger adults. This discrepancy was a result of availability of animals at the onset of each study. As demonstrated in chapter 2, there is an effect of aging on CVR and endothelium-dependent dilation in the isolated perfused heart. There is an interesting finding which further extends the overall conclusions regarding changes in glutathione content and endothelial function. When the control animals are compared across studies, there is a reduction in GSH:GSSG from chronic thiol depletion (chapter 2, 11.68±1.16) compared to the acute GSH supplementation study (chapter 3, 9.60±0.33) and from the acute depletion study (chapter 4,

16.01±1.54). The age and GSH dependent effects have been discussed and summarized above. When the sensitivity to BK is also examined, there is a similar trend seen such that the youngest animals had the lowest EC_{50} (-8.42±0.08 LogM) compared to the 37.5 week old animals (-8.06±0.04 Log M) and the 65 week old animals (-8.20±0.15 Log M). Taken together, despite the apparent age discrepancy across the studies, this allowed the unique finding that GSH,GSSG and the GSH:GSSG ratio may influence sensitivity to endothelium-dependent dilation in an age-dependent manner. This conclusion may help to explain the reduced dilation to NO and H_2O_2 seen in aging (95), possibly as a function of altered GSH:GSSG ratio and therefore redox signalling (163, 173). Furthermore, despite the age-effects seen in chapter 2 it could be postulated that the age-dependent effects observed are not as robust as they could be had younger rats been utilized instead of the adult rats. Despite this limitation the array of ages used in the current studies allowed for the comprehensive analysis provided throughout this discussion, and allowed certain age-dependent observations to be made.

The Langendorff apparatus also provides a potential for limitation as the specific effects of the agents across the entire vascular network is assessed. Thus changes that occur within the coronary microcirculation cannot be distinguished from changes that occur in the larger coronary arteries or arterioles (115, 153, 168, 176). For example Szekeres found that NOS inhibition was more effective in the arterioles compared to the arteries (168), this could explain the inability of LNAME to significantly impair maximal dilation to BK as other vascular segments could be dilating to a greater extent to compensate for the effect of NOS inhibition in the arterioles. Additionally Sellke and colleagues (1991) demonstrated that larger coronary microvessels were sensitive to EA whereas smaller coronary microvessels were not (153). It could be postulated that the responsiveness of certain branch orders of the coronary circulation in the current preparation were more or less responsive to the different manipulations used. The inhomogeneity in responsiveness in the coronary circulation could make the results difficult to interpret, given the multiple and redundant pathways that exist (115, 176).

However the advantage of the isolated perfused heart is in fact the ability to assess changes across the entire vascular bed as would occur *in vivo*. While this technique may lack precise mechanistic insights into a specific portion of the coronary vascular network, it provides great detail on the global responses of the coronary circulation. These results provide an integrative description of how changes in GSH and/or GSSG alter total coronary vascular resistance and endothelial function.

5.4.2 Future directions

The results obtained in the presence of thioredoxin (TRx) inhibition are intriguing as altered TRx may be responsible for several different cardiovascular diseases (86, 191) including hypertension (55). Most of the focus on endothelium-derived contracting factors has suggested a role for H_2O_2-mediated alterations in prostacylin/IP receptor pathway and dysfunction (48, 73, 75, 169). In the current experiments, work with selective COX inhibitors did not abolish the recontraction seen in the presence of CDNB. However, using the SOD mimetic TEMPOL, which is believed to increase H_2O_2 (188), it was demonstrated that the recontraction was enhanced. This suggests that the recontraction was independent of the COX pathway and enhanced by H_2O_2. More rigorous experiments are required to determine if these effects are specific to H_2O_2 as the results presented here are speculative. Additionally, downstream targets involved in the H_2O_2 pathway need to be investigated including the ERK pathway and Rho Kinase as both appear to be involved in ROS mediated coronary artery constriction (69). Therefore, future work should examine if the presence of catalase and selective Rho Kinase inhibitor Y27632 can attenuate the recontraction seen with CDNB. Changes in TRx are observed in the heart and aorta of hypertensive rats (170). Additionally there are several pathophysiological pathways of hypertension that TRx influences (55), and TRx-2 overexpression (mitochondrial specific) can be used to prevent angiotensin II mediated hypertension (187). Taken together TRx appears to be a key regulator of vasomotor function. The current study outlines a marked dysfunction in the intact

coronary circulation. Future work needs to clarify the pathway leading to this dysfunction as well as how changes in TRx may influence vasomotor dysfunction in cardiovascular disease states. Potential experiments that would provide further insight into this include examining the effects of CDNB on the vasomotor responses in the hearts from SHR and WKY animals.

In the study of chronic GSH depletion there was an increased eNOS protein content which was attributed to elevated H_2O_2 (53, 195). H_2O_2 causes upregulation of arginase activity (172), which has been shown to be partially responsible for the age-mediated reduction in endothelial function (20, 43, 186). Therefore, if arginase is increased the reduction in L-arginine would result in eNOS uncoupling and greater ROS production. The increase in eNOS activity and reliance on NO in the BSO treated animals may in fact be deleterious long term. Despite the increase in catalase expression, H_2O_2 production was still elevated likely because of altered GSH content. Thus, the initial increase in eNOS may represent a early adaptive response that has deleterious consequences long-term as reductions in eNOS cofactors contribute to endothelial dysfunction through uncoupling of eNOS and increased ROS production (173). Thus, the effects of longer term BSO treatment on coronary artery endothelial function needs to be addressed to determine whether this is a transient effect. Increased reliance on NO has been reported to occur in isolated perfused hearts in hypertension (96, 97); however, as disease progresses, reductions in endothelial function are observed (17). Thus, the long-term effects of GSH depletion on the coronary circulation need to be addressed.

Another potential line of investigation would be an analysis of the age-dependent changes that occur in GSH:GSSG and how these changes influence endothelium-dependent dilation in the coronary circulation. As discussed above differences were observed in the GSH:GSSG ratio across the different studies, and it appears these changes are associated with altered sensitivity to endothelium-dependent dilation, as well as total dilatory capacity. This would add greater insight into the age dependent changes that occur in healthy human aging (11, 114). Included in this could be an

examination of how changes in GSH:GSSG contribute to the altered dilatory pattern seen with aging (95) with a specific focus on the H_2O_2 dependent component.

In the acute GSH administration study, there appears to be a ROS-dependent component that is required for GSH to exert its effects. Future studies could utilize ROS generating pathways (xanthine/xanthine oxidase, or direct H_2O_2 administration) to further examine these effects. Similarly a chronic model of oxidative stress could be employed by using SHR and WKY rats, although the 10 µM concentration utilized in this study may attenuate the baseline CVR in the WKY not the SHR (Figure A9). It was postulated that a potential effect was a result of the thiolation of SERCA or through a non-NO non-prostanoid-dependent pathway. Interestingly, both could require the formation of RSNO. Given the effects observed with the GSH alkylating agent EA which putatively prevents the *de novo* synthesis of RSNO, it would be interesting to examine if EA augments the GSH dependent effect or conversely if GSH infusion can augment the EA effect.

The influence of GSH infusion on the ability to blunt coronary constriction was not examined. Given that GSH attenuates endothelium-dependent contraction in humans with spastic angina (103), the effects of GSH infusion on agonist mediated vasoconstriction would also be warranted. This would allow further assessment of the ROS requiring effects of GSH to be examined on a non-dilatory pathway.

CHAPTER 6

6.0 Summary and conclusions

The results of the present studies provide novel mechanistic insights into the interaction between GSH, GSSG, and the GSH:GSSG on the intact coronary vascular bed. It was demonstrated that aging and thiol depletion differentially impact coronary vascular resistance and endothelial-dependent dilation. Aging is associated with a reduced ability to generate spontaneous vascular tone, and as a result, total dilatory capacity is reduced. This does not appear due to an alteration in NO production as when these hearts are exposed to flow rates designed to mimic *in vivo* flow rates LNAME induced a similar degree of elevated CVR in the adult and old animals. Conversely, thiol depletion resulted in an age-dependent increased reliance on NO. This was demonstrated by the enhanced constriction (increased CVR) and greater impairment in BK-mediated dilation compare to all other groups. Additionally, it was determined that H_2O_2 does not appear to modulate the preserved vasomotor function with BSO treatment that has been recently reported (90). Unlike the conduit vasculature (13) the coronary circulation differentially adapts to chronic thiol depletion by increasing reliance on nitric oxide. Thus chronic thiol depletion results in an age-dependent increased reliance on NO and does not augment the vascular responses seen with increased age.

In response to acute GSH infusion, at a physiologically relevant concentration that does not alter baseline CVR, it was demonstrated that the enhanced BK-mediated dilation was independent of NO and its downstream target sGC, as enhanced sensitivity persisted despite NOS and/or sGC inhibition. It was determined that ROS was required for GSH to exert its effect. This is in agreement with previous studies (5, 104, 138) and extends them by suggesting that the elevated ROS seen in cardiovascular disease allows the GSH effect to be more pronounced.

The method of acute thiol depletion is a major determinant of how alterations in the GSH:GSSG ratio impact coronary vascular resistance and endothelium-dependent dilation. In the presence of both thioredoxin inhibition and GSH alkylation there were increases in baseline CVR and marked reductions in endothelial-dependent dilation. In the presence of CDNB, a recontraction was observed at the higher concentrations of BK which were independent of COX, yet enhanced in the presence of ROS scavenging. Conversely, the altered dilation in the presence of EA was not altered to the same extent with LNAME as the control condition, suggesting that *de novo* NO synthesis does not account for the altered dilation. This finding extends earlier work regarding the role of RSNO in the dilation to BK in the intact coronary circulation (39) and suggests that in this preparation the inhibition of RSNO formation and replenishment play a role in determining both baseline CVR and endothelium-dependent dilation in the intact coronary vascular bed. In response to BCNU, there was a marked reduction in total dilatory capacity as baseline CVR was slightly attenuated, and minimal CVR was slightly elevated.

Cross study analysis revealed strong inverse correlations between GSH and GSSG and both baseline and minimal CVR, while no correlations were found between either oxidized or reduced glutathione and any of the curve parameters measured. Therefore it is likely that changes in overall glutathione content play a role in determining baseline and minimal coronary vascular resistance. One intriguing finding is that it was either GSH or GSSG that were highly correlated with these measures, the GSH:GSSG ratio was not as strongly correlated with either baseline or minimal CVR. This likely reflects the age-dependent changes that were observed in this ratio that occurred across the studies.

6.1 Implications

The primary focus of this collective work was on NO bioavailability in response to changes in GSH. The results of these studies clearly demonstrated that changes in GSH influence baseline, and minimal CVR, and posses the ability to augment BK-mediated dilation. Therefore, the primary conclusion is that GSH possess the ability influences several aspects of the production and destruction of NO and that depending on the nature of GSH manipulation, the effects are divergent. It was further demonstrated that alterations in the content of both GSH and GSSG play significant role in the setting of baseline and minimal coronary vascular resistance, thus impacting total dilatory capacity. The nature of the availability of GSH and altered GSH:GSSG ratio is complex as is evident from the studies described within. The importance of these findings extends beyond the *in vitro* nature of these studies as the results have implications for understanding disease process and potential therapeutic effects of GSH. The importance of regulating GSH, GSSG and the GSH:GSSG is highlighted by the potential impact that its reduction has on minimal CVR which has implications for adequate cardiac perfusion. The ability of GSH to augment endothelium-dependent dilation suggests that GSH may be under-utilized as an adjunct for therapy for individuals with cardiovascular disease. There is an age-dependent adaptation that occurs during GSH depletion. This highlights the importance of healthy aging and suggests that the ability of the vasculature to adapt to an additional oxidative stress in conditions of aging is reduced. The ability of GSH to have such divergent affects on both endothelium-dependent dilation and coronary vascular resistance highlights the importance of this work.

A reduction in endothelium-dependent dilation is a hallmark sign of cardiovascular disease states and is often attributed to a reduction in NO bioavailability. This loss of NO bioavailability is also associated with cardiovascular disease progression as NO plays a significant role in maintaining vascular wall homeostasis. These studies clearly illustrate the ability of GSH to enhance, or reduce

vasomotor dysfunction, but also highlight the ability of GSH to alter coronary vascular resistance. The role that glutathione plays within the vascular wall is complex and this collective work has provided additional mechanistic insight into the role that changes in the GSH content plays within the coronary circulation.

These findings provide a new framework and understanding of how manipulations of GSH impact that pathophysiology of coronary vascular resistance and endothelium-dependent dilation in the intact coronary circulation. Thus providing novel insights into the understanding both the pathological consequences of altered GSH, and the ability of GSH to augment endothelium dependent dilation in cardiovascular disease states.

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APPENDIX

Supplementary Data and Figures

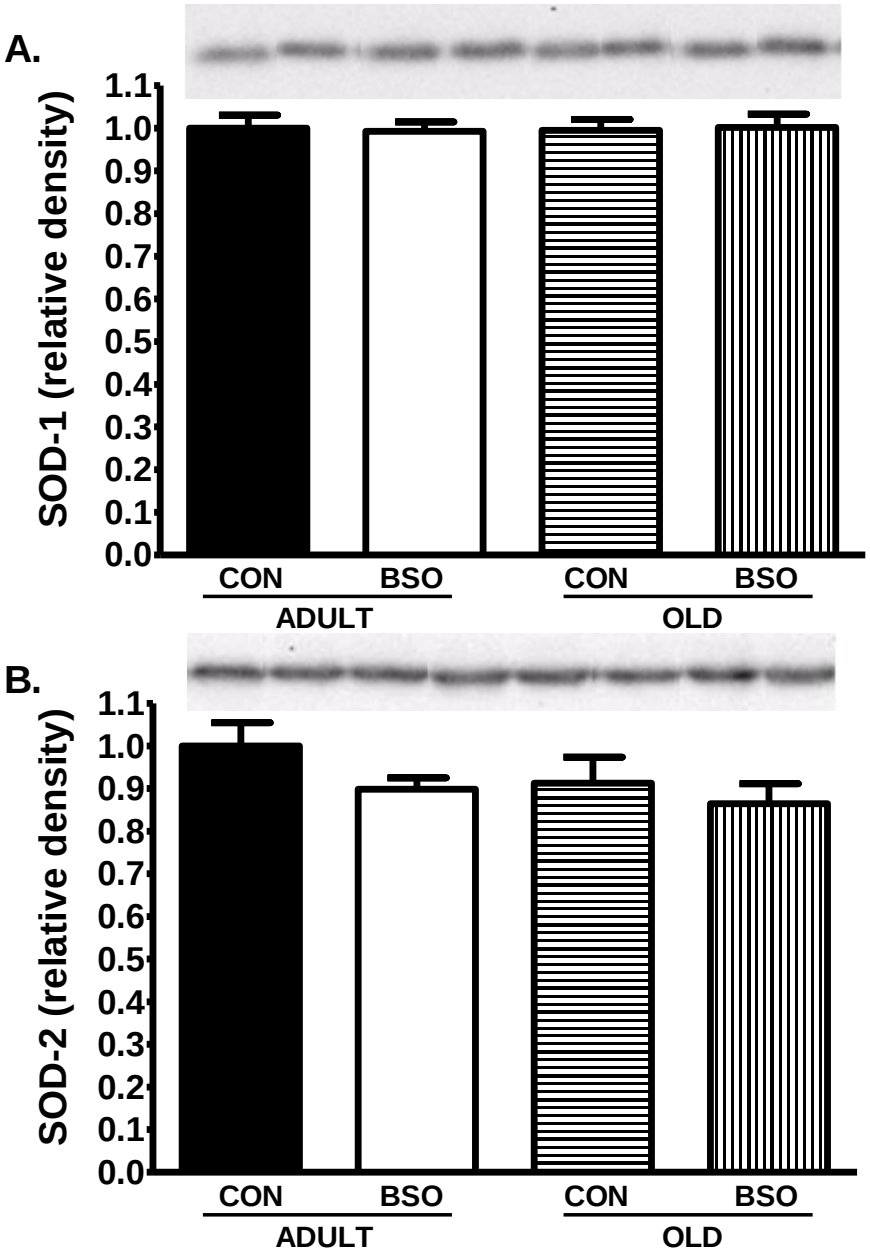

Figure A1 Immunoblots analysis for superoxide dismutase.

No Differences were seen across all groups for either SOD1 (A) or SOD2 (B) Above each graph is a representative immunoblot of n=2/group in the order that they appear on the graph. Data are presented as mean±SE (n=9/group).

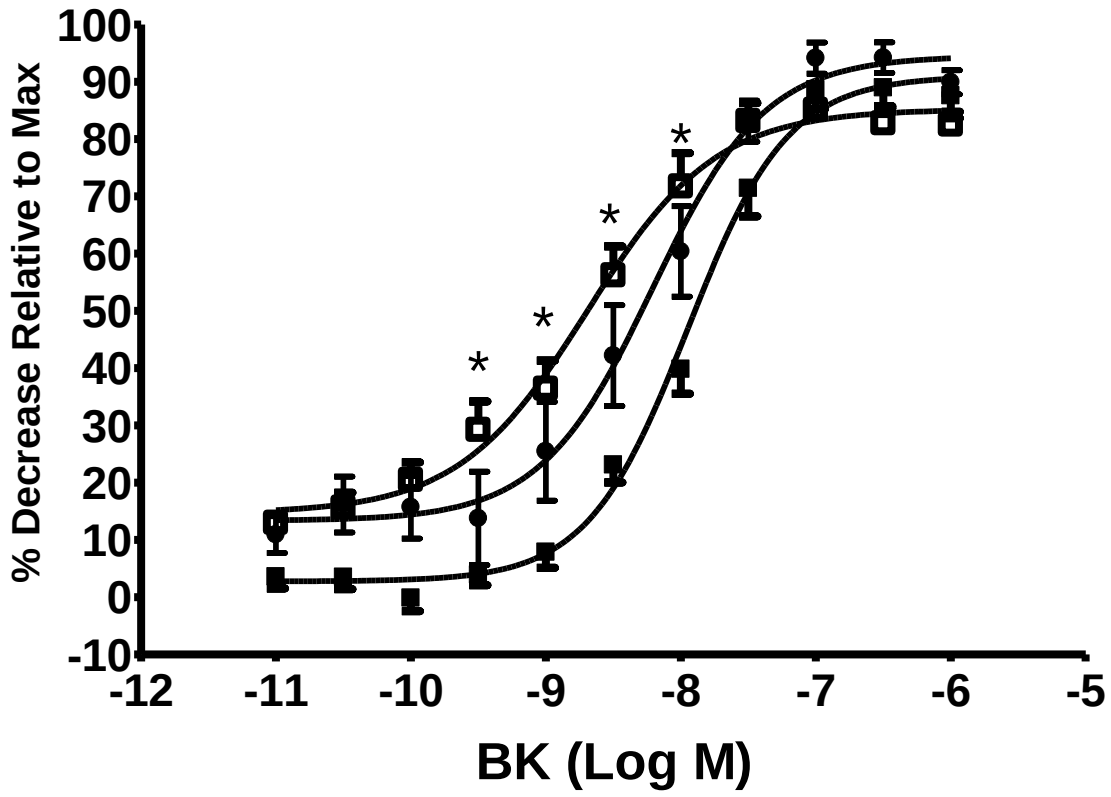

Figure A2. BK Dose response curve in the Con, 1 and 10 µM GSH

Dilatory curves from the control (■), GSH_{10} (□) and 1 µM GSH (●). GSH_1 was not statistically different from the control group although this concentration did slightly enhance the EC_{50} and AUC but not max. *, $p<0.05$ vs control. Data are presented as mean±SE (n=7/group).

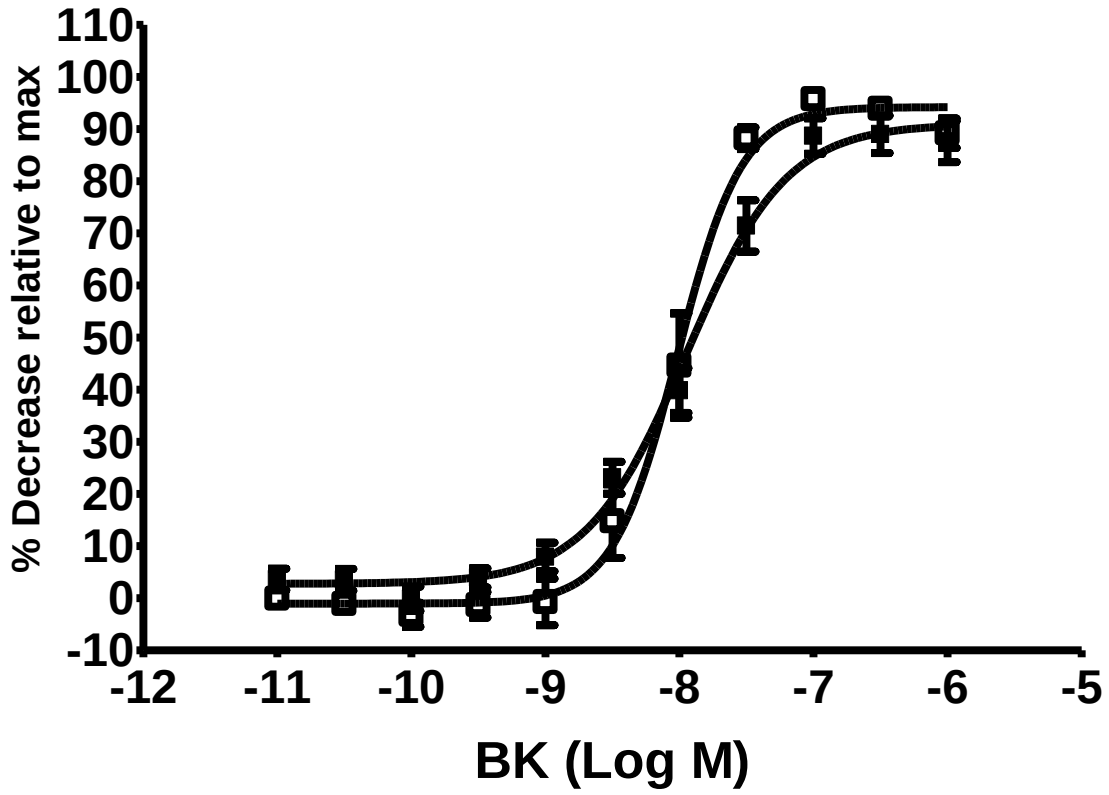

Figure A3 Bradykinin induced dilation in the presence of ODQ.

Data are mean±SE (n=6-7/group), for Control (■) and ODQ 10 μM (□). No Differences were observed in maximal dilation, sensitivity or EC_{50}.

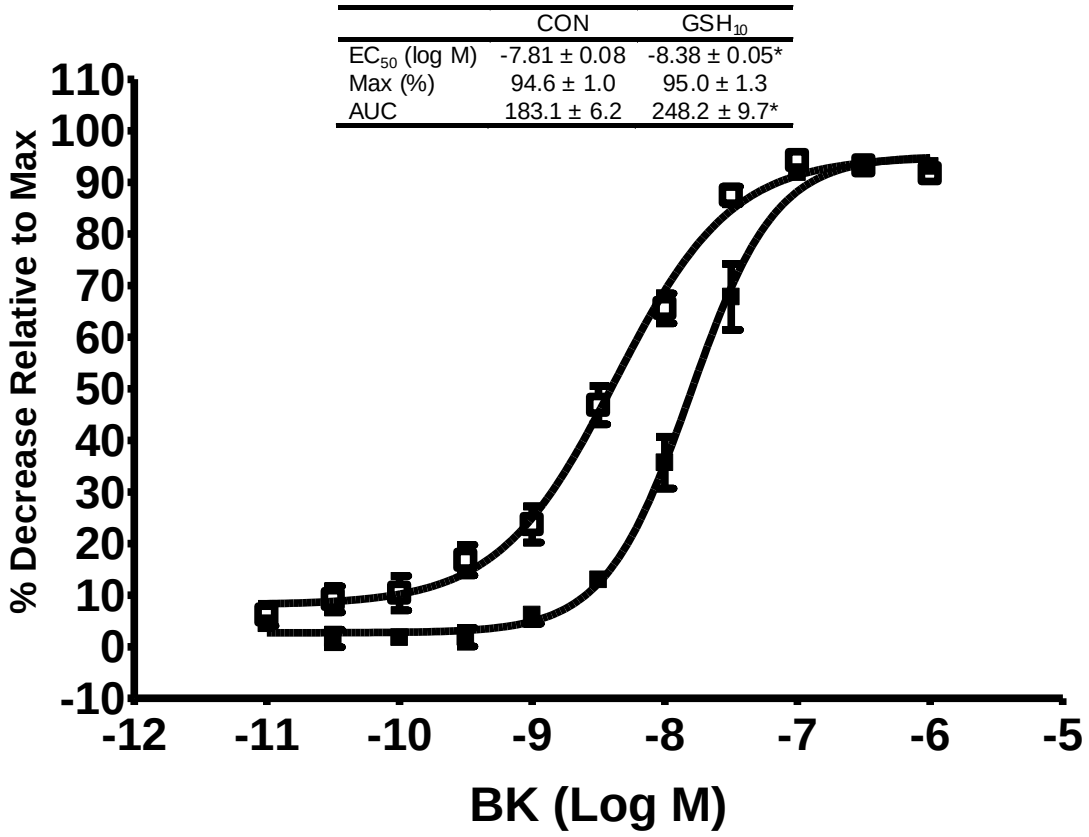

Figure A4. BK Dose response curve in the presence of LNAME and ODQ.

Dilatory curves from the control (■) and GSH_{10} (□) in the presence of LNAME (0.1 mM) and ODQ (3 μM) the GSH_{10} effect persisted. Data are presented as mean±SE (n=6/group).

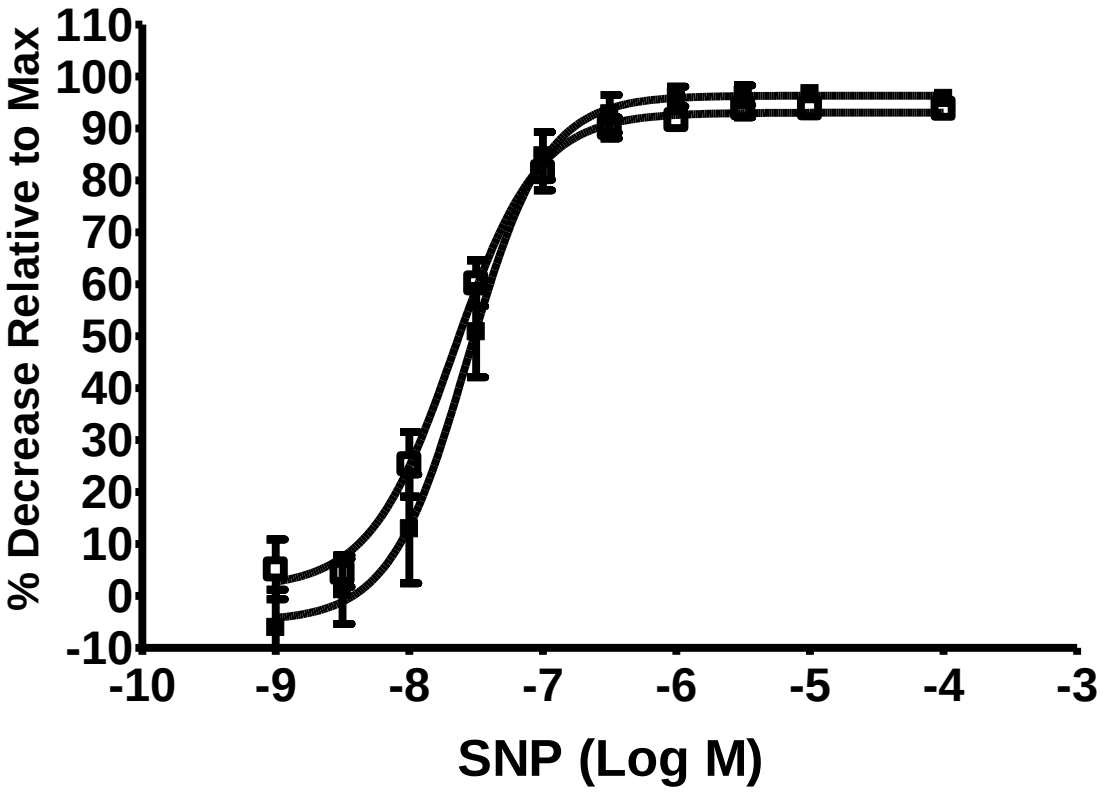

Figure A5 SNP induced dilation in the presence of TEMPOL

The presence of TEMPOL 0.1 μM did not significantly alter the SNP mediated dilation in either the Control (■) or GSH_{10} (□) groups (n=3/group).

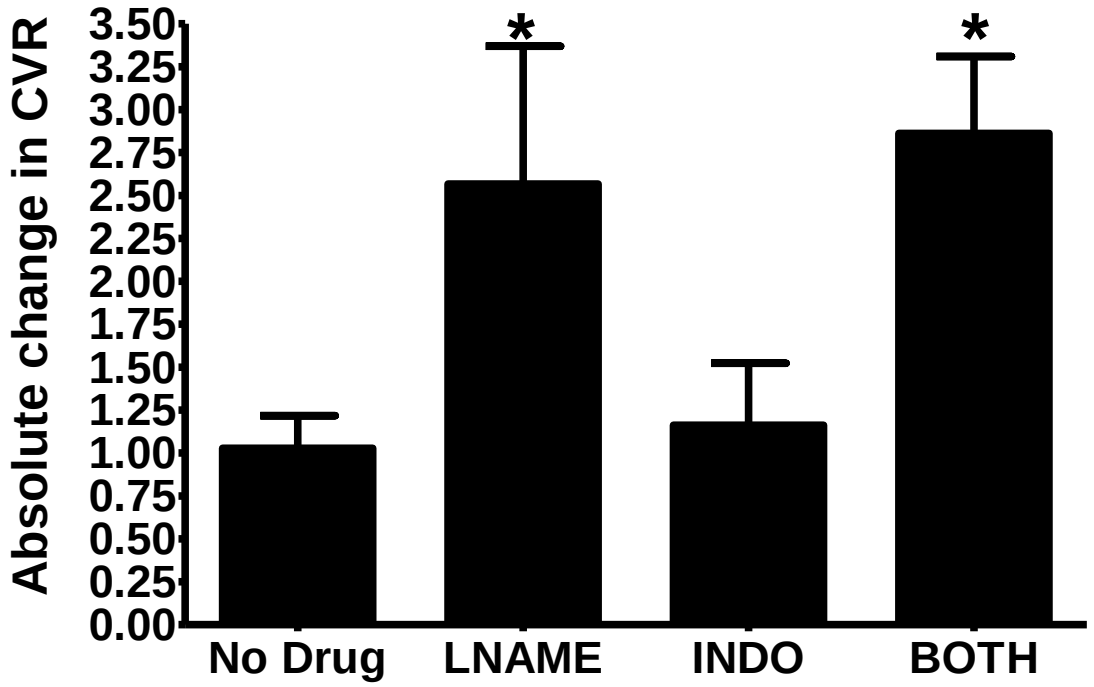

Figure A6 Effect of combined LNAME and indomethacin on change in CVR

BK (0.1 μM) induced decrease in CVR. In the presence of INDO there was no difference compared to the control (no drug) response. LNAME resulted in an increased change in CVR (owing to the higher starting point). In the presence of both INDO and LNAME (BOTH) the decrease in CVR was similar to LNAME alone. *$p<0.05$ vs No Drug. Data are mean±SE (n=7-17/group).

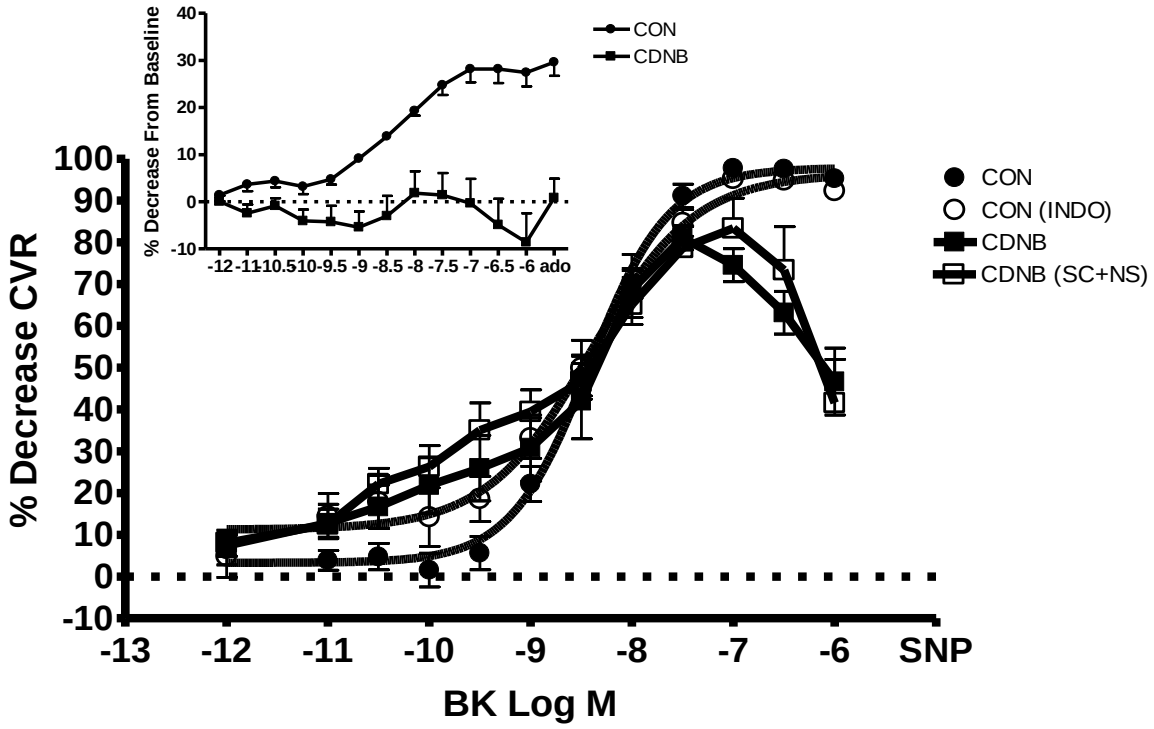

Figure A7. Effect of cyclooxygenase inhibition on BK dose-response in control and CDNB treated hearts

Hearts were allowed to equilibrate for 30 minutes in the presence of INDO (10^{-5} M) followed by dose-response curve to BK. Data are from the CON (●), CDNB (■) in the absence of INDO or presence INDO control (○) or SC560 (3×10^{-7} M) + NS398 (10^{-6} M) CDNB (□). In the presence of dual COX-inhibition in both groups the responses are not statistically different from the respective no drug condition. The insert shows the dilation relative to baseline from CON (●), CDNB (■) both in the presence of INDO (10^{-5}M). As is evident from this graph CDNB was unresponsive to endothelium-dependent and independent dilation in the presence of INDO. All data is presented as mean±SE (n=5-7/group).

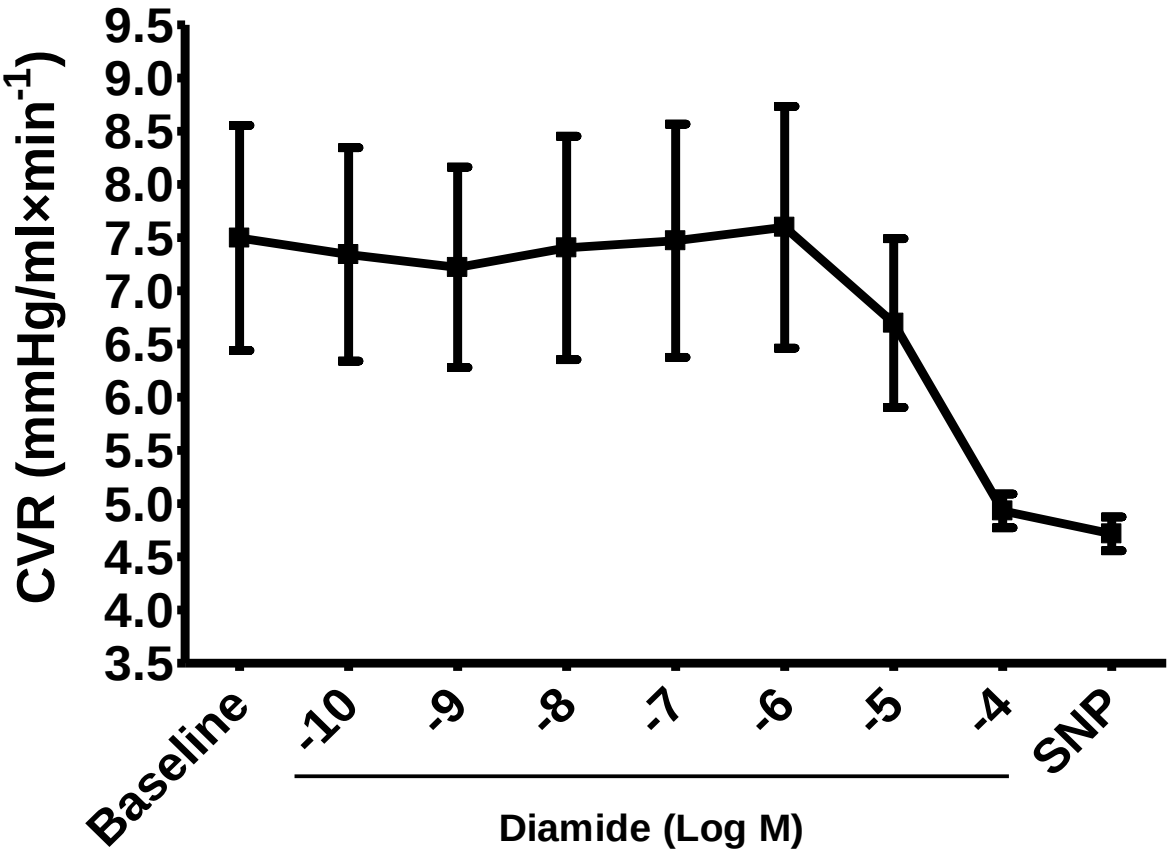

Figure A8 Diamide dose-response curve

Hearts were allowed to equilibrate for 30 minutes followed by dose-response curve to diamide. Following the highest concentration of diamide, SNP was used to dilate the coronary vasculature. Similar to the data from the BK dose-response curves in the presence of diamide at either 1 or 10 µM, minimal CVR was ~4.7±0.16 mmHg/ml×min^{-1}. All data are presented as mean±SE (n=3).

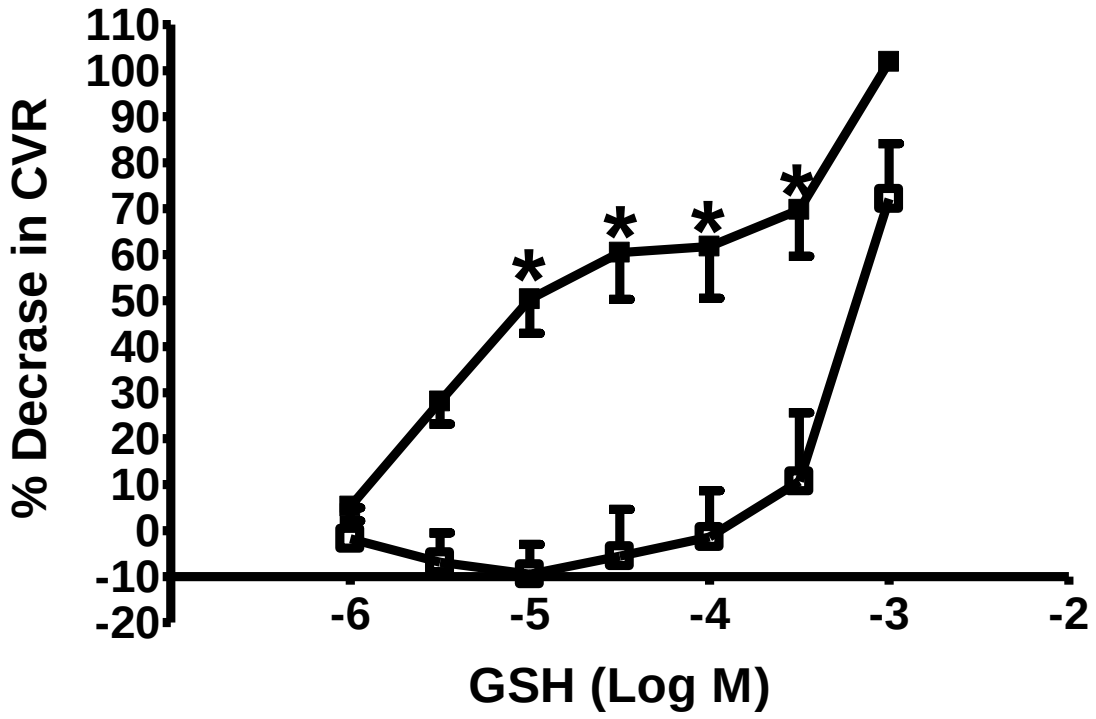

Figure A9. GSH dose-response curve from SHR and WKY rats

Dilatory curves from the control WKY (■) and hypertensive SHR rats (□). *p<0.05 vs WKY. Data are presented as mean±SE.