THE ROLE OF PUTATIVE NOX INHIBITORS IN HOMOCYSTEINE-INDUCED VASCULAR DYSFUNCTION

By

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Doctor of Philosophy

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I. Abstract:

Excess plasma homocysteine (Hcy; hyperhomocysteinemia, HHcy) remains an independent risk factor for cardiovascular disease (CVD) and treatments remain elusive. The source of Hcy, methionine, is an essential amino acid acquired by ingestion of animal foods. Normal methionine metabolism effectively removes Hcy via recycling back into methionine or excretion via the kidney. However, in aberrant methionine metabolism, Hcy accumulates and causes damage to the vascular system by increasing oxidative stress; the exact mechanism of how this occurs is unknown. Importantly, the B vitamins B_6, B_9 and B_{12} are essential to proper methionine/Hcy metabolism and are often found in low levels in patients presenting with HHcy; this has provided a potentially viable treatment strategy in the clinical setting. Disappointingly, clinical trials administering B vitamins to reduce HHcy have been unsuccessful in reducing CVD and treatments continue to be sought.

The NADPH oxidase (Nox) family of enzymes are expressed in a broad range of cell types throughout the body and are the primary source of superoxide (Nox1, Nox2) and hydrogen peroxide (Nox4) within the vasculature under both physiological and pathological conditions. Nox1, 2 and 4 are of primary interest in vascular disease, as there is evidence that Hcy can interfere with the proper function of Nox1, 2 and 4 signalling, potentially leading to an over-expression of pro-oxidants. Nox1, 2 and 4 have been implicated in vascular disease (endothelial dysfunction), hypertension, vascular inflammation, stroke, diabetes, and atherosclerosis, and putative inhibitors of these enzymes are now available. Additionally, nitric oxide (NO) is also measured as a marker of proper vascular function; indeed, the current gold standard of assessing NO availability is by indirectly measuring vascular responses to acetylcholine. Accordingly, a loss of NO bioavailability is linked to the development of many of the same vascular pathologies caused by HHcy and also potentially increased Nox1, 2, and 4 activity.

Thus, this thesis examined if current putative Nox inhibitors could prevent vascular dysfunction caused by homocysteine (as indirectly measured by acetylcholine-mediated vasorelaxation). Using New Zealand white rabbits, C57BL/6 mice and a $Nox2^{-/-}$ (C57BL/6 background) mouse models, we observed that pharmacological intervention with single Nox1, 2 and 4 inhibitors reduced the effect of acetylcholine on vasorelaxation. In 1% methionine-fed $Nox2^{-/-}$ mice, we observed an improvement in function. We also assessed combinations of Nox1, 2 and 4 inhibitors and found that, although function was not restored to control levels, it was improved compared with single Nox inhibition. Due to these results, we performed a gp91ds-tat dose response in rabbit aorta. We found that in our models of vascular dysfunction, lower doses of gp91ds-tat significantly improved acetylcholine-mediated vasorelaxation. These results showed for the first time that in both pharmacological and diet-induced HHcy, high dose putative Nox inhibitors might not be effective.

In conclusion, the observations made in this thesis highlight the important role that Hcy plays in the redox balance in the context of vascular function. Future work in this area should focus on low dose Nox inhibition in Hcy induced disease in *in vivo* models, in order to better determine which drug can be used to HHcy induced vascular damage.

II. Doctor of Philosophy Student Declaration

"I, Renee Melissa Smith, declare that the PhD thesis entitled The Role Of Nox1, 2 And 4 Inhibition In Homocysteine-Impaired Acetylcholine-Mediated Vasorelaxation is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work".

Signature  **Date** 03/09/2018

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To Leon

This thesis would not have been possible without your
constant guidance, companionship, and support.
I will be forever grateful, thank you.

Contents

IV. List of Abbreviations

Abbreviation	Explanation
ACh	Acetylcholine
ANOVA	Analysis of variance
CβS	Cystathionine β synthase
CaCl	Calcium chloride
CAD	Coronary artery disease
CVD	Cardiovascular disease
DAB	3,3'-Diaminobenzidine
DPI	Diphenylene iodonium
EC_{50}	Half maximal effective concentration of a drug
ED	Endothelial dysfunction
eNOS	Endothelial nitric oxide synthase
g	Grams
GKT	GKT137831
GP	Gp91ds-tat
H_2O	Hydrogen dioxide
H_2O_2	Hydrogen peroxide
HDL	High-density lipoprotein
Hcy	Homocysteine
HHcy	Hyperhomocysteinemia
iNOS	Inducible nitric oxide synthase
I/U	International units
HUVECs	Human umbilical vein endothelial cells
KCl	Potassium chloride
KH2PO4	Potassium dihydrogen phosphate
L	Litre
LDL	Low-density lipoprotein
µM	Micromolar concentration
mM	Millimolar concentration
M	1 Molar concentration

MgSO4	Magnesium sulfide
ML	ML-090
MTHFR	Methylenetetrahydrofolate reductase
NaCl	Sodium chloride
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NaHCO3	Sodium hydrocarbonate
NO	Nitric oxide
NOS	Nitric oxide synthase
Nox	Nicotinamide Adenine Dinucleotide Phosphate Oxidases
NOX	$Nox2^{-/-}$ transgenic mice
O_2	Oxygen
O_2^-	Superoxide
OS	Oxidative stress
PBS	Phosphate buffer solution
PFA	Paraformaldehyde
Phen	Phenylephrine
Pi3K/Akt	Phosphatidylinositol 3-kinase/protein kinase B
ROS	Reactive oxygen species
RT	Room temperature
SEM	Standard error of the mean
Ser^{1177}	$Serine^{1177}$
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
Thr^{495}	$Threonine^{495}$
TrisHcl	Trisaminomethane
WT	Wild type
VAS	VAS2870

Chapter 1

1.1 Thesis summary

The pathological effect on the vasculature due to excess plasma Hcy has been known since the early 20th century, yet although efforts have been made to treat this disease, there is still no treatment to improve CVD. Work by Dr. Zulli and others had already provided evidence that Hcy causes vascular dysfunction in an *ex vivo* rabbit model. Further, Zulli and colleagues also reported that Hcy and eNOS are inextricably linked, with Hcy hypothesised to interfere with eNOS enzymatic activity, although the mechanism remains unknown. The finding that reductions in vascular function were related to the endothelial layer highlight the essential role of NO in normal vascular function. The evidence that membranous signalling Nox enzymes are the primary source of ROS within the vasculature under both physiological and pathological conditions, led us to hypothesise that if putative Nox inhibitors are used in HHcy, this could improve function in the vasculature and thus be used to treat HHcy. Separately, Hcy and Nox, particularly Nox2, have been implicated in the development and ongoing dysfunction in impaired acetylcholine-mediated vasorelaxation. We hypothesised that Nox might be directly implicated in Hcy-induced endothelial dysfunction. We tested these hypothesises with different models: pharmacological Nox inhibition in rabbits and mice and Nox2 deletion in mice using acute (in vitro) and chronic (dietary) HHcy. In rabbits, in both acute and chronic models, Hcy-impaired acetylcholine mediated vasorelaxation, which worsened with putative Nox inhibitors. This was dependent on the inhibitor used. We subsequently found that when a superoxide donor or Akt activator was used, the effect of gp91ds-tat was blunted. Interestingly, Nox2$^{-/-}$ mice were protected from impaired function when fed a 1% methionine-rich diet, whereas in other mice we observed reduced acetylcholine-mediated vasorelaxation. We also investigated, using semi-quantitative immunohistochemistry, the effect that Hcy and/or gp91ds-tat has on eNOS phosphorylation. We found that Hcy plus gp91ds-tat reduces

eNOS phosphorylation in the acute Hcy rabbit at Ser^{1177}, an position that requires increased phosphorylation with concomitant reduction in phosphorylation at Thr^{495}. Interestingly, in the acute Hcy rabbit model, phosphorylation was decreased at Thr^{495}.

As the thesis evolved, we reduced the dose of gp91ds-tat in our acute model, which improved aortic function.

In conclusion, the observations made in this thesis establish the foundation to further study low dose gp91ds-tat in a pathological model, which could be a promising avenue for reducing CVD in HHcy.

1.2 General Introduction

Cardiovascular disease (CVD) is the leading cause of mortality and morbidity in the world (Lozano et al. 2012). CVD is the umbrella term that describes the major diseases of the heart and blood vessels; including coronary artery disease (CAD), increased blood pressure, peripheral artery disease and atherosclerosis (Epstein 1996). Classic CVD lifestyle risk factors contributing to the development and augmentation of morbidity and mortality outcomes include smoking (Bild et al. 2014), obesity (Coppieters & von Herrath 2014), insulin resistance and Type 2 Diabetes Mellitus (T2DM),(Cannizzo et al. 2012; Hills et al. 2004) and lack of physical activity (CADTH 2014). Diet is a major factor in CVD risk, with public policy advising on a reduction of foods high in saturated fat, refined sugars and starches, advice which has been in place since 1980 (HHS & USDA 1980; NHMRC 2017). Dietary guidelines such as these are part of a CVD prevention strategy, as by reducing the risk of CVD events, such as heart attack and stroke, the economic burden may also be lessened (Baer et al. 2014; Bowen et al. 2018; Flueckiger et al. 2016; Roth et al. 2017). It is established that diet contributes to the risk of CVD incidence (Iacono et al. 1975; Kannel & McGee 1979), and cholesterol was highlighted as a potential associated factor (Doyle et al. 1957; Jolliffe 1959; Lawry et al. 1957; Mann et al. 1955). From this evidence, the targeting of single macronutrients, such as the recommendation to reduce saturated fats with carbohydrates, justified the implementation of the dietary guidelines. This advice has now been replaced with a broader guideline, focusing instead on improving the dietary pattern as a whole, as it is now thought that a wide variety of healthy foods can improve CVD risk from a dietary perspective (Baer et al. 2014; Liu et al. 2017). Importantly, homocysteine (Hcy), a sulphur-containing amino acid by-product of methionine metabolism, remains an independent risk factor for increased CVD risk and dietary intervention has not proven successful in clinical trials to date (Clarke, Robert et al. 2014; Debreceni, Balazs & Debreceni 2012; Hodis et al. 2009; Lonn et al. 2006; Saposnik et al. 2009;

Toole et al. 2004). Strategies to reduce risk and improve outcomes involve pharmacological administration and lifestyle changes, and, depending on the severity of the disease, surgical intervention. Lifestyle modifications aimed at reducing CVD symptoms complement pharmacological treatments such as statins to reduce cholesterol (Lee, H-Y et al. 2014), diuretics to reduce blood pressure (Schaffer et al. 2014), in addition to policies for dietary modification, such as reducing saturated fatty acids and carbohydrates to improve the total HDL and LDL cholesterol ratio (Baer et al. 2014).

The burden of CVD across the world has varying impacts. A recent large-scale study assessing the relationship between sociodemographic regions and CVD risk found that while there has been a sharp decline in CVD in high socio-economic regions over the last 25 years, lower socio-economic regions showed either no change or an increase in incidents (Roth et al. 2017). This brings into focus the increasing economic burden of CVD, which is of particular importance to nations without the means to investigate preventative measures aimed at reducing CVD development. Importantly, a recent report using modelling to investigate the cost benefit of preventative screening for CVD in the USA, found that hypertension screening and aspirin counselling saved money per patient (Dehmer et al. 2017), while a 2009 review reported that combined for the USA, Canada and Europe, approximately 600 billion was spent on the total cost of CVD for 2006 (Tarride et al. 2009).

1.3 Pathogenesis of Atherosclerosis

Atherosclerosis is essentially an inflammatory condition, caused by events that result in excess oxidative stress (Sorescu 2002) leading to vessel remodelling (Moraes et al. 2010). Initially, damage occurs at the site of attack and triggers a normal inflammatory response involving monocyte and leukocyte recruitment, which repair the area, and, in a normal response, leave the area (Raggi et al. 2018). As repeated attacks occur, the vascular wall undergoes modification which triggers ongoing aggregation and proliferation of lipoprotein-filled

macrophages which first become foam cells and then, over time, become fibrotic (Burke et al. 2001; Miida et al. 2014). Acting in a positive feedback mechanism, damaged cells continue to cluster in the arterial vessels and provoke an aggressive and repetitive immune response, resulting in deposition of lipid and smooth muscle actin (Moraes et al. 2010). The pro-inflammatory response further endangers susceptible plaque caps via degradation of the cap by proteolytic enzymes, and after rupture the contents travel to the heart, lungs or brain (Andreou et al. 2015; Burke et al. 2001; Miida et al. 2014). Plaque instability increases the risk of stroke and myocardial infarction (Crucet et al. 2013) and is used as a predictive risk of CHD events (Amato et al. 2017; Nambi et al. 2010). The significant damage to the endothelium results in an overall change in the ratio of the delicate ROS balance, causing increased O_2^- and reduced NO, characterised as endothelial dysfunction (ED) (Cai, H & Harrison 2000; Quesada et al. 2015). ED directly affects the normal function of acetylcholine by reducing the amount of available NO and impairing vasodilation (Kobayashi et al. 2004; Lee, J et al. 2016).

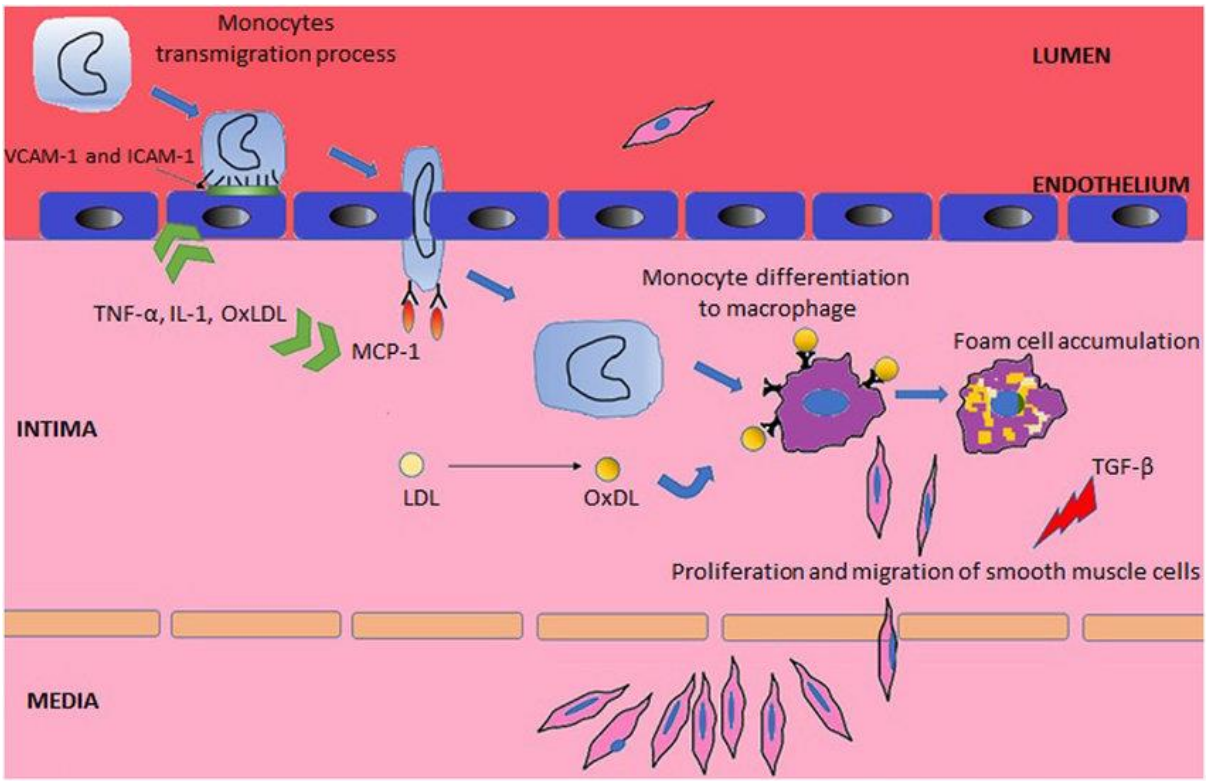


Figure 1.1. Atherosclerosis can occur as a result of aberrant inflammatory regulation. The steps of atherosclerosis are varied and rely on several factors, including the deposition of oxidised low-density lipoproteins and the differentiation of monocytes into macrophages (Teixeira & Tam 2018).

1.4 Risk Factors for Atherosclerosis

As a multi-factorial vascular disease, atherosclerosis can develop under a number of conditions, indeed this contributes to the difficulty in both treating and reducing its burden. There are multiple risk factors associated with developing atherosclerosis: T2DM, smoking, hypertension, obesity, sedentarism and hyperlipidaemia; recently, changes to gut bacteria have also been identified as a novel risk (Bays 2014; Bild et al. 2014; Khan et al. 2014; Mudau et al. 2012; Nyyssonen et al. 2012; Steed & Tyagi 2011; Tan et al. 2012). There are currently no effective treatments in place for atherosclerosis, only preventative policies addressing lifestyle behaviours, such as smoking cessation, physical activity and dietary modification, particularly to reduce low-density lipoprotein cholesterol (LDL-C) (Bild et al. 2014; Bowles & Laughlin

2011; Eijsvogels et al. 2016; Kalanuria, Nyquist & Ling 2012; Nyyssonen et al. 2012; Tejani et al. 2014; Thompson et al. 2003). Establishing an exact pathophysiological mechanism from these risk factors has been very difficult. HHcy is supported as a causal factor involved in the development of atherosclerosis, although how impaired methionine metabolism impacts on the development of atherosclerosis remains unclear (Hoffman 2011; Jiang et al. 2012; Julve et al. 2013). Importantly, despite the indication of a relationship between atherosclerosis and HHcy, the available evidence does not support the hypothesis that attempting to reduce HHcy levels with B vitamins reduces disease (Debreceni, Balazs & Debreceni 2012; Lonn et al. 2006). Elevated plasma Hcy levels, both moderate and severe, might increase the risk of adverse atherothrombotic events in humans: animal studies support this, such as in apolipoprotein E knockout ($ApoE^{-/-}$) mice where there is a proposed link to atherosclerosis (Carluccio et al. 2007). In addition, dietary intervention in a rabbit model has been shown to produce HHcy and ED relatively quickly (4 weeks) along with stable plaque formation after 12 weeks of a normal diet (Rai, Hare & Zulli 2009), which has enabled study of the larger role that HHcy might play in the progression of atherosclerosis (Smith, R et al. 2015; Zulli & Hare 2009). Despite their widespread use in pharmacological research, wild-type mice are resistant to developing heart disease and thus genetically modified and intensive diet-fed models are routinely employed to mimic human pathophysiology (Liao, Huang & Liu 2017; Russell & Proctor 2006; Zaragoza et al. 2011).

1.5 Homocysteine

Homocysteine (Hcy), an intermediary product of methionine metabolism, has been shown to play an important role in CVD development. Hcy was first associated with disease in children presenting with vascular lesions, thrombosis, and severe mental retardation in the early 1960s; homocystinuria (Hcy in the urine) was noted and a genetic defect in methionine metabolism

proposed as the cause (Carson & Neill 1962; Gerritsen & Waisman 1964; Gibson, Carson & Neill 1964). Several decades after these ground-breaking studies, idiopathic excess Hcy (hyperhomocysteinemia; HHcy) was implicated as an independent risk factor for coronary artery disease (CAD), stroke and heart attack (Beard, Reynolds & Bearden 2012; Erol et al. 2007; Sen et al. 2010). HHcy is characterized by blood vessel dysfunction and proposed to exacerbate plaque accumulation via an upregulation of inflammatory mediators and thus impair endothelial function via reduced nitric oxide (NO) bio-availability (Steed & Tyagi 2011; Wilson et al. 2007; Zulli et al. 2004). Therefore, treatments aimed at reducing Hcy plasma levels have been focused on reducing the burden of impaired methionine metabolism by increasing critical B vitamins (Kaul, Zadeh & Shah 2006). However, attempts to reduce Hcy through B vitamin administration, which are essential for re-methylating Hcy to methionine (Castro et al. 2006), have been unsuccessful in reducing CVD (Debreceni, Balazs & Debreceni 2012) and novel treatments to directly reduce the detrimental cardiovascular effects of Hcy are still required.

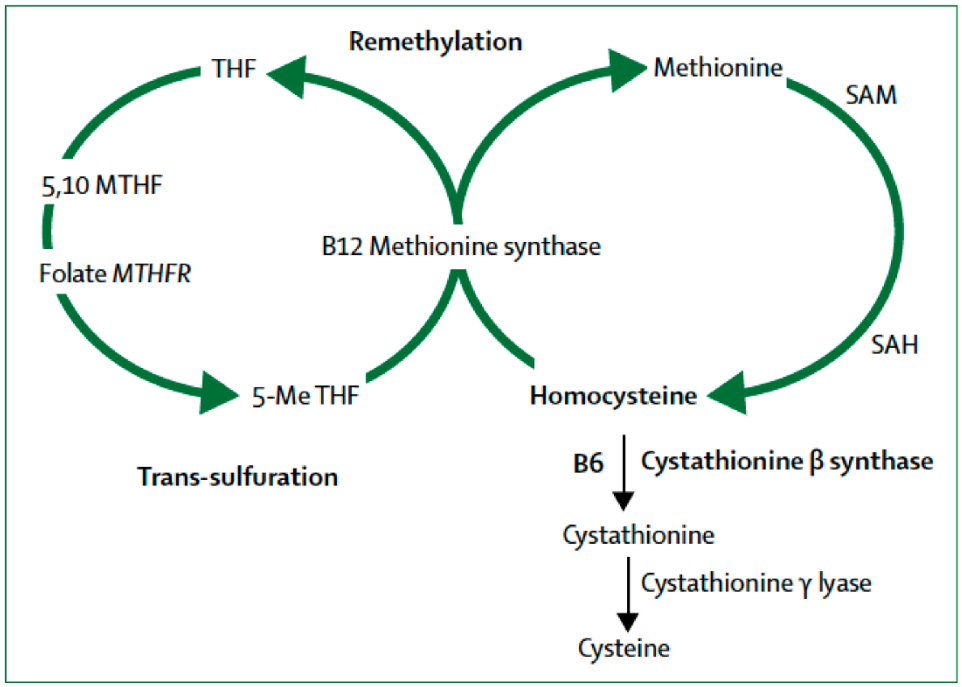


Figure 1.2. Methionine requires the B Vitamins B_6, B_9, and B_{12} for the metabolism of Hcy. Excess homocysteine leads to redox stress and promotion of inflammation (Roman, Mancera-Paez & Bernal 2019)**.**

Methionine is a sulphur-containing amino-acid first isolated and described in the early 1920s (Mueller 1923). Methionine is provided by ingestion of animal products in the diet and its metabolism into homocysteine, regulated by Vitamin B_6, B_9 (folate), and B_{12} (Joseph & Loscalzo 2013). HHcy often accompanies low B-vitamin levels (Gao et al. 2003), however, B-vitamins are not a predictor of mortality in either all-cause or CVD-related deaths (Dangour et al. 2008) and moderate increases in total Hcy levels are generally attributed to genetic mutations to the gene coding the enzyme methylenetetrahydrofolate reductase (MTHFR) (Frosst et al. 1995). Importantly, the MTHFR enzymes is required for folate metabolism and HHcy is present in individuals with homozygous mutated MTHFR (Liew & Gupta 2015). Normal methionine metabolism metabolises Hcy to cysteine via cystathionine via trans-sulfuration or back into methionine via methyl donation (Ansari et al. 2014). In healthy individuals, the kidney excretes excess Hcy (about 6 μmol/day) via the glomerulus and it is estimated that 99% of Hcy is reabsorbed (van Guldener 2006). In patients with end-stage renal disease, the incidence of HHcy is 85-100%, however the exact nature of how Hcy becomes dysregulated and increased is still unclear (van Guldener 2006). Importantly, proper Hcy regulation relies upon a correct ratio of Hcy between in its re-methylated form and its trans-sulfuration form (Ansari et al. 2014) and a recent study reported that poor ratio distribution could be related to its negative influence on pro-inflammatory markers (Fang et al. 2014). Dysregulated methionine metabolism, such as reduced B vitamins, or genetic anomalies such as cystathionine β synthase (CBS) deletion results in less Hcy excretion, which eventually overwhelms the rate of clearance and thus Hcy accumulates in the plasma (Castro et al. 2006).

In addition to catastrophic vascular events at young ages, genetically impaired HHcy presents with associated problems including mental retardation (Carson & Neill 1962), fatty liver (Gibson, Carson & Neill 1964) and displacement of the lens (Yap 2003). Importantly, these deficiencies present early in development, providing evidence that normal methionine metabolism is essential not only for proper development of the brain, vascular, and optic system but for long-term vascular health. Plasma Hcy levels indicate risk: mild HHcy is 13-30 μM, moderate 31-100 μM, and severe >100 μM (Beard, Reynolds & Bearden 2012; Friedman et al. 2001; Lim & Cassano 2002). Plasma Hcy levels in the normal population is reported as approximately 9 μM (Friedman et al. 2001; Lim & Cassano 2002). In clinical trials, reducing plasma Hcy to mild levels in those with the disease or other risk factors rarely see improvements (Clarke, R. et al. 2011; Debreceni, Balazs & Debreceni 2012; Hoffman 2011; Lonn et al. 2006). However it is revealing that plasma levels were typically not reduced below 9.5 μM, still within a risk category; it has also been reported that >85% dialysis patients show HHcy in the mild to moderate state (Friedman et al. 2001), indicating that for damage to be reduced, Hcy levels would ideally be below 9 μM.

HHcy is involved in clot formation through pathological platelet aggregation driven by collagen and thrombin adhesion, which have been modified by protein thiol groups. Malinowska, Tomczynska and Olas (2012) conducted a study measuring the impact of HHcy on platelet adhesion. They found significant differences between control (resting, not treated) and experimental (thrombin-treated) cells when exposed to concentrations of Hcy of 0.1, 0.2 and 1.0 μM for 15 minutes at 37°C. Platelet aggregation was not affected by collagen unless treated with thrombin first, indicating that the cells need to be compromised before platelet aggregation attributed to Hcy becomes detrimental. These results support the hypothesis that Hcy contributes to the dysfunction of the vessel wall, but importantly that it promotes a

worsened state if already compromised. These results are in line with those previously reported (Jakubowski 1999, 2000) showing changes to the proteins that have had an impact on the vasculature. HHcy has also been reported to have contributed to reduced blood flow via endothelial damage in mice (Tyagi et al. 2011). There are conflicting reports of Hcy's influence on NO, where a reduction in plasma HHcy has not resulted in an improvement in disease/risk factors (Sharma, M et al. 2008). However, Zivkovic and colleagues (2012) report that cardiac cells showed reduced coronary flow when treated with a 10 μM dose of Hcy. The complex nature of these interactions indicate that there are many factors at play in the changes of expression in regulatory signalling. For example, HHcy has been shown in separate studies to upregulate NO (Upchurch et al. 1997) and down-regulate NO (Ann De Groote et al. 1996).

1.6 Impaired ACh-Mediated vasorelaxation as a sensor for reduced nitric oxide bioavailability

An important mitigating factor in the development of CAD is a reduction in NO bioavailability, which is measured by impaired acetylcholine (ACh)-mediated vasorelaxation, (Kruzliak, Sabo & Zulli 2015). Proper endothelial function is essential for normal blood flow, blood pressure (Dharmashankar & Widlansky 2010) and its dysfunction is a key aspect in the development of CAD and an important predictor of disease which can be measured directly via *in vitro* isometric tension testing of arteries, as well as *in vivo* measurements of forearm flow mediated vasodilatation (Rees et al. 1989). HHcy-impaired ACh-mediated vasorelaxation appears to be a catalyst for increasing CVD risk, however the mechanism of action is unclear (Ren et al. 2016).

1.7 Nox family of enzymes: Focus on Nox1, 2 and 4

The nicotinamide adenine dinucleotide phosphate oxidases (NADPH; Nox1, 2, 3, 4, 5, Duox1 & 2) generate ROS as signalling molecules and this is reported be their sole purpose (Rivera et

al. 2010; Selemidis et al. 2008; Shafique et al. 2013). Nox have been implicated in CAD when their normal ROS production becomes dysregulated and increased, essentially causing a signal fault (Gimenez et al. 2016; Judkins et al. 2010; Sampson, N. et al. 2011). Although the literature indicates that ROS attenuate vascular function, their vasculoprotective capacity is becoming better understood (Barbosa et al. 2013; Shafique et al. 2013; Silva et al. 2016).

The reduction in basal levels of essential vascular relaxing factors, such as NO, (Aurora, Hare & Zulli 2012) and upregulation of ROS, generated as a by-product of mitochondrial respiration and important signalling molecules for vascular cells (Bedard & Krause 2007a; Rochette et al. 2013) could be key in the development of impaired ACh-mediated vasorelaxation (Cai, H & Harrison 2000; Suematsu et al. 2007). ROS are important both for the health and pathology of the vasculature (Rochette et al. 2013; Shafique et al. 2013), but if dysregulated, are associated with a reduction in vascular function (Cai, H & Harrison 2000; Förstermann & Sessa 2012).

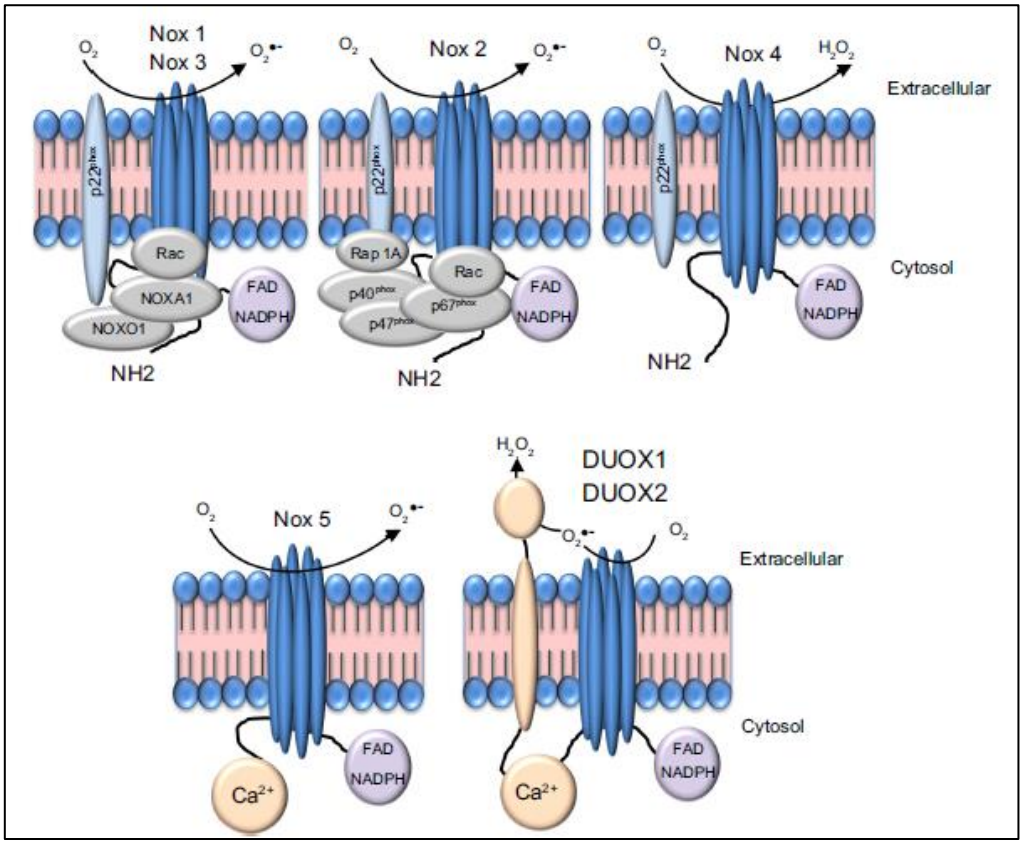


Figure 1.3. The Nox family of membrane-bound enzymes are made up of 7 different sub-types. Their main function is the generation of essential ROS. Additionally, they all have unique sub-catalytic formation requirements that require conformation to be activated (Downs & Helms 2013).

The focus of our work is on Nox1, 2 & 4 and how they might interact with and influence Hcy in the vasculature, as putative pharmacological inhibitors have been developed. Briefly, to inhibit Nox1 production, we used ML-090 (ML); for Nox2 inhibition, we used apocynin, gp91ds-tat (GP) and PhoxI2; and for Nox4 inhibition, we used VAS2870 (VAS). They will be discussed in greater detail in a later section, keeping in mind the possibility of non-specificity and other 'off target' effects.

Nox1 and 2 transport electrons across the cell membrane to reduce levels of free oxygen (O_2) and increase superoxide (O_2^-), whereas Nox4 directly produces hydrogen peroxide (H_2O_2), although recent evidence suggests that Nox4 also produces small amounts of O_2^- (Ahmad et al. 2010). Nox1, 2 and 4 can all promote the development of endothelial dysfunction, hypertension and inflammation when they become dysregulated (Rivera et al. 2010). Risk of adverse thrombotic events is increased plasma H_2O_2 (Dayal et al. 2013). In addition to its role in cardiovascular disease, elevated Hcy levels are also proposed to play an important but unclear role in chronic renal disease (van Guldener 2006); there is also evidence to suggest that an interaction with the NADPH oxidases is involved in the pathophysiology of thrombosis (Abais et al. 2013; Yi et al. 2004). Further, previous work by Edirimanne and co-workers (2007) found that a Nox inhibitor was able to improve endothelial relaxation in hyperhomocysteinemic rats.

1.8 Studies Linking Hcy to Nox

Inflammation increases the activity of Nox, however they are implicated in CAD pathogenesis due to oxidative stress caused by dysregulated signalling and electron uncoupling (Schramm et al. 2012); evidence suggests that HHcy can activate Nox to release ROS and cause oxidative stress, whether directly or indirectly (Takeno et al. 2016; Takeno et al. 2015; Tyagi et al. 2005; Yi et al. 2004). There have been several studies reporting associations between HHcy and Nox. Zhang and colleagues (2010) reported that in HHcy-induced rat mesangial cells, inhibiting O_2^- (via NDMA-receptor Nox-dependent inhibitor MK-801; 200μM; 2 hours) reduced glomerular injury and extracellular matrix deposits were reduced, as was Hcy-induced Nox-dependent O_2^- generation. Also in the renal system, Yi et al (2004), reported that Nox is involved in glomerular injury, via Hcy-dependent activation of Nox subunits. In the cardiovascular system, Edirimanne and co-workers (2007) found that the Nox inhibitor apocynin (4mg/kg; 7 days) was able to improve endothelial relaxation in the aortae of HHcy rats; whereas Bao et al. (2010) reported that inhibition of Nox with diphenylene iodonium (DPI; 10μM; 30 minutes) in human endothelial progenitor cells blunted the formation of ROS induced by Hcy via Nox. In studies assessing pathway links between Hcy and Nox in endothelial dysfunction, Carluccio et al (2007) showed that in human umbilical vein endothelial cells (HUVECs) increased levels of Hcy (100μM) induced translational changes to Nox and increased ROS and oxidative stress. In their review, Kassab and Piwowar (2012) reported findings that Hcy increased Nox activity and ROS generation, via several proposed mechanisms in different models of T2DM, including mitochondrial damage and Hcy-induced apoptosis. Thus, the evidence points to a possible link between Hcy and Nox in pathology. In addition to this, there is recent evidence of HHcy producing immune-related "inflammasomes" in renal podocytes in the presence of Nox (Abais et al. 2013). It was found that when Nox was inhibited, either by a chemical inhibitor or by translational interference, immune-complexes and cytokines were otherwise reduced and surrounding infrastructure was spared further insult. However, as the overwhelming evidence

shows that inhibiting the oxidative stress induced by Nox appears to be beneficial the cellular environment, it is unclear if this the due to cause or effect.

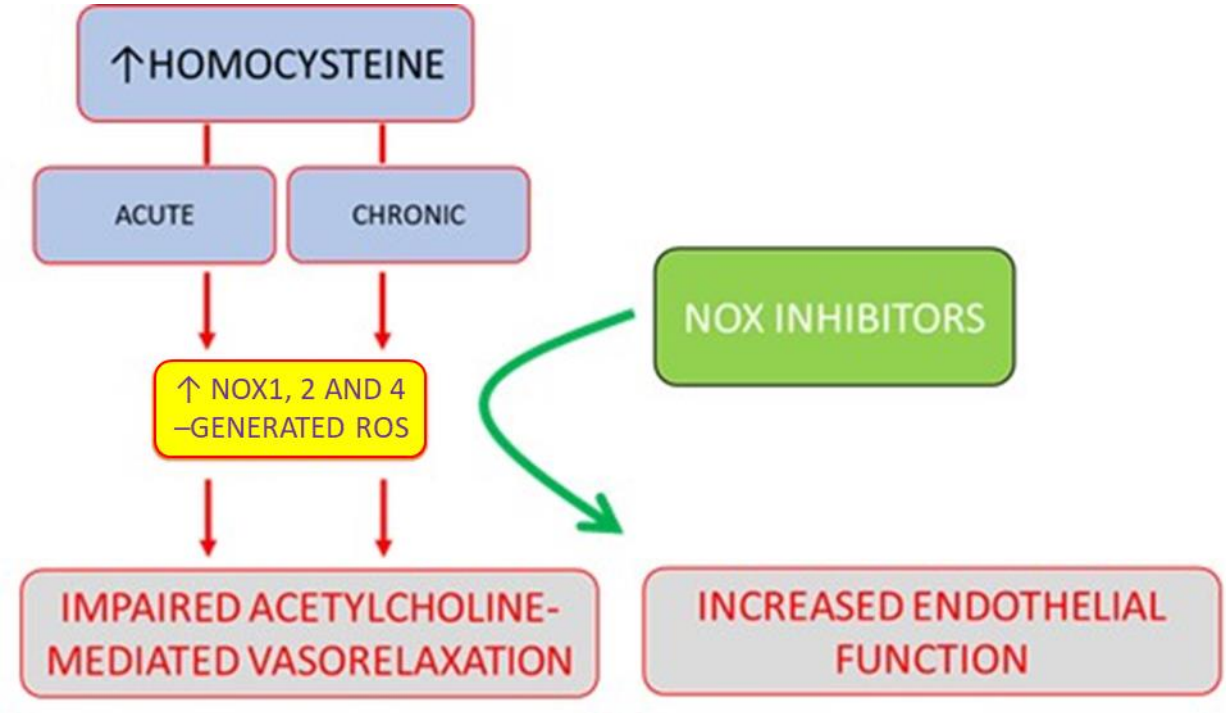


Figure 1.4. Homocysteine may increase ROS generated from Nox1, 2 and 4. Therefore we hypothesised that by using Nox inhibitors, this would reduce ROS, and improve ACh-mediated vasorelaxation.

1.9 Clinical studies: Disappointing Outcomes.

The relationship between methionine metabolism and HHcy is linked to the dysregulated vitamin levels required for proper methionine metabolism (Hoffman 2011), and this formed the rationale that targeted vitamin treatment might reduce plasma HHcy and improve the risk of embolism and myocardial infarction. Additionally, the proposed upregulation of oxidative stress by HHcy justified the use of antioxidants to reduce plasma HHcy to normal levels (Au-Yeung et al. 2004). However, studies investigating the administration of B vitamins and

antioxidants have been disappointing (Clarke, Robert et al. 2014; Debreceni, Balazs & Debreceni 2012; Hodis et al. 2009; Kaul, Zadeh & Shah 2006; Lonn et al. 2006; Pettigrew et al. 2008), suggesting that increased CVD risk in HHcy is not caused by low B-vitamins or increased oxidative products. However, it's most likely that no reduction in morbidity and mortality was due to Hcy levels not being reduced low enough to make a significant difference. For example, in the Heart Outcomes Prevention Evaluation 2 (HOPE2) study, which was attempting to improve morbidity and mortality by reducing Hcy levels using vitamin B therapy, Hcy levels were only reduced to approximately 9.5 μM in the 2 different treatment groups, respectively (Lonn et al. 2006), and are both above normal Hcy blood levels. It is thus tempting to speculate that either vitamin therapy does not reduce Hcy far enough or may be better used as a preventative measure against future HHcy.

1.10 Current Treatments: Statins

There is a range of therapeutic interventions currently advised for those either suffering with CVD or at risk of developing CVD symptoms; statins are the most widely administered for both groups and their extensive use over the years has resulted in large body of literature supporting their efficacy (Ray, KK et al. 2014). Statins target the liver and, depending on the statin, the dose is retained anywhere from 46 - >80% (Stancu & Sima 2001). Functionally, statins target hepatocytes to reversibly bind an essential enzymatic cholesterol precursor, HMG-CoA reductase, and alter its conformational structure to render it ineffective; the effect of this is reduced plasma cholesterol (Stancu & Sima 2001). Statin therapy reduces risk by reducing detrimental levels of LDL-c, which are established as associated with atherosclerotic plaque development (Bild et al. 2014; Magalhaes et al. 2016). However, statin therapy is not suitable treatment for many patients and in some cases, subjects have ceased statin administration due to intolerance (Ray, KK et al. 2014). In addition to this, there is conflicting

evidence on the efficacy of treating low-risk populations with statin therapy for those attempting to prevent onset of CAD; this is a common recommendation (Debreceni, B. & Debreceni 2014). As the authors point out, there has been a major study offering contradictory advice on the use of statins as a prevention for CAD. The study by Taylor et al. (2011) initially advised no improvement to low-risk population and implied unnecessary risk with administration; 2 years later Taylor et al. (2013) revised their conclusion to indicate that there is no risk to low risk population and administration is more likely to be followed by a drop in mortality rate across all groups. The update was met with some controversy, with suggestions that such recommendations are dangerous.

HHcy has been shown in both cell culture and animal studies to interfere with the mechanism of statins at the molecular level. Simvastatin was not able to reduce HHcy levels or rescue HHcy-induced increased iNOS expression in human hepatocarcinoma cells and murine macrophages and in $MTHFR^{-/-}$ and $MTHFR^{+/+}$ female and male murine models, Hcy levels were higher in the over-expressed models, but there were no differences attributed to simvastatin (Mikael & Rozen 2008). The authors suggest that Hcy can interfere with both anti-atherogenic and anti-inflammatory properties of statins, providing a rationale that statin therapy may not be suitable for treating HHcy (Mikael & Rozen 2008).

1.11 Nox1, 2 and 4 Inhibition

There are several pharmacological inhibitors acting on the different Nox isoforms that have shown to be effective in reducing ROS production in pathophysiological states (Kahles & Brandes 2012). These inhibitors are relevant to understanding the complex role that Nox1, 2 and 4 have on the vasculature.

The research on ML is limited but some work has shown its efficacy against Nox1-generated ROS; currently our lab has the only publication on ML in Hcy-impaired acetylcholine-mediated vasorelaxation. Another study showed that ML provided a protective effect on murine oxygen and glucose deprived retinas, where ML treated cells attenuated ROS production and were protected from ischaemic death (Dvoriantchikova et al. 2012). This study provides novel evidence that ROS production is reduced by ML, by a proposed mechanism of action of Nox1 inhibition, particularly via its role in contributing to ischaemic injury. They also found no significant difference between two Nox inhibitors (ML and VAS) ability to reduce ROS after ischaemia, suggesting that Nox1 was responsible for generating ROS. Their work provides further evidence of an increase in ROS production following ischaemia. In contrast, work from our lab provides the novel evidence that ML reduces the effect of Hcy thiolactone (HcyT; the atherogenic form of Hcy) in impaired ACh-mediated vasorelaxation in rabbit aortae; we attributed these results to HcyT-induced increased Nox1 activity, contributing to vascular dysfunction, via a mechanism possibly related to its product O_2^- (Smith, R et al. 2015).

The putative Nox2 inhibitor GP has been used more widely as a Nox2 inhibitor; it has been reported to have attenuated vascular O_2^- production which was attributed to Nox2 inhibition but GP's effect as a pan-Nox inhibitor remains controversial (Drummond et al. 2011; Kahles & Brandes 2012). In a study by Rey and colleagues (2001), GP (50 µM; 30 mins) applied to mice aorta cells was found to attenuate angiotensin-II induced O_2^-, attributed to GP's capacity to inhibit Nox2. More recently, Meyer and co-workers (2014) reported that in the carotid vessels of young and old mice, GP was effective at reducing contraction in vitro, suggesting a role for Nox2 in ageing vessels. In renal cells, GP was reported to have attenuated Nox-induced O_2^- and improving vasodilation in an HHcy environment (Abais et al. 2013). Further, work in our own lab revealed that a combination of inhibiting Nox2 with GP plus Hcy impaired blood

vessel relaxation to acetylcholine (Smith, R et al. 2015). This is significant for our work, as we propose that mechanisms involving both HHcy and Nox are intrinsically involved in endothelial dysfunction.

Apocynin is a purported Nox2 inhibitor and potential antioxidant (Heumuller et al. 2008), which has been shown to attenuate vascular lesions and signs of oxidative stress, and its efficaciousness is well-documented (Drummond & Sobey 2014; Kahles & Brandes 2012), however, its use is not without controversy. Apocynin is a 'pro-drug' and requires activation via myeloperoxidase to elicit its effect; it has been reported to only be effective as an inhibitor in leukocytes (Heumuller et al. 2008). Kelly et al. (2009) report that apocynin treatment (5mg/kg; 30 minutes) worsens stroke outcome in mice. In addition to this, Huemueller et al. (2008) report that apocynin is in fact an antioxidant and should not be used as a Nox inhibitor in endothelial cells and VSMCs. There are several studies highlighting the efficacy of apocynin as a Nox2 inhibitor. Dayal et al. (2013) report that apocynin (600 µM; 15 minutes) inhibited fibrinogen binding in platelets of 18 month old mice thus reducing blood clotting. Importantly, there is evidence of apocynin's positive effect on hypertensive animals: Zhang et al.(2014) found that apocynin (1µM; 2 weeks) reduced O_2^- production in macula densa cells of hypertensive mice; Hu et al. (2006) reported that apocynin (1.5mM in drinking water (DW); 4 days) reversed dexamethasone-induced hypertension in rats and Baumer et al. (2007) reported reduced blood pressure in hypertensive rats using apocynin (2mM in DW; 4 weeks). Studies involving Nox2 genetically deficient mice have reported different findings, with the majority showing improvements in pathophysiological markers. A few studies show the benefit of Nox2 in pathology. In a model of aortic aneurysm, Kigawa and colleagues (2014) reported that Nox2 deficiency enhances both macrophage recruitment and MMPs, which then disrupts tissue remodelling and concluded that Nox2 inhibition would not be beneficial in treating aortic aneurysm.

The overwhelming majority of studies, however, point to an up-regulation of ROS-generation by Nox2 that deletion of which effectively removes and thus function improves (extensively reviewed by Rivera et al. (2010). Of the few studies looking at genetic overexpression of Nox2, results were in contrast: Shafique et al. (2013) found that Nox2 overexpression improved function and pointed to a beneficial role for excess O_2^-. However, Douglas et al. (2012) found that Nox2 overexpression plus $ApoE^{-/-}$ resulted in possibly earlier plaque initiation and also pointed to increased oxidative stress. Pharmacological studies mentioned previously point to an important role for oxidative stress in the development of endothelial dysfunction, however whether this will be determined as positive or negative remains unclear.

PhoxI2 is described as a specific Nox2 inhibitor and it functions by disrupting the two catalytic subunits essential for Nox2 conformational binding, $p67^{phox}$ and Rac (Jo & Luo 2012). It has been reported as successfully blocking O_2^- production in neutrophils, presumably via Nox2 inhibition (Bosco et al. 2012). Other studies have reported the efficacy of PhoxI2 in reducing oxidant production from Nox2: in mice and human platelets, PhoxI2 reduced ROS and platelet aggregation (Akbar et al. 2018).

VAS, which is reported as non-selective for Nox, is purported to block Nox1 and 2 action via its ability to inhibit oxidised LDL generated ROS but its effect on Nox4 is controversial (Kahles & Brandes 2012). Data from our lab supports its role as a possible Nox4 inhibitor, as putative Nox1 or Nox2 inhibitors yielded differing results. Support for its efficacy in blocking Nox4 has been reported by Wind and co-workers (2010), where they discuss that in spontaneously hypertensive rat (SHR) aortae, vascular ROS production was reduced to healthy, control levels and that ROS-induced signalling was inhibited by VAS, suggesting an inhibition of superoxide. In addition to this, aortic relaxation was increased in both the control and experimental rats, highlighting an improvement in endothelial function. Further support for VAS inhibiting the effects of Nox has been reported by d'Uscio and colleagues (d'Uscio et al. 2012), where they

report that VAS inhibits superoxide formation generated by Nox in the aortae of mice with mutated amyloid-β precursor protein. In contrast, in mesenteric microvessels of morbidly obese human subjects with insulin-resistance, VAS was not found to have attenuated the effects of Nox as there was no reported improvement in the vessels that had shown reduced vasorelaxation in the presence of bradykinin, a potent vasodilator (El Assar et al. 2013). However there are differences to this study's methodology: the vessels treated were from the mesentery, whereas previously described studies have tested coronary vessels. Microvessels therefore might not be a suitable model to study as they may respond differently than aortae in disease (Karabag et al. 2007); indeed in the recent RESOLVE trial, it was reported that exercise training improved arterial function but not microvascular function in metabolic syndrome (Vinet et al. 2018). It is unknown why microvessels respond differently than larger vessels but could be due to exposure to comparatively lower shear stresses (Green, DJ et al. 2004), requiring a longer adaptation period to intervention (Olver & Laughlin 2016) or the presence of a metabolic disease such as T2DM (Caballero et al. 1999). HHcy appears to contribute to microvascular dysfunction, especially in T2DM, rather than act as a causal factor, suggesting that microvascular complications arise independently of plasma Hcy levels (Karabag et al. 2007).

As Nox1 and 2 generate O_2^- (Bedard & Krause 2007a) and the main product of Nox4 is H_2O_2 (Nisimoto et al. 2014), it has therefore been tempting to measure responses to the vascular system using combinations of the available Nox inhibitors, results from our lab were unsuccessful in the context of HHcy (Smith, R et al. 2015). However, a recent development in Nox inhibition is the introduction of a novel, specific Nox1 & 4 inhibitor GKT137831 (GKT; also called GKT831) and has shown promise in reducing the inflammation associated with vascular disease, T2DM and cancer (Gray et al. 2017; Sampson, Natalie et al. 2018). GKT efficacy has been assessed in both animal studies and clinical trials are currently ongoing, but

not yet published. In animals, administration of GKT (10-40 mg/kg/day) has been effective at reducing vascular disease-associated morbidities: in a murine model of T2DM, albuminuria was reversed (Gray et al. 2017), a murine model of Type 1 Diabetes Mellitus had reduced diabetes-induced glomerular hypertrophy and mesangial matrix accumulation (Gorin, Yves et al. 2015) and in a rat model of chronic hypoxia, oxidative stress was significantly reduced (Lu et al. 2017). In cell culture, results have been equally promising in cancer treatment where GKT (30 μM)-treated prostate fibroblasts had reduced ROS and impaired ability to migrate (Sampson, Natalie et al. 2018). Additionally, in retinal vascular disease, GKT (100 nM) reduced ROS generation and supported normal cell growth under oxidative stress (Appukuttan et al. 2018). The literature suggests that the combination of O_2^- and H_2O_2 inhibition, potentially leading to a homeostatic balance of pro- and anti-oxidants, are the key to their efficacy.

1.12 Inducible Nitric Oxide Synthase (iNOS)

Our interest in the inflammatory markers that characterise atherosclerosis extend to inducible nitric oxide synthase (iNOS), a product upregulated in vascular disease (Depre et al. 1999). iNOS appears to be present only in inflammatory and pathological conditions, especially atherosclerosis, where it produces high amounts of NO, possibly in an attempt to maintain vascular homeostasis (Ponnuswamy et al. 2009). This can exacerbate an already compromised environment when NO reacts with O_2^- to produce the powerful oxidant peroxinitrite ($ONOO^-$) and the delicate balance between pro- and anti- oxidants may be lost (Pacher, Beckman & Liaudet 2007). NO is an essential and regulatory vasodilator, synthesised by the conversion of the amino acid L-arginine to L-citrulline and mediated by the family of nitric oxide synthase (NOS) isoforms and their cofactors, especially tetrahydrobiopterin (BH_4) (Lee, J et al. 2016). In pathological states, the inability of iNOS to bind with the essential cofactor BH_4 results in ROS production instead of NO, resulting in NOS uncoupling (Lee, J et al. 2016) and leading to a pro-oxidant state. In the context of Hcy, this is of particular interest given that BH_4 can be

reduced by excess Hcy (Bendall et al. 2014), providing the potential for iNOS uncoupling and free radical production. In addition, excess Hcy may also increase Nox-derived O_2^-, (Kassab & Piwowar 2012), resulting in accumulating oxidative stress, which also contributes to the unbalanced redox state.

Chapter 2

2.1 General Methods

All procedures were carried out in relation to the Victoria University Animal Experimental Ethics Committee (VUAEEC) #13/011, #12/019, and #14/005. Procedures were carried out at Western Centre of Research Excellence (WCHRE) and Werribee Animal Holding Facility (WAHF).

White New Zealand rabbits were obtained from Nanowie Small Animal Production Unit (Bellbrae, Victoria).

C57BL/6 mice were obtained from Animal Resource Centre (ARC) and $Nox2^{-/-}$ mice were obtained from Monash Animals Services (Prof. Grant Drummond).

All animals were transported by a commercial animal transport carrier to either WCHRE or WAHF, according to the Australian Code of Practice for the Housing and Care of Laboratory Mice, Rats, Guinea-pigs and Rabbits "Standards for transportation" in adequately ventilated, vermin- and escape-proof containers with food and water provided during transportation. The containers were supplied with cushioning for thermoregulation, impact absorption and stress reduction. The number of animals was the maximum per container to provide sufficient space and prevent injury. All containers were clearly labelled. Animals were transferred on arrival to standard cages (rabbits 1 per cages; mice 5 per cage) with clean water. Food and bedding was then provided according to AEEC GUIDELINE G005 (2013).

Once onsite at WAHF, all animals were acclimatised for at least 5 days prior to use.

WCHRE: Once the rabbit arrived at WCHRE, it was taken to Level 4, Animal Procedures Room (room 6.050), given food and water and killed as soon as practical. The time between delivery of animal and killing did not exceed 3 hours.

WAHF: VU Animal Facility Standard Feeding, Bedding and Enrichment. Rabbits were held onsite according to AEEC GUIDELINE G005 (2013). For rabbits, each male was housed separately. They were able to see each other and animals were not left alone in the animal for more than 48 hours. For mice, each held a maximum of 5 animals. Additionally, no vegetables or fruit was given to animals when they were fed a specialised diet designed to increase plasma homocysteine, as this has been clearly shown to inhibit the initiation and progression of blood vessel disease in animals and humans (i.e. beneficial effects of Mediterranean diet). As well, no hay or straw was given as this adds fibre to the diet and fibre has well established anti-lipid effects (MacMahon 1999). Further, non-edible items of environmental enrichment (wooden blocks and non-edible plastic for gnawing) were provided. In previous applications, there was no observation of deleterious health impacts to animals as a result of removing hay, vegetables and fruit, in this time period.

Special formulated feeds for rabbits and mice designed to increase plasma homocysteine were sourced from Glen Forrest Stockfeeders (Glen Forrest, Perth, WA). Rabbits were fed an atherogenic chow of (5% Peanut Oil, 0.5% cholesterol and 1% methionine) (Zulli & Hare 2009) and mice were provided a 1% methionine-rich chow. All food and water was provided *ad libitum*.

2.2 Experimental Protocol: Anaesthesia and Culling

Rabbits: New Zealand rabbits (12-24 weeks, male, n=63) were weighed. Rabbits were weighed, swaddled in a towel to restrict movement, and then injected SC with 0.25mg/kg medetomidine in the neck scruff. The animal was left to rest in a covered cage for a minimum

of 10 minutes. The animal was then removed from the cage and placed in an airtight box attached to an isoflurane and oxygen pump (4% isoflurane). The animal stayed in the cage, covered, for at least 10 minutes or until unable to right itself after being gently tilted. The animal was then laid out on the bench on a bluey and a mask attached to the same isoflurane/oxygen pump placed over its face for 5 minutes or until there were no reflexive responses from pinching the foot between the toes, paw and ear. Whilst the mask was still on, the ventral side of the body was wetted, and the area just above the groin was then cut opened (approx. 10 cm) and the intestines moved out of the cavity onto the bench. The inferior vena cava was located and an opening cut (approx. 2-3 cm). For all rabbits, bleed out for a minimum of 3 minutes or until animal was dead (no reflexes at paw, ear or eye, cold to the touch, iris fully dilated). The diaphragm was also cut to ensure brain death.

Mice: C57BL/6 (n=80) and $Nox2^{-/-}$ (n=16). Each mouse was weighed and placed in an airtight box attached to an isoflurane and oxygen pump (4% isoflurane) for a minimum of 5 minutes or until loss of consciousness. The mouse was then removed from the box and immediately underwent cervical dislocation. The protocol for removing the aorta was the same with the exception of the amount for flushing, which was 3 x 5mL.

Dissection: The body was cut open with a scalpel near the groin and then opened from chest to groin. The intestines were lifted up and placed out of the cavity in order to expose the fat beneath. Using gauze, the fat was gently pulled away from the vena cava. The thoracic aorta was located under the heart and a small tube inserted and clamped so that solution would not leak into cavity. The vena cava was open with a small cut down near the groin and then ice-cold Krebs was very slowly flushed through the aorta (3 x 60 mL for rabbits; 3 x 5 mL for mice). After flushing, the aorta (thoracic to iliac at the bifurcation) was cleaned of fat and connective tissue, removed from the animal and placed in ice-cold Krebs. The aorta was then placed on ice in Krebs under a microscope, further cleaned and cut into aortic rings (2-3 mm

lengths for rabbits; 1-2 mm for mice) and placed in organ baths with Krebs, kept at a constant temperature of 37 °C and continuously bubbled with carbogen (95% O_2 /5% CO_2). Baths hold 5 mL. After a 30 minute equilibration period where rings were left unmounted in the bath, rings were then mounted on 2 metal hooks attached to force displacement transducers (OB8, Zultek Engineering, Australia), taking care not to scrape the endothelium along the hook and stretched (2 g for rabbits; 0.5 g for mice) then rinsed with fresh Krebs and left to plateau for 30 mins. Once they reached plateau, they were re-stretched to the first tension, refreshed with fresh Krebs and left another 20 mins to plateau a second time.

Separate, adjacent aortic rings were incubated with either VAS, apocynin, GP, ML, GKT, 1400W (all 10^{-6}) or combinations thereof. Once applied, they were left to incubate for 30 minutes. After 30 minutes, the inhibitor was reapplied to compensate for drug metabolism. For rings also incubated with Hcy, this was applied now and left to incubate for 1 hour. In total, rings were incubated with the inhibitor for 1.5 hours and Hcy for 1 hour. Baselines were taken at the end of 1 hour and phenylephrine (rabbits) or thromboxane analogue (mice; U46619) added. Then, to assess endothelial dependent relaxation to ACh, aortic rings were pre-contracted with Phenylephrine (Phen, 0.3µM). After the contraction reached a plateau, a concentration-response (CR) curve to acetylcholine-induced relaxation was obtained. For pyrogallol (1µM) and SOD (1000 I/U), this was added 5 minutes before the first ACh dose.

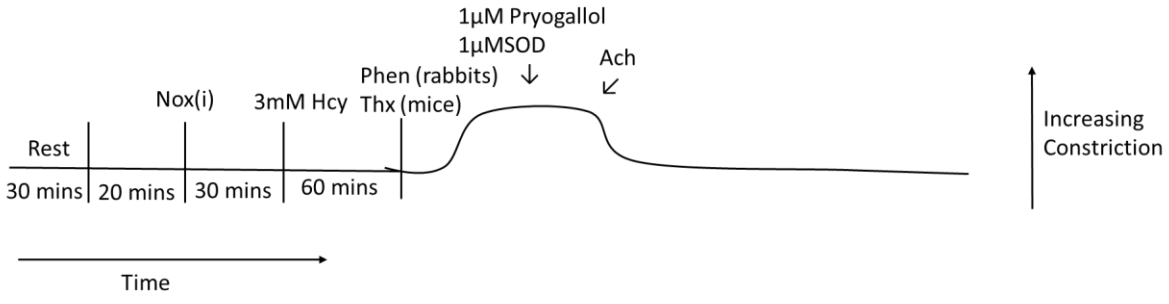


Figure 2.1. Schematic illustrating the timeline of the experimental drug protocol. This schedule remained unchanged for all experiments, unless otherwise indicated.

2.3 Immunohistochemistry

Several sections of the abdominal aortae were removed from the organ bath immediately after the last dose of ACh and fixed in 4% paraformaldehyde in 1x PBS, pH 7.3 for the immunohistochemical detection of eNOS at Ser1177 and Thr495 (Envision kit system using the monocyte/macrophage monoclonal antibody (DAKO Corporation, Carpentaria, USA), the eNOS antibody (monoclonal IgG1, Transduction Laboratories, USA).

Rings were embedded vertically in single paraffin blocks to maintain equal cutting thickness throughout all vessels. Paraffin ribbons were cut at 5 µm and placed on a 40°C water bath to allow the sections to expand to their original size before they were collected on microscope slides and dried for 72 hours at 37°C.

Immunohistochemical analysis was performed using the 'Envision' kit system (Cat#K4006, Dako, USA) following manufacturer's protocol. Monoclonal antibody (dilutions all 1:100 in 10mM TrisHCl, pH7.3, overnight incubation) to eNOS, $eNOS_{ser1177}$, $eNOS_{thr495}$ antibody (Cat#610297, Cat#612393, Cat#612707 respectively, Transduction Laboratories, USA) were used (3) and diaminobenzidine (DAB) was used to identify phosphorylation sites (10mM TrisHCl, pH7.3 was used as wash buffer). Images of tissue for quantification (4 per ring, x400 magnification) were randomly selected using a light microscope (BX53, Olympus, USA) and analysed using the MCID analysis software (MCID, UK). To capture the phosphorylation sites, which were stained brown, the 'ribbon' tool was used to trace around the edge.

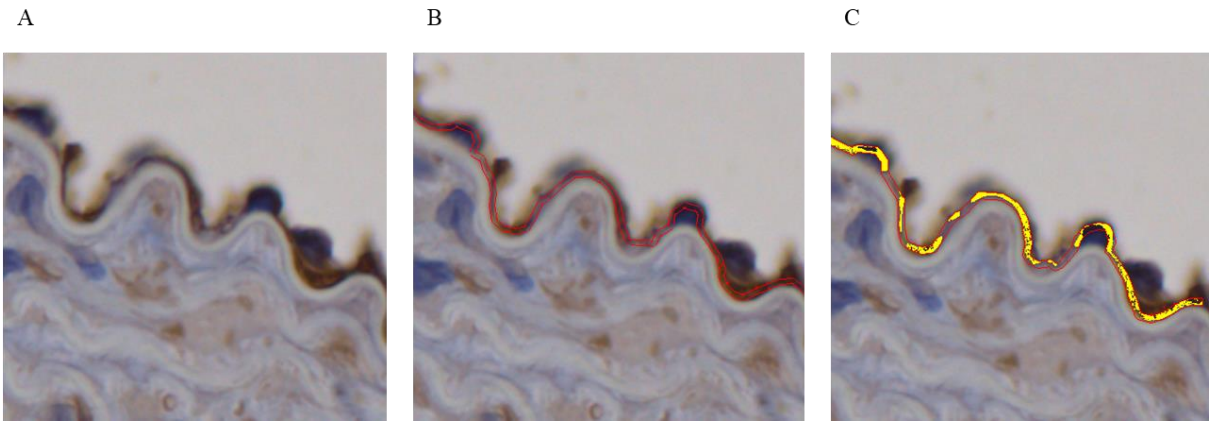


Figure 2.2. The 'ribbon' tool was used to trace the edge of phosphorylated sites for each image and then the proportional intensity of these areas were divided by the colour intensity to ascertain the extent of phosphorylation. Figure 2.2A shows the site of phosphorylation. The red line indicate examples of where the sites have been captured (Figure 2.2B) and the yellow line shows the trace of the captured phosphorylated sites that were then calculated (Figure 2.2C).

The hue, saturation and intensity was adjusted to highlight all the DAB (brown) stain, and the proportional area and intensity recorded (Arora, Hare & Zulli 2012). The mean was calculated for all four sections of eACh ring and this value was used for statistical analysis (for n=1).

2.4 Statistics

All data were analysed using either a one or two -way ANOVA (where appropriate and unless otherwise indicated) followed by Sidak's Multiple Comparisons test. Data are represented as mean ± SEM.

2.5 Solutions

Krebs Buffer Solution: (mM): NaCl 118.4, KCl 4.7, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 2.5, glucose 11.1 + ddH_2O.

KPSS: (mmol/L): KCl 123.7, $MgSO_4$ 1.17, KH_2PO_4 1.18, $NaHCO_3$ 25, glucose 5.5) + ddH_2O.

100 mL 4% Paraformaldehyde solution (PFA): dissolve 4 g paraformaldehyde in 50 mL ddH_2O, add 10 drops of 1 M NaOH and heat on a block with a magnetic stirring action until clear and 55C, ensuring not to go over 55 C. Once clear, add 10 mL 10 x PBS and 30 mL of RT ddH_2O. Check pH and adjust to 7.3. ddH_2O was added to 100 mL.

ACh: Acetylcholine (Sigma) was made into a 10^{-2} stock with ddH_2O and then serially diluted to into a 10^{-3}, 10^{-4}, and 10^{-5} solutions for the ACh curve. Final bath concentrations were 10^{-8}-10^{-5}.

Phen: Phenylephrine (Sigma) solution was made into a 10^{-3} stock with ddH_2O. Final bath concentration was $3x10^{-7}$.

Thromboxane (U46619; Sigma): stock 10^{-4}, added to bath (5 uL into 5mL), final concentration was 10^{-7}.

10 x PBS: NaCl 118 mM, KCl 4.7 mM, Na_2HPO_4, KH_2PO_4 1.2 mM + ddH_2O.

10 mM TrisHCl pH 7.3.

Chapter 3

3.1 Putative Nox2 Inhibitors Apocynin, Gp91ds-Tat, and PhoxI2 Worsen Homocysteine-Induced Impaired Acetylcholine-Mediated Relaxation

3.2 Abstract

Background: Increased homocysteine (Hcy) is a risk factor for coronary artery disease (CAD). Hcy increases reactive oxygen species (ROS) via NADPH oxidases (Nox), reducing acetylcholine-mediated vasorelaxation. We aimed to determine if putative Nox2 inhibitors prevent Hcy-impaired acetylcholine-mediated vasorelaxation.

Methods: New Zealand White rabbit and wild-type (C57BL/6) and $Nox2^{-/-}$ (NOX) mice aortic rings were mounted in organ baths. Rabbit rings were incubated with either apocynin (10μM), gp91ds-tat (GP, 1μM) or PhoxI2 (1μM) and mice rings GP (1μM) only. Some rabbit rings were incubated with 3mM Hcy, before pre-contraction, followed by dose-response relaxation to acetylcholine (ACh; 0.01μM-10μM). In rabbit rings treated with Hcy and GP, O_2^- donor pyrogallol (1μM) or Akt activator SC79 (1μM) was added 5 minutes before ACh. Mice rings were used to compare Nox2 deletion to normal ACh-mediated relaxation.

Results: In rabbits, Hcy reduced ACh-mediated relaxation vs. control ($p < 0.0001$). Treatment + Hcy reduced relaxation compared with treatment alone ($p < 0.0001$). Pyrogallol and SC79 reversed the response of GP + Hcy ($p = 0.0001$). In mice, Nox2 deletion reduced ACh-mediated vasorelaxation.

Conclusions: Apocynin, GP and PhoxI2 worsens ACh-mediated vascular relaxation in rabbit aorta, which is supported by results from mouse Nox2 deletion data. These inhibitors worsen Hcy-induced vascular dysfunction, suggesting that current putative Nox2 inhibitors might not be useful in treating HHcy.

3.3 Background

Cardiovascular disease (CVD) is a leading cause of mortality and morbidity (Steed & Tyagi 2011), and impaired acetylcholine-mediated vasorelaxation is a classical risk for the development of CVD (Gheissari et al. 2018). Hyperhomocysteinemia (HHcy) is an independent risk factor for CAD and atherosclerosis (Zulli & Hare 2009) via initiating impaired acetylcholine-mediated vasorelaxation (Song et al. 2015; Zulli et al. 2003). Thus, restoration of endothelial function is a vital step in the prevention of CVD (Santos-Parker, LaRocca & Seals 2014). Low risk individuals report plasma Hcy between 6-9µmol/L, risk is increased >10µmol/L, with levels reported as high as 300 µM in chronic kidney disease (CKD) (Beard, Reynolds & Bearden 2012). Endothelial function is measured by acetylcholine mediated vasorelaxation, and is reliant upon concomitant endothelial nitric oxide synthase (eNOS) phosphorylation of Ser^{1177} and dephosphorylation at Thr^{495} (Schmitt et al. 2009), resulting in nitric oxide (NO) release (Zhao, Vanhoutte & Leung 2015). Impaired eNOS phosphorylation results in reduced acetylcholine mediated vasorelaxation, and the development of atherosclerosis, a key factor in CVD (Feletou, Kohler & Vanhoutte 2010). Pharmacological targeting of eNOS to improve phosphorylation has been effective in reducing atherosclerotic lesions in $apoE^{-/-}$ mice (Xing, SS et al. 2015) and improving acetylcholine mediated vasorelaxation in obese C57BL/6 mice (Han, F et al. 2015),

In the Heart Outcomes Prevention Evaluation 2 (HOPE2) study and the Vitamin Intervention for Stroke Prevention (VISP) study, Hcy levels were not reduced to the low risk level and indeed risk was still elevated for these patients (Beard, Reynolds & Bearden 2012; Lonn et al. 2006). Additional findings of the HOPE2 study found that overall risk of stroke was reduced but not the severity or disability (Saposnik et al. 2009). Taken together, novel treatments for HHcy are necessary to reduce CVD.

Nox2 is a member of the nicotinamide adenosine diphosphate (NADPH) oxidases family (Nox1-5, Duox1, 2). These trans-membranous enzymes produce functional reactive oxygen species (ROS) essential for cell signalling and proper endothelial function (Bedard & Krause 2007b; Rivera et al. 2010). Increased O_2^- has also been reported in HHcy and cell culture studies show that Hcy promotes cellular damage, presumably from ROS production via Nox2 (Zou, T et al. 2010). Further, Hcy has been reported to stimulate the Nox2 subunit $p47^{phox}$ and increase apoptotic ROS production in rats (Sipkens et al. 2011).

Pharmacological inhibitors that target enzymes which produce ROS are a potential therapeutic avenue in CVD. We hypothesised that apocynin, GP, and PhoxI2 are potentially novel treatments to reduce the detrimental effects of Hcy on blood vessel disease.

3.4 Methods

All experiments were carried out according to the National Health and Medical Research Council "Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (8^{th} Ed. 2013). Apocynin, PhoxI2, pyrogallol, SC79, ACh, Hcy, and phenylephrine) were purchased from Sigma-Aldrich, (Merck), Darmstadt, Germany. Gp91ds-tat was purchased from Australian Biosearch, Perth, WA, Australia and U46619 (thromboxane analogue, Cayman Chemical) was purchased from Sapphire Biosciences, Sydney, NSW, Australia.

Rabbits

New Zealand rabbits (12 weeks, male, n=15, VUAEC #12/019) were housed in separate cages and maintained at a constant temperature of approximately 23°C and were provided water and food *ad libidum*. Whilst in our care, animals were fed a normal chow diet. Animals were anaesthetised (3mg/kg xylazine + 2mg/kg ketamine), exsanguinated and the abdominal aorta excised and flushed with cold oxygenated Krebs buffer (NaCl 118.4, KCl 4.7, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$, 2.5, glucose 11.1, mM).

Mice

Wild-type (WT) and Nox2$^{-/-}$ (NOX) mice (12-14 weeks old, male, n=8, VUAEC #14/014) (Zulli & Hare 2009). Animals were housed in cages with a maximum of 5 mice per cage and maintained at a constant temperature of approximately 23°C. Food and water were provided *ad libidum*. At sacrifice, mice were anaesthetised with isoflurane (4% O_2) followed by cervical dislocation.

The aortae were cleaned of fat and connective tissue, cut into rings (2-3 mm lengths) and placed in organ baths (OB8, Zultek Engineering, Australia), filled with Krebs, kept at a constant temperature of 37°C and continuously bubbled with carbogen (95% O_2 + 5% CO_2). These rings were left to rest, unmounted, for 30 mins. Rings were then mounted between two metal hooks attached to force displacement transducers, stretched to 2g (rabbits) or 0.5g (mice) and allowed to reach resting tension plateau. Rings were then re-stretched and allowed to reach resting tension plateau a second time.

Experimental Protocol

Rings were incubated with the Nox2 inhibitors apocynin (10µM; (Tang et al. 2012)), gp91ds-tat (GP, 1µM; (Rey et al. 2001)) or PhoxI2 (1µM; (Bosco et al. 2012)) for 30 minutes; NOX rings were not incubated with Nox2 inhibitors. Drugs were re-introduced into the baths immediately prior to the addition of 3mM Hcy for 1 hour where used. Control rings had neither Hcy nor Nox2 inhibitor added. To assess ACh mediated relaxation (ACh; 0.01-10µM (Zulli et al. 2003)), rings were pre-contracted with either phenylephrine (rabbits; Phen; 0.1-0.3µM (Zulli et al. 2003)) or a thromboxane analogue (mice; thx; 0.3 µM (Gauthier et al. 2011)). After the contraction reached a plateau, a concentration-response curve to ACh-induced relaxation was obtained. Pyrogallol (1µM) (an O_2^- donor) or SC79 (1µM) (an Akt activator) were added 5 minutes before the first ACh dose in rabbit rings treated with Hcy and GP.

Immunohistochemistry

Aortic rabbit rings were removed from the organ bath after the last dose of ACh and fixed in 4% paraformaldehyde for 24hr at RT and then maintained in 1x PBS at 4°C, pH 7.3 for immunohistochemical detection of eNOS at Ser^{1177} and Thr^{495} (Envision kit system, monoclonal antibody (DAKO Corporation, Carpentaria, USA), eNOS antibody monoclonal IgG1, Transduction Laboratories, USA), as described previously (Arora, Hare & Zulli 2012). Slides were prepared using established methods (Montes & Junqueira 1991). Images for all slides were taken with an Olympus microscope (x40 magnification) and the computer program Leica (Leica Microsystems GmbH, Wetzlar, Germany). For eNOS quantification, the endothelial layer was traced with the 'ribbon' tool (MCID Core; InterFocus Imaging, Linton, UK). The endothelial layer was quantified using MCID by setting the hue, saturation and intensity to detect the brown DAB reaction. The intensity and proportional area were recorded for all proteins and tracings were averaged and used for data analysis. All data points are arbitrary units and normalized to control as '1', described previously (Arora, Hare & Zulli 2012).

Statistical Analysis

Isometric tension data were analysed using normal or two-way repeated measures ANOVA, measuring differences in response to drug dose between and within groups, followed by Sidak's Multiple Comparisons Test. Control values for each vasorelaxation graph were all pooled. An ordinary one-way ANOVA followed by Dunnett's multiple comparisons test was used to determine significances for $EC_{50.}$ All data were analysed using GraphPad Prism (version 7.01 for Windows, GraphPad Software, La Jolla, California USA). Data are represented as mean ± SEM. Significance was accepted at $p < 0.05$.

3.5 Results

Acetylcholine mediated vasorelaxation

Rabbits: Aortic ACh-mediated relaxation was significantly reduced by the addition of Hcy compared to control (68.5±7.0% vs. 99.8±0.53%, $p < 0.0001$; Figure 1A-C). To investigate whether putative Nox2 inhibitors could restore this effect, three different pharmacological putative Nox2 inhibitors were used.

Apocynin worsened relaxation compared to control (93.1±1.7% vs. 99.8±0.53%, in the early doses ($p < 0.0001$, Figure 3.1A). The combination of Hcy plus apocynin further reduced relaxation compared to apocynin (93±1.4% vs. 37.2±12.9%, $p < 0.0001$) and 3mM Hcy alone. GP reduced relaxation compared with control (83.4±3.3 vs. 99.8±0.53%, $p < 0.0001$, Figure 3.1B). The addition of Hcy further reduced aortic relaxation compared with GP (43.5±7.1% vs. 83.4±3.3%, $p < 0.0001$, Figure 3.1B) and 3mM Hcy alone.

PhoxI2 reduced function compared with control (65.4±6.5 vs. 99.8±0.53%, $p < 0.0001$, Figure 3.1C), similar to Hcy. Aortic relaxation was further reduced by the combination of PhoxI2 and Hcy compared with PhoxI2 alone (4.8±28.8% vs. 65.4±6.5, $p < 0.0001$, figure 3.1C) and 3mM Hcy alone.

To investigate if O_2^- participated in changes to vascular relaxation, pyrogallol, an O_2^- donor, was added. The addition of pyrogallol inhibited the effect of GP in treated rings (71.2±6.4% vs 43.5±7.1%, $p < 0.01$; Figure 3.1D). Secondly, to determine if O_2^- participated in signal transduction through the Pi3k/Akt pathway, as it is also involved in NO release (Auger 2010), we added SC79 (1µM). SC79 negated the effects of GP in rings incubated with Hcy plus GP (68.9.4±4.5% vs. 43.5±7.1%, $p < 0.0001$, Figure 3.1D).

Mice: NOX showed reduced vasorelaxation compared with WT (38.8±5.8% vs. 73.7±2.5%, $p < 0.0001$) but showed no difference compared with WT + GP (Figure 3.2). However, WT + GP was significantly impaired compared with WT (42.4±12.8% vs 73.7±2.5%, $p < 0.0004$; Fig. 3.2).

3.6 Figures

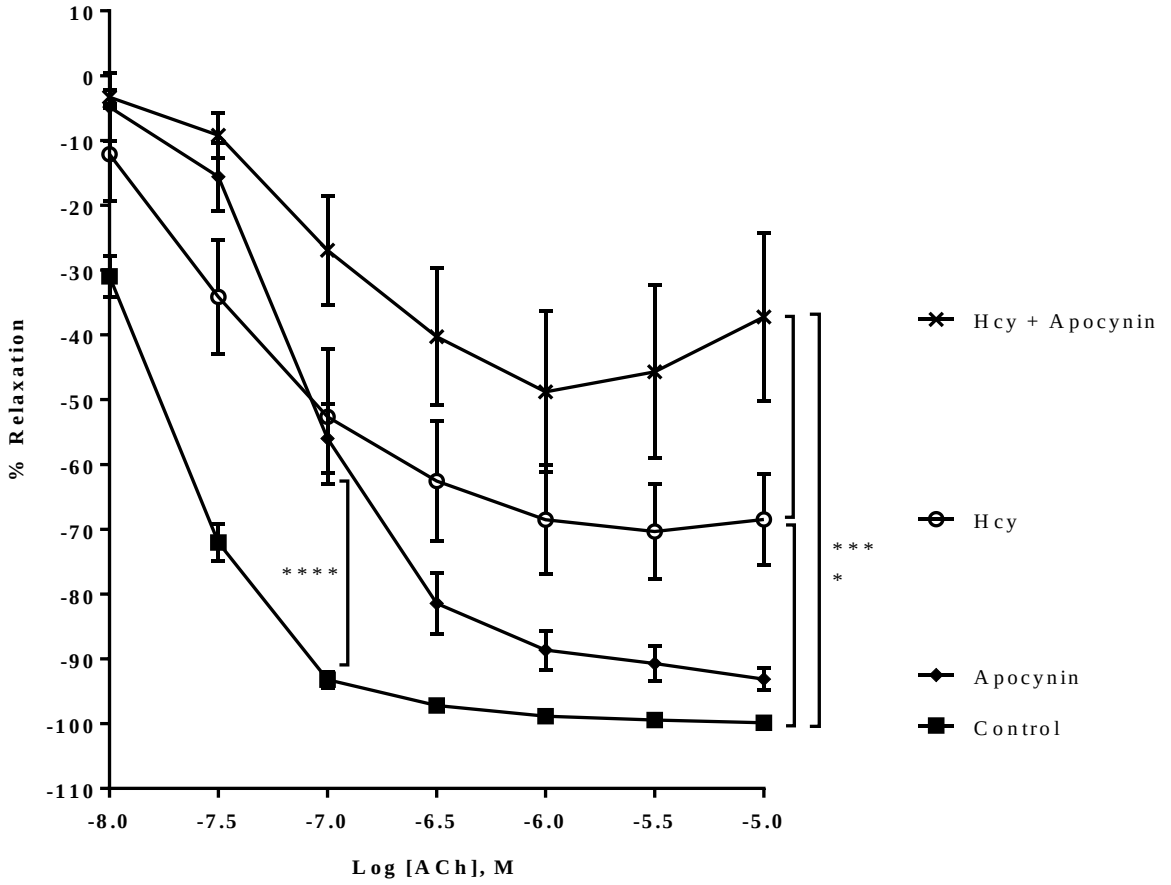
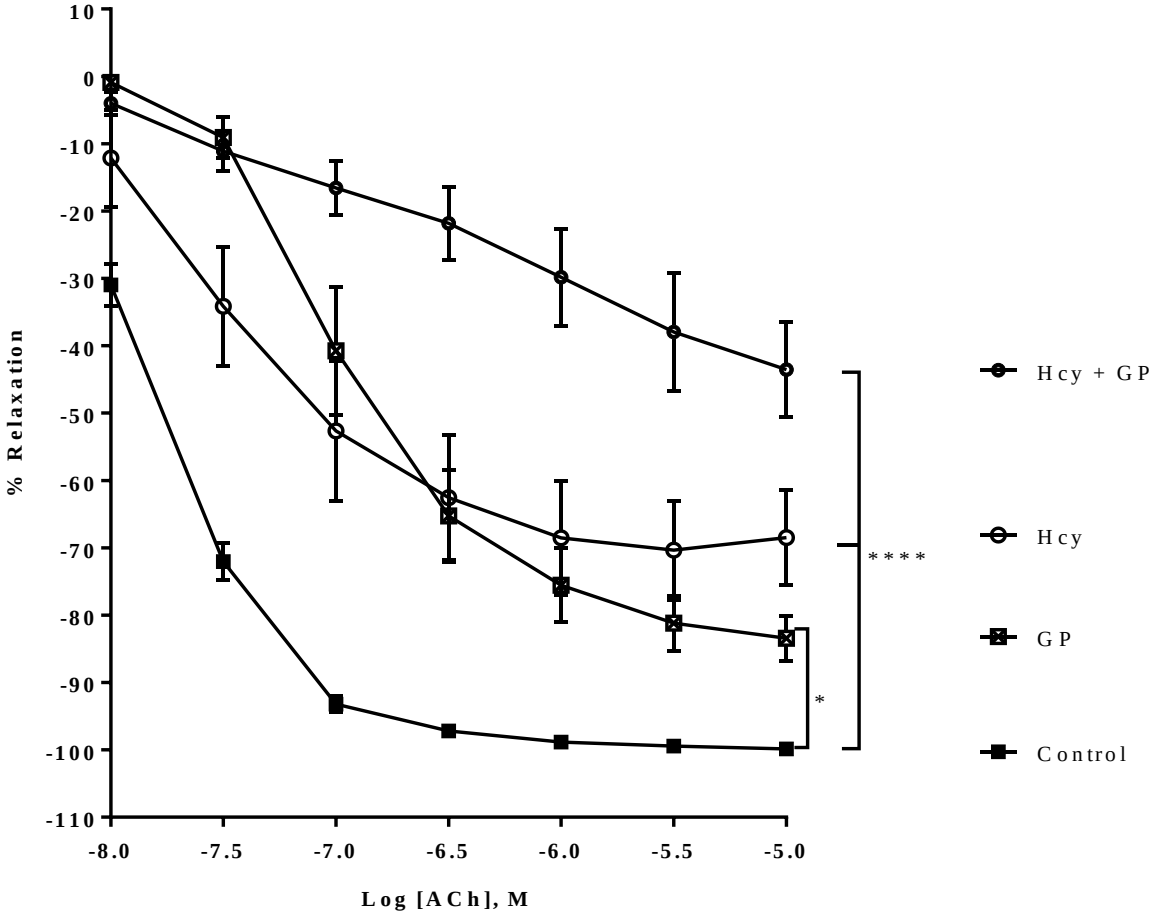
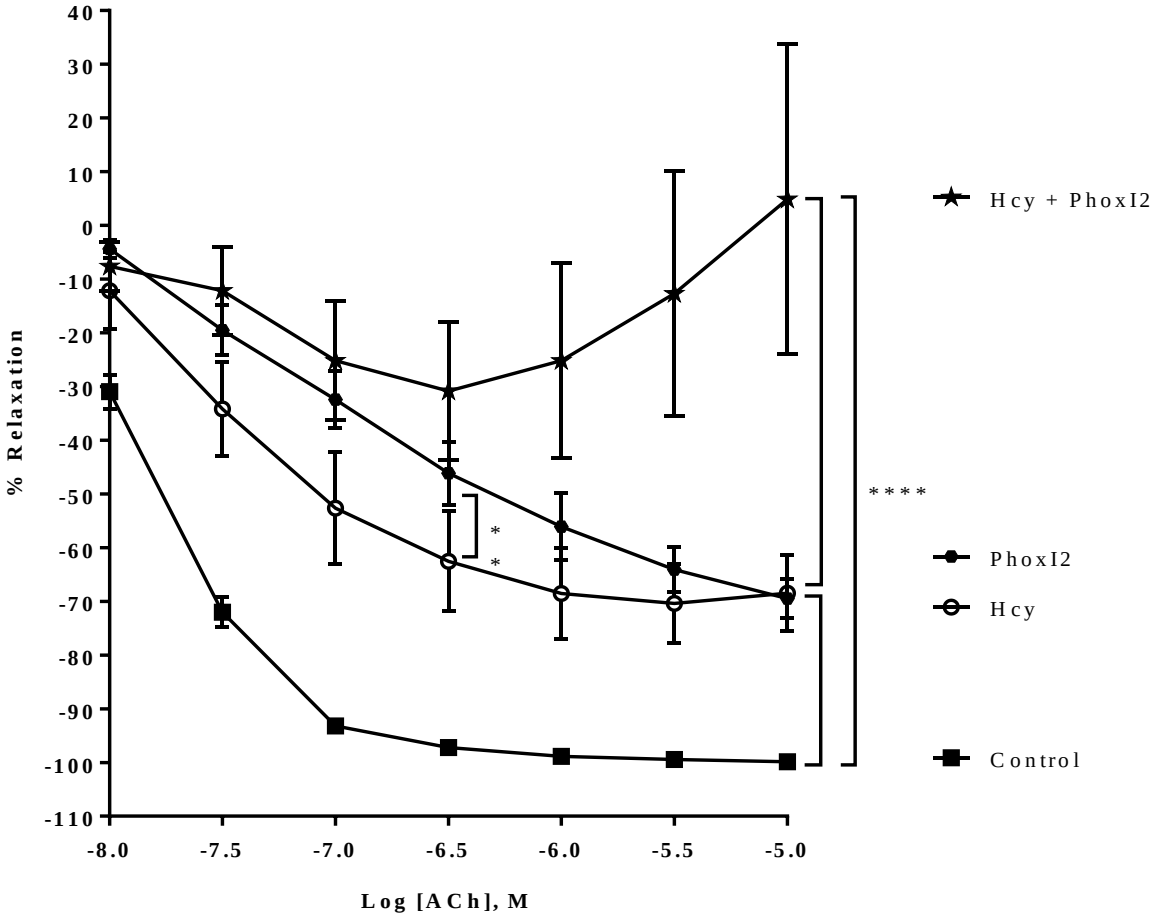


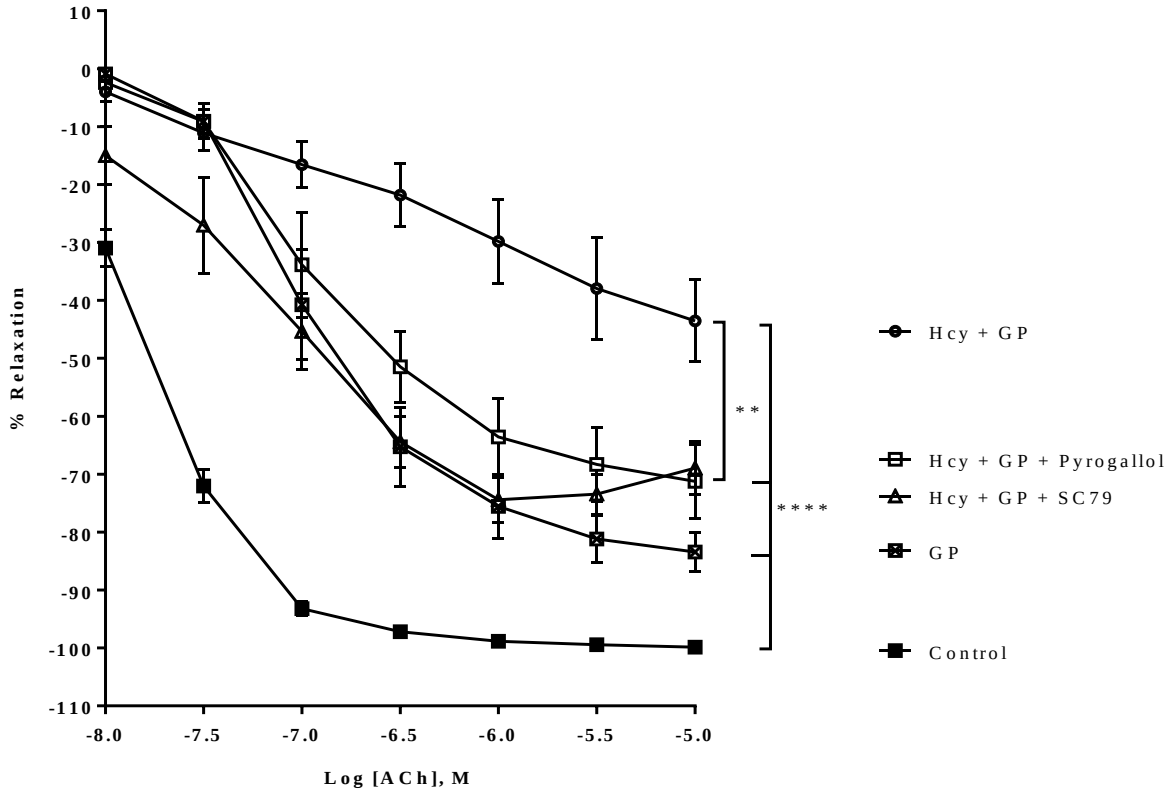
Figure 3.1: A) In control rabbit rings, incubation with 3mM Hcy for 1 hour significantly impaired function compared with control. Rings incubated with both Hcy and apocynin resulted in a severe impairment of relaxation, compared with control, Hcy treatment and apocynin treatment alone (** $p < 0.0001$). Results are mean ± SEM (n=5 per group). All multiple comparisons were performed using Sidak's Multiple Comparisons Test. Only the highest significance has been recorded.**



B) In control rabbit rings, GP significantly impaired function compared with control. Rings incubated with Hcy alone and in combination with GP resulted in a severe impairment of relaxation, compared with control and GP treatment alone (* $p < 0.05$, **** $p < 0.0001$). Results are mean ± SEM (n=5 per group). All multiple comparisons were performed using Sidak's Multiple Comparisons Test. Only the highest significance has been recorded.



C) In control rabbit rings, the addition of PhoxI2 or Hcy significantly impaired function compared with control. Rings incubated with both Hcy and PhoxI2 resulted in a severe impairment of relaxation, compared with control, Hcy alone and PhoxI2 alone ($p < 0.01$, **** $p < 0.0001$). There was no significant difference between treatment with Hcy and PhoxI2. Results are mean ± SEM (n=5 per group). All multiple comparisons were performed using Sidak's Multiple Comparisons Test. Only the highest significance has been recorded.**



D) The addition of pyrogallol and SC79 in rabbit rings incubated with Hcy and GP, resulted in an improvement in relaxation, compared with Hcy and Nox2 inhibition alone (** $p < 0.0001$). We also report an improvement in function between rings treated with Hcy + GP and Hcy + GP + Pyrogallol (** $p < 0.01$). Results are mean ± SEM (n=5 per group). All multiple comparisons were performed using Sidak's Multiple Comparisons Test. Only the highest significance has been recorded.**

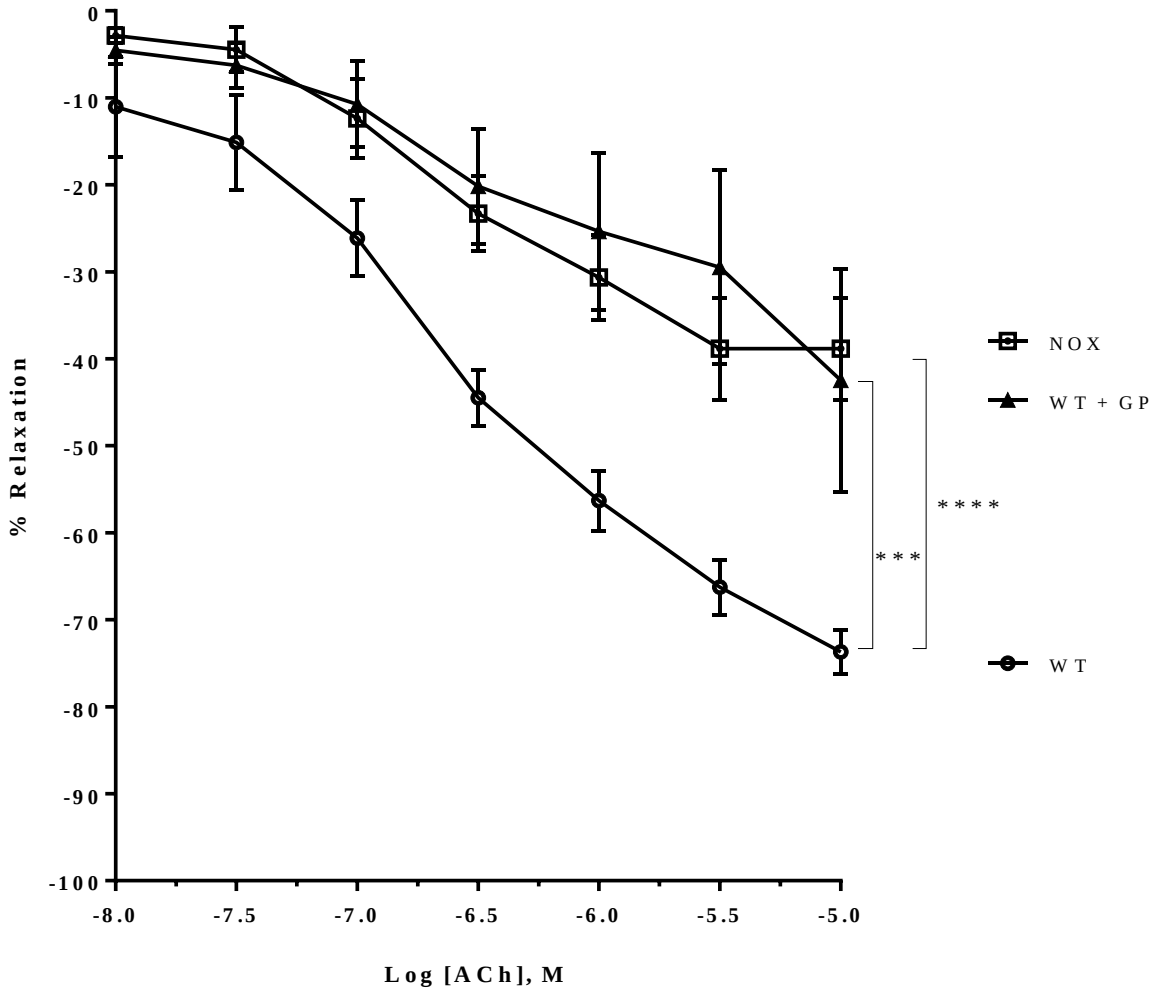


Figure 3.2: In mice, NOX (n=8) and WT + GP (n=4) significantly worsened relaxation vs WT (n=8) (** $p < 0.0001$ and *** $p < 0.0004$, respectively). Interestingly, there was no difference in function between NOX and WT + GP. Results are mean ± SEM. All multiple comparisons were performed using Sidak's Multiple Comparisons Test. Only the highest significance has been identified.**

Table 3.1. EC_{50} for rabbits and mice

Rabbits	Control	Apocynin	Apocynin + Hcy**	PhoxI2*	PhoxI2 + Hcy***	
EC_{50}	-7.8	-7.1	-7.1	-7.6	-7.4	
SEM	0.2	0.1	0.3	1.9	1.0	
Rabbits	Hcy	GP	GP + Hcy***	GP + Pyrogallol*	GP + Hcy + Pyrogallol**	GP + Hcy + SC79
EC_{50}	-7.6	-7.0	-5.5	-6.9	-7.0	-7.1
SEM	1.0	0.1	15.2	0.2	0.2	0.2
Mice	WTChow	WTChow + GP	NOXChow			
EC_{50}	-5.3	1.3	-6.8			
SEM	5.1	446.3	0.4			

There were significant differences in ACh response in rings compared with control. Significant differences between the control and treated groups indicate the reduced efficacy of ACh. All groups compared against control using one-way ANOVA followed by Dunnett's Multiple Comparisons Test. * $p = 0.01$; ** $p = 0.001$; *** $p = 0.0002$. Results are presented as mean ± SEM.

3.7 Discussion

The present study provides direct evidence that apocynin, GP, and PhoxI2 worsens ACh-mediated vasorelaxation, and that these drugs further reduce the acetylcholine mediated vasorelaxation observed after Hcy incubation. Interestingly, NOX2 deletion in mice also show reduced acetylcholine mediated vasorelaxation. As well, histochemical analysis of rabbit aorta further revealed that incubation with Hcy decreased phosphorylation at Thr^{495} while increasing phosphorylation at Ser^{1177} (Fig 3A), suggesting an underlying upregulation of eNOS to maintain homeostasis. In HHcy, GP reduced eNOS phosphorylation at Ser^{1177}, suggesting that GP can affect acetylcholine medicated vasorelaxation via this pathway.

The rabbit model showed increased acetylcholine mediated relaxation in the presence of Hcy (Figure 3.1A-C). The drugs employed in this study all act on Nox2 assembly differently: apocynin is a small molecule Nox2 inhibitor that blocks the migration of $p47^{phox}$ to the membrane (Rastogi et al. 2016), GP is a chimeric 18 amino-acid sequence peptide which blocks assembly of $p47^{phox}$ and $gp91^{phox}$ (Rastogi et al. 2016) and PhoxI2, another small molecule inhibitor, exerts its inhibitory action on Nox2 by binding to $p67^{phox}$, disrupting the binding of $p67^{phox}$ to Rac (Bosco et al. 2012; Jo & Luo 2012); they are also structurally different from

each other (Fig. 3.4). Interestingly, despite differences in structure and function, apocynin, GP and PhoxI2 all impaired function in both healthy and HHcy vessels, in both rabbits and (GP) in mice.

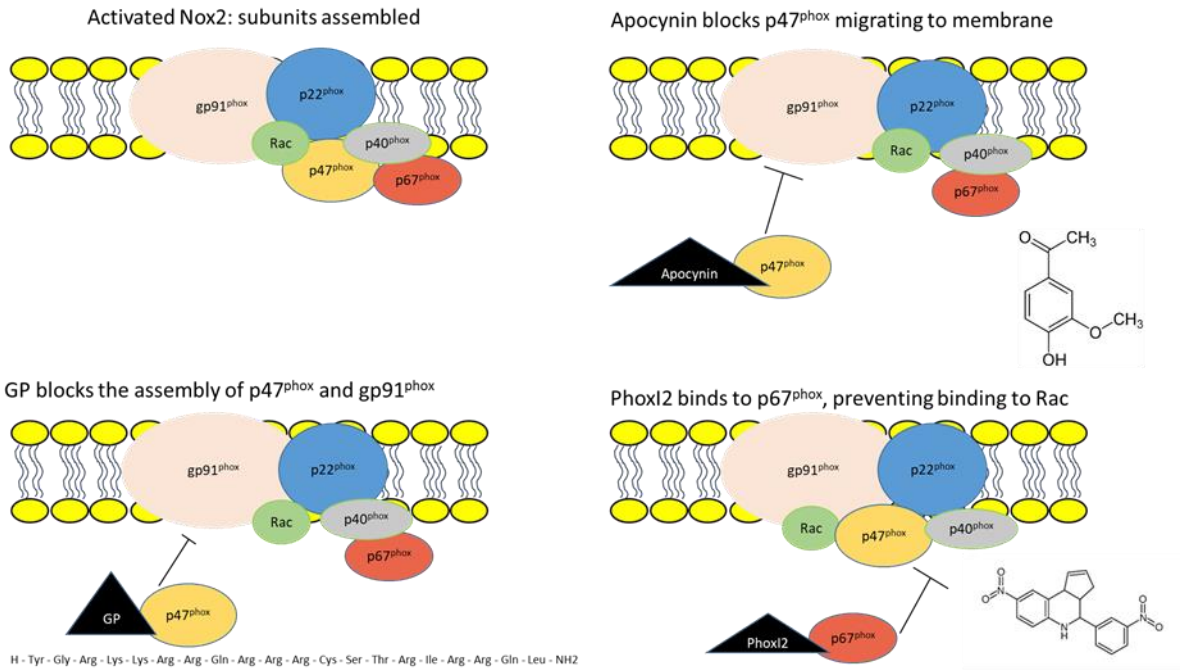


Figure 3.4. Nox2 assembly, inactivation by apocynin, GP or PhoxI2 and their structure. Nox2 inactivation results in reduced O_2^- production (Rastogi et al. 2016).

Thus, here we report that these drugs might not be suitable to impair the detrimental effects of homocysteine on blood vessel function, and suggest further development of novel Nox2 inhibitors for this purpose. We also showed reduced vascular function in NOX mice (Fig. 2), which could be due to failure of genetic compensatory mechanisms. For example, in an eNOS mouse knock-out, it was recently reported that vasodilatory responses in the ophthalmic artery were kept stable by a compensatory mechanism of endothelium-derived hyperpolarising factors, specifically lipoxygenase and with some participation from calcium-controlled K+ channels, which are essential for vasodilation (Manicam et al. 2017). Additionally, gene deletion has resulted in significant upregulation of "non-essential" genes to perform the same or similar function (Bergmiller, Ackermann & Silander 2012), suggesting that compensation

is an essential function. Additionally, there was no difference in response between the WT GP treated and NOX mice, which suggests that the effect of GP on blood vessels is similar to the genetic deletion of Nox2 in this model. This indicates a possible specificity of GP for Nox2 (Fig. 3.2). The major finding in this study is that Nox2 inhibition worsens Hcy-induced dysfunction. The failure of apocynin, GP and PhoxI2 to improve Hcy-induced dysfunction is surprising, given that it has been widely reported that acetylcholine mediated vasorelaxation has been restored with these drugs (Drummond & Sobey 2014), indeed as have genetic knockout models of Nox2 (Rivera et al. 2010).

SC79 is a recently described specific intracellular Akt-activator which has been shown to suppress neuronal ischaemic excitotoxicity (Jo et al. 2012). Activation of the Akt pathway is essential for endothelial function (Ren et al. 2016); it's role as an eNOS and NO modulator is well-reported (Barbosa et al. 2013; Wang, R et al. 2015). For Akt to function as a mediator of endothelial-dependent vasodilation, Nox2 assembly and activation is essential (Abid et al. 2007). Additionally, ROS in the cardiovascular system can activate Akt (Abeyrathna & Su 2015), although the exact impact on vasodilation remains unclear. SC79 has been shown to have other effects. It has been reported to reverse doxycycline-mediated anti-histamine effects in mast cells (Su et al. 2014), improved myelination in damaged neuronal cells (da Silva et al. 2014) and in human T regulatory cells, SC79 activated Akt to rescue Foxp3-induced Glut1 repression (Samik, Britany & M. 2015). Here we have shown that SC79 reduced the effect of Hcy + GP and improved vasodilation (Figure 3.1D). SC79 elicits its effect by directly binding to the Akt PH domain and inducing a favourable conformation, thus improving downstream signalling (Jo et al. 2012) and eNOS-stimulated NO release (Taguchi et al. 2016). Crucially, SC79 allows for Akt activation in the cytosol, avoiding the need for targeting Akt on the membrane (da Silva et al. 2014).

Our results indicate that apocynin, PhoxI2 and GP might not be suitable to normalize impaired acetylcholine-mediated vasorelaxation caused by Hcy-induced stress as they decrease the level of ROS required for NO release (Rifkind et al. 2018). Pyrogallol is a benzenetriol ('Final Report on the Safety Assessment of Pyrogallol' 1991), and has been studied (in doses > 100 µM) as an inducer of impaired ACh-mediated vasorelaxation (Demirci, McKeown & Bayraktutan Dvm 2008; Lee, SF & Lin 1994) and cardiomyocyte impairment (Esberg & Ren 2004). Pyrogallol generates O_2^-, H_2O_2 and the hydroxyl radical (Lee, SF & Lin 1994). Surprisingly, we found that putative Nox2 inhibitors worsened acetylcholine mediated relaxation, and exacerbated HHcy induced reduction in ACh-mediated relaxation and significantly lowers the potency (EC_{50}) of ACh; this was somewhat mitigated by incubation with pyrogallol (Fig. 3.1D). This implies that a reduction in O_2^-, and not drug structure, that impaired acetylcholine-mediated vasorelaxation.

Support for an essential vasodilatory role for O_2^- has been shown in a novel Nox2 over-expressed mouse, where the authors reported improved coronary vasodilation, eNOS activation and NO synthesis (Shafique et al. 2013). Additionally, exercise training in rats was shown to increase O_2^- and consequently eNOS phosphorylation, which is essential for proper ACh mediated vasodilation (Barbosa et al. 2013). It is currently unknown whether the improvement in endothelial function in the presence to O_2^- was due to increased SOD activity or increased signalling directly activating eNOS; together both could have augmented downstream NO release (Zhao, Vanhoutte & Leung 2015).

We used ACh to measure endothelial-dependent relaxation, which is an established method of assessing NO release (Förstermann & Sessa 2012; Rai, Hare & Zulli 2009). These isometric tension results are in line with previous studies from our lab, wherein a recent paper used the NO donor sodium nitroprusside (SNP) to show that normal relaxation occurs to the same extent

in HHcy incubated vessels, and that a novel peptide of the renin angiotensin system can restore normal ACh mediated vasorelaxation after exposure to Hcy (Qaradakhi et al. 2017).

3.8 Limitations

Pharmacological Nox inhibitors were used in this study based on previous findings (Bosco et al. 2012; Rey et al. 2001; Tang et al. 2012). Additionally, pharmacological Nox inhibitors are not 100% specific (Bedard & Krause 2007b) and have not been fully ascertained (Smith, R et al. 2015), therefore may have off-target effects.

3.9 Conclusion

The results presented here provide evidence that the current putative Nox2 inhibitors, are unlikely to reduce HHcy induced vascular damage, and might even worsen damage. Further research into developing new Nox2 inhibitors should be sought for HHcy-induced disease.

3.10 The Effect of Nox4 Inhibitor VAS2870 in Acute and Chronic Homocysteine-Induced Endothelial Dysfunction

3.11 Abstract

Introduction: Plasma homocysteine (Hcy) is a metabolic by-product of methionine, an essential amino acid supplied by the diet. Excess Hcy (HHcy) is associated with endothelial dysfunction and is an independent risk factor for cardiovascular disease (CVD), stroke and heart attack. Clinical-based therapy aimed at normalising Hcy have been unsuccessful, thus novel treatments are necessary to protect against Hcy-induced vascular dysfunction. Hcy stimulates the NADPH oxidase (Nox) family of enzymes to produce excess reactive oxygen species (ROS). Antioxidants are scavengers of excess ROS. There is evidence that antioxidant properties present in nuts may interact with Nox, thereby participating in the release of ROS production. The aim of the study was to investigate inhibiting Nox4 as a novel therapeutic approach to treating HHcy.

Objectives: To determine if the antioxidant effect of putative Nox4 inhibitor VAS prevents acute Hcy-induced endothelial dysfunction in the rabbit and to assess the effect of Hcy-dysfunction in a chronic mouse model of endothelial dysfunction induced with a 1% methionine diet.

Methods: New Zealand White rabbits (Rchow) and wild-type mice fed chow (WTchow) or a 1% methionine diet (WTmeth; 4 weeks) had their aortae excised, cut into 2-3mm rings and mounted in organ baths. Rabbit rings were incubated with either 1µM VAS and/or 3mM Hcy for 1 hour; mice rings were incubated with VAS only. A bolus dose of superoxide dismutase (SOD; 1000 I/U, H_2O_2 donor) or the Akt activator SC79 (1µM) was added to potentially negate the effects of VAS in rabbit rings. To measure vasodilation, all rings were subjected to a dose response curve to acetylcholine (ACh; 0.01µM-10µM).

Results: In Rchow, acute HHcy induced endothelial dysfunction in the aorta vs control ($p<0.0001$). The addition of VAS in acute HHcy worsened function, compared with control ($p<0.0001$), however the effect of VAS was improved by the addition of superoxide dismutase (SOD; $p<0.001$). In WTmeth, diet intervention or VAS alone significantly reduced relaxation compared to control ($p<0.001$), while in combination, slightly improved endothelial function ($p<0.05$) compared to intervention in combination.

Conclusion: Nox4 inhibition accentuates acute and chronic Hcy-induced endothelial dysfunction in rabbits, but paradoxically improves function in chronic HHcy in mice. Compounds present in nuts might have properties similar to Nox inhibitors. This mechanism operates via the essential free radical H_2O_2 and further research is necessary to determine a suitable treatment for Hcy-induced vascular dysfunction.

3.12 Introduction

An increased level of homocysteine (Hcy) is an independent risk factor for coronary artery disease (CAD) and atherosclerosis (Jiang et al. 2012; Julve et al. 2013), typified by arterial intimal thickening and increasing risk of stroke and myocardial infarction (Miida et al. 2014). Hyperhomocysteinemia (HHcy), classified as >9 µM (Beard, Reynolds & Bearden 2012), is caused by aberrant methionine metabolism; in severe cases it has been recorded as high as 300µM (Beilby, John & Rossi). HHcy is associated with reduced levels of vitamin B6, B12 and folate (Beilby, John & Rossi), which are required for catalysing enzymes to ensure proper metabolism (Hoffman 2011). Rabbits fed a high (1%) methionine diet develop HHcy and display intimal proliferation, a vascular pathology important for the development of atherosclerosis (Zulli et al. 2004). However, numerous studies investigating B vitamin administration to treat HHcy have been disappointing, with little to improvement in risk for patients (Hodis et al. 2009; Kaul, Zadeh & Shah 2006; Lonn et al. 2006; Pettigrew et al. 2008). The outcomes of such studies may be influenced by the relatively small reduction in plasma Hcy. For example, the healthy range of Hcy is reported to be 6-9µM (Beard, Reynolds & Bearden 2012), whereas these trials failed to reduce Hcy levels into the healthy range, usually reaching around 13µM, which could explain the disappointing outcomes.

Nicotinamide adenosine diphosphate (NADPH) oxidases (Nox) are trans-membranous enzymes producing functional reactive oxygen species (ROS) comprising of 7 different types: Nox1-5 and Duox1 & 2. In CVD, Nox1, 2 and 4 are relevant; especially as the ROS they generate are considered essential for signalling (Bedard & Krause 2007b; Drummond & Sobey 2014; Manolescu et al. 2010). Dysregulated ROS have been implicated in the progression of cardiovascular disease (Howitt et al. 2014), atherosclerosis (Judkins et al. 2010), ischaemic injury (Dvoriantchikova et al. 2012; Kahles & Brandes 2012) and systemic hypertension (Wind et al. 2010). In addition to this, it has been shown that in cancer proliferating cells, genetic

silencing of Nox1 and Nox4 directly inhibits ROS production and reduces the associated DNA damage (Illeperuma et al. 2015). Central to this study, Nox4 is highly expressed in the vasculature and produces H_2O_2 continuously (Schurmann et al. 2015); this isoform generates approximately 90% H_2O_2 and 10% O_2^- (Nisimoto et al. 2014). Nox4 is the most highly expressed of the Nox family (Breton-Romero & Lamas 2014), and is unique among the other Nox as it does not require activation via several intracellular subunits, only depending on subunit $p22^{phox}$, thus simplifying its activation. Paradoxically, the evidence for Nox4 inhibition in CVD suggests both beneficial and deleterious outcomes in CVD. An increased risk of adverse thrombotic events with excess plasma H_2O_2 has been reported (Dayal et al. 2013) and Nox4 inhibition has been shown to relax hypoxic bovine pulmonary arteries (Ahmad et al. 2010). In ischaemic reperfusion, Nox4 inhibition was cardio-protective (Siu et al. 2014). On the other hand, Nox4-generated H_2O_2 might be protective, as over-expression of Nox4 has been shown to increase endothelial nitric oxide synthase (eNOS) activity, leading to enhanced vasodilation (Drummond & Sobey 2014). Further, Nox4 over-expressed mice were protected against contractile dysfunction, hypertrophy and dilation during hypoxia, in contrast *null* Nox4 mice were not protected (Zhang, M et al. 2010).

Several properties of nuts important in CVD research include antioxidant capacity, which have led to nuts being included in diet (Estruch et al. 2016; Hernaez et al. 2017). Indeed the best known plant compound studied in CVD research is resveratrol, present in red wine grapes, berries and peanuts (Spanier. et al. 2009). Although the research investigating a direct relationship between nuts and Nox is limited, there is preliminary support for investigating nuts in relation to Nox in Hcy-induced endothelial dysfunction, particularly relating to the important presence of antioxidants. For example, it has been reported ellagic acid, a polyphenolic compound present in nuts and fruit, is successful in inhibiting Nox-induced O_2^- and contributing to a downstream effect of increased antioxidant defences (Lee, WJ et al. 2010).

Further, selenium, present in Brazil nuts and an important antioxidant modulator, has been shown, in low levels, to exhibit anti-diabetic properties (Steinbrenner et al. 2011). Importantly, Nox4 is reportedly activated by insulin and participates in the insulin signalling cascade (Steinbrenner et al. 2011).

VAS is a reported putative Nox4 inhibitor (Wind et al. 2010) and has been effective in ischaemic stroke (McLeod et al. 2010). It is proposed to control ROS signalling in spontaneously hypertensive rats to improve endothelial function (2010) and abolish NO inhibition, via T-type calcium channels, in mice (Howitt et al. 2014). In contrast, a $Nox4^{-/-}$ /$ApoE^{-/-}$ double knockout murine model on a Western-style (high-fat) diet had increased atherosclerotic plaque in the aortic sinus, thoracic and abdominal aorta compared to wild-type controls (Schurmann et al. 2015). These conflicting results highlight that it is still unclear exactly how Nox4, and its product H_2O_2, might be protective in the cardiovascular system (Schurmann et al. 2015).

The Akt pathway is highly complex and participates in the regulation of multiple cellular processes including proliferation, apoptosis and antioxidant generation and is of particular importance to cardiovascular function (Abeyrathna & Su 2015). Akt can be activated by several different effectors, including shear stress (Balligand, Feron & Dessy 2009) and ROS (Abeyrathna & Su 2015); SC79 activates Akt by inducing the conformational change necessary for activation (Jo et al. 2012; Moreira et al. 2015). Whilst the data is scant, there is evidence for an important role for Akt in Hcy-induced endothelial dysfunction. Akt activation is essential for eNOS activation and function, via its role in catalysing phosphorylation of eNOS at Serine 1177 (Ser^{1177}) (Heiss & Dirsch 2014). Hcy has been shown to reduce phosphorylation of both and eNOS and Akt, resulting in reduced NO expression, but this was reversed by activating the Akt pathway with the trace element selenium (Ren et al. 2016), essential for proper antioxidant-driven ROS scavenging (Sampson, N. et al. 2011).

The aim of this study was to determine whether pharmacological inhibition of Nox4 with the putative inhibitor VAS would improve ACh-dependent endothelial relaxation in acute Hcy dysfunction and chronic Hcy dysfunction in a mouse model of fed a 1% methionine diet. To assess whether ACh-dependent relaxation also involved the Akt pathway, we also used a pharmacological Akt activator, SC79.

3.13 Methods

All animals were housed and experiments carried out according to the National Health and Medical Research Council "Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (8^{th} Ed. 2013).

Rabbits: New Zealand rabbits (12 weeks, male, n=15, VUAEC #12/019) were housed in separate cages and maintained at a constant temperature of approximately 23°C and were provided water and food *ad libitum*. Animals arrived between 9 and 12 weeks of age and were used in order of oldest to youngest over a 3 week period. Whilst in our care, animals were fed a normal chow diet. Animals were anaesthetised with isoflourane (4% O_2), exsanguinated and the abdominal aorta excised and flushed with cold oxygenated Krebs buffer (NaCl 118.4, KCl 4.7, NaHCO3 25, MgSO4 1.2, CaCl2, 2.5, glucose 11.1, mM).

Mice: Wild-type mice were fed chow (WTchow; n=4) or a 1% methionine diet for 8 weeks (WTmeth; n=4; VUAEC #14/014). Animals were housed in cages and maintained at a constant temperature of approximately 23°C. Food and water were provided *ad libitum*. At sacrifice, mice were anaesthetised with isoflourane (4% O_2) followed by cervical dislocation, and the aorta excised and flushed as above.

The aortae were cleaned of fat and connective tissue, cut into rings (2-3 mm lengths) and placed in organ baths (OB8, Zultek Engineering, Australia), filled with Krebs, kept at a constant temperature of 37°C and continuously bubbled with carbogen (95% O_2 + 5% CO_2). These rings were left to rest, unmounted, for 30 mins. Rings were then mounted between two metal hooks attached to force displacement transducers, stretched to 2g (rabbits) or 0.5g (mice) and allowed to reach resting tension plateau. After this, rings were again stretched to 2g (rabbits) or 0.5g (mice) and allowed to reach resting tension plateau a second time.

Experimental Protocol

Rabbits: Experimental rings were incubated with 1µM VAS for 30 minutes and/or 3mM Hcy for 1 hour, before being pre-contracted with phenylephrine (Phen) (0.1-0.3µM). Once contraction reached 50% of maximum tension, in some rings, superoxide dismutase (SOD; 1000 I/U) or SC79 (Akt activator; 1µM) was added, prior to a concentration-response curve to ACh-induced relaxation (0.01µM-10µM).

Mice: Control rings were incubated with Krebs only. Experimental rings were incubated with 1µM VAS for 30 minutes before being pre-contracted with thromboxane analogue (U4; 0.1µM), after the contraction reached a plateau (approx. 50% of its maximum tension). A concentration-response curve to ACh was then performed.

Statistical Analysis

All data were analysed using a two-way repeated measures ANOVA, measuring differences in response to drug dose between and within groups, followed by Sidak's Multiple Comparisons Test. Data are represented as mean ± SEM. Significance was accepted at $p<0.05$.

3.14 Results

As expected, Hcy-induced endothelial dysfunction in the aorta vs control (Figure 3.3A; 63.0±7.9% vs 97.9±1.0%, $p<0.0001$), while treatment with VAS alone also significantly impaired vasorelaxation compared to control (Figure 3.3B; 89.1±2.7% vs. 97.9±1.0%, $p<0.0001$), although not to the same extent as Hcy (63.0±7.9 vs 89.1±2.7%, $p<0.0001$). To investigate the impact that Nox4 inhibition might have on Hcy-induced endothelial dysfunction, we treated some rings with both Hcy and VAS. In rings with combined Hcy and Nox4 inhibition, function was significantly impaired compared with controls (Figure 3.3B; 19.0±16.3% vs. 97.9±1.0%, $p<0.0001$) or either treatment alone. To investigate if the removal of H_2O_2 was the direct cause of reduced relaxation in Nox4 inhibition, SOD, an antioxidant which catalyses H_2O and O_2^- into H_2O_2 and O_2 , was added to the organ bath. The addition of SOD 5 minutes before the construction of the ACh curve significantly reversed the effect of VAS in Hcy-treated vessels (Figure 3.3C; 55.9±8.7%, $p<0.001$). To investigate the role of the Akt pathway in Hcy-induced endothelial dysfunction, we used the Akt activator SC79 to selectively activate the Akt pathway (Jo et al. 2012). To assess the function in the healthy vessel, we applied SC79 alone to rings, which resulted in impaired function (Figure 3.3D; 69.3±1.9%, $p<0.0001$). In induced dysfunction, adding SC79 significantly restored vasodilatory function to the vessel compared with VAS and Hcy alone (Figure 3.3E; 61.3±6.8% vs. 19.0±16.3%, $p<0.0001$).

Treating WTmice (C57/BLK6) fed chow with the putative Nox4 inhibitor VAS significantly reduced endothelial function compared with control (Figure 3.4A; 77.9±0.8% vs. 60.3±2.6%, $p<0.001$). To assess the effect of a high methionine diet on endothelial dysfunction in mice, we fed WTmice a 1% methionine diet. Mice fed a 1% methionine diet exhibited significantly reduced vasodilation compared to control diet (Figure 3.4B; 59.1±4.1 vs 77.9±0.8%, $p<0.001$). Interestingly, the addition of VAS to mice fed a 1% methionine diet improved endothelial function compared with VAS treatment alone (Figure 3.4C; 69±5.4% vs. 60.3±2.6%, $p<0.05$).

3.15 Figures

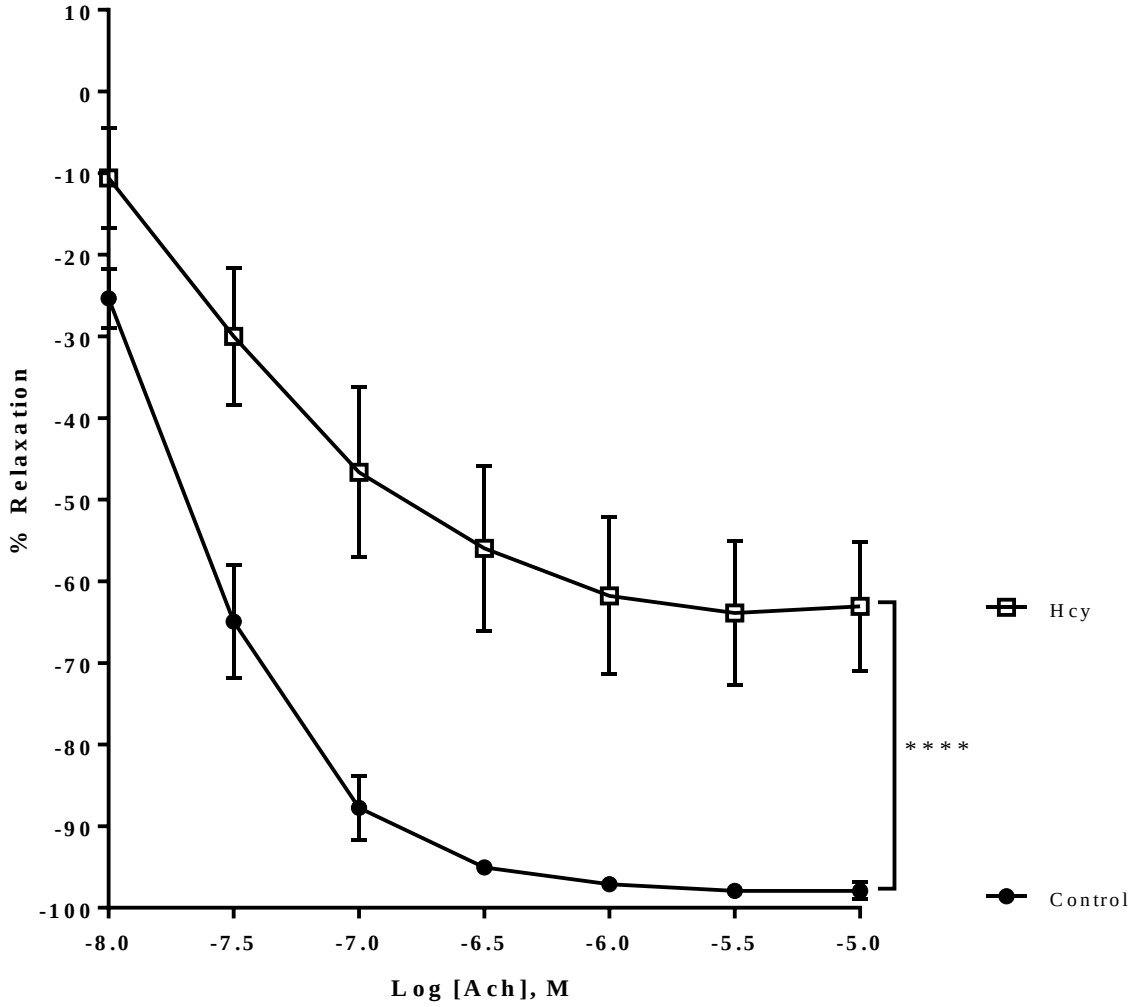
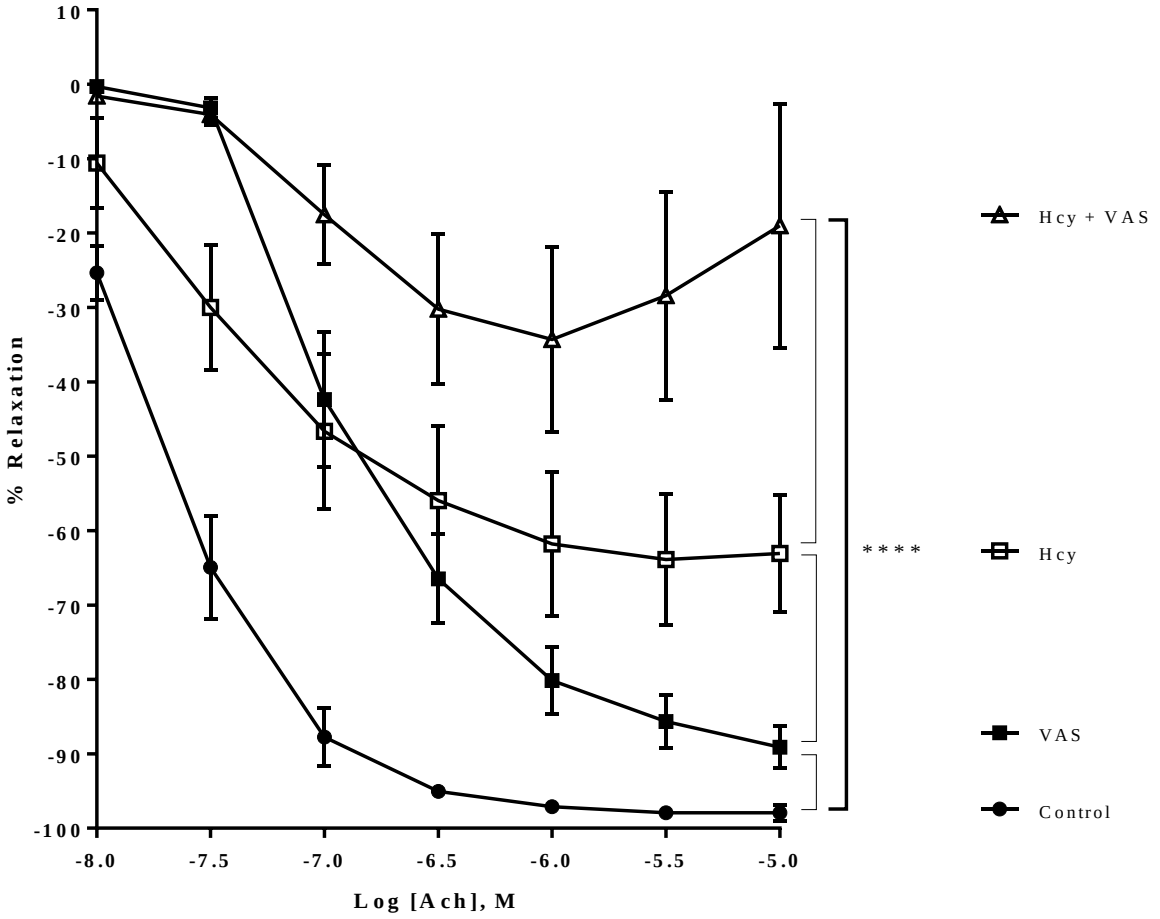
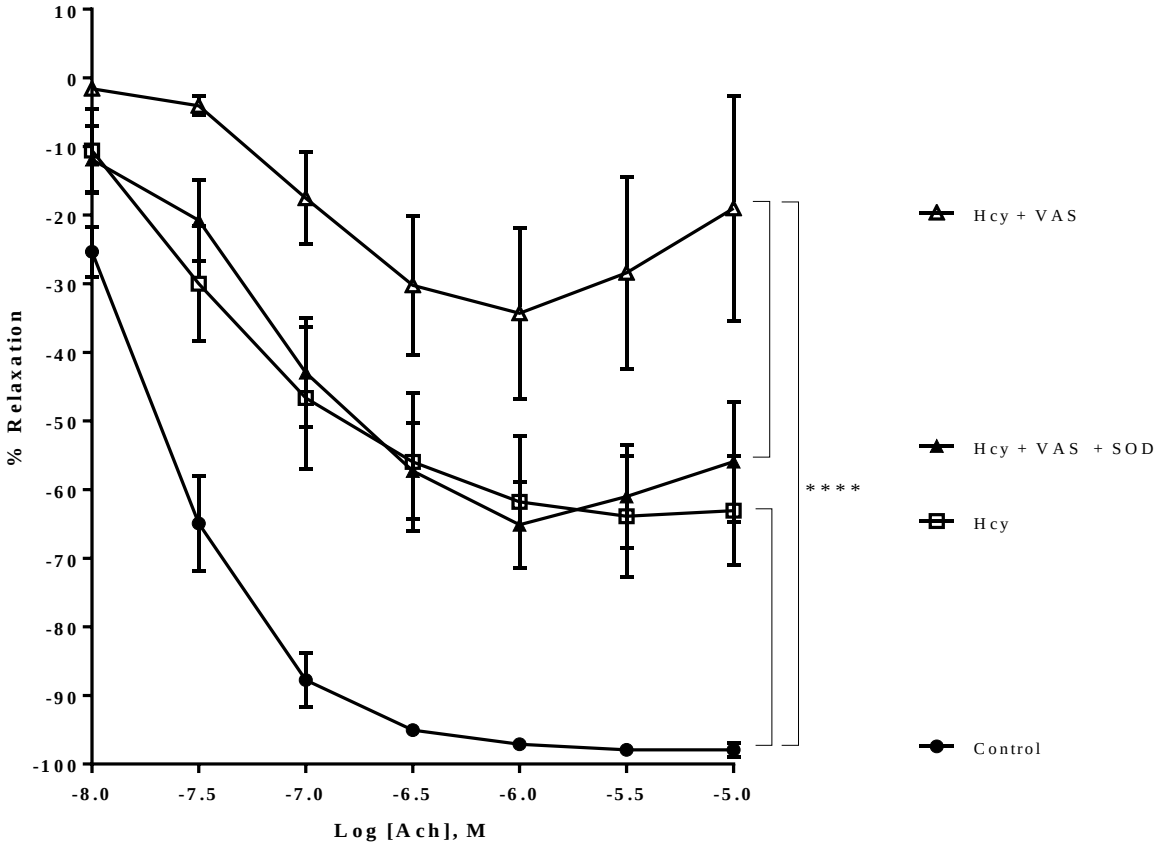


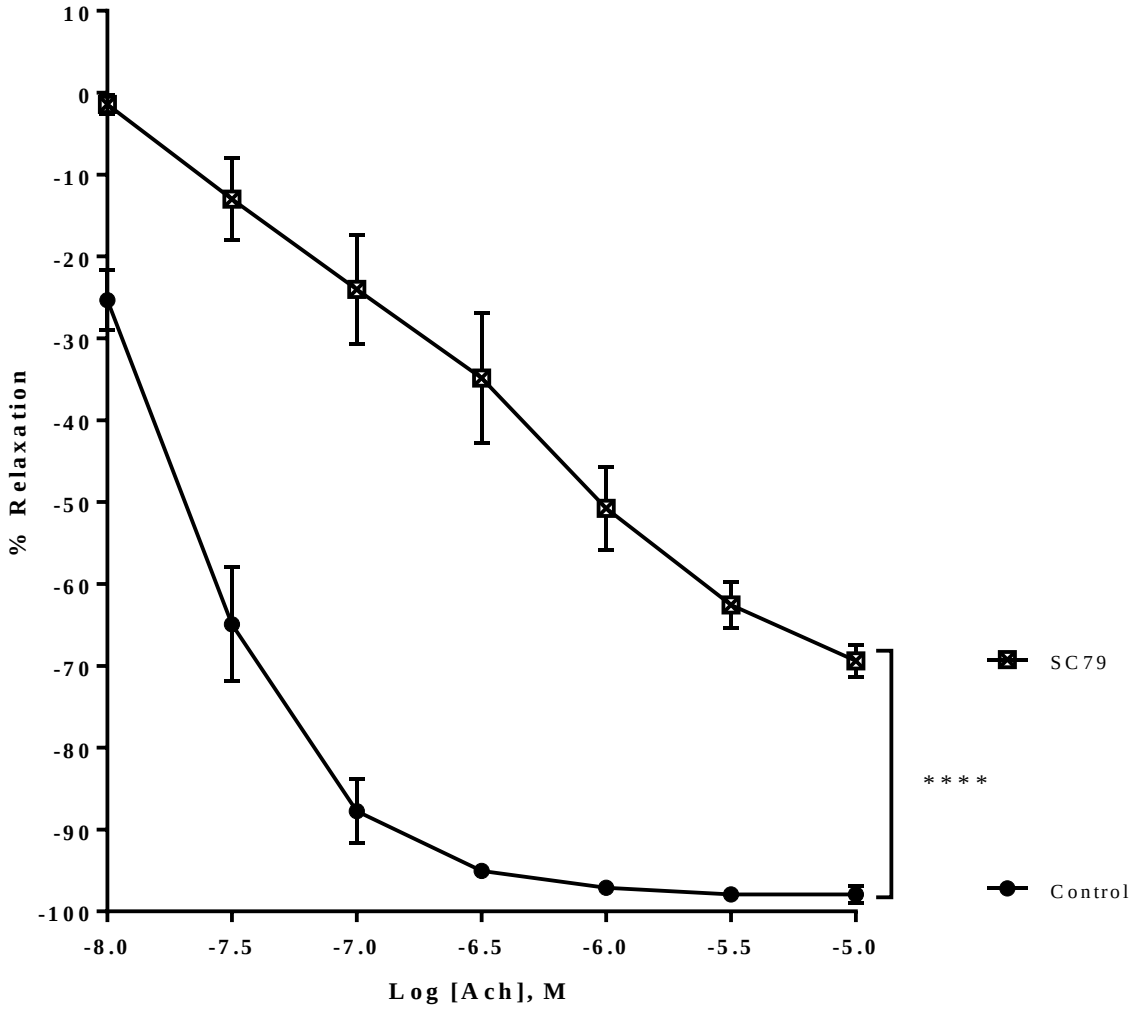
Figure 3.3A) Rabbit aortic rings were treated with 3mM Hcy for 1 hour before being subject to an ACh dose curve. Hcy treatment significantly impaired ACh-dependent endothelial relaxation compared with control (p<0.0001). Results expressed as mean ± SEM (n=5 per group). Only the highest significance has been recorded.



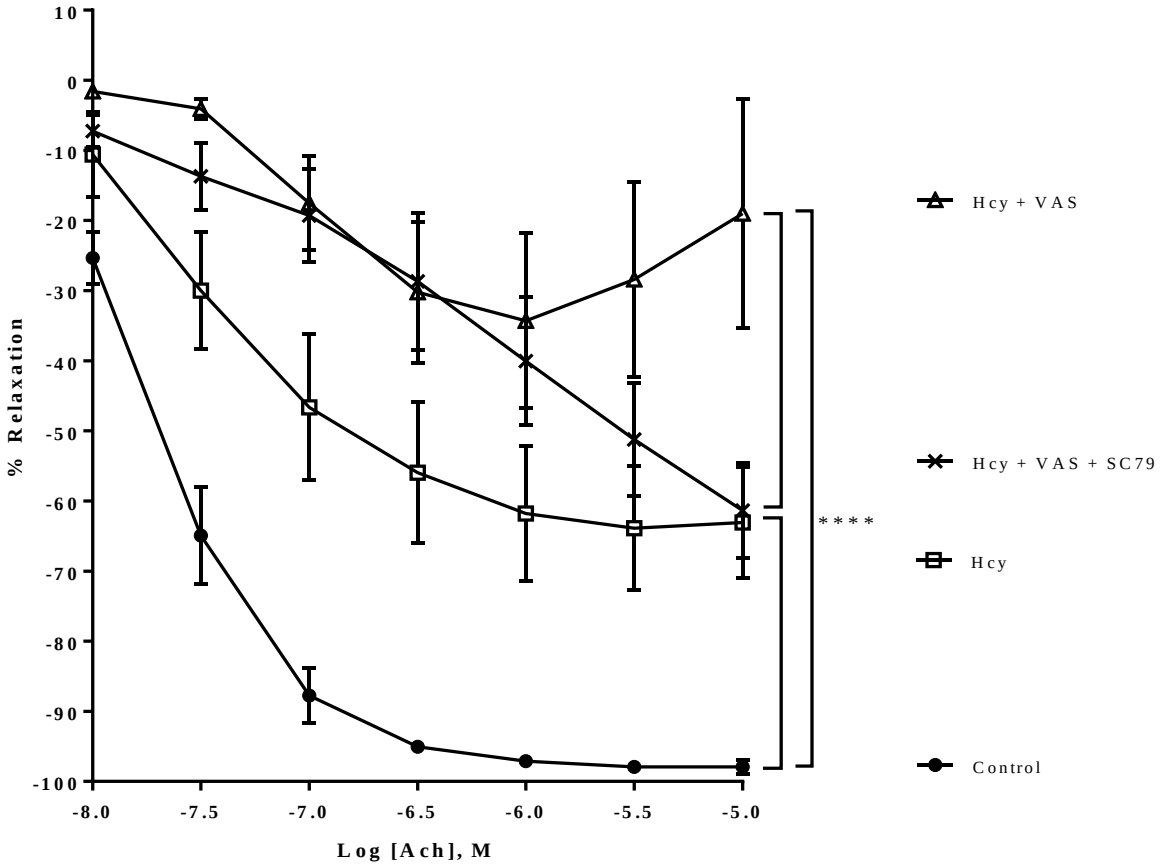
B) In control rabbit vessels, applying VAS to inhibit Nox4 impaired function compared with control in early doses (p<0.0001). Application of VAS in vessels treated with Hcy further worsened vascular function, compared to control and singular Hcy and VAS interventions (p<0.0001). Results expressed as mean ± SEM (n=5 per group). Only the highest significance has been recorded.



C) To measure the effect of H_2O_2 on endothelial dysfunction, we used the H_2O_2 donor superoxide dismutase (SOD) to increase H_2O_2 levels. In dysfunctional rabbit rings treated with Nox4 inhibition and Hcy, the addition of H_2O_2 significantly improved vasodilation compared with VAS and Hcy in combination (p<0.0001). Results expressed as mean ± SEM (n=5 per group). Only the highest significance has been recorded.



D) In healthy rabbit vessels, the stimulation of the Akt pathway with SC79 significantly impaired vascular relaxation compared with control (p<0.0001). Results expressed as mean ± SEM (n=5 per group). Only the highest significance has been recorded.



E) In rabbit rings treated with Hcy and VAS, the addition of SC79 significantly negated the effect of VAS in Hcy-induced dysfunction in healthy rabbits (p<0.0001), however there was no difference compared to Hcy treatment alone. Despite the improvement in function, treating with VAS + Hcy + SC79 resulted in significantly impaired function compared with control (p<0.0001). Results expressed as mean ± SEM (n=5 per group). Only the highest significance has been recorded.

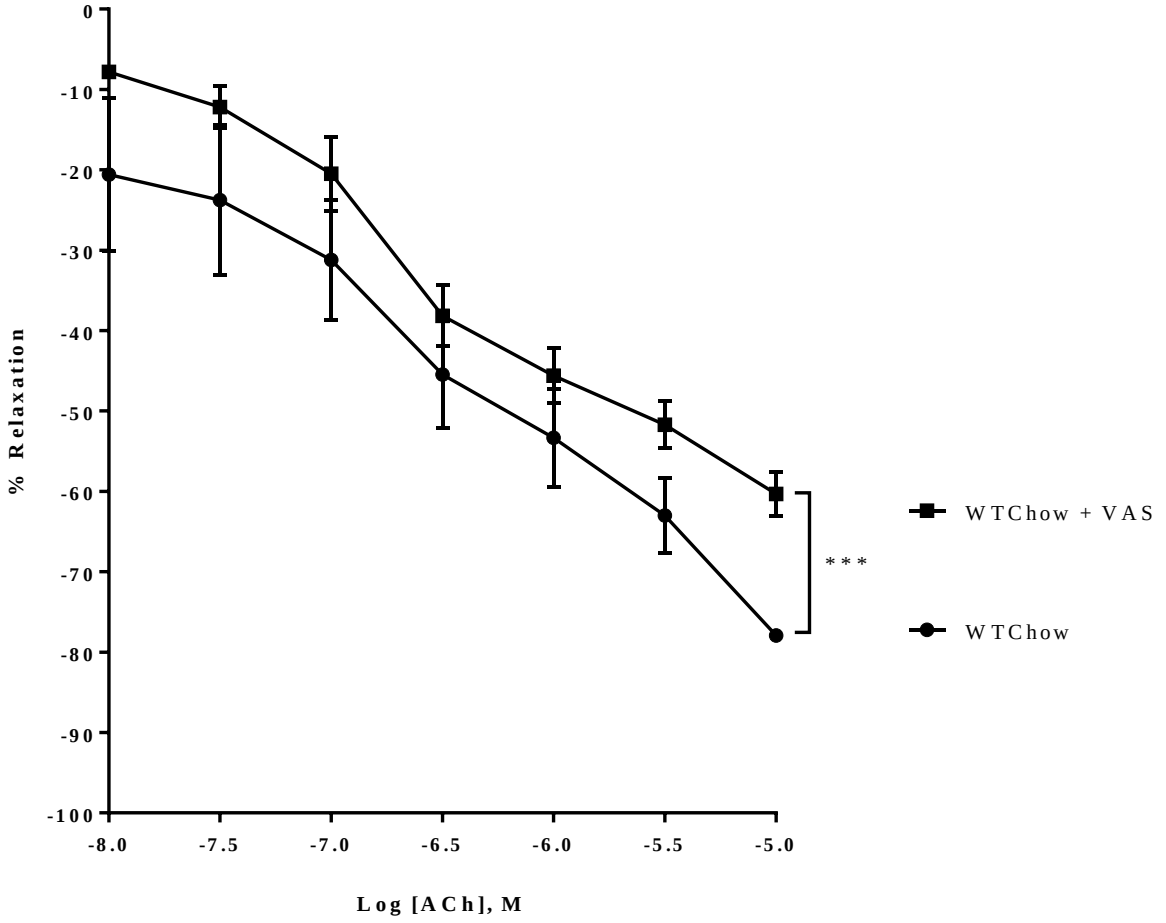
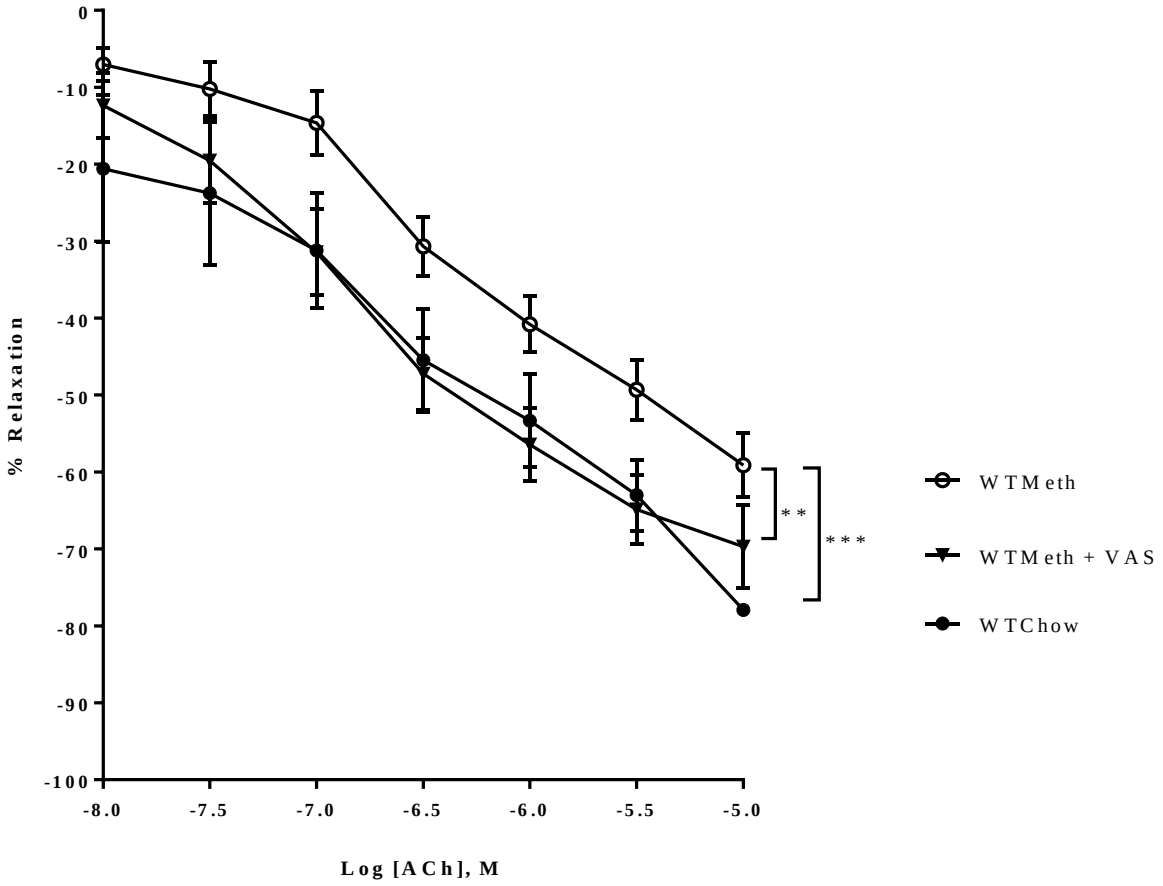
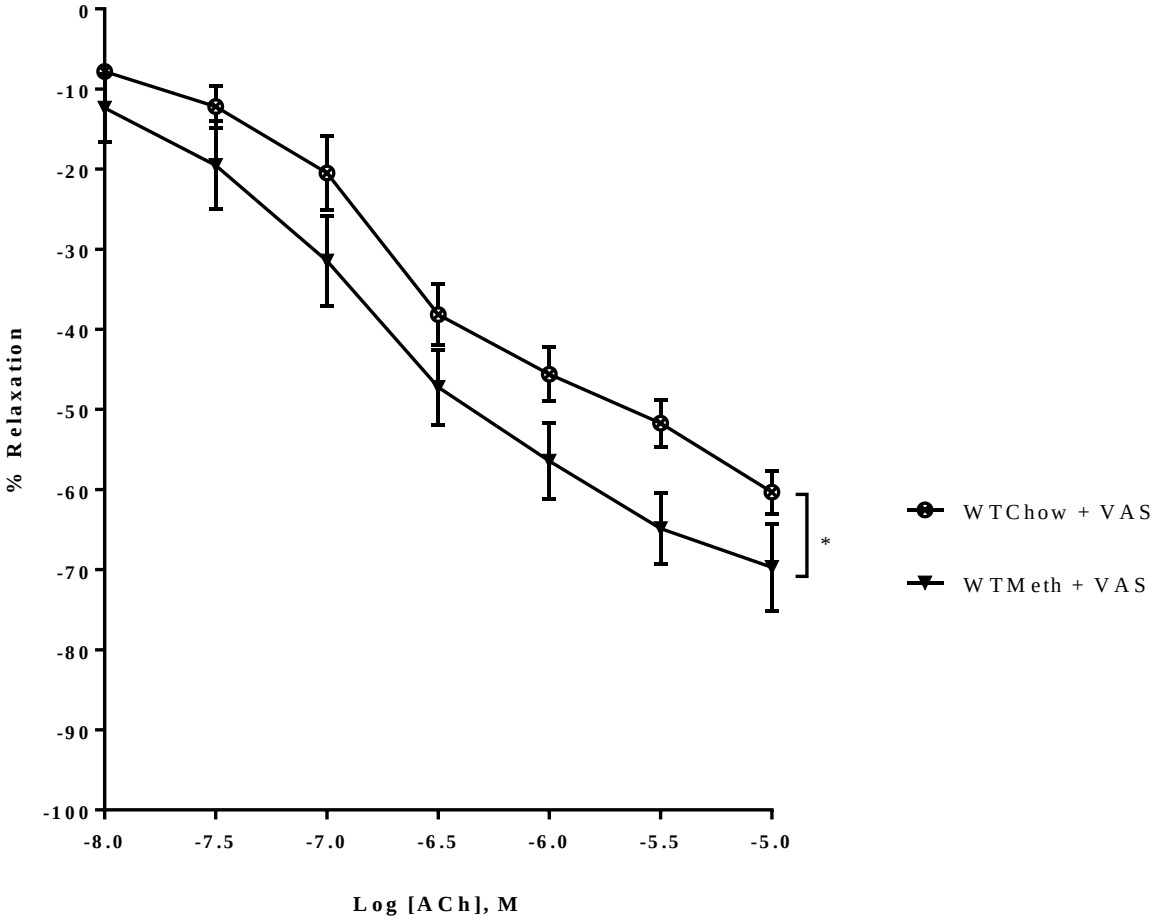


Figure 3.4A: Treating rings from WTChow mice with VAS resulted in impaired function (p<0.001). Results are mean ± SEM (n=4 per group). All values compared using Sidak's Multiple Comparisons Test. Only the highest significance has been recorded.



B) Rings from WTMeth mice had reduced function compared with WTchow (p<0.001). Treating WTMeth rings with VAS improved function vs. WTMeth (p<0.01). Results are mean ± SEM (n=4 per group). All values compared using Sidak's Multiple Comparisons Test. Only the highest significance has been recorded.



C) VAS treatment improved function in WTMeth rings compared with WTChow rings (p<0.05). Results are mean ± SEM (n=4 per group). All values compared using Sidak's Multiple Comparisons Test. Only the highest significance has been recorded.

3.16 Discussion

The major findings of this study are 1) Nox4 inhibition by the putative inhibitor VAS impaired ACh-dependent relaxation in acute Hcy-induced endothelial dysfunction in rabbit rings, 2) the addition of H_2O_2 via SOD in Hcy-treated rabbit rings improved ACh-dependent relaxation, 3) activating the Akt pathway with SC79 partially rescued function in rabbit rings treated with 3mM Hcy + VAS and, 4) VAS treatment improved endothelial function in methionine-fed

mice rings. These results suggest that Nox4 and its product H_2O_2 are involved in maintaining function in acute Hcy-induced endothelial impairment, as VAS worsened function whereas additional H_2O_2 reversed the effect of VAS and improved function. We propose that this protective effect is due to an increase the availability of eNOS and NO, directly upregulated in the short-term by increased basal ROS. Under certain conditions, ROS has been shown to improve endothelial-dependent vasodilation (Shafique et al. 2013). In contrast, the improvement seen in WTmeth and VAS treatment indicates that chronic HHcy has a different effect on the endothelium and this is also directly attributed to ROS levels. Further, SC79 also reversed the effects of VAS, via activation of the Akt pathway (Jo et al. 2012), suggesting a regulatory role for both Hcy and Nox4 in this pathway.

The progression of cardiovascular disease is characterised by excess oxidative stress, reducing NO bioavailability and impairing vasodilation (Howitt et al. 2014). Along with smoking cessation and exercise, nut consumption is recommended (Eilat-Adar et al. 2013) and has been found to reduce oxidative stress and inflammatory markers in haemodialysis patients (Stockler-Pinto et al. 2014) and depress postprandial glycaemia in type 2 diabetics (Jenkins et al. 2008). HHcy is an independent risk factor for cardiovascular disease and there are currently no treatments to normalise HHcy levels. HHcy can stimulate ROS production (Sharma, S, Singh & Sharma 2013; Topal et al. 2004), mechanisms of which including via Nox4 (Drummond & Sobey 2014; Kahles & Brandes 2012). Increases in H_2O_2 levels can lead to NOS uncoupling and ROS accumulation (Siu et al. 2014). Yet, the results presented here suggest that H_2O_2 is essential for maintaining endothelial function in Hcy-induced dysfunction. Further, adding SC79 to rabbit vessels treated with both Hcy and VAS significantly improved relaxation (Figure 3.3E), suggesting a regulatory effect on eNOS activation and enhanced NO production.

In rabbits, Nox4 inhibition with VAS impaired normal relaxation to ACh and further impaired relaxation when Hcy was added (Figure 3.3B), which implies an important role for H_2O_2 in at

least the short-term maintenance of endothelial function. Importantly, chronic methionine feeding caused a decrease in endothelial function in mice (Figure 3.4B), which was improved by Nox4 inhibition with VAS (Figure 3.4C), suggesting that the Nox4 product H_2O_2 plays an important role in long-term vascular homeostasis. This result is in line with our hypothesis but in contrast with the results of our rabbit studies, as Nox4 expression, and thus H_2O_2 generation, may be increased in the presence of HHcy (Bao, XM, Wu, CF & Lu, GP 2010); this informed the hypothesis that removing H_2O_2 might prevent Hcy-induced dysfunction. Instead, we found that the presence of H_2O_2 improved endothelial function in our rabbit model of acute Hcy-induced dysfunction, and suggest that it is essential for endothelial-dependent relaxation. On the other hand, chronic Hcy-induced dysfunction, replicated by the WTmeth model showed that reduced dysfunction was restored by VAS (Figure 3.4C).

Although H_2O_2 has traditionally been associated with oxidative damage, the burgeoning evidence suggests that its role in signalling and homeostasis is essential (Burgoyne et al. 2013). In rabbits, we attribute the difference between control, Hcy, VAS and SOD to the changing levels of H_2O_2. VAS application resulted in reduced function which was essentially restored by the addition of SOD, particularly in the Hcy treated rings. SOD is an important antioxidant which reduces O_2^- and H_2O to H_2O_2. Despite reports that VAS is a non-specific Nox inhibitor (Wingler et al. 2012), we have shown that there are differences in dysfunction between different Nox inhibitors (Smith, R et al. 2015), indicating that they do affect ROS differently. The redox relationship involving ROS is still misunderstood, owing largely to the difficulty in measuring changes in the natural redox status (Albrecht et al. 2011). As we rely on indirect methods of altered endothelial function, we can only speculate as to why VAS worsened function in our rabbit model and improved it in our mouse diet model.

The contrasting results can be partially explained by altered ROS homeostasis between models, however it may be due to differences in antioxidant production between mice and rabbits,

specifically the presence of Nox5 in rabbits. While the data is limited, there is evidence of significant differences between the amount of the antioxidant CoQ (also known as ubiquinone), an important ROS scavenger; compared with rabbits, mice have a much higher CoQ9:CoQ10 ratio (Lass, Agarwal & Sohal 1997). Nuts are a rich source of CoQ10 (Pravst, Zmitek & Zmitek 2010) and are recommended to confer benefits as part of a healthy diet (Eilat-Adar et al. 2013). CoQ is important in exercise-generated ROS (Balligand, Feron & Dessy 2009), which is involved in mitochondrial energy metabolism and ROS generation. Importantly, the ratio of CoQ9:CoQ10 appears to be important for antioxidant levels and radical scavenging (Yang et al. 2015). A study assessing a high-fat diet in rats found that the diet increased the CoQ9 ratio and this was attributed to increased oxidative stress (Bravo et al. 2012). An increase of oxidative stress from the diet in the mouse model might have induced excess O_2^- into H_2O_2, thus bypassing the H_2O_2 dampening effect of VAS in this model and enabling downstream NO release. H_2O_2 has been shown to increase NO stimulation, co-induced by the inducible form of nitric oxide synthase, iNOS (Shimizu et al. 2003), and iNOS itself is upregulated in mice with HHcy (Dayal et al. 2014). The responses to VAS observed in rabbits versus mice could be attributed to differences in basal O_2^- availability, which may impact NO levels. In rabbits, naturally occurring higher levels of CoQ10 present in tissue might have increased O_2^- scavenging, enough to reduce H_2O_2 levels and diminish NO release. Although the exact function of Nox5 remains unclear (Fulton 2009), it appears that Nox5 produces functional ROS in a manner similar to Nox1-4 (Weyemi et al. 2015). There is also evidence that Nox5 mediates bile-acid induced H_2O_2 (Li & Cao 2016) and is involved in radiation-induced DNA damage (Weyemi et al. 2015). In the cardiovascular system, Nox5 is implicated in CAD and atherosclerotic lesions (Guzik et al. 2008) and vascular remodelling in hypertension (Montezano et al. 2015). Strikingly, Nox5 does not require the $p22^{phox}$ for activation, whilst this is an essential requirement for Nox1-4 (Fulton 2009; Prior et al. 2016), and this presumably

results in different cellular calcium requirements between rabbits and mice. Unlike Nox1-4, Nox5 is not bound by catalytic subunit regulation and is able to produce ROS by changes in calcium (Chen et al. 2015; Qian, J et al. 2012). In our rabbit model, the presence of Nox5 might have mediated an upregulation of ROS; Nox 5 is reported to be upregulated in several cardiovascular disease states, such as acute myocardial infarction and hypertension (Montezano et al. 2015). There is currently no research published assessing a potential relationship between Nox5 and Hcy, however, it is interesting to speculate that, like Nox2, Nox5 and its ROS products could be involved in contributing to increased oxidative stress in the presence of increased plasma Hcy; clearly, more investigation is warranted.

While H_2O_2 inhibition is reported to be beneficial for cell viability and eNOS activity (Xing, S et al. 2014), there is evidence of a regulatory role for H_2O_2 presence in endothelial cells, where it has been shown to increase NO activity (Cai, WX et al. 2014); indeed these results support that. A decrease of NO activation could explain our results in rabbits, particularly if Nox inhibition had a simultaneous negative effect on the Akt pathway, which is dependent upon H_2O_2-directed eNOS phosphorylation at Serine^{1177} and essential for eNOS activation and vasorelaxation (Hien et al. 2011; Lan et al. 2011). Indeed, the improvement we saw when we added SC79 supports this. Interestingly, selenium, found in Brazil nuts, has been shown to participate in Akt activation and induce eNOS phosphorylation (Ren et al. 2016). Perhaps most strikingly, selenium has also been shown to improve the cellular environment damaged by acute Hcy, via both increased endothelial cell viability and apoptosis (Ren et al. 2016). This highlights the important role that nuts may be able to play in Hcy-induced dysfunction. Our results show that H_2O_2 was also involved in relaxation and is involved in cell signalling to restore endothelial function in Hcy-induced dysfunction. However, the same results were not seen in the WTmeth mice. In chronic Hcy dysfunction, the endothelial layer may be too deranged for rescue, as long term HHcy exposes cells to increasing risk of damage and

apoptosis (Kruman et al. 2000), hence the improvement in function via the removal of H_2O_2. This suggests that redox status has a point at which balance cannot be restored, only removal of ROS may be beneficial.

The exact role of Nox4 both acute and chronic Hcy dysfunction is still unclear but these results point to regulation of the cellular environment. As a signalling enzyme, proper functioning Nox4 seems essential in regulating vasorelaxation: Ray, R et al. (2011) recently developed an over-expressed Nox4 mouse which had greater vasorelaxation to ACh and reduced blood pressure compared to wild-type littermates; this was attributed directly to H_2O_2 production.

3.17 Limitations

As pharmacological Nox inhibitors are not specific (Bedard & Krause 2007b; Heumuller et al. 2008), the effects of the drug might explain the differences in responses in the different animal models used. In addition to this, our animal and *ex vivo* model might not necessarily represent the true pathophysiological state in humans.

3.18 Conclusion

It is well established that HHcy, atherosclerosis and oxidative stress are inextricably linked, however the precise biochemical pathways require further analysis. The results presented here provide evidence that VAS might not be suitable to prevent Hcy-induced CVD.

Chapter 4

4.1 eNOS phosphorylation is impaired in HHcy plus GP incubated tissue.

4.2 Abstract

Background: Increased homocysteine (Hcy) is a risk factor for coronary artery disease (CAD), a direct result of which is reduced nitric oxide (NO), an essential vascular relaxing factor. Further, Hcy increases reactive oxygen species (ROS) via NADPH oxidases (Nox), reducing acetylcholine-mediated vasorelaxation, which is reliant on NO. We aimed to determine if the putative Nox2 inhibitor GP91ds-tat (GP) prevents Hcy-impaired acetylcholine-mediated vasorelaxation via impaired eNOS phosphorylation.

Methods: New Zealand White rabbit aortic rings were mounted in organ baths. Rabbit rings were incubated with GP, 10^{-6} M). Some rabbit rings were also incubated with 3mM Hcy, before pre-contraction, followed by dose-response relaxation to acetylcholine (ACh; 0.01µM-10µM). After ACh responses, rabbit rings incubated with Hcy + GP were immediately fixed and embedded in paraffin for immunohistochemical analysis.

Results: Rabbit tissue analysis revealed that Hcy reduced eNOS phosphorylation at Thr^{495} and increased eNOS phosphorylation at Ser^{1177}; no further alteration at Thr^{495} was observed with GP. In contrast, GP prevented increased phosphorylation at Ser^{1177}.

Conclusions: Acute Hcy causes an upregulation of eNOS phosphorylation at Ser^{1177}. In contrast, GP reduced essential eNOS phosphorylation at Ser^{1177}, suggesting that Nox2 plays a role in NO release and availability.

4.3 Introduction

Cardiovascular disease (CVD) and atherosclerosis are associated with excess plasma homocysteine (Hcy), which continues to be an independent risk factor in morbidity and mortality (Debreceni, B. & Debreceni 2014; Fang et al. 2014). NO release causing vasorelaxation is reliant upon concomitant eNOS dephosphorylation at Thr^{495} and phosphorylation at Ser^{1177} (Mount, Kemp & Power 2007) and there is evidence that Hcy can reduce NO output (Becker et al. 2005). Additionally, there is also evidence that Hcy can interfere with the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) family of signalling enzymes to impair essential NO functions, especially acetylcholine-mediated vasorelaxation (Edirimanne et al. 2007).

To that end, our aim was to assess via immunohistochemistry whether 3mM Hcy and Nox2 inhibition by GP impacted eNOS phosphorylation.

4.4 Methods

Immunohistochemical analysis was performed as described in Chapter 2.2.4.

Statistical Analysis

eNOS phosphorylation was analysed using ordinary one-way ANOVA followed by Tukey's Multiple Comparisons Test. . All data were analysed using GraphPad Prism (version 7.01 for Windows, GraphPad Software, La Jolla, California USA). Data are represented as mean ± SEM. Significance was accepted at $p < 0.05$.

All proteins and tracings were averaged and used for data analysis. All data points are arbitrary units and normalized to control as '1', described previously (Arora, Hare & Zulli 2012).

4.5 Results

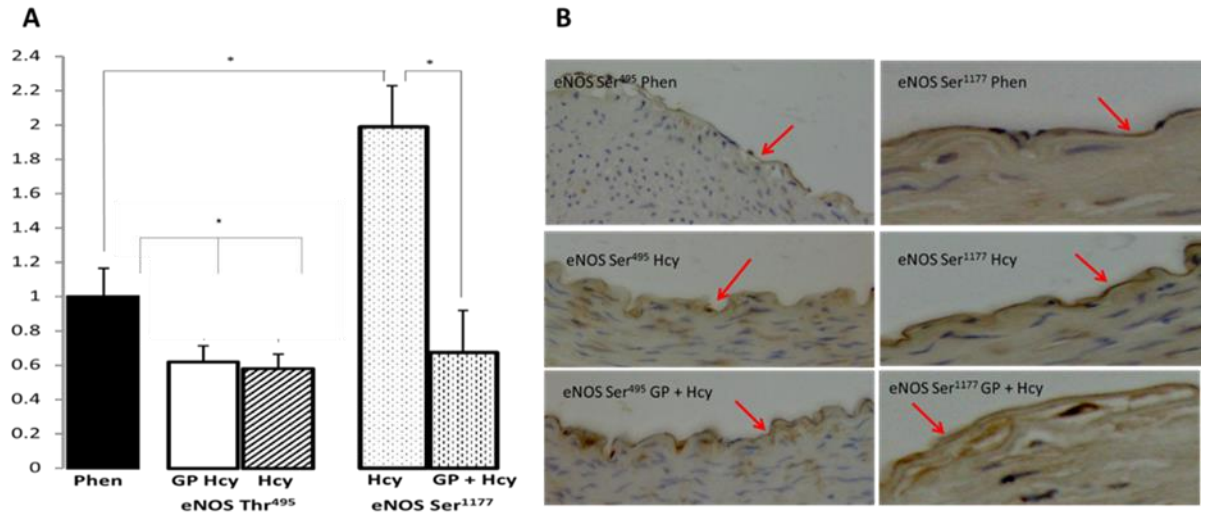


Figure 4.1A) Quantification of eNOS phosphorylation at Thr^{495} and Ser^{1177} in response to Phen and with pre-treatment of Hcy or GP + Hcy in chow rings; * = $p < 0.05$). B) Comparison of phosphorylation of eNOS to Phen at Thr^{495} and Ser^{1177}with pre-treatment of Hcy or GP + Hcy. Arrows indicate the brown stain showing phosphorylation and presence of the eNOS protein. (Hcy = homocysteine, GP = gp91ds-tat, Phen = phenylephrine, eNOS = endothelial nitric oxide synthase, Ser = serine, Thr = threonine).

In Ser^{1177}, vehicle vessel did not respond to ACh in the expected manner, with phosphorylation reduced to ACh and no significant difference between ACh and Phen (Figure 4.1A). We expected that phosphorylation would be increased to indicate eNOS phosphorylation and subsequent relaxation. However, in vessels pre-incubated with 3mM Hcy, phosphorylation of eNOS was significantly increased, compared to Nox inhibition alone and Nox2 inhibition with 3mM (Figure 4.1A). Images of eNOS phosphorylation (Figure 4.1B) support these findings.

4.6 Discussion

We performed immunohistochemical analysis on rabbits incubated with acute HHcy. To assess eNOS phosphorylation, we saved tissue as soon as the ACh curve was complete, so as to

preserve phosphorylation. Importantly, there was clear presence of endothelial eNOS, indicating that there was no loss of tissue to either apoptosis or necrosis. Reduced phosphorylation of eNOS at Thr^{495} and increased phosphorylation at Ser^{1177} is required for proper eNOS activation (Lan et al. 2011). At Thr^{495}, Hcy alone or in combination with GP significantly reduced phosphorylation compared to control (Figure 4.1A, B). This is in line with the literature however does not correlate with our isometric tension studies (please refer to Ch. 3.2), where we saw a reduction in relaxation from these treatments. This implies that, while important, dephosphorylation of eNOS at Thr^{495} may play a smaller role in vascular relaxation than phosphorylation at Ser^{1177}. There was increased phosphorylation of eNOS at Ser^{1177} in HHcy, suggesting eNOS activation as a compensatory mechanism to normalise vasodilation, perhaps by activation of the Akt pathway, which stimulates eNOS phosphorylation at Ser^{1177} to increase NO availability (Jo et al. 2012; Wang, R et al. 2015). Paradoxically, we saw a reduction in ACh-dependent relaxation in HHcy, therefore we expected to see reduced eNOS phosphorylation at Ser^{1177}, suggesting that O_2^- is essential for downstream eNOS activation. This is supported by the improvement in relaxation when pyrogallol and SC79 were added; GP + Hcy virtually abolished acetylcholine mediated relaxation, suggesting that other Nox isoforms cannot compensate for this effect (please refer to Ch. 3.2). However, GP + Hcy were not significantly reduced at Ser^{1177} compared to Phen, which supports the ACh constriction curve outcome. This was unexpected, as it has been well reported that a reduction in Nox2 activation is related to an increase in endothelial-dependent relaxation (Han, XB et al. 2014). The increase in phosphorylation of Ser^{1177} on Hcy and concomitant reduction in GP + Hcy provides evidence that Nox2 is essential for eNOS activation. Reduced eNOS phosphorylation could be explained by an inactivation of Akt due to reduced activation of Nox2, which could imply that activation of this pathway in the development of arterial pathogenesis could be partially regulated by Nox2. Further analyses

in the changes to phosphorylation at these sites could be expanded to include an eNOS overexpressing murine model. To our knowledge, this has not yet been investigated and would provide further insight into the mechanisms of Hcy-induced dysfunction.

4.7 Limitations

Relying on phosphorylation data from immunohistochemical analysis is not always a reliable indicator of the underlying molecular pathways and requires supporting evidence from other methodologies, such as protein analysis and RNA/DNA quantification.

4.8 Conclusion

There is evidence to suggest that HHcy is able to upregulate eNOS phosphorylation at Ser^{1177}, suggesting that it may play a regulatory role in maintaining proper eNOS activation and NO availability in the short-term. Additionally, the reduction of eNOS phosphorylation at Ser^{1177} in the presence of GP suggests that in HHcy, Nox2 is also required for eNOS activation.

Chapter 5

5.1 Combinations of Nox1, 2 and 4 inhibition improves HHcy-impaired acetylcholine-mediated vasorelaxation, whereas iNOS inhibition worsens it.

5.2 Abstract

Introduction: Increased plasma homocysteine (Hcy) remains an important and independent risk factor for cardiovascular disease (CVD). Treatments aimed at reducing hyperhomocysteinemia (HHcy) have been disappointing, thus novel treatments are a necessary strategy. Hcy stimulates the production of excess reactive oxygen species (ROS), possibly via the free radical donators NADPH oxidases (Nox) and the uncoupling of inducible nitric oxide synthase (iNOS). Inhibiting Nox and iNOS could thus be a novel therapeutic approach to treating hyperhomocysteinemia (HHcy).

Methods: New Zealand White rabbits (n=6, male, 16 weeks, VUAEC#14/005) were anaesthetised, the abdominal aortae excised, cut into 2-3mm rings and mounted in organ baths attached to force displacement transducers. Rings were incubated with iNOS inhibitor, Nox1 inhibitor, Nox2 inhibitor, Nox4 inhibitor or combinations thereof for 30 minutes. Prior to incubation with 3mM Hcy for 1 hour. Dose-response curves to acetylcholine (ACh; 0.01µM-10µM) were then performed.

Results: Hcy treatment impaired endothelial-dependent relaxation to ACh, compared with control (no treatment; $p < 0.0001$). On its own, iNOS inhibition significantly impaired function compared with no treatment ($p < 0.0001$); there was no significant difference between treating with iNOS inhibitor and Hcy alone, whereas treating Hcy plus iNOS inhibitor significantly impaired function compared with control ($p < 0.0001$). Treating Hcy with combined iNOS and single Nox inhibition resulted in reduced function compared with controls: iNOS + Nox1 + Hcy ($p < 0.0001$); iNOS + Nox2 + Hcy ($p < 0.0001$) and iNOS + Nox4 + Hcy

($p < 0.0001$). Treating Hcy with combined iNOS and Nox1, 2 and 4 inhibition likewise impaired relaxation compared with control ($p < 0.0001$).

Discussion: The ROS generators iNOS and Nox1, 2 and 4 are important in vascular pathophysiology. Here we have examined their role in endothelial dysfunction, as there is evidence that their ROS products are important in vascular homeostasis. Inhibiting both iNOS and Nox resulted in endothelial impairment, thus we have provided direct evidence that they are essential to vasodilation in the acute phase of Hcy-induced endothelial dysfunction.

Conclusion: The data presented here indicate a vitally important role for iNOS and Nox1, 2 and 4 in proper ACh-dependent endothelial function. Excess Hcy and its related endothelial dysfunction remains a treatment challenge, however iNOS inhibition is unlikely to be of benefit in this context.

5.3 Introduction

An increased level of homocysteine (Hcy) is an independent risk factor for coronary artery disease (CAD) and atherosclerosis (Jiang et al. 2012; Julve et al. 2013), typified by arterial intimal thickening and increasing risk of stroke and myocardial infarction (Miida et al. 2014). Severe hyperhomocysteinemia (HHcy) is classified as >15 μmol/L (Beard, Reynolds & Bearden 2012) and is thought to be caused by dysregulated methionine metabolism, although the exact mechanism is unclear. Vitamin B6, B12 and folate are essential to catalyse the enzymes required for proper metabolism (Hoffman 2011), leading to the postulation that B-vitamin therapy might be an effective treatment at reducing plasma Hcy levels. However, targeted B vitamin therapy has not proven an effective treatment in clinical trials (Hodis et al. 2009; Kaul, Zadeh & Shah 2006; Lonn et al. 2006; Pettigrew et al. 2008).

Nicotinamide adenosine diphosphate (NADPH) oxidases (Nox) are trans-membranous enzymes producing functional reactive oxygen species (ROS) comprising of 7 different types: Nox1-5 and Duox1 & 2. In CVD, Nox1, 2 and 4 are relevant; especially as the reactive oxygen species (ROS) they generate are considered essential for signalling: Nox1 and 2 generate O_2^- and Nox4 generates H_2O_2 (Bedard & Krause 2007b; Drummond & Sobey 2014; Manolescu et al. 2010). Dysregulated ROS are implicated in the development of cardiovascular disease (Howitt et al. 2014), atherosclerosis (Judkins et al. 2010), ischaemic injury (Dvoriantchikova et al. 2012; Kahles & Brandes 2012) and systemic hypertension (Wind et al. 2010).

Inducible nitric oxide synthase (iNOS) appears to be present only in inflammatory and pathological conditions, especially atherosclerosis, where it produces high amounts of nitric oxide (NO) in an attempt to maintain vascular homeostasis (Ponnuswamy et al. 2009). This can exacerbate an already compromised environment when NO reacts with O_2^- to produce the powerful oxidant peroxinitrite ($ONOO^-$) and the balance between oxidant and relaxant may be

lost (Pacher, Beckman & Liaudet 2007). NO is an essential and regulatory vasodilator, synthesised by the conversion of the amino acid L-arginine to L-citrulline and mediated by the family of nitric oxide synthase (NOS) isoforms and their cofactors, especially tetrahydrobiopterin (BH4) (Lee, J et al. 2016). In pathological states, the inability of iNOS to bind with the essential cofactor BH4 results in ROS production instead of NO, resulting in NOS uncoupling (Lee, J et al. 2016) and leading to a pro-oxidant state. In the context of Hcy, this is of particular interest given that BH4 can be reduced by excess Hcy (Bendall et al. 2014), providing the potential for iNOS uncoupling and free radical production. In addition, excess Hcy may also increase Nox-derived O_2^-, (Kassab & Piwowar 2012), creating a vicious cycle of increasing oxidative stress.

Several pharmacological agents have been established as iNOS and Nox1, 2 and 4 inhibitors. 1400W is a selective iNOS inhibitor, inhibiting iNOS in aortic rat rings (Garvey et al. 1997), and reducing ischaemic reperfusion injury in porcine kidneys (Hosgood, Yates & Nicholson 2014). ML090 is a novel Nox1 inhibitor, reported as reducing the oxidative burst from murine retinal cells (Dvoriantchikova et al. 2012) and work from our lab has shown that ML090 blocks the effect of homocysteine-thiolactone (HcyT, the atherogenic form of Hcy) in response to ACh in rabbits (Smith, R et al. 2015). The Nox2 inhibitor PhoxI2 is a specific Nox2 inhibitor, eliciting its action by disrupting the two catalytic subunits essential for its conformational binding (Jo & Luo 2012). It is reported as successfully inhibiting Nox2-mediated O_2^- production in neutrophils (Bosco et al. 2012). The Nox4 inhibitor VAS has been shown to be effective in ischaemic stroke (McLeod et al. 2010) and Wind et al. (2010) reported that in spontaneously hypertensive rat aortae, VAS reduced vascular ROS production to control levels and inhibited ROS-induced signalling. Interestingly, recent data from our own lab (data not shown) using $Nox2^{-/-}$ mice with pharmacological Nox1 and 4 inhibition (ML090 andVAS, respectively) or guinea pigs with iNOS inhibition (1400W), indicate that ROS, presumably

from increased Hcy levels, confer some protection to the endothelium in chronic diet-induced HHcy, which reveals a potential role for ROS homeostasis in chronic pathology.

To that end, our aim was to examine the role of iNOS and Nox inhibition in the context of acute Hcy-induced endothelial dysfunction an attempt to restore vasodilation.

5.4 Methods

All experiments were carried out according to the National Health and Medical Research Council "Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (8th Ed. 2013).

Animals

Male, New Zealand White rabbits (16 weeks, male, n=6, VUAEC #14/005) were housed in separate cages, maintained at a constant temperature of approximately 23°C and were provided water and normal chow *ad libidum*. Animals were anaesthetised (medetomidine 0.25mg/kg), exsanguinated and the abdominal aorta excised and flushed with cold (on ice) oxygenated Krebs buffer (NaCl 118.4, KCl 4.7, NaHCO3 25, MgSO4 1.2, CaCl2, 2.5, glucose 11.1, mM). The aorta was cleaned of fat and connective tissue, cut into rings (2-3 mm lengths) and placed in organ baths (OB8, Zultek Engineering, Australia), filled with Krebs, kept at a constant temperature of 37°C and continuously bubbled with carbogen (95% O_2 + 5% CO_2). Rings were left to rest, unmounted, for 30 mins, and then mounted between two metal hooks attAChed to force displacement transducers, stretched to 2g and allowed to relax for 20 mins, twice.

Experimental Protocol

Rings were incubated with 1400W (iNOS inhibitor; 1 µM), ML090 (Nox1 inhibitor; 1µM), gp91ds-tat (Nox2 inhibitor; 1µM) or VAS (Nox4 inhibitor; 1µM) for 30 minutes. Drugs were

re-introduced into the baths immediately prior to the addition of 3mM Hcy where used. Then, to assess endothelial dependent relaxation to acetylcholine (ACh; 0.01µM-10µM), rings were pre-contracted with phenylephrine (Phen; 0.3-1.0µM). After the contraction reached a plateau, a concentration-response curve to ACh-induced relaxation was obtained.

All data were analysed using a repeated measures two-way ANOVA followed by Sidak's Multiple Comparisons Test. Data are represented as mean ± SEM.

5.5 Results

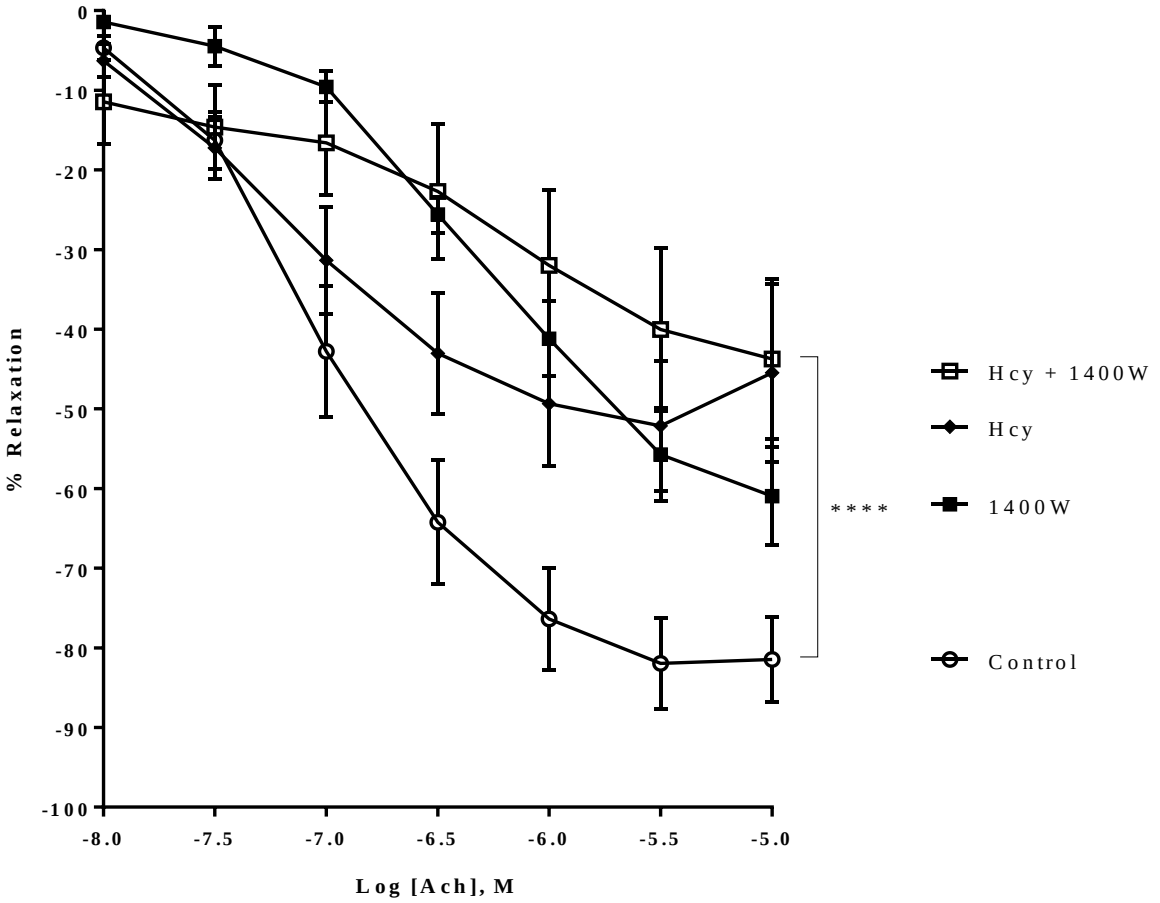
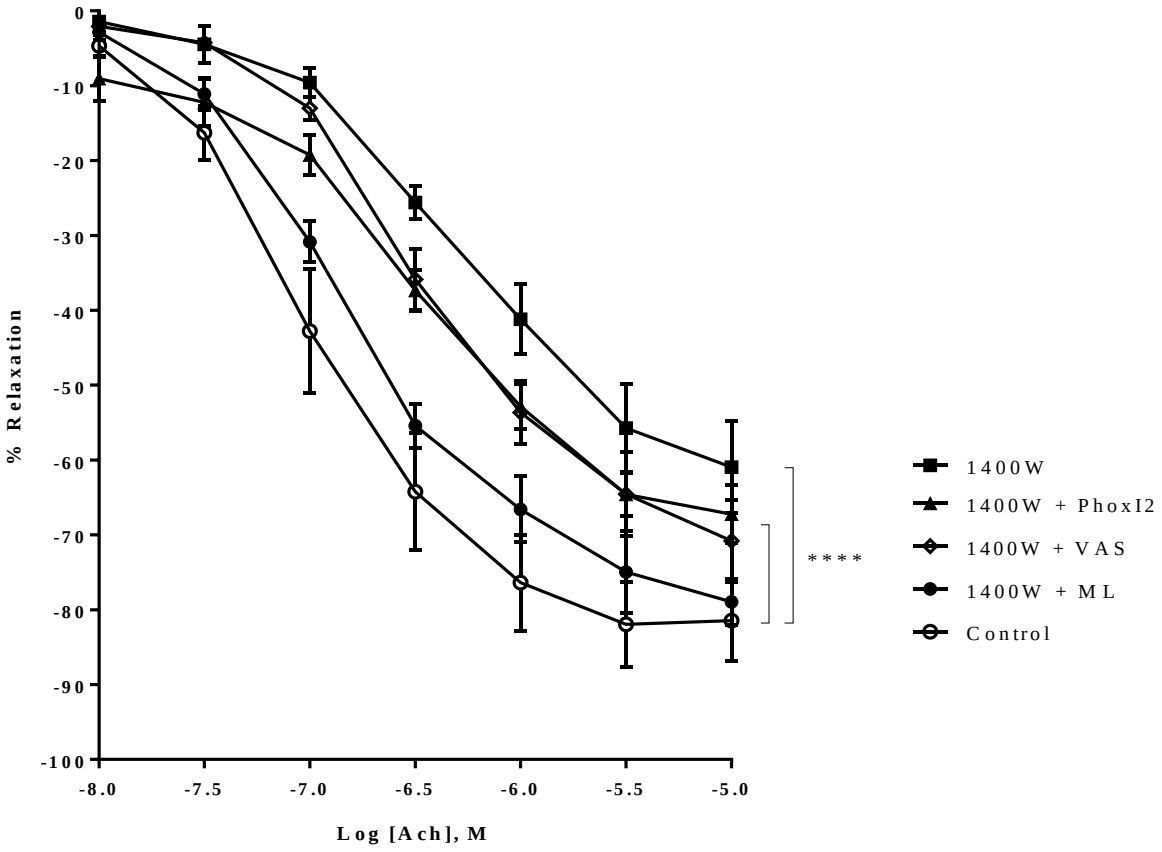


Figure 5.1. Healthy rabbit aortic rings were treated with 3mM Hcy treatment and iNOS inhibition (1400W). All of these treatments significantly impaired endothelial dependent

relaxation to ACh, compared with control. **** $p < 0.0001$; HCY, homocysteine; 1400W (iNOS inhibitor); control (no treatment). All data were analysed using a repeated measures two-way ANOVA followed by Sidak's Multiple Comparisons Test. Data are represented as mean ± SEM (n=6 per group). Only the highest significance has been recorded. As expected, Hcy treatment impaired endothelial-dependent relaxation to ACh, compared with control (no treatment; 45.4±11.1% vs 81.4±5.3%, $p < 0.0001$, Figure 5.1). To assess the effect of iNOS on the aorta, we used 1400W to selectively inhibit iNOS. On its own, 1400W significantly impaired function compared with no treatment (55.7±5.8%, $p < 0.0001$), yet there was no significant difference between 1400W and inducing endothelial dysfunction with 3mM Hcy. Although the combination of 1400W and Hcy treatment together significantly impaired function compared with control (43.7±10.1%, $p < 0.0001$, Figure 5.1), separately they resulted in similar impairment of the vessel.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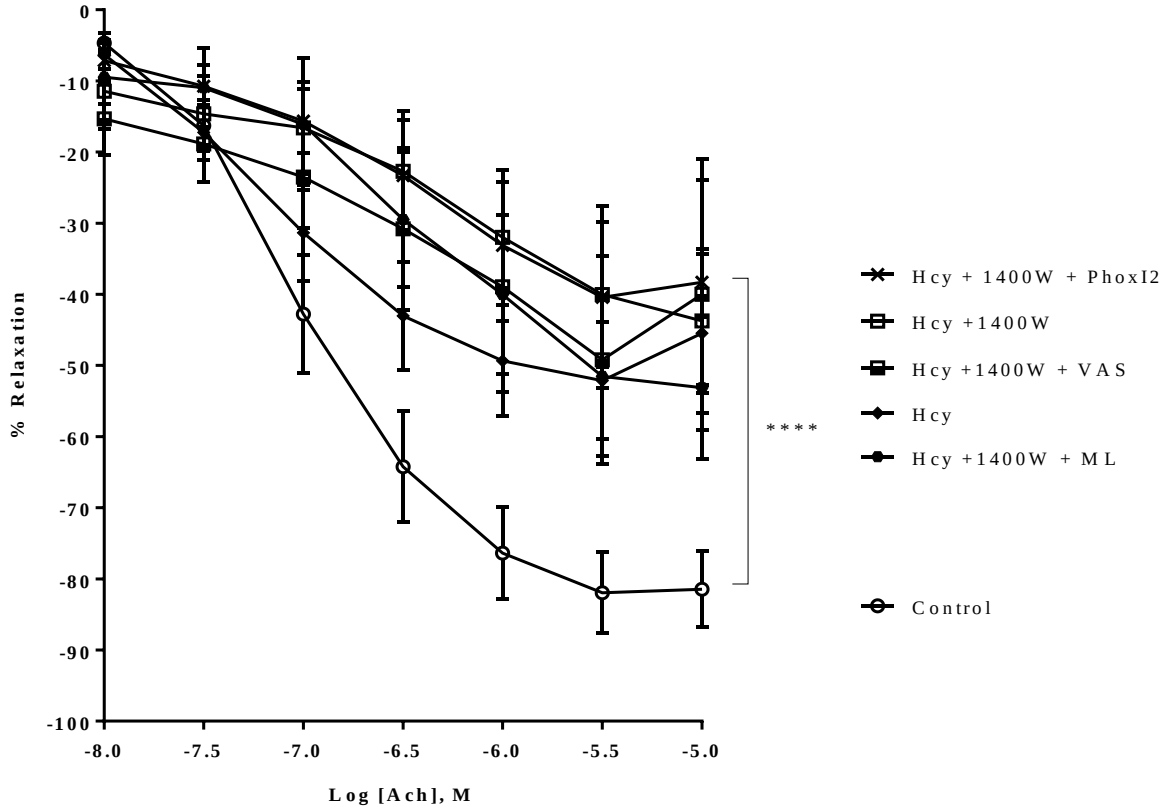
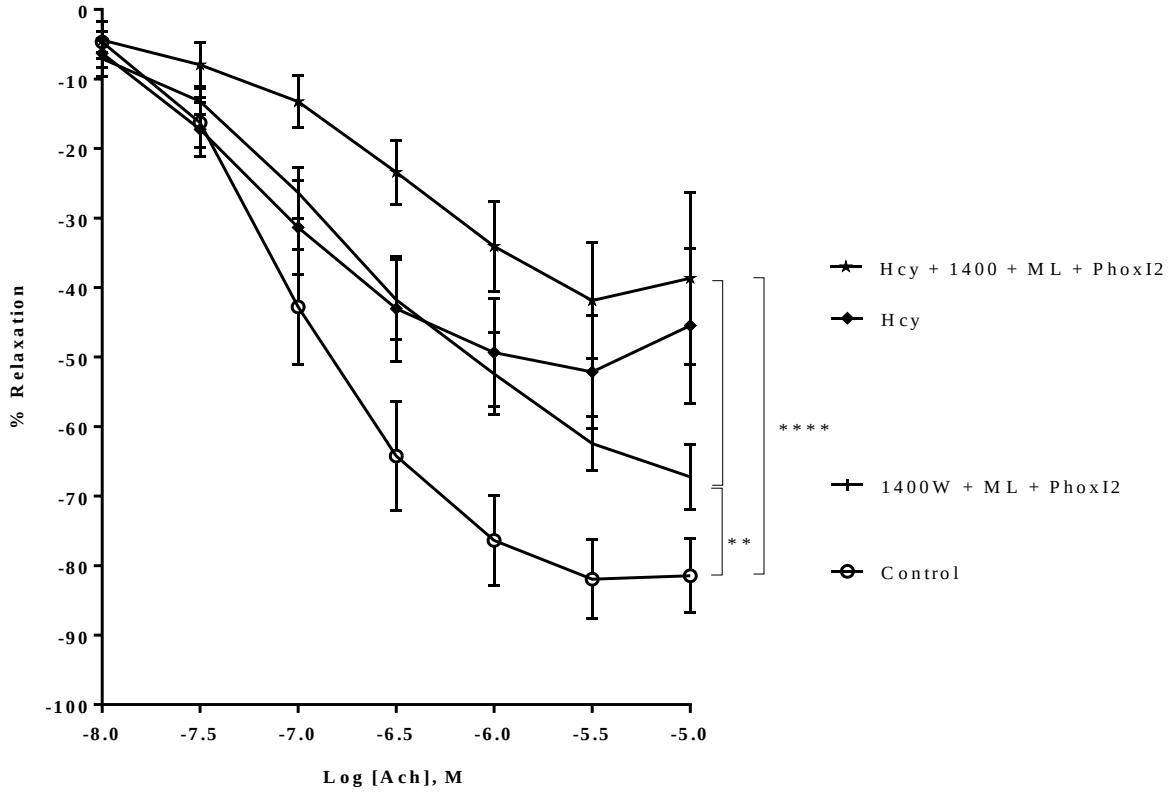


B

Figure 5.2A. In healthy rabbit aortic rings, inhibition of iNOS and Nox2 or Nox4 resulted in significant impairment to vasodilation compared with control (no treatment). Figure 2B. The addition of 3mM Hcy treatment and iNOS inhibition with 1400W plus single Nox1, 2 and 4 inhibition impaired endothelial dependent relaxation to ACh, compared with control. **** $p < 0.0001$; HCY, homocysteine; 1400W (iNOS inhibitor); ML090 (Nox1 inhibitor); PhoxI2 (Nox2 inhibitor); VAS (Nox4 inhibitor); control (no treatment). All data were analysed using a repeated measures two-way ANOVA followed by Sidak's Multiple Comparisons Test. Data are represented as mean ± SEM (n=6 per group). Only the highest significance has been recorded. In healthy vessels there was a significant decrease in function when 1400W alone and a Nox2 or Nox4 inhibitor was used in conjunction.

The combination of iNOS and Nox2 inhibition or iNOS and Nox4 inhibition reduced function (1400W + PhoxI2 vs control, 23.3±7.8 vs 64.2±7.8%, $p < 0.0001$; 1400W + VAS vs control, 35.8±4.0% vs 64.2±7.8%, $p < 0.0001$, Figure 5.2A); however the combination of ML090 and 1400W had no significant effect on control (Figure 5.2A). To assess the effect of iNOS and Nox inhibition in treating Hcy-induced dysfunction, we combined iNOS and single Nox1, 2 or 4 inhibitors. Combining these treatments in Hcy resulted in significantly reduced function compared with controls: 1400W + ML090 + Hcy (53.1±10.0%, $p < 0.0001$, Figure 5.2B); 1400W + PhoxI2 + Hcy (38.2±14.4%, $p < 0.0001$, Figure 5.2B) and 1400W + VAS + Hcy (39.9±19.0%, $p < 0.0001$, Figure 5.2B). Interestingly, none of the combinations with Nox inhibitors were significantly different to Hcy treatment alone.



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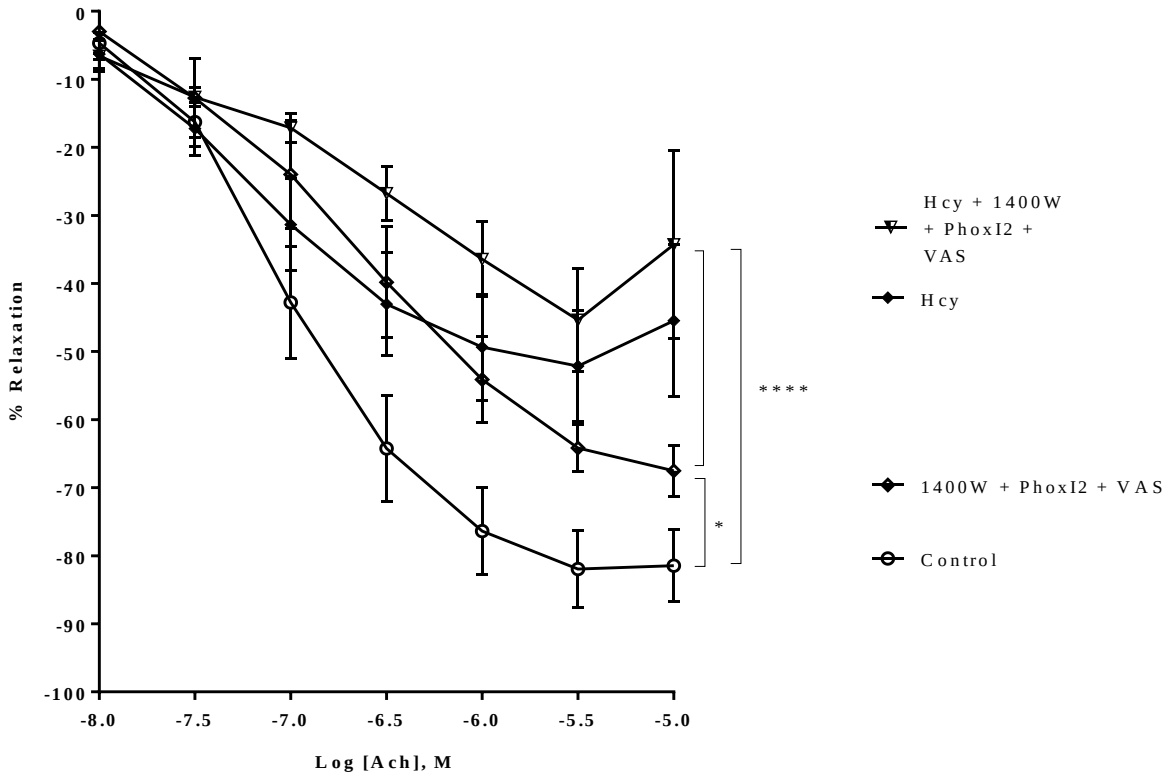
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Hcy + 1400W + PhoxI2 + VAS
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1400W + PhoxI2 + VAS
Control

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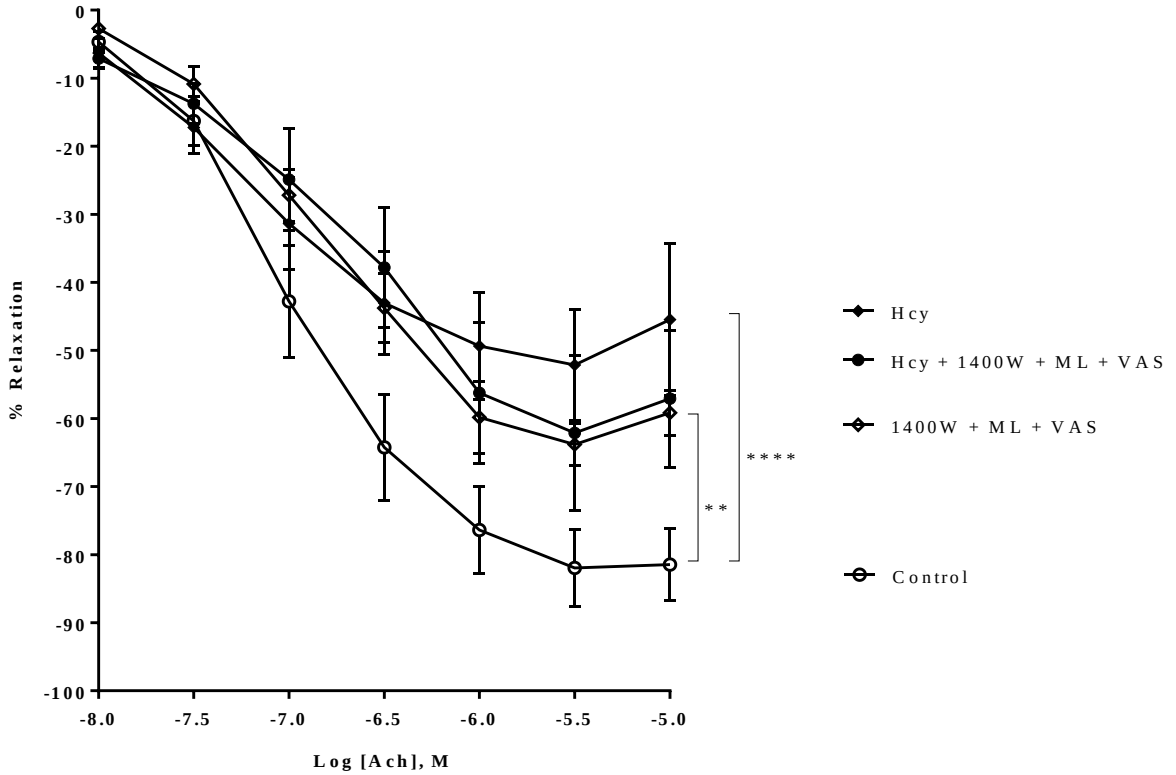
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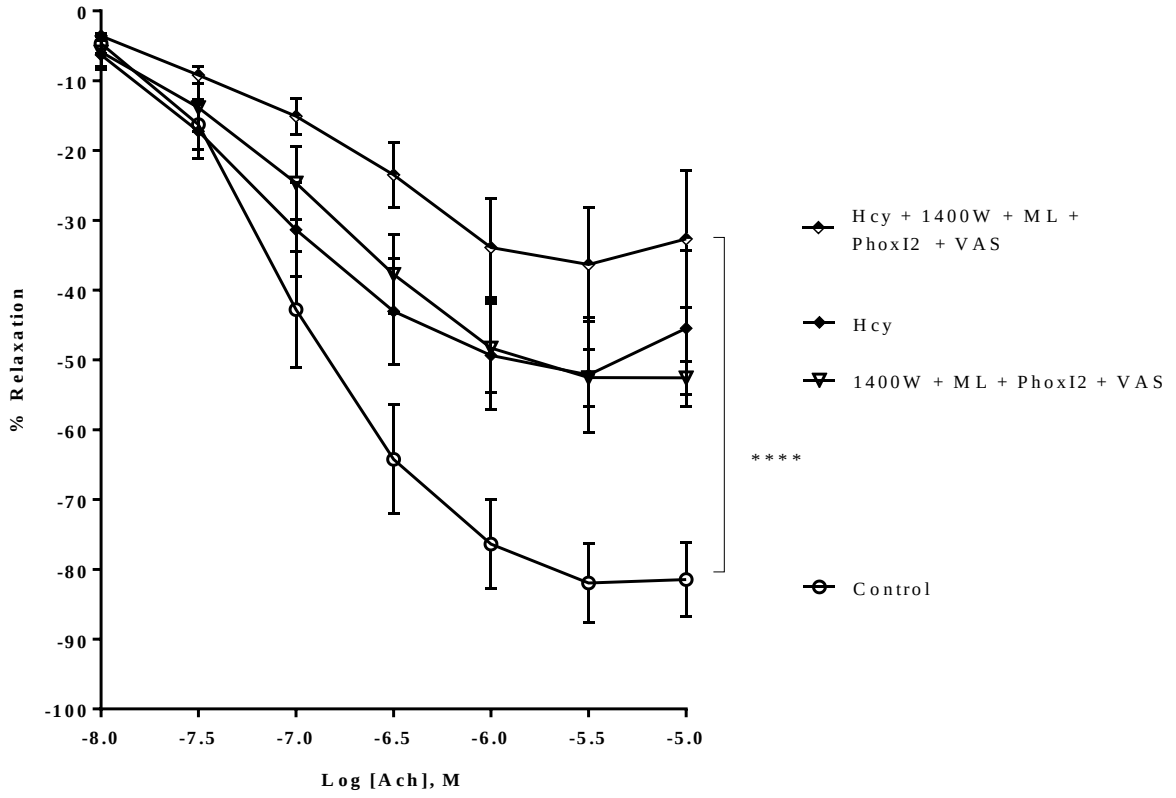


D

Figure 5.3A-D. In healthy rabbit aortic rings, treatment with iNOS inhibition plus combinations of Nox 1 and 2 inhibition significantly impaired endothelial dependent relaxation to ACh compared with control; compared to these groups, the addition of 3mM Hcy further worsened function. *** $p < 0.001$; **** $p < 0.0001$; HCY, homocysteine; 1400W (iNOS inhibitor); ML090 (Nox1 inhibitor); PhoxI2 (Nox2 inhibitor); control (no treatment). All data were analysed using a repeated measures two-way ANOVA followed by Sidak's Multiple Comparisons Test. Data are represented as mean ± SEM (n=6 per group). Only the highest significance has been recorded. To assess how iNOS and the different Nox isoforms might contribute to dysfunction via ROS production, we combined 1400W with Nox inhibitors and Hcy. Without Hcy treatment, inhibiting iNOS, Nox1 and Nox2 (ML090 and PhoxI2) was not significantly different to control, however the

addition of Hcy worsened function (38.6±12.3%, $p < 0.0001$) compared with control but was not significantly different to Hcy treatment alone (Figure 5.3A).

To assess differences between the ROS generated Nox2 and Nox4, we combined 1400W with PhoxI2 and VAS both with and without Hcy treatment. Combined iNOS and Nox2 and Nox4 inhibition significantly impaired function compared to controls (39.8±8.1% vs 64.2±7.8%, $p=0.0004$). The addition of Hcy further worsened function, compared to both the control (34.2±13.8, $p < 0.0001$) and the combination without Hcy ($p < 0.0001$, Figure 5.3B).

To measure differences between Nox1 and Nox4, which produce different ROS, we used a combination of 1400W, ML090 and VAS. This combination significantly impaired function compared with control (59.1±3.2%, $p=0.002$, Figure 5.3C), however the addition of Hcy to that combination did not further worsen function.

To assess the effect that removing all ROS from iNOS and Nox1, 2 and 4, we used all inhibitors together. The combination of 1400W and all three Nox inhibitors reduced function compared with control (52.5±2.3%, $p < 0.0001$, Figure 5.3D) as did the addition of Hcy to this treatment (32.6±9.8%, $p < 0.0001$, Figure 5.3D).

5.6 Discussion

During oxidative stress, iNOS has the propensity to become uncoupled and form O_2^- rather than increase NO, thus it has been considered a potent activator of oxidation and vascularly detrimental (Hollingsworth et al. 2016; Pacher, Beckman & Liaudet 2007). Under these same conditions, Nox1, 2 and 4 have also been reported as exacerbating the problem by producing yet more ROS (Bedard & Krause 2007b). Because excessive Hcy can also increase ROS under pathological conditions (Leung et al. 2013; Xiao et al. 2014), we hypothesised that iNOS and Nox inhibition might protect against endothelial dysfunction in excess Hcy. To test this, we

employed the use of an iNOS inhibitor and Nox1, 2 and 4 inhibitors under acute Hcy-induced dysfunction. The major finding of our study is that iNOS inhibition alone and in conjunction with Nox1, 2 and 4 inhibition fails to protect against dysfunction and, if anything, exacerbates it. Contrary to our hypothesis, these findings demonstrate that instead of mediating pathophysiological conditions in acute Hcy-induced dysfunction as has been reported, iNOS and Nox1, 2 and 4 are essential for proper endothelial function, keeping in mind 'off target' effects.

The results assessing iNOS inhibition in healthy rabbit aortae suggest that, if 1400W is specific for iNOS, endogenous basal levels of iNOS are required for proper function, as singular iNOS inhibition impaired function (Figure 5.1). This is consistent with previous work showing that iNOS is necessary to protect against thrombotic occlusion injury (Dayal et al. 2014; Upmacis et al. 2011). Further, work by Yan and Hansson (1998) show that iNOS is continuously expressed in neointimal smooth muscle cells in response to injury and pro-inflammatory mediators which results directly in increased NO levels. This suggests a relationship between iNOS and Hcy; indeed Mayo and colleagues (2014) reported that Hcy increased iNOS levels in murine choroid explants during angiogenesis. In pathological states, iNOS is upregulated to increase NO levels (Iwakiri & Kim 2015).

In our study, iNOS inhibition was implicated in contributing to impaired aortic vasodilation, with both Nox inhibition alone (Figure 5.2A) and Hcy treatment (Figure 5.2B). The exact mechanism is unclear but could be a function of reduced Nox activation and impaired iNOS binding (Feng & Tollin 2009; Smith, BC et al. 2013). For NO production, iNOS must be bound tightly to calmodulin which requires electron donation from NADPH (Hollingsworth et al. 2016). Our results indicate that when Nox and iNOS are inhibited, not only is there a reduction in electron transfer ability from Nox due to its inhibition, but also a critical reduction in iNOS itself.

Our results show a greater reduction in function when iNOS inhibition was paired with double and triple Nox inhibition in Hcy-treated aortae (Figures 5.3A-D). It was recently reported that cardiac iNOS mRNA is upregulated in iNOS($^{+/+}$) mice with HHcy which protects against oxidative stress and ischaemic reperfusion injury; indeed iNOS($^{-/-}$) mice with HHcy were not protected from increased ROS production and showed greater infarct size (Dayal et al. 2014), suggesting the iNOS as the central factor. Further, lung injury was attenuated in wild-type but not in iNOS$^{-/-}$ mice, but this was resolved when iNOS was restored to the *null* mice (D'Alessio et al. 2012), providing direct evidence of iNOS interaction. Our findings confirm that when basal levels of iNOS and Nox1, 2 and 4 are removed with their essential ROS, the protective effect of these is reduced and the vessel is more susceptible to a reduction of relaxing factors and impairment.

5.7 Limitations

There are limitations to our study. Whilst the use of a rabbit model for endothelial dysfunction has repeatedly been used as an analogue for cardiovascular pathologies in humans, it is not without its limitations. In addition to this, the specificity of Nox inhibitors remains a controversial issue, especially as they may have off-target effects. Hence, the potential that the inhibitors used in the present study may be acting on other Nox isoforms or other molecules must also be considered. Also, 1400W could have 'off target' effects (possible eNOS and nNOS)

5.8 Conclusion

The data presented here indicate the possibility of a vitally important role for both iNOS and Nox1, 2 and 4, in Hcy-induced pathology, especially in the context of proper ACh-dependent endothelial function. Treatments to reduce the effects of excess Hcy remains a challenge.

Chapter 6

6.1 The Effect of Combined Nox Inhibitors in Homocysteine-Induced Endothelial Dysfunction

6.2 Abstract

Objective: Elevated plasma homocysteine (Hcy) is an independent risk factor cardiovascular disease (CVD) and is associated with endothelial dysfunction and risk of thromboembolism. Treatments aimed at reducing plasma Hcy have not produced positive clinical outcomes, thus novel treatments are required to protect against Hcy-induced vascular dysfunction. Hcy increases reactive oxygen species via NADPH oxidase (Nox) enzymes, thus Nox inhibition could provide a therapeutic avenue to treat Hcy-induced CVD. Thus, the objective was to determine the impact of putative Nox inhibitors on Hcy-induced endothelial dysfunction in rabbits and mice.

Methods: New Zealand White rabbits (n=15; VUAEC #12/019) were fed a normal chow diet and wild-type (WT) and $Nox2^{-/-}$ (NOX) were mice fed normal chow (n=4) or a 1% methionine diet (n=4). The abdominal aortae were excised, cut into rings and mounted in organ baths. Aortic rings were incubated with combinations of ML090 (Nox1 inhibitor), gp91ds-tat (Nox2 inhibitor), VAS2870 (Nox4 inhibitor) (all 10^{-6} M). Some rings were incubated with 3mM Hcy for 1 hour to induce endothelial dysfunction and then dose-response curves to acetylcholine (ACh; 0.01µM-10µM) were performed.

Results: In rabbits aorta, Hcy incubation impaired aortic relaxation compared with control (97.9±1.0% vs. 63.0±7.9%, $p < 0.0001$). In Hcy-induced endothelial dysfunction, Nox1 + Nox2 inhibition reduced function compared with control (97.9±1.0% vs. 67.6±4.1%, **** $p < 0.0001$), as did Nox1 + Nox4 inhibition (97.9±1.0% vs. 69.7±1.8%, **** $p < 0.0001$). Likewise, Hcy + Nox2 + Nox4 inhibition significantly impaired relaxation (97.9±1.0% vs.

48.8±3.8%, **** $p < 0.0001$). In vessels treated with Hcy, the addition of triple Nox inhibition improved the response to ACh (61.7±9.6% vs. 78.1±3.6%, ** $p < 0.01$).

Wild type (WT) mice fed a 1% methionine-rich diet had impaired endothelial function compared with WT fed a normal chow diet (77.9±0.8% vs. 59.1±4.1%, $p = 0.001$). Triple Nox1, 2 and 4 inhibition in WT fed a 1% methionine diet mice worsened function compared with control (no treatment; 77.9±0.8% vs. 57.5±10.2%, $p < 0.0001$). $Nox2^{-/-}$ (NOX) mice were otherwise pharmacologically inhibited with Nox1 and Nox4 inhibitors. Compared with WT controls, Nox2 deletion plus pharmacological Nox1 and 4 inhibition worsened function (77.9±0.8% vs. 22.1±9.5, $p < 0.0001$). Nox2 deletion plus pharmacological Nox1 and 4 inhibition with a 1% methionine diet also reduced function (77.9±0.8% vs. 35.7±7.9%, $p < 0.0001$).

Discussion: This study reports that pharmacologically inhibiting Nox1 and 2 and 4 improves the effect of HHcy-impaired acetylcholine-mediated vasorelaxation in acute Hcy-induced endothelial dysfunction in healthy rabbits but is detrimental in a $Nox2^{-/-}$ mouse model. These results provide clear evidence of a complex relationship between Nox1, 2 and 4 and their oxidative products in HHcy-impaired acetylcholine-mediated vasorelaxation.

Conclusion: These data suggest a complex interplay between the different Nox isoforms in Hcy-impaired acetylcholine-mediated vasorelaxation. Further work is required to elucidate the suitability of Nox inhibitors for treating Hcy-induced CVD.

6.3 Introduction

Cardiovascular disease (CVD) remains a leading cause of mortality with deaths from stroke and heart attack increasingly affecting the population (Heart Foundation). Initiation and progressive development of vascular dysfunction is driven by coronary artery disease (CAD) and atherosclerosis (Jiang et al. 2012; Julve et al. 2013; Vacek et al. 2015), whereby the arterial environment is damaged by intimal thickening, leading to reduced nitric oxide (NO) availability and impaired vasorelaxation.

Homocysteine (Hcy) is a sulphur-containing amino acid obtained through dietary methionine and increased levels are an important and independent risk factor for CVD (Sen et al. 2014; Veeranna et al. 2011). Normal physiological Hcy is 6-9 µM, whilst in hyperhomocysteinemia (HHcy) levels reach >15 µM (Beard, Reynolds & Bearden 2012). Normal methionine metabolism synthesises and recycles Hcy into cysteine and methionine; this mechanism requires adequate vitamin B_6, B_{12} and folate (Carmel, Melnyk & James 2003; Hoffman 2011). Dysregulated methionine metabolism results in excess Hcy, which increases inflammation and contributes to endothelial damage (Hoffman 2011; Veeranna et al. 2011). Folate administration in cell culture and animal studies report lowered Hcy levels and improved vascular function (Qipshidze et al. 2011; Tousoulis et al. 2014). In contrast, reports from Hcy-lowering clinical trials imply that B vitamin administration may not be an effective therapy (Hodis et al. 2009; Kaul, Zadeh & Shah 2006; Lonn et al. 2006; Pettigrew et al. 2008). However, in these studies Hcy levels were not reduced into the healthy range, presumably leading to the disappointing outcomes. Thus, there remains a lack of suitable Hcy-lowering therapy to reduce CVD risk.

Nicotinamide adenosine diphosphate (NADPH) oxidases (Nox) are located in the cell membrane and generate functional reactive oxygen species (ROS). Nox1, 2 and 4 are expressed throughout the vasculature (Ray, R et al. 2011; Rivera et al. 2010) and generate superoxide (O_2^-; Nox1, Nox2) (Drummond & Sobey 2014) and hydrogen peroxide (H_2O_2; Nox4) (Schurmann et al. 2015). Nox1, 2 and 4 are important in CVD development in the context of uncontrolled ROS regulation (Bedard & Krause 2007b; Drummond & Sobey 2014; Manolescu et al. 2010) and dysregulated ROS are further implicated in atherosclerosis (Judkins et al. 2010), ischaemic injury (Dvoriantchikova et al. 2012; Kahles & Brandes 2012) and systemic hypertension (Wind et al. 2010).

HHcy may increase Nox-derived O_2^-, impairing vascular function (Kassab & Piwowar 2012). Pharmacological Nox inhibition in cell and animal studies have yielded mixed results: Nox2 inhibition by apocynin (4mg/kg; 7 days) improved endothelial relaxation in HHcy rat aortae (Edirimanne et al. 2007) and reduced Hcy-induced ROS in human endothelial progenitor cells (DPI; 10µM; 30 minutes) (Bao, X-M, Wu, C-F & Lu, G-P 2010), but Nox2 (gp91ds-tat) and Nox4 (Plumbagin) inhibition worsened acetylcholine-dependent relaxation in Hcy-thiolactone (HcyT; the atherogenic form of Hcy) treated rabbit aortae (Smith, R et al. 2015). The putative Nox4 inhibitor VAS2870 and has been shown to be effective in ischaemic stroke (McLeod et al. 2010) and Wind et al. (2010) reported that in spontaneously hypertensive rat aortae, VAS2870 reduced vascular ROS production to control levels and inhibited ROS-induced signalling. Howitt et al. (2014) have shown that VAS2870 abolishes NO inhibition in both Nox2 knock-out and wild-type mice, via T-type calcium channels thought to drive NO-derived relaxation. The novel pharmacological Nox1/4 inhibitor GKT137831 (GKT) has been shown to reduce collagen and fibronectin the renal cortex of diabetic mice (Gorin, Y. et al. 2015). Atherosclerotic plaque contains irregular matrix deposition and HHcy is involved in fatty

lesion formation (Roos et al. 2016; Thampi et al. 2008). GKT targets both Nox1 and Nox4 to reduce O_2^- and H_2O_2, and has shown promise in animal studies treating diabetic nephropathy (Green, DE et al. 2012; Sedeek et al. 2013). A recently developed triple Nox knock-out (Nox1, 2 and 4) mouse has also shown to have lower diastolic blood pressure than wild-type littermates but also lower levels of eNOS (Rezende et al. 2015). However, recent data from our own lab (not shown) using $Nox2^{-/-}$ mice with pharmacological inhibitors (VAS2870 and ML090) indicate that ROS, presumably from increased Hcy levels, confer some protection to the endothelium in chronic diet-induced HHcy, which reveals a potential role for ROS homeostasis in chronic pathology.

To that end, we hypothesised that pharmacological inhibition of Nox1, 2 and 4 might improve aortic relaxation in HHcy-induced endothelial dysfunction. To our best knowledge, this is the first study assessing these effects.

6.4 Methods

All experiments were carried out according to the National Health and Medical Research Council "Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (8th Ed. 2013).

Rabbits: New Zealand rabbits were housed in separate cages and maintained at a constant temperature of approximately 23°C and were provided water and food ad libidum. Animals were fed a normal chow diet (12 weeks, male, n=15, VUAEC #12/019). Animals were anaesthetised (n=15: medetomidine 0.25mg/kg), exsanguinated and the abdominal aorta excised and flushed with cold (on ice) oxygenated Krebs buffer (NaCl 118.4, KCl 4.7, NaHCO3 25, MgSO4 1.2, CaCl2, 2.5, glucose 11.1, mM).

Mice: Wild-type (WT) and $Nox2^{-/-}$ (NOX) mice fed normal chow (n=4) or a 1% methionine diet for 8 weeks (n=4) to elevate plasma Hcy. Animals were housed in cages with a maximum of 5 mice per cage and maintained at a constant temperature of approximately 23°C. Food and water were provided ad libidum. At sacrifice, mice were anaesthetised with isoflourane (4% O_2) in a sealed chamber for a minimum of 5 minutes and then underwent cervical dislocation and exsanguinated as per rabbits.

The aorta was cleaned of fat and connective tissue, cut into rings (2-3 mm lengths) and placed in organ baths (OB8, Zultek Engineering, Australia), filled with Krebs, kept at a constant temperature of 37°C and continuously bubbled with carbogen (95% O_2 + 5% CO_2). Rings were left to rest, unmounted, for 30 mins, and then mounted between two metal hooks attached to force displacement transducers, stretched to 2g (rabbits) or 0.5g (mice) and allowed to relax for 20 mins, twice.

Experimental Protocol

Rings were incubated with ML090 (Nox1 inhibitor; 1µM), gp91ds-tat (Nox2 inhibitor; 1µM) or VAS2870 (Nox4 inhibitor; 1µM) for 30 minutes; NOX rings were not incubated with Nox2 inhibitor. Drugs were re-introduced into the baths immediately prior to the addition of 3mM Hcy where used. Control rings had no Hcy or treatments added but were otherwise treated as experimental rings. Experimental rings without diet intervention were incubated in Hcy for 1 hour. Then, to assess endothelial dependent relaxation to acetylcholine (ACh; 0.01µM-10µM), rings were pre-contracted with either phenylephrine (rabbits; Phen; 0.3µM) or the thromboxane analogue U46619 (mice; thx; 0.3 µM). After the contraction reached a plateau, a concentration-response curve to ACh-induced relaxation was obtained.

All data were analysed using a repeated measures two-way ANOVA followed by Sidak's Multiple Comparisons Test. Data are represented as mean ± SEM.

6.5 Results

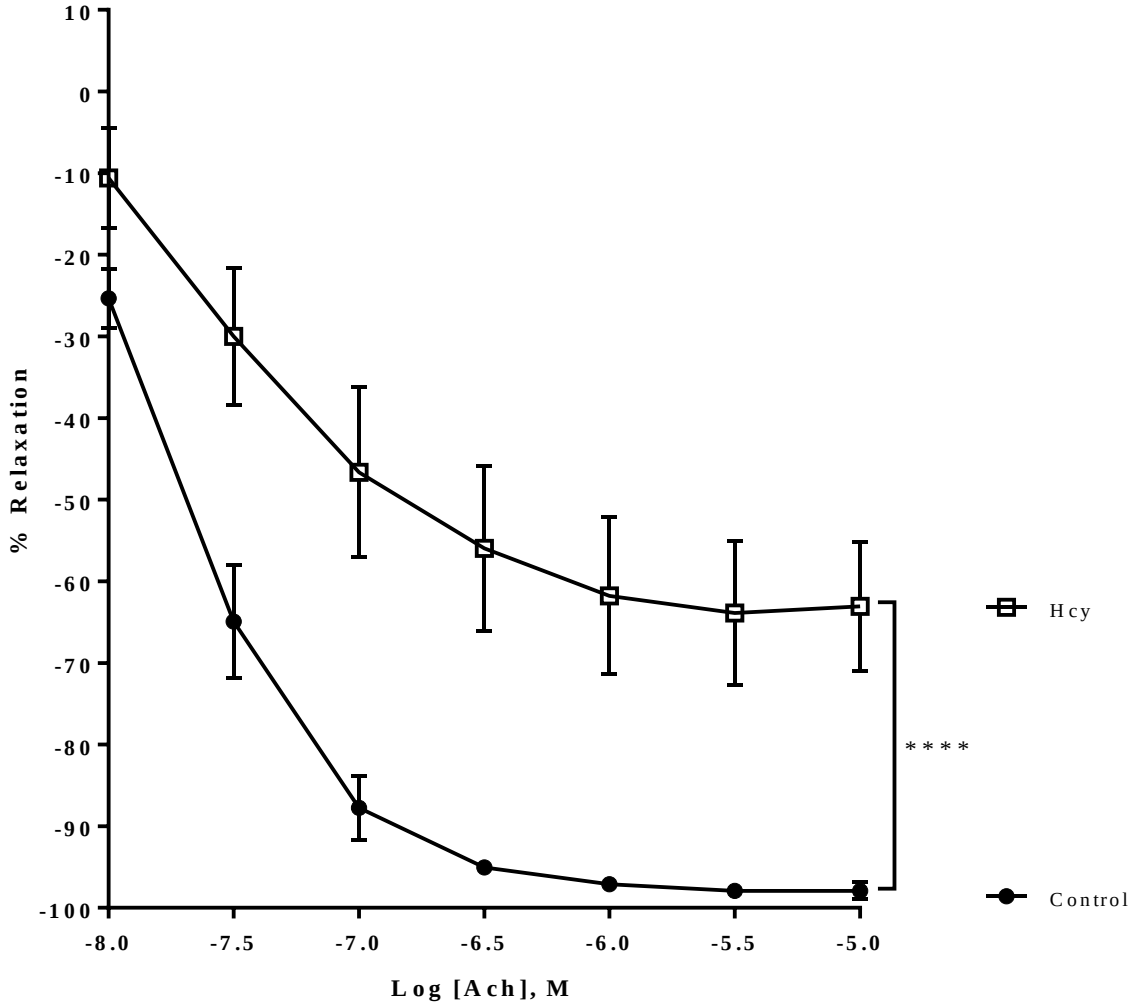


Figure 6.1. To measure the extent of impaired function and responses of potential pharmacological treatments for increased plasma homocysteine (Hcy), we induced endothelial dysfunction in ex vivo rabbit aorta sections with 3mM Hcy. The application of Hcy significantly impaired ACh-dependent relaxation in the aorta. (Control vs. Hcy 97.9±1.0% vs. 63.0±7.9%, $p < 0.0001$). Data are represented as mean ± SEM (n=5 per group). **(Taken from Figure 1A as as this was performed in the same group of rings).**

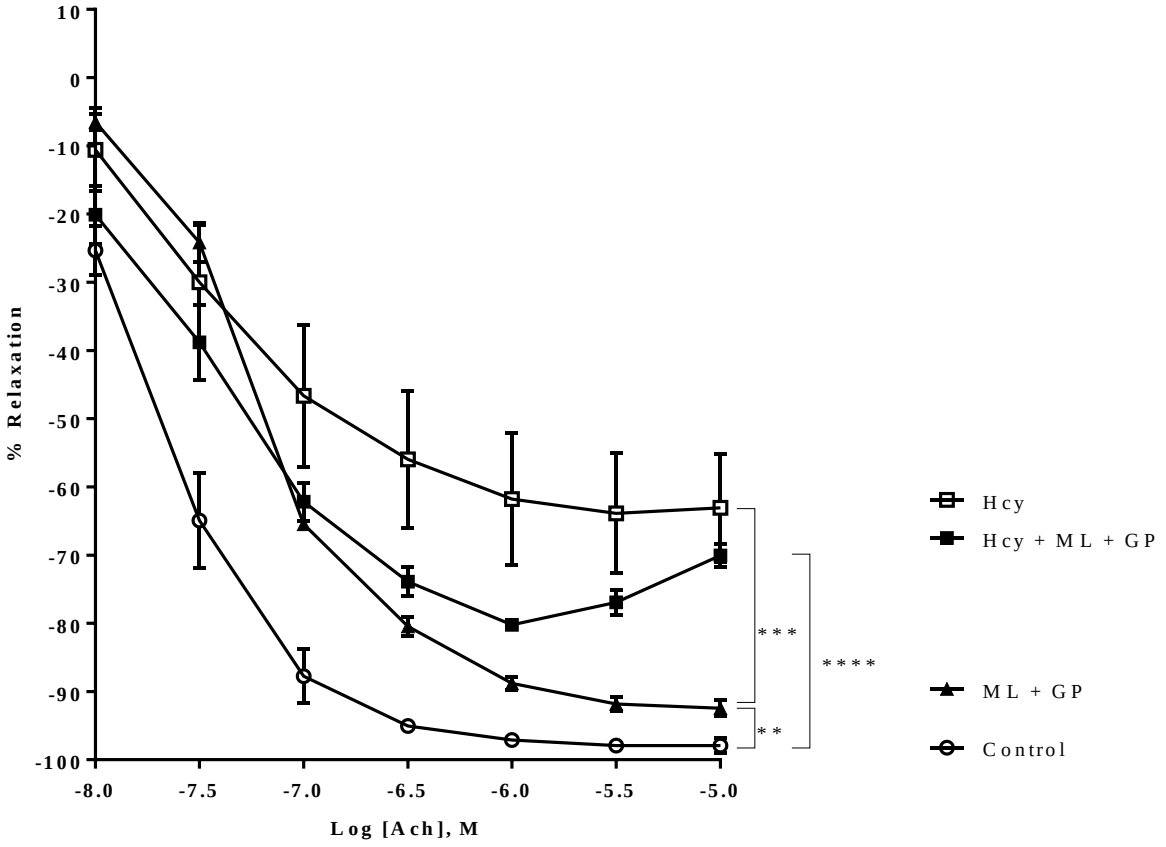
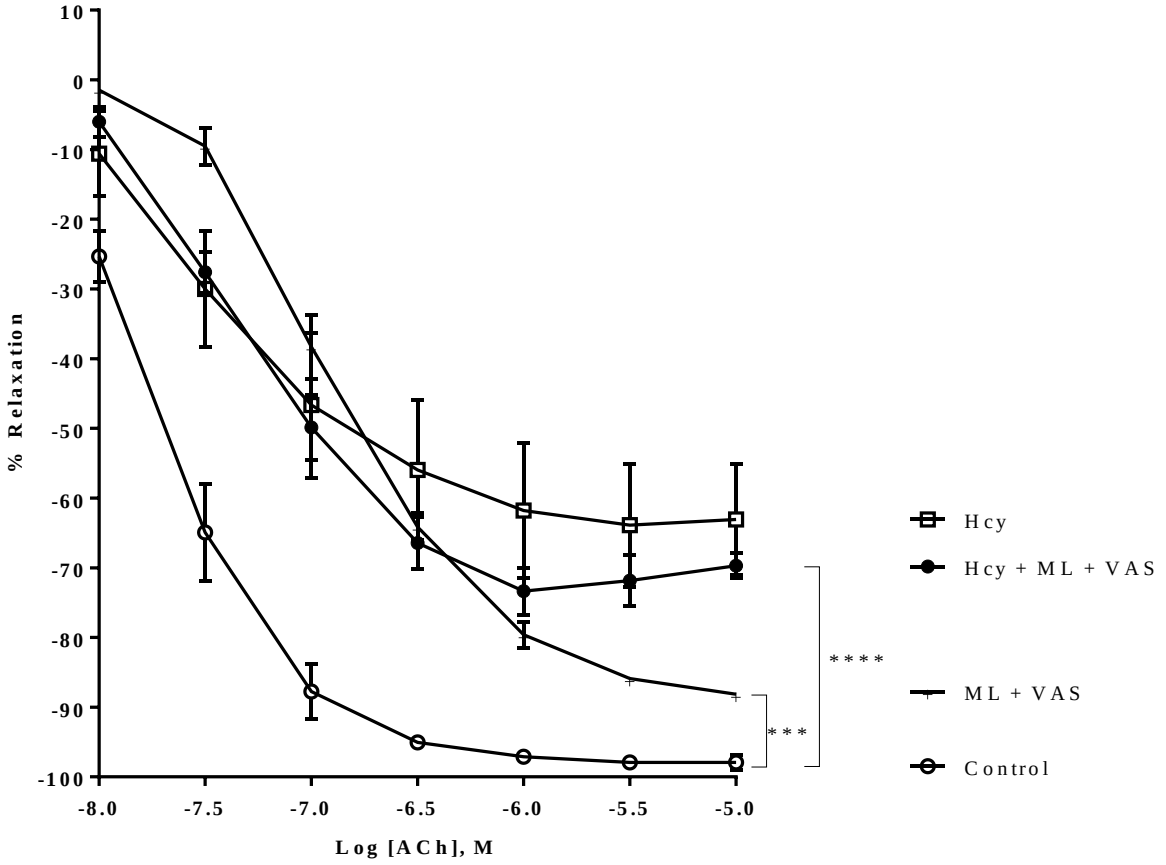
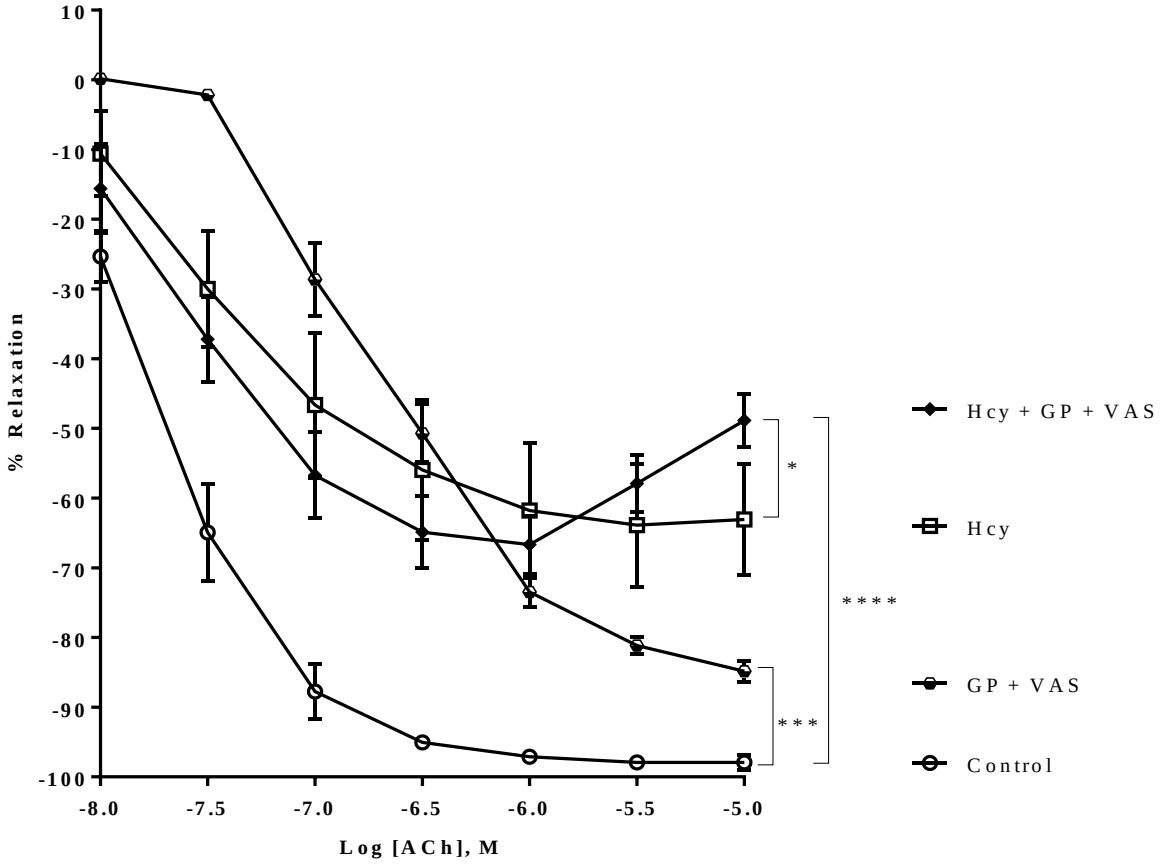


Figure 6.2A) To test the extent that double combinations of Nox1 and 2 are involved in endothelial responses to ACh in the healthy and diseased vessel, we applied Nox1 and 2 inhibitors to non-treated control and Hcy-treated vessels. In healthy rabbit aortae, combinations of Nox1, 2 inhibitors (ML090 and gp91ds-tat, respectively) significantly impaired endothelial function. Control vs. ML090 + gp91ds-tat (95.0±0.8% vs.80.4±1.3%, ** $p < 0.01$). In vessels treated with Hcy, the addition of Nox1 and 2 inhibitors further blunted the response compared with control (Control vs Hcy + ML090 + gp91ds-tat (97.9±1.0% vs. 67.6±4.1%, **** $p < 0.0001$), however Nox1 and 2 inhibition in Hcy-treated vessels improved function compared with Hcy treatment alone (61.7±9.6% vs. 78.1±3.6%, *** p 0.001). Data are represented as mean ± SEM (n=5 per group).



B) To test the extent that double combinations of Nox1 and Nox4 inhibitors are involved in endothelial responses to ACh in the healthy and diseased vessel, we applied Nox1 and 4 inhibitors to non-treated control and Hcy-treated vessels. In healthy rabbit aortae, combinations of Nox1 and 4 inhibitors (ML090 and VAS2870, respectively) significantly impaired endothelial function. Control vs. ML090 + VAS2870 (97.0±0.8% vs.79.6±1.8%, *** $p = 0.001$). In vessels treated with Hcy, the addition of Nox1 and 4 inhibitors impaired the response to ACh, compared with control (97.9±1.0% vs. 69.7±1.8%, **** $p < 0.0001$). There was no difference in function between Nox1 and 4 inhibitors in Hcy-treated vessels compared with Hcy treatment alone). Data are represented as mean ± SEM (n=5 per group).



C) To test the extent that double combinations of Nox2 and 4 are involved in endothelial responses to ACh in the healthy and diseased vessel, we applied Nox2 and 4 inhibitors to non-treated control and Hcy-treated vessels. In healthy rabbit aortae, combinations of Nox2 and 4 inhibitors (gp91ds-tat and VAS2870, respectively) significantly impaired endothelial function. Control vs. gp91ds-tat + VAS2870 (97.9±0.8% vs.81.1±1.1%, *** $p = 0.001$). In vessels treated with Hcy, the addition of Nox2 and 4 inhibitors significantly impaired function compared with control (97.9±1.0% vs. 48.8±3.8%, **** $p < 0.0001$). Adding the Nox2 and 4 inhibitors to vessels treated with Hcy further impaired function, compared with Hcy treatment alone (63.0±7.9% vs. 48.8±3.8%, * $p = 0.01$). Data are represented as mean ± SEM (n=5 per group).

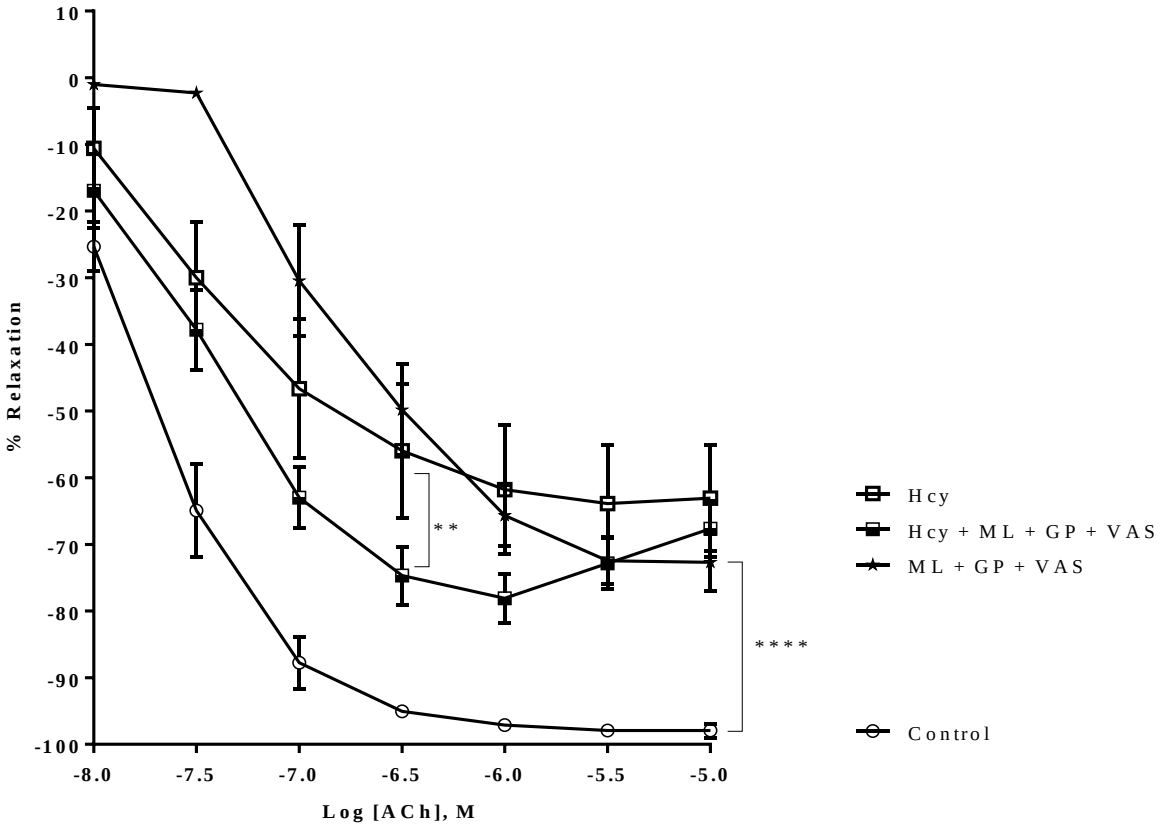


Figure 6.3. To test the extent of Nox1, 2 and 4's involvement in normal endothelial responses to ACh, we treated healthy and Hcy-treated rabbit vessels with all a combination of all three Nox inhibitors (ML090, go91ds-tat and VAS2870, respectively). In healthy rabbit aortae, triple Nox inhibition significantly blunted the response to ACh compared with control (97.9±1.0% vs. 72.6±4.2%, **** $p < 0.0001$). In vessels treated with Hcy, the addition of triple Nox inhibition improved the response to ACh (61.7±9.6% vs. 78.1±3.6%, ** $p < 0.01$). Data are represented as mean ± SEM (n=5 per group).

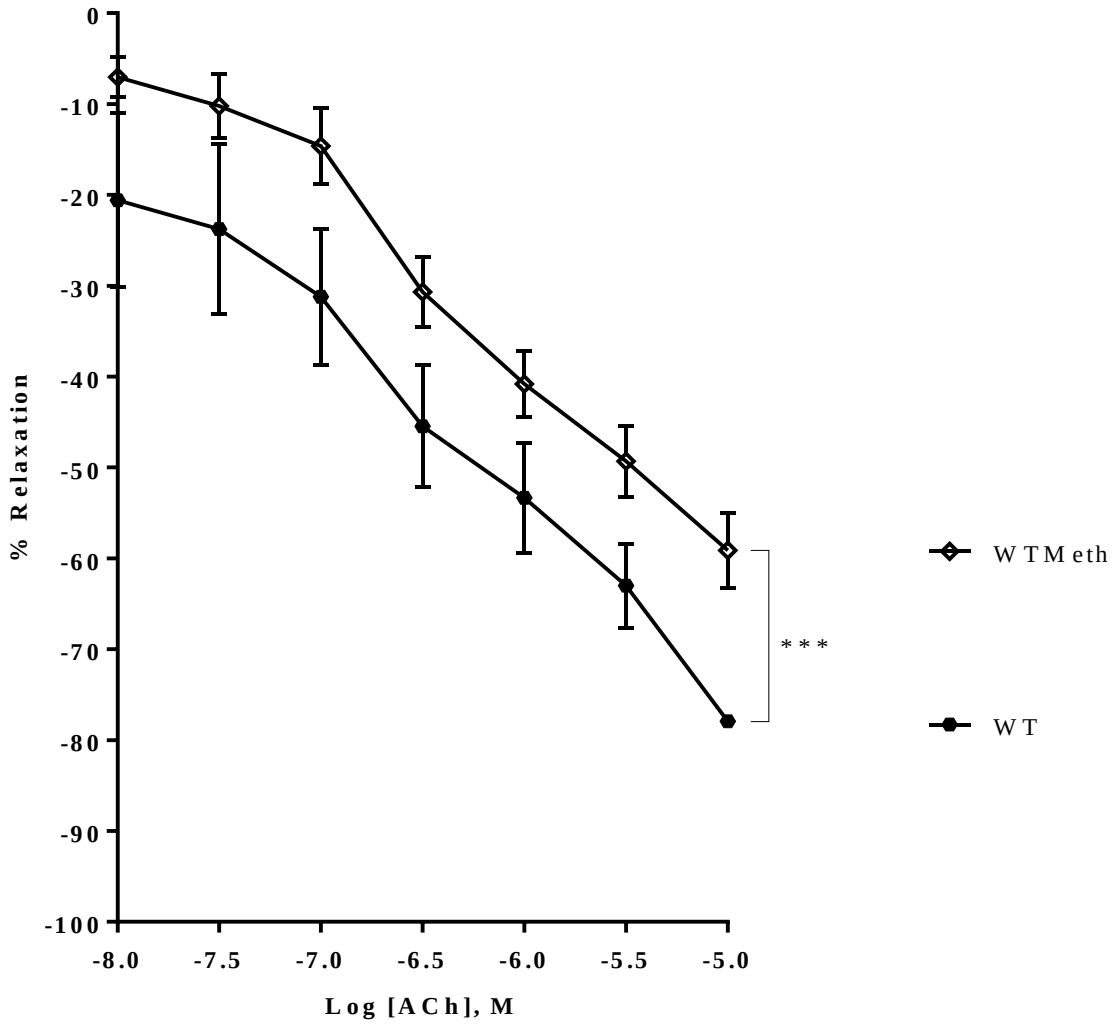
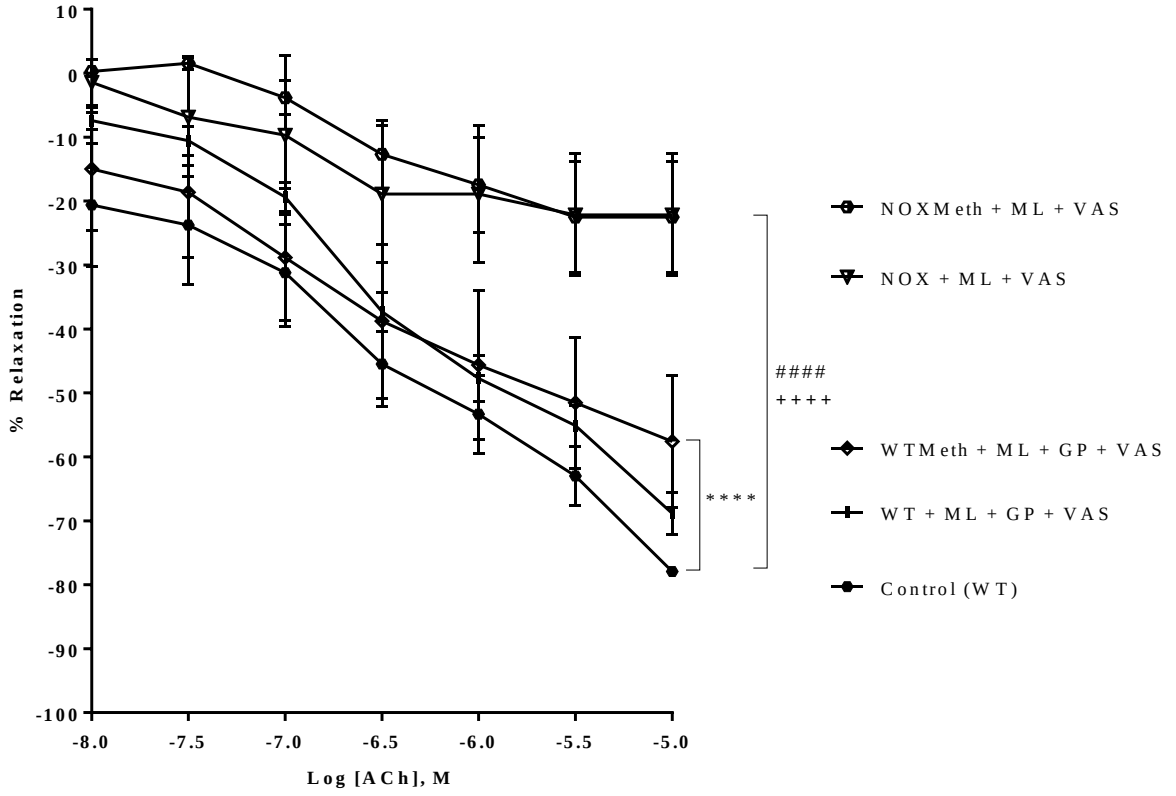


Figure 6.4A. To induce Hcy-dependent endothelial dysfunction in WT (C57/BLK6), we administered a 1% methionine diet (*ad libidum*) for 4 weeks to increase plasma Hcy. WT rings treated with the 1% methionine diet had decreased relaxation compared with controls (no treatment; 77.9±0.8% vs. 59.1±4.1%, *** p = 0.001). Data are represented as mean ± SEM (n=4 per group).



B) To assess the effect of combined Nox1, 2 and 4 inhibition in WT mice, we used combined ML090, gp91ds-tat and VAS2870 and a $Nox2^{-/-}$(NOX) model and administered ML090 and VAS2870 only. Triple Nox1, 2 and 4 inhibition in WT fed a 1% methionine diet mice worsened function compared with control (no treatment; 77.9±0.8% vs. 57.5±10.2%, **** $p <$ 0.0001). Nox2 deletion plus pharmacological Nox1 and 4 inhibition worsened function (77.9±0.8% vs. 22.1±9.5, ++++ $p < 0.0001$). Nox2 deletion plus pharmacological Nox1 and 4 inhibition with a 1% methionine diet also reduced function (77.9±0.8% vs. 35.7±7.9%, #### $p < 0.0001$). Data are represented as mean ± SEM (n=4 per group).

6.6 Discussion

The central finding of this study is that pharmacologically inhibiting Nox1 and 2 and 4 improves the effect of ACh in acute Hcy-induced endothelial dysfunction in healthy rabbit

aortae but is detrimental in a Nox2$^{-/-}$ mouse model. These results provide clear evidence of a complex relationship between Nox1, 2 and 4 in Hcy-induced vascular disease.

There are currently no reliable methods of reducing Hcy levels in those patients who pose a risk of developing or worsening CVD. ROS are essential for intracellular signalling, and although the exact mechanisms are not fully understood (Ullrich & Kissner 2006), Nox are the main generators of functional ROS in the vasculature (Rivera et al. 2010). In addition to this, endothelial function assessed ex vivo is measured by a response to ACh, reductions in which are attributed directly to a decrease in NO availability (Smith, R et al. 2015). In the acute model, Hcy-treated rabbit vessels with combined Nox1, 2 and 4 inhibition improved ACh-dependent relaxation (Figure 6.1A, B). In diet-treated vessels, Nox inhibition failed to mimic these effects and instead reduced relaxation (Figure 6.1C, D and 6.2).

The role of Nox in homocysteine-induced vascular disease is not well understood and these results add more uncertainty to underlying Hcy pathology. Reports suggest that Nox inhibition confers a protective result (Gonzalez et al. 2014; Miller et al. 2013; Troiano et al. 2016) but these have so far failed to characterise precise mechanisms. ROS inhibition is generally perceived as the main protective factor in Nox inhibition, as it is thought to drive inflammation (Martinon 2010), however, ROS over-expression has also been shown to be protective (Ray, R et al. 2011; Shafique et al. 2013) and our lab has previously showed that Nox inhibition failed to protect against Hcy thiolactone, the atherogenic form of Hcy (Smith, R et al. 2015). Our findings suggest that, like in adaptation to acute exercise or wound repair (Barbosa et al. 2013; Roy, Khanna & Sen 2008), ROS is likely essential in acute HHcy to establish maintenance of intracellular homeostasis impaired by excess Hcy (Wang, S, Song & Zou 2012), possibly in the developmental phase of dysfunction. The reasons for this are unclear and in fact pose more questions about the mechanisms involved, but it points to physiological differences in the way that ROS are used in endothelial function. One possible explanation is that intracellular ROS

levels change depending on the Nox inhibitor used; this could be attributed to the differences between murine and rabbit models of antioxidant production, which might influence ROS levels (Kizhakekuttu & Widlansky 2010). The antioxidant CoQ (also known as ubiquinone) is involved in ROS generation; the ratio of CoQ9:CoQ10 appears to be important for antioxidant levels, and thus radical scavenging (Yang et al. 2015). A low CoQ9:CoQ10 ratio might be important for oxidative stress (Bravo et al. 2012); mice have been reported as having a high CoQ9:CoQ10 ratio compared with rabbits (Lass, Agarwal & Sohal 1997), which could be interpreted as mice having more naturally occurring oxidative stress, and thus may be naturally able to maintain homeostasis in the presence of increased levels of ROS. In the NOX murine model of chronic HHcy, inhibiting all 3 Nox (Nox2 genetically and Nox1 and Nox4 pharmacologically), reduced relaxation compared to controls (WT without treatment), but a 1% methionine restored function (figure 2B). We attribute these results to an increase of available essential ROS that has been catalysed to effect downstream NO stimulation.

6.7 Study Limitations

The use of putative Nox inhibitors remains controversial and has not been fully elucidated in in vitro models of disease. Further, it is unknown if the drugs used herein have additional effects that could influence ACh dependent relaxation. The conclusion to draw from these data is that there is a role for pharmacological Nox inhibitors in response to ACh in Hcy-induced endothelial dysfunction. More targeted and specific research must be done in animal models order to establish any other apparent effects of these treatments.

6.8 Conclusion

The potential for Nox1, 2 and 4 inhibition to be used as a therapeutic agent in Hcy-induced endothelial damage warrants serious consideration, as there appears to be different responses depending on the model used. The combination of all three putative Nox inhibitors improved

relaxation in the healthy rabbit in Hcy-treated vessels and in healthy WT mice. However, using these putative inhibitors in an atherogenic diet in rabbits and $Nox2^{-/-}$ deletion resulted in further reductions in relaxation. This is suggestive of a fundamental requirement of free radicals, especially O_2^-, for proper endothelial function.

6.9 An atherogenic diet causes imapired acetylcholine-mediated vasorelaxation which is not rescued by combined Nox1, 2 and 4 inhibition

6.10 Abstract

Objective: Elevated plasma homocysteine (Hhcy) is an independent risk factor for cardiovascular disease (CVD), yet clinical trials aimed at reducing Hcy levels using targeted vitamin therapy have not met with success, and in some cases worsened outcomes. Thus novel pharmacological intervention to treat Hhcy is lacking. Hhcy increases reactive oxygen species (ROS) via NADPH oxidase (Nox) enzymes, thus Nox inhibition might be a therapeutic target. We sought to determine the effect of putative Nox inhibitors in an atherogenic rabbit model of endothelial dysfunction.

Methods: New Zealand White rabbits fed chow (n=3) or atherogenic diet (n=3) to elevate plasma Hcy. Aortae were excised, mounted in organ baths and incubated with ML090 (Nox1 inhibitor), gp91ds-tat (Nox2 inhibitor), VAS (Nox4 inhibitor) or combinations thereof (all 10^{-6} M). Dose-response curves to acetylcholine (ACh; 0.01µM-10µM) were performed.

Results: An atherogenic diet reduced function compared with a normal diet ($p < 0.001$). In atherogenic rabbits, Nox2 inhibition significantly impaired function versus control ($p < 0.001$) as did Nox4 inhibition ($p < 0.0001$), whereas Nox1 inhibition had no effect. Double combinations of Nox inhibitors also impaired function versus control: Nox1 + Nox2 ($p < 0.01$),

Nox1 Nox4 ($p < 0.0001$) and Nox2 + Nox4 ($p < 0.0001$). Using all three Nox inhibitors in combination also impaired aortic relaxation compared with control ($p < 0.0001$).

Conclusion: These data suggest a complex interplay between the different Nox isoforms in diet-induced endothelial dysfunction, and that current Nox inhibitors might not be a beneficial treatment for dietary induced endothelial dysfunction.

6.11 Introduction

An increase in plasma homocysteine (Hcy) and LDL-C cholesterol have been established as increased risk factors for developing cardiovascular disease (CVD) (Silverman et al. 2016; Tribouilloy et al. 2000). Longer term effects of these risks are the development of coronary artery disease (CAD), atherosclerosis and ischaemic stroke (Jiang et al. 2012; Julve et al. 2013; Vacek et al. 2015; Wang, C-y et al. 2014). Hcy is a non-protein sulphur containing amino acid produced as a normal by-product of methionine metabolism (Hu, Y et al. 2016; Pacana et al. 2015); it's mechanism of action is currently unknown.

Normal methionine metabolism requires adequate vitamin B6, B12 and folate (Carmel, Melnyk & James 2003; Hoffman 2011; Hu, Y et al. 2016), when dysregulated it results in excess Hcy, contributing to endothelial damage (Hoffman 2011; Veeranna et al. 2011). To that end, folate administration has been employed in studies attempting to reduce excess plasma Hcy (hyperhomocysteinemia; HHcy); indeed cell and animal studies have reported lowered plasma Hcy levels and improved vascular function (Qipshidze et al. 2011; Tousoulis et al. 2014). However, in human clinical trials, B vitamin therapy has not been successful (Ebbing et al. 2010; Hodis et al. 2009; Kaul, Zadeh & Shah 2006; Lonn et al. 2006; Pettigrew et al. 2008). The reasons for this are unclear, complex and point to an arterial environment complicated by inflammatory and chemical modulators. Reports from the Vitamin Intervention for Stroke Prevention (VISP) Efficacy Analysis postulate that the inclusion fortified food (mandated by

the time the trial began), low B12 dose and inability of uptake in renal impairment are among the potential factors (Spence et al. 2005). Further, low total Hcy level (approximate mean for all participants is given as 14 µM) is also suggested as a possible reason for the disappointing result by the authors of the trial (Toole et al. 2004). Given that in the rare genetic disorder, homocystinuria, where patients record 300-500 µM Hcy and present with premature atherosclerosis and the substantial risk of stroke only once Hcy is >100 µM (Beilby, J. & Rossi 2000), the suggestion that relatively low HHcy could account for those results makes sense. Normal physiological plasma Hcy levels are 6-9 µM; hyperhomocysteinemia (HHcy) is characterised as mild 15-30 µM, moderate 31-100 µM and severe >100 µM (Beard, Reynolds & Bearden 2012). A meta-analysis of 8 clinical trials assessing B vitamin supplementation for lowering Hcy levels found that there was no significant effect of B vitamin application on the outcomes of CVD events (Clarke, Robert et al. 2014). In addition to clinical trials employing B vitamins, antioxidants, as ROS scavengers, have been investigated as another novel approach to reduce HHcy. In the large-scale Heart Outcomes Prevention Evaluation (HOPE) Study (Yusuf et al. 2000), high-risk CVD patients were administered Vitamin E or placebo and followed up for 4.5-6 years. The investigators reported no significant difference in risk of CVD events or death between the groups; this is a phenomenon seen across several other vitamin E or antioxidant clinical trials (Yusuf et al. 2000). Low-density lipoprotein cholesterol (LDL-C), is another established risk factor for CVD (Silverman et al. 2016) which contributes to the inflammatory response by reportedly increasing ROS generation (Mazière et al. 2013). Statins are the common treatment to decrease LDL-C, but often come with negative side-effects (Beckett, Schepers & Gordon 2015). A recent strategy employing the use of a statin, which has the potential benefit of concomitant LDL-C reduction, reduced overall CVD risk but did not reduce Hcy (Evans et al. 2014). Thus, there remains a lack of suitable Hcy-lowering therapy to reduce CVD risk.

Nicotinamide adenosine diphosphate (NADPH) oxidases (Nox) are located in the cell membrane and generate functional reactive oxygen species (ROS). Of the 7 Nox in the family (Nox1-5, Duox1 and 2), Nox1, 2 and 4 are currently the most relevant for vascular pathologies (Altenhofer et al. 2012). Nox1, 2 and 4 are expressed throughout the vasculature (Ray, R et al. 2011; Rivera et al. 2010) and generate superoxide (O_2^-; Nox1, Nox2) (Drummond & Sobey 2014) and hydrogen peroxide (H_2O_2; Nox4) (Schurmann et al. 2015). Despite an essential role as functional ROS generators, Nox1, 2 and 4 are implicated in uncontrolled and dysregulated ROS generation (Bedard & Krause 2007b; Drummond & Sobey 2014; Manolescu et al. 2010) which might exacerbate atherosclerosis (Judkins et al. 2010), ischaemic injury (Dvoriantchikova et al. 2012; Kahles & Brandes 2012) and hypertension (Wind et al. 2010).

HHcy may directly upregulate Nox-derived O_2^-, leading to impaired vascular function (Kassab & Piwowar 2012). Pharmacological Nox inhibition in cell and animal studies suggest that removal of ROS is beneficial under experimental conditions. Nox1 inhibition with ML090 rescued retinal cells from ischaemia (Dvoriantchikova et al. 2012) and improved acetylcholine-dependent relaxation in acute plasma Hcy-thiolactone (HcyT; the atherogenic form of Hcy) in rabbits (Smith, R et al. 2015). Endothelial relaxation in HHcy rat aortae was improved by Nox2 inhibition with apocynin (Edirimanne et al. 2007), which was also shown to reduce Hcy-induced ROS in human endothelial progenitor cells (Bao, X-M, Wu, C-F & Lu, G-P 2010). Nox4 inhibition with VAS is reported as effective in ischaemic stroke (McLeod et al. 2010) and Howitt and colleagues (2014) have shown that VAS abolishes NO inhibition in both Nox2 knock-out ($^{-/-}$) and wild-type mice.

To that end, we hypothesised that pharmacological inhibition of Nox1, 2 and 4 might restore aortic relaxation to normal in an atherogenic model of diet-induced endothelial dysfunction, leading to novel therapeutic strategies to reduce CVD.

6.12 Methods

All animals were housed and experiments carried out according to the National Health and Medical Research Council "Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (8^{th} Ed. 2013). New Zealand rabbits fed either a normal chow diet (n=3, 12 weeks, male, VUAEC #14/005) or an atherogenic diet for 4 weeks (n=3, 16 weeks, male, SF00-218 Rabbit Diet: 5% Peanut Oil, 0.5% cholesterol and 1% methionine, VUAEC #13/011) were anaesthetised (0.25mg/kg medetomidine via subcutaneous injection into scruff of neck) prior to 4% isoflourane/oxygen induction in a chamber for a minimum of 10 minutes oxygen (flow rate: 4L/min non-rebreathing circuit). Once unconscious (no reflex at foot, ear and eye), the animal was culled by exsanguination. The aorta was then flushed with cold (on ice) oxygenated Krebs buffer (NaCl 118.4, KCl 4.7, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$, 2.5, glucose 11.1, mM), removed, cleaned of fat and connective tissue, cut into rings (2-3 mm lengths) and placed in organ baths (OB8, Zultek Engineering, Australia), filled with Krebs, kept at a constant temperature of 37°C and continuously bubbled with carbogen (95% O_2 + 5% CO_2). Rings were left to rest, unmounted, for 30 mins. Rings were then mounted between two metal hooks attached to force displacement transducers, stretched to 2g and allowed to relax for 20 mins, twice.

Experimental Protocol

Control rings were incubated with Krebs only. Experimental rings were incubated with either 1µM ML090, gp91ds-tat, VAS or combinations thereof for 30 minutes before being pre-contracted with phenylephrine (Phen) (0.3-1µM). Rings were then subjected to cumulatively increasing concentrations of acetylcholine (ACh) (0.01µM-10µM) to measure the ability of the ring to relax, as an analogue of NO release.

Statistical Analysis

Data were subject to a 2 way repeated measures ANOVA and compared using Sidak's Multiple Comparisons Test. Data are presented as mean ± SEM.

6.13 Results

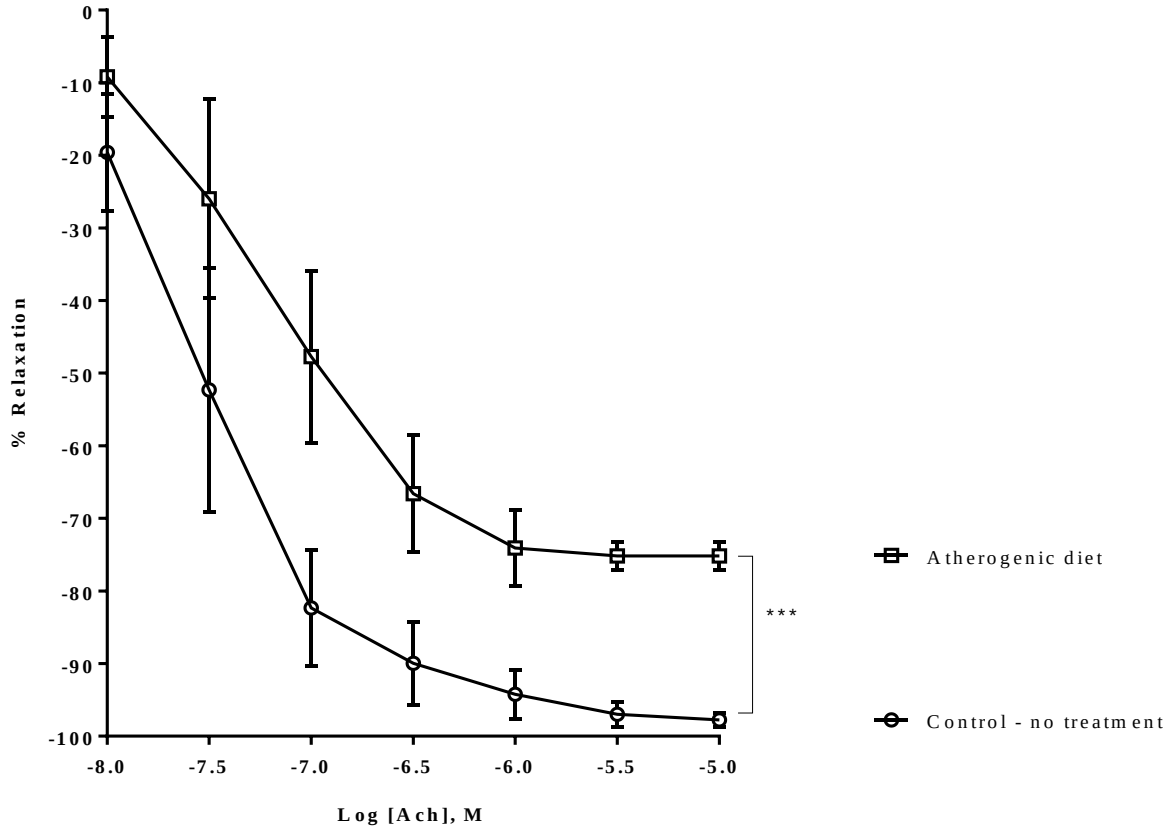
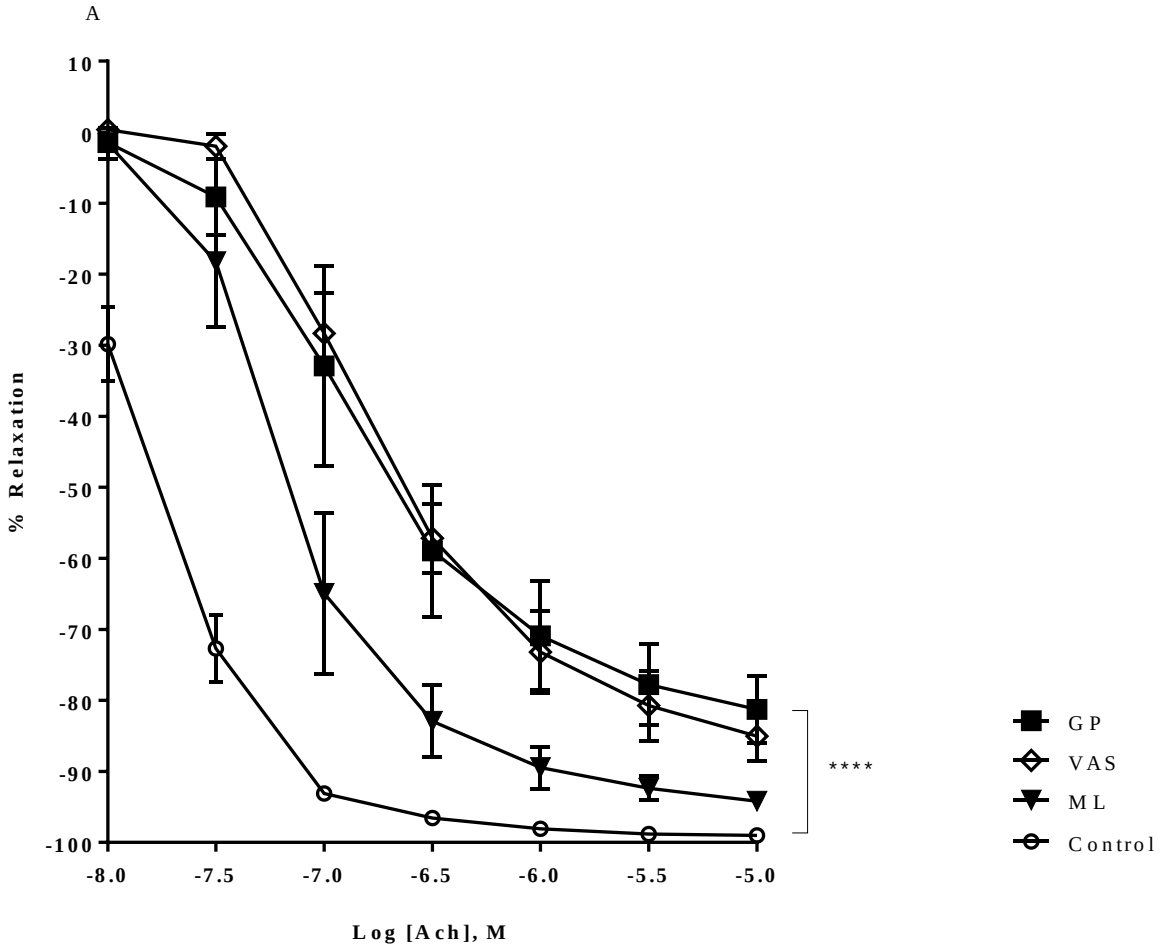


Figure 6.5: An atherogenic diet significantly impaired vasorelaxation in rabbits. Results are mean ± SEM (n=3 per group), * *p* 0.001. All values compared using Sidak's Multiple Comparisons Test.** We have previously reported that normal endothelial relaxation to ACh in the rabbit aorta is impaired by a physiologically relevant atherogenic diet on vasodilation (Rai, Hare & Zulli 2009; Zulli et al. 2003). To assess the ability of this diet high to impair vascular function, we fed rabbits a physiologically relevant atherogenic diet for 4 weeks. The diet rabbits significantly impaired acetylcholine-dependent relaxation compared with rabbits on a normal chow diet (75.1±1.8% vs 97.7±1.0%, *p* 0.001, Figure 6.5).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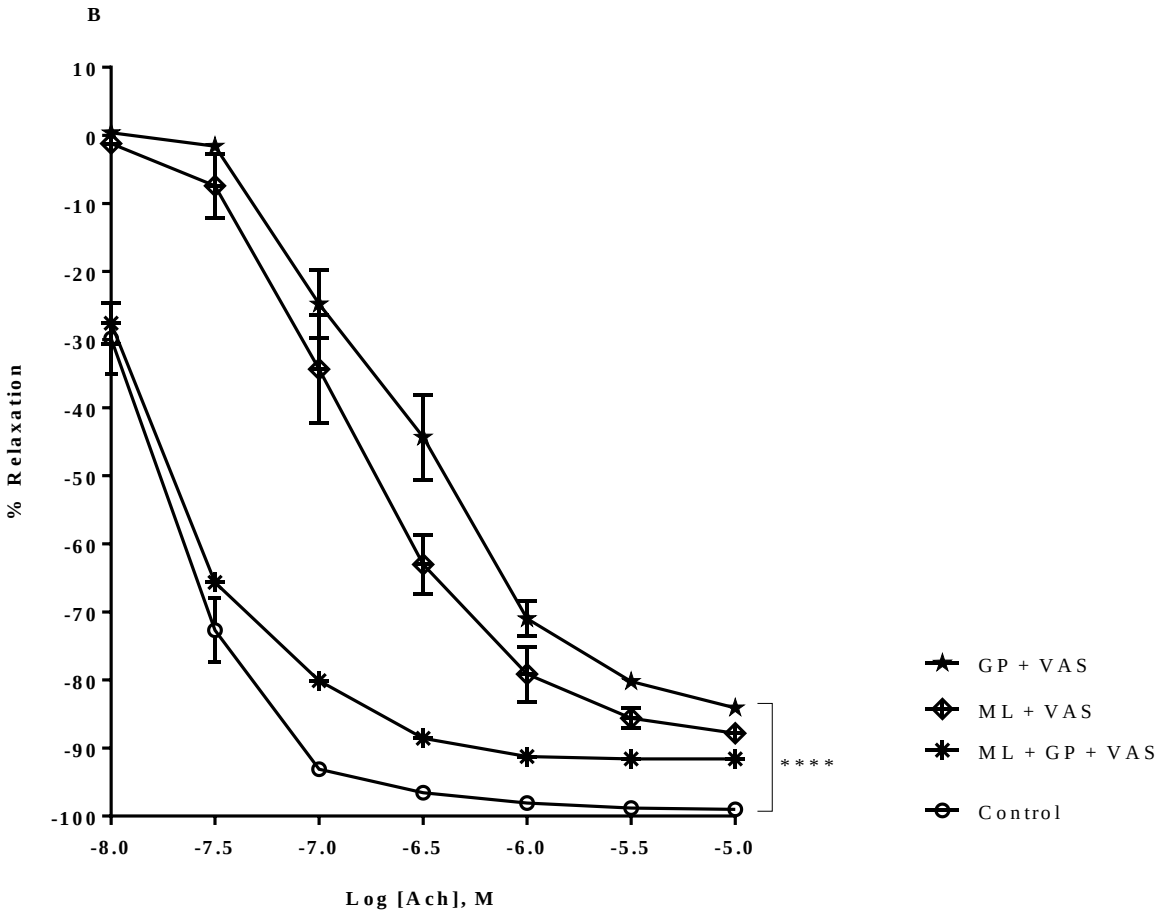
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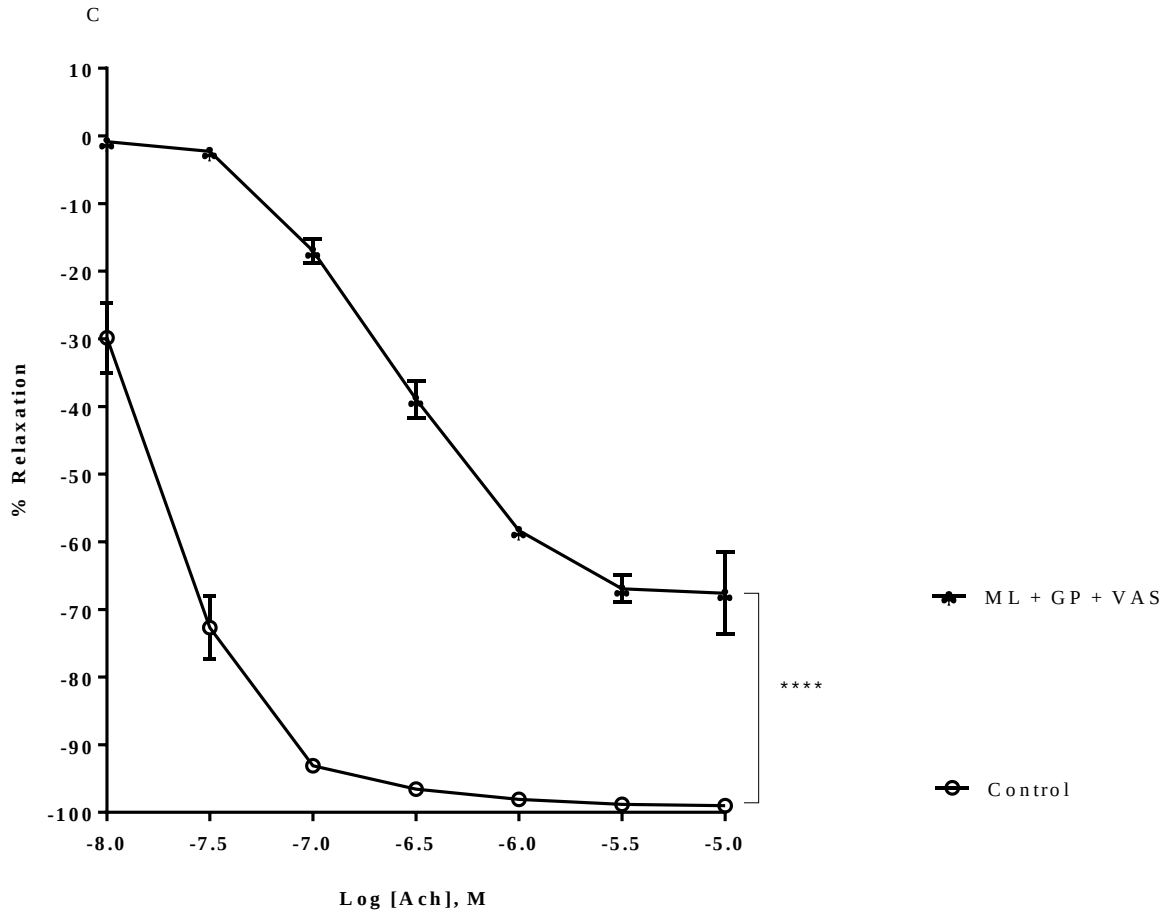
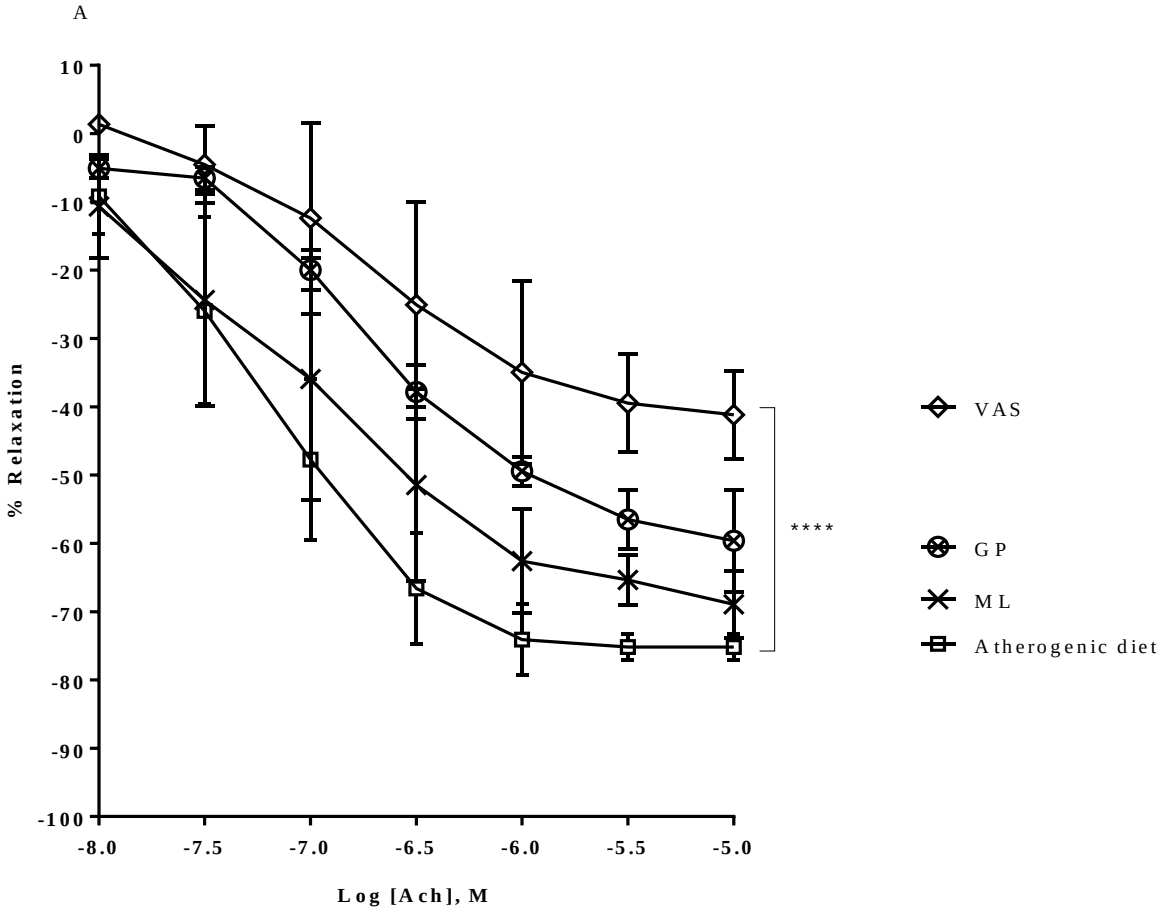
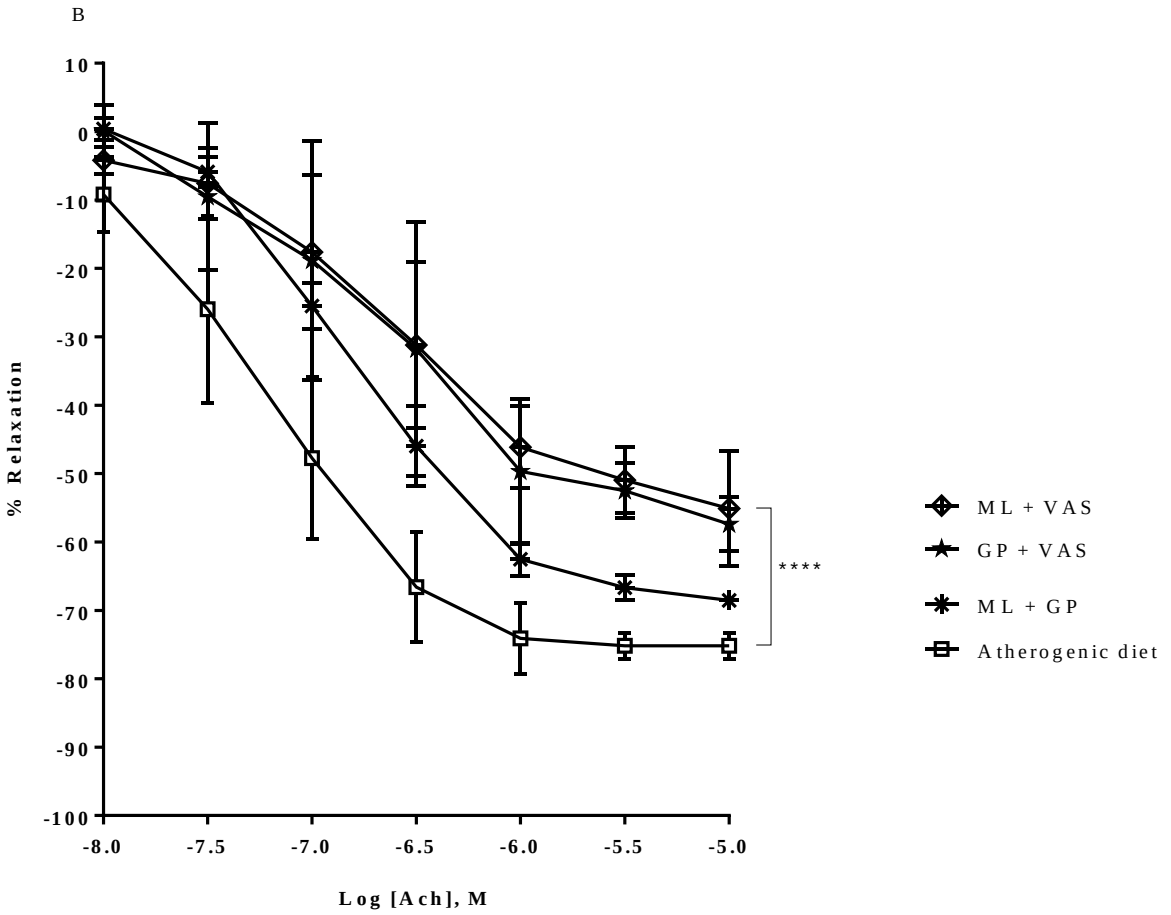


Figure 6.6A. (single Nox inhibition in chow diet), B (double Nox inhibition in chow diet) and C (triple Nox inhibition in chow diet): Nox1, 2 and 4 inhibition resulted in impaired vasorelaxation in normal diet control rabbits. Results are mean ± SEM (n=3 per group), ** *p* 0.0001. All values compared using Sidak's Multiple Comparisons Test.** To assess the effect of Nox inhibition in healthy rabbit vessels, several Nox inhibitors were employed. We used ML090 to inhibit Nox1, which reduced function compared to control (18.2±9.1% vs 72.6±4.7%, *p* 0.0001). Nox2 inhibition with gp91ds-tat reduced function (32.9±14.1% vs 93.1±0.8%, *p* 0.0001) and Nox4 inhibition with VAS2870 also reduced function (28.3±5.6% vs 93.0±0.8%, *p* 0.0001) (Figure 6.6A). Double combinations of Nox inhibitors also significantly impaired function compared with control: ML090 + gp91ds-tat (80.1±0.4% vs 93.1±0.8, *p* 0.01), ML090 + VAS2870 (63.0±4.3% vs 96.5±0.6%, *p* 0.0001) and gp91ds-tat +

VAS2870 (44.3±6.2% vs 96.5±0.6%, *p* 0.0001, Figure 6.6B). To assess the effect of broad Nox inhibition in this model, we used all three Nox inhibitors together, which reduced function compared with control (67.6±6.0% vs. 99.0±0.1%, *p* 0.0001, Figure 6.6C).

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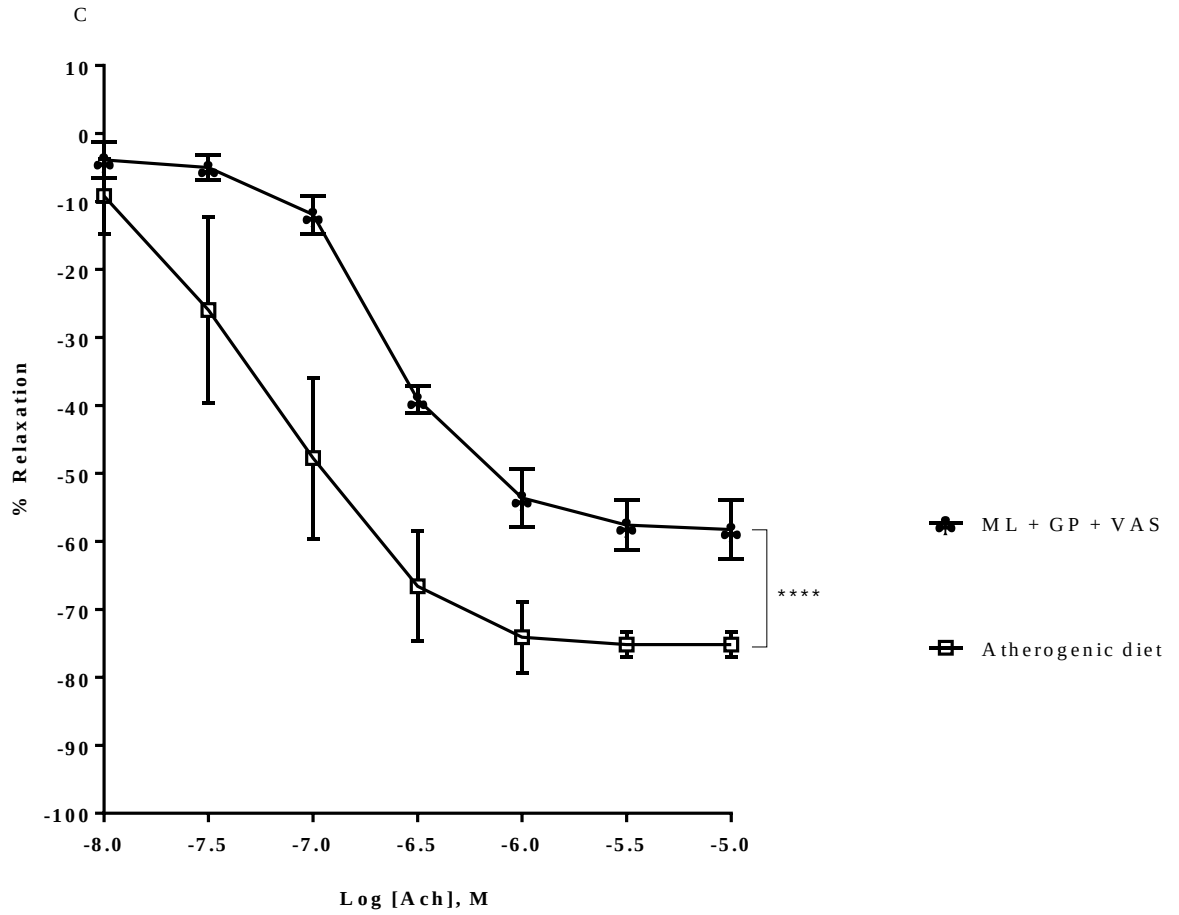
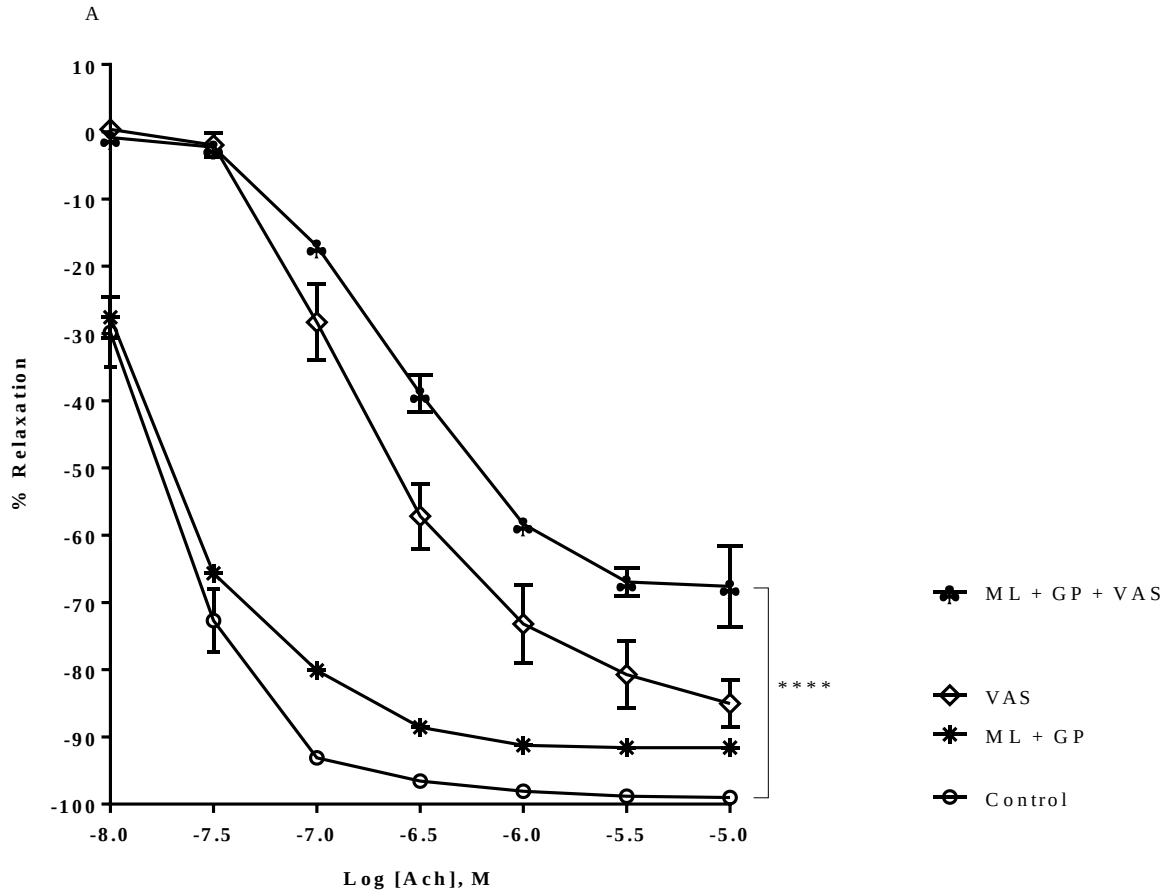


Figure 6.7A (single Nox inhibition in atherogenic diet), B (double Nox inhibition in atherogenic diet) and C (triple Nox inhibition in atherogenic diet): Nox1, 2 and 4 inhibition significantly impaired vasorelaxation in atherogenic diet-fed rabbits. Results are mean ± SEM (n=3 per group), ** *p* 0.01, * *p* 0.001, **** *p* 0.0001. All values compared using Sidak's Multiple Comparisons Test.** To assess the effect of Nox inhibition in diet-induced dysfunction in rabbits, several Nox inhibitors were employed. We used ML090 to inhibit Nox1; in contrast to our previous report on acute HcyT (Smith, R et al. 2015), ML090 alone failed to have an impact on the vessel in this model; in combination with both other inhibitors it is unclear if it contributed to an impairing effect on relaxation (Figures 6.7B, 6.7C). In contrast, Nox2 inhibition with gp91ds-tat significantly impaired function compared with control (37.8±3.9% vs 66.6±8.0%, *p* 0.001, Figure 6.7A) as did Nox4 inhibition with VAS2870

(34.9±13.4% vs. 74.0±5.2%, *p* 0.0001, Figure 6.7A). Double combinations of Nox inhibitors also significantly impaired function compared with control: ML090 + gp91ds-tat (25.5±3.3% vs 47.7±11.8, *p* 0.01, Figure 6.7C), ML090 + VAS2870 (31.2±12.1% vs 66.6±8.0%, *p* 0.0001, Figure 6.7C) and gp91ds-tat + VAS2870 (31.7±18.6% vs 66.6±8.0%, *p* 0.0001, Figure 6.7B). To assess the effect of broad Nox inhibition in this model, we used all three Nox inhibitors together, which failed to improve relaxation compared with control (11.9±2.8% vs. 47.7±11.8%, *p* 0.0001, Figure 6.7C).



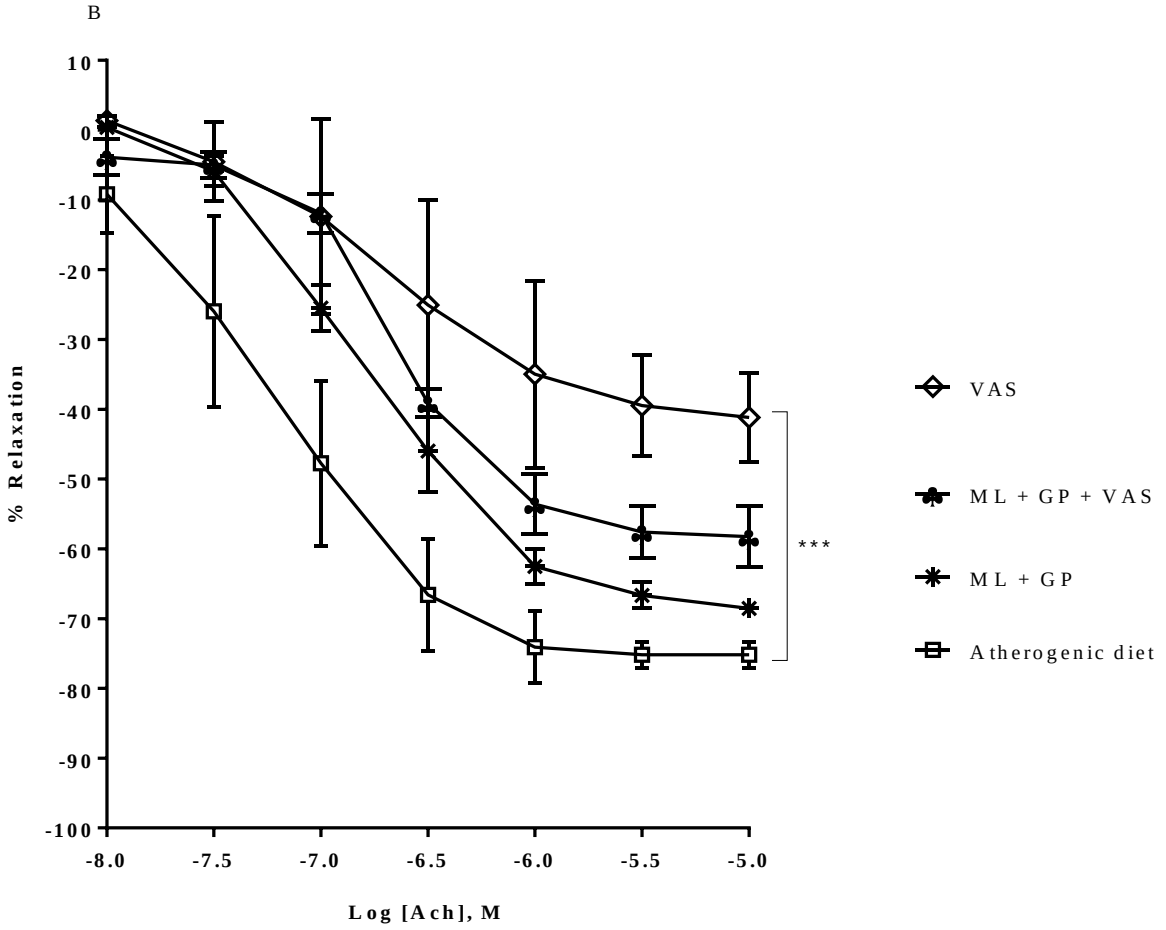
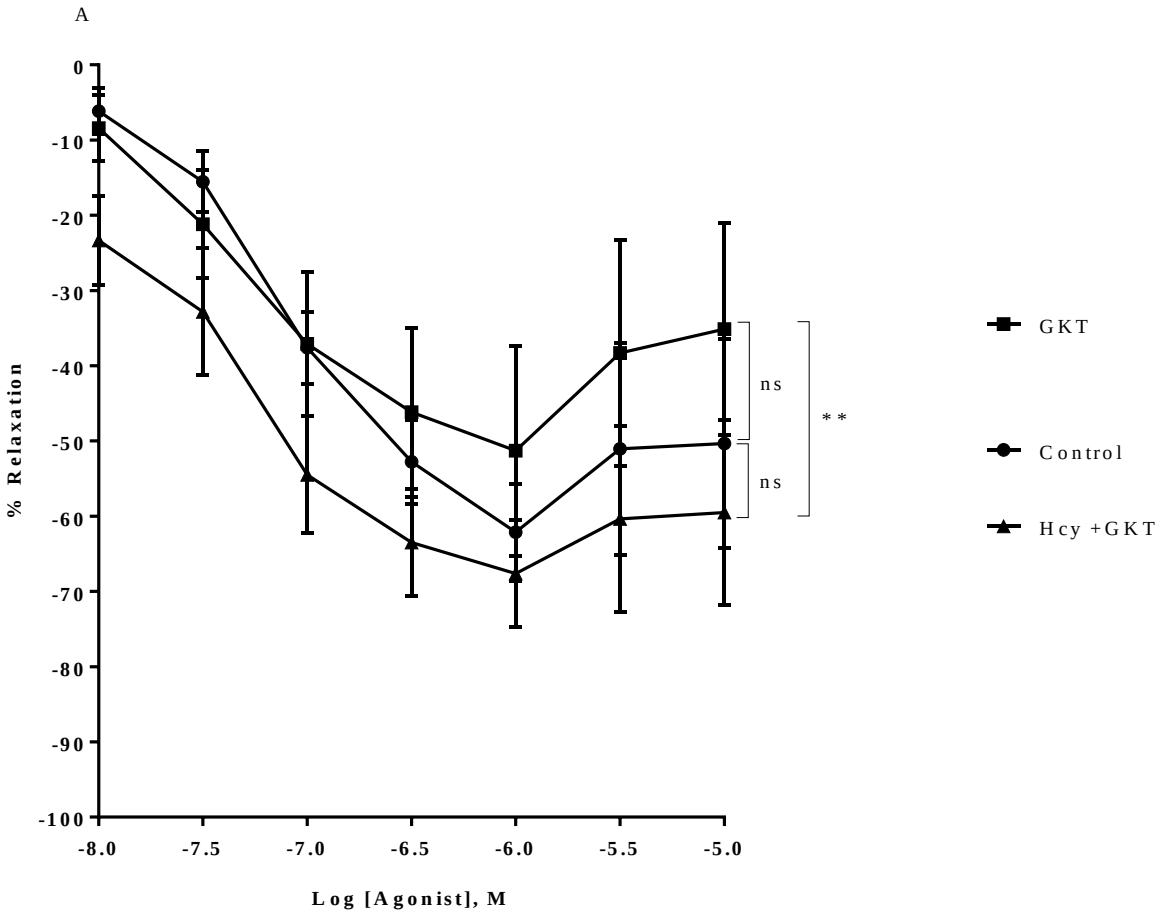


Figure 6.8A (chow diet) and B (atherogenic diet): Combined Nox1 + 2 and Nox1, 2 + 4 inhibition was not as detrimental to vascular function as single Nox4 inhibition with VAS2870 in both diet-fed and healthy rabbit vessels. Results are mean ± SEM (n=3 per group), * *p* 0.05, ** *p* 0.01, * *p* 0.001, **** *p* 0.0001.** All values compared using Sidak's Multiple Comparisons Test. The results indicate that there is an important role for Nox4 in endothelial-dependent relaxation, in both healthy and atherogenic rabbit vessels. In healthy vessels, compared to VAS2870, the combination of ML090 + gp91ds-tat improved function (85.0±3.5% vs 91.5±0.9%, *p* 0.0001). In contrast, the triple combination worsened function (85.0±3.5% vs 67.6±6.0%, *p* 0.05). In atherogenic vessels, VAS2870 alone was improved by both of these combinations; ML090 + gp91ds-tat (68.5±1.1% vs 41.1±6.3%, *p* 0.001) and triple Nox inhibition: (58.2±4.3% vs 41.1±6.3%, *p* 0.05).


A
0
-10
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GKT
Control
Hcy +GKT

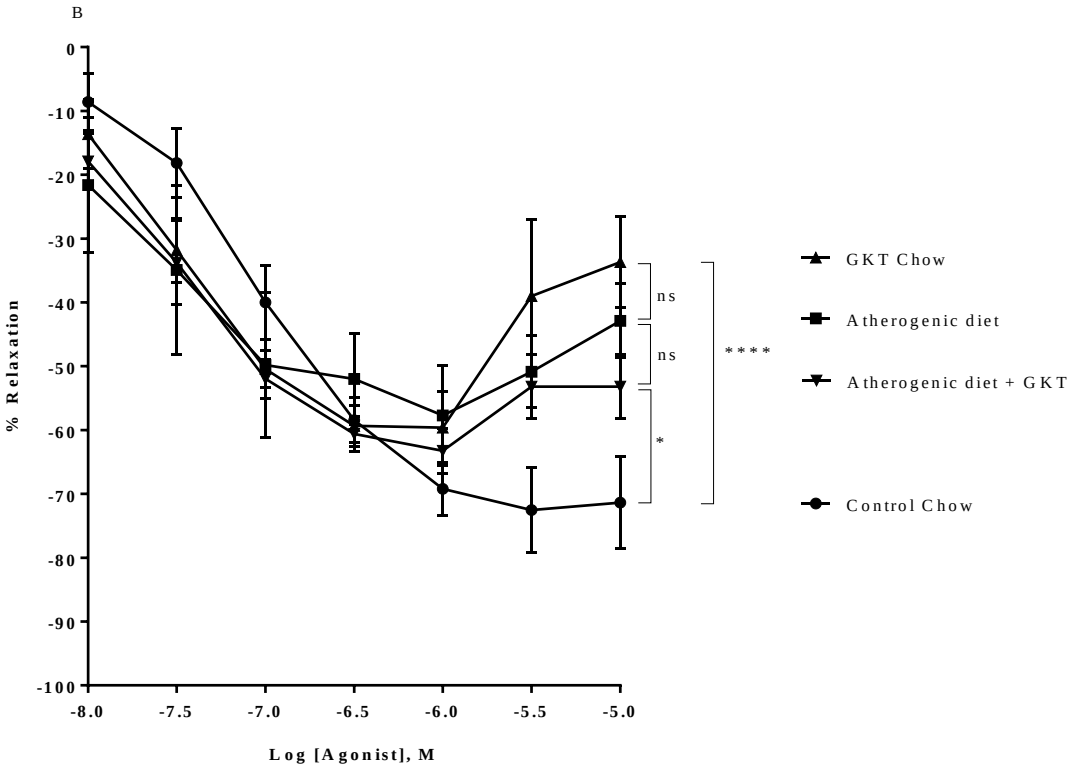


Figure 6.9: Combined Nox1+4 inhibition using the specific Nox1+4 inhibitor GKT was able to rescue function in acute 3mM HHcy fed normal chow (A, n=5) but not in an atherogenic model of HHcy (B, n=3). Results are mean ± SEM, ** *p* 0.002, ** *p* 0.0001. Significance was accepted at $p < 0.05$.**

6.14 Discussion

The major finding of this study is that in a physiologically relevant atherogenic diet in rabbits, single or combined pharmacological Nox1, 2 and 4 inhibition reduces the acetylcholine-dependent endothelial responses.

First, the 4 week dietary intervention worsened acetylcholine-dependent aortic relaxation compared to a normal diet (Figure 6.5). This further supports an established hypothesis that feeding rabbits a methionine and cholesterol-rich diet induces endothelial dysfunction (Rai, Hare & Zulli 2009; Zulli & Hare 2009; Zulli et al. 2003). We suggest that the dysfunction

induces by this diet is influenced by the increased ROS generated by the increase in methionine in the diet. There is evidence that methionine in proteins acts as a ROS target and presence of methionine offer protection from oxidative stress over cells with reduced methionine (Luo & Levine 2009). Further, targeted methionine by ROS might protect residues that would otherwise be targeted by oxidants, acting effectively as an antioxidant (Luo & Levine 2009). Thus, excess methionine in the diet may have caused an upregulation of

Interestingly, there is evidence that cholesterol itself is a direct activator of Nox-derived O_2^- (Masoud, Bizouarn & Houée-Levin 2014) and can therefore influence the amount of available ROS. In the atherogenic diet used in this study, it's possible that the increase of cholesterol lead to protective levels of ROS in the vehicle, where there was no Nox inhibition (Figure 6.6A).

In healthy rabbit vessels, singular inhibition of Nox2 and Nox4 resulted in significantly worsened function, whereas singular Nox1 inhibition also had a significant effect but only in the early doses of ACh (Figure 6.6A). In contrast, in atherogenic rabbit vessels, Nox2 and Nox4 inhibition worsened function and we found no significant difference between Nox1 inhibition and controls (Figure 6.7A). This suggests that the ROS produced by Nox1, 2 and 4 is important for endothelial-dependent relaxation and that Nox1 expression might be comparatively less than Nox2 and Nox4 in the rabbit aorta and therefore not as involved in endothelial-dependent relaxation.

Nox1 is highly expressed in colon epithelial cells and an important contributor of ROS in that region and ML090 is reported as a selective Nox1 inhibitor (Brown SJ et al. 2010). In the endothelial and medial vascular smooth muscle layer, Nox1 is present in low levels and has been shown to increase in mice models of hypertension and atherosclerosis and rat models of diabetes (Rivera et al. 2010). Hcy has been shown to upregulate the expression of Nox1 and

Nox2 levels in osteocytes (Takeno et al. 2015) and in methionine-fed rats, Hcy increased the expression of the essential Nox conformational subunit $p22^{phox}$ (Becker et al. 2005), suggesting a role for HHcy in Nox-driven ROS overproduction; there remains a lack of evidence showing this in the aorta. Our results suggest that an absence of effect by ML090 could be explained by its low expression (Figure 6.7A). Interestingly, our lab showed that ML090 was able to block the effect of acute HcyT-induced dysfunction in rabbits (Smith, R et al. 2015), which suggests that Nox1 might be able to produce ROS in an acute homeostatic mechanism to maintain signalling, but given the results herein it's likely that this effect is short-term.

Inhibition of Nox2 by gp91ds-tat significantly reduced function in the atherogenic model (Figure 6.7A), suggesting an important role for O_2^- in maintenance of vascular function in chronic Hcy-induced dysfunction. Interestingly, Nox2 inhibition in the healthy vessels also impacted on function (Figure 6.6A), again implying that Nox2-generated ROS is also highly important for healthy vascular function via its signalling role. Nox2 is highly expressed throughout the vasculature (Rivera et al. 2010); we used the gp91ds-tat peptide to inhibit Nox2 (Rey et al. 2001). Studies have shown that Nox2 inhibition to be beneficial in pathological states such as atherosclerosis (Quesada et al. 2015), and overexpression is purported to increase cardiac fibrosis and systolic dysfunction (Murdoch et al. 2014). However, there are also reports of Nox2 overexpression being beneficial, especially in adaptation to stressors (Shafique et al. 2013). For example, Shafique et al. (2013) reported that Nox2 overexpression increased ROS which in turn increased autophagy; in the atherogenic model, the lack of Nox2 might have had the exact opposite effect thereby creating an environment with too many dysfunctional cells. This mechanism could explain the detrimental effect on function in the atherogenic model when we inhibited Nox4 with VAS (Figure 6.7A). Importantly, this implies an essential role for H_2O_2. H_2O_2 has been shown to be protective in the endothelial layer by participating in a

concomitant decrease in apoptosis and maintenance of the pathways that result in relaxation (Tuo et al. 2004); the same effect could be occurring here.

Double combinations of Nox inhibitors also worsened relaxation, in both healthy and atherogenic vessels (Figures 6.6B and 6.6B). These results point to an interaction between the three Nox and the ROS in maintaining tone in chronic dysfunction. Triple Nox1, 2 and 4 inhibition resulted in significantly reduced function in both healthy vessels (Figure 6.6C) and the atherogenic model (Figure 6.7C). These results further support the proposed necessary role for Nox-generated ROS in normal function and chronic endothelial dysfunction. Despite triple Nox inhibition in the healthy vessels being less severe than the atherogenic model, the results suggest that removal of ROS in either condition is unlikely to be beneficial. To our knowledge, this is the first study of its kind, therefore there is no published evidence to explain why inhibiting these Nox in combination would have different effects. We speculate that there is a basal level of ROS that is important for function that upregulates in the remaining Nox when the others are inhibited. Interestingly, there was a significant difference between singular Nox4 inhibition and some of the combined inhibitors, namely Nox1 + 2 and triple Nox inhibition (Figure 6.8A and 6.8B), where these combined inhibitors actually improved function when compared to Nox4 inhibition alone. Nox4, and its product H_2O_2, might have upregulated when the other Nox were inhibited, which could explain why singular Nox4 inhibition was more detrimental than the other inhibitors. It is, however, more difficult to explain why triple Nox inhibition should also see improvement compared to VAS alone. We suggest that in these results, the absence of H_2O_2 appears to be important for the correct functioning of the vessel and indeed its presence in the combinations could explain the lessened reduction in function when compared to single Nox inhibition. In addition to combining the separate Nox1, 2 and 4 inhibitors, we were able to test the novel, specific Nox1+4 inhibitor GKT. In our acute 3mM model of HHcy, we found that in the vessels treated with Hcy, GKT was able to reduce

impairment almost to control levels (Figure 6.9A). In contrast, we found that GKT was not able to rescue the dysfunction caused by an atherogenic diet (Figure 6.9B). The reasons for these results remain unclear, however, like the combination Nox inhibitors, they also point to an imbalance to ROS homeostasis. More work is required for the underlysing mechanisms of GKT to become better understood.

6.15 Limitations

Current Nox inhibitors available tend to have non-specific effects (Bedard & Krause 2007b; Heumuller et al. 2008) that are not yet fully explained (Smith, R et al. 2015). For example, VAS2870 is described herein as a non-specific Nox4 inhibitor (Drummond et al. 2011; Sun et al. 2012). Whilst this description is apt, our results show that the pharmacological Nox inhibitors employed do interact with their targeted enzymes differently, therefore they cannot be fully discounted. Further, *in vitro* modelling might not translate to the human response.

6.16 Conclusion

There is evidence to suggest that Nox1, 2 and 4 are integral to the maintenance of proper vascular function, both in healthy and chronic disease models, therefore we would propose that inhibition of these enzymes are contraindicated in rabbit models of Hcy-induced endothelial dysfunction.

Chapter 7

7.1 The Benefit of GP91DS-TAT in Improving Homocysteine-Induced Impaired Acetylcholine-Mediated Vasorelaxation is Dependent on Concentration

7.2 Abstract

Introduction: Increased plasma homocysteine (hyperhomocysteinemia; HHcy) is an independent risk factor for coronary artery disease (CAD) and pharmacological treatments have not been identified. NADPH oxidases (Nox) are essential signalling enzymes, but can increase reactive oxygen species (ROS) if dysregulated. HHcy can increase ROS, which can impair nitric oxide (NO) release, an essential factor in vasorelaxation. We have previously demonstrated that gp91ds-tat (GP; 10^{-6} M) is detrimental in acute Hcy-induced impaired acetylcholine-mediated vasorelaxation.

Objectives: To determine if different concentrations of GP (10^{-6} - 10^{-12} M) prevents Hcy-induced reductions in acetylcholine-mediated relaxation, in both acute and chronic models of HHcy-impaired function in rabbits.

Methods: Male New Zealand White rabbits were fed either chow (n=16) or atherogenic diet (5% Peanut Oil, 0.5% cholesterol and 1% methionine) for 4 weeks (n=16) (VUAEC#14/005). The aortae were excised, cut into 2-3 mm rings and mounted in organ baths. In chow fed rabbits, rings were incubated with GP (10^{-12} - 10^{-6} M) for 30 minutes and further incubated with 3mM Hcy for 1 hour; rings from atherogenic-fed rabbits were incubated with GP only. All rings were then pharmacologically pre-contracted with phenylephrine (Phen; $3x10^{-7}$ M) and subjected to dose response relaxation to acetylcholine (ACh; 10^{-8} - 10^{-5} M, half log units).

Results: In rabbit aortic rings, 3mM Hcy incubation reduced acetylcholine-mediated relaxation in the aorta vs. control (no treatment; 62.0±5.7 vs. 84.5±2.7, $p < 0.0001$, n=7 for each dose).

In acute Hcy, only incubation with GP at 10^{-8} M was able to improve function (27.7±16 vs. 63.2±3.4 $p < 0.02$, n=8). In the atherogenic rabbit model, GP at doses 10^{-7} (87.1±1.6) and 10^{-9} (86.8±1.4) were able to improve function compared with the control (74±2.4, $p < 0.0008$, and 0.001, respectively; n=11 for each dose).

Conclusion: At higher doses, GP worsens acute Hcy-induced impaired acetylcholine-mediated vascular relaxation in rabbit aorta. However, at lower doses, the effect is protective in both acute and chronic HHcy, suggesting that small, physiologically relevant doses of GP might be a useful treatment strategy in HHcy-impaired acetylcholine-mediated vasorelaxation.

7.3 Background

Cardiovascular disease (CVD) continues to be a challenging public health concern, responsible for approximately 70% of deaths worldwide (Organization 2017). Among the classical risk factors for CVD is excess plasma homocysteine (Hcy) (Steed & Tyagi 2011), a sulphur-containing amino-acid by-product of methionine metabolism (McCully Kilmer 2005). Increased plasma Hcy (hyperhomocysteinemia; HHcy) is associated with increased risk of atherogenesis and thrombosis (Racek et al. 2005). HHcy is classified as mild (10-30 μM), moderate (31-100 μM) and severe (> 100 μM) (Beard, Reynolds & Bearden 2012); in chronic kidney disease (CKD), levels have reported HHcy as high as 300 μM (Beilby, J. & Rossi 2000). Crucially, HHcy has been shown to participate in the development and acceleration of atherosclerosis (Jiang et al. 2012; Julve et al. 2013; Zulli & Hare 2009) and involved in impairing acetylcholine-mediated vasorelaxation (Jia et al. 2011; Song et al. 2015; Wu et al. 2015; Zulli et al. 2003). Preventing CVD is an important strategy in reducing economic and social burden (Hu, F, Liu & Willett 2011; Weintraub et al. 2011), and thus research is focused on finding ways to restore endothelial function (Gu et al. 2013; Santos-Parker, LaRocca & Seals 2014; Vasdev, Stuckless & Richardson 2011) as treatment for HHcy is still to be determined. Understanding the role the B vitamins play in methionine metabolism (Castro et al. 2006) has justified their application in several clinical trials aimed at reducing HHcy plasma levels (Clarke, Robert et al. 2014; Lonn et al. 2006; Saposnik et al. 2009). However, the results of these trials have been disappointing and unable to deliver a treatment strategy to reduce HHcy.

The nicotinamide adenosine diphosphate (NADPH) oxidases family (Nox1-5, Duox1, 2) (Bedard & Krause 2007b) are trans-membranous enzymes that produce functional reactive oxygen species (ROS) essential for cell signalling and proper endothelial function (Bedard & Krause 2007b; Rivera et al. 2010). Of these, Nox2 is the most-widely studied, especially in

the context of vascular function (Drummond & Sobey 2014), and produces O_2^- as its main product (Selemidis et al. 2008). ROS from Nox, especially O_2^-, have been identified as important factors in both atherosclerosis development (Douglas et al. 2012) and reduced vascular function (Konior et al. 2013). Increased $O2^-$ has also been reported in HHcy (Becker et al. 2005) and HHcy could be involved in promoting cellular damage via Nox2 (Zou, CG et al. 2009). Further, Hcy has been reported to stimulate the Nox2 subunit p47phox and increase apoptotic ROS production in rats (Sipkens et al. 2011).

The putative Nox2 inhibitor gp91ds-tat (GP) is an 18 amino-acid peptide that accesses the cell via its TAT tail and inhibits Nox2 by blocking the assembly of $p47^{phox}$ and $gp91^{phox}$ (Rastogi et al. 2016). Previously, we have shown that GP, in combination with other Nox inhibitors, is involved in worsening acetylcholine-mediated vasorelaxation impaired by Hcy thiolactone (the atherogenic form of Hcy (Jakubowski 2000)) (Smith, R et al. 2015)(also Nox2 and Nox4 paper once published). This finding is not supported by most studies assessing genetic or pharmacological Nox2 inhibition in vascular disease, where GP and/or Nox2 knock-outs generally improve vascular function in different models of dysfunction (Bendall et al. 2002; Deng et al. 2012; Guemez-Gamboa et al. 2011; Judkins et al. 2010; Qian, C et al. 2016). Given that differences across animal species and genetics can result in variations to vascular disease susceptibility (Van Craeyveld et al. 2012), our aim was to test the efficacy of GP across a range of doses in an established rabbit model of HHcy-impaired acetylcholine-mediated vasorelaxation (Zulli & Hare 2009),

7.4 Methods

All handling of animals and experiments were carried out according to the National Health and Medical Research Council "Australian Code of Practice for the Care and Use of Animals for Scientific Purposes" (8th Ed. 2013) and approved under Victoria University's Animal Ethics

Committee (VUAEC) #14/005. Acetylcholine, homocysteine, and phenylephrine were purchased from Sigma-Aldrich, (Merck), Darmstadt, Germany. Gp91ds-tat was purchased from Australian Biosearch, Perth, WA, Australia. Special rabbit chow was purchased from Specialty Feeds (Glen Forrest Stockfeeders), Perth, WA, Australia.

Animal Procedure

New Zealand rabbits (male, 12-18 weeks, n=24, VUAEC #14/005) were housed in separate cages, maintained at a constant temperature of approximately 23°C and provided water and food *ad libidum*. Whilst in our care, 12 rabbits were fed a normal chow diet and the other 12 fed an atherogenic diet (SF00-218 Rabbit Diet: 5% Peanut Oil, 0.5% cholesterol and 1% methionine) for 4 weeks (Rai, Hare & Zulli 2009). The rabbit was securely wrapped in a towel and was anaesthetised with a subcutaneous scruff injection of medetomidine (0.25mg/kg) then left to rest, caged and covered, for 10 minutes. Once the animal appeared languid and docile, it was then moved to a chamber attached to circulatory gas system and subjected to further anaesthetic (isoflurane + 4% O_2) for 10 minutes. Once the animal was unable to right itself upon being turned over (chamber tipped gently), it was removed, placed on its back with its head placed back so that the oesophagus was not occluded and a mask containing the same anaesthetic mixture was held over its face for 5 mins. After 5 mins, the reflexes (pinching into space between toes, edge of ear, touching eye with a gloved finger) were tested and if no response, the rabbit was exsanguinated via the lower vena cava for a minimum of 3 minutes. Once life signs were not exhibited by the rabbit (fully dilate iris, no reflexive responses), the diaphragm was cut and the mask removed.

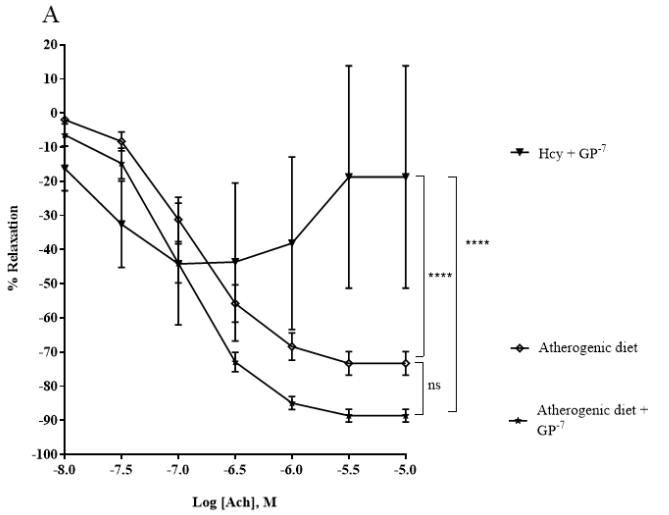
Isometric Tension Studies

The aortae were cleaned of fat and connective tissue and placed in cold (on ice) Krebs solution. The abdominal section of the aorta was cut into rings (2-3 mm lengths) and placed in organ

baths (OB8, Zultek Engineering, Australia), filled with Krebs, kept at a constant temperature of 37°C and continuously bubbled with carbogen (95% O_2 + 5% CO_2). These rings were left to rest, unmounted, for 30 minutes. Rings were then mounted between two metal hooks attached to force displacement transducers, stretched to 2g and allowed to reach resting tension plateau (30 minutes). Rings were then re-stretched and allowed to reach resting tension plateau a second time (20 minutes). Rings were incubated with the GP (10^{-6} - 10^{-12} M (Rey et al. 2001)) for a total of 1 hour 30 minutes. Some rings from chow-fed rabbits were also incubated with 3mM Hcy for 1 hour. Control rings had neither Hcy nor Nox2 inhibitor added. To assess acetylcholine mediated relaxation (ACh; 10^{-8} - 10^{-5} M (Zulli & Hare 2009)), rings were pre-contracted with phenylephrine (Phen; $3x10^{-7}$ M (Zulli & Hare 2009)). After the contraction reached a plateau, a concentration-response curve to ACh-mediated vasorelaxation was performed (10^{-8} - 10^{-5} M, half log units).

Statistical Analysis: Isometric tension data were analysed using a two-way repeated measures ANOVA, measuring differences in response to drug dose between and within groups, followed by Sidak's Multiple Comparisons Test. Control values for each vasorelaxation graph were all pooled. All data were analysed using GraphPad Prism (version 7.01 for Windows, GraphPad Software, La Jolla, California USA). Data are represented as mean ± SEM. Significance was accepted at $p < 0.05$.

7.5 Results


A
% Relaxation
Log [Ach], M
Hcy + GP-7
Atherogenic diet
Atherogenic diet + GP-7
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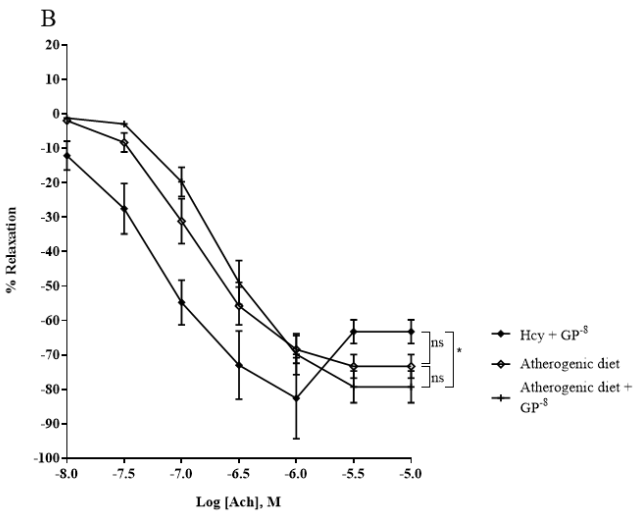
B
% Relaxation
Log [Ach], M
Hcy + GP-8
Atherogenic diet
Atherogenic diet + GP-8
ns
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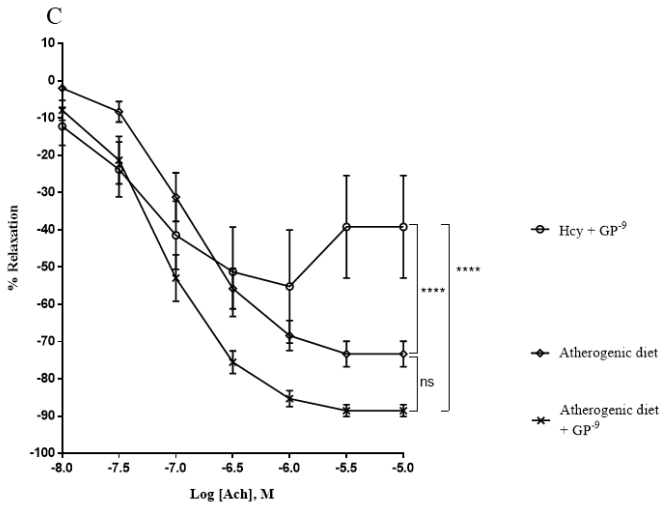
C
% Relaxation
Log [Ach], M
Hcy + GP-9
Atherogenic diet
Atherogenic diet + GP-9
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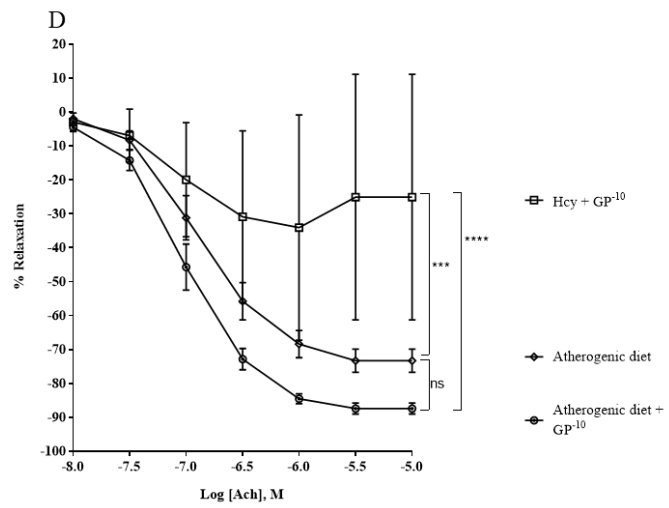
D
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Log [Ach], M
Hcy + GP-10
Atherogenic diet
Atherogenic diet + GP-10
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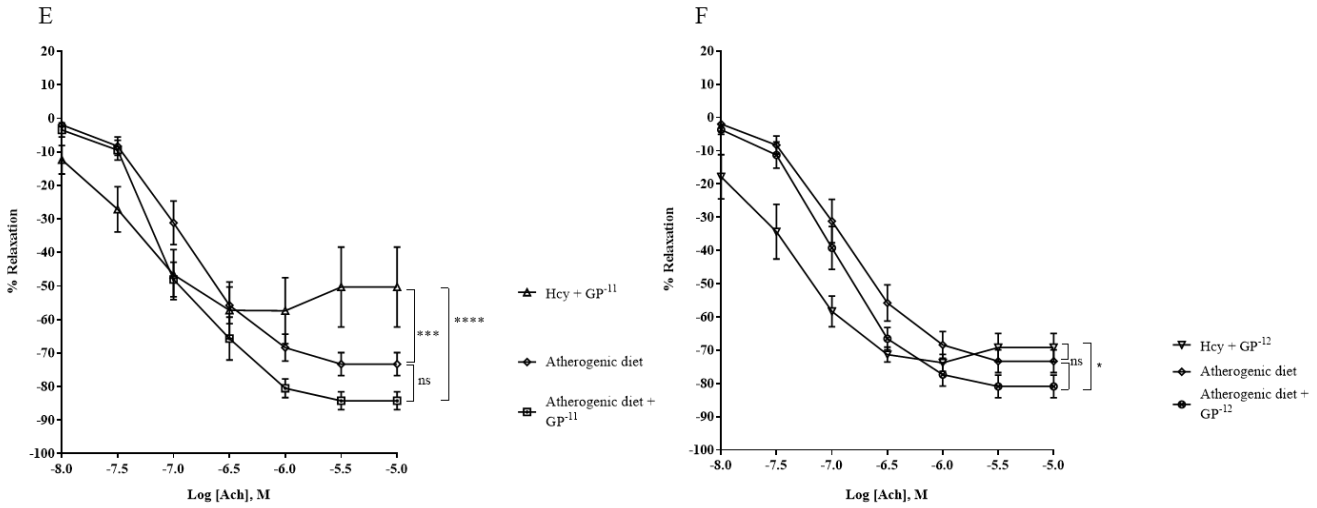
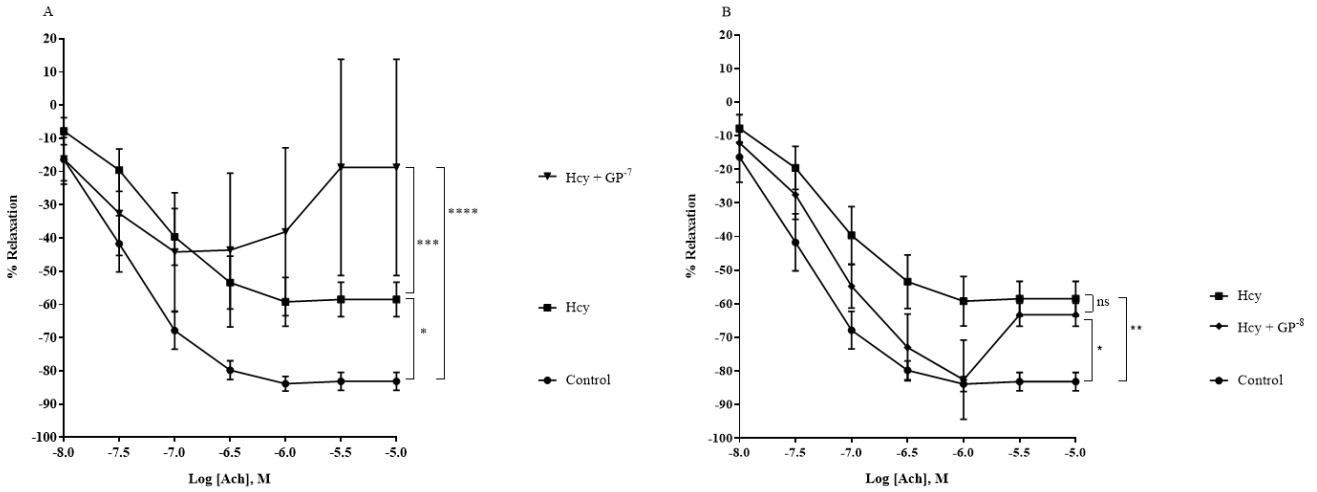


Figure 7.1A-F. In acute 3mM Hcy, GP at different doses (10^{-7} – 10^{-12} µM) worsens HHcy-induced vascular dysfunction. Data are presented as mean ± SEM (n=7 for Hcy and controls; n=8 for GP^{-8}; n=11 for $GP^{-7,9-12}$. * = 0.04; * = 0.004; **** = 0.0001; ns = no significance. Significance was accepted at $p < 0.05$.**



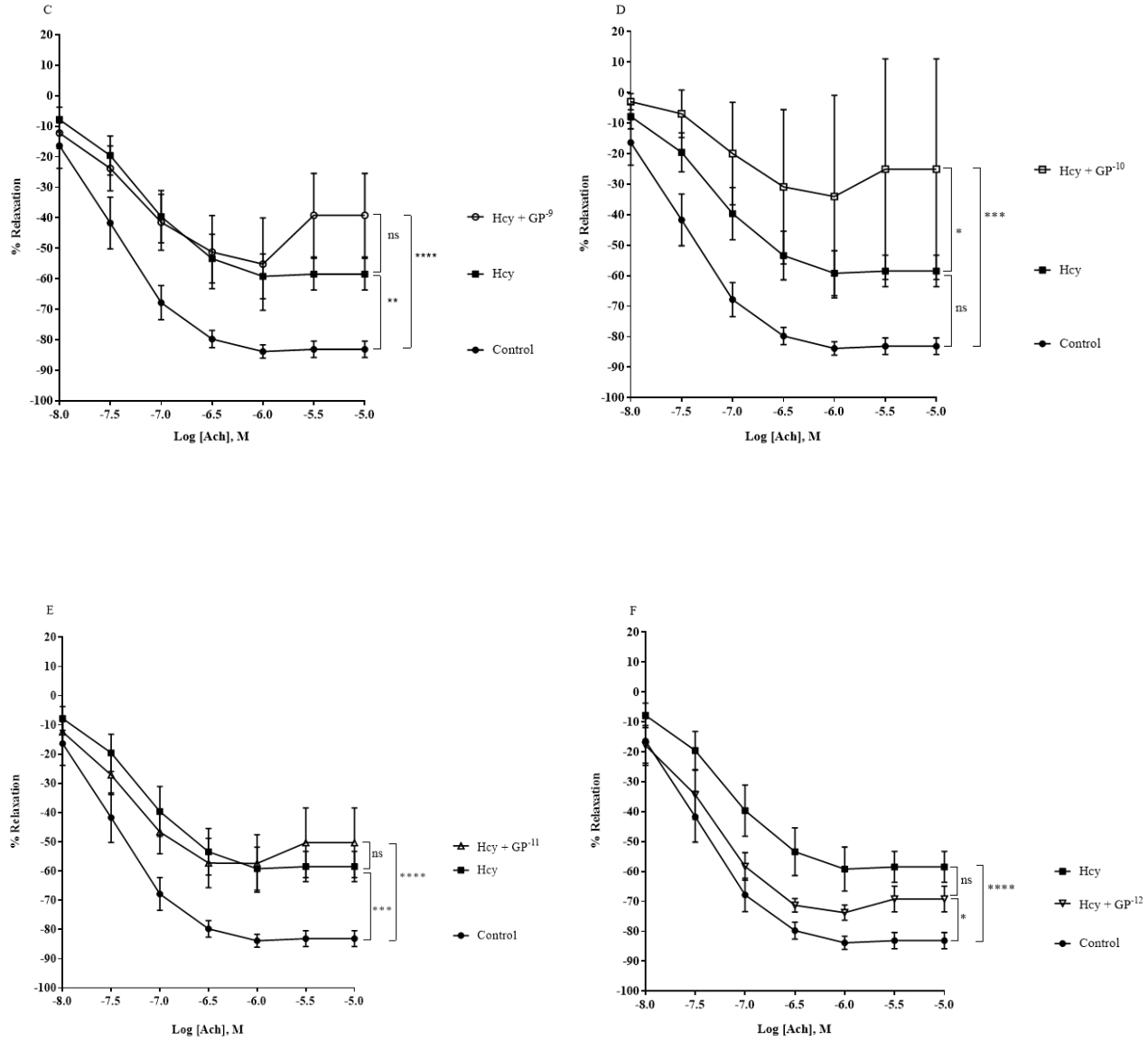


Figure 7.2A-F. In an atherogenic model of HHcy, GP at multiple doses 10^{-7} – 10^{-12} µM worsens acetylcholine-dependent vasorelaxation. Data are represented as mean ± SEM (n=7 for Hcy and controls; n=8 for GP^{-8}; n=11 for $GP^{-7,9-12}$. * = 0.02; * = 0.001; **** = 0.0001; ns = no significance. Significance was accepted at $p < 0.05$.**

7.6 Discussion

In this study, we provide direct evidence that GP improves *ex vivo* acetylcholine-mediated vasorelaxation in both acute and chronic models of HHcy in a dose-dependent manner.

We have shown previously that incubation with putative Nox2 inhibitors GP, apocynin and PhoxI2 (all 10^{-6} M), are detrimental in HHcy-impaired acetylcholine-mediated vasorelaxation (Smith, R et al. 2015), despite their structural differences, suggesting that the dose, not the drug structure, determines these drug's efficacy in Nox2 inhibition. The use of 3mM Hcy to induce impaired-ACh-mediated vasorelaxation has been previously established by work from our lab (Smith, R et al. 2015).

Initially we used a 10^{-6} M concentration of GP, based on the published literature (Rey et al. 2001). In our previous work, we were unable to replicate the responses with the published dose, therefore we investigated a gradual reduction in the dose to measure improvements to HHcy-impaired acetylcholine-mediated vasorelaxation. To our knowledge, Nox and HHcy has not been studied under these conditions, and so the published dose may have been inappropriate for our model. In the healthy rabbits, we used 3mM Hcy in *ex vivo* tissue to mimic an acute impaired response to ACh. As expected, Hcy reduced ACh-mediated vasorelaxation in healthy controls (Fig. 7.1). In the acute HHcy model, none of the different doses were able to restore function back to control levels (Figure 7.1A-F).

In the atherogenic model, the long-term feeding was to mimic a chronic impairment to ACh-mediated vasorelaxation. We have previously shown this to be an effective model of endothelial dysfunction (Zulli & Hare 2009; Zulli et al. 2004). In this model we found that GP^{-8} was able to slightly improve acetylcholine-mediated vasorelaxation, (Fig. 7.2B).

These results suggest that there is a change in ROS balance in the context of excess plasma Hcy. Future treatment strategies should be focused on ascertaining the efficacy of doses of Nox inhibitors in *in vivo* models of Hcy-impaired acetylcholine-mediated vasorelaxation.

7.7 Limitations

The pharmacological Nox2 inhibitor used in this study, GP, was based on established work (Qian, C et al. 2016; Smith, R et al. 2015), however the specificity of this drug is debatable (Bedard & Krause 2007b) and requires further clarification (Smith, R et al. 2015) as it may have off-target effects.

7.8 Conclusion

In conclusion, the delicate balance of ROS that Nox2 generates to maintain effective signalling appears to be impaired in HHcy disease. It would be worthwhile pursuing treatment strategies that allow for the dosage of Nox inhibitors, especially GP, to be developed in *in vivo* models of HHcy-impaired acetylcholine-mediated vasorelaxation.

Chapter 8

8.1 General Discussion

Summary of Major Findings

This thesis examined several putative pharmacological Nox1, 2 and 4 inhibitors in rabbits and mice, and further investigated Nox2 deletion in mice, under acute HHcy-induced impaired acetylcholine-mediated vasorelaxation and an atherogenic diet. The major observations made in this thesis contribute to the understanding that the dose of Nox inhibitors is vital to the success of treating HHcy. Some of these key observations include:

8.2 Single Nox1, 2 and 4 inhibition worsens HHcy-impaired acetylcholine-mediated vasorelaxation, but this can be partially rescued by activating the Akt pathway or inducing O_2^- generation.

In chapter 3, we assessed the effect of single Nox1, 2 and 4 inhibition in HHcy-induced impaired acetylcholine-mediated vasorelaxation. In both rabbits and mice and using the gold standard to measure acetylcholine-mediated vasorelaxation, isometric tension, we found that the Nox2 inhibitors apocynin, GP and PhoxI2, and the Nox4 inhibitor VAS, in the published dose (10^{-6} M) was not effective at reducing impaired vasodilation, and in fact significantly worsened it. Interestingly, in GP + Hcy treated rings, activating the Akt pathway or inducing O_2^- production, was able to negate this effect. We propose that the basis for these findings indicate the positive role for O_2^- in this model in activating Akt. (1). In support of this, a previous in human umbilical vein endothelial cells (HUVEC), insulin was used as an Akt activator and was able to partially restore endothelial dysfunction attributed to increased plasma Hcy (2). Whilst we did not directly measure Akt phosphorylation, the improved vasodilation is a marker of Akt activation.

8.3 eNOS phosphorylation is reduced in HHcy plus GP incubated tissue.

Semi-quantitative immunohistochemistry was performed on aorta from the isometric tension studies, which is outlined in chapter 4. In order for eNOS to be activated, it requires phosphorylation at Ser1177 and dephosphorylation at Thr495 (3). In tissue incubated with HHcy and GP, we found that the combination of GP and acute Hcy resulted in a loss of phosphorylation at Ser1177. Acute Hcy alone caused an upregulation of Ser1177, whereas there was no change to Thr495 dephosphorylation. HHcy-induced changes to eNOS phosphorylation has been reported before in HUVEC, where reduced eNOS phosphorylation at Ser1177 was attributed to increased plasma Hcy (2).

8.4 Combinations of Nox1, 2 and 4 inhibition improves HHcy-impaired acetylcholine-mediated vasorelaxation, whereas iNOS inhibition worsens it.

In chapter 5, we examined the effect of combining the Nox inhibitors. Interestingly, we found that although single Nox inhibition worsened HHcy-induced impaired acetylcholine-mediated vasorelaxation, combinations of the inhibitors improved vasorelaxation. From these results, we inferred that blocking multiple Nox enzymes is required to improve acetylcholine-mediated vasorelaxation. As full relaxation was not restored, we hypothesised that iNOS could be a causative factor. Thus, we assessed the effect of reducing iNOS, an established promotor of atherosclerosis where it has only been found elevated in inflammatory disease and potentially important in the redox balance.

In contrast, we found that when iNOS was inhibited, either alone or in combination with Nox inhibition, acetylcholine-mediated vasorelaxation was significantly reduced. We attribute this impaired vasorelaxation (keeping in mind ‘off target’ effects of 1400W) to a disturbance to the delicate homeostatic redox balance that essential signalling factors that ultimately support.

8.5 An atherogenic or 1% methionine diet worsens acetylcholine-mediated vasorelaxation in rabbits and wild-type (WT) mice, but improves function in a Nox2$^{-/-}$ mouse model.

In chapter 6, to confirm the results of the acute pharmacological HHcy dysfunction, we used an established rabbit and mouse diet model of HHcy. In rabbits fed an atherogenic diet and WT mice fed a 1% methionine diet, single or combined Nox1, 2 and 4 inhibition was not effective at rescuing impaired acetylcholine-mediated vasorelaxation. In contrast, the Nox2$^{-/-}$ mouse had rescued acetylcholine-mediated vasorelaxation, compared with WT mice, suggesting that the delicate redox balance was AChieved in the endothelium. The results here suggest an important role for basal ROS in vasorelaxation.

8.6 The efficacy of GP in HHcy-impaired acetylcholine-impaired vasorelaxation is dependent on the dose.

If basal ROS is important, we hypothesised that improvement in function should be observed by reducing the dose of GP for Nox2 inhibition. Here we used a dose-response curve of 10^{-6} – 10^{-12} M to assess any changes in HHcy-impaired acetylcholine-dependent vasorelaxation. This dose-response curve was carried out in both acute and chronic HHcy in rabbits. We found that in contrast to the higher doses, GP administered in the 10^{-10} – 10^{-12} range was effective at restoring function. We attribute this to the delicate balance of O_2^- that is required at the basal level and suggest that those minimal levels of Nox2-generated ROS are essential for maintaining proper acetylcholine-mediated vasorelaxation.

8.7 Summary

It is established that restoring normal NO bioavailability will reduce the burden of CVD. High plasma homocysteine (a risk factor for CVD) can reduce NO bioavailability via activation of Nox enzymes. Our laboratory is interested in restoring NO bioavailability via inhibition of these Nox enzymes. The choice of using putative Nox inhibitors was based on a myriad of published data indicating a detrimental role for oxidative stress.

A major finding of this study is that singular use of ML090 (Nox1), Gp91ds-tat (Nox2) and VAS2870 (Nox4) use does not improve, and worsens, blood vessel function in normal and pathological aorta. Yet, combination of these drugs do inhibit the effect of homocysteine. As well, it is identified that low dose Gp91ds-tat could be a novel avenue for HHcy treatment. Further, the vasodilatory effect of low-dose Gp91ds-tat provides further evidence that the other Nox inhibitors we employed herein are not 100% specifc for Nox1, 2 and 4 and also supports the argument that basal levels of Nox-derived oxidative stress are essential for maintaining Ach-dependent vasodilation in Hcy-induced endothelial dysfunction.

8.8 Future Directions

The positive results of the GP dose-response curve suggest that a potential return to basal ROS levels may be important for understanding the relationship between Nox2 and HHcy. These results represent a real need to characterise proper treatment regimens in 'partial' knock-out models, which may help to develop a treatment strategy suitable for humans. Additionally, the small but clear improvement in some combinations of Nox1, 2 and 4 inhibitors are an interesting find, and these results support current findings of the novel and specific Nox1/4 inhibitor, GKT. Given the apparent safety of GKT, future treatment strategies in our models of HHcy-induced dysfunction clearly warrants investigating GKT further. Additionally, research going forward should include work on the recently described triple Nox knock-out mouse, which could provide more insight into the pathophysiology of HHcy.

8.9 Concluding Remarks

In conclusion, this thesis has made a number of observations which significantly contribute to our understanding of HHcy and putative Nox inhibitors. The studies herein serve as the foundation to further investigate lower doses of these inhibitors in HHcy induced vascular disease to prevent HHcy induced CVD.

List of Presentations and Manuscripts Arising from this Thesis

Presentation: Korea Society of Lipidology and Atherosclerosis (ICLA), Korea September 2016: The Effect of Nox Inhibition on Homocysteine-induced Endothelial Dysfunction.

Presentation: Victoria University Health and Biomedicine Post Graduate Student Conference 2016: The Effect of Nox Inhibition on Homocysteine-induced Endothelial Dysfunction.

Presentation: Graduate Research Centre (Victoria University) 2017: Nox2, Nox4 Inhibition and HSP72 Activation in Homocysteine-induced Endothelial Dysfunction.

Manuscript accepted 07th May 2019: Putative Nox2 Inhibitors Worsen Homocysteine-Induced Impaired Acetylcholine-Mediated Relaxation, Journal of Nutrition, Metabolism, and Cardiovascular Diseases.

Chapter 9

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