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Doctoral Thesis

The use of the β-blocker Nebivolol and the naturally derived compounds Stevia, Goji berry, and Epicatechin to prevent cardiovascular damage in the DOCA-salt rat model of hypertension

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Declaration of Authorship

I, Douglas Jackson, author of this thesis titled, 'The use of the β-blocker Nebivolol and the naturally derived compounds Stevia, Goji berry, and Epicatechin to prevent cardiovascular damage in the DOCA-salt rat model of hypertension' declare the following in full disclosure:

- The research and discussion presented in this thesis are the original work of the author.
- The research has built upon preliminary work performed for the completion of an undergraduate honours project.
- All of the laboratory work involved in this thesis has been completed during the term of this candidature.
- Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work.
- I have acknowledged all main sources of help.
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"I have no special talents. I am only passionately curious."

Albert Einstein

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Abstract

School of Medical and Applied Sciences

Doctor of Philosophy

The use of the β-blocker Nebivolol and the naturally derived compounds Stevia, Goji berry, and Epicatechin to prevent cardiovascular damage in the DOCA-salt rat model of hypertension

by Douglas Jackson

Oxidative stress is a significant factor in the pathogenesis of the DOCA-salt hypertensive rat, producing significantly diminished cardiovascular function. Treatments targeting oxidative stress have been very effective in this model, providing improvements in oxidative stress, cardiac function, vascular function and also remodelling within these tissues. Many plant derived compounds have intrinsic antioxidant properties, and can achieve very significant effects within disease models of oxidative stress. Extracts from the plants *Stevia rebaudiana* and *Lycium barbarum* are two compounds that exhibit promise in targeting the pathological processes underlying hypertension within the DOCA-salt model. The key finding of the studies within this thesis are that extracts from both of these plants improve electrophysiological remodelling within the DOCA-salt heart. Epicatechin, a compound found in cocoa and green tea, has been shown to reduce blood pressure in hypertensive human patients. However, its ability to provide cardioprotection in hypertension, especially within the DOCA-salt model, had previously not been assessed. This thesis presents evidence that epicatechin was effective in preventing an increase in diastolic stiffness associated with the DOCA-salt model and significantly reduce blood pressure. It is not only natural compounds that provide novel avenues for targeting oxidative stress, but the newer generations of β-blockers have also been shown to affect antioxidant pathways. The 3^{rd} generation β-blocker Nebivolol is a β_1 selective antagonist that is also able to increase nitric oxide bioavailability by mechanisms that are not fully elucidated but are currently attributed to β_2 and β_3 effects. The work contained in this thesis provides evidence that although 0.5mg.kg^{-1} of Nebivolol daily is sufficient to induced a sustained hypotensive effect in the hypertensive DOCA-salt rat, it was not able to prevent cardiovascular damage and remodelling from occurring.

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Abbreviations

2-factor ANOVA	Two-factor analysis of variance
ABS	Australian Bureau of Statistics
ACE	Angiotensin-converting enzyme
ACEi	Angiotensin-converting enzyme inhibitor/s
ACh	Acetylcholine
AIHW	Australian Institute of Health and Welfare
Ang II	Angiotensin II
APA	Action potential amplitude
APD	Action potential duration
AT_1R	Angiotensin II receptor type 1
AT_2R	Angiotensin II receptor type 2
BP	Blood pressure
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CFR	Coronary flow reserve
CRC	Concentration response curve
CSA	medial cross-sectional area
CVD	Cardiovascular disease
DC/s	Dendritic cell/s
Dev. P	Developed Pressure
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOCA	Deoxycorticosterone-acetate
DOX	Doxorubicin
EC/s	Endothelial cell/s

EC50	Effective concentration for 50% response
ECM	Extracellular matrix
EGCG	Epigallocatechin-3-gallate
ELISA	Enzyme-linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
EPI	Epicatechin
ESP	End systolic pressure
GP/s	General practitioner/s
GSH	Glutathione
GSH-Px	Glutathione peroxidase
HDL	High-density lipoprotein
HO	Heme oxygenase
HPLC	High-performance liquid chromatography
$\boldsymbol{I}_{K1}$	Inward rectifier potassium current
$\boldsymbol{I}_{Kr}$	Rapidly activating delayed rectifier potassium current
$\boldsymbol{I}_{Ks}$	Slowly activating delayed rectifier potassium current
$\boldsymbol{I}_{Ksus}$	Sustained outward potassium current
$\boldsymbol{I}_{Kur}$	Delayed rectifier potassium current
$\boldsymbol{I}_{Na}$	Sodium current
$\boldsymbol{I}_{to}$	Transient outward potassium current
IL-1β	Interleukin-1 beta
i.p.	Intraperitoneal
IP$_3$	Inositol trisphosphate
I/R	Ischemia-reperfusion
KPSS	Potassium physiological salt solution
LBP	*Lycium barbarum* polysaccharide
LDL	Low-density lipoprotein
L-NAME	L-Nitro-Arginine-Methyl ester
LV	Left ventricle
LVH	Left ventricular hypertrophy
MAP	Mean arterial pressure

MDA	Malondialdehyde
MI	Myocardial infarction
MMP	Matrix metalloproteinase
NA	Noradrenaline
NADPH	Nicotinamide adenine dinucleotide phosphate
NaNO	Sodium nitroprusside
Nrf2	Nuclear factor E2-related factor 2
OECD	Organisation for Economic Co-operation and Development
PI3K	Phosphatidylinositol 3-kinase
PRA	Plasma renin activity
PSS	Physiological salt solution
RAS	Renin angiotensin system
RAAS	Renin-angiotensin-aldosterone-system
RMP	Resting membrane potential
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RV	Right ventricle
SBP	Systolic blood pressure
s.c.	Subcutaneous
SOD	Superoxide dismutase
TAC	Total antioxidant capacity
TF3	Theaflavin-3,3'-digallate
TGA	Therapeutic Goods Administration
TLC	Thin-layer chromatography
TNF-α	Tumor necrosis factor alpha
UNX	Uninephrectomy
VEGF	Vascular endothelial growth factor
VSMC/s	Vascular smooth muscle cell/s

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Chapter 1

A review of hypertension and its associated remodelling of the cardiovascular system, and the potential for Nebivolol, *Stevia rebaudiana*, *Lycium barbarum*, and (-)-epicatechin to provide cardioprotection from the disease

1.1 Cardiovascular disease in Australia

Cardiovascular disease (CVD) was the leading cause of death during 2008 in Australia (Australian Institute of Health and Welfare, 2011)(AIHW). In 2007, 49% of CVD related deaths were from coronary heart disease (AIHW, 2011). Not only is it the leading cause of death, but it is also one of the most preventable causes of death within Australia. Furthermore, the economic cost associated with CVD is significant. During 2003, CVD contributed 18% of the overall burden of disease (years of health lost to disease or injury) in Australia (AIHW, 2011). Seventy-eight percent (78%) of the CVD burden resulted from years lost due to premature death (AIHW, 2011). All of these statistics show that cardiovascular disease is a major health problem for Australians. In comparison to other countries that form the Organisation for Economic Co-operation and Development (OECD), Australia had fewer deaths per 100,000 population than the United Kingdom, New Zealand, and the United States of America (AIHW, 2011). The latest data from the Australian Bureau of Statistics (ABS) shows that ischaemic heart disease was the cause of 13% of all deaths for 2012 (Australian Bureau of Statistics, 2012); the highest cause of death in Australia.

Total expenditure on CVD has increased since 2004-05, when CVD-related expenditure was 11% of the all healthcare expenditure (AIHW, 2011). The most recent publication of health care expenditure on cardiovascular disease shows that during 2008-09 the total cost of health expenditure ($7,605 million) made up 12% of total health care expenditure (AIHW, 2011). Ad-

ditionally, prescription pharmaceuticals were estimated to account for 22%, $1,648 million, of the CVD-related expenditure. (Australian Institute of Health and Welfare, 2014). This statistic only accounts for prescription medicines, and does not give any indication of the money that patients spend yearly on supplements and complementary medicines. The Australian Bureau of Statistics (2014) published survey data showing that over a quarter of Australians consume dietary supplements, with 29% of the population reporting to having taken a supplement on the day preceding the survey. With such a significant portion of Australians consuming dietary supplements it is understandable that there is a large commercial interest in the area.

In Australia, dietary supplements come under the umbrella of 'complementary medicines', a classification defined by the Therapeutic Goods Administration (2014). When considering the listing of low-risk complementary medicines, the TGA does not require efficacy of the compound to be proven, only safety (Therapeutic Goods Administration, 2014). Therefore, compounds and supplements available for purchase over the counter do not necessarily have scientific evidence supporting their health-claims and may in fact provide no benefit to the consumer. This does not preclude the use of natural compounds in treating and managing various diseases. Indeed, traditional medicines do provide novel areas of research, but it does highlight the need for continued research and evaluation into these compounds.

One of the properties of natural compounds that is often investigated is their antioxidant ability and potential to reduce oxidative stress. This is the case with the natural compounds found in *Stevia rebaudiana* and *Ly-*

cium barbarum, as well as the compound (-)-epicatechin, which is found in green tea and cocoa. However, targeting of the antioxidant and oxidative stress mechanisms is also being developed within existing pharmacological drug classes that had previously never exhibited such properties. The 3^{rd} generation beta-blocker, Nebivolol, is one such drug.

The role of oxidative stress in hypertension, both it's pathogenesis and pathology, is significant and exploitation of its mechanism is a worthy goal in the prevention and management of hypertension and cardiovascular disease in general.

1.2 Oxidative stress and antioxidants

Oxidative stress occurs when the cellular environment loses the equilibrium between oxidative molecules and antioxidant safeguards. Imbalance in the redox dynamic can either be caused by an increase in reactive species, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), a reduction of antioxidant components, such as down-regulation or modulation of antioxidant enzymes, or a combination of the two. The final result is an increased number of reactive molecules, which can interact with a number of cellular components, causing damage, including DNA, RNA, proteins, cell membrane components and NO (Chrissobolis and Faraci, 2006; Miao and St. Clair, 2009; Rees et al., 2008; Rojas et al., 2006).

Many cellular processes are influenced by ROS, and in cases of oxidative stress they can operate "abnormally". These include cell growth, cell differen-

tiation, extracellular matrix production and breakdown, and the stimulation of a number of kinases (Paravicini and Touyz, 2006). The interaction of ROS with NO is one of the most studied processes for oxidative stress causing diminished vascular compliance. Reactive oxygen species, such as superoxide ($O_2^{\bullet -}$), readily react with nitric oxide to form peroxynitrite ($ONOO^-$), a RNS. This process is not only deleterious in oxidative stress as it reduces NO, but the product is linked with increased expression of vascular endothelial growth factor (VEGF), a promoter of angiogenesis (Platt et al., 2005). Increased expression of VEGF is linked with the growth of dysfunctional vessels in a number of conditions, such as diabetic microangiopathy, atherosclerosis and tumor angiogenisis, all of which are also associated with producing ROS and elevating oxidative stress (Platt et al., 2005).

Vascular smooth muscle cells (VSMCs), often found to be hypertrophied in hypertension, are also affected by ROS modulated signal transduction (Lyle and Griendling, 2005). In a review by Lyle and Griendling (2005), it is suggested that superoxide does not serve a paracrine effect within the vasculature, rather it produces more of a direct local effect within the cell, and it is the metabolite H_2O_2 that is more suited to crossing the cell membranes and effecting neighbouring cells. This provides a mechanism by which oxidative stress within endothelial cells (ECs) lining the lumen of blood vessels could affect VSMC remodelling and proliferation. Endothelial cells are highly responsive to blood pressure and shear stress damage, making them susceptible to oxidative stress (Matlung et al., 2009).

The VSMCs are an important group of cells in the pathogenesis of hy-

pertension as they often undergo significant remodelling which also impairs function. Such remodelling is a result of a number of factors, all which exert some form of "remodelling pressure" on the cells. Reactive oxygen species are one of these factors, as one study has shown that they can potentiate the hypertrophic actions of angiotensin II (Ang II) (Weber et al., 2005). Transgenic mice over-expressing $p22^{phox}$ ($Tg^{p22phox}$), a gene involved in NADPH oxidase, were infused with either saline or Ang II for 13 days (Weber et al., 2005). Wall thickness and cross-sectional wall area were both increased in the $Tg^{p22phox}$ mice compared to the control strain (C57BL/6J mice)(Weber et al., 2005). The $Tg^{p22phox}$ mice given Ang II had a 65% increase in wall thickness compared to the 39% observed in the C57BL/6J mice infused with Ang II (Weber et al., 2005). Given that NADPH oxidase is considered a contributing source of ROS, this research provides supportive evidence of the interaction of oxidative stress in promoting pro-hypertensive changes.

The full extent to which ROS, RNS and oxidative stress play in the pathology, or even pathogenesis, of hypertension is not yet known. Currently ROS appears to be instrumental in potentiating and modulating the effects of other effector molecules at a molecular level. This link between oxidative stress and hypertension is supported by a number of review papers (Banday and Lokhandwala, 2008; Hirooka, 2008, 2011; Nozoe et al., 2007; Romero and Reckelhoff, 2000, 1999)

Animal models that are used to investigate hypertension show an increased incidence of oxidative stress. Spontaneously hypertensive rats demonstrate increased oxidative stress and reduced NO bioavailability, as do DOCA-

salt hypertensive rats and mice (Fujii et al., 2006; Suzuki et al., 1998; Viel et al., 2008). The L-nitro-arginine-methyl ester (L-NAME) rat is a unique model of hypertension that is based on the inhibition of NOS, reducing available NO to promote oxidative stress, endothelial dysfunction, hypertension, as well as increased systemic and vascular inflammation (Pechánová et al., 1999; Török, 2008).

Additionally, studies have used antioxidant compounds to treat experimental hypertension. L-arginine, an amino-acid which is a precursor to NO, has been shown to reduce oxidative stress and improve cardiac functional parameters when administered to hypertensive rats (Fenning et al., 2005). DOCA-salt rats were treated with L-arginine (5% in food) for 28 days after which oxidative stress and other functional and biochemical parameters were measured (Fenning et al., 2005). Plasma malondialdehyde was shown to be increased in the DOCA-salt hypertensive rats compared to the uninephrectomy (UNX) control animals (Fenning et al., 2005). Treatment with L-arginine was shown to be associated with a reduced plasma malondialdehyde (MDA) level in the hypertensive animals (Fenning et al., 2005). Given that L-arginine is a substrate for NO synthesis, it can be logically inferred that there may be an increase in the production of NO as a result of increased substrate availability and this may lead to increased ROS scavenging as a result. It is this mechanism that could explain the reduced MDA concentration seen in the treated rats, indicating that pro-antioxidant interventions, such as L-arginine supplementation, can effectively control oxidative stress. Although this study did show L-arginine correlated with a

reduced systemic oxidative stress state, it was unable to provide evidence of the effect on localised oxidative stress within the heart, blood vessels or kidneys.

Naturally derived compounds such as resveratrol and quercetin have also shown antioxidant properties and have helped in reducing oxidative stress in animal models of hypertension and cell culture models of oxidative stress (Arredondo et al., 2010; Celik and Arinç, 2010; Jiménez et al., 2007). One study showed that quercetin was able to prevent DNA breakage associated with chemotherapy treatment (Celik and Arinç, 2010). Idarubicin is a chemotherapeutic drug that is reduced by NADPH-cytochrome P450 reductase, resulting in the production of ROS molecules (Celik and Arinç, 2010). The study investigated the effect of various antioxidant compounds on preventing ROS mediated DNA breakage associated with idarubicin treatment (Celik and Arinç, 2010). Although this was done *in vitro* it allowed for greater specificity in controlling cellular damage production and preventative mechanisms, and therefore showed to great effect the protective function of quercetin in preventing ROS mediated DNA damage. At a concentration of 50μM quercetin was able to produce a 58% reduction in the amount of single-strand breaks (Celik and Arinç, 2010). At higher levels of 100μM and 200μM, damage was greatly reduced (>95% reduction)(Celik and Arinç, 2010). Quercetin has also been shown to increase the levels of antioxidant molecules in the cell. Glutathione (GSH) was shown to be increased in primary cerebellar granule neurones subjected to treatment with 60μM quercetin; activation of the Nrf2 (nuclear factor E2-related factor 2)

signalling pathway lead to increased expression of enzymes associated with GSH synthesis (Arredondo et al., 2010).

Whilst neither of the preceding studies are directly associated with hypertension, they demonstrate the effectiveness of natural compounds in preventing and treating oxidative stress and it's associated deleterious effects. They also represent recent examples of a current trend towards identifying and testing naturally derived compounds as tools in research studies and as potential treatments for a variety of disorders and diseases. Oxidative stress is a target for preventative therapies because of its involvement with a number of pathological changes that occur before, during, and following the development of hypertension and other CVDs. One of the most significant changes observed in hypertension is the remodelling of the vasculature.

1.3 Hypertension and the vasculature

Hypertension produces vascular damage through a number of mechanisms, which the body attempts to negate by remodelling. This remodelling is often beneficial in the short term but over time is maladaptive. Before discussing the hypertrophy, it is pertinent to review the vascular system and the damage responsible for the remodelling process.

It is well understood that the vascular system is highly innervated, and consequently under substantial control of the sympathetic nervous system. Vasomotor tone is largely dependent on the contribution of sympathetic drive and vasodilatory function is generally mediated by reduction in sympathetic

or via endothelial cell signalling to the smooth muscle cells of the vasculature.

Endothelial cells (EC), the cells lining the lumen of vessels, are central to the proper functioning of the vasculature. These cells produce NO, a potent vasodilator and antioxidant, which is released directly to the surrounding smooth muscle, initiating vasorelaxation (Förstermann and Münzel, 2006). This form of relaxation is termed endothelium-dependent relaxation, as functioning ECs are required. When production of NO is inhibited, or bioavailability is low, vessel relaxation is impaired and often there will be increased vascular tone in the vessels (Förstermann and Münzel, 2006). This general effect results in increased peripheral resistance, increasing overall systolic blood pressure. Endothelium-dependent relaxation can be lost through a number of mechanisms. Firstly, inhibition of endothelial NOS will stop local production of NO thereby decreasing relaxation. This process has been well described in studies where L-Nitro-Arginine-Methyl Ester (L-NAME) was used to block NOS, producing decreased sensitivity to acetylcholine along with severely diminished endothelium-derived relaxation (Kalliovalkama et al., 1999; Küng et al., 1995). However, inhibiting production of NO is not the only mechanism by which bioavailability of NO is reduced.

When $O_2^{\bullet -}$ is available in larger concentrations than NO, it quickly reacts to leave very little NO available release to the smooth muscle, therefore diminishing relaxation. It is this mechanism that is proposed to underlie much of the loss in vascular function that occurs during hypertension. The mechanism is particularly prevalent in conditions of oxidative stress. Additionally, ROS and RNS can react with NOS, uncoupling the enzyme, and leading to

the production of superoxide as opposed to NO, further exacerbating oxidative stress (Zweier et al., 2011).

The Renin-Angiotensin-Aldosterone-System (RAAS) also has a role in stimulating and initiating vascular remodelling. Angiotensin II (Ang II), a circulating peptide hormone, is widely associated with CVD and hypertension because of it's ability to increase systemic blood pressure (Rajagopalan et al., 1996), promote extra-cellular matrix remodelling (Guo et al., 2008), and increase oxidative stress (Sanchez et al., 2007).

Guo et al. (2008) demonstrated that Ang II increased the expression of matrix metalloproteinase-9 (MMP-9) in vascular smooth muscle cells and cited other studies by Eagleton et al. (2006) and Luchtefeld et al. (2005), in which it induced expression of MMP-3 and MMP-2. These enzymes are responsible for breaking down the extracellular matrix (ECM), allowing remodelling to take place (Hadler-Olsen et al., 2011). Therefore, Ang II overexpression is a concern as increasing levels of MMPs could perceivably lead to increased breakdown and re-organisation of the ECM, resulting in uncontrolled remodelling. Guo et al. (2008) indicates that MMP-9 is responsible for breaking down the intimal ECM, promoting atherosclerotic plaques, and continued ECM degradation would lead to plaque destabilisation. Coupling unstable plaques with the increased wall shear stress present in hypertension, it is easily understood why hypertension is a risk factor for thrombosis and other occlusive conditions.

Angiotensin II has specific receptors, the angiotensin receptors (AT_1R and AT_2R), and it is the AT_1R that is associated with vasoconstriction,

cellular proliferation, and pro-hypertensive and pro-remodelling pathways (Atlas, 2007). Interestingly, a study by Sparks et al. (2011) demonstrated, with the use of transgenic mice, that the AT_1R did not play any role in the Ang II mediated vascular changes. Although Ang II does exert influence on MMP activity, other stimuli such as ROS, inflammation and haemodynamic forces also influence the activity and expression of MMPs (Chen et al., 2013). Therefore, vascular remodelling is not solely dependent on a single factor, but is often multi-factorial.

1.3.1 Vascular remodelling

Oxidative stress damages the cellular environment of the endothelium, resulting in reduced functionality. Increased systolic blood pressure also impacts on the endothelium of vessels, particularly through increased wall shear stress. In order to compensate, the vascular tissue remodels, initially providing increased wall strength, but over time the changes become maladaptive. Remodelling occurs in both large conduit arteries and also in the smaller high resistance vessels, each initially providing benefit but eventually further exacerbating the problem.

The large conduit arteries undergo mainly hypertrophic remodelling, which results in increased smooth muscle cell size and increased extra-cellular components, including collagen and fibrinogen (Arribas et al., 2006). These changes lead to stiffening of the vessels, decreased compliance, and increased pulse pressure (Arribas et al., 2006). Pulse pressure is a measure of the

change in pressure during contraction of the heart, and equals the difference between systolic and diastolic blood pressure (Assmann et al., 2005) and has been identified as a predictor for coronary heart disease in elderly men. It has been shown to provide greater risk prediction than systolic blood pressure levels in men >60 years of age who were not undergoing antihypertensive treatment (Assmann et al., 2005).

It was subsequently shown in a more recent paper that the predictive property of pulse pressure was weaker than that of SBP in elderly patients who were receiving antihypertensive treatment (Bangalore et al., 2009), suggesting that each predictor of heart disease needs to be assessed within the context of the individual patient, in order to gain the most accurate predictive outcome. Pulse pressure has been shown to correlate well with the calcification of aortas, with peripheral pulse pressure giving r values around 0.02 (McEniery et al., 2009). This means that pulse pressure is also a good predictor for aortic stiffness, as arterial calcification is now becoming accepted as contributing to the stiffening process (McEniery et al., 2009). Aortic stiffening has been implicated as one possible mechanism by which isolated systolic hypertension may occur (Yasmin et al., 2005). Yasmin et al. (2005) have suggested that MMP-9 and MMP-2 may be involved with the progression and pathogenesis of isolated systolic hypertension, through their influence on the ECM. Changes in the ECM are not only implicated in the remodelling of the large conduit arteries, but also the smaller resistance arteries of the peripheral vasculature. Remodelling of small resistance vessels can have a profound effect on the systemic circulation, as well as the ability for the

body to maintain good blood flow to vital organs. Therefore, understanding the changes that occurs within the small resistance arteries as a result of hypertension, and how treatment options affect the function of these vessels is important. This also provides a comparison between hypertension-induced pathological changes to conduit vs. resistance vessels.

There are two forms of remodelling in the resistance arteries. The first is inward eutrophic remodelling, where an increased media/lumen ratio is observed without any change to the medial cross-sectional area (cell density in the wall) (Intengan and Schiffrin, 2001). The media/lumen ratio gives an indication to the size of the lumen compared to the thickness of the vessel wall. In hypertensive patients this ratio is increased, indicating the lumen diameter is narrower than normal (Intengan et al., 1999). The medial cross-sectional area (CSA) allows determination as to whether there has been an increase in cell density or population to account for this increased wall size. If the CSA has not changed then the increased wall thickness is due to re-organisation of the present cells and extracellular matrix. This unchanged CSA is characteristic of eutrophic remodelling and is not seen in hypertrophic remodelling. Both genetically based hypertensive animal models and essential hypertensive patients demonstrate eutrophic remodelling (Arribas et al., 2006; Intengan and Schiffrin, 2001). For the hypertensive patients this can progress to hypertrophic remodelling.

Hypertrophic remodelling causes the smooth muscle cells of the resistance arteries to increase in size, and promotes cellular proliferation and recruitment, both through direct and indirect mechanisms (Intengan et al., 1999;

Intengan and Schiffrin, 2001; Schiffrin, 1996). The CSA of a hypertrophic artery will be increased, indicating that cell proliferation and recruitment has taken place. This remodelling results in the wall "encroaching" into the lumen, reducing lumen diameter and therefore increasing systolic blood pressure. Hypertrophic remodelling is commonly seen in patients suffering secondary hypertension, and also in animal models of hypertension such as the DOCA-salt and the 1-kidney, 1-clip (1K1C) rat models (Arribas et al., 2006; Intengan and Schiffrin, 2001). Even though vascular remodelling is being unravelled, there is still not consensus as to whether vascular insult comes before hypertension or rather is a result of the changed haemodynamics. Although this order is still to be decided, there is evidence showing that changes to vascular function do have a dynamic impact on blood pressure and even inflammation, circulating cytokines and other humoral factors. Similar to the remodelling seen in the vasculature, the cardiac tissue undergoes remodelling to cope with the resultant change in haemodynamics, ultimately leading to maladaptive changes in the structure and function of the heart.

1.4 Hypertension and the cardiac system

Heart rate, stroke volume and total peripheral resistance are some of the factors that determine cardiac output. Cardiac output, the amount of blood pumped from the heart per minute, is often simply classified as the product of heart rate and stroke volume. Although this formula is easy to understand it does not take into account the effect that peripheral resistance has on cardiac

output, an important consideration in hypertension.

An increase in total peripheral resistance causes a decrease in cardiac output unless other factors are changed. It is this relationship that inevitably leads to cardiac hypertrophy, an attempt to increase pumping pressure to deal with the increased resistance encountered. A number of studies show the occurrence of cardiac hypertrophy in hypertension (Baker et al., 2004; Brown et al., 1999, 2000; Chan et al., 2006; Cohuet and Struijker-Boudier, 2006; Cuspidi et al., 2010; Khan and Sheppard, 2006). In addition to increased peripheral resistance, other factors act on cardiac tissue to induce hypertrophy. The peptide Ang II has shown to be produced within the cardiac tissue, independent of circulating levels (de Lannoy et al., 1998), providing a mechanism for cardiac hypertrophy even when plasma levels of Ang II are not significantly elevated. Additionally, blockade of the RAAS in DOCA-salt rats by captopril, candesartan or spironolactone can reverse cardiac fibrosis (Brown et al., 1999). DOCA-salt rats develop severe cardiac hypertrophy along with significantly increased fibrosis of the heart (Allan et al., 2005; Brown et al., 1999, 2000; Chan et al., 2006; Schenk and McNeill, 1992). Similarly SHR rats also exhibited significant cardiac hypertrophy (Carlstrom et al., 2007; Cerbai et al., 2000; Conrad et al., 1995). A number of reviews have examined the mechanisms for left ventricular hypertrophy and it is generally accepted that the myocardium thickens and shortens resulting in reduced ability to contract and relax (Cohuet and Struijker-Boudier, 2006; Gajarsa and Kloner, 2011; Raman, 2010). Studies by Chan et al. (2006) and Brown et al. (2000) showed DOCA-salt rats developed significant LVH by 4 weeks of treatment.

Treatment with aminoguanidine, an inhibitor of collagen crosslinking, was able to reduce LVH in DOCA-salt rats (Chan et al., 2006). Diastolic stiffness and left ventricular hypertrophy were also reduced in DOCA-salt rats by treatment with the anti-fibrotic drug Pirfenidone (Mirkovic et al., 2002). These studies demonstrate that regression or attenuation of LVH can result in improved cardiac functionality, highlighting the maladaptive nature of cardiac remodelling.

Hypertrophy of the cardiomyocte initially provides some physiological benefit, enhancing the ability of the cell to produce force and thus can be termed physiological hypertrophy. Ultimately these changes in the heart are maladaptive, leading to undesired side-effects and contributing to pathologies of the cardiovascular system. Once this occurs the hypertrophy is no longer referred to as physiological, but rather pathological hypertrophy. Over time the hypertrophied tissue requires an increased blood supply, producing a disparity between the required supply and the available supply that can be effectively delivered and distributed between the muscles. This is termed ischemia, a condition where blood supply is not adequate enough to meet the energy demands of the working muscle. In more severe cases this is often experienced as angina pectoris in patients and depending on the severity can be treated with simple pharmacological intervention. Angina is also a symptom of coronary artery disease and therefore is a risk factor for myocardial infarction and cardiac arrest. Myocardial infarction is the occurrence of cellular death within the heart and results in scarring of the affected area. Myocardial infarction and angina are not the only situations in which reduced

coronary flow reserve (CFR), or the inability for the heart to increase blood flow in response to increased oxygen demand, is observed. Prehypertensive patients also show reduced CFR, although not as severely as hypertensive patients (Erdogan et al., 2007), and this demonstrates that energy mismatch can occur much earlier than the onset of angina. In addition to increased energy demand from the muscle, changes in coronary vasculature function are also observed in patients who are suffering CFR (Hamasaki et al., 2000). The endothelial-dependent and independent vasodilatory responses are significantly reduced in patients suffering from hypertension and LVH, which Hamasaki et al. (2000) suggest may be a result of the vessels already exhibiting maximal vasodilation in order to compensate for the increased energy demand.

It is not only cardiomyoctye hypertrophy that remodels the heart; scarring and deposition of fibrotic compounds also affect the structure of the heart. It is these components that often impede cardiac function as they can interfere with the propagation of electrical signals through the tissue, resulting in lethal arrhythmias and dysrhythmias (González et al., 2002; Khan and Sheppard, 2006; Lu et al., 2011; Massare et al., 2010).

Humoral factors can also reduce functionality of the heart on a cellular level by altering gene transcription and expression (Baker et al., 2004; Domenighetti et al., 2007). Changes to cellular components via external signals or genetic mutations can also cause functional changes. Cellular components such as ion channels are very important in cardiac function, allowing for the generation and control of action potentials and contractile functions,

as well as pacing.

1.4.1 Electrophysiology and associated changes

Central to cardiac function is the ability of the muscles to continuously maintain correct contraction and relaxation cycles. There are a number of changes that occur in cardiac cells that can cause this cardiac rhythm to be lost. Such abnormal changes in rhythm are typically classified as dysrhythmias, with the serious situation of a complete loss of rhythm being termed as an arrhythmia. The rhythmic and coordinated contraction of the heart is dependent on the ability of individual myocytes to contract and relax in concert with their neighbours. Underpinning their function is the movement of ions across the plasma membrane that occurs in a highly organised fashion, which is characterised by four distinct phases.

The first phase is termed phase 0 and is characterised by the activation of the I_{Na} current (Tamargo et al., 2004). The I_{Na} is a result of voltage-gated sodium channels opening and allowing influx of Na^+ from neighbouring cells. Phase 1 is the beginning of repolarization, as I_{to} and I_{Kur} now activate, causing a net efflux of ions from the cell (Tamargo et al., 2004). Throughout phase 0 and 1, the L-type calcium channels (LTCC) are open, allowing influx of calcium ions into the cell, which stimulate the release of Ca^{2+} from the sarcoplasmic reticulum (Brette et al., 2006). As the I_{to} slows and stops, the efflux and influx of ions reaches an equilibrium, resulting in a plateau of the depolarisation, identified as phase 2 (Brette et al., 2006; Tamargo

et al., 2004). The plateau is lost once delayed rectifier currents (I_{Kur}, I_{Kr} and I_{Ks}) and the inward rectifier current (I_{K1}) begin, resulting in further repolarisation (phase 3) and reaching the resting membrane threshold of -70 to -80mV (Tamargo et al., 2004). It is these currents which can be lost by either changes in the genetic expression or even oxidative stress-mediated structural changes of the ion channels.

Phenotype changes, such as cardiomyocyte hypertrophy, are often accompanied by changes to gene expression, especially the activation of the "fetal gene program" as described by Hill (2003). Here, gene expression is altered and resembles the expression profiles commonly observed during embryo development (Hill, 2003). This may lead to changes in ion channel density that would have a significant effect on ion flux, potentially leading to prolongations in action potential duration (APD). Furthermore, these changes to gene expression can effect how Ca^{2+} ions are handled within the cell, resulting in further alterations to Ca^{2+}-mediated signalling cascades (Hill, 2003).

Cardiac hypertrophy is not only a result of the enlargement of myocytes but also a result of increased deposition of fibrotic tissues such as collagen and fibrinogen. A study by Lu et al. (2011) showed that in cell culture experiments, extracellular collagen was able to increase expression of AT_1R in HL-1 cardiomyocytes. The treatment with collagen led to an increase in the levels of transient, systolic and diastolic intracellular Ca^{2+} concentrations as well as showing larger I_{to} and I_{Ksus} levels (Lu et al., 2011). The change in $[Ca^{2+}]_i$ response would have an effect on electrical functionality of the cardiomyocyte and most likely manifest as altered electrophysiological

parameters. Indeed APD was shortened at 20%, 50% and 90% of repolarisation, a result of increased activity of I_{to} and I_{Ksus} rather than any effect on the $[Ca^{2+}]_i$ (Lu et al., 2011). Lu et al. (2011) proposed that such changes in the $[Ca^{2+}]_i$ may lead to increased cardiomyocyte contractility, compensating for the fibrosis present. However, this ultimately will result in cardiac hypertrophy and reduced CFR, which will further stress the cardiac tissue. Given that collagen upregulated AT_1R expression, it follows that Ang II therefore plays some role in electrical remodelling. Lu et al. (2011) tested the role of Ang II by treating the HL-1+collagen cells with Losartan, an Ang II receptor antagonist. Losartan administration resulted in attenuation of the $[Ca^{2+}]_i$ in systolic, diastolic and transient measurements (Lu et al., 2011). Shortening of the action potential duration allows for earlier depolarisation and these could lead to increased pacing of the contraction cycle.

1.5 Traditional treatments for cardiovascular disease

With the high prevalence of cardiovascular disease, and it's multifactorial origins, there are multiple pharmacological interventions to choose from. The strategy for treatment must take into account the severity of the condition, any contra-indications for the individual treatment options and also drug tolerability. A full understanding of this is not the aim of this review, but rather it is a brief overview of the classes of drugs and their mechanisms of

action.

A report by the Australian Institute of Health and Welfare (2007) showed during the period from 2000 through to 2006, drugs targeting the RAAS were the most prescribed classes by GPs in Australia. The data also revealed an upward trend in the use of RAS agents, while diuretics were less prescribed during 2006 than 2000 (Australian Institute of Health and Welfare, 2007).

1.5.1 Renin-angiotensin-system targeted treatments

The Angiotensin-converting enzyme (ACE) is responsible for the conversion of angiotensin I to angiotensin II. Inhibiting this process, via inhibitors of ACE (ACEi), results in a decrease in the production of Ang II, reducing available ligand for the AT_1R. Similarly, Ang II receptor blockers prevent Ang II from binding and stimulating the Ang II receptor. A third treatment option targeting the RAAS is to inhibit the enzyme renin. Renin is at the beginning of the angiotensin cascade, cleaving angiotensinogen into angiotensin I and allowing the process of Ang II synthesis to begin (Gradman and Kad, 2008). The direct renin inhibitor provides promise for treatment of hypertension and suppression of Ang II expression as current trials show it to be well tolerated and more effective than diuretics at reducing blood pressure (Schmieder et al., 2009).

The use of ACEi in treating hypertension has been shown to be just as effective as combined β-blocker/diuretic therapy (Hansson et al., 1999). The captopril prevention project (CAPPP) trial also revealed that captopril

(an ACEi) was more effective at reducing mortality in diabetics than the β-blocker/diuretic combination (Hansson et al., 1999). Captopril (50mg/kg) has also been shown to improve endothelium-dependent responses in the aorta of Zucker rats, which are used as a model of metabolic syndrome (Duarte et al., 1999). This study also showed that ACEi were able to reverse insulin resistance (Duarte et al., 1999). Both of these studies demonstrate that ACEi were effective in a diabetes setting also and therefore may be effective choices for treating patients who are suffering both diabetes and hypertension. In a study by Pechánová et al. (1997), captopril (100mg/kg/day) was investigated in the L-NAME rat model of hypertension and was shown to reduce cardiac remodelling. Captopril administration prevented the increase in collagen concentration within the left ventricle of the L-NAME rats (Pechánová et al., 1997). Overall, angiotensin-converting-enzyme inhibitors provide antihypertensive effects by reducing the pro-hypertrophy and pro-oxidative stress stimuli on the cardiovascular system (Duarte et al., 1999; Pechánová et al., 1997).

Angiotensin II receptor blockers should produce a similar pharmacological treatment outcome to ACEi as they target the terminal effector compound of Ang II in the RAAS cascade. Blockade of the ATR should prevent the negative actions of ATR binding such as vasoconstriction and cellular proliferation, reducing blood pressure and cardiovascular remodelling. A clinical trial, the Losartan Intervention For Endpoint reduction in hypertension study (LIFE), investigated the effectiveness of losartan compared to atenolol (β-$_1$-adrenoceptor antagonist) (Lindholm et al., 2002). The results showed that

losartan was more effective at reducing mortality and morbidity compared to atenolol, and also showed that left ventricular hypertrophy was improved by losartan treatment, which was not achieved by atenolol (Lindholm et al., 2002).

The report by the AIHW (2007), discussed earlier, also observed that even though the prescription of RAAS agents by GPs had increased, the prescription of β-blockers remained stable across the same period, with 19.2% of patients suffering from hypertension using a β-blocker (Australian Institute of Health and Welfare, 2007). A number of studies have investigated the effectiveness of beta-blockers compared to other classes of CVD treatments, and the results have been well reviewed by Wiysonge et al. (2012). The systematic review carried out by Wiysonge et al. (2012) only included studies that investigated the use of β-blockers as first-line therapy for hypertension, and did not focus on other CVDs such as heart failure. They note that three quarters of the total number of participants involved in the studies were on atenolol, a β_1-selective blocker (Wiysonge et al., 2012). Of the 13 randomly controlled trials (RCTs) reviewed by Wiysonge et al. (2012) atentol was a primary treatment in 8 of the studies. Of the 13 RCTs only 6 studies had β-blockers other than atenolol; metoprolol (2) , propranolol (3) and oxprenolol (1) (Wiysonge et al., 2012). None of the β-blockers used were from the latest generation of drugs, and therefore conclusions derived from studies such as those by Wiysonge et al. (2012) and Lindholm et al. (2002) need to be considered in light of this. The most recent study included was the 2005 ASCOT study, more recent studies, using later generation β-blockers,

were excluded from the analysis for a variety of reasons, including their focus on heart failure rather than hypertension (Wiysonge et al., 2012). This is one demonstration of the shifting focus of newer generation β-blockers from hypertension to other CVDs such as heart failure. The newer generation of β-blockers also show improved treatment profiles. Bhosale et al. (2014) compared the safety and efficacy of nebivolol with atenolol, and observed that nebviolol produced significantly fewer adverse effects, such as fatigue, dizziness, and bradycardia, in patients compared with atenolol.

1.5.2 β-adrenoceptor antagonists

The early β-blockers were non-selective and blocked β_1, β_2 and β_3, but with advances in drug discovery and synthesis, newer generations of these compounds are far more selective (Ram, 2010). The β-receptors are largely associated with heart tissue (β_1, β_2), blood vessels and bronchi (β_2), skeletal muscle (β_2, β_3), and adipocytes β_3) (Anderson, 2006; Grujic et al., 1997; Ram, 2010; Rehsia and Dhalla, 2010). The traditional β-blockers, typified by propranolol and metoprolol, achieved blood pressure reduction by primarily reducing cardiac output via negative chronotropic and inotropic effects (Ram, 2010). Newer generations of β-blockers such as carvedilol and nebivolol achieve blood pressure reduction via vasodilatory mechanisms (Ram, 2010). The use of β-blockers in the treatment of hypertension has come under scrutiny recently, as they do not appear to provide any advantage over other treatments (Che et al., 2009); in fact they are no longer

classed as frontline therapies (National Heart Foundation of Australia (National Blood Pressure and Vascular Disease Advisory Committee) (NHFA), 2010). However, the newer generations of β-blockers may require a softening of this stance, with Che et al. (2009) indicating a number of studies used atenolol with once daily dosing, which is less than the pharmacokinetic and pharmacodynamic properties of the drug would suggest is necessary for appropriate treatment of the patient. Che et al. (2009) cite a study by Neutel et al. (1990), in which one daily dosing with atenolol left the patient unprotected in the last 6 hours of a 24-hour period. Therefore, improvements in β-blocker functionality and mechanism of action may open the door for increased use of β-blockers within CVD.

Table 1.1: Generations of β-blockers

	Antagonist targets	**Examples**
1^{st} Gen	β_1, β_2 ("non-selective")	Propranolol
2^{nd} Gen	β_1 ("selective")	Atenolol & Metoprolol
3^{rd} Gen	β_1, β_2 & α_1	Carvedilol
4^{th} Gen	β_1, pro-vasodilator effects	Nebivolol

1.6 β-blocker (Nebivolol)

As mentioned earlier, the use of β-blockers in the treatment of hypertension is still debated, with controversy surrounding the design of studies comparing β-blockers with other treatments (Che et al., 2009; Giles et al., 2008). Although β-blockers are not at the front for hypertensive treatments, they still provide benefits in the treatment of CVD. A study examined the use of metoprolol in treating atherosclerotic plaques in healthy patients (Östling et al., 2011). The study showed that treatment with metoprolol increased plaque stability and the authors concluded that this may be involved in the cardioprotective effect of β-blockers (Östling et al., 2011). Beta-blockers have also shown promise and effectiveness in the treatment of heart failure, as they are able to prevent ventricular remodelling, reduced arrhythmic generation and even reduce sympathetic activity in the failing heart (Rehsia and Dhalla, 2010).

Nebivolol is a third-generation β-blocker that has additional NO stimulating properties. The NO enhancing mechanisms presents a number of new avenues for pharmacotherapy. As discussed earlier, oxidative stress and ROS/RNS levels play an important role in the pathology of hypertension. In a review on the pharmacology of nebivolol, various studies were cited which demonstrated both the acute hypotensive effect and the endothelium-dependent effect of nebivolol (Mangrella et al., 1998). The hypotensive effect of nebivolol was compared to other β-blockers (propranolol, atenolol and pindolol) and showed a significantly larger and quicker hypotensive result than the those achieved by the other compounds (Mangrella et al., 1998). This is

thought to be due to increased release of NO causing vasodilation, especially in the peripheral vascular beds, which have a great influence over blood pressure. Other studies are looking at non-conventional uses for this β-blocker, such as cardioprotection in diabetes (Peter et al., 2006).

Patients suffering from type 2 diabetes and recently diagnosed with mild to moderate hypertension were recruited and those with specific complications were rejected from the study (Peter et al., 2006). The patients were then given 5mg nebivolol once a day and had follow-up visits at 3 and 6 months (Peter et al., 2006). Results indicated that nebivolol treatment was able to reduce ambulatory blood pressure, both systolic and diastolic, as well as improving the high-density lipoprotein (HDL) subfractions (Peter et al., 2006). Additionally, indicators of possible atherosclerotic risk were increased and total antioxidant capacity (TAC) was reduced after nebivolol treatment (Peter et al., 2006). Both these changes are disturbing as a reduction in TAC may indicate that antioxidant mechanisms are depleted or reduced, hence increasing the risk of oxidative stress and consequently the oxidation of the LDL subfractions. Low-density lipoprotein oxidation contributes to atherosclerosis progression as oxidised LDLs are taken up by macrophages, prompting them to change their phenotype and become foam cells. Foam cells become incorporated into plaques and secrete cytokines and chemokines that attract more macrophages and the process continues in a circular fashion, effectively growing the plaque. However, these specific effects observed by Peter et al. (2006) may be isolated to the sequalae associated with diabetes and not applicable to other cardiovascular related disorders.

Nebivolol has also been assessed in a nitric oxide-deficient animal model of hypertension, the L-NAME rat, and shown to prevent the associated development of arterial hypertension (Fortepiani et al., 2002). Nebivolol ($1 mg.kg^{-1}$) was given to 3 groups of male Sprague-Dawley rats who also received daily doses of 0.1, 1 or 10 $mg.kg^{-1}$ N^{ω}-nitro-L-arginine methyl ester (L-NAME), an inhibitor of eNOS (Fortepiani et al., 2002). In the L-NAME control groups, mean arterial pressure (MAP) was significantly increased and showed a dose-dependent relationship (Fortepiani et al., 2002). This increased MAP was normalised by nebivolol in the 0.1 and 1 mg/kg groups, and the 10 mg/kg group showed a reduced increase in BP when treated with nebivolol (Fortepiani et al., 2002). Plasma renin activity (PRA) was also increased in the L-NAME only groups, with nebivolol treatment normalising this effect in all treated groups (Fortepiani et al., 2002). The authors of this paper suggest that nebivolol does not work through increasing NO production but rather through PRA-suppression (Fortepiani et al., 2002). It is most likely that nebivolol either upregulates eNOS or increases signalling for eNOS activation rather than directly causing release of NO. Therefore, these results published by Fortepiani et al. (2002) cannot be used to explain the actions of nebivolol in all cases, but rather explain the additional benefits of nebivolol in an NO deficient model.

The mechanism by which nebivolol increases nitric oxide appears to be via a metabolite that is an agonist of the β_2-andrenoceptor (Broeders et al., 2000). In the experiment by Broeders et al. (2000), mice were injected with nebivolol via the tail vein, and after 45 minutes the mice were anesthetised,

their abdomen opened and blood collected. Aortas from the mice were taken and the release of nitric oxide in response to either nebivolol, metoprolol, mouse plasma (containing the metabolites of nebivolol) or acetylcholine was measured (Broeders et al., 2000). The results showed that nebivolol and metoprolol alone did not increase NO release from baseline, established from the control animals, but rather plasma from nebivolol injected rats did cause a significant increase in NO (Broeders et al., 2000). This increase was not present in preparations free of calcium or in aortas incubated with the β_2-blocker butoxamine (0.1 mmol/L) (Broeders et al., 2000), indicating that the response seen with the nebivolol metabolites was dependent on both calcium and the stimulation of the β_2-adrenoceptor.

Overall, Broeders et al. (2000) conclude that nebivolol achieves increased NO concentrations via the actions of one or several of its metabolites, especially via activation of the β_2-adrenoceptor that leads to an increase in free cytosolic calcium ($[Ca^{2+}]_i$). This proposed mechanism was further supported by a study on mouse renal arteries, which investigated vasodilation in response to increasing doses of nebivolol (Georgescu et al., 2005). The results showed nebivolol induced relaxation in the arteries, but after treatment with EGTA (a chelator of Ca^{2+}) the effectiveness was reduced (Georgescu et al., 2005). Additionally, the β_2-adrenoceptor antagonist butoxamine, the gap junction blocker 18 beta-glycyrrhetinic acid, the Ca^{2+}-activated K^+ channel inhibitors tetraethylammonium and iberiotoxin all reduced the degree of vasodilation produced by nebivolol administration (Georgescu et al., 2005). These results confirm that the β_2-adrenoceptor along with a Ca^{2+}-mediated

signalling cascade are involved with nebivolol-mediated vasodilation. The β_3-adrenoceptor has also been implicated in the vasodilatory effect of nebivolol, with β_3-adrenoceptor-deficient mice showing diminished relaxation in response to nebivolol compared to their wild-type counterparts (Dessy et al., 2005).

Cardiac remodelling is a major factor in the progression of cardiovascular disease, as explained earlier, and therefore it is important that potential treatment and preventative measures address this issue. Nebivolol has been shown to protect the heart from the remodelling that takes place after an ischemic event such as myocardial infarction (MI) (Mercanoglu et al., 2012). In a study on 12 week old Sprague-Dawley rats subjected to ligation of the left anterior descending coronary artery, nebivolol was shown to reduce left ventricular remodelling both acutely (2 days post MI) and chronically (28 days post MI) (Mercanoglu et al., 2012). Untreated MI rats showed significant decreases in cardiac functionality, as well as significant remodelling as confirmed by the increased heart and ventricle weights (Mercanoglu et al., 2012). All these results were obtained using sub-clinical doses of nebivolol, therefore the rats did not show any significant difference in mean arterial blood pressure between the MI and MI-nebivolol groups (Mercanoglu et al., 2012). This demonstrates that nebivolol is able to produce a cardioprotective effect independent of blood pressure. Nebivolol also reduced the rise in serum NO and $ONOO^-$ observed 28 days following the MI (Mercanoglu et al., 2012).

One study compared the effects of nebivolol and carvedilol (3^{rd} generation

β-blocker + α_1 antagonist) on left ventricular function and found that there was no significant difference between the two (Lombardo et al., 2006). Another study compared bisoprolol (β_1-selective), carvedilol and nebivolol and concluded that there was very little difference between the three in terms of their clinical relevance or effect on quality of life (Stoschitzky et al., 2006). In another comparative study, nebivolol and metoprolol both showed reductions in blood pressure and heart rate, however only nebivolol improved oxidative stress and insulin resistance (Celik et al., 2006). Nebivolol does differentiate itself from the other β-blockers due to its ability to effect not just heart rate and blood pressure, but improve other contributing factors to CVD such as oxidative stress and insulin resistance.

1.6.1 Novel avenues for hypertension prevention and management

Most of the current treatments for hypertension are focused on either the RAAS, β-adrenergic mechanisms, or blocking calcium channels. In a systematic review of calcium channel blockers, which covered eighteen randomised controlled trials (14 focused on dihydropyrinides), Chen et al. (2010) concluded that there was evidence to support the use of calcium channel blockers over β-blockers in treating hypertension. It should be noted that the clinical trials dealing with β-blockers that were analysed by Chen et al. (2010) mainly utilised the β_1 selective antagonists atenolol or metoprolol and therefore the conlcusions drawn should not be applied to the latest generation of

β-blockers. A few new generation pharmaceutical compounds are starting to diversify their pharmacological actions and target other systems and pathways such as the vasodilator β-blockers (carvedilol & nebivolol). However, an important part of the mechanisms of hypertension are oxidative stress and inflammation.

An ability to reduce oxidative stress has been discussed in the media and on advertising campaigns for tea and some forms of coffee, and presents a novel avenue for treating hypertension. Already there are a number of natural compounds being investigated in relation to cardiovascular and metabolic disease such as resvertrol, quercetin and olive leaf (Poudyal et al., 2010; Scheffler et al., 2008; Thirunavukkarasu et al., 2007). The involvement of oxidative stress in promoting and inducing cardiovascular remodelling makes it a promising target for preventative and interventional therapy. By using antioxidants to reduce oxidative stress and bring the equilibrium back into balance, it might be possible to prevent ROS-mediated remodelling from taking place.

Three novel compounds being investigated for their effect in hypertension and conditions of oxidative stress and inflammation are extracts from *Stevia rebaudiana*, *Lycium barbarum* (Goji berries) and the compound (-)-epicatechin, which is found in green tea and cocoa. These compounds are already commonly consumed by the public and, are often spruiked as "superfoods" by the media.

1.7 *Stevia rebaudiana*

Stevia rebaudiana is a plant native to South America, with anecdotal evidence suggesting stevia's putative medicinal properties were harnessed by native tribes through herbal and tea preparations (Chatsudthipong and Muanprasat, 2009). Extracts from *Stevia rebaudiana* have been shown to possess antioxidant, antihypertensive and anti-glycemic properties, which can help to explain the medicinal applications of the plant in native cultures (Chatsudthipong and Muanprasat, 2009). Currently, stevia preparations are being sold as sugar replacements, and also in so called "smart sugars"; a combination of sugar and stevia extract designed to allow for a reduction in sugar consumption but maintaining the overall sweetness and taste. Stevia does not contain any calories and also does not generate an increase in blood glucose, therefore making it an attractive sweetener for diabetic patients. Work by Jeppesen et al. (2003) showed the stevia extracts, stevioside and sterol, initiated a first-phase insulin response, a response lost in diabetic patients. Even though there is controversy as to whether a first-phase insulin response occurs *in vivo*, considering the absorption from the stomach into the blood would never resemble an intravenous injection of glucose, it is still an important diagnostic measurement for diabetes (Caumo and Luzi, 2004).

This response was seen in both diabetic [Goto-Kakizaki (GK)], and non-diabetic rats (Jeppesen et al., 2003). Diabetic GK rats were treated with stevioside ($2.5 mg.kg^{-1}.day^{-1}$) for 6 weeks, with intra-arterial catheters being surgically implanted at week 5 (Jeppesen et al., 2003). At the end of the

treatment period rats were given an intra-arterial glucose tolerance test, a bolus injection of D-glucose ($2g.kg^{-1}$ dissolved in 0.9% saline) was administered and blood samples taken for 180 minutes post injection (Jeppesen et al., 2003). Blood glucose levels were significantly increased in both the GK controls and the GK treated, but there was an observed suppression of the plasma glucose in the stevia treated rats (Jeppesen et al., 2003). Plasma insulin levels of the GK control rats started to significantly rise 30 minutes post-challenge, whereas the GK-treated rats showed a large, quick increase in plasma insulin before 30 minutes post-challenge (Jeppesen et al., 2003), showing a first-phase-like response. Insulin levels in the GK-treated rats continued to rise over the time course (Jeppesen et al., 2003).

This effect of stevioside was also observed in cell cultures of INS-1 cells (a pancreatic beta cell line), where incubation of 1 μmol/L of stevioside or steviol significantly increased the insulin content of the cells (Jeppesen et al., 2003). This study supports the claimed anti-hyperglycemic properties of stevia as well as providing the basis for a mechanism of action. It appears the consumption of stevia is able to stimulate the first-phase insulin response, especially in the diabetic animal (Jeppesen et al., 2003). Although controversy surrounds the existence of a "first-phase" response, there is consensus that after consumption of food, insulin is released within 5-10 minutes. Given that this is lost in diabetes sufferers and stevia is able to induce such a response, it provides a new avenue for helping to manage diabetes and possibly metabolic syndrome.

Stevia has not only been investigated in relation to diabetes but there

has also been some work performed regarding its cardiovascular relevance. A number of studies have investigated the hypotensive effect of stevia treatment in both animal models and human hypertensive patients (Chan et al., 2000, 1998; Hsieh et al., 2003). In the first study, Chan et al. (1998) gave male SHR rats intravenous doses of stevioside at 50, 100 and 200 $mg.kg^{-1}$, and analysed their plasma for any change in the catecholamine profile as well as alterations in blood pressure. There was no observed change in the levels of adrenaline, noradrenaline or dopamine, but there were significant decreases in both systolic and diastolic blood pressures (Chan et al., 1998).

A study in human volunteers, those with mild to moderate hypertension without any secondary complications, used a dose of 250mg stevioside three times a day for one year and demonstrated a reduction in blood pressure without any observed changes in biochemical parameters (Chan et al., 2000). This multi-centre, randomised, double-blind, placebo-controlled study investigated the effect of stevioside dosing on 106 Chinese patients, and showed a mean reduction in systolic blood pressure of 12mmHg and a mean 8mmHg reduction in diastolic blood pressure (Chan et al., 2000). Serum levels of creatinine (elevated in renal damage), aspartate aminotransferase and alanine aminotransferase (both elevated in liver distress) were assessed, as were plasma concentrations of glucose, triglycerides and total cholesterol, none of which were changed by stevioside treatment (Chan et al., 2000). Furthermore, stevioside does not appear to increase the lipid profile of patients (Chan et al., 2000), which has important implications in the treatment of hypertensive and diabetic patients who may be obese.

Another multi-centre, double-blind, placebo-controlled study used a 500mg three times a day dose in a two year study of the efficacy of stevioside in recently diagnosed mild hypertension sufferers (Hsieh et al., 2003). The mean systolic and diastolic blood pressure was 150mmHg and 95mmHg respectively at the initiation of the study, with a significant reduction to 140mmHg systolic and 89mmHg diastolic (Hsieh et al., 2003). Again, this study assessed biochemical parameters for liver, kidney and lipid profiles, and supporting the conclusions made by (Chan et al., 2000) showed stevioside is well tolerated by human hypertensive patients (Hsieh et al., 2003).

Stevioside has been shown to generate relaxation in noradrenaline precontracted rat aortic rings, through both endothelium-dependent and independent mechanisms (Bornia et al., 2008). Bornia et al. (2008) demonstrated that stevia was unable to relax aortic rings in the presence of either the eNOS inhibitor N^G-nitro L-arginine (L-NOARG) or the guanylate cyclase inhibitor 1H-[1,2,4]-oxidiazolo [4,3-a] quinoxaline-1-one (ODQ), demonstrating that stevia achieves vasodilation via the NO pathway. However, in endothelium-denuded vessels, stevia was still able to achieve a vasodilatory response, which is suggested to be achieved through Ca^{2+} channel antagonism (Bornia et al., 2008). Work by Lee et al. (2001) supports this mechanism of action, with the authors also concluding that stevioside's effect was mediated via Ca^{2+} influx inhibition.

Stevia extracts have also been shown to possess antioxidant properties, with Shivanna et al. (2013) demonstrating both *in vitro* antioxidant activity of stevia leaves and pro-antioxidant effects *in vivo*. Powder made from stevia

leaves, when supplemented into the diet of STZ-diabetic rats, was able to increase the expression of antioxidant enzymes, including superoxide dismutase and catalase (Shivanna et al., 2013).

To date there has been no research into the vascular and cardiac responses to stevia supplementation in rat models of hypertension, particularly the DOCA-salt model.

1.8 *Lycium barbarum* (goji berry)

Goji berry, also known as wolf berry, is a bright red fruit from the *Lycium barbarum* plant and has been consumed in Chinese diets since biblical times (Gruenwald, 2009). It possesses some of the highest antioxidant properties of any plant (Gruenwald, 2009), and as such has potential for protecting against oxidative stress. Goji berries appear to also have immuno-modulatory properties, with a number of studies showing this effect (Chen et al., 2009a,b, 2008). It is the polysaccharide-protein complexes found in the plant, known as *Lycium barbarum* polysaccharides (LBPs), that are believed to provide the specific benefits of goji berry consumption.

The immuno-modulatory effect of LBPs was investigated in an experiment where splenocytes from 6-week old C57BL/6 mice were exposed to increasing concentrations of 1μg/mL through to 300μg/mL LBPs (Chen et al., 2008). The LBPs were separated into fractions (LBPF) via a chromatographic process which yielded 5 distinct fractions, and these were also used as separate treatments in the study, as well as a crude LBP preparation

(Chen et al., 2008). Exposure to LBPs was able to stimulate the splenocytes significantly in a dose-dependent manner (Chen et al., 2008). Additionally, $CD3^+$ T-cells, but not $CD19^+$ B-cells, were also stimulated by LBPs, by either a homogenous mixture or the tested fractions LBPF4 and LBPF5 (Chen et al., 2008). This demonstrates that the LBPs in goji berries are able to modulate the immune system, specifically the adaptive immune component. The study also investigated the effect of LBPs on the activation maker CD25 in T-cells, and LBPs did increase the number of $CD25^+$ T-cells observed, although 3 of the LBP fractions did not increase expression when given alone (Chen et al., 2008). The *in vivo* effect of LBPs were also investigated with BALB/c mice being administered LBPs either via intra-peritoneal injection or orally for 7 weeks (Chen et al., 2008). The cells where then cultured and those that came from LBPs-treated mice showed increased proliferation over those from vehicle-treated mice (Chen et al., 2008). The study concluded that LBPs were able to stimulate and activate T-cells, therefore improving immune function and supporting the hypothesis that LBPs possess immunoenhancement functions (Chen et al., 2008).

A year later the same lead author investigated the effect of LBPs on macrophages and found that it increased both mRNA expression and production of TNF-α (Chen et al., 2009b). It was also shown to increase mRNA expression of IL-1β by 1600-4700 fold and IL-12p40 by 1,360,00-6,810,000 fold (Chen et al., 2009b). Unlike TNF-α, IL-1β and IL-12p40 cytokines were not detected by ELISA (Chen et al., 2009b), showing mRNA expression dose not always correlate with an increase in actual protein. *Lycium*

barbarum polysaccharide was further shown to stimulate and induce maturation of dendritic cells (DCs) (Chen et al., 2009a). Dendritic cells treated with LBPs are highly immunogenic, and are more potent in inducing Th1 and Th2 responses in T-cells *in vitro* (Chen et al., 2009b). Administration of LBPs to BALB/c, either via sub-cutaneous injections (s.c.), i.p or p.o. showed increased Th1 priming, with s.c. administration giving the highest results (Chen et al., 2009a).

These studies demonstrate that the LBPs present in goji berries are able to enhance the immune system at a number of levels. This provides evidence for the basis of LBPs in cancer, immunology, and a number of other research areas closely linked with immune modulation. Goji berries also possess high concentrations of antioxidants, a property that holds promise for hypertension research. One study has shown that treatment with LBPs can improve NO bioavailabilty in the male Wistar rat both in normal control animals and also in those subjected to ischema reperfusion injury (Shao-Ping and Pin-Ting, 2010).

In an effort to determine whether the antioxidant properties of LBPs were sufficient in preventing oxidative damage from occurring in rats treated with the chemotherapeutic drug Doxorubicin (DOX), male Sprague-Dawley rats were pretreated with 200mg.kg^{-1} of LBP (Xin et al., 2011). Doxorubicin is an effective chemotherapeutic, however it does increase the formation of $O_2^{\bullet -}$ and hydrogen peroxide (H_2O_2) that leads to cardiotoxicity (Xin et al., 2011). Cardiac cells are more susceptible to oxidative stress as they do not tend to express as many antioxidant components, such as catalase and GSH-

peroxidase (GSH-Px), as other cells in the body (Xin et al., 2011). The study by Xin et al. (2011) used male Sprague-Dawley rats that were given either water+saline, LBP+saline, water+DOX or LBP+DOX (Xin et al., 2011). On the seventh day of the study, the rats were injected intra-venously with either saline (0.9%, 10mg/kg) or DOX (10mg/kg) depending on their group (Xin et al., 2011). Three days after the injection, electrocardiograms (ECGs) were performed and then the rats were euthanised and terminal experiments performed (Xin et al., 2011). The results of the experiment showed that LBP was able to reduce MDA levels and increase SOD and GSH-Px levels *in vivo*, with GSH-Px being normalised (Xin et al., 2011). The treatment of LBP was also able to partially attenuate the histopathological changes observed in the DOX-treated rat hearts (Xin et al., 2011). Treatment with DOX showed significant vacuolisation and myofibrillar disorganisation in rat heart sections compared to control animals (Xin et al., 2011). Additionally, given DOX is a chemotherapeutic agent, LBP treatment was assessed to determine if there was any effect on the cytotoxicity of DOX to human A549 lung carcinoma cells, and there was none (Xin et al., 2011).

1.9 Flavanols (Flavan-3-ols)

(-)-Epicatechin (EPI) and its stereoisomer catechin are naturally occurring flavanols (or flavan-3-ols), a subgroup of the larger polyphenol family. These two compounds are noticeably associated with the health benefits of green tea and also dark chocolate. In a review of the current research into cocoa and

its cardiovascular effects, Corti et al. (2009) cited its positive effects on blood pressure, insulin resistance, vascular function, and platelet function. These effects are believed to be primarily related to the ability of the cocoa polyphenols to increase NO bioavailabilitiy, although this mechanism was unclear at the time (Corti et al., 2009). Flavanols in general appear to possess antioxidant properties with epigallocatechin-3-gallate (EGCG) and theaflavin-3,3'-digallate (TF3) showing direct ROS scavenging ability (Dreger et al., 2008). Both of these compounds were shown to not affect gene regulation of either the SOD1, GPx3, or catalase genes (Dreger et al., 2008), indicating that the observed antioxidant effects were not attributable to upregulation of the tested antioxidant enzymes. Dreger et al. (2008) did note that EGCG was able to upregulate the expression of heme oxygenase-1 (HO-1), the inducible isoform of heme oxygenase (HO). Heme oxygenase-1 is induced in response to stresses, such as oxidative stress, inflammation, shear stress, endotoxin, and ultraviolet radiation (Dawn and Bolli, 2005). Other polyphenols are also able to enhance mRNA expression of HO-1, with resveratrol upregulating HO-1 expression in streptozotocin-induced diabetic rats (Thirunavukkarasu et al., 2007). Given that EGCG and not TF3 upregulated HO-1 expression, it is likely that other catechin entities may also induce such a response, whereas the theaflavins would not. This may be related to structural differences, with the theaflavins possessing more phenol groups, therefore changing the spatial configuration of the compound possibly impacting on receptor binding.

1.10 Epicatechin

In 2010, Ramirez-Sanchez et al. (2010) published a paper suggesting a possible mechanism for the observed increase in NO associated with EPI consumption. By analysing the phosphorylation of endothelial nitric oxide synthase (eNOS), epicatechin was shown to have a direct effect on eNOS activation (Ramirez-Sanchez et al., 2010). Phosphorylation at serene residues 633 and/or 1177 and dephosphorylation of threonine 495 are associated with activation of eNOS (Ramirez-Sanchez et al., 2010). These sites were observed to be phosphorylated and dephosphorylated respectively when subjected to 1μmol/L EPI *in vitro* (Ramirez-Sanchez et al., 2010). Given that EPI does not possess kinase-like action, it is highly likely that a binding site exists which activates either a kinase cascade or some other signalling pathway to cause eNOS activation. Ramirez-Sanchez et al. (2010) suggest that both the phosphatidylinositol 3-kinase (PI3K) pathway and the Ca^{2+}/CaMKII pathway mediated EPI-associated eNOS activation. Using inhibitors specific for downstream kinases within the PI3K pathway, cAMP-dependent protein kinase (PKA) and Akt, it was shown that EPI-associated phosphylation of eNOS was diminished (Ramirez-Sanchez et al., 2010). Additionally, by inhibiting the cell membrane protein Phospholipase C, the cell membrane-associated protein responsible for initiating IP_3-mediated release of Ca^{2+} from the endoplasmic reticulum, eNOS activation was diminished. Furthermore, by inhibiting CaMKII itself, which is responsible for activation and phosphorylation of CaM (a downstream effector), eNOS activation was also

diminished (Ramirez-Sanchez et al., 2010).

This proposed mechanism explains the short-term effects of EPI, but does not go far enough to elucidate and explain the possible long-term effects. Other studies have used epicatehcin (1 $mg.kg^{-1}$, daily) to protect cardiac tissue from myocardial infarction and oxidative stress in models of ischemia-reperfusion injury and myocardial infarction (Yamazaki et al., 2008, 2010). Epicatechin treatment was able to significantly attenuate myocardial oxidative stress within the infarct area and surrounding tissue in rats subjected to coronary artery ligation for forty-five minutes before reperfusion was allowed (Yamazaki et al., 2008). Treatment with epicatechin was also able to reduce the infarct area in rats subjected to permanent coronary occlusion (Yamazaki et al., 2010). These two studies show the pro-antioxidant and anti-remodelling properties of EPI, and provide a solid basis for further work on EPI-mediated anti-oxidant effects in cardiovascular disease.

Epicatechin also appears to inhibit ACE via competitive binding at the active site (Actis-Goretta et al., 2003). This discovery adds another layer to the cardioprotective properties of EPI, with ACE being the rate-limiting step for angiotensin II production. Angiotensin II involvement is a major factor in cardiac remodelling, with the local angiotensin system particularly being involved and initiating the hypertrophy of the myocytes and other cardiac cells (Bader, 2002; de Lannoy et al., 1998; Müller et al., 1998). This mechanism may be part of the long-term effect of epicatechin dosing. Therefore, it is hypothesised that epicatechin will provide protection to the cardiovascular system of hypertensive rats, via a number of mechanisms all working

in concert to prevent the adverse remodelling in response to the increased blood pressure.

1.11 Animal Models

There are a number of animal models that model cardiovascular diseases, in particular hypertension. The variation in models is indicative of the multi-factorial nature of hypertension with the population, and both genetic and non-genetic animal models are used in cardiovascular research. One of the more extensively used rat models of hypertension is the spontaneously hypertensive rat (SHR). The SHR is a genetic based animal model of chronic hypertension that progresses through various stages of hypertension towards heart failure (Conrad et al., 1995). Although genetic models may be easier to relate to human populations other models offer more specific causes of pathological progression. One such model is the DOCA-salt rat model of volume overload.

The DOCA-salt rat model is often referred to as an artificial model of hypertension. The basis for the hypertension is not a naturally occurring phenomenon, but rather intervention with a mineral-corticoid that leads to increased fluid retention. Deoxycorticosterone-acetate (DOCA), increases the reabsorption of sodium ions in the kidney and consequently water retention is also increased (Iyer et al., 2010). The model also requires that the animals drink a 1% salt water solution in replacement for water, increasing the sodium content and thus resulting in increased retention of fluid. As

fluid is retained plasma volume increases, leading to volume overload within the rat, producing hypertension. The DOCA-salt model also undergoes a uninephrectomy, which reduces the ability of the renal system to compensate the changes in Na^+ handling within the kidneys. Although this does not model real-world circumstances completely, the changes to the cardiovascular system mimics those that are seen in pressure overload and severe chronic hypertension. DOCA-salt rats show increased levels of pro-inflammatory cytokines (Seifi et al., 2010); lipid peroxidation and increased ROS generation (Viel et al., 2008); increased production of collagen I and III within the heart (Brown et al., 1999); reduced endothelium-dependent relaxation of thoracic aorta (Allan et al., 2005); and increased endothelin-1 expression (Gómez-Guzmán et al., 2012; Yemane et al., 2009).

There is some uncertainty about the role that sympathetic nerve activity (SNA) may play within the model, with a number of studies showing contradictory results when attempting to elucidate the role of SNA (Yemane et al., 2009). Although SNA may be involved with the DOCA-salt model a study by Kandlikar and Fink (2011) has demonstrated that global SNA was not increased during the development of the model. The study measured noradrenaline spillover at days 2, 7 and 14 of the DOCA-salt treatment, with plasma NA levels showing no significant difference to that observed in normotensive rats (Kandlikar and Fink, 2011). Although global elevations in SNA were not observed the authors make note that their results do not discount the potential for local SNA to be increased (Kandlikar and Fink, 2011).

The DOCA-salt model has been used to assess the effect of red wine polyphenols in hypertension, particularly the effect of oxidative stress (MDA & Iso-PGF2α) (Jiménez et al., 2007). DOCA-salt rats showed extensive increases in both MDA and Iso-PGF2α, as well as showing significant localisation of $O_2^{\bullet -}$ in aortic rings (Jiménez et al., 2007). Additionally, their endothelial-dependent relaxation response to acetylcholine was diminished (Jiménez et al., 2007). The treatment of DOCA-salt rats with RWP improved these hypertensive parameters (Jiménez et al., 2007). Apocynin, an inhibitor of NADPH oxidase, was also tested in the DOCA-salt model and found to significantly reduce oxidative stress and production and localisation of O_2, and also improved response to endothelial-mediated relaxation of aortas (Jiménez et al., 2007). Other studies have shown the extensive involvement of oxidative stress in the DOCA-salt pathology, with Viel et al. (2008) showing levels of xanthine oxidase-derived $O_2^{\bullet -}$ being significantly increased in DOCA-salt rats compared to their UNX counterparts.

Not only is oxidative stress increased in the DOCA-salt rats but functional aspects of the cardiovascular system are severely diminished (Chan et al., 2011, 2006; Fenning et al., 2005; Jiménez et al., 2007; Mirkovic et al., 2002; Momtaz et al., 1996). Studies have shown loss of vascular reactivity and endothelium-dependent relaxation (Allan et al., 2005; Nunes et al., 2000), and these same studies have also shown diminished cardiac functionality in DOCA-salt rats. In one study by Mirkovic et al. (2002), a significant increase in diastolic stiffness was observed in the DOCA-salt rats, but treatment with either pirfenidone or amiloride attenuated this change. This increase in dias-

tolic stiffness is reported in a number of other papers (Chan et al., 2011, 2006; Fenning et al., 2005). In addition to the gross functional changes observed in the hearts DOCA-salt rats there is also significant electrophysiological remodelling. A number of studies show the DOCA-salt model associated with significant prolongation in action potential duration (Chan et al., 2006; Fenning et al., 2005; Loch et al., 2006) and other studies have shown significant changes in sodium currents (Liu et al., 2013) and calcium handling (Momtaz et al., 1996) occur within the hypertrophied hearts of the DOCA-salt rat.

As has been shown, the DOCA-salt rat model of hypertension is a valuable model for understanding the contribution of oxidative stress within hypertension and cardiovascular damage. In addition to the strong link between oxidative stress and cardiovascular damage that is characteristic of the DOCA-salt model, the model mimics, within 4 weeks, cardiovascular changes that are seen in chronic cardiovascular remodelling in humans (Iyer et al., 2010). As such it is an appropriate model to first assess potential cardiovascular protective compounds, such as those investigated in this thesis.

1.12 Conclusion

Cardiovascular disease, and specifically hypertension, affect a large number of people and also require a large commitment to treatment by both the individual, their family, the doctors, and ultimately the government. Therefore, effective treatments for hypertension need to be well tolerated by the body, easy to administer, and something people feel comfortable taking. Natural

compounds tend to fit these criteria and those that are high in antioxidants, pro-hypotensive and are easy consumable should be investigated. This is the rational behind the following investigations into *Stevia rebaduiana*, *Lycium barbarum*, and (-)-epicatechin. All of these compounds have previously shown some potential beneficial mechanisms in diseases that are often comorbidities of hypertension and CVD. Similarly, Nebivolol is one of the more recent β-blockers which has potential to not only affect health benefits via antagonism of β_1 receptors, but to also address the one of the underlying pathologies associated with hypertension and CVD, oxidative stress. By investigating these four compounds, not only can a greater understanding of where natural compounds sit within addressing CVD and hypertension, but also it is possible to target oxidative stress on a variety of pathways/mechanisms.

1.13 Aims

The aims of this study were to assess, using the DOCA-salt rat model of hypertension, the potential cardiovascular protective effects of: (1) Nebivolol, (2) *Stevia rebaduiana*, (3) *Lycium barbarum*, and (4) (-)-epicatechin.

1.14 References

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Chapter 2

Comprehensive materials and methods

Disclaimer

This chapter contains a complete description of the materials and methods used to perform the experiments contained within this thesis. The relevant chapters covering each experimental study (Ch. 3, 4, 5, 6) have been written as standalone papers, ready for submission to journals. Therefore they will contain a comprehensive methods section pertaining to the experiments relevant to their respective study. It should be noted that similarities between this chapter and the individual methods sections of each experimental paper will occur and should therefore be noted as such. The methods described within this chapter are methods that are commonly used within the laboratory in which this thesis and required experimental work were completed and performed, and therefore will share resemblance with journal articles and/or publications from work performed in the laboratory. These methods have been written and described by myself and where appropriate acknowledgment and references have been provided.

2.1 Chemicals and Treatments

2.1.1 Anaesthesia

Zoletil (Zoletil-100) and Xylazil (Ilium Xylazil-20) were purchased from Pharmazo (QLD, Australia).

2.1.2 Euthanasia

Lethabarb euthanasia injection (sodium pentobarbitone, 375mg.mL^{-1}) was purchased from Pharmazo (QLD, Australia).

2.1.3 Experimental compounds

Noradrenaline (99%), acetylcholine (≥99%, TLC), sodium nitroprusside (≥99%), nebivolol hydrochloride (≥98%, HPLC), epicatechin (≥90%, HPLC, Sigma), dimethyl sulfoxide (≥99.9%, Sigma)(DMSO), N,N-dimethylformamide (≥99%, Sigma)(DMF), and deoxycorticosterone-acetate (D7000, Sigma)(DOCA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). *Lycium barbarum* extract in capsule form (Nature's Way Goji Berry 500mg (60 Veggie Caps), normalised to 40% *Lycium barbarum* polysaccharides) was purchased from Evidencia Fitness & Health (VIC, Australia). Powdered *Stevia rebaudiana* extract (stevioside 90% and rebaudioside A 10% - HPLC verified) was obtained from Cargill Australia by the School of Science and Environment at CQUniversity, Rockhampton, Australia.

2.1.4 Dosing preparation

Nebivolol

Ten milligrams (10mg) of nebivolol hydrochloride was dissolved in 10mL of pure water (Milli-Q Water purification system, Merk Millipore, Australia) to produce a preparation of 1mg.mL^{-1} Nebivolol.

Lycium barbarum extract

Lycium barbarum extract capsules were broken apart and the contents used to make up a 200mg.mL^{-1} LBP solution in pure water.

Stevia rebaudiana extract

Two grams (2g) of *Stevia rebaudiana* extract powder was dissolved in pure water to a final concentration of 200mg.mL^{-1}.

(-)-epicatechin

Ten milligrams (10mg) of (-)-epicatechin was first dissolved in 100μL DMSO and then diluted using 9.9mL pure water, producing a final concentration of 1mg.mL^{-1}.

2.2 Animals

Male Wistar rats were purchased from the Animal Resource Centre (Perth, Australia) at six weeks of age, under approval from the CQUniversity Animal

Ethics Committee (A11/03-268). The rats were housed in a temperature controlled room, set at 25±2°C, and a 12hr light/dark cycle was maintained. All rats had *ad libitum* access to food and water prior to and during the experimental period. Rats were fed with commercially available rat chow pellets and housed in cages of 2 or 6 rats. Surgery was not performed until the rats had reached 8 weeks of age and were also over 300g.

2.3 Surgical procedure: uninephrectomy

Uninephrectomy is required in the development of the DOCA-salt model, as it allows for renal insufficiency to be attained much more easily, particularly once treatment has begun. Effects of the mineral corticoid (DOCA) are much more pronounced and exacerbated in rats that are renal deficient, leading to greater and faster development of the DOCA-salt rat pathology.

Following the methods of Fenning et al. (2005) and Chan et al. (2006), each rat was anaesthetised by an intraperitoneal (i.p.) injection of zolazepam and tiletamine (25mg.kg^{-1}, Zoletil) together with xylazile (10mg.kg^{-1}, Rompun). Once the rat was fully under surgical anaesthesia (showing no signs of eye or pedal movement in response to painful stimuli) a small section of the left flank was shaved and cleaned of fur. A small incision was then made through the hide and abdominal wall, exposing the left kidney. The renal blood vessels and ureter were clamped and ligated, allowing the kidney to be safely removed. The kidney was removed using a scalpel, the ligated vessels and ureter were returned into the abdominal cavity and the inci-

sion site cleaned and sterilised. The abdominal wall was sutured closed and the hide stapled using surgical staples. Pain management, using meloxicam (0.1mg.kg^{-1}, Metacam) was administered following surgery and maintained for two days following.

2.4 Treatment protocols

After all of the rats had undergone uninephrectomy, they were randomised into the relevant groups for each study. Drug doses were delivered by oral gavage. The groups were as follows.

- UNX - Normotensive controls (no treatment)
- UNX+N - Nebivolol treated normotensive (Nebivolol, 0.5mg.kg^{-1})
- UNX+S - Stevia treated normotensive (Stevia, 200mg.kg^{-1})
- UNX+G - Goji treated normotensive (Goji, 200mg.kg^{-1})
- UNX+E - Epicatechin treated normotensive (Epicatechin, 1mg.kg^{-1})
- DOCA - Hypertensive control (no treatment)
- DOCA+N - Nebivolol treated hypertensive (Nebivolol, 0.5mg.kg^{-1})
- DOCA+S - Stevia treated hypertensive (Stevia, 200mg.kg^{-1})
- DOCA+G - Goji treated hypertensive (Goji, 200mg.kg^{-1})
- DOCA+E - Epicatechin treated hypertensive (Epicatechin, 1mg.kg^{-1})

All rats that were allocated to the UNX groups received vehicle treatment of 0.4mL dimethylformamide (DMF) subcutaneous every fourth (4^{th}) day, and were allowed to drink standard tap water. The rats allocated to

the DOCA groups received subcutaneous DOCA injections (25mg DOCA in 0.4mL of DMF) every 4^{th} day, and 1% NaCl in their drinking water (Chan et al., 2006; Fenning et al., 2005). The use of 1% NaCl in the drinking water for the DOCA-treated rats, is aimed at increasing sodium levels within the animal to assist in the development of volume overload. The DOCA injection dose was maintained for the entirety of the experimental period, and was not adjusted to changing animal body weights.

The majority of the animal work was performed at the same time. If further samples were required outside of the primary experimental period the required groups were accompanied by UNX and DOCA control animals. All data was then pooled to provide maximum sample values and account for any variation in experimental periods. Due to the requirements of various techniques, sample sizes vary across the data. Both the Langendorff and Intracellular electrophysiology techniques require dedicated cardiac tissue samples, and therefore cannot be performed on the same animals. In order to obtain a statistically significant sample size (n= 12) for each parameter the total number of rats in each group was larger than the individual sizes for those techniques. Due to this, initial sample sizes for each study are larger than those reported for various parameters. Additionally effect sizes vary depending on each parameter and as a result the required n= values are different. Animal numbers were chosen to achieve a n average sample size of 12 for each group.

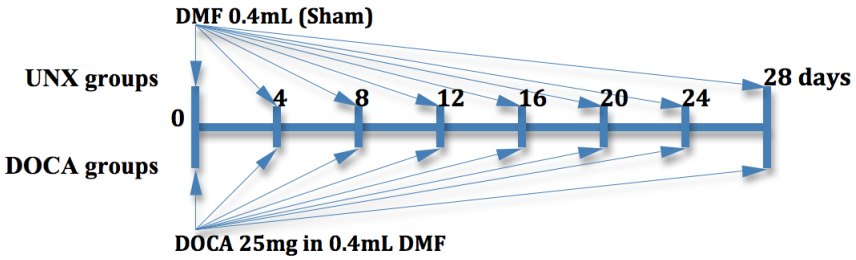

Figure 2.1: Injection timeline

2.5 Systolic blood pressure measurement

Systolic blood pressure was measured at three time points during the experimental period (0, 2 & 4 weeks). The initial baseline measurements (0 weeks) were performed after uninephrectomy, but before randomisation to treatment groups. The baseline results are presented as UNX. The tail-cuff plethysmography method of blood pressure determination, as previously described by Fenning et al. (2005), was used. Rats required to undergo blood pressure analysis were immobilised by a light anaesthetic (Tiletamine 15mg.kg^{-1} and Zolazepam 15mg.kg^{-1} i.p.). A tail pulse transducer (MLT1010), connected to a PowerLab data acquisition unit, was attached to the tail to measure pulse within the tail. After this, an inflatable tail cuff was attached; this was connected to the PowerLab via a Capto SP844 physiological pressure transducer (MLT844/D). The PowerLab was connected to an iMac G4 computer running LabChart software to record the data. Blood pressure traces were performed by inflating the cuff until the systolic blood pressure pulse was lost from the tail, and then slowly emptying the cuff and measuring the pressure at which pulse returned to the tail. A minimum of three separate

recordings for each rat were collected, allowing for a three sample average to be determined. Results from each group were pooled to produce a group average.

2.6 Euthanasia

After 28 days of treatment the rats were euthanised by an intraperitoneal injection of sodium pentobarbitone (*Lethabarb* 275mg.mL^{-1}). The rats were deemed to have been adequately euthanised one they showed no signs of response to painful stimuli.

2.7 Blood sample collection and analysis

Following euthanasia, while the heart was still beating and providing sufficient systemic circulation, blood was drawn using a syringe and needle from the thoracic *vena cava* and collected in serum separator tubes. A minimum of 5mL of blood was collected from each successful draw. Blood was allowed to clot at room temperature and then centrifuged for 15 mins at 3000g. The supernatant was then pipetted off and stored in 1.5mL eppendorf tubes at -80°C until required for analysis.

2.7.1 Serum total nitric oxide

Determination of serum total nitric oxide was performed using a commercially available colorimetric assay (Total Nitric Oxide kit (R&D Systems,

Minneaplis, MN, USA)). This assay allows for determination of plasma/serum concentrations of endogenous nitrate, an end product of nitric oxide metabolism. This is an indirect estimation of nitric oxide bioavailability and is used as a marker for oxidative stress. A standard curve was generated from the supplied standards using a 4-parameter logistic curve, from which unknown samples were interpolated.

2.7.2 Serum malondialdehyde

Determination of serum malondialdehyde concentration was performed using a commercially available ELISA kit (OxiSelect MDA Adduct ELISA kit (Cell Biolabs Inc. San Diego, CA, USA)). A standard curve was generated from the supplied standards using a 4-parameter logistic curve, which was used to interpolate the unknown samples.

2.8 Biometric analysis and indices

Body weight was measured weekly and immediately following euthanasia. This final weight was used to normalise organ weights. Organs were removed from the animal after all required experimental tissue was taken, and blotted dry before being weighed. The heart weight was taken after the experiments were performed. Briefly following the microelectrode experiment, the left ventricle and papillary muscle were blotted dry and weighed together, then the right ventricle was blotted dry and weighed. Following the whole-heart function experiment, the atria were removed and the right ventricle removed,

blotted dry and weighed. The left ventricle was opened with a cut through the intra-ventricular septum, blotted dry and then weighed. The kidney, liver, and spleen, were also dissected out and blotted dry. These were used as measure of potential systemic or toxicological effects.

2.9 Thoracic aorta organ bath analysis

Thoracic aortic function was assessed using an isolated organ bath technique (Fenning et al., 2005), and is described below. Following euthanasia, and once the blood draw and removal of the heart was performed, the thoracic aorta was dissected out and submerged in cold Tyrode's physiological salt solution (Tyrode's PSS) (mM: NaCl 136.9, KCl 5.4, $MgCl_2.H_2O$ 1.0, $NaH_2PO_4.2H_2O$ 0.4, $NaHCO_3$ 22.6, $CaCl_2.2H_2O$ 1.8, glucose 5.5, ascorbic acid 0.3, Na_2EDTA 0.05). The tissue was then cleaned of fat and cut into 5mm ring segments. One segment was mounted onto a force transducer (FT03, Grass Technologies, Middleton, WI, USA) and submerged in warm (37°C) Tyrodes solution, which was continuously bubbled with carbogen gas (95% O_2/5%CO_2). The transducer was connected to an iMac computer via a PowerLab 8/30 - PowerLab ML228 bridge amp combo (ADInstruments, NSW, Australia). Vessels were allowed to acclimatise for 30 min before being pretensioned to 10mN. Concentration-response curves were performed to noradrenaline (NA), acetylcholine (ACh) and sodium nitroprusside (NaNO). For the ACh and NaNO curves a pre-contraction was generated by a sub-maximal concentration of NA. These three curves assessed adrenergic-induced contractile

function, and endothelium-dependent and endothelium-independent relaxation, respectively. The results were normalised as a percentage of total relaxation in order for the effective concentration needed to induce a 50% response (EC50) to be calculated.

2.10 Mesenteric wire-myography analysis

Small vessel myograph was performed on second order mesenteric arteries. The method was adapted from the DMT 610M user manual (Danish Myo Technology, 2008). A 10cm long segment of the ileum with the associated mesenteric vasculature was removed from each rat, after all other experimental tissues were taken. The sample was pinned out in a dissection tray submerged in cold Tyrode's PSS. The blood vessels were cleaned of fat and the mesenteric artery was identified and dissected out. A two millimetre (2mm) segment was cut and threaded with 40μm stainless steel wire. Once one wire was successfully threaded through the vessel lumen, it was attached to the force transducer tooth of a DMT 610M wire-myograph system (DMT, Denmark) and the second wire was threaded and attached to the non-force transducer tooth. The myograph was connected to a PowerLab 8/30 (ADInstruments, NSW, Australia) and an iMac computer running LabChart Pro 7 software recorded the data. Once all tissues had been normalised following the procedure outlined by DMT, they were subjected to a KCl challenge with a modified potassium Tyrode's solution (KPSS) (mM: NaCl 37mM, KCl 100, $MgCl_2.H_2O$ 1.0, $NaH_2PO_4.2H_2O$ 0.4, $NaHCO_3$ 22.6, $CaCl_2.2H_2O$

1.8, glucose 5.5, ascorbic acid 0.3, Na_2EDTA 0.05). After the tissues had achieved a sufficient contraction they were rinsed with standard Tyrode's physiological salt solution and rested. Tissue that did not show a contractile response was removed and a replacement tissue strung up in its place. Concentration-response curves to NA, ACh and NaNO were performed, separated by washout periods. Pre-contractions for ACh and NaNO curves were produced by sub-maximal concentrations of NA.

2.11 Cardiac intracellular electrophysiology (single cell microelectrode)

Cardiac electrophysiology was assessed following the single cell microelectrode technique described by Fenning et al. (2005). Briefly, the heart was dissected out and submerged in cold Tyrode's PSS that was continuously bubbled with carbogen gas. The atria and right ventricle were removed and the intraventricular septum cut down the middle. The papillary muscle was removed and one end of it was pierced with a stainless steel hook. The other end of the papillary muscle was secured by a stainless steel pin into a 1mL experimental chamber between two platinum electrodes. The hook was attached to a modified micro-force transducer (SensoNor AE 801) and the chamber was continuously perfused with warm (37$\pm$0.5°C) gassed Tyrode's PSS at a rate of 3mL.min^{-1}. The micro-force transducer was connected to an iMac computer running Chart v5.5 via an amplifier (World Precision

Instruments TBM-4). Once the papillary tissue was set up, it was slowly stretched to a pre-load of 5-10mN over 1 min. Electrical field stimulation (Grass SD-9) was used to induce contractions at a frequency of 1Hz, with a pulse width of 0.5msec and a stimulus strength of 20% above threshold. A potassium chloride filled glass electrode (World Precision Instruments, filamentated borosilicate glass; outer diameter 1.5mm; tip resistance 5-15mΩ when filled with 3M KCl) was used to impale the muscle, and electrical readings were recorded. A minimum of three separate impalements from different regions of the muscle were recorded and used to determine means for each tissue. Electrical recordings were captured using a Cyto 721 electrometer (World Precision Instruments, USA) connected to an iMac running LabChart Pro v5.5 (ADInstruments, NSW, Australia) via a PowerLab 4/25. This method allowed the recording of resting membrane potential (RMP), action potential amplitude (APA), action potential durations at 20, 50, 90% of depolarisation, and force of contraction.

2.12 Isolated whole heart functional analysis (Langendorff method)

Whole heart functional analysis was performed via a non-recirculating constant-pressure retrograde-perfused isolated heart system, similar to the methods published by Fenning et al. (2005) and Chan et al. (2011). Following euthanasia, the heart was rapidly dissected out and submerged in ice-cold

modified Krebs-Henseleit buffer (KHB) (mM: NA 119.1, KCl 4.75, $MgSO_4$ 1.19, KH_2PO_4 1.19, $NaHCO_3$ 25.0, glucose 11.0 and $CaCl_2$ 2.16). The aorta was cleaned of fat and cannulated via the dorsal root before the heart was perfused with warm (37°C), carbogen-gassed KHB at a constant pressure of 100mmHg. A latex balloon, connected to a Capto SP844 physiological pressure transducer (MLT 844/D), was inserted into the left ventricle via the mitral valve, and the heart paced at 250bpm by electrical stimulation of the right atria. The presssure transducer was connected to a PowerLab 4/30 (ADInstruments, NSW, Australia), which was connected to an iMac computer running LabChart Pro v5.5. The balloon was filled with water until a starting pressure of 0mmHg was achieved. Pressure-response curves of 5mmHg increments were performed, with each pressure step recorded for a minimum of 20 seconds. This was continued until a maximum diastolic pressure of 30mmHg was achieved. A linear plot of the tangent elastic modulus (E, dyne/cm^2) and the stress (σ, dyne/cm^2) was used to define the myocardial stiffness constant (k, dimensionless). Rate of LV pressure changes (dP/dT), end systolic pressure (ESP), and developed pressure were also recorded.

2.13 Data capture and statistical analysis

All *in vivo*, *ex vivo*, and *in vitro* data was recording using LabChart Pro software running on Apple Mac computers. Statistical analysis was carried out using GraphPad Prism 6 statistical analysis software, with 2-factor ANOVA, One-way ANOVA and Student's T-tests being used to test for significance

where appropriate. *Post hoc* analysis, where required, was performed in GraphPad Prism 6 using the Bonferroni correction. All data is reported as the mean ± standard error of the mean (SEM). Where data is presented in the thesis, numbers in parenthesis denote the sample (n) number of the measure or group. For all tests a $P<0.05$ was taken to indicate significance.

2.14 References

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Chapter 3

Low dose nebivolol reduces blood pressure, improves myocardial compliance and promotes a shift in adrenergic sensitivity of thoracic aortic rings within the DOCA-salt rat model of hypertension

Abstract

Nebivolol is a 3^{rd} generation beta-blocker shown to possess β_1 specific antagonist actions as well as β_2 & β_3 agonist effects. This allows nebivolol to increase NO production, promoting antioxidant effects and improvements in vascular function. These effects have been investigated in numerous studies but none have utilised the DOCA-salt model of hypertension and oxidative stress. The aim of this study was to assess the effects that nebivolol, at a dose of 0.5mg.kg^{-1}, has on the cardiovascular remodelling and functional changes associated with the DOCA-salt hypertensive rat. Eight week old male Wistar rats underwent uninephrectomy before being treated with either vehicle or 25mg DOCA (in 0.4mL of DMF) every 4 days for 28 days. Each group was further divided into nebivolol-treated (0.5mg.kg^{-1}.day^{-1}) and non-treated. Thoracic aorta and mesenteric organ baths were used to assess vascular function, and cardiac function was assessed by single cell microelectrode electrophysiology and non-recirculating retrograde-perfused isolated whole heart Langendorff. Blood pressure was significantly increased in the DOCA treated groups compared to UNX controls, however Nebivolol did reduce BP in both DOCA and UNX rats (UNX - 130 ± 6mmHg, UNX+N - 103 ± 8mmHg, DOCA - 194 ± 5mmHg, DOCA+N - 157 ± 6mmHg). Vascular reactivity to NA in rats was reduced in all DOCA rats (including Nebivolol treated), with the thoracic aorta showing reduced maximal contractions and DOCA+N rats showing an EC50 shift to the right compared to

control DOCA rats. Mesenteric arteries did not show this reduced response to NA however, indicating the effect may be more prominent within the larger conduit vessels. Nebivolol, at this dose, was not able to confer cardioprotection to the heart, with DOCA+N animals showing similar reductions in cardiac function, increased cardiac hypertrophy, and prolonged action potentials as DOCA control rats. This study demonstrates the potent haemodynamic effect of Nebivolol, at a dosage of $0.5\text{mg.kg}^{-1}\text{.day}^{-1}$, within the DOCA-salt rat, as well as showing evidence of a potential α_1-adrenoceptor antagonistic effect within thoracic aorta tissue.

3.1 Introduction

Pharmacological interventions in the treatment of cardiovascular disease (CVD) have come a long way since the first generations of compounds, such as propanolol, with research continuing to improve efficacy, side-effect profiles, and most importantly patient outcomes. The first β-blocker, propanolol, was a non-selective antagonist, inhibiting both β_1 and β_2 adrenergic receptors (Che et al., 2009; Ram, 2010). Blood pressure lowering effects were primarily due to the chronotropic and inotropic actions of β-adrenergic antagonists (Ram, 2010). Since those early days the β-blocker class of drugs has improved, with later generations having greater specific β_1 receptor binding profiles, such as Metoprolol and Atenolol (Che et al., 2009; Ram, 2010). In addition to the β-antagonism, newer compounds possess peripheral vasodilatory effects that are achieved either through antagonism of the α_1-adrenergic receptor (Carvedilol), or by enhancing nitric oxide (NO) release (Nebivolol) (Che et al., 2009).

Nebivolol is a third-generation β-blocker possessing β_1-selective antagonist action along with vasodilator effects, believed to be mediated by a metabolite of nebivolol that possess β_2 agonist activity (Broeders et al., 2000; Dessy et al., 2005; Münzel and Gori, 2009; Ram, 2010). Although the vasodilatory mechanism has not been completely elucidated, both the β_2 and β_3 receptors have been implicated. Broeders et al. (2000) demonstrated that nebivolol had very little direct effect on mouse thoracic aorta, but plasma from nebivolol-injected rats did induce a β_2-mediated increase in endothe-

lial $[Ca^{2+}]_i$. Additionally, work by Dessy et al. (2005) in β_3 knockout mice, showed the nebivolol-induced relaxation in the coronary microvasculature of wild-type controls was significantly reduced in the β_3 knockouts. One paper has suggested that nebivolol may have an α_1-blocking function as well (Rozec et al., 2006). Phenylephrine (a selective α_1 agonist) concentration-response curves carried out in aortas incubated with varying concentrations of nebivolol showed a dose-dependent shift towards the right, indicating an α_1-antagonistic effect (Rozec et al., 2006).

Nebivolol has also been shown to improve cardiac function, cardiac remodelling and oxidative stress (Heeba and El-Hanafy, 2012; Mercanoglu et al., 2012; Oelze et al., 2006; Troost et al., 2000; Zhou et al., 2010). There appears to be very little, if any, published literature investigating the effect of nebivolol on cardiovascular function and oxidative stress within the DOCA-salt rat model. Given the significant contribution of oxidative stress to damage and remodelling within the cardiovascular system, and the fact that the DOCA-salt model shows significant involvement of oxidative stress in terms of its pathology, there is a clear benefit in pursuing this avenue of inquiry. When determining a dose of nebivolol for this study, we considered the findings of a number of publications that had used doses of between $2mg.kg^{-1}$ and $20mg.kg^{-1}$, which often led to significant reductions in blood pressure (Górska et al., 2010; Guerrero et al., 2006, 2003; Mason et al., 2009; Oelze et al., 2006; Susic et al., 2012; Wang et al., 2013). Therefore, we chose a lower dose of $0.5mg.kg^{-1}$ in order to reduce the potential for significant blood pressure reduction. The aim of this study was to determine if Nebivolol at

this dose was able to confer cardioprotective benefit in the DOCA-salt model of hypertension. It was hypothesised that at a level of 0.5mg.kg^{-1} blood pressure would not be significantly effected, but there would be an improvement in cardiac and vascular function.

3.2 Materials and Methods

3.2.1 Drugs and treatment

Deoxycorticosterone-acetate (DOCA), N,N-dimethylformamide (DMF), acetylcholine (≥99% TLC), noradrenaline (99%), sodium nitroprusside (≥99%) and nebivolol hydrochloride (≥98% HPLC) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Xylazil (Ilium Xylazil-20), Zoletil (Zoletil-100) and Lethabarb (Lethabarb euthanasia injection) were purchased from Pharmazo (Australia). All drugs were, excluding DOCA and DMF, dissolved in Milli-Q filtered water (Merk Millipore, Australia) before being used. A stock solution of nebivolol of 1mg/mL were prepared by dissolving 10mg of nebivolol hydrochloride in 10mL of pure water (Milli-Q water purification system, Merk Millipore Australia).

3.2.2 Animal preparation

All animals were purchased from the Animal Resource Centre (ARC) (Perth, Western Australia) under the approval of the CQUniversity Animal Ethics Committee (A11/03-268). Six week old male Wistar rats, purchased from ARC, were housed at the CQUniversity animal house under a 12hr light/dark cycle. They were provided with water and standard rat chow *ad libitum* for the duration of the treatment protocol. Each rat underwent a uninephrectomy to reduce renal capacity and assist with development of DOCA-induced volume overload. Pain management with meloxicam (0.1mg.kg^{-1}, Metacam)

was provided for 2 days following the procedure. Once recovered they were randomised into two groups, UNX and DOCA. All UNX rats received a sub-cutaneous injection of 0.4mL of dimethylformamide (DMF) every fourth day for 28 days as a vehicle control. They were either given no further treatment (UNX, n=22) or nebivolol ($0.5mg.kg^{-1}.day^{-1}$) (UNX+N, n=24). DOCA rats received 25mg of deoxycorticosterone-acetate (DOCA) in 0.4mL of DMF sub-cutaneously every fourth day for 28 days. Their water was replaced with 1% saline for the treatment period. This was to increase Na^+ intake and fluid retention, exacerbating the volume overload. Non-DOCA treated groups had access to *ad libitum* normal water. Half of the DOCA rats then received nebivolol ($0.5mg.kg^{-1}.day^{-1}$) (DOCA+N, n=20) or no further treatment (DOCA-salt, n=24).

3.2.3 Systolic blood pressure

Systolic blood pressure readings were performed at time points of 0, 2 and 4 weeks of treatment. Blood pressure was measured via the non-invasive tail cuff method, outlined by Fenning et al. (2005). Initial baseline measurements (0 weeks) were performed after uninephrectomy and before randomisation into treatment groups. The baseline results are reported as UNX 0 weeks. Before each rat was measured, they were immobilised by a light anaesthetic that was a combination of tiletamine ($15mg/kg^{-1}$ ip.) and zolazepam ($15\ mg/kg^{-1}$ ip.). A tail pulse transducer (MLT1010) was attached to the tail, after which an inflatable cuff [connected to a Capto SP844 physio-

logical pressure transducer (MLT844/D)] was attached. Pressure traces were captured using a PowerLab (ADInstruments, Sydney, Australia) connected to an iMac G4 computer. Each rat assessed was measured by 3 individual readings. Blood pressure results from each rat were grouped to generate a group mean.

3.2.4 Serum malondialdehyde determination

On day 28 of treatment, immediately following euthanasia, while the heart was still beating, blood samples from the abdominal vena cava were collected. This was via a syringe and needle and transferred into serum separator tubes, where it was allowed to clot. Once clotting had occurred, the tubes were centrifuged and the supernatant was removed and stored at -80°C until required for analysis. A commercially available kit for MDA analysis was used to test the samples (OxiSelect^{TM} MDA adduct competitive ELISA kit (*Cell Biolabs*, Inc, San Diego, CA, USA)). Manufacturer's instructions were followed and a standard curve was produced from the supplied standards using a 4-parameter logistic curve, against which the serum samples were interpolated.

3.2.5 Thoracic Aorta isolated organ baths

Following the methods of Fenning et al. (2005) the thoracic aorta was removed, submerged in cold Tyrode's physiological salt solution (NaCl 136.9, KCl 5.4, $MgCl_2.H_2O$ 1.0, $NaH_2PO_4.2H_2O$ 0.4, $NaHCO_3$ 22.6, $CaCl_2.2H_2O$ 1.8, glucose 5.5, ascorbic acid 0.3, Na_2EDTA 0.05) (TPSS) and carefully

dissected free of fat and connective tissue. Standardised segments were then cut and strung up in 30mL organ baths, where they were submerged in warm (37°C) carbogen (95% O_2/5% CO_2) gassed TPSS. Concentration-response curves were performed for noradrenaline (NA), acetylcholine (ACh) and sodium nitroprusside (NaNO). Pre-contractions for the ACh and NaNO relaxation curves were produced by sub-maximal concentrations of NA.

3.2.6 Mesenteric wire-myograph

Small vessel myograph was performed on the second order mesenteric arteries, as described by Danish Myo Technology (2008). Briefly, two millimetre (2mm) segments of artery were dissected out and threaded with 40μm stainless steel wire while submerged in cold Tyrode's physiological salt solution. Once one wire was successfully threaded through the vessel lumen it was attached to the force transducer tooth of a DMT 610M wire-myograph system (DMT, Denmark) and then the second wire was threaded and attached to the non-force transducer tooth. The DMT 610M was connected to a PowerLab 8/30 (ADInstruments, NSW, Australia) and an iMac computer running LabChart Pro 7 software recorded the data. Once all tissues had been normalised and subjected to a KCl challenge with a modified potassium Tyrode's solution (KPSS) (mM: NaCl 37mM, KCl 100, $MgCl_2.H_2O$ 1.0, $NaH_2PO_4.2H_2O$ 0.4, $NaHCO_3$ 22.6, $CaCl_2.2H_2O$ 1.8, glucose 5.5, ascorbic acid 0.3, Na_2EDTA 0.05) they were rinsed with standard Tyrode's physiological salt solution and rested. Concentration-response curves to NA, ACh

and NaNO were performed, separated by washout periods. Pre-contractions for ACh and NaNO curves were produced by sub-maximal concentrations of NA.

3.2.7 Single cell microelectrode

Action potential durations were assessed using a single cell microelectrode technique, as described by Fenning et al. (2005). A summary is provided below.

The heart was dissected out and submerged in cold Tyrode's (TPSS) that was continuously bubbled with carbogen gas. The atria and right ventricle were dissected off and the intraventricular septum cut. The papillary muscle was removed; one end was pierced with a stainless steel hook and the other was secured by a stainless steel pin into a 1mL chamber. The hook was attached to a modified micro-force transducer (SensoNor AE 801) so that the tissue was between two platinum electrodes. This chamber was continuously perfused with warm (37±0.5°C) gassed Tyrodes physiological salt solution at a rate of 3mL.min^{-1}. The micro-force transducer was connected to an iMac computer running Chart v5.5 via an amplifier (World Precision Instruments TBM-4). Once the papillary tissue was set up in the chamber, it was slowly stretched to a pre-load of 5-10mN. Electrical field stimulation (Grass SD-9) was used to induce contractions at a frequency of 1Hz, with a pulse width of 0.5msec and a stimulus strength of 20% above threshold. A potassium chloride filled glass electrode (World Precision Instruments, filamentated borosil-

icate glass; outer diameter 1.5mm; tip resistance 5-15mΩ when filled with 3M KCl) was used to impale the muscle, allowing electrical potential readings from a single cell to be recorded. A minimum of three separate impalements from varying regions of the muslce were recorded to determine mean action potential duration and related measures. Electrical recordings were captured using a Cyto 721 electrometer (World Precision Instruments) connected to an iMac running LabChart Pro v5.5 (AdInstruments) via a PowerLab 4/25.

3.2.8 Langendorff isolated heart preparation

Whole heart functional analysis was performed via a non-recirculating constant-pressure retrograde-perfused isolated heart system, similar to the methods published by Fenning et al. (2005) and Chan et al. (2011). Following euthanasia, the heart was rapidly dissected out and submerged in ice-cold modified Krebs-Henseleit buffer (KHB) (mM: NA 119.1, KCl 4.75, $MgSO_4$ 1.19, KH_2PO_4 1.19, $NaHCO_3$ 25.0, glucose 11.0 and $CaCl_2$ 2.16). The aorta was identified and cannulated via the dorsal root and perfused with warm (37°C), carbogen gassed KHB, at a constant pressure of 100mmHg. A latex balloon, connected to a Capto SP844 physiological pressure transducer (MLT 844/D), was inserted into the left ventricle before pacing the heart at 250bpm. Pacing was achieved by electrode stimulation of the right atria. The Capto SP844 was connected to a PowerLab 4/30 (ADInstruments, NSW, Australia), which was connected to an iMac computer running LabChart software. The balloon was filled until a starting pressure of 0mmHg was achieved. Pressure-

response curves of 5mmHg increments were performed, each pressure step being recorded for a minimum of 20 seconds. This was maintained until a maximum pressure of 30mmHg was achieved, at which point the ballon was emptied and removed from the heart. A linear plot of the tangent elastic modulus (E, dyne/cm^2) and the stress (σ, dyne/cm^2) was used to define the myocardial stiffness constant (k, dimensionless). The rate of pressure change (dP/dT), end systolic pressure (ESP) and developed pressure were also recorded via this technique.

3.2.9 Data capture and statistical analysis

All *in vivo*, *ex vivo*, and *in vitro* data was recorded using LabChart Pro software v5.5 running on Apple Mac computers. Statistical analysis was carried out using GraphPad Prism 6 statistical analysis software, with 2-factor ANOVA, One-way ANOVA and Student's T-tests being used to test for significance where appropriate. Comparison between groups was performed using 2-factor ANOVA, with Bonferroni *post hoc* analysis. Where individual comparisons were required, such as in assessing difference between specific organ bath response Studet's T-test was used. All data is reported as the mean $\pm$ standard error of the mean (SEM). For all tests, $P<0.05$ was considered as significant.

3.3 Results

3.3.1 Hypertension induction

Hypertension was successfully induced by DOCA-salt treatment in the control rats, with a mean increase in systemic BP of 75 ± 8.2 mmHg above baseline(Table 3.1). This increase in blood pressure was reduced by 4 weeks of treatment with nebivolol (DOCA+N 37 ± 7.8 mmHg lower than comparative controls) (Table 3.1). Nebivolol also induced a reduction in UNX BP, with UNX+N having a final BP of 102 ± 8.1 mmHg compared to 130 ± 6.4 mmHg (UNX) (Table 3.1).

3.3.2 Biometric measurements

Final body weights were significantly reduced at the termination of the experiment with DOCA-salt rats, irrespective of nebivolol treatment, having mean body weights of 351 ± 9.6g (DOCA) and 349 ± 6.9g (DOCA+N) compared to 444 ± 13.3g (UNX) and 463 ± 10.9g(UNX+N) (Table 3.1). The hearts of DOCA-salt rats showed significant hypertrophy, which was confined to the left ventricle (Table 3.1). Nebivolol treatment did not improve the LV hypertrophy observed in DOCA-salt hearts.

3.3.3 Serum malondialdehyde concentration

Serum concentration of madoldialdehyde was significantly increased in both the DOCA-salt and the DOCA+N rats, with values increased by 58.08 ± 22.99pmol/mL and 59.83 ± 19.60pmol/mL compared to UNX controls respectively (Table 3.1).

Table 3.1: Nebivolol: Effects on biometric, biochemical and haemodynamic parameters

	UNX	UNX+N ($0.5mg.kg^{-1}$)	DOCA	DOCA+N ($0.5mg.kg^{-1}$)
Body weight (g)				
	444 ± 13 (22)	463 ± 11 (24)	351 ± 10 (24) ‡	349 ± 7 (20) ‡
Organ weights (normalised to body weight, $g.kg^{-1}$)				
LV	2.15 ± 0.12 (14)	2.15 ± 0.09 (14)	2.91 ± 0.10 (18) ‡	3.05 ± 0.10 (15) ‡
RV	0.49 ± 0.03 (14)	0.48 ± 0.04 (14)	0.43 ± 0.05 (18)	0.58 ± 0.03 (15)
Liver	33.3 ± 0.8 (14)	34.4 ± 0.7 (14)	37.4 ± 1.4 (18)	40.9 ± 2.0 (15) ‡
Kidney	4.88 ± 0.10 (14)	4.91 ± 0.12 (14)	8.31 ± 0.33 (18) ‡	8.55 ± 0.28 (15) ‡
Spleen	2.66 ± 0.11 (14)	2.91 ± 0.15 (14)	3.11 ± 0.16 (18)	3.92 ± 0.19 (15) ‡ †
Blood Pressure (mmHg)				
0 Weeks	119 ± 6 (12)			
2 Weeks	116 ± 6 (11)	130 ± 13 (9)	162 ± 11 (5) ∗	134 ± 9 (9) †
4 Weeks	130 ± 6 (6)	103 ± 8 (7) ∗	194 ± 5 (7) ‡	157 ± 6 (6) † ‡
Biochemistry				
MDA (pmol/mL)	125.5 ± 4.1 (4)	144.5 ± 9.5 (6)	183.6 ± 22.6 (4) ∗	185.4 ± 15.5 (6) ∗

The numbers in parenthesis are the n= values.
LV - Left Ventricle, RV - Right Ventricle, NO - Nitric Oxide, MDA - Malondialdihyde.
∗ = $p<0.05$ vs UNX, § = $p<0.05$ vs UNX+N, ‡ = $p<0.05$ vs UNX/UNX+N,
† = $p<0.05$ vs DOCA.

Table 3.2: Nebivolol: Aorta EC50 values

	UNX (15)	UNX+N (14)	DOCA (13)	DOCA+N (13)
Noradrenaline	-6.67 ± 0.10	-6.16 ± 0.05 *	-7.24 ± 0.08 ‡	-6.73 ± 0.07 * †
Acetylcholine	-6.52 ± 0.09	-6.55 ± 0.07	-5.98 ± 0.11 ‡	-6.78 ± 0.17 †
Sodium nitroprusside	-7.64 ± 0.04	-7.32 ± 0.04 *	-6.80 ± 0.04 ‡	-6.95 ± 0.05 † ‡

All values are the logarithm of the EC50 (log[EC50]). Numbers in parenthesis are the minimum n= values for the group.
$*$ = p<0.05 vs UNX, ‡ = p<0.05 vs UNX/UNX+N, † = p<0.05 vs DOCA

Table 3.3: Nebivolol: Mesenteric artery EC50 values

	UNX (14)	UNX+N (13)	DOCA (15)	DOCA+N (11)
Noradrenaline	-5.90 ± 0.05	-5.96 ± 0.05	-5.40 ± 0.04 ‡	-5.84 ± 0.06 †
Acetylcholine	-6.90 ± 0.10	-6.55 ± 0.13 *	-6.98 ± 0.15 ‡	-6.17 ± 0.27 † *
Sodium nitroprusside	-6.50 ± 0.10	-6.68 ± 0.07 §	-7.34 ± 0.09 ‡	-6.29 ± 0.13 †

All values are the logarithm of the EC50 (log[EC50]). Numbers in parenthesis are the minimum n= values for the group.
$*$ = p<0.05 vs UNX, ‡ = p<0.05 vs UNX/UNX+N, † = p<0.05 vs DOCA, § = p<0.05 vs DOCA+N

3.3.4 Vascular function

Thoracic aorta functional analysis

Vascular function was altered by both DOCA-salt and nebivolol treatment. DOCA control rat aortas produced lower maximal contractions in response to NA (Fig. 3.1). Nebivolol-treated groups also showed reduced NA-induced maximal contractions (Fig. 3.1). Accompanying these results were shifts in EC50 values with nebivolol-treated rats showing a significant shift to the right (Table 3.2).

Vasodilation to ACh and NaNO was not improved in nebivolol-treated hypertensive animals; apart from the UNX+N response to ACh only the DOCA controls demonstrated any significant deviation from UNX control responses. Endothelium-dependent relaxation was significantly increased in DOCA aortas compared to the other groups (Fig 3.2), and endothelium-independent relaxation similarly showed greater relaxation (Fig. 3.3).

Mesenteric artery functional analysis

Concentration-response curves from mesenteric arteries showed no differences in maximal contraction or relaxation between any of the groups (Figs. 3.4, 3.5 & 3.6). The EC50s of NA, ACh and NaNO were all affected in DOCA controls; these effects were attenuated by nebivolol treatment (Table 3.3).

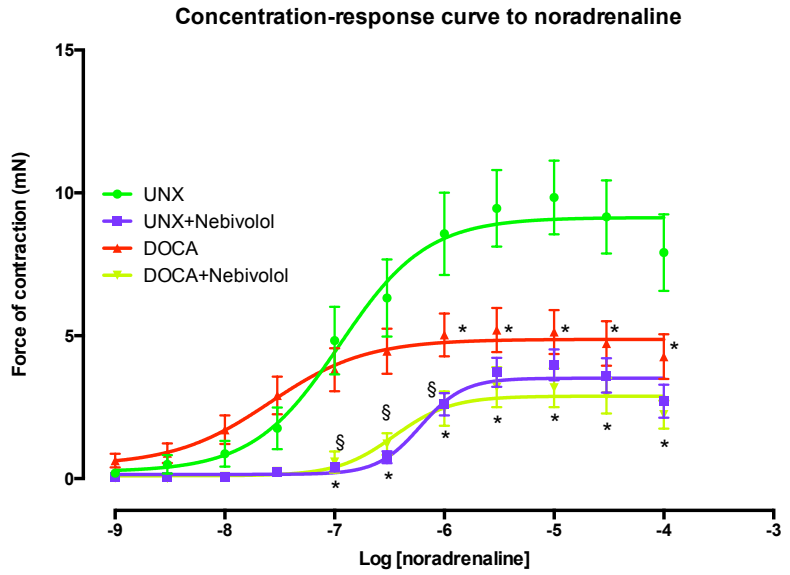

Figure 3.1: Thoracic aorta noradrenaline concentration-response curves. Lines of best fit produced by a non-linear four parameter regression analysis. UNX (n=17), UNX+N (n=14), DOCA (n=15), DOCA+N (n=14) $*$ = $p<0.05$ vs UNX, § = $p<0.05$ vs DOCA

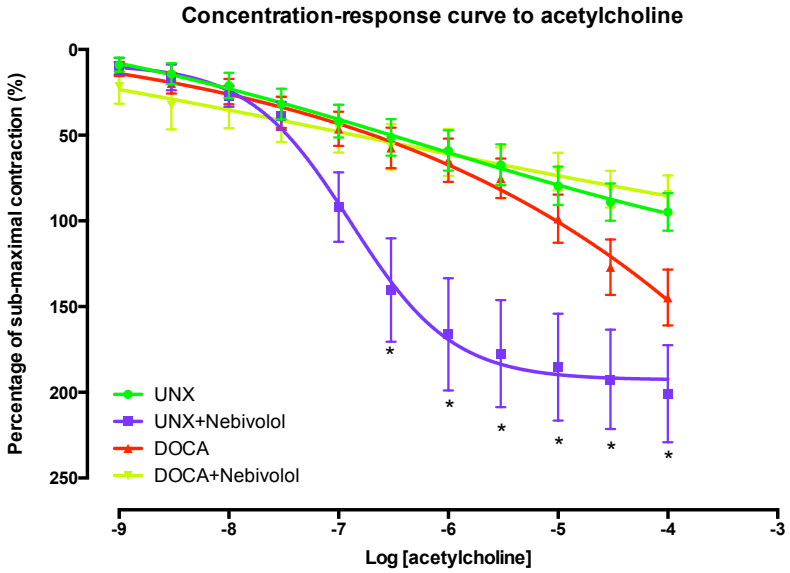

Figure 3.2: Thoracic aorta acetylcholine concentration-response curves, shown as a percentage of pre-contractile response. Lines of best fit produced by a non-linear four parameter regression analysis. UNX (n=18), UNX+N (n=15), DOCA (n=11), DOCA+N (n=7) $*$ = $p<0.05$ vs UNX

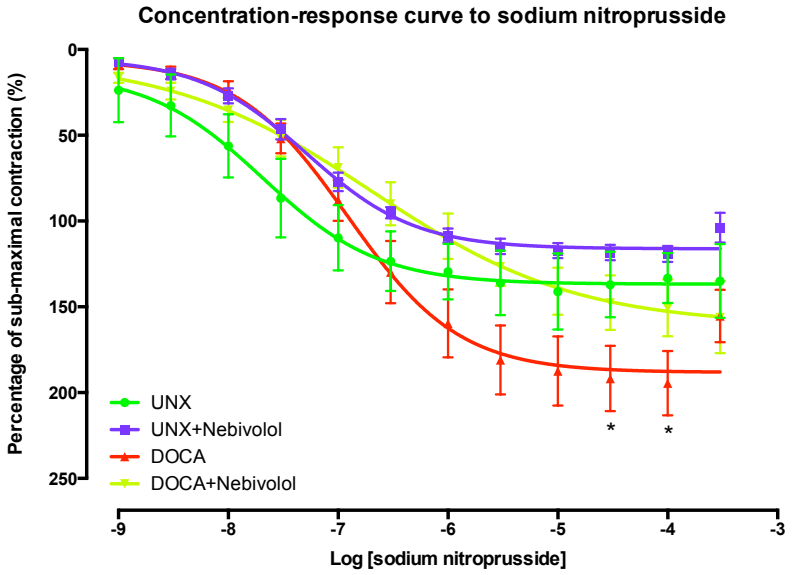

Figure 3.3: Thoracic aorta sodium nitroprusside concentration-response curves, shown as a percentage of pre-contractile response. Lines of best fit produced by a non-linear four parameter regression analysis. UNX (n=18), UNX+N (n=16), DOCA (n=12), DOCA+N (n=12) $*$ = p<0.05 vs UNX

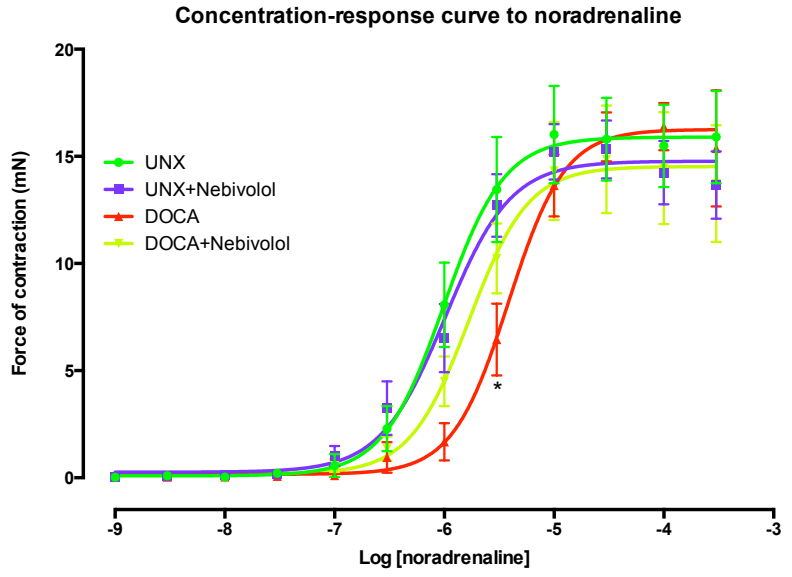

Figure 3.4: 2^{nd} order mesenteric artery noradrenaline concentration-response curve. Lines of best fit produced by a non-linear four parameter regression analysis. UNX (n=14), UNX+N (13), DOCA (n=15), DOCA+N (n=13) $*$ = p<0.05 vs UNX

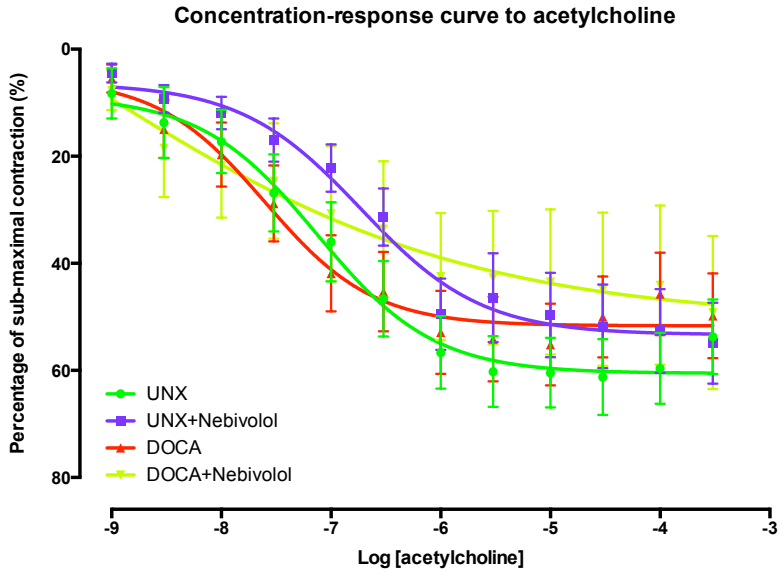

Figure 3.5: 2^{nd} order mesenteric artery acetylcholine concentration-response curve. Lines of best fit produced by a non-linear four parameter regression analysis. UNX (n=20), UNX+N (n=13), DOCA (n=18), DOCA+N (n=12)

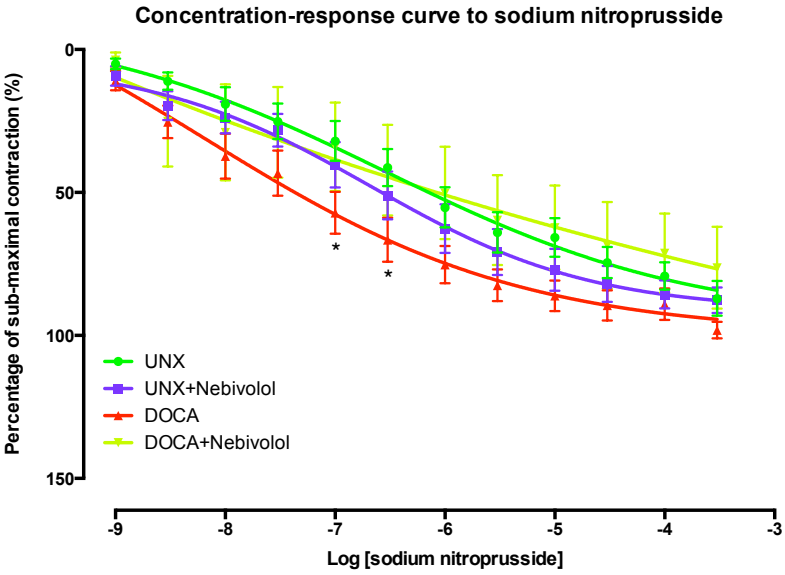

Figure 3.6: 2^{nd} order mesenteric artery sodium nitroprusside concentration-response curve. Lines of best fit produced by a non-linear four parameter regression analysis. UNX (n=19), UNX+N (n=8), DOCA (n=17), DOCA+N (n=6) $*$ = $p<0.05$ vs UNX

3.3.5 Cardiac function

Single cell microelectrode analysis revealed papillary muscles from DOCA rats had increased action potential durations. This was observed across all three points of measurement; 20%, 50% and 90% of repolarisation (Table 3.4), with the values observed being over double the duration of UNX rats. Nebivolol treatment did not reduce this prolongation, nor did it improve the diastolic stiffness constant of the myocardium (Table 3.4).

Myocardial compliance was improved by nebivolol treatment, with developed pressure, rate of contraction (max +dP/dT), rate of relaxation (max -dP/dT), and end systolic pressure all showing a significant improvement compared to the hypertensive controls (DOCA) (Table 3.4). These measures of myocardial function showed no significant difference to UNX values, showing that nebivolol was able to normalise myocardial compliance within the DOCA-salt rat, independent of left ventricular hypertrophy.

Table 3.4: Nebivolol: Cardiac Functional Analysis

	UNX	UNX+N ($0.5mg.kg^{-1}$)	DOCA	DOCA+N ($0.5mg.kg^{-1}$)
Electrophysiological Measurements				
APD 20% (ms)	11.90 ± 0.56 (10)	12.10 ± 0.69 (7)	23.77 ± 2.81 (10)‡	19.78 ± 2.38 (9)*
APD 50% (ms)	18.63 ± 1.34 (10)	19.10 ± 1.75 (7)	46.93 ± 6.36 (10)‡	38.33 ± 5.32 (9)*
APD 90% (ms)	54.80 ± 5.26 (10)	67.76 ± 5.52 (7)	120.3 ± 10.51 (10)‡	115.9 ± 12.38 (9)*
RMP (mV)	-63.15 ± 4.61 (10)	-73.01 ± 1.70 (7)	-58.43 ± 3.28 (10)	-53.89 ± 7.13 (9)‡
APA (mV)	58.07 ± 3.00 (10)	65.71 ± 2.15 (7)	60.63 ± 4.78 (10)	63.74 ± 6.95 (9)
Force (mN)	1.33 ± 0.29 (10)	0.99 ± 0.14 (7)	1.88 ± 0.49 (10)	1.90 ± 0.35 (9)
df/dt ($V.s^{-1}$)	0.38 ± 0.08 (7)	0.33 ± 0.05 (7)	0.52 ± 0.16 (5)	0.67 ± 0.08 (9)
dv/dt ($V.s^{-1}$)	14.19 ± 0.56 (7)	15.23 ± 0.42 (7)	14.73 ± 1.22 (5)	13.82 ± 1.42 (9)
Langendorff Measurements				
Diastolic Pressure (mmHg)	10.75 ± 0.50 (8)	10.50 ± 0.61 (10)	10.78 ± 0.47 (8)	10.82 ± 0.45 (10)
Dev. Pressure (mmHg)	133 ± 17 (8)	119 ± 19 (10)	81 ± 16 (8)*	122 ± 9 (10)†
Max +dP/dT ($mmHg.s^{-1}$)	2484 ± 327 (8)	2225 ± 360 (10)	1409 ± 283 (8)*	2136 ± 160 (10)†
Max -dP/dT ($mmHg.s^{-1}$)	-1762 ± 251 (8)	-1489 ± 271 (10)	-1031 ± 226 (8)*	-1521 ± 131 (10)†
ESP (mmHg)	135 ± 18 (8)	130 ± 19 (10)	92 ± 16 (8)*	132 ± 10 (10)†
Diastolic Stiffness	30.17 ± 1.43 (8)	31.16 ± 1.34 (10)	33.93 ± 1.01 (8)‡	34.31 ± 1.14 (10)‡

Numbers in parenthesis are the minimum n= values for the group.
$*$ = $p<0.05$ vs UNX, ‡ = $p<0.05$ vs UNX/UNX+N, † = $p<0.05$ vs DOCA.
APD = action potential duration, RMP = resting membrane potential, APA = action potential amplitude, df/dt = rate of force change as measured by micro-force transducer using electrical impedance measurements, dv/dt = rate of voltage change, max +dP/dT = maximal rate of contraction, max -dP/dT = maximal rate of relaxation, ESP = end systolic pressure

3.4 Discussion

As a 3^{rd} generation β-blocker, nebivolol is highly selective for the β_1 receptor and only shows non-selective properties once the dose exceeds 10mg in poor metabolisers (Münzel and Gori, 2009). As a racemic mixture of D- and L-isomers it is the D-isomer, that shows high selectivity for the β_1 receptor (Münzel and Gori, 2009), however it has also been shown to improve NO bioavailability. This effect has been attributed to a metabolite of nebivolol acting on the β_2 receptor to promote increased intracellular free $[Ca^{2+}]_i$ as well as increased expression of eNOS (Broeders et al., 2000; Georgescu et al., 2007). The involvement of the β_3 receptor has also been implicated, with nebivolol shown to evoke relaxation in the coronary microvasculature in the presence of β_{1-2} receptor blockade (Dessy et al., 2005). This effect was diminished in β_3 knockout mice, providing further evidence of a β_3 agonist effect (Dessy et al., 2005).

Nebivolol has also been extensively studied in rodent models, including spontaneously hypertensive rats (SHR) (Górska et al., 2010; Guerrero et al., 2006, 2003; Susic et al., 2012; Wang et al., 2013), Angiotensin II-treated rats (Oelze et al., 2006) and streptozotocin (STZ) diabetic rats (Mason et al., 2009). These studies have shown that nebivolol produces significant hypotensive effects, associated with decreased cardiac hypertrophy and collagen deposition (Guerrero et al., 2006, 2003); improvements in vascular response to ACh mediated relaxation and significant reductions in serum and left ventricular Ang II (Wang et al., 2013); reductions in Ang II-mediated superoxide

production by reducing the expression of NADPH-oxidase subunits $p67^{phox}$ and Rac1 (Oelze et al., 2006); and the improvement in bioavailability of NO in the vasculature of diabetic-hypertensive rats (Mason et al., 2009).

We have found very little published literature investigating nebivolol's use within the DOCA-salt hypertensive rat. The DOCA-salt hypertensive rat has shown to produce significant cardiovascular remodelling in a very short time, which is related to the renin-angiotensin system, sympathetic nervous system activation, and increased oxidative stress and inflammation (Beswick et al., 2001; Chan et al., 2011; Fenning et al., 2005; Landmesser et al., 2003; Somers et al., 2000).

Previous studies investigating nebivolol have used doses ranging from 2–20mg.kg^{-1}, a range associated with significant reductions in blood pressure (Górska et al., 2010; Guerrero et al., 2006, 2003; Mason et al., 2009; Wang et al., 2013). The aim of our study was to determine if nebivolol provided cardioprotective effects at a lower dose of 0.5mg.kg^{-1}.

Mean systolic blood pressure was significantly elevated within the DOCA-salt control animals. The magnitude of the hypotensive response induced by 0.5mg.kg^{-1}.day^{-1} nebivolol was greater than we had anticipated (after 4 weeks of treatment, 37 $\pm$ 7.8mmHg lower). In comparison to both Propranolol, a non-selective β-blocker, and Celiprolol, a β_1 selective antagonist that also exhibits eNOS and NO promoting actions, nebivolol showed a much greater propensity to induce a hypotensive response in the DOCA-salt rat. In a study by Tsuda and Masuyama (1991) DOCA-salt rats were administered Propranolol via drinking water, achieving an average dose of

16.6mg.kg^{-1}.day^{-1} and 21.7mg.kg^{-1}.day^{-1} across the two treatment groups. Neither of these doses achieved any significant effect on blood pressure (Tsuda and Masuyama, 1991). In two studies by Kobayashi et al. (2003, 2001), Celiprolol at a dose of 10mg.kg^{-1}.day^{-1} did not significantly reduce blood pressure in the DOCA-salt rat. Our results demonstrate the significant antihypertensive effect of nebivolol within the DOCA-salt rat model, however this was not associated with improvements to cardiac hypertrophy.

Left ventricular hypertrophy was observed in all DOCA-salt rats, including those that were treated with nebivolol. This demonstrates that remodelling within the DOCA-salt heart is not solely a compensatory mechanism to deal with elevated blood pressure, but rather the result of the underlying pathology of the model. Work by Kobayashi et al. (2003, 2001) also demonstrated this, showing Celiprolol improved LV hypertrophy without producing a significant hypotensive response. The contribution of oxidative stress to remodelling of the DOCA-salt heart was shown by Fenning et al. (2005) when they prevented the increase in BP and development of LV hypertrophy via L-Arginine supplementation. L-Arginine is a natural precursor of nitric oxide and by increasing it's availability, Fenning et al. (2005) were able to diminish the extent of LV hypertrophy observed in DOCA-salt rat hearts. Nebivolol also promotes NO production (Broeders et al., 2000; Georgescu et al., 2007), however our results demonstrate that at a dose of 0.5mg.kg^{-1}, any positive impact on LV hypertrophy associated with promoting NO production was not significant enough to prevent all of the cardiac remodelling within the DOCA-salt hypertensive rat.

Inspite of the significant effect on blood pressure by nebivolol, our study did not observe any significant improvement in electrophysiological parameters. Action potential duration was significantly prolonged within both the DOCA and DOCA+N hearts, indicating that the observed changes were independent of blood pressure elevation. Previous studies have observed that such changes in action potential duration may be a result of the increased hypertrophy of the cardiac tissue, where increased mass leads to decreased ion channel density, thereby reducing ion flux across the membrane and directly affecting the action potential (Fenning et al., 2005; Iyer et al., 2010; Loch et al., 2006a,b). Unlike those studies, in which moderate improvements in APD were achieved by regression of LVH (Fenning et al., 2005; Iyer et al., 2010; Loch et al., 2006a,b), nebivolol at a dose of 0.5mg.kg^{-1} was unable to achieve improvements in APD or LVH.

Notwithstanding this, myocardial function was improved by nebivolol treatment, with developed pressure, rates of contraction and relaxation, and end systolic pressure all showing a return to the values observed in the normotensive rats. In the absence of any observed improvement in oxidative stress, left ventricular hypertrophy or the diastolic stiffness constant, it can be inferred that the improvement in myocardial function is a result of reduced pressor load on the heart. This reduced workload of the heart is a direct effect of nebivolol's significant antihypertensive effect.

Aortic tissues from DOCA, DOCA+N and UNX+N rats showed significant impairment in contractility. The loss of contractile function within the DOCA groups is consistent with previous DOCA-salt studies (Brown et al.,

2000; Fenning et al., 2005), however nebivolol treatment did not show any improvement in contractile function. This is in contrast to Fenning et al. (2005), in which the NO precursor L-arginine was able to improve contractile function within the aortic tissue preparations. Aortic samples from DOCA-salt rats have been shown to be under significant oxidative stress (Somers et al., 2000) and considering our results showed serum MDA was not improved in DOCA+N rats, it can be concluded that nebivolol, at a dose of $0.5 mg.kg^{-1}$, does not sufficiently improve the state of oxidative stress, particularly within the vasculature of the DOCA-salt rat. Consequently, nebivolol is unable to prevent the vascular damage associated with oxidative stress. This conclusion is further supported by the fact that within our study nebivolol did not improve endothelium-dependent relaxation in response to ACh, an effect that is also consistent with the vascular damage that occurs within the DOCA-salt model (Fenning et al., 2005; Loch et al., 2006a). Concentration-response curves to ACh in aortic tissues showed nebivolol treatment to have enhanced relaxation in UNX rats only; potentially a result of nebivolol's ability to increase cellular NO levels (Georgescu et al., 2005).

The explanation for nebivolol's inability to promote improvements in the contractile response of aortic tissues to NA cannot be applied to the observed impairment in UNX+N rats. Therefore, there must be another mechanism by which adrenergic induced contraction is being affected, and this may also have implications for the observed DOCA+N responses. It is known that noradrenaline achieves smooth muscle contraction via stimulation of the α_1-adrenergic receptor. An *in vitro* study investigating the mechanistic actions

of nebivolol on thoracic aorta rings has shown it attenuates phenylephrine induced contraction (Rozec et al., 2006). The concentration-response curve of NA was shifted to the right in the presence of nebivolol; this was a dose-dependant effect (Rozec et al., 2006). Rozec et al. (2006) successfully demonstrated that nebivolol had an antagonistic effect on α_1-adrenergic receptors; an effect noted within our study also with EC50s in both DOCA+N and UNX+N aortas shifted to the right. The presence of an α_1 antagonistic action of nebivolol provides a simple explanation for the observed decrease in maximal contractile response observed within our UNX+N aortic tissue experiments.

The idea of nebivolol having an α_1-blocking ability is not one universally agreed upon. The review published by Ram (2010) stated that nebivolol did not have α_1-blocking activity, whereas an earlier review stated it did (Münzel and Gori, 2009). Our data, both the EC50 and the reduced maximal contractions achieved by NA, support the concept that nebivolol possesses α_1-antagonistic properties. Our study appears to be one of the first to establish this effect *in vivo*, and within the DOCA-salt rat. Future directions for this research include investigating if this *in vivo* effect is dose dependent, as well as comparing the contractile responses of chronically treated aortas to various contractile stimulants.

We conclude from this study that nebivolol is able to effectively reduce blood pressure within the DOCA-salt hypertensive rat, at a dose lower than has previously been published ($0.5 mg.kg^{-1}$). This was able to prevent the loss in myocardial compliance and whole heart function seen in the DOCA rats,

however cardiac remodelling was still maintained with LVH, diastolic stiffness and changes to APD not improved by treatment. Therefore, we conclude that the observed improvements are independent of the underlying pathology and are a result of a decrease in pressor load related to the reduction in blood pressure. Indeed, this shows just how potent the haemodynamic effects of nebivolol are. Additionally we have provided possible evidence for a *in vivo* α_1-antagonist effect, supporting work by Rozec et al. (2006). Given the contrasting opinions relating to the existence of an α_1-antagonist effect for nebivolol, further research is required before a conclusive decision can be formed.

3.4.1 Limitations

This study did not measure fibrosis within the cardiac tissues, data that would help elucidate the extent of nebivolol's actions within the myocardium. Understanding whether fibrosis was altered in the DOCA+N hearts would provide a greater understanding of how cardiac function was improved and why it did not translate to improvements in LV mass, diastolic stiffness or APD. Additionally, not only does collagen content affect compliance, but also collagen cross linking, something that could be assessed by using modified hydroxyproline assays.

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Chapter 4

Stevia rebaudiana extract reduces oxidative stress and attenuates cardiac electrophysiological and functional changes within the DOCA-salt hypertensive rat, without modulating left ventricular hypertrophy or blood pressure

Abstract

Stevia rebaudiana is a native South American plant that has been traditionally used as a sweetener, however it also possesses anti-diabetic and antihypertensive properties that may have beneficial effects for those using it. Stevia has been shown to significantly impact blood pressure within hypertensive patients. Our aim was to determine the extent to which stevia provides protection to the cardiovascular system of the DOCA-salt hypertensive rat.

Hypertension was induced and sustained by treatment with subcutaneous injections of deoxycorticosterone-acetate (DOCA) over a 28 day period following uninephrectomy. Stevia-treated rats received 200mg.kg^{-1}.day^{-1} via oral gavage. Blood pressure was measured at 2 and 4 weeks of treatment. After the 28 day treatment period the rats were euthanised for terminal experimentation. Vascular organ baths, intracellular electrophysiology and isolated whole heart studies were carried out. Serum concentrations of total nitric oxide (NO) and malondialdehyde (MDA) were assessed as markers for oxidative stress.

Stevia treatment did not alter blood pressure in the treated hypertensive rats (DOCA 194 ± 5mmHg v DOCA+stevia 178 ± 10mmHg), neither did it affect the development of left ventricular hypertrophy in the hypertensive rats. Markers of oxidative stress were significantly improved by stevia administration, with NO and MDA serum concentrations normalised to levels

comparable to the UNX groups. Action potential duration, developed pressure, rate of contraction and end systolic pressure were all improved in the DOCA+S rats. Despite these changes vascular function within DOCA+S rats was not improved by stevia treatment.

In conclusion *Stevia rebaudiana* extract was able to significantly improve oxidative stress, cardiac functionality and action potential duration within the hypertensive rat, independent of changes in blood pressure and left ventricular hypertrophy.

4.1 Introduction

Cardiovascular diseases is a leading cause of death within Australia and contributes to 18% of the overall burden of disease in Australia (Australian Institute of Health and Welfare, 2011). Lifestyle modification is strongly advised in the management of all patients with hypertension, regardless of drug therapy (National Heart Foundation of Australia (National Blood Pressure and Vascular Disease Advisory Committee) (NHFA), 2010). Not only is 30 minutes of moderate-intensity physical activity advised, but also dietary changes that include limiting salt intake to $\leq$ 4g/day (NHFA, 2010). Patients who are undertaking lifestyle changes may look towards dietary supplements, especially those that advertise potential cardiovascular health effects.

Stevia rebaudiana Bertoni extract (stevia) is a popular natural substitute for sugar and possesses many beneficial properties apart from being a non-caloric sweetener. Stevia shows remarkable antiglycemic, glucose transport modulation and pro-antioxidant properties (Rizzo et al., 2013; Shivanna et al., 2013) as well as antihypertensive effects in both animal and human studies (Chan et al., 2000, 1998; Hsieh et al., 2003). In spontaneously hypertensive rats (SHRs), stevia concentrations of 50, 100 and 200mg.kg^{-1}, when administered intravenously, caused acute reductions in blood pressure (Chan et al., 1998). At a dose of 200mg.kg^{-1}, stevia achieved a 31% decrease in blood pressure and the hypotensive response persisted for the duration of the study (60 min) (Chan et al., 1998). At the lower doses of 50 and 100mg.kg^{-1}, stevia still induced an acute drop in blood pressure, with systolic blood pres-

sure returning to normal levels within 30 and 60 mins respectively (Chan et al., 1998).

Vascular organ bath experiments have shown stevia's direct vasodilator effects in both aorta and mesenteric arteries (Bornia et al., 2008; Yesmine et al., 2013), providing evidence of a potential antihypertensive mechanism. Antioxidant properties have also been suggested, with Tadhani et al. (2007) showing *in vitro* antioxidant activity of the leaves and callus of the plant. However, no previous work has been performed on the effect of stevia within an oxidative stress-dominant model of hypertension.

The deoxycorticosterone-acetate (DOCA) hypertensive rat and mouse are widely utilised models of oxidative stress and hypertension, especially in relation to cardiovascular remodelling (Brilla et al., 1990; Fenning et al., 2005; Galisteo et al., 2004; Loch et al., 2007; Mirkovic et al., 2002). These models have allowed researchers to develop a greater understanding of the role molecular systems play in promoting oxidative stress and cardiovascular remodelling. Hallmarks of the model include significant blood pressure elevation, renal hypertrophy, left and right ventricular hypertrophy (Brown et al., 2000), as well as pro-oxidative stress mechanisms including superoxide production (Somers et al., 2000) and nitric-oxide synthase (NOS) uncoupling (Landmesser et al., 2003).

A number of studies have used the DOCA model to assess antioxidant targets in regards to treating hypertension and cardiovascular remodelling, including L-Arginine (Fenning et al., 2005), aminoguanidine (Chan et al., 2006), as well as flavanoids and polyphenols (Chan et al., 2011; Galisteo

et al., 2004; Jiménez et al., 2007). L-Arginine, the precursor for nitric oxide, was shown to significantly improve oxidative stress, left ventricular hypertrophy, cardiac scarring, diastolic stiffness, action potential duration and vascular function within the DOCA-salt rat (Fenning et al., 2005). These results provide evidence for the involvement of NO deficiency and oxidative stress in the pathology of DOCA-salt-induced hypertension. The dietary flavonoid quercetin was also able to improve oxidative stress within the DOCA-salt rat (Galisteo et al., 2004). Quercetin treatment lowered blood pressure and improved oxidative stress, demonstrated by decreased TBARS levels in the heart and plasma, (Galisteo et al., 2004). Similarly, red wine polyphenols were able to to reduce plasma MDA, plasma ET-1, urinary iso-$PGF_{2\alpha}$, NADPH oxidase activity, and decrease $O_2^{\bullet -}$ production within aortic rings (Jiménez et al., 2007).

The aim of this study was to assess the effect of stevia extract on a number of parameters within the DOCA-salt rat model including:

1. Blood pressure and cardiac function.
2. Electrophysiological changes within the cardiomyocyte.
3. Vascular reactivity and function within both large-conduit and small-resistance vessels.
4. Systemic oxidative stress and nitric oxide levels.

4.2 Materials and Methods

4.2.1 Drugs and treatment

Xylazil (Ilium Xylazil-20), Zoletil (Zoletil-100), and Lethabarb (Lethabarb euthanasia injection) were purchased from Pharmazo (QLD, Australia).

Deoxycorticosterone-acetate (DOCA), N,N-dimethylformamide [DMF $\geq$ 99%(Sigma)], acetycholine ($\geq$99% TLC), noradrenaline (99%), and sodium nitroprusside ($\geq$99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All drugs were dissolved in Milli-Q filtered water (Merk Millipore, Australia). Powdered *Stevia rebaudiana* extract (stevioside 90% and rebaudioside A 10% - HPLC verified) was obtained from Cargill Australia by the School of Science and Environment at CQUniversity, Rockhampton, Australia. Stock solutions were prepared by dissolving 2g of stevia extract in 10mL of Milli-Q filtered water, achieving a desired concentration of 200mg.mL^{-1}.

4.2.2 Animal preparation

Six week-old male Wistar rats were purchased from the Animal Resource Centre (Perth, Western Australia) under approval from the CQUniversity Animal Ethics Committee (A11/03-268). The rats were housed in a temperature controlled room (25$\pm$ 2°C) with a 12 hour light/dark cycle, and had *ad libitum* access to water and rat chow pellets. The rats did not undergo any treatment or surgical procedure until they were 8 weeks of age

and over 300g in weight. A uninephrectomy is performed to impair renal function, and aid in the development of DOCA-induced volume overload. At 8 weeks of age, each rat was anaesthetised by an intraperitoneal injection of zolazepam and tiletamine (25mg.kg^{-1}, Zoletil) together with xylazine (10mg.kg^{-1}, Rompun). A flank incision was made, the left renal vessels and ureter were ligated and the kidney was removed. Following the procedure, the incision site was sutured and the hide stapled. Pain management using meloxicam (Metacam 0.1mg.kg^{-1} sc.) was provided following the surgery for two days. Animals where then randomised into either DOCA-salt or UNX groups. Rats within the DOCA group were provided with 1% NaCl in the drinking water and received subcutaneous injections of deoxycorticosterone-acetate (25mg in 0.4mL of dimethylformamide (DMF) every 4^{th} day). UNX rats were given tap water to drink and received vehicle injections every 4^{th} day (0.4mL DMF sc.).

4.2.3 Experimental design

Rats were allocated to either DOCA control (n=23), DOCA+Stevia (n=22), UNX control (n=22) or UNX+Stevia (n=22). Stevia-treated rats received 100mg.kg^{-1} twice a day (morning and evening), via oral gavage, for a total of 200mg.kg^{-1}.day^{-1}. Treatment was maintained for 28 days, at which point animals were euthanised via a single intraperitoneal injection of sodium pentobarbitone (*Lethabarb* 375mg.mL^{-1}). A total dose of 200mg.kg^{-1}.day^{-1} was chosen, and administered in 100mg.kg^{-1} aliquots morning and night in order

to reduce the acute impact on blood pressure that occurred in response to 200mg.kg^{-1}, while also increasing the frequency of treatment allowing for variations in metabolism throughout the day. For a 300g rat, 150μL of stock solution would be administered at each dosing period (morning and evening), giving an overall dose of 200mg.kg$^{-1]}$.day^{-1}.

4.2.4 Systolic blood pressure

Blood pressure measurements were carried out at 0, 14 and 28 days of treatment. Baseline (0 days) BP measurements were carried out after uninephrectomy and before the rats were randomised into treatment groups. The results are reported as UNX 0 Weeks. The rats were lightly anaesthetised by a combination of tiletamine (15 mg/kg ip.) and zolazepam (15 mg/kg ip.). After rats were immobilised, a tail pulse transducer (MLT1010) and an inflatable tail cuff connected to a Capto SP844 physiological pressure transducer (MLT844/D) were attached. A PowerLab (ADInstruments, NSW, Australia) was used to capture the pressure traces.

4.2.5 Blood serum analysis

Immediately following euthanasia, while the heart was still beating and providing adequate blood circulation, a minimum 5mL blood was collected from the abdominal vena cava by syringe and needle. The sample was transferred to, and allowed to clot within, serum separator tubes. It was then centrifuged at 3000g for 15min and the supernatant removed and stored at -80°C until re-

quired for analysis. Serum malondialdehyde (MDA) analysis was performed using a commercially available kit (OxiSelect MDA Adduct ELISA Kit; Cell Biolabs, Inc, USA) and following the instructions provided by the supplier. Serum nitric oxide (NO) was also analysed using a commercially available kit (R&D Systems Total Nitric Oxide kit (R&D Systems, Minneapolis, United States of America)) and the supplier's instructions were followed.

4.2.6 Thoracic aorta organ bath

The thoracic aorta was dissected out, submerged in cold (4°C) Tyrode's physiological salt solution (mM: NaCl 136.9, KCl 5.4, $MgCl_2.H_2O$ 1.0, $NaH_2PO_4.2H_2O$ 0.4, $NaHCO_3$ 22.6, $CaCl_2.2H_2O$ 1.8, glucose 5.5, ascorbic acid 0.3, Na_2EDTA 0.05) and cleaned of fat. It was then cut into a 5mm segment that was mounted onto a force transducer and submerged in warm (37°C) Tyrode's solution, continuously bubbled with carbogen gas (95% O_2/5%CO_2). Vessels were allowed to acclimatise for 30 mins before being pretensioned to 10mN. Concentration-response curves (CRCs) were performed to noradrenaline (NA), acetylcholine (ACh) and sodium nitroprusside (NaNO). Pre-contractions for the ACh and NaNO curves were achieved by inducing a sub-maximal contraction, determined from the NA concentration-response curve. These curves assessed adrenergic-induced contractile function, endothelium-dependent and -independent relaxation. The results were normalised as a percentage of total relaxation in order for the effective concentration needed to induce a 50% response (EC50) to be calculated.

4.2.7 Mesenteric wire-myograph

Secondary branch mesenteric arteries were identified and dissected out while submerged in carbogen gassed Tyrode's physiological salt solution. The artery was cut to a 2mm length and then mounted using 40μm stainless steel wire within a DMT 610M multi wire-myograph system (DMT, Denmark), which was connected to a PowerLab 8/30 (ADInstruments, NSW, Australia) using LabChart Pro 7 software on an iMac computer. Once all vessels were mounted, heating was switched on and all baths were continuously bubbled with carbogen for 30 min until a target temperature of 37°C was reached. Vessels were then normalised following the normalisation protocols provided by DMT. After normalisation, the vessels were rested before each was subjected to a potassium challenge by flushing with potassium Tyrode's solution (mM: NaCl 136.9, KCl 100, $MgCl_2.H_2O$ 1.0, $NaH_2PO_4.2H_2O$ 0.4, $NaHCO_3$ 22.6, $CaCl_2.2H_2O$ 1.8, glucose 5.5, ascorbic acid 0.3, Na_2EDTA 0.05). This was to determine functionality of the tissues. The tissues were again rested before commencing cumulative concentration-response curves to NA, ACh, and NaNO. Pre-contraction for the acetylcholine and sodium-nitroprusside curves was achieved through a sub-maximal dose, determined from the noradrenaline concentration-response.

4.2.8 Single cell microelectrode

Single cell microelectrode analysis was carried out in the manner of Fenning et al. (2005) and is briefly described here. Immediately after euthanasia,

the left ventricular papillary muscle was rapidly excised while submerged in cold gassed (95% O_2/5% CO_2) Tyrode's physiological salt solution (described above) and a stainless steel hook was inserted into one end of the muscle. The hook and tissue were then placed in a 1.0mL experimental chamber between two platinum electrodes, which was continuously perfused with warm ($37 \pm 0.5°C$) gassed Tyrode's solution at a rate of 3 $mL.min^{-1}$. The hook was connected to a modified sensor element (SensoNor AE 801), connected to an amplifier (World Precision Instruments TBM-4) and the free end of the muscle was pinned in place using a stainless steel pin. The muscle was slowly stretched to a pre-load of 5-10 mN, over a period of one-minute. Once this was achieved, electric-field stimulation (EFS) (Grass SD-9) was used to induce contractions at a frequency of 1 Hz; pulse width 0.5msec and a stimulus strength of 20% above threshold. A potassium chloride-filled glass electrode (World Precision Instruments, filamentated borosilicate glass; outer diameter 1.5mm; tip resistance of 5-15 mΩ when filled with 3M KCl) was used to impale the muscle, with a silver/silver chloride electrode being used as reference. Readings were taken for a minimum of 20 min with at least 3 separate impalements for each tissue. Electrical activity was recorded using a Cyto 721 electrometer (World Precision Instruments) connected to an iMac running PowerLab Chart v5.5 (ADInstruments, NSW, Australia) via an analogue digital converter (PowerLab 4/25). The specific parameters recorded were, resting membrane potential (RMP), action potential amplitude (APA), action potential durations at 20%, 50%, 90% of repolarisation, and the force of contraction.

4.2.9 Langendorff isolated heart preparation

The heart was excised and placed in ice-cold modified Krebs-Henseleit buffer (mM: NaCl 119.1, KCl 4.75, $MgSO_4$ 1.19, KH_2PO_4 1.19, $NaHCO_3$ 25.0, glucose 11.0 and $CaCl_2$ 2.16), and then the aorta was canulated via the dorsal root. The heart was perfused with gassed (95% O_2/5%CO_2) modified Krebs-Henseleit buffer at constant pressure (100mmHg) and constant temperature (37°C). A latex balloon catheter connected to a Capto SP844 physiological pressure transducer (MLT 844/D), which was connected via a PowerLab 4/30 (ADInstruments, NSW, Australia) to an iMac computer running LabChart software. The balloon was inserted into the left ventricle via the mitral valve. The heart was paced at 250bpm by electrode stimulation of the right atria. Starting at 0mmHg, the end-diastolic pressure was increased by 5mmHg every 20 seconds, by increasing the balloon's volume, until a maximum of 30mmHg was achieved. Once an end-diastolic pressure of 10mmHg was achieved, the perfusate emptying from the heart was collected in order to determine a rate of perfusion of each heart. A linear plot of the tangent elastic modulus (E, dyne/cm^2) and stress (δ, dyne/cm^2) defined and provided the stiffness constant of the myocardium (k, dimensionless). Recordings of diastolic pressure, developed pressure, rate of change in pressure (+dP/dT -dP/dT), end systolic pressure, and the diastolic stiffness constant (k).

4.2.10 Statistical analysis

Statistical analysis included two-factor analysis of variance (2-ANOVA) with Bonferroni's *post hoc* analysis, one-way ANOVA with Bonferroni's *post hoc* analysis and Student's t-test where appropriate. All statistical analysis was performed using GraphPad Prism 6 software. Group comparisons were performed using 2-factor anova with *Bonferroni* correction. When comparison was required between two groups two-tailed Student's T-test was used. All results are presented as mean ± standard error of the mean (SEM). For all test, $P<0.05$ was classed as significant.

4.3 Results

4.3.1 Systolic blood pressure

Blood pressure was significantly increased after two weeks in DOCA-treated groups compared to UNX control animals (Table 4.1), and at the conclusion of the study, both DOCA and DOCA+S groups showed mean blood pressures in excess of 170mmHg. The healthy treated control rats (UNX+S) did not show any significant change in mean blood pressures, with both UNX and UNX+S groups having mean blood pressures of 130 ± 6mmHg and 136 ± 5 respectively at the conclusion of the study (Table 4.1).

4.3.2 Biometric measurements

DOCA-salt groups showed decreased weight gain, with final body weights 83 ± 11g lower than their UNX counterparts. When normalised for their body weights, both DOCA and DOCA+S rats showed significant hypertrophy of the heart. The left ventricular weights were significantly increased compared to the UNX control hearts (Table 4.1). Kidney hypertrophy was also present, with DOCA-treated animals showing over a 1.5-fold increase in kidney weight per kilogram of body weight (Table 4.1). DOCA-salt treatment also resulted in hypertrophy of the liver and spleen, which was not improved by stevia treatment (Table 4.1).

4.3.3 Serum markers

Total serum NO concentration was significantly diminished within the DOCA-salt group (Table 4.1). Serum concentrations of MDA were 2.5-fold higher in the DOCA compared to the UNX group. Both of these changes were prevented by stevia treatment, with DOCA+S rats showing serum concentrations comparable with the UNX controls (Table 4.1).

4.3.4 Vascular function

Aortic response to noradrenaline stimulation was diminished in the DOCA groups, with both DOCA and DOCA+S maximal contractions significantly reduced compared to the UNX groups (Fig. 4.1). The DOCA treatment resulted in the EC50 for acetylcholine shifting to the right (Table 4.2). This was accompanied by a significant increase in endothelial-dependent relaxation within the DOCA controls, compared to UNX controls (Fig. 4.2). Endothelium-independent relaxation was also significantly increased within the DOCA group, achieving a maximal relaxation of 195 $\pm$ 19% ($p<0.05$) of the pre-contraction (Fig. 4.3). Both DOCA and DOCA+S showed significant reduction in NaNO sensitivity, with EC50 values shifting to the right (Table 4.2).

Table 4.1: Stevia: Effect on biometric, biochemical and haemodynamic parameters

	UNX	UNX+S (200mg.kg^{-1})	DOCA	DOCA+S (200mg.kg^{-})
Body weight (g)				
	444 ± 13 (22)	406 ± 10 (20)	351 ± 10 ‡ (23)	334 ± 9 (22)‡
Organ Weights (normalised to body weight, g.kg^{-1})				
LV	2.15 ± 0.12 (14)	2.11 ± 0.23 (20)	2.91 ± 0.10 (18) ‡	3.22 ± 0.23 (22)‡
RV	0.49 ± 0.03 (14)	0.42 ± 0.04 (20)	0.43 ± 0.05 (18)	0.54 ± 0.04 (22)
Liver	33.3 ± 0.8 (14)	35.9 ± 0.7 (20)	37.4 ± 1.4 (18)*	40.0 ± 2.4 (22)*
Kidney	4.88 ± 0.10 (14)	4.71 ± 0.16 (20)	8.31 ± 0.33 (18)‡	8.60 ± 0.52 (22)‡
Spleen	2.66 ± 0.11 (14)	2.78 ± 0.20 (20)	3.11 ± 0.16 (18)*	3.54 ± 0.22 (22)‡
Blood Pressure (mmHg)				
0 Weeks	119 ± 6 (12)			
2 Weeks	116 ± 6 (11)	135 ± 4 (13)	162 ± 11 (5)*	153 ± 8 (11)*
4 Weeks	130 ± 6 (6)	136 ± 5 (12)	194 ± 5 (7)‡	178 ± 10 (8)‡
Biochemical Markers				
NO (μmol/L)	55.12 ± 6.24 (7)	56.22 ± 12.26 (6)	38.43 ± 4.97 (6)‡	66.01 ± 13.04 (5)†
MDA (pmol/mg)	14.80 ± 5.32 (5)	No data	39.35 ± 9.23 (4)∗	10.32 ± 2.55 (4)†

Numbers in parenthesis are the minimum n= values for the group.
∗=p<0.05 vs UNX, ‡=p<0.05 vs UNX/UNX+S, †=p<0.05 vs DOCA.
LV - Left Ventricle, RV - Right Ventricle, NO - Nitric Oxide, MDA - Malondialdihyde.

Table 4.2: Stevia: Aorta EC50 values

	UNX (15)	UNX+S (15)	DOCA (13)	DOCA+S (14)
Noradrenaline	-6.67 ± 0.10	-6.97 ± 0.10 *	-7.24 ± 0.08 ‡	-7.15 ± 0.11 ‡
Acetylcholine	-6.52 ± 0.09	-6.58 ± 0.07	-5.98 ± 0.11 ‡	-6.47 ± 0.07 †
Sodium nitroprusside	-7.64 ± 0.04	-7.41 ± 0.04 *	-6.80 ± 0.04 ‡	-6.93 ± 0.04 ‡

All values are the mean logarithm of the EC50 (log[EC50]). Numbers in parenthesis are the minimum n= values for the group.
∗=p<0.05 vs UNX, ‡=p<0.05 vs UNX/UNX+S, †=p<0.05 vs DOCA

Table 4.3: Stevia: Mesenteric artery EC50 values

	UNX (14)	UNX+S (13)	DOCA (15)	DOCA+S (17)
Noradrenaline	-5.90 ± 0.05	-5.64 ± 0.07 *	-5.40 ± 0.04 *	-5.38 ± 0.03
Acetylcholine	-6.90 ± 0.10	-7.22 ± 0.11 *	-6.98 ± 0.15	-6.39 ± 0.12 * †
Sodium nitroprusside	-6.50 ± 0.10	-6.68 ± 0.06	-7.34 ± 0.09 *	-6.97 ± 0.06 *†

All values are the mean logarithm of the EC50 (log[EC50]). Numbers in parenthesis are the minimum n= values for the group.
∗=p<0.05 vs UNX, †=p<0.05 vs DOCA

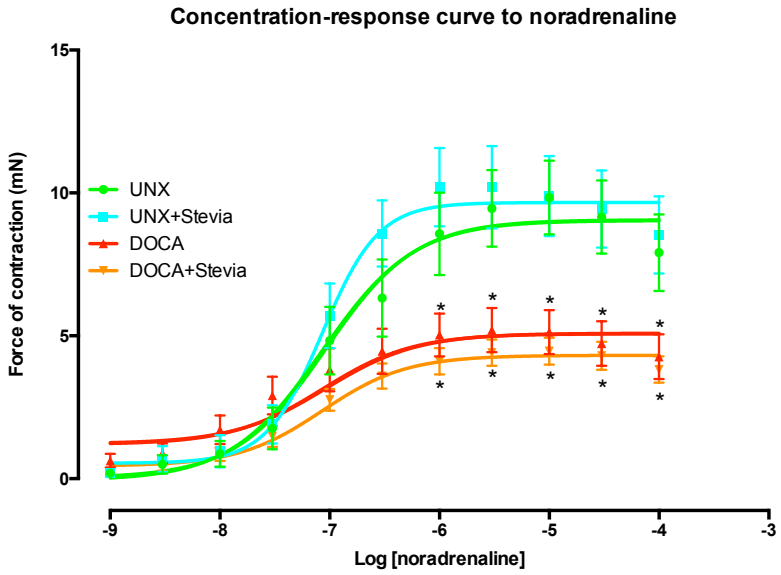

Figure 4.1: Thoracic aorta noradrenaline concentration-response curves. Lines of best fit produced by a non-linear four parameter regression analysis. UNX (n=17), UNX+S (n=16), DOCA (n=15), DOCA+S (n=14). $*$ = $p<0.05$ vs UNX/UNX+S

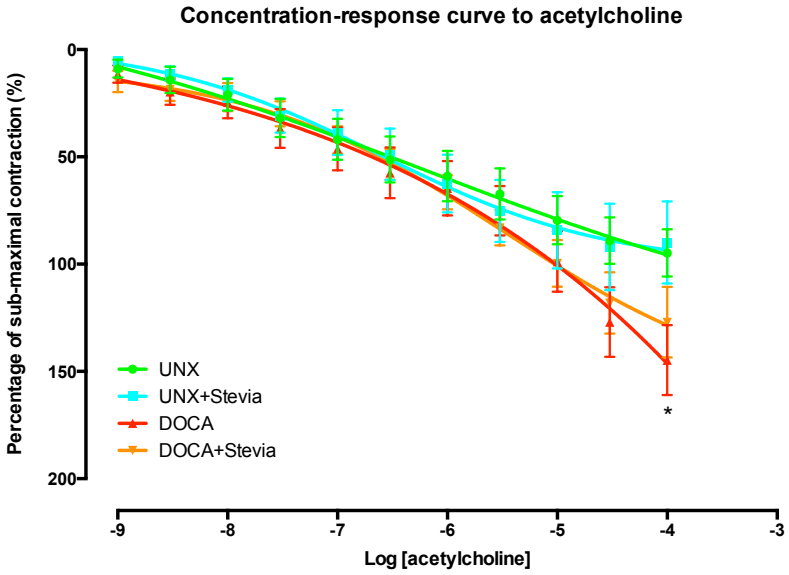

Figure 4.2: Thoracic aorta acetylcholine concentration-response curves. Lines of best fit produced by a non-linear four parameter regression analysis. UNX (n=15), UNX+S (n=18), DOCA (n=15), DOCA+S (n=19) $*$ = $p<0.05$ vs UNX/UNX+S

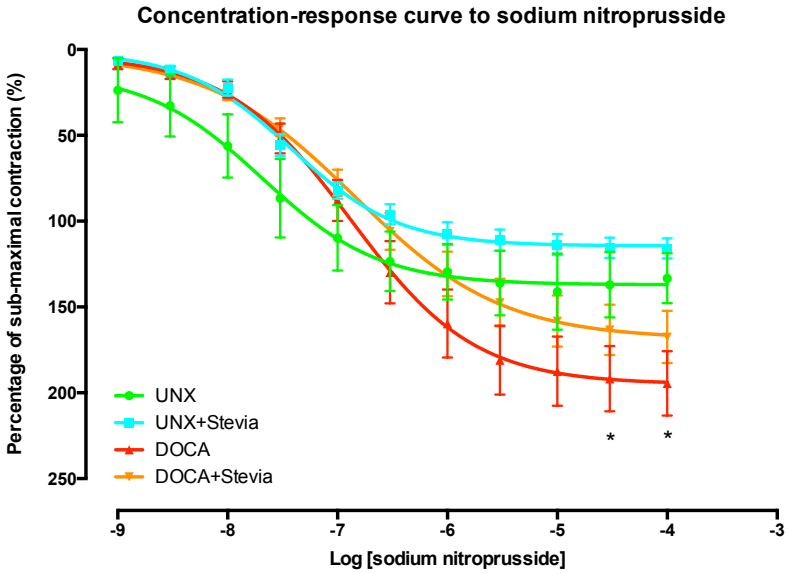

Figure 4.3: Thoracic aorta sodium nitroprusside concentration-response curves. Lines of best fit produced by a non-linear four parameter regression analysis. UNX (n=18), UNX+S (n=15), DOCA (n=13), DOCA+S (n16). $*$ = p<0.05 vs UNX/UNX+S

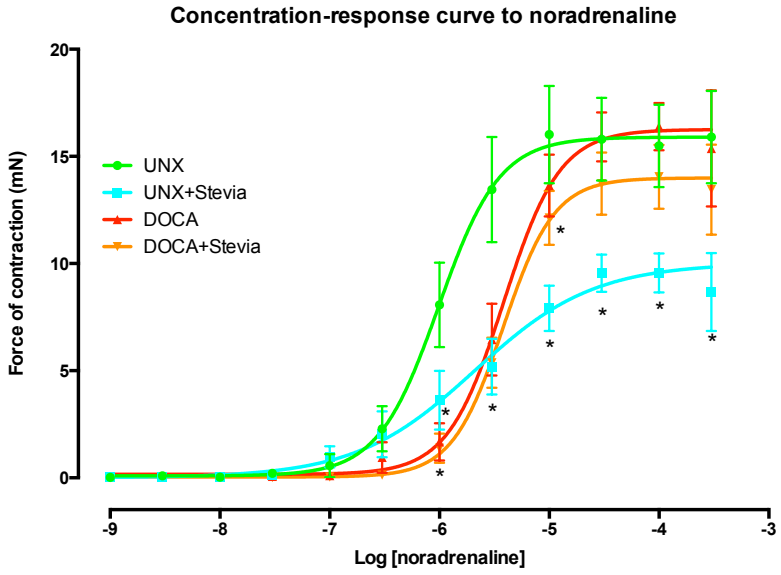

Figure 4.4: 2^{nd} order mesenteric artery noradrenaline concentration-response curve. Lines of best fit produced by a non-linear four parameter regression analysis. UNX (n=14), UNX+S (n=15), DOCA (n=15), DOCA+S (n=17). $*$ = p<0.05 vs UNX

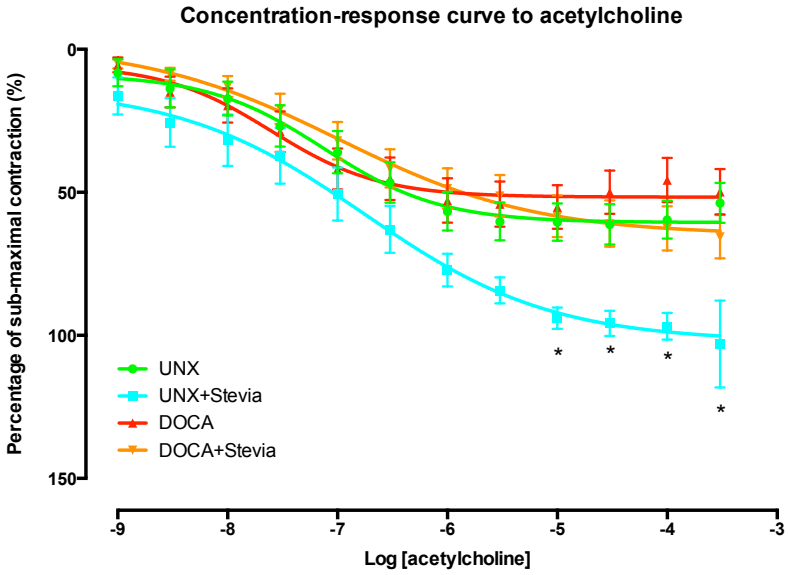

Figure 4.5: 2^{nd} order mesenteric artery acetylcholine concentration-response curve. Lines of best fit produced by a non-linear four parameter regression analysis. UNX (n=14), UNX+S (n=13), DOCA (n=18), DOCA+S (n=17) $*$ = p<0.05 vs UNX

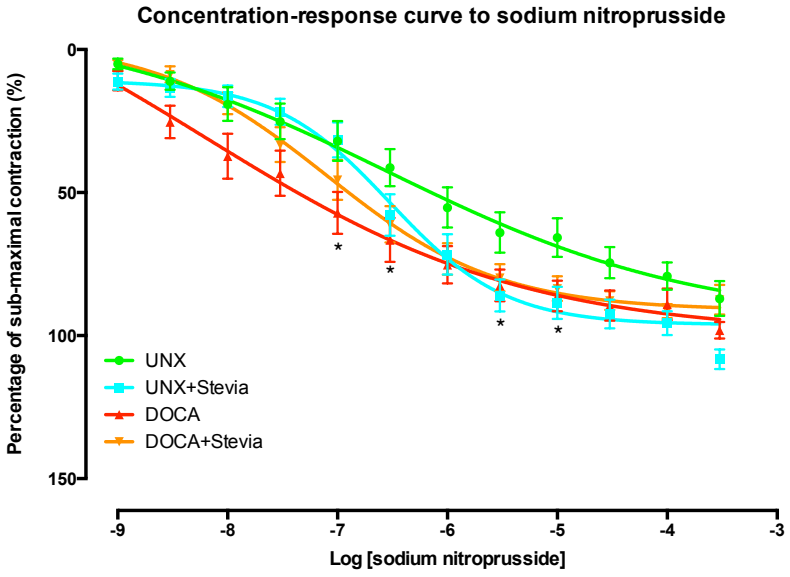

Figure 4.6: 2^{nd} order mesenteric artery sodium nitroprusside concentration-response curve. Lines of best fit produced by a non-linear four parameter regression analysis. UNX (n=15), UNX+S (n=14), DOCA (n=18), DOCA+S (n=17) $*$ = p<0.05 vs UNX

Mesenteric function was not adversely affected by DOCA-salt treatment; both DOCA and DOCA+S groups showed no significant reduction in maximal contraction (Fig. 4.4), endothelial-dependent relaxation (Fig. 4.5) or endothelial-independent relaxation (Fig. 4.6). A significant reduction in maximal contractile force was observed in stevia-treated UNX rats (Fig. 4.4), as was a significant increase in endothelial relaxation (Fig. 4.5). Endothelium-independent responses were unchanged by stevia treatment. Stevia treatment did alter EC50 values (Table 4.3), notably the UNX+S rats showed an increased sensitivity towards acetylcholine.

4.3.5 Cardiac function

Cardiac electrophysiology within the DOCA-salt control animals was severely affected, with action potential duration (APD) doubling in comparison to UNX control animals (Table 4.4). Measures of APD at 20, 50 and 90% of repolarisation demonstrated significant prolongation across the entirety of the repolarisation period (Table 4.4). Stevia extract prevented the prolongation of APD at 20 and 50% of repolarisation, and significantly reduced the increase of APD90 (Table 4.4). Assessment of cardiac function via the Langendorff isolated heart method revealed whole heart compliance was significantly diminished in the hypertensive DOCA-salt rats compared to the normotensive UNX rats (Table 4.4). Developed pressure from DOCA-salt control hearts showed an average decrease of 52 $\pm$ 24mmHg compared to UNX control hearts, and the speed of contraction (+dP/dT) and relaxation (-dP/dT) was

reduced by 1075 ± 433mmHg.s^{-1} and 731 ± 338mmHg.s^{-1} respectively (Table 4.4). Stevia treatment successfully prevented the decrease in contraction and relaxation rates (Table 4.4). Increased diastolic stiffness was observed in both the DOCA and DOCA+S hearts, indicating that 200mg.kg^{-1}.day^{-1} of stevia was not sufficient to reduce the development of myocardial stiffness (Table 4.4).

Table 4.4: Stevia: Cardiac functional analysis

	UNX	UNX+S (200mg.kg^{-1})	DOCA	DOCA+S (200mg.kg^{-1})
Electrophysiological Measurements				
APD 20% (ms)	11.90 ± 0.56 (10)	14.60 ± 0.45 (11)	23.77 ± 2.81 (10)‡	15.66 ± 0.56 (11)†
APD 50% (ms)	18.63 ± 1.34 (10)	23.55 ± 1.16 (11)	46.93 ± 6.36 (10)‡	28.91 ± 1.34 (11)†
APD 90% (ms)	54.80 ± 5.26 (10)	62.80 ± 3.98 (11)	120.3 ± 10.51 (10)‡	90.76 ± 5.48 (11)† ‡
RMP (mV)	-63.15 ± 4.61 (10)	-71.57 ± 2.16 (11)†	-58.43 ± 3.28 (10)	-60.86 ± 2.86 (11)
APA (mV)	58.07 ± 3.00 (10)	61.90 ± 2.52 (11)	60.63 ± 4.78 (10)	61.14 ± 4.49 (11)
Force (mN)	1.33 ± 0.29 (10)	1.71 ± 0.33 (11)	1.88 ± 4.88 (10)	1.15 ± 0.25 (11)
Langendorff Measurements				
Diastolic (mmHg)	10.71 ± 0.45 (8)	10.34 ± 0.14 (8)	10.78 ± 0.47 (8)	11.08 ± 0.45 (9)
Dev. P (mmHg)	133 ± 17 (8)	99 ± 16 (8)	81 ± 16 (8)*	119 ± 11 (9)†
+dP/dT (mmHg.s^{-1})	2312 ± 336 (8)	1824 ± 297 (8)	1409 ± 283 (8)*	2103 ± 186 (9)†
-dP/dT (mmHg.s^{-1})	-1628 ± 259 (8)	-1223 ± 226 (8)	-1031 ± 226 (8)*	-1496 ± 167 (9)
ESP (mmHg)	135 ± 18 (8)	109 ± 16 (8)	92 ± 16 (8)*	130 ± 11 (9)†
Stiffness (k)	30.33 ± 1.28 (8)	23.43 ± 4.72 (8)	33.93 ± 1.01 (8)‡	34.13 ± 1.90 (8)‡

Numbers in parenthesis are the minimum n= values for the group.
*=p<0.05 vs UNX, ‡=p<0.05 vs UNX/UNX+S, †=p<0.05 vs DOCA.
APD = action potential duration, RMP = resting membrane potential, APA = action potential amplitude, max +dP/dT = maximal rate of contraction, max -dP/dT = maximal rate of relaxation, ESP = end systolic pressure

4.4 Discussion

Hypertension was induced in the DOCA and DOCA+S rats, with both groups showing blood pressures in excess of 170mmHg. Studies within humans showed that long-term dosing of stevioside produced a significant drop in systolic and diastolic BP, an effect that was observed within 7 days of the patients starting treatment (Chan et al., 2000; Hsieh et al., 2003). Within the SHR animal model of hypertension, stevia induced an acute BP drop that was observed in response to doses of 50, 100 and 200mg.kg^{-1} (Chan et al., 1998). In contrast to the antihypertensive effect observed in genetic models of hypertension, our results showed stevia (200mg.kg^{-1}) had no effect on blood pressure within the hypertensive DOCA-salt rat (a non-genetic model).

Accompanying the elevation in blood pressure were significant changes to serum concentrations of nitric oxide and malondialdehyde. Nitric oxide is an antioxidant and vasodilator that reacts with reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet -}$), to produce peroxynitrite ($ONOO^-$). The accumulation of $ONOO^-$ is usually very low due to the relatively quick scavenging of superoxide by superoxide dismutase (SOD), and the rapid movement of NO into red blood cells and subsequent conversion to nitrate by oxyhemoglobin (Pacher et al., 2007). However, in a system under oxidative stress, superoxide concentrations are much higher (Ghosh et al., 2004) leading to a higher probability of NO being converted into $ONOO^-$ rather than nitrate. Both these molecules ($O_2^{\bullet -}$ and $ONOO^-$) are reactive and can have profound ef-

fects on many cellular molecules including nucleic acids, proteins and lipids (Pacher et al., 2007). The peroxidation of lipids, by reactive species, results in the production of malondialdehyde (MDA) and therefore it is an excellent marker of the level of oxidative stress within a system.

Our results show that the DOCA-salt rats were under significant oxidative stress, with serum concentrations of MDA significantly elevated and bioavailability of NO reduced. Stevia extract was able to normalise these changes, suggesting a significant direct effect on oxidative stress and NO bioavailability. In another study, L-Arginine, the precursor of NO, was shown to reduce oxidative stress and blood pressure within the DOCA-salt rat, indicating a central role for NO deficiency and oxidative stress in the pathology of the model (Fenning et al., 2005). By supplementing the rats with L-Arginine, Fenning et al. (2005) were able to promote increased production of NO, essentially promoting and exploiting nitric oxide's antioxidant capacity to reduce circulating oxidants such as superoxide. A study by O'Brien et al. (2009) targeted the source of ROS production, the mitochondrial respiratory chain, by supplementing the animals with L-Carnitine. L-Carnitine is a co-factor in the transport of free fatty acids to the mitochondria, and by increasing it's availability, O'Brien et al. (2009) were able to prevent the increase in oxidative stress and blood pressure associated with the DOCA-salt rat model. In contrast to both of these studies, our treatment significantly improved oxidative stress and increased NO bioavailability without impacting on blood pressure development over the period of the study.

Oxidative stress has been shown to contribute to vascular remodelling,

promoting collagen deposition and consequently increased stiffness in the thoracic aorta of DOCA-salt rats (Chen et al., 2013). Oxidative stress and ROS (notably O_2^-) have clearly been shown to increase aortic spontaneous tone in DOCA-salt rats (Ghosh et al., 2004) and it is this mechanism that may provide an explanation for the reduced maximal contractions observed in aortas from the DOCA-salt groups. As the aorta is already contracted by a significant amount, the ability for it to contract further is reduced, whereas the UNX groups started with a lower pre-contraction tone and therefore the tissue was able to generate more force. This mechanism also explains why the DOCA and DOCA+S groups were able to produce greater relaxations to ACh and NaNO. This effect was not observed within the mesenteric arteries, with UNX, DOCA and DOCA+S groups showing similar CRC profiles. The significant reduction in maximal contraction within the UNX+S rats may be a result of calcium channel modulation.

Previous research has shown stevia elicits a direct effect on both large conduit and small resistance vasculature (Bornia et al., 2008; Yesmine et al., 2013). The mechanism through which vasodilation is achieved appears to involve both endothelium-dependent and -independent pathways. Research by Bornia et al. (2008) demonstrated that nitric oxide synthase (NOS) inhibition reduced the ability of stevioside (a stevia extract) to induce vasodilation, but also noted that in denuded vessels, stevioside was still able to achieve relaxation. Following on from this, research conducted by Yesmine et al. (2013) demonstrated the mechanism of action may be dependent on vascular localisation. They showed that aortic tissue response could be reduced

by inhibiting NOS, while mesenteric arteries showed no significant loss of vasodilatory response to stevia in the presence of NOS inhibition (Yesmine et al., 2013). Stevia has also been shown to inhibit Ca^{2+} influx, but had no effect on intracellular Ca^{2+} release (Lee et al., 2001). This provides an explanation for the NOS-independent vasodilation described by both Bornia et al. (2008) and Yesmine et al. (2013), as well as the reduced mesenteric maximal contractions observed within the current experiment. Chronic modulation of Ca^{2+} influx would ultimately reduce the ability of the cell to "source" calcium from the extracellular environment, limiting intracellular Ca^{2+} available to produce a forceful contraction. This mechanism of action for stevia also explains the enhanced response to endothelial-dependent relaxation observed in the UNX+S mesenteric vessels. By limiting influx of Ca^{2+} into the cell during ACh-induced relaxation the interplay between vasodilation and contraction (resulting from NA in the organ bath) would be further pushed towards vasodilation, and consequently a greater vasodilatory response.

Cardiomyocyte analysis provides further evidence for a significant calcium channel modulation effect of stevia extract. Action potential durations, as measured by the single cell microelectrode, were shown to be prolonged within the DOCA control group. Work by Bénitah and Vassort (1999) has shown that mineralocorticoid receptor stimulation upregulates Ca^{2+} current (I_{Ca}) in adult rat cardiomyoctes, providing a mechanism for increased Ca^{2+} influx. Further research has shown that electrical remodelling occurs before cardiac hypertrophy, with upregulation of I_{Ca} and downregulation of K^+ transient outward current (I_{to}) contributing to the prolongation of APD

(Perrier et al., 2004). The fact that LV hypertrophy was still present in DOCA+S hearts removes the possibility that APD improvements were a result of decreased hypertrophy, and suggests that the observed prevention of prolongation is based on ionic channel modulation. Considering both I_{Ca} and I_{to} are affected by mineralocorticoid treatment, it is possible that stevia may achieve APD improvements through either calcium and/or potassium channel modulation. It is widely accepted that I_{to} is a prominent component of repolarisation (Aimond et al., 1999; Apkon and Nerbonne, 1991; Nerbonne and Kass, 2005; Wettwer et al., 1993), with I_{Ca} only contributing to the start of repolarisation. The ventricular myocytes of the rat show a very fast repolarisation, without the characteristic plateau seen in other species (Apkon and Nerbonne, 1991; Wettwer et al., 1993). This would suggest that if stevia was to act on K^+ channels to increase I_{to}, an effect would be seen across all points of repolarisation, rather than just across APD20 and 50, which can be argued are affected more by I_{Ca} than APD90 is.

The ability for stevia to attenuate and improve action potential duration independently of any reduction in LV hypertrophy is significant because in other studies the improvement of APD is observed in conjunction with improvements in LV hypertrophy and cardiac remodelling (Fenning et al., 2005; Iyer et al., 2010b; Loch et al., 2006a,b). In two papers by Loch et al. (2006a,b), improvements in APD and LV hypertrophy were observed in DOCA-salt rats treated with either Rosuvastatin or Omapatrilat. In both cases, Loch et al. (2006a,b) suggests that the observed reductions in APD are a result of decreased LV hypertrophy, resulting in increased I_{to} chan-

nel density. Furthermore, in both of these studies, treatment was only able to significantly improve APD90 within the DOCA-salt hearts (Loch et al., 2006a,b), whereas our study achieved normalisation of APD20 and APD50 and a significant improvement in APD90, showing an effect across the whole repolarisation cycle. In addition to improvements in the electrophysiological parameters, stevia also improved functional dynamics of the hypertensive hearts.

Compliance of the heart muscle was significantly decreased within the DOCA-salt rats, consistent with other DOCA-salt studies (Allan et al., 2005; Chan et al., 2006; Fenning et al., 2005). Stevia treatment improved the contractile capacity of the DOCA+S hearts by increasing rate of contraction as well as developed pressure within the LV. The rate of relaxation of the myocardium, though not statistically significant, did show a trend towards normalisation. Despite these positive changes in cardiac function, myocardial stiffness was not affected by stevia extract. Cardiac remodelling in the DOCA-salt rat, particularly cardiac fibrosis and inflammation, is independent of blood pressure elevation (Ammarguellat et al., 2002), and is susceptible to drugs that suppress inflammation (Iyer et al., 2010a,b; Mirkovic et al., 2002). Improving oxidative stress within the DOCA-salt rat has been shown to decrease the development of LV hypertrophy and associated changes in cardiac function, however it was unable to normalise the decrease in rate of contraction (+dP/dT) that occurred (Fenning et al., 2005). Therefore, it can be inferred that oxidative stress may not be solely responsible for this change, and stevia's ability to improve both +dP/dT and -dP/dT is

not achieved solely via modulating NO and eNOS, but may involve anti-inflammatory and ion channel effects.

4.4.1 Conclusion

Stevia's ability to attenuate pathological changes within the DOCA-salt hypertensive rat is more pronounced within the heart than in the vasculature. Oxidative stress plays a central role in the pathogenesis of the DOCA-salt rat, and given the inability of 200mg.kg^{-1} of stevia to prevent the development of hypertension or LV hypertrophy, we suggest that the observed reduction in oxidative stress was not a result of acute direct preventative actions by stevia, but rather through increased expression of antioxidant mechanisms mediated by stevia. Therefore, the full antioxidant effect of stevia was achieved via a time-dependent mechanism such as gene expression, rather than a direct effect on eNOS activity or ROS production. Furthermore, stevia extract provides cardioprotection within the DOCA-salt rat model, improving cardiac function as well as attenuating changes in cardiac electrophysiology. These changes significantly reduce the risk of dysrythmias occurring, and generally improve cardiovascular health. In conclusion, *Stevia rebaudiana* provides significant cardioprotection independent of improvements in blood pressure or LV hypertrophy within the DOCA-salt rat model of hypertension.

4.4.2 Study limitations and future directions

A limitation of this study is the fact that it did not investigate the effect of stevia on inflammation. Given the significant involvement inflammatory processes have in mediating collagen deposition within the myocardium, it would help to illuminate the potential reasons for the lack of LV hypertrophy regression that was observed. Additionally, now that significant end-organ protective and antioxidant effects have been demonstrated within this model of hypertension, future research into the specific molecular consequences of stevia treatment should be pursued. Assessment of potential impacts on eNOS gene expression and protein production, as well as eNOS phosphorylation states, would allow for a better understanding of how the antioxidant effects are achieved.

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Chapter 5

Lycium barbarum (goji berry) extract improves oxidative stress, prevents cardiac electrophysiological remodelling and preserves myocardial compliance within the DOCA-salt hypertensive rat

Abstract

Lycium barbarum (goji berries), traditionally used in Chinese medicine, possesses antioxidant, neuroprotective and cardioprotective abilities. This study investigated the ability of an extract of goji berries to prevent oxidative stress and cardiovascular remodelling in the DOCA-salt rat model of hypertension. Goji berry extract, at a dose of 200mg.kg^{-1}.day^{-1} for 28 days, reduced blood pressure (DOCA, 194 ± 5mmHg v DOCA+Goji, 160 ± 7mmHg) and normalised serum levels of MDA and NO. Cardiac electrophysiological remodelling was prevented, with action potential durations normalised by goji treatment (DOCA; APD20 - 24 ± 3ms, APD50 - 47 ± 6ms, APD90 - 120 ± 11ms v DOCA+G; APD20 - 16 ± 1ms, APD50 - 27 ± 2ms, APD90 - 81 ± 6ms [$p<0.05$]). Myocardial function was also improved by goji treatment, with DOCA+Goji hearts having greater rates of contraction and relaxation as well as improved developed and end systolic pressures when compared to hypertensive controls (DOCA). These improvements were produced without regression of left ventricular hypertrophy (LVH).

Vascular function, however, was not improved by goji extract, with aortic tissues from both DOCA and DOCA+Goji rats showing reduced responses to adrenergic stimuli. This study demonstrates that goji berry extract is able to reduce oxidative stress, attenuate the changes in cardiomyocyte electrophysiology, as well as preserve myocardial function despite persistent LVH, but fails to prevent the vascular damage and stiffening of the myocardium associated with the DOCA-salt model.

5.1 Introduction

Hypertension is characterised by blood pressure in excess of 140mmHg systolic and/or 90mmHg diastolic (National Institute of Health, 2004), and is a contributing factor to a number of cardiovascular diseases (Wang et al., 2006), as well as a co-morbidity in chronic and uncontrolled diabetes sufferers (Australian Institute of Health and Welfare, 2014). High blood pressure also leads to vascular damage, especially within the kidneys, where this can reduce blood supply and consequently kidney function (Australian Institute of Health and Welfare, 2014). It is universally acknowledged that hypertension, especially untreated, leads to the development of hypertrophy of the left ventricle. This is associated with changes in the function of the coronary vasculature, particularly the impairment of both endothelium-dependent and -independent vasodilation (Hamasaki et al., 2000). These changes appear to be a result of maximal vasodilation of these vessels in an attempt to increase blood flow and compensate for the increased energy demand of the hypertrophied heart (Hamasaki et al., 2000). This disparity between energy demand and supply is a result of abnormal coronary flow reserve, which is the maximal increase in coronary flow that can be achieved above a normal resting state.

Significant remodelling of the cardiovascular system is a hallmark of hypertension (Maron and Leopold, 2014; Nadruz, 2014), as is increased levels of oxidative stress (Schulz et al., 2008). The DOCA-salt rat is a model of hypertension characterised by rapid pathological progression, producing sig-

nificant electrophysiological and myocardial remodelling, as well as a significant elevation in oxidative stress (Brown et al., 2000; Chan et al., 2011, 2006; Chen et al., 2013; Fenning et al., 2005; Ghosh et al., 2004; Jiménez et al., 2007; Mirkovic et al., 2002). Results from DOCA-salt studies have shown the model to produce significant left ventricular hypertrophy (Brown et al., 2000; Fenning et al., 2005; Jiménez et al., 2007), increased oxidative stress (Chen et al., 2013; Galisteo et al., 2004), vascular dysfunction (Chan et al., 2006; Fatehi-Hassanabad et al., 2004; Ghosh et al., 2004), and cardiac electrophysiological and functional impairments (Fenning et al., 2005; Iyer et al., 2010b; Loch et al., 2006b). Treatments targeting the antioxidant and pro-nitric oxide mechanism have been able to attenuate many of these changes. When L-arginine, the precursor to nitric oxide, was supplemented into the food of DOCA-salt rats, improvements in oxidative stress, LVH, APD and vascular function were observed (Fenning et al., 2005). Furthermore, the flavonoid quercetin, which possesses strong antioxidant properties, was able to significantly reduce blood pressure and oxidative stress within the DOCA-salt rat, specifically elevating the antioxidant defences in the liver, kidney and heart tissues (Galisteo et al., 2004).

Extract from the goji berry (*Lycium barbarum*) has long been used in traditional Chinese medicines (Chang and So, 2008; Potterat, 2010) and research has shown it to provide antioxidant, neuroprotective and cardioprotective benefits (Amagase et al., 2009; Chang and So, 2008; Li, 2007; Li et al., 2007; Ming et al., 2009; Shao-Ping and Pin-Ting, 2010). The fruit itself contains a group of water-soluble glycoconjugates that are believed to

confer the medicinal properties (Amagase et al., 2009). The compounds, termed *Lycium barbarum* polysaccharides (LBPs), are often used as a standardised measure when utilising commercially-produced juices and power preparations. In humans, a daily dose of 1632mg (120mL of GoChi *Lycium barbarum* juice) of LBPs over 30 days produced significant increases in serum levels of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), as well as a corresponding decrease in malondialdehyde (MDA) (Amagase et al., 2009). *Lycium barbarum* polysaccharides have also been studied in the streptozotocin-diabetic rat (STZ), providing further evidence of LBP's ability to improve oxidative stress and increase antioxidant defences (Li, 2007). In the STZ study varying doses of LBP were administer over 30 days, resulting in improved blood glucose levels in a dose-dependent manner, with 200mg.kg^{-1} showing the greatest effect across all of the biochemical measures (Li, 2007). Very little research has been performed to determine if chronic treatment with goji berry extract is effective in conferring cardiovascular protection to the DOCA-salt heart and vasculature.

The aim of this study was to determine if goji berry extract (goji) provides cardioprotection to the DOCA-salt rat, particularly it's ability to prevent the loss of vascular and cardiac function seen within the DOCA-salt model. A dose of 200mg.kg^{-1} was chosen based on both the previous literature (Li, 2007), which showed it produced significant improvements in oxidative stress, and the need to maintain consistency between the two plant extracts within the larger study framework of this thesis.

5.2 Materials and Methods

5.2.1 Drugs and treatment

Zoletil (Zoletil-100), Xylazil (Ilium Xylazil-20) and Lethabarb (Lethabarb euthanasia injection) were sourced from Pharmazo (QLD, Australia). Noradrenaline (99%), acetylcholine (≥99% TLC), sodium nitroprusside (≥99%), N,N-dimethylformamide (DMF ≥99%, Sigma), and deoxycorticosterone-acetate (DOCA)(D7000, Sigma), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Capsules of *Lycium barbarum* extract (Natures Way - Goji Berry Standardized - 60 vegetarian capsules, standardised to 40% polysaccharides) were purchased from Evidencia Fitness & Health (VIC, Australia). The capsule contents were used to produce a stock solution of 200mg.mL^{-1} LBPs. All drugs, except DMF and DOCA, were made up using Milli-Q filtered water (Merk Millipore, Australia).

5.2.2 Animal Model

This experiment was performed under the approval of the CQUniversity Animal Ethics Committee (A11/03-268). Six week-old males Wistar rats were purchased from Animal Resource Centre (Perth, Western Australia) and housed in the animal house. A 12 hour daylight cycle was simulated and the temperature was maintained at 25 ± 2°C for the duration of the experiment. The rats had *ad libitum* access to both food and water while in the animal house. All rats underwent a uninephrectomy, which is required in

the DOCA-salt model in order to facilitate the development of volume overload. Surgery was only performed on rats that were over 300g in weight and 8 weeks of age. Surgical anaesthesia was induced by an intraperitoneal injection of zolazepam and tiletamine (25mg.kg^{-1}, Zoletil) and Xylazine (10mg.kg^{-1}, Rompun). After this, signs of a pain-response were tested and in the absence of any, the surgical procedure began. An incision was made in the flank of the rat, exposing the left kidney. The renal vessels and ureter were clamped, ligated, and the kidney removed using a scalpel. The ligation was checked to make sure that it was holding before the incision site was sutured and the hide stapled using surgical staples. Subcutaneous injections of meloxicam (Metacam, 0.1mg.kg^{-1}) were provided for pain management following surgery and maintained 2 days post surgery. Each rat was randomly assigned to one of the following groups; UNX, UNX+G, DOCA or DOCA+G. Rats in the UNX group were given vehicle injections of 0.4mL dimethylformamide (DMF), subcutaneously every 4^{th} day. The rats allocated to the DOCA groups were given 1% NaCl in their drinking water and received subcutaneous injections of 25mg deoxycorticosterone-acetate (DOCA) in 0.4mL DMF every 4^{th} day.

5.2.3 Experimental design

Experimental groups at the end of the treatment period were as follows: UNX (=20), UNX+G (n=22), DOCA (n=18), DOCA+G (n=25). Goji-treated rats received a standardised treatment of 200mg.kg^{-1} each day. The goji

berry extract was made up from commercially available capsules (Nature's Way, Goji Berry Standardized 60 Vcaps) to a standardised 200mg polysaccharides per mL (200mg LBP/mL). Dosing by oral gavage was carried out daily for the 28 day experimental period. Once the treatment period ended, the rats were euthanised via a single intraperitoneal injection of sodium pentobarbitone (Lethabarb 375mg.mL^{-1}). For a 300g rat a single dose of 300μL of stock solution would be administered via oral gavage. As the experiment progressed the amount of stock per dose was changed to match the change in weight of the rat.

5.2.4 Systolic blood pressure

Blood pressure was analysed using the tail-cuff method of Fenning et al. (2005), and performed at week 0, 2 and 4 of the treatment period. Baseline (0 week) measurements were performed after the rats recovered from the uninephrectomy and before they were randomised into treatment groups. The results are reported as UNX (0 week). Each rat undergoing blood pressure measurement was immobilised with an intraperitoneal injection of Tiletamine (15mg.kg^{-1}) and Zolazepam (15mg.kg^{-1}). A tail pulse transducer (MLT1010) and an inflatable tail cuff connected to a Capto SP844 physiological pressure transducer (MLT844/D) were attached, which was all connected to an iMac G4 via a PowerLab (ADInstruments, NSW, Australia). A minimum of three separate recordings per animal were captured and analysed.

5.2.5 Blood serum analysis

Immediately after death, an abdominal *vena cava* blood draw was performed while the heart was still beating and providing adequate circulation. Collection was performed using a syringe and needle, with a minimum of 5mL being obtained and transferred to a serum-separater tube. The samples were allowed to clot and spun down in a centrifuge at 3000g for 15min. The supernatant was removed and stored at -80°C until analysed. Commerically available assays were used to measure serum concentrations of malondialdehyde (MDA) (OxiSelect MDA Adduct ELISA KIT [Cell Biolabs, Inc]) and nitric oxide (NO) (R&D Systems Total Nitric Oxide kit [R&D Systems, Minneapolis, United States of America]). Each assay was performed according to the instructions provided by the manufacturer.

5.2.6 Thoracic aorta organ baths

Thoracic aorta tissue was analysed using an isolated organ bath system. Following euthanasia and blood collection, the thoracic aorta was dissected out and submerged in cold (4°C), carbogen-gassed (95% O_2,5% CO_2) Tyrode's physiological salt solution (mM: NaCl 136.9, KCl 5.4, $MgCl_2 \cdot H_2O$ 1.0, $NaH_2PO_4 \cdot 2H_2O$ 0.4, $NaHCO_3$ 22.6, $CaCl_2 \cdot 2H_2O$ 1.8, glucose 5.5, ascorbic acid 0.3, Na_2EDTA 0.5). It was then cleaned of fat and cut into 5mm segments before being threaded with stainless steel wire and submerged into warm (37°C) gassed (carbogen) Tyrode's physiological salt solution. The aortic tissue was allowed to acclimatise for 30 min before they were pretensioned

to 10mN. Concentration-response curves (CRCs) were then performed for noradrenaline (NA), acetylcholine (ACh) and sodium nitroprusside (NaNO). The results for ACh and NaNO were normalised to percentage of total response in order to determine EC50 values.

5.2.7 Mesenteric wire-myograph

Concentrations response curves were performed on 2^{nd} order mesenteric arteries that had been dissected out, while submerged in cold, carbogen-gassed Tyrode's physiological salt solution. Two millimetre (2mm) lengths were mounted within the myograph system (DMT 610M multi wire-myograph system (DMT, Denmark)), which was connected via a PowerLab 8/30 (ADInstruments, NSW, Australia) to an iMac computer running LabChart Pro 7 software. After all tissue samples were mounted within the myograph, the temperature was elevated to, and maintained at 37°C. Tissues were normalised following the protocols outlined by Danish Myograph Technologies (DMT, Denmark) and after a period of recovery were stimulated by flushing the bath with potassium Tyrode's solution (mM: NaCl 136.9, KCl 100, $MgCl_2 \cdot H_2O$ 1.0, $NaH_2PO_4 \cdot 2H_2O$ 0.4, $NaHCO_3$ 22.6, $CaCl_2 \cdot 2H_2O$ 1.8, glucose 5.5, ascorbic acid 0.3, Na_2EDTA 0.05). Following the KCl Tyrode's flush, fresh, normal Tyrode's physiological salt solution was used to fill the baths and the tissues were again rested before concentration-response curves (CRCs) to NA, ACh and NaNO were performed.

5.2.8 Single cell microelectrode

Intracellular electrophysiological analysis was performed as described by Fenning et al. (2005). This required the left ventricular papillary muscle to be rapidly dissected from the rat heart while submerged in cold Tyrode's physiological salt solution that was gassed with carbogen. Once the papillary muscle was excised from the heart, a stainless steel hook was inserted into one end and the other end was pinned to the base of a 1mL experimental chamber. This chamber was continuously perfused (3mL.min^{-1}) with warm (37°C), carbogen-gassed, Tyrode's physiological salt solution. The hook was connected to a modified SensoNor AE 801 sensor element, connected to a World Precision Instruments TBM-4 amplifier. The muscle was slowly stretched to a pre-load of 5-10mN. Contractions were then induced using a Grass SD-9 EFS system, at a frequency of 1Hz, pulse width of 0.5 msec and a stimulus strength 20% above threshold. A glass electrode (World Precision Instruments, filamentated borosilicate glass, outer diameter 1.5mm, tip resistance - 5-15mΩ when filled with 3M KCl) was filled with 3M KCl and used to impale individual cells within the papillary muscle. A silver/silver chloride electrode was used as a reference. Electrical readings were recorded for a minimum of 20 mins with at least 3 separate impalements taken per tissue. Recordings were captured by a Cyto 721 electrometer (World Precision Instruments) connected via a PowerLab 4/25 analogue digital converter to an iMac running PowerLab Chart v5.5 (ADInstruments, NSW, Australia). Parameters of resting membrane potential (RMP), action potential amplitude

(APA), action potential duration at 20%, 50%, 90% of repolarisation, and force of contraction, were all recorded and analysed.

5.2.9 Langendorff isolated heart preparation

The non-recirculating, constant-pressure Langendorff isolated heart technique was performed to assess whole-heart function and obtain the diastolic stiffness constant (k, dimensionless). This method followed the technique described by Fenning et al. (2005). Following euthanasia, the heart was dissected from the rat and placed in ice-cold modified Krebs-Henseleit buffer (mM: NaCl 119.1, KCl 4.75, $MgSO_4$ 1.19, KH_2PO_4 1.19, $NaHCO_3$ 25.0, glucose 11.0 and $CaCl_2$ 2.16), where it was rapidly cleaned of fat before being canulated via dorsal root of the aorta. The heart was then continuously perfused by warm (37°C), carbogen-gassed modified Krebs-Henseleit buffer at a constant pressure of 100mmHg. A latex balloon catheter was then inserted into the left ventricle via the mitral valve. The catheter was connected to a Capto SP844 physiological pressure transducer (MLT 844/D) which connected to an iMac computer via a PowerLab 4/30. LabChart software was used to record the data for analysis. Once the balloon catheter was in the left ventricle, the heart was paced at 250bpm via right atria electrode stimulation. The balloon was filled until an end diastolic pressure of 0mmHg was reached. End diastolic pressure was increased in 5mmHg increments by increasing the volume of water within the balloon. Each step was recorded for 20 seconds before moving to the next increment. This was continued until a

maximum of 30mmHg end diastolic pressure was achieved. A linear plot of the tangent elastic modulus (E, dyne/cm^2) and stress (δ, dyne/cm^2) defined and provided the stiffness constant of the myocardium (k, dimensionless). Recordings of diastolic pressure, developed pressure, rate of pressure change (+dP/dT -dP/dT), end systolic pressure, and the diastolic stiffness constant (k) were made and analysed.

5.3 Results

5.3.1 Systolic blood pressure measurement

DOCA-salt administration significantly increased blood pressure in both the control (DOCA) and goji-treated (DOCA+G) groups (Table 5.1). After 4 weeks of treatment, goji berry extract significantly ameliorated the rise in BP compared to untreated DOCA rats (Table 5.1). No significant hypotensive effect was observed in the UNX+G rats.

5.3.2 Biometric measurements

The body weight of the DOCA-salt groups was significantly lower than UNX controls ($\Delta = 67 \pm 11$g). Goji-treated rats showed a lower mean body weight compared with UNX controls (390 ± 12.47g v 444 ± 13.30g). DOCA treatment also produced hypertrophy of the left ventricle, which was not negated by goji treatment (Table 5.1). Goji berry extract produced a significant effect on right ventricle weights, with 2-way ANOVA finding a significant effect between control and goji-treated groups (Table 5.1). Kidney weights means were increased by 4.25 ± 0.19 g/kg from UNX values and mean liver weight was increased within goji-treated DOCA rats (Table 5.1).

5.3.3 Serum markers

Serum markers of oxidative stress were adversely affected by DOCA-salt treatment, with MDA more than doubling in concentration (Table 5.1). Ni-

tric oxide was also affected, with serum levels in the DOCA-salt controls decreased by 16.69 ± 8.2μmol/L (Table 5.1). Treatment with goji extract normalised circulating serum levels of MDA, and increased total serum NO, indicating it had a significant beneficial effect to reduce oxidative stress (Table 5.1).

5.3.4 Vascular function

Thoracic aorta functional analysis

Thoracic aorta function was diminished in both DOCA and DOCA+G groups, with reduced maximal contractions observed in CRCs to NA (Fig. 5.1). Associated with this decrease in maximal contraction was an increase in the EC50 concentration of NA, indicating increased sensitivity to adrenergic stimulation (Table 5.2). Acetylcholine-induced relaxation was not impaired by DOCA or goji treatment, with no statistically significant alterations in maximal relaxation when normalised to pre-contractile tension (Fig. 5.2). Goji treatment did result in a decrease in the EC50 concentration of ACh in both UNX and DOCA-treated rats when compared to their respective control groups (Table 5.2). Endothelium-independent relaxation was significantly greater in the DOCA control group when compared to UNX controls and no significant difference was established between DOCA or DOCA+G (Fig. 5.3).

Mesenteric artery functional analysis

arteries showed very little difference in overall function, with all groups achieving similar maximal contractions and relaxations across all three functional assessments (Figs. 5.4, 5.5, 5.6). Noradrenaline sensitivity was reduced in all treatment groups compared to UNX controls; this was most significant in DOCA controls rats (Table 5.3). Goji-treated rats, both UNX+G and DOCA+G, showed a similar noradrenaline EC50 (Table 5.3). Both DOCA and DOCA+G rats showed decreased EC50s for sodium nitroprusside compared to UNX groups (Table 5.3).

5.3.5 Cardiac function

Action potential duration at 20, 50 and 90% of repolarisation were prolonged in the DOCA group, with each time point showing a greater than doubling of the duration (Table 5.4). This prolongation was fully attenuated by goji treatment, with all measures of repolarisation showing no significant difference to healthy UNX controls (Table 5.4).

An increase in the diastolic stiffness constant was observed in the DOCA-salt rats and this was not prevented or improved by goji treatment (Table 5.4). Other functional measures were also diminished in the DOCA controls, with developed pressure, rates of contraction and relaxation, and end systolic pressure all showing significant decreases in their values (Table 5.4). All of these functional measures were normalised by goji extract, except for rate of relaxation (Table 5.4). Although the change in rate of relaxation of

DOCA+G hearts was not statistically significant, a trend towards significance was observed (p=0.056, one-tailed t-test).

Table 5.1: Goji: Effect on biometric, biochemical and haemodynamic parameters

	UNX	UNX+G (200mg.kg^{-1})	DOCA	DOCA+G (200mg.kg^{-1})
Body Weight (g)				
	444 ± 13 (22)	390 ± 13 (22) ∗	351 ± 10 (23) ‡	350 ± 7 (25) ‡
Organ Weights normalised to body weight (g.kg^{-1})				
LV	2.15 ± 0.12 (14)	2.12 ± 0.08 (22)	2.91 ± 0.10 (18)‡	3.22 ± 0.23 (25)‡
RV	0.49 ± 0.03 (14)	0.52 ± 0.03 (22)§	0.43 ± 0.05 (18)	0.56 ± 0.03 (25)§
Liver	33.3 ± 0.8 (14)	33.5 ± 0.8 (22)	37.4 ± 1.4 (18)	40.6 ± 1.9 (25)‡
Kidney	4.88 ± 0.10 (14)	4.75 ± 0.12 (22)	8.31 ± 0.33 (18)‡	8.47 ± 0.39 (25)‡
Spleen	2.66 ± 0.11 (14)	2.96 ± 0.13 (22)	3.11 ± 0.16 (18)	3.33 ± 0.16 (25)∗
Blood Pressure (mmHg)				
0 Weeks	119 ± 6 (12)			
2 Weeks	116 ± 6 (11)	111 ± 6 (4)	162 ± 11 (5)‡	180 ± 8 (3)‡
4 Weeks	130 ± 6 (6)	129 ± 4 (8)	194 ± 5 (7)‡	160 ± 7 (11)‡ †
Biochemical Markers				
NO (μmol/L)	55.12 ± 6.24 (7)	61.68 ± 7.99 (4)	38.43 ± 4.97 (6)‡	66.82 ± 10.98 (5)†
MDA (pmol/mL)	14.80 ± 5.32 (5)	No data	39.35 ± 9.23 (4)*	15.79 ± 4.53 (6)†

Numbers in parenthesis are the minimum n= values for the group.
* = p<0.05 vs UNX, ‡ = p<0.05 vs UNX/UNX+G, † = p<0.05 vs DOCA, § = p<0.05 (Goji effect).
LV - Left Ventricle, RV - Right Ventricle, NO - Nitric Oxide, MDA - Malondialdehyde,

Table 5.2: Goji: Aorta EC50 values

	UNX (n=15)	UNX+G (n=18)	DOCA (n=13)	DOCA+G (n=13)
Noradrenaline	-6.67 ± 0.10	-6.61 ± 0.06	-7.24 ± 0.08 ‡	-7.11 ± 0.20 ‡
Acetylcholine	-6.52 ± 0.09	-7.26 ± 0.09 *	-5.98 ± 0.11 ‡	-6.51 ± 0.09 †
Sodium nitroprusside	-7.64 ± 0.04	-7.41 ± 0.04 *	-6.80 ± 0.04 ‡	-6.93 ± 0.04 † ‡

All values are the logarithm of the EC50 (LogEC50). The minimum n= value is shown in parenthesis for each group.
$*$ = $p<0.05$ vs UNX, ‡ = 0.05 vs UNX/UNX+G, † = $p<0.05$ vs DOCA

Table 5.3: Goji: Mesenteric artery EC50 values

	UNX (n=14)	UNX+G (n=17)	DOCA (n=15)	DOCA+G (n=19)
Noradrenaline	-5.90 ± 0.05	-5.75 ± 0.05 *	-5.40 ± 0.04 ‡	-5.68 ± 0.03 * †
Acetylcholine	-6.90 ± 0.10	-6.73 ± 0.09	-6.98 ± 0.15	-6.87 ± 0.15
Sodium nitroprusside	-6.50 ± 0.10	-6.55 ± 0.04	-7.34 ± 0.09 ‡	-7.03 ± 0.08 † ‡

All values are the logarithm of the EC50 (LogEC50). The minimum n= value is shown in parenthesis for each group.
$*$ = $p<0.05$ vs UNX, ‡ = 0.05 vs UNX/UNX+G, † = $p<0.05$ vs DOCA

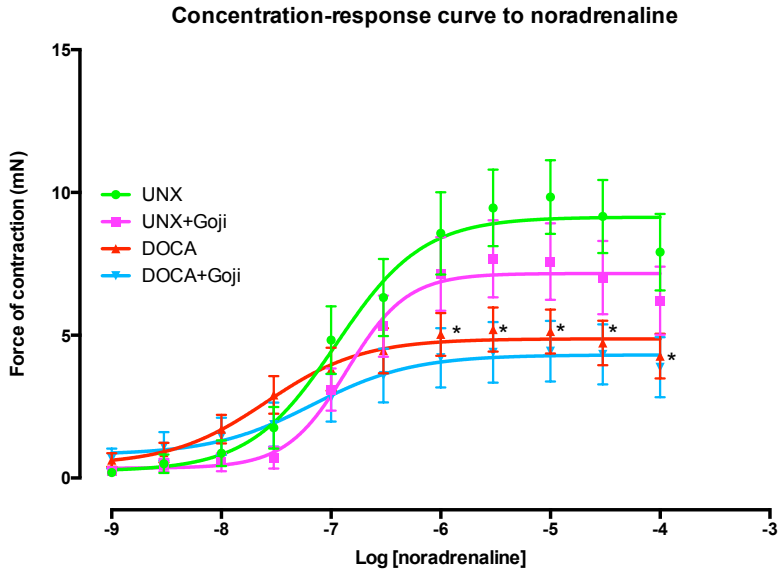

Figure 5.1: Thoracic aorta noradrenaline concentration-response curves. Lines of best fit produced by a non-linear four parameter regression analysis. UNX (n=17), UNX+G (n=18), DOCA (n=15), DOCA+G (n=13).
$*$ = $p<0.05$ vs UNX

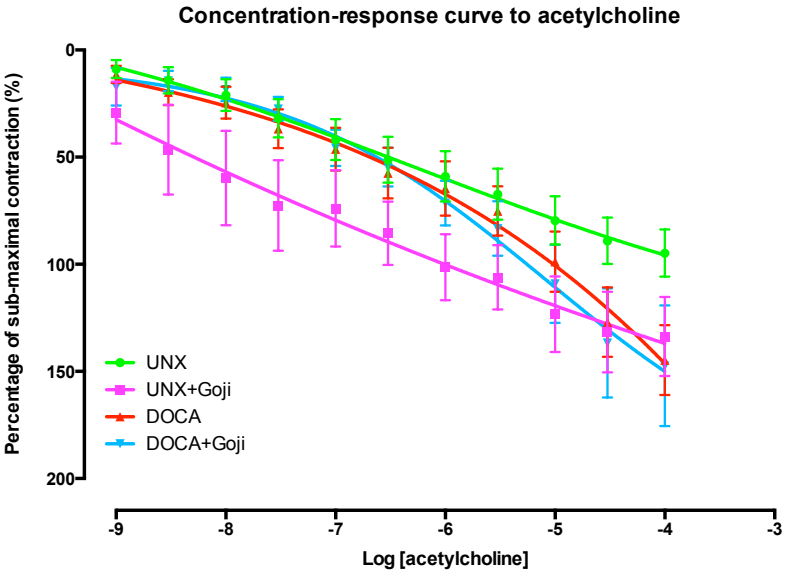

Figure 5.2: Thoracic aorta acetylcholine concentration-response curves. Lines of best fit produced by a non-linear four parameter regression analysis. UNX (n=15), UNX+G (n=19), DOCA (n=15), DOCA+G (n=17).

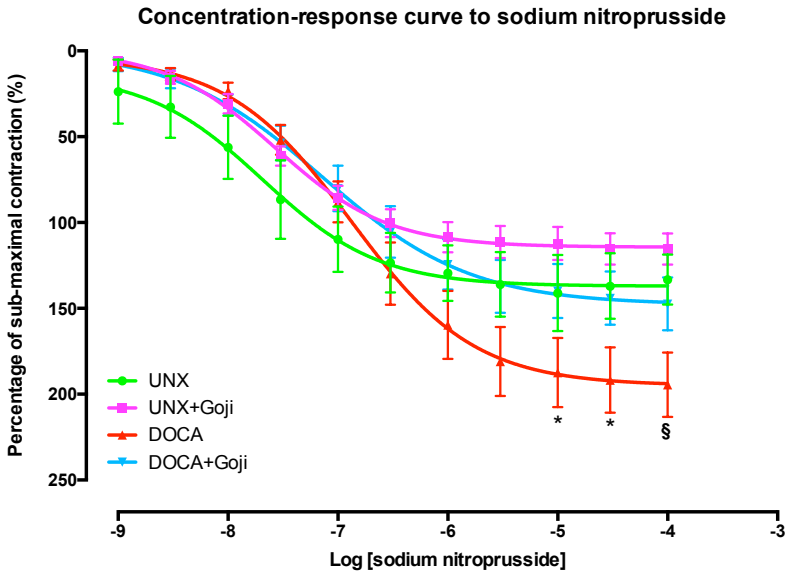

Figure 5.3: Thoracic aorta sodium nitroprusside concentration-response curves. Lines of best fit produced by a non-linear four parameter regression analysis. UNX (n=18), UNX+G (n=18), DOCA (n=13), DOCA+G (n=15)
$*$ = $p<0.05$ vs UNX+G, § = $p<0.05$ vs UNX/UNX+G

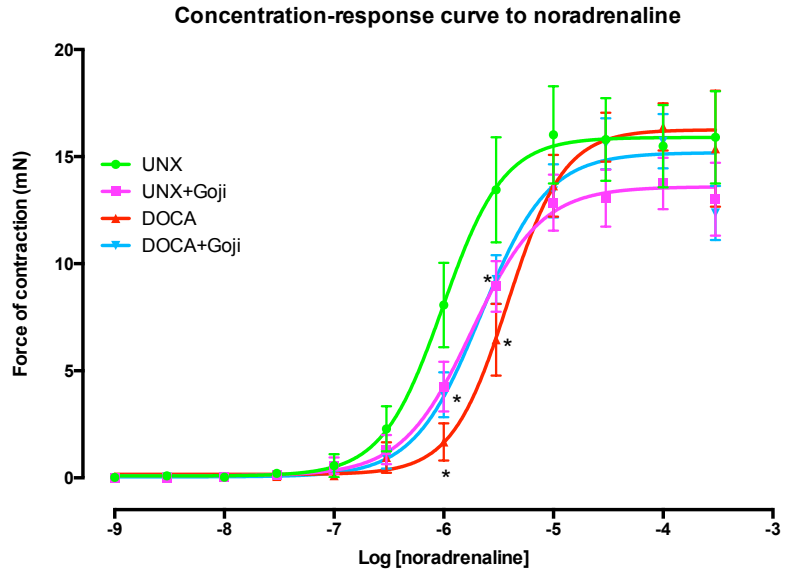

Figure 5.4: 2^{nd} order mesenteric artery noradrenaline concentration-response curve. Lines of best fit produced by a non-linear four parameter regression analysis. UNX (n=14), UNX+G (n=18), DOCA (n=15), DOCA+G (n=20).
$*$ = $p<0.05$ vs UNX

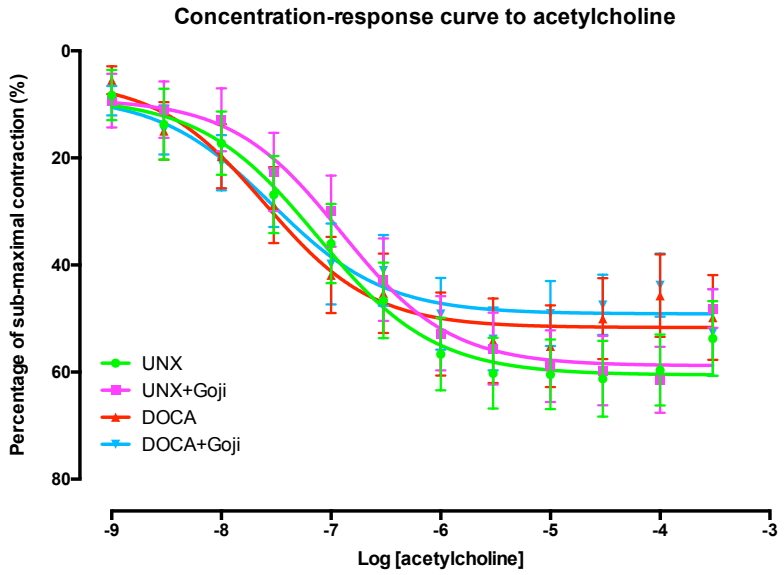

Figure 5.5: 2^{nd} order mesenteric artery acetylcholine concentration-response curve. Lines of best fit produced by a non-linear four parameter regression analysis. UNX (n=14), UNX+G (n=17), DOCA (n=18), DOCA+G (n=20).

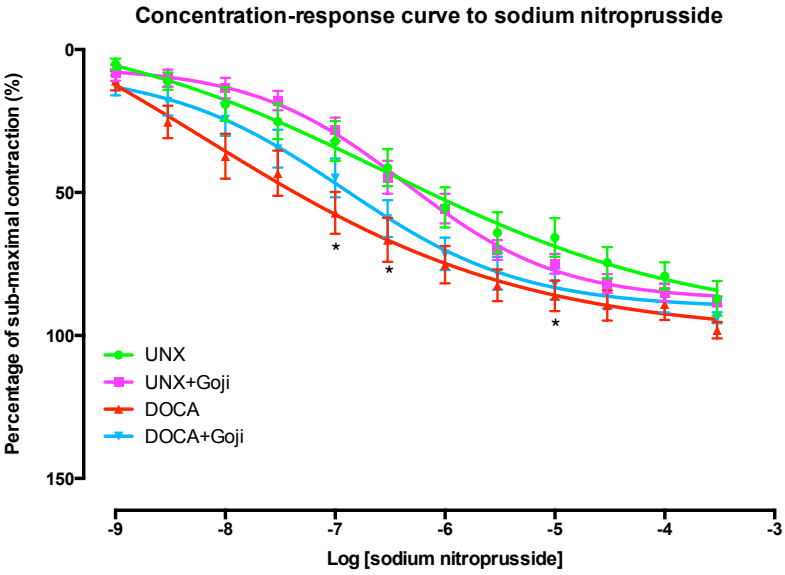

Figure 5.6: 2^{nd} order mesenteric artery sodium nitroprusside concentration-response curve. Lines of best fit produced by a non-linear four parameter regression analysis. UNX (n=15), UNX+G (n=17), DOCA (n=18), DOCA+G (n=19).
$*$ = $p<0.05$ vs UNX

Table 5.4: Goji: Cardiac functional analysis

	UNX	UNX+G (200mg.kg^{-1})	DOCA	DOCA+G (200mg.kg^{-1})
Electrophysiological Measurements				
APD 20% (ms)	11.90 ± 0.56 (10)	14.80 ± 0.88 (9)	23.77 ± 2.81 (10)‡	15.58 ± 0.92 (9)†
APD 50% (ms)	18.63 ± 1.34 (10)	22.69 ± 1.80 (9)	46.93 ± 6.36 (10)‡	27.27 ± 2.43 (9)†
APD 90% (ms)	54.80 ± 5.26 (10)	61.69 ± 6.07 (9)	120.30 ± 10.51 (10)‡	80.90 ± 5.86 (9)†
RMP (mV)	-63.15 ± 4.61 (10)	-70.31 ± 4.83 (9)	-58.43 ± 3.28 (10)	-66.98 ± 2.50 (9)
APA (mV)	58.07 ± 3.00 (10)	61.65 ± 2.52 (9)	60.63 ± 4.78 (10)	61.11 ± 3.08 (9)
Force (mN)	1.33 ± 0.29 (10)	1.57 ± 0.50 (9)	1.88 ± 4.88 (10)	1.65 ± 0.15 (9)
Langendorff Measurements				
Diastolic Pressure (mmHg)	10.71 ± 0.45 (8)	11.30 ± 0.35 (10)	10.70 ± 0.42 (8)	10.46 ± 0.58 (8)
Dev. Pressure (mmHg)	133 ± 17 (8)	124 ± 7 (10)	81 ± 16(8) ∗	126 ± 16 (8)†
+dP/dT (mmHg.s^{-1})	2312 ± 336 (8)	2279 ± 138 (10)	1409 ± 283 (8)∗	2296 ± 308 (8)†
-dP/dT (mmHg.s^{-1})	-1628 ± 259 (8)	-1580 ± 119 (10)	-1031 ± 226 (8)∗	-1628 ± 271 (8)
ESP (mmHg)	135 ± 18 (8)	135 ± 7 (10)	92 ± 16 (8)∗	136 ± 15 (8)†
Stiffness (k)	30.33 ± 1.28 (8)	30.64 ± 0.90 (10)	33.93 ± 1.01 (8)‡	34.04 ± 1.18 (8)‡

Numbers in parenthesis are the minimum n= values for the group.
∗ = p<0.05 vs UNX, ‡ = p<0.05 vs UNX/UNX+G, † = p<0.05 vs DOCA.
APD = action potential duration, RMP = resting membrane potential, APA = action potential amplitude, max +dP/dT = maximal rate of contraction, max -dP/dT = maximal rate of relaxation, ESP = end systolic pressure

5.4 Discussion

Many studies have investigated goji berry (*Lucium barbarum*) extract's antioxidant and immunomodulatory capabilities (Amagase et al., 2009; Chen et al., 2009a,b, 2008; Li, 2007; Li et al., 2007; Ming et al., 2009; Shao-Ping and Pin-Ting, 2010; Song et al., 2011; Xin et al., 2011), but very few have investigated it's function and relevance in models of hypertension. Previous work has shown that goji extract is able to improve oxidative stress within both diabetic rats (Li, 2007) and aged mice (Li et al., 2007). In the streptozotocin-diabetic rats goji extract improved measures of both liver and kidney SOD, GSH-Px, catalase, glutathione reductase and MDA (Li, 2007). Furthermore, this study observed that these effects were dose-dependent (Li, 2007). In the second study, aged mice showed a significant increase in MDA levels within heart tissue, concurrent with decreased expression of SOD, GSH-Px, catalase and total antioxidant capacity (TAOC) (Li et al., 2007). Treatment with goji extract improved these measures in a dose-dependent manner (Li et al., 2007), a finding that provided a strong theoretical basis for our study. Changes in oxidative stress and antioxidant mechanisms are key components to the pathological remodelling that occurs within the DOCA-salt rat model (Fenning et al., 2005; Ghosh et al., 2004; Iyer et al., 2010a), making it a model well-suited to investigating goji extract's effects.

Hypertension was successfully induced in both the DOCA and DOCA+G groups, with goji treatment producing a decrease in BP by week 4. Though this decrease was significant in comparison to the DOCA control group, hy-

pertension was not normalised in the DOCA+G rats. In addition, blood pressure within the DOCA+G rats was significantly elevated by week 2 of treatment, suggesting that goji extract's antihypertensive effect may develop over a longer period of time, which was not able to be fully captured by this study. Levels of NO and MDA were also improved by goji extract, and at the end of the study, DOCA+G rats had normalised serum concentrations of both compounds. This is an important finding, as it demonstrates that although goji extract had improved oxidative stress by the end of the study, the immediate or acute benefit provided was not sufficient to prevent the development of hypertension within the DOCA-salt rat.

Although *Lycium barbarum* possesses direct antioxidant activity (Donno et al., 2014), studies have shown that it also increases expression of a number of pro-antioxidant enzymes (Amagase et al., 2009; Li, 2007; Li et al., 2007; Ming et al., 2009). It is via this mechanism of action that we believe goji berry extract normalised the oxidative stress markers. Further research on *Lycium barbarum* (goji berry) extract has shown that it is able to enhance expression of peroxisome proliferator activated receptor-γ (PPAR-γ) (Song et al., 2011), which has been implicated in promoting antioxidant mechanisms (Hamblin et al., 2009). Of significance to the present study is the finding that PPAR-γ activation stimulates NO production and release (Polikandriotis et al., 2005). PPAR-γ is able to bind both oxidatively-modified lipids and oxidised phospholipids (Hamblin et al., 2009). Therefore, increasing expression of PPAR-γ within states of oxidative stress may increase the production and expression of eNOS and NO, itself a ligand for PPAR-γ (Ptasinska et al., 2007).

The increased serum NO observed at the end of the study did not correlate with improved vascular function in the hypertensive rats. The DOCA-salt-induced reduction in maximal contractile force was maintained despite goji treatment. The inability for the DOCA and DOCA+G aortic tissues to produce maximal contractile responses similar to their normotensive counterparts is likely due to increased spontaneous tone developed before the CRCs were performed. Increased spontaneous tone generation has been observed within the DOCA-salt rat (Ghosh et al., 2004), supporting our hypothesis. This increased basal tone also explains the observation that both DOCA and DOCA+G groups were able to produce maximal relaxations greater than their pre-contractions in both ACh and NaNO CRCs. This does not explain however, the increased ability for the UNX+G aortic tissue to produce a similar "enhanced" relaxation to ACh. The increased serum NO levels suggest a greater production and/or bioavailability of NO within the UNX+G aortic tissue, leading to greater NO-mediated responses such as those elicited by ACh. The mean maximal contraction of UNX+G aortas was not statistically significant, but did show a trend towards a lower maximal contraction. The EC50 concentration of acetylcholine was significantly affected by goji treatment, with the UNX+G group showing increased sensitivity compared to the UNX controls.

Mesenteric CRCs showed no significant changes in maximal contraction or maximal relaxation, with no group relaxing greater than their pre-contraction. Changes to EC50 concentrations were observed in the DOCA and DOCA+G groups, with decreased sensitivity to noradrenaline, the opposite to that ob-

served in the aortas from the same groups. This leads us to propose that the mechanisms behind the changes in EC50 are dependent on vascular localisation. Unlike the aorta, noradrenaline EC50 concentration was improved in the mesenterics by goji treatment. Conversely, UNX+G rats showed an increase in NA required to achieve the EC50 concentration. We hypothesise that treatment with goji extract is promoting an increase in NO production and release, which is affecting the ability for the UNX mesenterics to contract (shifting the EC50 to the right of the UNX), but is improving the oxidative state of the DOCA mesenterics, promoting improvements in adrenergic induced contraction. The effect of goji extract on mesenteric vasculature is an area of potential future research, so as to adequately explain this observed effect.

Previous studies have demonstrated the significant cardiac remodelling that occurs in the DOCA-salt rat heart (Chan et al., 2006; Fenning et al., 2005). Our results demonstrated a similar remodelling of the heart, with the diastolic stiffness constant (k) and action potential duration both showing significant increases compared to the UNX rats. Functional measures of the myocardium were also diminished in the DOCA rats, with developed pressures, rates of contraction and relaxation, as well as end systolic pressure all showing significant reductions compared to the UNX controls. Treatment with goji extract attenuated the diminished myocardial function, showing that it was able to preserve myocardial functionality, despite the development of hypertension, LVH and diastolic stiffness (k).

Cardiac electrophysiological remodelling, observed in the DOCA rats as

prolongations in APD, was attenuated by treatment with goji extract. Action potential durations at all three measured time points (APD 20, 50 & 90% of repolarisation) were normalised by goji treatment. This is in contrast to the results from our stevia study, in which APD showed improvements across all time points, but only APD20 and APD50 were normalised. In DOCA-salt rats treated with the NO precursor L-arginine, prolongation of APD was prevented at 20% and 90% of repolarisation, an effect that the authors attributed to potential NO-mediated restoration of the early transient outward current or a result of LVH regression (Fenning et al., 2005). This relationship between improved APD and LVH regression was also observed in DOCA-salt rats treated with the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor Rosuvastatin, where prolongation of APD90 was prevented (Loch et al., 2006b). Omapatrilat, a vasopeptidase inhibitor, was shown to improve, but not normalise, APD at 90% repolarisation in DOCA-salt hypertensive rats (Loch et al., 2006a). Left ventricular hypertrophy was reduced, but also not normalised, by Omapatrilat treatment (Loch et al., 2006a), further demonstrating the link between LVH and electrophysiological remodelling in the DOCA-salt rat. Our results are significant in light of this relationship, as APD was improved across all measured time points without regression of LVH.

Goji extract has been demonstrated to improve activity of Na^{+}-K^{-}-ATPase and Ca^{2+}-ATPase within a model of ischemia/reperfusion injury (Shao-Ping and Pin-Ting, 2010). The improvement in activity of Ca^{2+}-ATPase, an enzyme that is crucial for intracellular handling of Ca^{2+} ions, is

a potential mechanism for the improvements in APD observed in DOCA+G rats. Boardman et al. (2014) reported that cardiac-specific SERCA2 knock-out mice developed mechanical dysfunction within 4 weeks and the heart became mechanically inefficient. Calcium release from the sarcoplasmic reticulum is essential for activation of the small conductance calcium-activated potassium channels in ventricular myocytes (Terentyev et al., 2014). Further research is required in order to determine if goji extract does improve SERCA activity within the DOCA-salt heart, or whether the improved APD is a result of decreased myocardial-specific oxidative stress, as was observed in aged mice (Li et al., 2007), or potentially a combination of the two.

In conclusion, extract from *Lycium barbarum*, at a dose of $200\text{mg.kg}^{-1}\text{.day}^{-1}$, prevented many of the pathological changes associated with the DOCA-salt hypertension rat. Goji extract improved oxidative stress, produced a mild decrease in mean BP after 4 weeks of treatment, attenuated the prolongation of myocardial APD, and preserved myocardial functionality despite persistent LVH and diastolic stiffness. Although it is difficult to separate the reduction in BP from the other beneficial improvements in cardiovascular function observed, the fact that BP was still significantly elevated in the DOCA+G rats at the termination of the study, while other functional parameters were normalised, does suggest the *in vivo* effects of goji extracts are not solely dependent on BP. Rather they may relate to some direct anti-oxidant, cardioprotective effects. This built upon earlier work by Li et al. (2007) and Li (2007), where *Lycium barbarum* extract was shown to significantly improve oxidative stress within the heart of aged mice, and demonstrates relevance

for goji extracts in cardiovascular research.

5.4.1 Study limitations and future directions

Our study was unable to report on the effect that goji extract had on serum MDA levels within normotensive UNX rats. Although this study was assessing the preventative nature of goji extract, the blood pressure results over the treatment period suggest that goji extract's antihypertensive effect was starting to develop by the 4^{th} week of treatment. Therefore, a longer study would allow for any greater development of an antihypertensive effect to occur. Additionally, pre-treatment with goji extract may produce a better outcome in terms of preventing the development of oxidative stress, as it would allow for gene expression to be modulated before the oxidative stress was induced by DOCA-salt treatment. Further work is required to determine the mechanisms by which many of the reported effects are taking place, particularly the extent to which PPAR-γ and SERCA2 modulation may be involved.

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Chapter 6

(-)-Epicatechin reduces blood pressure and improves left ventricular function and compliance in hearts of the DOCA-salt hypertensive rat

Abstract

(-)-Epicatechin (epicatechin) is a flavanol found in green tea and cocoa, and has been shown to attenuate TNF-α-mediated inflammation, improve NO levels and eNOS activation, and inhibit NADPH oxidase. In this study we investigated the effect of chronic low dosing of epicatechin ($1mg.kg^{-1}.day^{-1}$) on the cardiovascular function of DOCA-salt hypertensive rats. Wistar rats (8 weeks of age) underwent uninephrectomy, with half then receiving 1% NaCl in their drinking water along with subcutaneous injections of 25mg deoxycorticosterone-acetate (DOCA) in 0.4mL of dimethylformamide (DMF) every 4^{th} day for 4 weeks. Epicatechin-treated rats received a daily dose of $1mg.kg^{-1}$ of epicatechin by oral gavage. Single cell cardiac electrophysiology was used to measure action potential durations (APD), providing an indication of the potential for cardiac dysrythmias. Left ventricular (LV) function was assessed using the Langendorff technique. Isolated organ baths and wire myograph organ baths were used to assess vasomotor responses to noradrenaline (NA), acetylcholine (ACh) and sodium nitroprusside (NaNO) in aortic and mesenteric rings. Serum malondialdehyde (MDA) concentration was used as a marker of oxidative stress. Epicatechin produced a significant reduction in blood pressure (BP) within DOCA-salt rats (194 ± 5mmHg vs 147 ± 6mmHg $p<0.0001$) and normalised serum concentrations of MDA. These changes did not translate to improvements in LV hypertrophy or the prolongation of APD. Myocardial stiffness (k) was increased and LV compliance significantly diminished in the DOCA hearts, changes

that were attenuated by epicatechin treatment ($p<0.05$). No improvement in DOCA-salt vascular function was observed in epicatechin treated rats, although changes in vascular reactivity were observed in healthy-treated rats. We have demonstrated the ability of (-)-epicatechin to reduce blood pressure, reduce oxidative stress, prevent myocardial stiffening, and preserve cardiac compliance in the hypertrophied hearts of DOCA-salt rats. However, these changes did not translate to improvements in APD or LV hypertrophy.

6.1 Introduction

Epicatechin, a flavanol found in green tea and cocoa, is being increasingly investigated for its therapeutic potential in metabolic syndrome (Vazquez-Prieto et al., 2012), myocardial ischema and infarction (Prince, 2013; Yamazaki et al., 2008, 2010) and vascular function (Ramirez-Sanchez et al., 2010, 2011; Schroeter et al., 2006). Counted among it's many actions is it's ability to improve endothelial nitric oxide synthase (eNOS) activation and NO levels (Ramirez-Sanchez et al., 2012, 2011), inhibit NADPH oxidase (Steffen et al., 2007), attenuate TNF-α-mediated inflammation and insulin resistance (Vazquez-Prieto et al., 2012), and attenuate mitochondrial damage (Prince, 2013). These properties indicate that epicatechin has the potential to lower blood pressure, reduce inflammation, improve oxidative stress and mitochondrial function, thereby attenuating vascular and cardiac functional damage. Epicatechin has also been shown to confer significant cardioprotection in animal models of myocardial infarction and ischemia-reperfusion, at a dose as low as $1 mg.kg^{-1}.day^{-1}$ (Yamazaki et al., 2008, 2010). These studies are extreme models of oxidative stress, clearly one of the key targets for epicatechin.

There has been very little research so far investigating the effects epicatechin in the DOCA-salt model of cardiovascular disease and hypertension. One study has been published (Gómez-Guzmán et al., 2012), however this was in older Wistar rats (12 vs 8 weeks) and did not assess measures of cardiac function, rather the vascular function and molecular investigations such

as oxidative stress, NADPH oxidase.

The current study, in comparison to the previous one by Gómez-Guzmán et al. (2012), is using the more traditional DOCA model starting at 8 weeks of age and progressing through for 4 weeks. The DOCA-salt rat model of hypertension is a very aggressive model of cardiovascular remodelling, with most animals demonstrating significant cardiac and vascular dysfunction by 4 weeks of treatment (Brown et al., 2000; Chan et al., 2011, 2006; Fenning et al., 2005; Ghosh et al., 2004; Loch et al., 2007; Mirkovic et al., 2002; Somers et al., 2000).

The aim of this study was to assess the ability of epicatechin, at a dose of $1mg.kg^{-1}.day^{-1}$, to provide cardioprotection in this hypertensive model. This was carried out through vascular and cardiac functional studies, including single cell microelectrode electrophysiology, and the whole heart isolated Langendorff heart technique.

6.2 Materials and Methods

6.2.1 Chemicals and treatment

Deoxycorticosterone (D7000, Sigma) (DOCA), N,N-dimethylformamide (≥99%, Sigma) (DMF), dimethyl sulfoxide (≥99%, Sigma) (DMSO), noradrenaline (99%), acetylcholine (≥99%, TLC), sodium nitroprusside (≥ 99%) and (-)-epicatechin (≥, HPLC, Sigma) were purchased through Sigma-Aldrich (St. Louis, MO, USA). With the exception of DOCA and DMF, all compounds were dissolved in purified water (Milli-Q water purification system, Merk Millipore, Australia). A stock solution of (-)-epicatechin (1mg.mL^{-1}) was made up by dissolving 10mg (-)-epicatechin in a combination of 9.9mL purified water and 100μL DMSO.

6.2.2 Animal preparation

Experimental Wistar rats (6 weeks of age) were purchased from the Animal Resource Centre (Perth, Western Australia) under the approval of the CQUniversity Animal Ethics Committee (A11/03-268). Upon arrival, the rats were housed within the CQUniversity animal house, where they were given water and food *ad libitum*. Temperature was maintained at 25±2°C with a 12 hour light/dark cycle. Once the rats had reached 8 weeks of age and weighed over 300g, they underwent surgery to remove the left kidney. Uninephrectomy assist with the development of the model, by reducing renal function and therefore helping to promote volume overload. This was

achieved by a small flank incision, which exposed the kidney and allowed for the renal vessels and ureter to be ligated. Once ligation was confirmed and blood flow to the kidney had ceased, the kidney was removed by scalpel. The incision was then sutured and the hide stapled. All rats received pain management (Metacam 0.1mg.kg^{-1}) on the day of surgery and for two days following surgery. They were then randomised into either uninephrectomy (UNX) or deoxycorticosterone-acetate (DOCA) groups. UNX rats received a vehicle injection of 0.4mL dimethylformamide (DMF), subcutaneous, every 4^{th} day for the entirety of the experimental period (4 weeks). DOCA-salt rats received 1% NaCl in their drinking water along with subcutaneous injections of 25mg DOCA (in 0.4mL DMF) every 4^{th} day for the 4 week treatment period.

6.2.3 Experimental design

Both UNX and DOCA rats were split into epicatechin-treated and untreated groups: UNX (n=22), UNX+E (n=21), DOCA (n=23) and DOCA+E (n=17). Epicatechin was administered, via oral-gavage, at a dose of 1mg.kg^{-1} per day for 28 days. For a 300g rat, 300μL of stock solution would be administered daily. As the rat aged and weight changed the amount of stock would be adjusted to match, and maintain the 1mg.kg^{-1} dose.

6.2.4 Systolic blood pressure

Blood pressure was analysed using a modified pressure cuff/pressure transducer system as described by Fenning et al. (2005). Baseline (0 week) measurements were carried out after the rats recovered from the uninephrectomy and before they were randomised into treatment groups. The results are reported as UNX (0 week). Rats undergoing BP analysis were immobilised by an intraperitoneal injection of Tiletamine (15mg.kg^{-1}) and Zolazepam (15mg.kg^{-1}). Once the rats were immobile, a tail pulse transducer (MLT1010) was attached to the tail, followed by an inflatable cuff connected to a Capto SP844 physiological pressure transducer (MLT844/D). This was connected to an iMac G4 via a PowerLab 4/30 (ADInstruments, NSW, Australia). Representative samples of the groups were assessed at 0, 2 and 4 weeks of treatment. A minimum of 3 blood pressure measurements were taken to produce a mean value from each assessed rat. This data was pooled into treatment groups for statistical analysis.

6.2.5 Serum malondialdehyde determination

Blood samples were drawn using a syringe and needle from the thoracic vena cava following euthanasia, while the heart was still beating and providing adequate circulation. Samples, a minimum of 5mL, were then transferred to serum separator tubes and allowed to clot before being centrifuged (3000g, 15min) and the supernatant removed and stored at -80°C until required for analysis. Serum MDA concentrations were determined using the commer-

cially available OxiSelect^{TM} MDA adduct competitive ELISA kit (Cell Biolabs, Inc, San Diego, CA, USA). Manufacture's instructions were followed and a standard curve was produced using a 4-parameter logistic curve.

6.2.6 Thoracic aorta organ baths

Following euthanasia and blood sample collection, the thoracic aorta was dissected out and cleaned while submerged in cold Tyrode's physiological salt solution (Tyrode's PSS) (mM: NaCl 136.9, KCl 5.4, $MgCl_2 \cdot H_2O$ 1.0, $NaH_2PO_4 \cdot 2H_2O$ 0.4, $NaHCO_3$ 22.6, $CaCl_2 \cdot 2H_2O$ 1.8, glucose 5.5, ascorbic acid 0.3, Na_2EDTA 0.05). Five millimetre (5mm) long ring segments were then threaded onto stainless steel hooks connected to an FT03 force displacement transducer (Grass Technologies, Middleton, WI, USA). The tissue was then anchored in a 30mL isolated organ bath, filled with warm (37°C) Tyrode's PSS, bubbled with carbogen (90% O_2/10% CO_2). Concentration-response curves were performed for noradrenaline (NA), acetylcholine (ACh) and sodium nitroprusside (NaNO). Results were also normalised as a percentage of total response in order that EC50 concentrations could be determined.

6.2.7 Mesenteric wire myograph

Mesenteric arteries were assessed using the multi-channel wire myograph system from DMT (Danish Myograph Technologies, DMT, Denmark). Second order mesenteric arteries were dissected and cleaned while submerged in cold Tyrode's before 2mm ring segments were mounted in the myograph system.

Each sample of mesenteric artery was mounted to the pressure transducer via a 40μm diameter stainless-steel wire. The tissues were acclimatised for 30 min while bath temperature was increased to 37°C, where it was maintained for the entirety of the experiment. Once temperature was achieved, vessels underwent normalisation procedures as prescribed by the manufacturers (DMT, Denmark). After normalisation and a subsequent rest period of 30 min, tissues were subjected to a potassium challenge by rinsing with KCl Tyrode's (mM: NaCl 37, KCl 100, $MgCl_2 \cdot H_2O$ 1.0, $NaH_2PO_4 \cdot 2H_2O$ 0.4, $NaHCO_3$ 22.6,chCaCl2.2H_2O 1.8, glucose 5.5, ascorbic acid 0.3, Na_2EDTA 0.05). Once contractions were achieved the baths were rinsed with normal Tyrode's and rested for 30 min. Concentration-response curves were performed for NA, ACh and NaNO. Results were normalised as a percentage of total response to determine EC50 concentrations.

6.2.8 Single cell microelectrode

Electrophysiology was performed using the single cell mirco-electrode method, described by Fenning et al. (2005). Following the collection of blood, the heart was rapidly dissected and placed in a dissection dish filled with cold Tyrode's PSS (carbogen gassed). The atria and right ventricle were dissected and the intraventricular septum cut. The cleanest papillary muscle was dissected out, pierced at one end with a stainless steel hook and the other end secured to the base of the electrophysiological experimental chamber (1mL volume). The chamber was continuously perfused with warm (37°C), car-

bogen gassed Tyrode's PSS. The papillary muscle was positioned between two platinum electrodes and the hook was attached to a modified SensoNor AE 801 micro-force transducer. The transducer was connected to an iMac computer via an amplifier (World Precision Instruments TBM-4). The papillary muscle was then slowly stretched to a pre-load of 5-10mN over one minute. Electrical field stimulation (Grass SD-9)(frequency of 1Hz, pulse width of 0.5msec, stimulus strength of 20% above threshold) was used to induce contractions within the tissue. Impalements were made with a potassium chloride (3M) filled glass electrode (World Precision Instruments, filamented borosilicate glass, outer diameter 1.5mm, tip resistance 5-15mΩ filled with 3M KCl). Reference readings were provided using a silver/silver chloride electrode. Recordings of electrical activity were recorded using a Cyto 721 electrometer (World Precision Instruments) connected to an iMac via a PowerLab 4/25 analogue digital converter. LabChart v5.5 was used to view and record the measurements of the electrophysiology instruments. Each papillary muscle was impaled a minimum of 3 times at three distinct locations, and activity was recorded for 30 min each. Recordings were analysed to determine action potential amplitude, action potential duration at 20, 50, and 90% or repolarisation, force of contraction, and the velocity of changes in voltage (dv/dT) and force (df/dT).

6.2.9 Langendorff isolated heart preparation

Left ventricular function was measured using the Langendorff technique adapted from Chan et al. (2006). Following the collection of blood, the heart was rapidly excised and submerged in ice-cold modified Krebs-Henseleit buffer (modified KHB) (mM: NaCl 119.1, KCl 4.75, $MgSO_4$ 1.19, KH_2PO_4 1.19, $NaHCO_3$ 25.0, glucose 11.0 and $CaCl_2$ 2.16). The aorta was briefly cleaned of fat and then canulated via the dorsal root and purfused with warm (37°C), gassed (carbogen) modified KHB at a constant pressure of 100mmHg. A latex balloon catheter connected to Capto SP844 physiological pressure transducer (MLT 844/D), was inserted into the left ventricle via the mitral valve. The pressure transducer was connected to an iMac G4 computer via a PowerLab 4/30 (ADInstruments, NSW, Australia) and pressure-volume traces were recorded by LabChart software. The heart was paced at 250bpm by electrode stimulation of the right atria and pressure-volume curves were recorded. Starting from 0mmHg, the end diastolic pressure was increased in 5mmHg increments every 20 seconds, until a maximum of 30mmHg was reached. Diastolic stiffness was assessed by determining the myocardial stiffness constant, k (dimensionless), obtained by calculating the slope of the linear relationship between stress (σ, dyn cm^{-2}) and tangent elastic modulus (E, dyn cm^{-2}) (Chan et al., 2006). Other parameters measured were diastolic pressure, developed pressure, velocity of contraction (+dP/dT) and relaxation (-dP/dT), and end systolic pressure (ESP).

6.2.10 Statistical analysis

Statistical analysis of the data was performed using two-factor analysis of variance (2-way ANOVA), using Bonferroni's *post hoc* testing where applicable. Statistical analysis of diastolic stiffness elevation between UNX and DOCA groups was performed using a one-tailed Student's t-test. This decision was based on previous literature that has shown DOCA-salt rats to exhibit increased diastolic stiffness (Chan et al., 2006). Therefore, the directional nature of this test was appropriate. The statistical software Graphpad Prisms 6 was used for all analysis. All data is presented as mean ± standard error of the mean (SEM). For all tests, $P<0.05$ was taken as the level of significance.

6.3 Results

6.3.1 Systolic blood pressure

Blood pressure was significantly elevated in the DOCA-salt control rats, with an average increase of 64 ± 7.7mmHg compared to UNX ($p<0.0001$) (Table 6.1). Epicatechin significantly reduced the average final BP in both UNX+E and DOCA+E groups when compared to their respective controls (Table 6.1).

6.3.2 Biometric measurements

Rats receiving DOCA-salt injections showed reduced weight gain compared to their UNX counterparts. The mean difference between DOCA and UNX groups was 93 ± 16g (Table 6.1). Left ventricular hypertrophy was observed in UNX+E, DOCA and DOCA+E group, with DOCA+E showing the greatest increase in LV weight (Table 6.1). Both DOCA and DOCA+E had increased kidney and liver weights when normalised to body weight (Table 6.1).

6.3.3 Serum malondialdehyde

DOCA-salt hypertensive control rats had serum MDA concentrations significantly increased compared to UNX controls (Table 6.1). Serum MDA showed an average increase of 58 ± 23 pmol/mL within the DOCA-salt controls, an increase that epicatechin treatment normalised to UNX values (Table 6.1).

Table 6.1: Epicatechin: Effects on biometric, biochemical and haemodynamic parameters

	UNX	UNX+E ($1mg.kg^{-1}$)	DOCA	DOCA+E ($1mg.kg^{-1}$)
Body Weight (g)				
	444 ± 13 (22)	452 ± 14 (21)	351 ± 10 (23)‡	343 ± 15 (17)‡
Organ Weights (normalised to body weight, $g.kg^{-1}$)				
LV	2.15 ± 0.12 (14)	2.74 ± 0.07 (12)*	2.91 ± 0.10 (18)*	3.48 ± 0.12 (10)‡†
RV	0.49 ± 0.03 (14)	0.55 ± 0.02 (12)	0.43 ± 0.05 (18)	0.53 ± 0.03 (10)
Liver	33.3 ± 0.8 (14)	32.8 ± 0.6 (12)	37.4 ± 1.4 (18)‡	38.6 ± 1.4 (10)‡
Kidney	4.88 ± 0.10 (14)	4.92 ± 0.05 (12)	8.31 ± 0.33 (18)‡	7.86 ± 0.19 (10)‡
Spleen	2.66 ± 0.11 (14)	2.86 ± 0.15 (12)	3.11 ± 0.16 (18)	3.16 ± 0.38 (10)
Blood Pressure (mmHg)				
0 Weeks	119 ± 6 (12)			
2 Weeks	116 ± 6 (11)	132 ± 9 (4)	162 ± 11 (5)∗	134 ± 28 (3)
4 Weeks	130 ± 6 (6)	104 ± 5 (7)∗	194 ± 5 (7)‡	147 ± 6 (7)‡ †
Biochemistry				
MDA (pmol/mL)	125.5 ± 4.1 (4)	107.3 ± 8.3 (6)	183.6 ± 22.6 (4)‡	134.6 ± 4.8 (6)†

LV - left ventricle, RV - right ventricle, MDA - malondialdehyde, * = $p<0.05$ vs UNX, ‡ = $p<0.05$ vs UNX/UNX+E, † = $p<0.05$ vs DOCA.
Numbers in parenthesis are the minimum n= values.

Table 6.2: Epicatechin: Aorta EC50 values

	UNX (17)	UNX+E (12)	DOCA (13)	DOCA+E (12)
Noradrenaline	-6.67 ± 0.10	-6.43 ± 0.07 *	-7.24 ± 0.08 ‡	-6.73 ± 0.07 § †
Acetylcholine	-6.52 ± 0.09	-6.92 ± 0.05 *	-5.98 ± 0.11 ‡	-6.01 ± 0.11 ‡
Sodium nitroprusside	-7.64 ± 0.04	-7.91 ± 0.05 *	-6.80 ± 0.04 ‡	-6.95 ± 0.05 ‡†

All values are the mean logarithm of the EC50 (log[EC50]). Numbers in parenthesis are the minimum n= value for that group.
$*$ = p<0.05 vs UNX, ‡ = 0.05 vs UNX/UNX+E, † = p<0.05 vs DOCA, § = p<0.05 vs UNX+E

Table 6.3: Epicatechin: Mesenteric artery EC50 values

	UNX (14)	UNX+E (14)	DOCA (15)	DOCA+E (12)
Noradrenaline	-5.90 ± 0.05	-5.87 ± 0.06	-5.40 ± 0.04 *	-5.79 ± 0.07 †
Acetylcholine	-6.90 ± 0.10	-5.74 ± 0.17 *	-6.98 ± 0.15 ‡	-5.55 ± 0.17 † *
Sodium nitroprusside	-6.50 ± 0.10	-6.41 ± 0.06	-7.34 ± 0.09 ‡	-6.76 ± 0.13 †

All values are the logarithm of the EC50 (log[EC50]). Numbers in parenthesis are the minimum n= value for that group.
$*$ = p<0.05 vs UNX, ‡ = 0.05 vs UNX/UNX+E, † = p<0.05 vs DOCA

6.3.4 Vascular function

Thoracic aorta functional analysis

Contractile function was impaired in DOCA-salt rats. The maximum force generated in response to NA was significantly less than that produced by UNX controls (Fig. 6.1). Epicatechin treatment was not able to improve DOCA+E responses to NA (Fig. 6.1). Furthermore, it resulted in a significant reduction in maximal contraction in UNX+E aortas (Fig. 6.1) and an increase in NA EC50 concentration (Table 6.2). Noradrenaline EC50 concentrations were reduced in the DOCA-salt controls (Table 6.2); epicatechin normalised this shift in DOCA+E rats, bringing the EC50 in-line with UNX control concentrations (Table 6.2).

Endothelium-dependent relaxation was diminished in DOCA+E rats, achieving only a 55 ± 13% reduction in pre-contracted tension (Fig. 6.2). Epicatechin treatment caused increased ACh-mediated responses in UNX rats, with the UNX+E group achieving a maximal relaxation of 169 ± 28% of the pre-contracted tension (Fig. 6.2). Relaxation to NaNO was significantly different between the DOCA and DOCA+E vessels, however none of the groups showed a significant difference when compared to the normotensive UNX rats (Fig 6.3).

Mesenteric artery functional analysis

Epicatechin-treated rats showed a similar noradrenaline contractile profile to thoracic aorta tissue, with maximal contractile tension decreased in compar-

ison to their control groups (Fig. 6.4). This diminished contractile response was not associated with any significant change to NA EC50 concentration (Table 6.3). DOCA-salt controls did have a strong shift in their EC50 concentration ($p<0.001$) showing reduced sensitivity to adrenergic stimulation (Table 6.3). Vasodilatory function was diminished in epicatechin-treated rats, with endothelium-dependent relaxation in both UNX+E and DOCA+E mesenterics impaired (Fig. 6.5). This is in contrast to epicatechin's effect in the aortic tissue, where it enhanced vasodilation within the UNX+E rats. A reduced sensitivity to ACh was also observed in these tissues, as EC50 concentrations were increased (Table 6.3). Endothelium-independent relaxation was also impaired in UNX+E and DOCA+E rats. Despite this, NaNO EC50 was only significantly affected in the DOCA-salt control rats (Table. 6.3).

6.3.5 Cardiac function

Electrophysiological impairment was present in the ventricular papillary muscles of DOCA-treated rats, with action potential duration showing significant prolongation (Table. 6.4). Measures of APD at the time points of 20, 50 and 90% of repolarisation showed a greater than doubling of the duration (Table 6.4); a change that was not prevented or improved by epicatechin treatment. Neither resting membrane potential, nor action potential amplitude were significantly altered by either DOCA-salt treatment or epicatechin treatment (Table 6.4).

Whole heart functional analysis demonstrated DOCA-salt-treated con-

trol hearts had increased diastolic stiffness, as measured by the myocardial stiffness constant (k). This effect of DOCA-salt treatment was completely attenuated by epicatechin (Table 6.4). Developed pressure and the rates of contraction and relaxation (+dP/dT and -dP/dT, respectively) were significantly reduced in the DOCA-salt hypertensive rats (Table 6.4), indicating a loss of myocardial function and compliance. Treatment with epicatechin was able to normalise these changes (Table 6.4), allowing DOCA+E rats to exhibited normalised myocardial function.

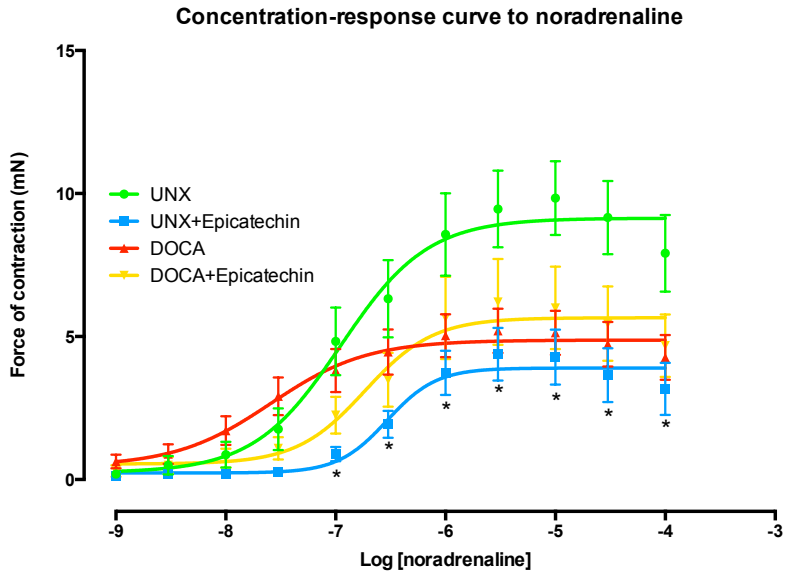

Figure 6.1: Thoracic aorta noradrenaline concentration-response curves. Lines of best fit produced by a non-linear four parameter regression analysis. UNX (n=17), UNX+E (n=19), DOCA (n=15), DOCA+E (n=12). $*$ = $p<0.05$ vs UNX

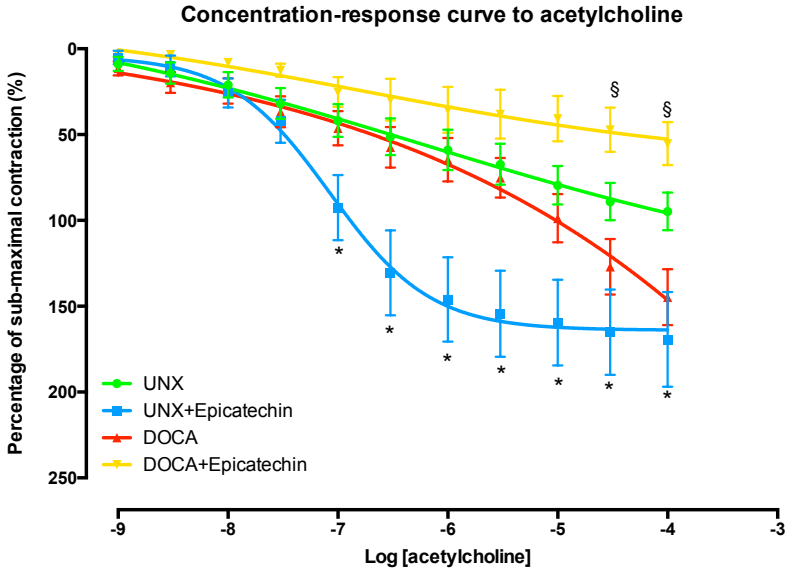

Figure 6.2: Thoracic aorta acetylcholine concentration-response curves. Lines of best fit produced by a non-linear four parameter regression analysis. UNX (n=19), UNX+E (n=15), DOCA (n=15), DOCA+E (n=14), $*$ = $p<0.05$ vs UNX, § = $p<0.05$ vs DOCA

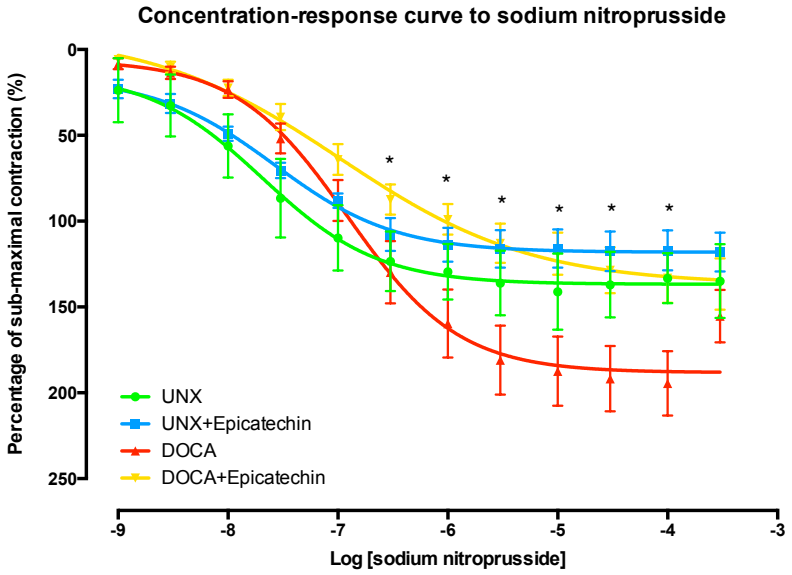

Figure 6.3: Thoracic aorta sodium nitroprusside concentration-response curves. Lines of best fit produced by a non-linear four parameter regression analysis. UNX (n=18), UNX+E (n=12), DOCA (n=13), DOCA+E (n=16). $*$ = $p<0.05$ vs DOCA

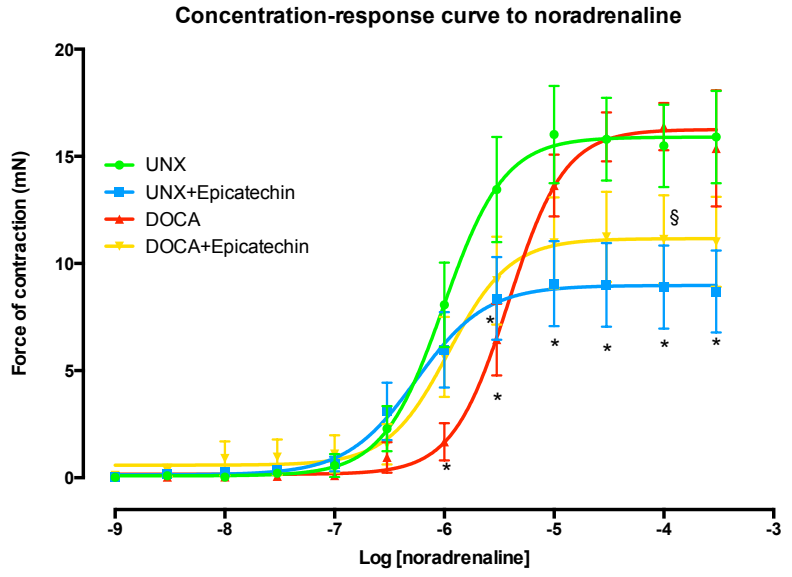

Figure 6.4: 2^{nd} order mesenteric artery noradrenaline concentration-response curve. Lines of best fit produced by a non-linear four parameter regression analysis. UNX (n=14), UNX+E (n=14), DOCA (n=15), DOCA+E (n=13). $*$ = $p<0.05$ vs UNX, § = $p<0.05$ vs DOCA

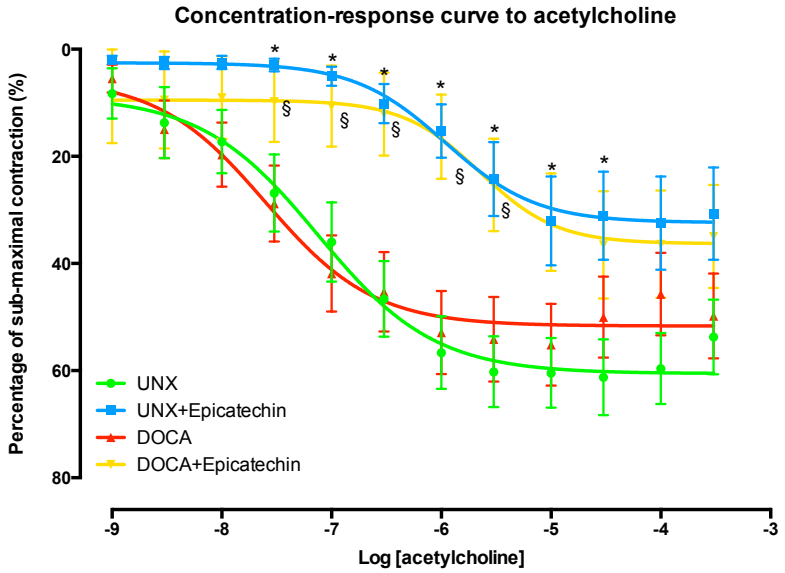

Figure 6.5: 2^{nd} order mesenteric artery acetylcholine concentration-response curve. Lines of best fit produced by a non-linear four parameter regression analysis. UNX (n=14), UNX+E (n=14), DOCA (n=18), DOCA+E (n=12). $*$ = p<0.05 vs UNX, § = p<0.05 vs DOCA

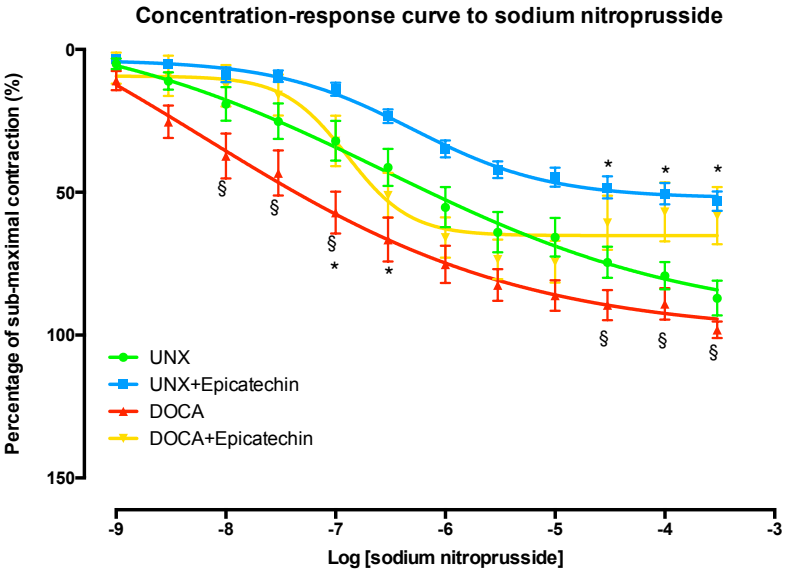

Figure 6.6: 2^{nd} order mesenteric artery sodium nitroprusside concentration-response curve. Lines of best fit produced by a non-linear four parameter regression analysis. UNX (n=15), UNX+E (n=14), DOCA (n=17), DOCA+E (n=13). $*$ = p<0.05 vs UNX, § = p<0.05 DOCA+E

Table 6.4: Epicatechin: Cardiac functional analysis

	UNX	UNX+E ($1mg.kg^{-1}$)	DOCA	DOCA+E ($1mg.kg^{-1}$)
Electrophysiological Measurements				
APD 20% (ms)	11.90 ± 0.56 (10)	13.26 ± 1.15 (9)	23.77 ± 2.81 (10)‡	22.58 ± 4.21 (8)*
APD 50% (ms)	18.63 ± 1.34 (10)	22.89 ± 2.76 (9)	46.93 ± 6.36 (10)‡	45.96 ± 10.40 (8)*
APD 90% (ms)	54.80 ± 5.26 (10)	85.11 ± 8.82 (9)	120.3 ± 10.51 (10)*	122.1 ± 17.14 (8)*
RMP (mV)	-63.15 ± 4.61 (10)	-52.89 ± 4.78 (9)	-58.43 ± 3.28 (10)	-53.51 ± 4.38 (8)
APA (mV)	58.07 ± 3.00 (10)	64.48 ± 5.05 (9)	60.63 ± 4.78 (10)	69.16 ± 5.03 (8)
Force (mN)	1.33 ± 0.29 (10)	1.33 ± 0.41 (9)	1.88 ± 4.88 (10)	2.62 ± 0.71 (8)
df/dt ($V.s^{-1}$)	0.38 ± 0.08 (7)	0.44 ± 0.08 (9)	0.52 ± 0.16 (10)	0.75 ± 0.18 (8)
dv/dt ($V.s^{-1}$)	14.19 ± 0.56 (7)	16.01 ± 0.48 (9)	14.73 ± 1.22 (10)	15.30 ± 1.18 (8)
Langendorff Measurements				
Diastolic Pressure (mmHg)	10.71 ± 0.45 (8)	10.43 ± 0.52 (10)	10.78 ± 0.47 (8)	9.29 ± 0.34 (8)
Dev. Pressure (mmHg)	133 ± 17 (8)	113 ± 13 (10)	81 ± 16 (8)*	131 ± 13 (8)†
+dP/dT ($mmHg.s^{-1}$)	2312 ± 336 (8)	2067 ± 244 (10)	1409 ± 283 (8) *	2580 ± 430 (8)†
-dP/dT ($mmHg.s^{-1}$)	-1628 ± 259 (8)	-1500 ± 187 (10)	-1031 ± 226 (8)*	-1827 ± 167 (8)†
ESP (mmHg)	135 ± 18 (8)	123 ± 13 (10)	92 ± 16 (8)*	140 ± 12 (8)†
Stiffness (k)	30.33 ± 1.28 (8)	30.30 ± 2.07 (10)	33.93 ± 1.01 (8) *	27.53 ± 1.54 (8)†

Numbers in parenthesis are the minimum n= values for the group.

$*$ = $p<0.05$ vs UNX, ‡ = $p<0.05$ vs UNX/UNX+E, † = $p<0.05$ vs DOCA.

APD = action potential duration, RMP = resting membrane potential, APA = action potential amplitude, df/dt = rate of force change as measured by micro-force transducer using electrical impedance measurements, dv/dt = rate of voltage change, max +dP/dT = maximal rate of contraction, max -dP/dT = maximal rate of relaxation, ESP = end systolic pressure

6.4 Discussion

The DOCA-salt rat model of hypertension is associated with extensive myocardial remodelling, including LV hypertrophy; increased LV interstitial inflammation and collagen deposition; increased diastolic stiffness; and decreased antioxidant and increased superoxide concentrations in the heart (Brown et al., 2000; Chan et al., 2011, 2006; Lee et al., 2012; Mirkovic et al., 2002; Somers et al., 2000). In the current study, epicatechin decreased systolic blood pressure, however this was not accompanied by a reduced LV mass or return to normotensive levels; the DOCA+E rats were still hypertensive. It can be inferred from this that the reduced blood pressure did not have a significant effect on the enlargement of the heart, despite improvements in functional parameters. This effect on blood pressure is in contrast to a study by Gómez-Guzmán et al. (2012) in which treatment of DOCA-salt rats with $2mg.kg^{-1}.day^{-1}$ was not able to significantly reduce blood pressure. However, a major difference between that experiment and the current study is the age of the rats. Gómez-Guzmán et al. (2012) started the DOCA and epicatechin protocol when the rats were 14 weeks of age, therefore the level of "cardiovascular" maturity may be significantly higher than the rats in the current study. Ageing has an effect on oxidative stress, with a study showing aged mice had a significant increase in oxidative stress and a significant decrease in antioxidant mechanisms (Li et al., 2007). Therefore, it is highly probable that the 14 week old rats would have a significantly different oxidative stress and antioxidant starting baseline those used in the current study.

Indeed, our study showed epicatechin treatment at 1mg.kg^{-1} was able to normalise serum MDA concentrations at the end of the experimental period, an effect that was only observed at a dose of 10mg.kg^{-1} in the study by Gómez-Guzmán et al. (2012).

Aortic tissue from epicatechin-treated rats showed a significant change in their ACh-mediated vasodilation in relation to all other groups. Although the DOCA+E rats showed a diminished vasoactive response, the UNX+E rats showed a significant increase in ACh-mediated responses, a result demonstrating the eNOS and NO enhancing properties of epicatechin that were observed by Ramirez-Sanchez et al. (2012, 2011). The diminished ACh-mediated response in the DOCA+E rats indicates vascular damage was still present. The increase in ACh-mediated response observed within the aortic tissue was not duplicated in the mesenteric tissue, with both UNX+E and DOCA+E responses showing a reduced magnitude. One study has shown that epicatechin and related compounds had an inhibitory effect on ACh-mediated responses in aortic ring segments (Sanae et al., 2002). In this paper, ACh-mediated relaxation was reduced in isolated thoracic aorta segments incubated with 10μM epicatechin for 15 min prior to phenylephrine-induced contraction (Sanae et al., 2002). The mechanism underlying the observations of Sanae et al. (2002) may explain the reduced ACh-mediated responses observed within the mesenteries, however it does not explain the reduction in contractile function observed in both the aortic and mesenteric tissues from our study. In the study by Sanae et al. (2002), contractile response to adrenergic stimuli was enhanced by epicatechin, a response not observed in either

the aortic or mesenteric segments in our study. The dose of (-)-epicatechin used in our study, whilst effective in the acute setting for reducing blood pressure, was not able to reverse remodelling of the vessels as there was still functional damage present.

Action potential duration was prolonged in the DOCA-salt hearts, an effect that has been observed in other DOCA-salt studies (Chan et al., 2011; Fenning et al., 2005). Unlike those studies however, in which treatment reduced oxidative stress, LV hypertrophy and diastolic stiffness (Chan et al., 2011; Fenning et al., 2005), epicatechin was unable to improve the prolongation of APD in the DOCA-salt rat. The ability for epicatechin to reduce blood pressure and improve oxidative stress appears to be more suited to improving cardiac function and stiffness rather than attenuating the electrophysiological changes in the DOCA-salt hypertensive heart. This observed disparity between epicatechin's effect on cardiac function and electrophysiology may be explained by the pathology of the DOCA-salt rat model itself. Electrophysiological changes have been shown to occur before morphological remodelling takes place (Perrier et al., 2004), suggesting that changes in APD may occur early in the pathogenesis of DOCA-salt hypertension and before epicatechin's effects are developed. Additionally, prolongation of APD has been attributed to changes in the density of I_{to}, resulting from cardiomyocyte hypertrophy without a concurrent increase in K^+ channel expression (Fenning et al., 2005; Loch et al., 2006a,b). This is a likely explanation for the APD prolongation observed in DOCA+E, particularly considering they showed greater LV hypertrophy than the DOCA control rats. The current

study did not measure fibrosis within the cardiac tissue; fibrosis and cardiac fibroblasts have shown the ability to alter electrophysiology within the diseased heart (Aguilar et al., 2014; ichi Sadoshima and Izumo, 1993; Lu et al., 2011; Massare et al., 2010). If cardiac fibrosis was significantly increased in the DOCA+E hearts, it may help to explain why cellular damage was still present within the myocardium, although the UNX+E hearts also showed significant LVH, but without accompanying prolongation of the APD. Further, considering that the DOCA+E hearts showed improved measures of diastolic stiffness the presence of fibrosis would not adequately explain the results.

Diastolic stiffness was increased in the DOCA-salt rats and was associated with diminished cardiac function; developed pressure, rates of contraction and relaxation all showed significantly reduced values compared to UNX normotensive controls. These changes were attenuated by epicatechin treatment, with DOCA+E rats having normalised values for these parameters. These results are significant because they were achieved despite increased LV hypertrophy in the DOCA+E rats; LV hypertrophy was significantly greater in DOCA+E than in DOCA and UNX control rats. In other studies that utilised the DOCA-salt model, improvements in diastolic stiffness and associated measures of cardiac compliance were accompanied by significant reductions in LV hypertrophy and APD (Chan et al., 2011, 2006; Fenning et al., 2005; Loch et al., 2006b; Mirkovic et al., 2002). This demonstrates that epicatechin is more protective of the myocardium than it was of the vasculature, despite significant improvements in oxidative stress and blood

pressure.

The pre-hypertrophic alteration of Ca^{2+} handling mediated by mineralocorticoid receptors (Perrier et al., 2004) may lead to an increased hypotrophic response via the activation of calcineurin. Increased I_{Ca} increases $[Ca^{2+}]_i$, promoting activation of calmodulin and leading to calcineurin phosphorylation (Harvey and Leinwand, 2011). This pathway of myocyte hypertrophy explains the presence of sustained LV hypertrophy despite epicatechin treatment. Further investigation is needed to elucidate if this mechanism is actually occurring and contributing to the LV hypertrophy of the DOCA-salt rat.

Studies of epicatechin pre-treatment in models of ischemia-referfusion (I/R) injury and permanent coronary occlusion (PCO) have demonstrated that epicatechin significantly reduced infarct area 48 hrs post insult (Yamazaki et al., 2008, 2010). Over the longer term, epicatechin was shown to sustain the reduction in infarct size for up to 3 weeks post PCO (Yamazaki et al., 2010). Many factors are implicated in the mechanism of cardiac remodelling, included in these are the matrix metalloproteinases (MMPs) (Hutchinson et al., 2010). The activity of these enzymes is controlled by tissue inhibitors of MMPs (TIMPS), with research by Yarbrough et al. (2014) showing that over-expression of TIMP-4 reduced collagen type IA1 and type IIIA1 in a model of LV pressure overload. The same study also showed that knocking out TIMP-4 resulted in significantly increased levels of both collagen isoforms (Yarbrough et al., 2014).

The activity of MMP-9 has been shown to increase in I/R, with Ya-

mazaki et al. (2008) demonstrating a potential inhibitory effect of epicatechin on MMP-9 within the border zones of the infarct site. This is not a definitive link between epicatechin and ECM management, but it does suggest that epicatechin may be achieving the improvement in diastolic stiffness observed in the current study by influencing the extracellular environment. Some studies have investigated the effect of other catechins, such as epigallocatechin-3-gallate (EGCG), on MMP expression. One study in a human endothelial cell line showed EGCG to reduce the expression of MMP-9 (Khoi et al., 2013). Another study, in the OVCAR-3 human ovarian carcinoma cell, demonstrated that EGCG could dose-dependently reduce expression of MPP-2 (Wang et al., 2014). Even though these studies are not related to the cardiomyocyte, they do provide further evidence supporting the hypothesis that epicatechin is able to modulate ECM turnover. The influence that epicatechin has on MMPs and ECM degradation is an area worthy of further research. Future directions for this research are to establish if collagen presence within the DOCA+E LV is significantly altered by epicatechin treatment and whether this is mediated via the MMP activity. Gelatinase activity (likely associated with MMP-2 and MMP-9) has been observed in sections of LV from DOCA-salt rats previously (Ammarguellat et al., 2002). Epicatechin treatment shows a much more specific cardiac response than vascular, which, although not improving electrophysiology, does improve stiffness and functional parameters of the heart.

In conclusion, we have demonstrated that epicatechin, when administered at a dose of $1 mg.kg^{-1}.day^{-1}$, was not able to prevent LV hypertrophy, but did

exhibit a significant improvement on systolic blood pressure and oxidative stress in hypertensive animals. Of further importance to the establishment of epicatehcin's cardioprotective properties was it's ability to prevent the increase in diastolic stiffness and changes in cardiac function in the DOCA-salt rat heart. The improvements in cardiac compliance may, in part, be a result of reduced blood pressure and improvements in oxidative stress, however the presence of increased LV mass within the DOCA+E rats suggests there may be another mechanism involved.

6.4.1 Limitations

A limitation of this study is the lack of data on fibrosis within the heart. Having this data would allow for a better understanding of how epicatechin may have achieved the improvements in diastolic stiffness and cardiac function, and why APD was not improved. Serum concentrations of nitric oxide would have helped in determining how MDA was improved, but also whether eNOS and NO bioavailability were improved, specifically within the DOCA-salt rat.

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Chapter 7

Conclusion

7.1 Overview

Cardiovascular damage and remodelling was successfully induced by the 4 week DOCA-salt treatment regime. Hypertensive control animals showed a mean blood pressure of 194mmHg, and this was associated with significantly increased serum concentrations of MDA, hypertrophy of the left ventricle, and changes in contractile and relaxant responses of aortic tissue segments. Mesenteric tissue rings did not show any significant change in maximal contractile and relaxant responses, however the EC50 for noradrenaline was increased, showing a diminished sensitivity to adrenergic stimuli. Cardiac function was also significantly affected by DOCA-salt treatment, with action potential durations showing more than a doubling in length. This effect was also observed in whole heart functional experiments, with rates of relaxation showing significant reductions. Similarly, the rate of contraction was also diminished in the hypertensive DOCA-salt-treated animals. Furthermore, mean LV developed pressure and end-systolic pressure were significantly reduced, demonstrating the impairment of the myocardium to generation contractile force. Lastly, as a measure of cardiac compliance, the diastolic stiffness constant was significantly increased in the DOCA-salt rats.

7.1.1 Nebivolol

Treatment with the 3^{rd}-generation β-blocker nebivolol ($0.5 mg.kg^{-1}$ daily for 4 weeks via oral-gavage) ameliorated the DOCA-salt effects on blood pressure across the entire treatment period, with final blood pressure showing

an overall reduction of 28 ± 15mmHg compared with DOCA-salt control animals. Furthermore, improvements were observed in developed pressure, rate of contraction, rate of relaxation, and end-systolic pressure. These observations are significant because, they were achieved despite there being no reduction or improvement in left ventricular hypertrophy (LVH), myocardial diastolic stiffness, action potential duration, or oxidative stress markers. The observed improvements of myocardial compliance are therefore most likely a direct result of the lower strain in the DOCA+N hearts compared to their untreated counterparts, who had much higher blood pressures and required a greater cardiac workload. The fact that nebivolol had no affect on the diastolic stiffness, LVH, or the action potential duration, indicates that although it was effective in lowering blood pressure and cardiac workload, it was not able to improve the underlying pathology within the model. Serum concentrations of MDA support this conclusion, with nebivolol-treated hypertensive rats showing comparative serum concentrations to the untreated hypertensive group (DOCA). Therefore, it is unlikely that nebivolol, at the chosen dose of 0.5mg.kg^{-1}, had any measurable effect on oxidative stress within the model, and was unable to improve pathological changes that were associated with oxidative stress.

Although improvements to the pathological remodelling of the cardiovascular system by treatment with 0.5mg.kg^{-1} nebivolol were observed, the study has shown that nebivolol is a potent anti-hypertensive drug. In comparison with other β-blocker studies in which the DOCA-salt rat model was used (Kobayashi et al., 2003, 2001; Tsuda and Masuyama, 1991), nebivolol

was able to achieve a significant reduction in blood pressure at a much lower dose than other β-blockers. It must be acknowledged however, that the compounds that make up the β-blocker class of drugs are very diverse, and their pharmacokinetic and pharmacodynamic profiles vary widely. The data also provided an interesting observation in relation to the mechanism of action of nebivolol. It was observed that normotensive rats treated with nebivolol showed an impaired contractile response to adrenergic stimuli within the aorta. A study by Rozec et al. (2006), suggests that nebivolol may also possess α_1 antagonist activity, an effect that provides an explanation for the results. The data from the current study potentially provides some of the first evidence of an α-adrenergic effect associated with chronic oral nebivolol treatment. However, further *in vitro* and *in vivo* experiments are required to confirm conclusively that α_1 is involved.

In conclusion, the NO enhancing properties of nebivolol, at a dose of 0.5mg.kg^{-1}, have no significant effect on the development of oxidative stress or the pathological remodelling that occurs within the DOCA-salt hypertensive rat.

7.1.2 *Stevia rebaudiana*

Stevia rebaudiana extract, at a daily dose of 200mg.kg^{-1} (via oral garage) for 4 weeks, did not prevent DOCA-salt-induced hypertensive rats, despite significant improvements in MDA and NO bioavailability at the end of the experiment. Although studies have shown that improving oxidative stress

can result in improvements in LVH and diastolic stiffness (Fenning et al., 2005; O'Brien et al., 2009), our data does not show this relationship. Such a response is expected if the antioxidant effects of stevia extract are mediated primarily through gene expression and modulation of antioxidant enzyme expression, rather than a direct oxidant scavenging and eNOS activation mechanism. This hypothesis allows for the development of oxidative stress within the short-term, promoting cardiovascular remodelling before stevia's effect on antioxidant expression levels is sufficient to normalise oxidative stress. This provides justification for further research into much longer periods of treatment with stevia, as well as pre-treatment with stevia before initiating the DOCA-salt model.

Even though LVH and diastolic stiffness were not improved by stevia treatment, other measures of cardiac function were. Developed pressure, rate of contraction, and end systolic pressure were all normalised. Interestingly, rate of relaxation did not show a significant improvement, however it did show a trend towards normalised values. Remodelling of the myocardium within the DOCA-salt model is not solely dependent on blood pressure or oxidative stress, but also involves aspects of inflammation, as previous studies have shown (Ammarguellat et al., 2002; Fenning et al., 2005; Iyer et al., 2010a,b; Mirkovic et al., 2002). Consequently, the difference in myocardial function between the DOCA and DOCA+Stevia animals may be partially a result of improved oxidative stress, but the normalisation of the values implies that stevia may also possess an anti-inflammatory effect. Stevia was also able to reduce and normalise cardiac electrophysiological remodelling, with action

potential durations in stevia-treated DOCA rats showing a significant shortening of the APD compared to their untreated counterparts. In view of the persistent left ventricular hypertrophy observed in the DOCA+S animals, this effect on APD is significant. Previous studies have observed improvements in APD in conjunction with regression of LVH, and have concluded that improved APD is a result of increased ion channel density secondary to reduced LVH (Fenning et al., 2005; Iyer et al., 2010a; Loch et al., 2006a,b). The data obtained from the stevia experiments shows that this is not the case in regards to stevia, rather changes in electrophysiology are not dependent on regression of LVH. This indicates that stevia is able to affect cardiac ion handling directly. However, whether this is through modulation of ion channels or modulation of channel expression remains to be seen. From this study, it can be concluded that *Stevia rebaudiana* provides significant cardioprotection within the DOCA-salt model, and this is independent of any reduction in blood pressure and left ventricular hypertrophy.

7.1.3 *Lycium barbarum* (goji berry)

The extract from *Lycium barbarum* (200mg.kg^{-1} via oral gavage, daily for 4 weeks) demonstrated a significant ability to provide cardioprotection within the DOCA-salt model, with improvements in oxidative stress, cardiac electrophysiological remodelling, and cardiac functional parameters. These improvements were accompanied by a significant reduction in blood pressure, however BP was not normalised and still showed a significant elevation from

the normotensive baseline. Similar to both stevia and nebivolol treatments, goji berry extract was unable to improve either diastolic stiffness or left ventricular hypertrophy. As with stevia, goji showed a significant decrease in MDA and a corresponding rise in NO bioavailability at the the time of terminal experiments. Therefore, it can be inferred that goji's effect on MDA and NO is not an acute effect, but rather a result of the chronic dosing regime. Previous research on goji extract has shown an ability to enhance expression of the peroxisome proliferator activated receptor-γ (PPAR-γ) (Song et al., 2011), which is able to stimulate NO production and release (Polikandriotis et al., 2005). This provides a mechanism for goji berry extract's ability to increase NO and therefore decrease oxidative stress, and also supports the hypothesis of an effect that is a result of chronic dosing rather than acute direct action.

Goji treatment was able to significantly improve APD within the DOCA-salt rats, with all three of the measured parameters showing no significant difference from normotensive values. This is in contrast to stevia, which was unable to normalise the APD at 90% of repolarisation, and it is also consistent with a previous study that found goji extract could modulate intracellular Ca^{2+} handling (Shao-Ping and Pin-Ting, 2010). Goji extract had very little effect on the vascular remodelling that occurred in the animals, and overall, goji extract appears to provide protection to the cardiac tissue much better than it does to the vasculature. Based on previous studies, it is most likely that this cardioprotection is a result of goji's ability to modulate the expression of PPAR-γ as well as modulating Ca^{2+} handling (possibly

through SERCA2 activity) within the cardiomyocytes.

7.1.4 (-)-Epicatechin

Epicatechin (1mg.kg^{-1} via oral gavage, daily for 4 weeks) shares a similar effect on cardiac function as nebivolol, stevia, and goji, as it is able to normalise the change in myocardial compliance associated with the DOCA-salt hypertension model. However, unlike the others, it also attenuated the increase in diastolic stiffness observed in the DOCA-salt rat model. This is in stark contrast to the other three compounds, which were unable to improve diastolic stiffness even though they improved cardiac compliance. Unlike the other compounds, epicatechin showed a significant effect on left ventricular mass, with both UNX+E and DOCA+E rats showing hypertrophy of the left ventricle. Furthermore, APD was increased in both the DOCA and DOCA+E rats, a change that is often observed in conjunction with LVH and proposed to be a result of decreased ion channel density due to increased LV mass (Fenning et al., 2005; Iyer et al., 2010a; Loch et al., 2006a,b). Diastolic stiffness was not increased in either of the epicatechin-treated groups, suggesting that the remodelling of the myocardium is not solely the result of increased collagen deposition and scar tissue formation. This hypothesis is based on previous research that showed catechins (epicatechin and epigallocatechin-3-gallate) may have an inhibitory effect on matrix metalloproteinases (MMPs) (Khoi et al., 2013; Wang et al., 2014; Yamazaki et al., 2008). It has been shown that by over-expressing TIMP-4 (TIMPs control

the activity of MMPs), collagen type IA1 and type IIIA1 were reduced in a ventricular pressure overload model (Yarbrough et al., 2014). Therefore, the myocardial remodelling observed in DOCA+E rats (also UNX+E rats to a lesser extent) may not show the same level of collagen deposition or MMP activity as has been observed in of DOCA-salt studies (Ammarguellat et al., 2002; Chan et al., 2006; Fenning et al., 2005; Iyer et al., 2010b).

Epicatechin has also been shown to increase NO bioavailability by activating eNOS (Ramirez-Sanchez et al., 2012, 2011). In healthy endothelial cells, this would lead to an increase in NO. However, within cells affected by high levels of oxidative stress, this may indeed have the opposite effect. Diminished levels of the eNOS cofactor tetrahydrobiopterin has been shown to lead to the uncoupling of eNOS, resulting in eNOS producing $O_2^{\bullet -}$ instead of NO (Schulz et al., 2008), an occurrence that has been observed within the DOCA-salt model (Landmesser et al., 2003). Therefore, epicatechin's ability to active eNOS may be detrimental within the DOCA-salt rat, especially in cells undergoing significant oxidative stress. This mechanism may be an explanation for the difference in acetylcholine-mediated relaxation of UNX+Epi and DOCA+Epi aortic tissues.

Overall, epicatechin was able to provide significant protection against cardiovascular damage within the DOCA-salt rat, as demonstrated by improvements in serum MDA, blood pressure, and cardiac function. Of particular importance was the finding that epicatechin treatment ($1 mg.kg^{-1}$) was able to preserve myocardial function independent of LVH.

7.1.5 Summary

The findings from these experiments show that each compound was able to affect the cardiovascular system within the DOCA-salt hypertensive rat model to differing degrees. Nebivolol, at a dose lower than many other studies have investigated, was shown to achieve a significant hypotensive effect, although this was not associated with any significant improvement in cardiovascular function. Even though cardiovascular function was not preserved or improved by Nebivolol within the study period, the significant haemodynamic effect observed suggests a potential for much better effects to be observed in a less aggressive model of hypertension. For example, the SHR model allows for a much longer period of treatment, and if the haemodynamic profile was similar over time the beneficial effects of significant BP reduction could may well be observed. Stevia and goji extracts both improved oxidative stress, promoting increased NO and normalised MDA levels, as well as attenuating the loss of myocardial functionality in the DOCA rats. Both of these compounds improved the change in APD, although goji berry extract was able to normalise APD across the entirety of the repolarisation cycle. This demonstrates that although stevia and goji achieved similar outcomes in terms of cardioprotection, their mechanisms may be vastly different. It should be noted though that the dose for both stevia and goji treatments was far greater than would be encounted via a normal balanced diet. The doses used are more closely linked to those that would be seen in terms of a supplementation or complimentary medicine approach. A future direction for the

research would be to assess these compounds at a lower dose, to determine if these cardioprotective effects are dose dependent. The results from these studies do raise an interesting question about the potential of combining a low-dose Nebivolol dose, such as 0.5mg.kg^{-1}, with a supplementary dose of stevia, goji berry extract or epicatechin. Unlike all of the other compounds, epicatechin was able to prevent the increase in diastolic stiffness that was observed in all of the DOCA groups, an effect that was independent of LVH. Such a result provides support for the hypothesis that epicatechin may be able to preserve cardiac function in situations of cardiac hypertrophy. It would be beneficial to assess epicatechin in either a longer model of DOCA-salt, or another more chronic model of hypertension to fully investigate it's ability to preserve cardiac function in situations where LVH is observed. It may be the case that epicatechin only delays the onset of the negative affects associated with LVH, or it may promote a more physiological hypertrophy pathway. Overall, these results demonstrate that targeting oxidative stress within hypertension, particularly within the DOCA-salt model, is an effective mechanism for providing cardioprotection.

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