INVESTIGATION OF THE MECHANISM UNDERLYING THE VASOPROTECTIVE EFFECTS OF RED YEAST RICE EXTRACT IN SPONTANEOUSLY HYPERTENSIVE RATS

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By

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ABSTRACT

INVESTIGATION OF THE MECHANISM UNDERLYING THE VASOPROTECTIVE EFFECTS OF RED YEAST RICE EXTRACT IN SPONTANEOUSLY HYPERTENSIVE RATS

Tan Jiunn Jye

Hypertension is linked to endothelial dysfunction, characterized by an imbalance between relaxing and constricting factors in the vasculature. Increased activity in the renin-angiotensin-aldosterone system (RAAS) is implicated in hypertension as it leads to increased vasoconstriction and oxidative stress to cause endothelial dysfunction. Red yeast rice (RYR) is a traditional Chinese medicine that contains monacolin K, similar to statins, which have been reported to inhibit RAAS activity. To add, RYR was reported to have anti-hypertensive effects as well. Hence, the present study aimed to elucidate the vasoprotective effects of RYR through RAAS suppression by oral administration in spontaneously hypertensive rats (SHR). SHR were randomly divided into 3 groups: SHR – Control; SHR – RYR (100 mg/kg/day); SHR – lovastatin (10 mg/kg/day). Wistar-Kyoto Rats (WKY) were used as normotensive controls. All animals were treated for 12 weeks by oral gavage. Systolic blood pressure was measured weekly by tail-cuff method. Vascular reactivity was determined using isolated aortic rings in an organ bath, Aortic levels of reactive oxygen species (ROS) and nitric oxide (NO) were determined by fluorescence assays in cryostat

sections of aorta, while tetrahydrobiopterin (BH_4) and cyclic guanosine monophosphate (cGMP) levels were measured by enzyme-linked immunosorbent assay (ELISA). Expression of vascular angiotensin II type 1 (AT_1) and type 2 receptor (AT_2) were evaluated by Western blot. It was observed that administration of RYR attenuated systolic blood pressure elevation and improved ACh-induced relaxation in aortic rings, thus suggesting the involvement of the eNOS-cGMP pathway. This suggestion is further supported by the observation whereby incubation of aortic rings from untreated SHR with a combination of RYR and N-Nitro-L-arginine methylester (L-NAME) resulted in a nearly complete inhibition of relaxation of the vascular tissue. In addition, RYR decreased ROS production and AT_1 receptor expression and significantly improved the levels of vascular NO, BH_4, cGMP and AT_2 receptor expression. These findings show that treatment with RYR extract for 12 weeks reduced the expression of AT_1 receptors, leading to attenuated oxidative stress that decreases eNOS uncoupling via improving the level of BH_4 and thus enhanced NO-cGMP signalling. These effects contribute to the improvement in vascular function and hence reduced systolic blood pressure observed in SHR. This study contributes new information regarding the blood pressure lowering mechanisms of RYR and its potential use as a complementary treatment for hypertension.

Keywords: Red yeast rice, oxidative stress, nitric oxide, vascular function, hypertension, functional food

Subject Area: RC666-701 Diseases of the circulatory (Cardiovascular) system

Subject Area: RM1-950 Pharmacology

Subject Area: RM300-666 Drugs and their actions

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DECLARATION

I TAN JIUNN JYE hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
ACh	Acetylcholine
AChE	Acetylcholinesterase
ARB	Angiotensin receptor blockers
AT_1	Angiotensin II type 1
AT_2	Angiotensin II type 2
BH_2	Dihydrobiopterin
BH_4	Tetrahydrobiopterin
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
COX-2	Cyclooxygenase-2
DAF-FM	4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate
DAG	Diacylglycerol
DHE	Dihydroethidium
EDCF	Endothelium-derived constricting factors
EDHF	Endothelium-derived hyperpolarising factor
EDRF	Endothelium-derived relaxing factors
eNOS	Endothelial nitric oxide synthase

ET-1	Endothelin-1
HMG-CoA	3-hydroxy-3-methyglutaryl coenzyme A
HRP	Horseradish peroxidase
INDO	Indomethacin
iNOS	Inducible nitric oxide synthase
IP	Prostacyclin receptor
IP_3	Inositol triphosphate
L-NAME	N-Nitro-L-arginine methylester
NADPH	Nicotinamide adenine dinucleotide phosphate
nNOs	Neuronal nitric oxide synthase
NO	Nitric oxide
NOx	NADPH oxidase
OCT	Optimal cutting compound
$ONOO^-$	Peroxynitrite
pEC_{50}	Potency (negative logarithm of the EC_{50})
PGI_2	Prostacyclin
PKA	Protein kinase A
PLC	Phospholipase C
PVDF	Polyvinylidene fluoride
RAAS	Renin angiotensin aldosterone system

RIPA	Radioimmunoprecipitation assay buffer
R_{max}	Maximal relaxation
ROS	Reactive oxygen species
RYR	Red yeast rice
SDS	Sodium dodecyl sulphate
sGC	Soluble guanylyl cyclase
SHR	Spontaneously hypertensive rats
SNP	Sodium nitroprusside
TBS	Tris-buffered saline
TP	Thromboxane-prostanoid receptor
TXA_2	Thromboxane
WKY	Wistar-Kyoto rats

CHAPTER 1

INTRODUCTION

Hypertension is a multifactorial disease that involves the interactions between genetically determined haemostatic control mechanisms and environmental factors (Das et al., 2023). It is a major risk factor for cardiovascular complications such as increased vascular tone and remodelling (Humphrey, 2021). Chronic elevation of systolic blood pressure is associated with endothelial dysfunction (Touyz et al., 2020). Endothelial dysfunction is characterised by an imbalance between the release of endothelium-derived relaxing factors (EDRF), such as nitric oxide (NO) and prostacyclin (PGI_2), and endothelium-derived constricting factors (EDCF) including endothelin-1 (ET-1), angiotensin II and thromboxane (Nappi et al., 2022). Overproduction of reactive oxygen species (ROS) quenches available NO to reduce vasodilation. The resulting increase in endothelium-dependent contractions results in the increased peripheral resistance seen in primary hypertension (Kostov, 2021).

The renin angiotensin aldosterone system (RAAS) is one of the physiological systems responsible for the regulation of arterial blood pressure (Swiderski et al., 2023). Briefly, this system is triggered in response to decreased blood volume in the circulatory system (Swiderski et al., 2023). Renin produced from the kidneys converts angiotensinogen produced by the liver into angiotensin I. Angiotensin I is then cleaved into angiotensin II by angiotensin converting enzyme (ACE). Being the primary effector peptide, angiotensin II is

responsible for the vasoactive effects by RAAS and sodium retention properties by promoting aldosterone secretion (Martyniak and Tomasik, 2022). Angiotensin II activates various signalling pathways by its binding to two distinct subtypes of G-protein coupled receptors: angiotensin II type 1 (AT_1) receptors and angiotensin II type 2 (AT_2) receptors (Martyniak and Tomasik, 2022). AT_1 receptors can activate phospholipase C (PLC) to recruit secondary messengers including inositol triphosphate (IP_3) and diacylglycerol (DAG) to eventually lead to the phosphorylation of Ca^{2+} channels, increasing the vascular tone (Eckenstaler et al., 2021). Also, AT_1 receptor activation also stimulates nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOx). These enzymes enhance the production of ROS, further contributing to oxidative stress, vasoconstriction, vascular remodelling and inflammation (Eckenstaler et al., 2021). In contrast, activation of AT_2 receptors have been reported to exert opposing functions to the activation of AT_1 receptors, such as promoting NO release to stimulate vasodilation and decrease ROS production through the inhibition of NOx, resulting in the attenuation of oxidative stress (Fatima et al., 2021).

Research on novel pharmacological agents for the management of hypertension has resulted in the creation of AT_1 receptor blockers. Pharmacological blockade of AT_1 receptors has been viewed as an important therapeutic approach as increased RAAS activity, leading to increased angiotensin II/AT_1 receptor signalling that is linked to the development of vascular abnormalities and pathological changes established during hypertension (Gallo et al., 2022a). In studies performed on hypertensive animal

models, AT_1 receptor antagonism has been demonstrated to improve acetylcholine-induced endothelium-dependent relaxations, decrease in AT_1 receptor and NOx expression and attenuate ROS production (Polina et al., 2020, Elseweidy et al., 2020, Zheng et al., 2022). Hence, these studies have shown the significance of angiotensin II/ AT_1 receptor signalling in modulating endothelial function.

In recent years, there has been increasing use of dietary foods and supplements that aid in the prevention and control of essential hypertension, including red yeast rice (RYR). RYR is a functional food product made from the fermentation of rice with a species of mould known as *Monascus purpereus* (Hu et al., 2020). Used as traditional medicine for thousands of years in the east, RYR has been recognised in traditional Chinese medicine for its anti-dyslipidaemia properties that is attributed to its most abundant active component monacolin K (Hu et al., 2020). Monacolin K shares similar structure to lovastatin, which inhibits 3-hydroxy-3-methyglutaryl coenzyme A (HMG-CoA) reductase involved in de novo cholesterol synthesis (Zhang et al., 2020a). This class of drugs, known as statins, have been reported to have suppressive effects on the RAAS. In spontaneously hypertensive rats (SHR), it was reported that orally administered pitavastatin had downregulated the expression of AT_1 (Zhang et al., 2017) receptor while simvastatin was observed to suppress its main activator enzyme renin (Huang et al., 2023). Furthermore, RYR was also reported to have antihypertensive effects in human subjects and murine model by improving endothelial function and attenuation of oxidative stress (Lin et al., 2015, Yuan et al., 2022). While the anti-hypertensive effects of RYR have been well-

documented, most studies have not fully explored its role in modulating RAAS activity, particularly through the inhibition of AT_1 receptor. This study aims to address this gap by evaluating the specific mechanisms underlying the blood pressure lowering properties of RYR, providing a better understanding of its potential therapeutic role in hypertension.

In this context, increased angiotensin II/AT1 receptor signalling plays a key role in the development of endothelial dysfunction in hypertension. RYR has been shown to lower blood pressure, and it contains monacolin K, an HMG-CoA reductase inhibitor, which have been reported to exert inhibitory effects on the RAAS. Taken together, it can be hypothesised that oral administration of RYR may improve vascular function through the suppression of RAAS activity by inhibition of AT_1 receptor.

Therefore, by using a genetically hypertensive animal model, SHR – the standard animal model for human primary hypertension, the present study aims to:

i) To examine if supplementation with RYR improves vascular function of SHR using functional study (organ bath method).

ii) To evaluate if supplementation with RYR improves the levels of reactive oxygen species (ROS), nitric oxide (NO), cyclic guanosine monophosphate (cGMP) and tetrahydrobiopterin (BH_4) in the vascular tissue of SHR.

iii) To evaluate if supplementation with RYR modulates the expression of AT_1 and AT_2 receptors in the vascular tissue of SHR.

Chapter 2

Literature review

2.1 Vascular endothelium

The vascular endothelium consists of a monolayer of cells found on the inner linings of the vascular system (Rahman and Siddik, 2023). It comprises of endothelial cells anchored to the basal lamina, forming the intima of the blood vessels (Figure 2.1) (Krüger-Genge et al., 2019). The endothelium maintains vascular function by releasing various vasoactive factors in response to numerous stimuli such as shear stress, hormones and cytokines (Rahman and Siddik, 2023). In addition, it is also involved in the upregulation of inflammatory cytokines, platelet aggregation and thrombosis (Baghai et al., 2018).

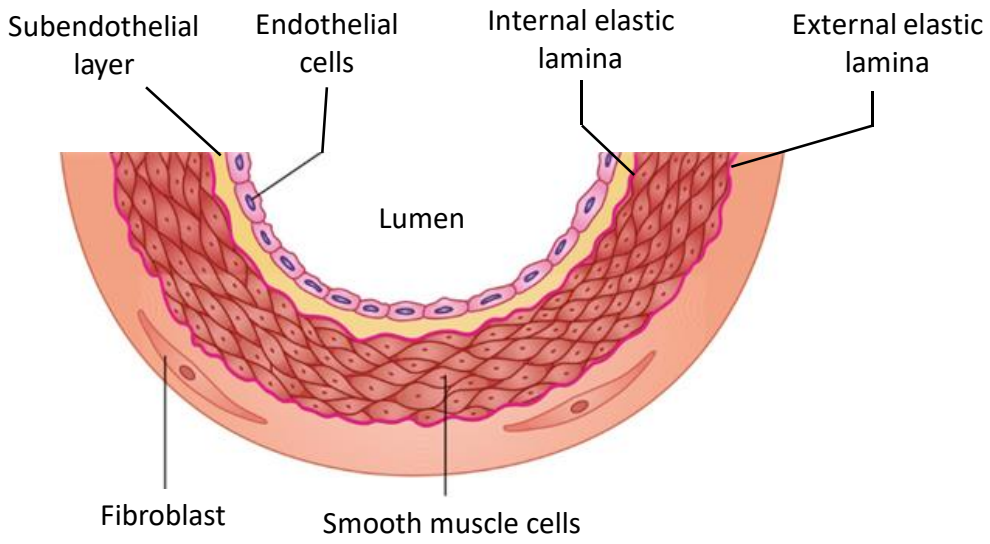

Figure 2.1: Cross section of an artery illustrating the location of the endothelium (Reproduced from Onyeweu et.al. 2021).

The vasoactive factors released by the endothelium can be either vasodilatory or vasoconstrictive. Vasodilating factors include nitric oxide (NO), prostacyclin (PGI_2) and endothelium-derived hyperpolarising factor (EDHF), which dilates blood vessels leading to increase blood flow and lowered pressure (Nappi et al., 2022). Vasoconstricting factors such as endothelin-1 (ET-1) and thromboxane (TXA_2) cause the opposite effect, leading to narrowed lumen and increased blood pressure (Ray et al., 2023).

2.1.1 Endothelium-derived relaxing and contracting factors

The presence of an endothelium-derived relaxing factor (EDRF) was first suggested by Furchgott and Zawadzki in the 1980s, when the pair demonstrated that acetylcholine relaxes isolated preparations of rabbit aorta only if the vascular endothelium is present, suggesting that endothelial cells produce a substance that causes relaxation in neighbouring smooth muscle cells (Furchgott and Zawadzki, 1980). Investigations by Palmer and colleagues then identified the substance to be NO (Palmer et al., 1987). Following this, two more EDRFs have been discovered, namely prostacyclin and EDHF (Kang, 2014). It is now known that the stimulation of endothelial cells by stimuli such as hormones, neurotransmitters, platelet-derived substances and shear stress release EDRFs that are readily diffused into adjacent vascular smooth muscle cells to initiate relaxation (Nappi et al., 2022).

NO generation in mammals is produced by nitric oxide synthase (NOS) which present in three different isoforms, all of which generate NO from L-arginine, molecular oxygen and nicotinamide adenine dinucleotide phosphate (NADPH) (Tran et al., 2022). Endothelial nitric oxide synthase (eNOS) is expressed in the endothelial cells lining the blood vessels; neuronal nitric oxide synthase (nNOS) is typically expressed in the synaptic cleft of neurons and inducible nitric oxide synthase (iNOS) is found in phagocytes or epithelial cells where expression is activated by proinflammatory cytokines (Król and Kepinska, 2021). Structurally, NOS are homodimeric heme-containing enzymes with an N-terminal oxygenase domain linked to a C-terminal reductase domain

via calmodulin-binding domain. Within the oxygenase domain is also tetrahydrobiopterin (BH_4) that serves as a vital cofactor for NO production (Król and Kepinska, 2021). Briefly, electrons from NADPH pass from the reductase domain to the heme iron in the oxygenase domain, enabling the binding of O_2 (Tenopoulou and Doulias, 2020). Another electron BH_4 is then donated to the heme-bound oxygen, enabling the reaction between O_2 and L-arginine to release NO (Tenopoulou and Doulias, 2020). Once produced, NO signalling is transduced by diffusion to its target sites, which may be in the same or adjacent cell. Paracrine signalling is possible in NO due to its small, uncharged and lipophilic characteristics, allowing it to pass through cellular membranes (Lundberg and Weitzberg, 2022). In the target cells, NO activates soluble guanylyl cyclase (sGC) which in turn increases the production of cyclic guanosine monophosphate (cGMP) that in converted from guanosine triphosphate (GTP) (Golshiri et al., 2020). In the vasculature, cGMP acts as a secondary messenger to induce smooth muscle relaxation by i) inhibition of Ca^{2+} entry to decrease intracellular Ca^{2+} concentration; ii) hyperpolarisation of smooth muscle cells and iii) activation of myosin light chain phosphatases that dephosphorylates myosin light chains to inhibit contraction (Silva et al., 2021).

In addition to NO, the endothelium also produces prostaglandins (PGI_2) and endothelium-dependent hyperpolarising factors (EDHF) to aid in vasodilation. PGI_2 is produced from the conversion of arachidonic acid catalysed by the enzymes cyclooxygenase-2 (COX-2) and prostacyclin synthase (Lau and Lui, 2022). PGI_2 binds to prostacyclin receptors (IP) and upon activation induces the production of cyclic adenosine monophosphate (cAMP) leading to activation

of protein kinase A (PKA) to result in vasorelaxation (Lau and Lui, 2022). EDHF hyperpolarises the underlying smooth muscle cells, causing an efflux of K^+ from smooth muscle cells. The change in membrane potential reduces intracellular Ca^{2+} concentration, causing relaxation (Garland and Dora, 2021). Typically, endothelium-derived NO regulates large conduit artery relaxation, while EDHF mediates vascular tone at smaller resistance arteries. However, there is currently no single universally accepted EDHF. Current candidates include K^+, arachidonic acid metabolites, hydrogen peroxide (H_2O_2), which act as signalling molecules to the smooth muscle cells and myoendothelial gap junctions that mediate the spread of endothelial cell hyperpolarisation (Garland and Dora, 2021).

Endothelial cells also initiate vasoconstriction through contractions on the underlying smooth muscle cells through the release of EDCFs. These EDCFs are namely thromboxane (TXA_2), endothelin-1 (ET-1) and reactive oxygen species (Poredos et al., 2021). Thromboxane is produced by COX enzymes and thromboxane synthase. As the ligand to thromboxane-prostanoid (TP) receptors found on platelets and smooth muscle cells, it leads to platelet aggregation and vasoconstriction by increasing intracellular Ca^{2+} levels of smooth muscle cells (Badimon et al., 2021). Since COX enzymes are responsible for the production of both TXA_2 and PGI_2, the balance of these two substances is vital for maintaining homeostasis in the healthy vasculature where PGI_2 activity outweighs TXA_2 (Mitchell et al., 2021, Knowles and Warner, 2019). The COX pathway produces ROS as a by-product as well, as TXA_2 synthesis up-regulates the activity and expression of NADPH oxidases (Wang et al., 2020). Endothelin-1 (ET-1) is a part of the endothelin peptide family. A potent vasoconstrictor

peptide, it interacts with two G-coupled protein receptors (GPCR); ET_A and ET_B (Enevoldsen et al., 2020). ET_A is expressed primarily vascular smooth muscle cells, promoting vasoconstriction, inflammation and cell proliferation (Barton and Yanagisawa, 2019). On the endothelial wall however, ET_B is expressed predominantly, and its activation leads to the release of NO and PGI_2 to cause vasorelaxation (Barton and Yanagisawa, 2019).

2.2 Endothelial dysfunction

The loss of normal endothelial function is associated with most forms of cardiovascular diseases, including hypertension (Chaudhary et al., 2020). Endothelial dysfunction is characterised by a shift from vasodilation to vasoconstriction due to imbalance between the release of EDRF and EDCF (Poredos et al., 2021).

Reduced NO is linked to impaired endothelial function due to the following factors. It results from the reduced activity of eNOS leading to the decrease in NO bioavailability. Synthesis of NO from L-arginine and molecular oxygen requires the cofactor tetrahydrobiopterin (BH_4) for functional eNOS activity (Janaszak-Jasiecka et al., 2023). During states of oxidative stress, ROS react with NO to form peroxynitrite ($ONOO^-$), which is a cytotoxic oxidant that also causes nitration of proteins to further causes endothelial damage (Poznyak et al., 2020). Also, peroxynitrite causes oxidative degradation of BH_4 to dihydrobiopterin (BH_2). When the levels of BH_4 are limiting, "eNOS uncoupling" occurs (Krüger-Genge et al., 2019). This causes the reductase function of eNOS to activate, leading to ROS being produced instead of NO (Tenopoulou and Doulias, 2020). This excess of ROS in the vascular endothelium decreases NO bioavailability and further aggravates the impairment of endothelial function (Figure 2.2).

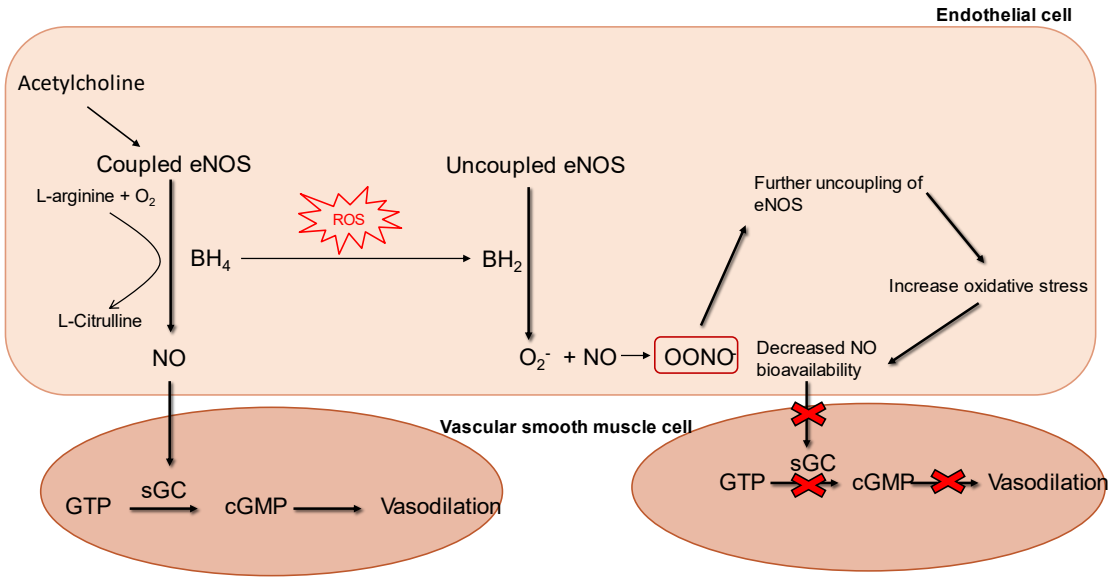

Figure 2.2: Schematic diagram illustrating the general mechanisms leading to endothelial dysfunction in the vascular endothelium and smooth muscle cells. Functional eNOS (coupled eNOS) requires the optimal concentration of tetrahydrobiopterin (BH_4) as cofactor to facilitate NO production from L-arginine. NO initiates vasodilation through cGMP-dependent downstream signalling cascade. Oxidative degradation of BH_4 by ROS accumulation leads to the uncoupling of eNOS causing superoxide (O_2^-) production instead. Peroxynitrite ($ONOO^-$), also a species of ROS, is produced from the interaction between NO and O_2^-. This results in a cycle of continuous eNOS uncoupling and increased oxidative stress, leading to the development of endothelial dysfunction (Reproduced from Hyunh et.al. 2019).

2.2.1 Endothelial dysfunction and ROS

Research from recent years has demonstrated that ROS are involved in endothelial dysfunction by activation of redox-signalling pathways related to hypertrophic remodelling, vasoconstriction, inflammation, and apoptosis in the cardiovascular system (Sarmiento et al., 2015, Poznyak et al., 2020 and Theofilis et al., 2021). The balance between ROS and antioxidant enzymes or ROS scavengers are regulated by the rate of production and the rate of its scavenging (He et al., 2017). Increased production of vascular ROS causes an imbalance in normal vascular homeostasis, leading to a condition known as oxidative stress. ROS are key signalling molecules that stimulate pathways involved in inflammation and vascular remodelling. These pathways include the activation of MAPK, NF-κB, AKT and JAK-2/STAT (Gutterman et al., 2016).

The major types of ROS are superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (•OH) and peroxynitrite ($ONOO^-$) (Jakubczyk et al., 2020). NADPH oxidase (NOx) is the major source of ROS formation in the vasculature (Zhang et al., 2020b). It is a family of transmembrane proteins that facilitates the electron transport from NADPH to molecular oxygen to produce superoxide and hydrogen peroxide. It is formed from subunits of the NOx family comprising of two cytosolic subunits (p47phox, p67phox), gp91phox, p22phox and a regulatory G protein Rac (Skonieczna et al., 2017). NOx1, NOx2, NOx4, and NOx5 are among its subtypes that are expressed in the endothelium and vascular smooth muscle cells. (Knock, 2019). These proteins are upregulated by shear stress, vasoactive factors (such as angiotensin II and ET-1) and growth factors

(such as epidermal growth factor (EGF) and platelet-derived growth factors (PDGF)) (Knock, 2019). Upregulation of NOx increases the production of ROS in the vasculature and subsequently the reduction of NO bioavailability as ROS reacts with NO to produce peroxynitrite (Piacenza et al., 2022).

Oxidative stress has been observed in hypertensive patients and animal models such as spontaneously hypertensive rats (SHR) (Wu et al., 2021, Khor et al., 2023). Oxidative stress not only causes NO to be depleted but also abnormal cell signalling and protein post-translational modifications (oxidation and phosphorylation). Protein phosphatases such as tyrosine phosphatase are inactive in the oxidised state (Touyz et al., 2020). This leads to additional downstream protein targets involved in inflammation being phosphorylated and activated, which can also further the development of hypertension and vascular remodelling (Touyz et al., 2020). In addition, oxidative stress is linked to a proinflammatory state of the vessel walls through upregulation of adhesion molecules (VCAM-1 and ICAM-1) that stimulate leucocyte adherence (Zhang et al., 2021).

2.3 Use of organ bath to study vascular function in isolated rat aorta

The organ bath technique is a widely used method to assess vascular function by isolating arterial segments, such as the rat aorta, and measuring changes in vascular tone. After sectioning the aorta into small rings, it is placed in a chamber filled with Kreb's buffer, that is aerated with 95% O_2 and 5% CO_2 at 37°C to mimic *in-vivo* conditions (Figure 2.3). The tissue is also connected to a force transducer that measures any changes in vascular tone to indicate constriction or dilation in response to different pharmacological agents (Robinson 2013). Isolation of the rat aorta was chosen for this study over smaller vessels as it is both larger and more accessible, allowing for more reliable measurements of vascular response. Additionally, as the aorta is a large vessel, it does not require the higher precision of wire myography, thus making the organ bath a more appropriate method for this study.

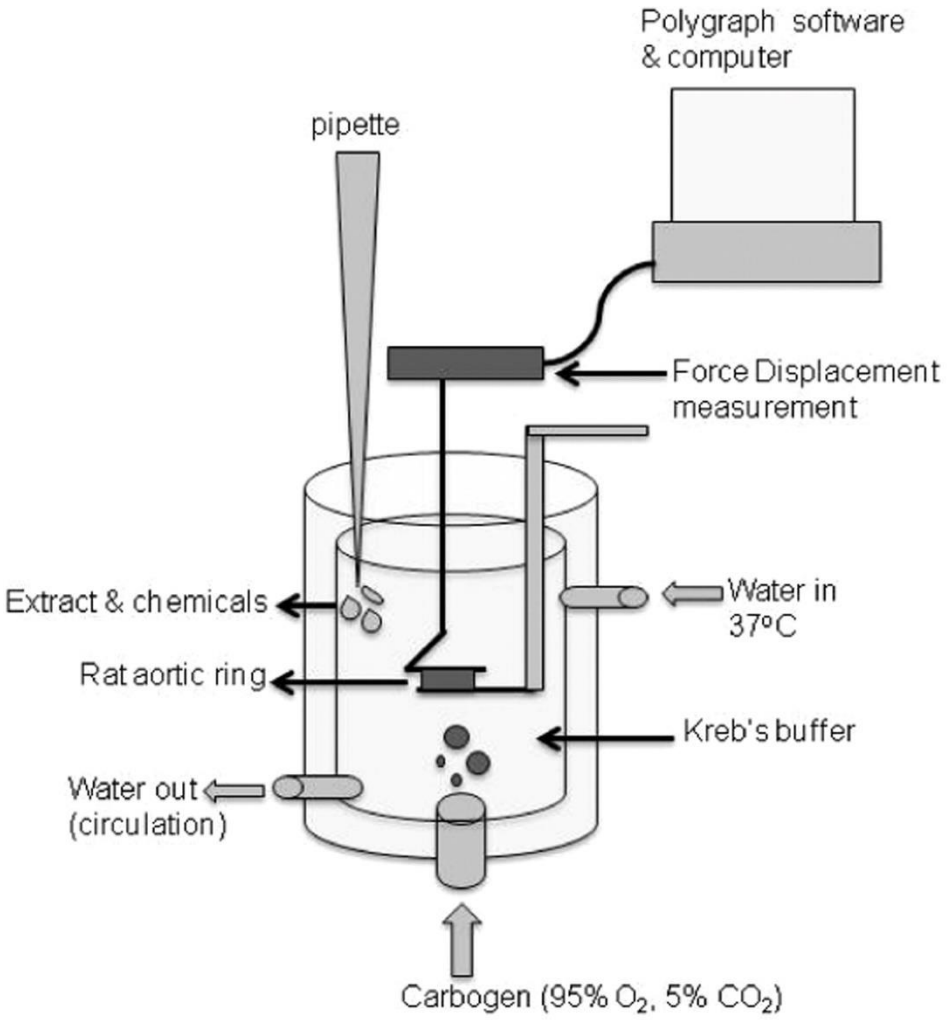

Figure 2.3: Schematic set-up of an organ bath (Shahlehi and Petalcorin, 2021).

2.4 Renin Angiotensin Aldosterone System

The renin angiotensin aldosterone system (RAAS) is an important regulator of electrolyte balance (water and sodium retention) and blood pressure (Azushima et al., 2020). Renin is the first and rate-limiting step that converts angiotensinogen to angiotensin I. Angiotensin I is then converted by angiotensin-converting enzyme (ACE) to angiotensin II. Angiotensin II is the main effector peptide of the RAAS. Angiotensin II mediates a variety of actions including vasoconstriction, water and sodium retention vascular remodelling and aldosterone production (Nwia et al., 2023). The actions of angiotensin II are mediated by 2 main receptor subtypes, angiotensin II type 1 (AT_1) receptor and angiotensin II type 2 (AT_2) receptor. Both subtypes are seven transmembrane G-protein coupled receptors and are differentiated by their biochemical and pharmacological properties, and their respective signalling pathways (Nwia et al., 2023) (Figure 2.4).

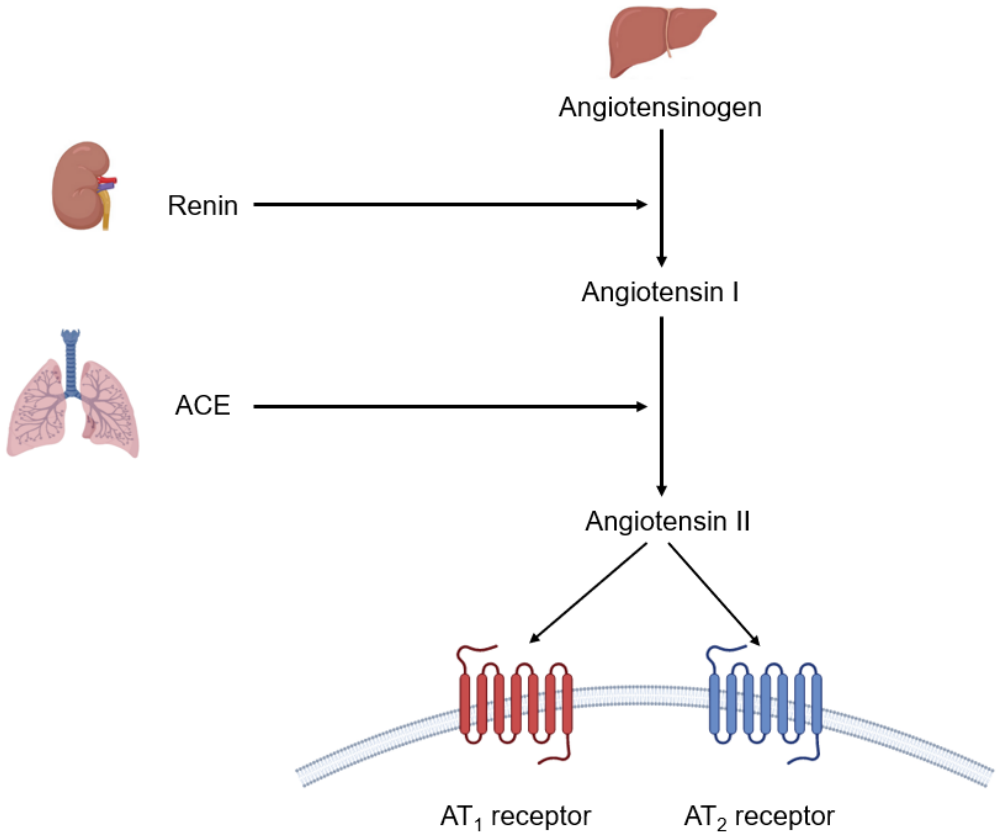

Figure 2.4: Illustration depicting the steps of the RAAS pathway. Renin released from juxtaglomerular cells of the kidney cleaves angiotensinogen produced from the liver into angiotensin I. Angiotensin I is further converted into angiotensin II by ACE secreted by the lungs. Effects mediated by angiotensin II are caused by the binding of angiotensin II to AT_1 and AT_2 receptor. ACE: angiotensin converting enzyme; AT_1: angiotensin II type 1; AT_2: angiotensin II type 2. (Reproduced from Gambaryan et al., 2023)

2.4.1 Angiotensin II Type 1 Receptor

The AT_1 receptor is responsible for classical actions of angiotensin II. These actions include vasoconstriction of arterioles, release of vasopressin to increase water reabsorption in the collecting ducts and secretion of aldosterone from the adrenal cortex to promote sodium and water retention in the distal tubules and collecting ducts (Nehme et al., 2019).

AT_1 receptors are widely expressed in the heart, kidneys, vascular smooth muscle cells, endothelium, brain, adrenal glands and adipocytes (Steckelings and Unger, 2019). The binding of angiotensin II to AT_1 receptor transduces phospholipase C (PLC) dependent signalling pathways, leading to the production of secondary messengers' inositol triphosphate (IP_3) and diacylglycerol (DAG) (Colin et al., 2023). These in turn release Ca^{2+} from vascular smooth muscle cells to activate myosin light chain kinase (MLCK) to trigger vasoconstriction (Figure 2.5) (Colin et al., 2023). Indeed, sustained AT_1 receptor signalling is a factor in primary hypertension and pharmacological antagonism of this receptor is a common approach to treating hypertension.

Activation of AT_1 receptors by angiotensin II also activates NADPH oxidases. These enzymes lead to the production of superoxide, which can also be converted to hydrogen peroxide by superoxide dismutase (Griendling et al., 2021). Notably, angiotensin II mediated ROS production can dephosphorylate eNOS to reduce production of NO (Ding et al., 2020). Furthermore, ROS also acts a secondary messenger to activate NF-κB (Lingappan, 2018). Angiotensin

II-mediated NF-κB activity is reported to be responsible for the expression of cell adhesion molecules (VCAM-1, ICAM-1) and proinflammatory molecules such as TNF-α by endothelial cells (Lingappan, 2018). Together, these molecular events result in responses such as vascular smooth muscle cell hypertrophy, proliferation and oxidation that lead to vascular stiffness, endothelial dysfunction and remodelling observed in hypertension (Colin et al., 2023).

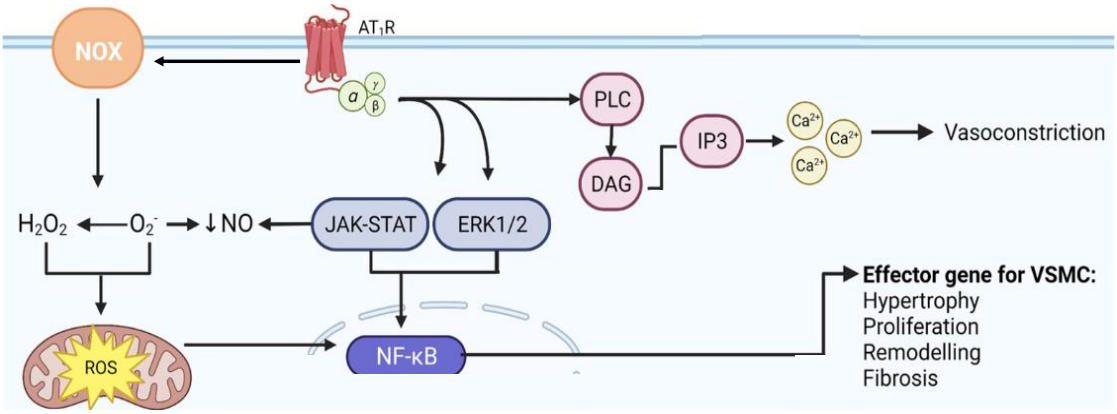

Figure 2.5 Signalling mechanisms of AT_1 receptor in the context of vasoconstriction and vascular remodelling. PLC: phospholipase C; DAG: diacylglycerol; IP_3: inositol triphosphate; Ca^{2+}: calcium ions; NO: nitric oxide; NOx: NADPH oxidase; H_2O_2: hydrogen peroxide; O_2^-: superoxide; ROS: reactive oxygen species (Reproduced from Swiderski et.al. 2019)

2.4.2 Angiotensin II type 2 receptor

Stimulation of AT_2 receptor has been reported to have opposing effects on the actions of RAAS mediated via the AT_1 receptor. The AT_2 receptor has been shown to exert vasodilatory, natriuretic, anti-fibrotic and anti-inflammatory effects (Fatima et al., 2021). AT_2 receptor expression has been demonstrated to be upregulated during pathological states such as cardiac failure, renal failure, diabetes, and atherosclerosis, even though it is highest during foetal development and decreases after birth. In adults, AT_2 receptors are expressed in the heart, kidney, vasculature, brain and pancreas (Fatima et al., 2021).

Activation of AT_2 receptors is coupled to G proteins as well. It has been shown to inhibit ERK 1/2 phosphorylation (pERK1/2) via stimulating MAPK phosphatase (MKP-1) and protein phosphatase 2 (PP2A), resulting in vasodilation and inhibition of remodelling in both vascular smooth muscle cells and cardiomyocytes (Figure 2.6) (Forrester et al., 2018). The relation between AT_2 receptor and the bradykinin receptor has been documented as well, whereby both receptors form functional heterodimers to increase eNOS activity, leading to increase NO production and promote vasodilation. (Kaschina et al., 2024). To add, a study performed by Abadir et. al reported that in bradykinin B_2-receptor-null mice, production of NO via the AT_2 receptor was increased when AT_1 receptor blockade was introduced (Abadir et al., 2003). Hence, these studies provide evidence that the AT_2 receptor may be able to activate the NO-cGMP pathway either dependent or independent of the bradykinin receptor. Furthermore, AT_2 receptor stimulation has been shown to mediate antioxidant

effects by inhibiting NADPH oxidase activity by activation of Src homology 2 domain phosphatase-1 (SHP-1) (Faria-Costa et al., 2014).

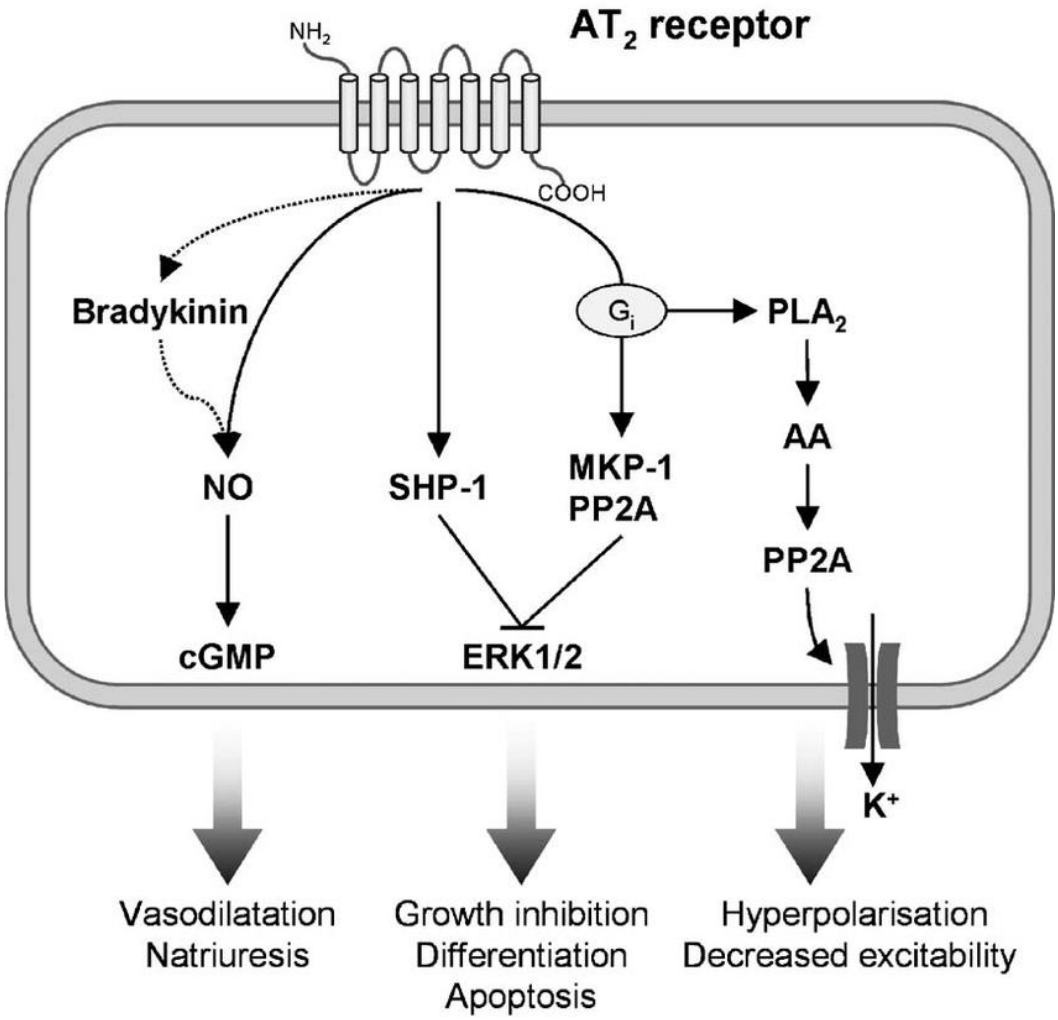

Figure 2.6: Signalling mechanisms of the AT_2 receptor. NO: nitric oxide; cGMP: cyclic guanosine monophosphate; SHP-1: Src homology 2 domain phosphatase-1; ERK1/2: extracellular-regulated kinase 1 & 2; MKP-1: MAP kinase phosphatase; PP2A: protein phosphatase 2A; PLA_2: phospholipase A_2; AA: arachidonic acid (Jöhren, 2004).

2.4.3 RAAS and endothelial dysfunction

The RAAS is implicated in in development of endothelial dysfunction and cardiovascular disease. Circulating angiotensin II activates the classical angiotensin receptors AT_1 receptor and AT_2 receptor (Eckenstaler et al., 2021). Most physiological effects of angiotensin II are mediated by AT_1 receptor activation. Effects such as vasoconstriction, sodium retention and vascular remodelling are promoted in several tissues such as the endothelium, vascular smooth muscle cells, heart and kidney (Eckenstaler et al., 2021). Dysregulation of NOx-dependent ROS production from AT_1 receptor activation leads to oxidative stress, which has been linked to endothelial dysfunction and increased blood pressure in SHR (Gillis et al., 2018, Kuczeriszka and Wąsowicz, 2022). Furthermore, intraperitoneal administration of angiotensin II that resulted in elevated blood pressure, hypertrophic vascular remodelling, increased levels of ROS, NOx-2 and NOx-4 and significantly elevated concentrations of pro-inflammatory cytokines TNF-α and IL-6 in the kidneys (Trejo-Moreno et al., 2021). To further supplement, NOx1-knockout mice displayed reduced aorta contractility in response to angiotensin II, attributed to a decrease in AT_1 receptor activation (Park et al., 2022). Thus, these observations suggest that increased RAAS activity is associated with endothelial dysfunction characterised by pro-oxidant and pro-inflammatory activity associated with vascular remodelling and hypertension.

2.5 Hypertension

2.5.1 Classification and statistics of hypertension

Hypertension or high blood pressure is a major risk factor of cardiovascular disease. It contributes to the rise in global mortality and morbidity and is estimated to impact 1.5 billion people by 2025 (Angeli et al., 2019). It is a multifactorial disease that stems from the combined action of environment, genetics and lifestyle, leading to an increase of total peripheral resistance with normal cardiac output (Angeli et al., 2019). Two types of hypertension have been categorised: i) primary or essential hypertension and ii) secondary hypertension (Oparil et al., 2018). Primary hypertension is the most frequent type of hypertension in adults. It is diagnosed when sustained elevation of BP greater than 140/90 mmHg is measured and when aetiology cannot be determined (Unger et al., 2020). In contrast, secondary hypertension is when the elevated pressure is caused by an identifiable underlying cause (Viera and Neutze, 2010). Up to 95% of hypertensive patients are diagnosed by primary hypertension while the remaining 5% accounts for secondary hypertension (Charles et al., 2017).

2.5.2 Hypertension and endothelial dysfunction

A change in the endothelium's behaviour that favours decreased vasodilation, cell proliferation, and a proinflammatory state is known as endothelial dysfunction. It is linked to risk factors for cardiovascular disease, such as hypertension (Gallo et al., 2022b). Endothelial dysfunction may contribute to the increased peripheral resistance seen in hypertension. In terms of mechanical stimuli, endothelial cells sense and respond to disturbed blood flow compared to steady, laminar flow (Abe and Berk, 2014). This causes reduced eNOS expression, increased ROS production by activating NOx-2 and leukocyte infiltration (Heo et al., 2013, Abe and Berk, 2014). These effects contribute to vascular inflammation and remodelling and reduced NO levels, in turn contributing to endothelial dysfunction. Furthermore, the rise of ROS levels also contributes to the dysregulation of normal endothelium function and stimulate signalling pathways such as NF-κB and MAPK that lead to inflammation and vascular remodelling (Touyz et al., 2020). In hypertension, excess ROS oxidizes and inactivates protein phosphatases associated with the regulation of endothelial function (Tabet et al., 2008, Touyz et al., 2020). A number of studies performed on hypertensive animal models have reported the causal link between endothelial dysfunction and hypertension. In SHR, decreased vasodilatory function of the aorta, thickening of aorta intima-media layer and increased in NOx expression were observed (Ribeiro et al., 2021, Fan et al., 2022). In ageing mice, endothelial dysfunction presenting as impaired vascular function and decreased NO bioavailability in plasma was accompanied by increased arterial blood pressure (Brunt et al., 2020). Lastly, hypertensive mice induced by angiotensin II infusion were observed to have increased ROS

production, macrophage infiltration in the aorta and decreased eNOS expression (Yan et al., 2020). In summary, these studies show that endothelial dysfunction, characterised by impaired vasodilation and increased oxidative stress, has a critical role to play in the development of hypertension.

2.5.3 Hypertension and RAAS system

Persistent overactivity of the RAAS leads to high levels of angiotensin II in the circulation, which further stimulates the constriction of blood vessels, increases NOx activity leading to overproduction of ROS and promote aldosterone secretion, leading to increased arterial blood pressure (Grassi and Drager, 2024). Aliskiren, an inhibitor of renin, was observed to decrease arterial blood pressure and increase the expression of eNOS in the aorta of SHR after 3 weeks of treatment (Pechanova et al., 2019). On the same page, by inhibiting the conversion of angiotensin I to angiotensin II, treatment with ACE inhibitors was observed to decrease systolic blood pressure, increased vascular reactivity and decrease AT_1 receptor expression in SHR (Li et al., 2020) as well as alleviated cardiac remodelling and reduced systolic blood pressure in N-Nitro-L-arginine methylester (L-NAME) induced hypertensive Wistar rats, (Stanko et al., 2024). Also, a recent study by Okuno and colleagues reported that in mice lacking AT_1 receptor in smooth muscle cells, angiotensin II infusion over 2 weeks did not induce vascular remodelling and cardiac hypertrophy compared to controls. This suggests that angiotensin II-AT_1 receptor activity is linked to the development of hyperplasia and hypertrophy in hypertension (Okuno et al., 2023). Furthermore, lorundrostat, an aldosterone synthase inhibitor, was reported to have systolic blood pressure lowering effects in patients with uncontrolled hypertension in phase 2 trials (Laffin et al., 2023). Collectively, these studies demonstrate the relationship between an overactive RAAS and hypertension, as pharmacological blockade of RAAS components has led to attenuation of hypertension and its effects on the cardiovascular system.

2.5.4 Pharmacological blockade of AT_1 receptors

Pharmacological RAAS intervention has been a common and successful strategy in the treatment of hypertension (Gallo et al., 2022a). One of the most effective methods is through the blockade of AT_1 receptors, or angiotensin receptor blockers (ARB). By inhibiting the binding of angiotensin II and AT_1 receptors, effects of its activation such as vascular smooth muscle contraction, increased sympathetic activity and sodium and water retention by the kidneys that lead to increased arterial blood pressure are prevented (Silva et al., 2019). Besides its effectiveness in lowering systolic blood pressure, ARBs have also shown beneficial effects in the regression of left ventricular hypertrophy (Ahmed et al., 2020). One of the ways that ARBs can be beneficial is through activating the RAAS's complementary protective axis. During selective blockade of AT_1 receptors, functional crosstalk between AT_1 and AT_2 receptors causes increased expression of the AT_2 receptor (Kaschina et al., 2017), as blockade of AT_1 receptors favours the binding of angiotensin II to AT_2 receptors. Activation of the AT_2 receptor is associated with vasodilation, raised production of NO and anti-inflammatory effects, leading to improved endothelial dysfunction (Steckelings et al., 2022). This is evident in a study conducted by Savoia and colleagues, whereby treatment with olmesartan for 2 weeks was shown to increase eNOS expression, improve NO bioavailability and attenuate ROS in SHR (Savoia et al., 2020). To further supplement, losartan was also observed to decrease markers of endothelial dysfunction and platelet activation in diabetic rats (Elseweidy et al., 2020). Thus, the available evidence supports that usage of ARBs is effective in the treatment of hypertension and endothelial dysfunction

most probably due to increasing NO bioavailability, prevention of oxidative stress and vascular inflammation induced by angiotensin II.

2.6 Red yeast rice

2.6.1 General description of red yeast rice

Red yeast rice (RYR) (Figure 2.7) is a natural product and functional food produced from the fermentation of rice with *Monascus purpereus (*Zhu et al., 2019). However, other species from the *Monascus* genus have been utilised in the production of RYR as well (Fukami et al., 2021). It has been a part of traditional Chinese medicine and diet for centuries and used as a colouring additive or flavouring for foods (meats and soybean products) and Chinese red wine (Fukami et al., 2021). More recently, it has been widely marketed as a health supplement with anti-dyslipidaemia properties (Younes et al., 2018). Its reddish colouration is derived from the pigments (rubropunctamine and monascorubramine) produced during its fermentation process. The constituents of RYR are as follows: 25-73% sugar (mainly comprised of starch), 14-31% proteins, 2-7% water and 1-5% of fatty acids, isoflavones, sterols and monacolins (Buzzelli et al., 2024, Fukami et al., 2021). Due to their cholesterol-lowering benefits, monacolins are of particular interest. Monacolins exists in various subtypes based on conditions such as fermentation process and strain of *Monascus* used (Zhang et al., 2020). Among the various subtypes, monacolin K is known to be the primary active component of RYR due to it having the highest concentration among monacolins and its similar chemical structure to lovastatin (Banach et al., 2022). Like the latter, monacolin K has hypocholesterolaemic properties through its ability to inhibit 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme of the endogenous cholesterol synthesis pathway (Cicero et al., 2021). To add, monacolin K requires conversion from its inactive (lactone) to active (hydroxyl acid) form.

Conversion *in-vivo* can occur enzymatically, by cytochrome P450 (CYP) 3A, or spontaneously from alkaline conditions during metabolism (Cicero et al., 2021).

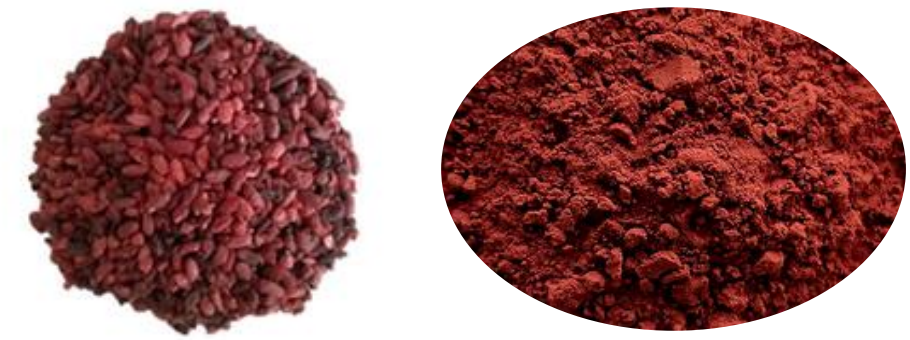

Figure 2.7: Images above show the original (left) and powdered (right) form of red yeast rice. (Hu et.al 2020).

2.6.2 Effects of red yeast rice consumption on cardiovascular diseases

There is some emerging body of knowledge regarding the efficacy of RYR preparations on hypertension and cardiovascular disease (CVD). The Chinese Coronary Secondary Prevention Study was a randomised controlled trial (RCT) that evaluated the efficacy of an RYR based supplement on CVD incidences. Participants that were assigned RYR daily over 4.5 years had 45% lowered CVD incidences when compared to placebo-assigned participants (Li et al., 2009). A recent meta-analysis across 30 RCTs using RYR preparations had also concluded that RYR usage had reduced mortality and major adverse cardiovascular events that was accompanied by improved total cholesterol, low density lipoprotein (LDL), blood glucose and mean arterial pressure (Yuan et al., 2022). Evidence also suggests that RYR supplementation improves endothelial function. In a study conducted on 25 hypercholesterolaemic subjects, 4 weeks of treatment with RYR had significantly improved pulse volume displacement, an indicator of endothelial function (Cicero et al., 2016). Furthermore, RYR was found to reduce ROS levels in streptozotocin-induced diabetic Wistar rats by raising the levels of superoxide dismutase in the kidneys, indicating its ability to reduce oxidative stress through its antioxidant properties (Rajasekaran and Kalaivani, 2015).

2.6.3 Potential inhibitory effects on RAAS signalling by monacolin K in red yeast rice

Studies on the ability of RYR to suppress the activity of RAAS was found to be limited. However, there is a considerable amount of evidence on the effects of statins in modulating RAAS. Renin, the initiator of RAAS pathway, is stimulated by cyclic adenosine monophosphate (cAMP), which can be stimulated by catecholamines and prostaglandins. This is evident in a study by Koh *et al*., whereby treatment with simvastatin was found to decrease catecholamine synthesis in isolated rat adrenal gland (Koh et al., 2018), while another study by Dong *et al.* reported an inhibition of prostaglandin rate-limiting enzyme, COX-2 after treatment with atorvastatin for 30 days in Sprague-Dawley rats (Dong et al., 2023). Statins have also been reported to influence AT_1 receptor expression. In a study performed on SHR, pitavastatin treatment for 8 weeks was found to decrease both angiotensin II and AT_1 receptor expression (Zhang et al., 2017). Similarly, treatment with simvastatin for 4 weeks was also reported to decrease AT_1 receptor expression in high-fructose diet model of programmed hypertension in Sprague Dawley rats (Chao et al., 2020). Adding on, increased aldosterone levels were observed to be associated with raised plasma concentrations of LDL, suggesting that its levels can be altered by statins. Evidence of this was reported by Huang and colleagues, whereby N-Nitro-L-arginine methylester (L-NAME)/angiotensin II induced hypertensive Wistar rats had significantly decreased plasma levels of aldosterone after 14 days treatment with either pravastatin or simvastatin (Huang et al., 2023). Based on these reported studies, the potential benefits of RYR treatment can be extrapolated to

have modulatory effects on RAAS, due to its inhibitory effect on HMG-CoA reductase by monacolin K.

2.7 Spontaneously Hypertensive Rat as an experimental model for primary hypertension

Spontaneously Hypertensive Rats (SHR) are genetically hypertensive models frequently used as experimental animal models in research related to primary hypertension and cardiovascular diseases. SHR are produced from the inbreeding between hypertensive Wistar-Kyoto Rats (Okamoto and Aoki, 1963). They typically develop hypertension around the age of 4-6 weeks spontaneously, without intervention (Jama et al., 2022). At the stage where hypertension is established, SHR exhibit increased peripheral resistance with normal cardiac output and cardiac remodelling occurs (Osada and Tsutsumi, 2020). SHR also show decreased NO bioavailability, sGC and cGMP, while having increased expression of AT_1 receptors and markers of oxidative stress when compared to WKY of identical age (Savoia et al., 2020, Li et al., 2023). Identical to patients with essential hypertension, the aetiology of hypertension in SHR remains unknown (Jama et al., 2022).

Chapter 3

Materials and Methodology

3.1 Experimental flow chart

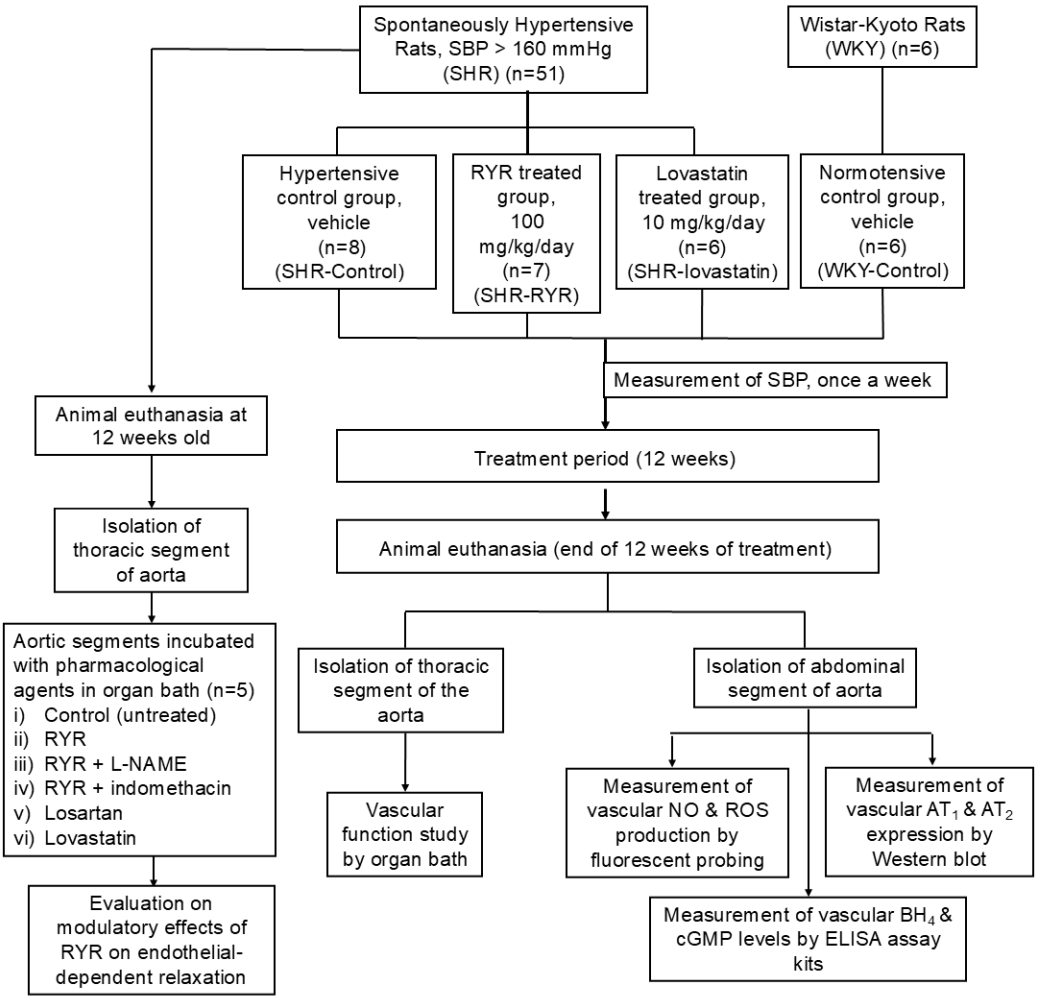

Figure 3.1: Experimental flow chart for the study.

3.2 Experimental animals

Male Wistar-Kyoto Rats (WKY) (n=6) and Spontaneously Hypertensive Rats (SHR) (n=6-8) age 10-12 weeks were obtained from the Universiti Malaya Animal Experimental Unit. Sample size was selected based on prior studies that evaluated natural compounds in SHR for hypertension research (Bareño et al., 2017, Moser et al., 2023). The Experimental animals were kept under controlled temperature (23 ± 2 °C) and light (12-hour light-dark cycle). The animals were fed with standard rat chow (Gold Coin Specialities Sdn Bhd, Selangor, Malaysia) with free access to tap water. Ethics approval for the study was obtained from the University Tunku Abdul Rahman Scientific and Ethical Review Committee under reference number U/SERC/253/2021. All experimental procedures were carried out according to the guidelines of University Tunku Abdul Rahman Scientific research ethics and code of conduct for the ethical care of experimental animals.

3.3 *In-vivo* treatment

For *in-vivo* study, the animals were randomly divided into four groups: WKY (vehicle – distilled water; strain control), SHR – Control (vehicle - distilled water; control group), SHR – RYR (100mg/kg/day; treatment group) and SHR – Lovastatin (10 mg/kg/day; positive control group). RYR and losartan were kept in room temperature and dissolved in distilled water prior administration into the animal. Animals were treated by oral gavage daily for 12 weeks. The dosage was chosen based on similar dosage reported in previous studies (Rehman et al., 2021, Lin et al., 2022).

3.4 Measurement of systolic blood pressure

Prior to the initiation of RYR treatment, the systolic blood pressure of the animals was measured by tail-cuff method (CODA® Monitor, Kent Scientific Corporation, CT, USA) and thereafter recorded every seven days after treatment started. The animals were trained to the restraint conditions to ensure accuracy of blood pressure readings. During measurement, rats were immobilised in a pre-warmed restrainer (28-30°C) for at least 15 minutes. The average value of 5 successive measurements were recorded. The tail-cuff method is utilised as it is a non-invasive procedure used to measure blood pressure in small animals.

3.5 Drugs and chemicals

Red yeast rice extract (RYR) was purchased from BiO-LiFE, Mega Lifesciences (Selangor, Malaysia). Lovastatin was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Acetylcholine chloride (ACh), phenylephrine, sodium chloride (NaCl), glucose, Nitro-L-arginine methyl ester (L-NAME), indomethacin and losartan were purchased from Sigma Chemicals (St Louis, Missouri, USA). Calcium chloride ($CaCl_2$), magnesium sulphate ($MgSO_4$), potassium dihydrogen phosphate (KH_2PO_4) and sodium nitroprusside (SNP) were purchased from BDH Laboratory Supplies (Poole, UK). Potassium chloride (KCl) and sodium bicarbonate ($NaHCO_3$) were purchased from EMSURE® Merck (Darmstadt, Germany). OCT compound was purchased from Sakura Finetek USA Inc. (Torrance, California, USA). All chemicals were dissolved in ultrapure water.

3.6 Preparation of Krebs physiological solution

This solution was prepared by dissolving the following compounds (mM): NaCl 118.93, $NaHCO_3$ 25.00, $MgSO_4$ 1.18, KCl 4.69, KH_2PO_4 1.03, Glucose 11.10, $CaCl_2$ 2.38 in one litre of Milli-Q water. The solution was prepared before use, oxygenated with 95% oxygen and 5% carbon dioxide and kept at 4°C.

3.7 Preparation of isolated aortic rings

The animals were sacrificed at the end of treatment by excess inhalation of carbon dioxide. The thoracic segment of the aorta was quickly isolated and placed ice-cold Krebs solution. The aorta was cleaned of any remaining perivascular and connective tissues without disrupting the vessel and sectioned into small segments (3-5 mm in length). Remaining segments of the aorta was also sectioned and snap-frozen in liquid nitrogen and stored in -20 °C for later processing.

3.8 Mounting and measurement of isometric tension of the thoracic aortic rings

To study the responses of the thoracic aortic rings to agonists, the segments were suspended between a L-shaped holder and the tissue hook of the organ bath. The organ bath was then filled with 5 mL of Krebs solution. The tissue hook was connected to a force displacement transducer (Grass Instrument Co., Quincy, Massachusetts, USA) and corresponding output was amplified and

recorded using PowerLab recoding system (AD Instrument, Sydney, NSW, Australia) throughout the entire experiment. The organ bath was constantly supplied with 95% oxygen and 5% carbon dioxide maintained at 37°C. Agonist-induced contractile and relaxant responses of the aortic rings were monitored on the computer as increase or decrease of the isometric tension (gram tension, gT), respectively.

3.9 Experimental protocol for the vascular function test

After mounting, the rings were first stretched to 1 gT and left to equilibrate for 30 minutes or until stabilised. During equilibrium, Krebs solution was replaced every 15 minutes. The aortic rings were then stretched to 2.5 gT for 30 minutes or until stabilised. After equilibrium, the aortic rings were primed with an isotonic potassium solution (high K^+, 60 mM) for 15 minutes to confirm vascular tissue viability. To validate the presence of functional endothelium, the rings were pre-contracted with 3 µM of phenylephrine before exposure to acetylcholine (ACh, 10 µM). Rings with more than 70% relaxation in response to acetylcholine were deemed to have viable endothelium (Ling et al., 2017).

To measure the cumulative concentration-response to ACh, the rings were precontracted with phenylephrine (0.3 - 1.0 µM) and exposed to increasing concentrations of ACh (3 nM - 10 µM) after a stable contraction was obtained.

The cumulative concentration-response to SNP was measured as well. After the response to increasing ACh concentrations were measured, the rings were then washed with Krebs solution every 10 minutes for 3 times before being pre-contracted with phenylephrine (0.3 – 1.0 μM). When a stable contraction was obtained, the rings were exposed to increasing concentrations of SNP (1 nM - 10 μM). ACh is used to induce endothelial-dependent relaxation by stimulating NO release from endothelial cells while SNP directly provides NO to act directly on vascular smooth muscle cells. This technique allows for the assessment of endothelial function and vascular smooth muscle response in isolated vessels.

3.10 *Ex-vivo* study on the modulatory effect of RYR on ACh-induced relaxation in SHR

In another set of experiments, aortic rings of 12-week-old SHR without RYR treatment were used. The rings were pre-incubated with RYR (100 μg/mL) (concentration of RYR was determined based on conversion of 100 mg/kg to μg/mL)

$$100 \text{ mg/kg} = \frac{100 \times 1000 \text{ μg}}{1000 \text{ mL}} = 100 \text{ μg/mL}$$

along with either one of the following pharmacological inhibitors: L-NAME (an eNOS inhibitor,10 μM) or indomethacin (non-specific cyclooxygenase inhibitor, 10 μM). Some of the rings were pre-incubated with pharmacological inhibitor losartan (an AT_1 inhibitor, 10 μM) or lovastatin (HMG-CoA inhibitor, 10 μM) without the presence of RYR. The concentration of inhibitors was selected based on previous studies investigating its effects in an organ bath (Tan et al., 2018, Fiorim et al., 2020, Stephens et al., 2021). The rings were pre-treated for 30

minutes prior to sustained contraction by phenylephrine (0.3-1.0 μM) and exposure to increasing concentrations of ACh (3 nM - 10 μM).

3.11 *In-situ* detection of vascular nitric oxide (NO) and reactive oxygen species (ROS)

DAF-FM and DHE are fluorescent probes that bind to their respective ligand to measure NO and ROS levels. Segments of cleaned isolated aorta from the treated animals were placed into round moulds made from aluminium foil containing optimal cutting temperature (OCT) compound (Sakura Finetik, California, USA) and frozen in liquid nitrogen. The embedded rings were cross sectioned at 5 μm thickness using a cryostat.

The level of intracellular NO level was determined using 4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate (DAF-FM) fluorescence assay while the level of intracellular ROS produced was measured with dihydroethidium (DHE) fluorescence assay. Sectioned aortas were incubated in phosphate buffered saline (PBS) containing either 10 μM DAF-FM (Invitrogen, Carlsbad, California, USA) or 5 μM DHE (Invitrogen, Carlsbad, California, USA) and incubated in the dark for 30 minutes at 37 °C. The dyes were then washed away with PBS before visualised and captured on ZEISS AXIO Observer A1 fluorescent microscope with ZEN imaging software (ZEISS Group, Oberkochen, Germany) with excitation/emission of 495/515 nm (DAF-FM); 515/585 nm (DHE). The fluorescent intensity of the images was analysed with ImageJ software. The NO and ROS levels were compared to that of WKY-Control.

3.12 Preparation and protein quantification of homogenised aortic lysates

Aortic segments from treated WKY and SHRs were homogenised in 200 µL of ice-cold RIPA lysis buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) using a Dounce Homogeniser. The lysates were then centrifuged at 20,000 g for 30 minutes at 4°C. The total protein concentration of the supernatants was then quantified using Pierce bicinchoninic acid (BCA) assay (Thermo Fisher Scientific Inc, Massachusetts, USA). Briefly, ten microlitres (10 µL) of each standard or unknown sample was pipetted into a 96-well microplate in duplicates. Then, 200 µL of working reagent was added to each well and mixed thoroughly for 30 seconds on a shaker. The plate was then covered and incubated at 37°C for 30 minutes. The absorbance of the wells was then measured at 562 nm using Tecan Infinite 200 PRO microplate reader (Tecan Group Ltd., Zurich, Switzerland).

3.13 Determination of vascular cyclic guanosine monophosphate (cGMP) levels

cGMP is the secondary messenger produced in response to NO signalling from the vasculature and thus measurement of its levels helps to evaluate the viability of the NO signalling pathway. Vascular cGMP levels were determined using cyclic GMP ELISA Kit (Cayman Chemical Company, Michigan, USA) based on the manufacturer's instructions. The assay principle is based on the competition between free cGMP and cGMP-acetylcholinesterase (AChE) conjugate (cGMP tracer) against cGMP antibody binding sites pre-coated onto the provided 96-well plate. As cGMP tracer concentration is made constant, the

amount of free cGMP in the sample will be inversely proportional to the absorbance obtained when cGMP tracer substrate solution is added.

Briefly, 50 µL of samples or standards was added to provided 96-well plate, followed by 50 µL each of cGMP tracer and cGMP antiserum. The plate was then covered with plastic film before incubated for 18 hours at 4°C. The wells were then washed and 200 µL of substrate was then added before being incubated again for 2 hours at 4°C. Finally, the plate was read at wavelength of 420 nm using Tecan Infinite 200 PRO microplate reader (Tecan Group Ltd., Zurich, Switzerland). Concentration of each sample was determined using the equation from the plotted standard curve. The levels of cGMP were compared to that of WKY-Control.

3.14 Determination of vascular BH_4 levels

BH_4 is an essential cofactor needed for eNOS to remain functional and thus measurement of its levels helps to evaluate the capability of NO production by eNOS. Vascular BH_4 levels were determined using Rat BH_4 ELISA Kit (Elabscience, Wuhan, China) based on the manufacturer's instructions. The assay principle is based on the detection of BH_4 antibody-HRP conjugated complex.

Briefly, 100 µL of samples or standards was added to provided 96-well plate, followed by 100 µL of detection antibody solution. The plate was then covered and incubated for 60 minutes at 37°C. The wells were then washed and 100 µL of HRP conjugate substrate was added before being incubated again for 30 minutes at 37°C. Finally, 50 µL of stop solution was added and the plate was

immediately read at wavelength of 450 nm using Tecan Infinite 200 PRO microplate reader (Tecan Group Ltd., Zurich, Switzerland). Concentration of each sample was determined using the equation from the plotted standard curve. The levels of BH_4 were compared to that of WKY-Control.

3.15 Western blotting

This method is used to detect and quantify the expression of AT_1 and AT_2 receptors, which regulate blood pressure through the RAAS. Total protein lysates (10 μg of total protein) were prepared in Laemmli loading buffer. The samples were loaded into a gel and were separated by 10% sodium dodecyl sulphate (SDS) – polyacrylamide gel electrophoresis and the transferred onto polyvinylidene fluoride (PVDF) membrane (GVS Group, Bologna, Italy) at 110 V for 90 minutes. The membranes were then blocked for 30 minutes in 3% bovine serum albumin (BSA) in Tris-buffered saline containing 0.2% Tween 20 (TBS-T) at room temperature for 30 minutes with light shaking. Following blocking, the membrane was incubated overnight with primary antibodies against β-actin (1:10,000; Cell Signalling, Massachusetts, USA), angiotensin type 1(AT_1) and angiotensin type 2 (AT_2) receptors (1:500; Santa Cruz Biotechnology, California, USA). The blots were then washed three times in TBS-T (3 x 10 minutes) and incubated with their corresponding HRP-conjugated secondary antibodies (1:1000; Santa Cruz Biotechnology, California, USA) for 2 hours at room temperature. The blots were washed again (3 x 10 minutes) and visualised using enhanced chemiluminescent detection kit (Pierce-Thermo Fisher Scientific, Massachusetts, USA) and captured with Azure 600 Western

Blot Imaging System (Azure Biosystems, California, USA). Band intensities were analysed using ImageJ software (U. S. National Institutes of Health, Bethesda, Maryland, USA) to quantify protein expression and protein levels were normalised to β-actin and compared to WKY – Control.

3.16 Statistical analysis

Results were represented as mean ± standard error of mean (SEM) for the number of animals (n). The responses to ACh and SNP were calculated as percentage relaxation to phenylephrine-induced contractions. Results were plotted and analysed using Graphpad Prism 9 (GraphPad Software, La Jolla, CA, USA). Concentration-response curve data were analysed using "log(agonist) vs response – variable slope (four-parameter)" model and EC_{50} values, defined as the concentration of producing 50% of the maximal response, were determined from the fitted curves. The pEC_{50} values, calculated as the negative logarithm of the EC_{50}, are used as a measure of potency of the drug. Additionally, the area under the dose-response curve (AUC) was calculated as an alternative method to evaluate the overall response to the drugs. Students t-test (comparison between two groups) or one-way ANOVA (comparison between more than 2 groups) was used followed by Bonferroni's multiple comparison test. A p-value of < 0.05 was considered statistically significant.

Chapter 4

Results

4.1 Effect of treatment with RYR extract on systolic blood pressure

Throughout the treatment period, there was a constant increase in systolic blood pressure of the SHR-Control group (Figure 4.1). At week 12, treatment with RYR had significantly attenuated the increase in SBP by 11.5 % when compared to SHR-Control (Table 4.1; SHR - Control vs SHR - RYR: 221.5 ± 3.6 mmHg vs 197.4 ± 1.7 mmHg). A similar trend was also observed in SHR treated with lovastatin. In contrast, the systolic blood pressure of WKY-Control remained relatively constant throughout the treatment period.

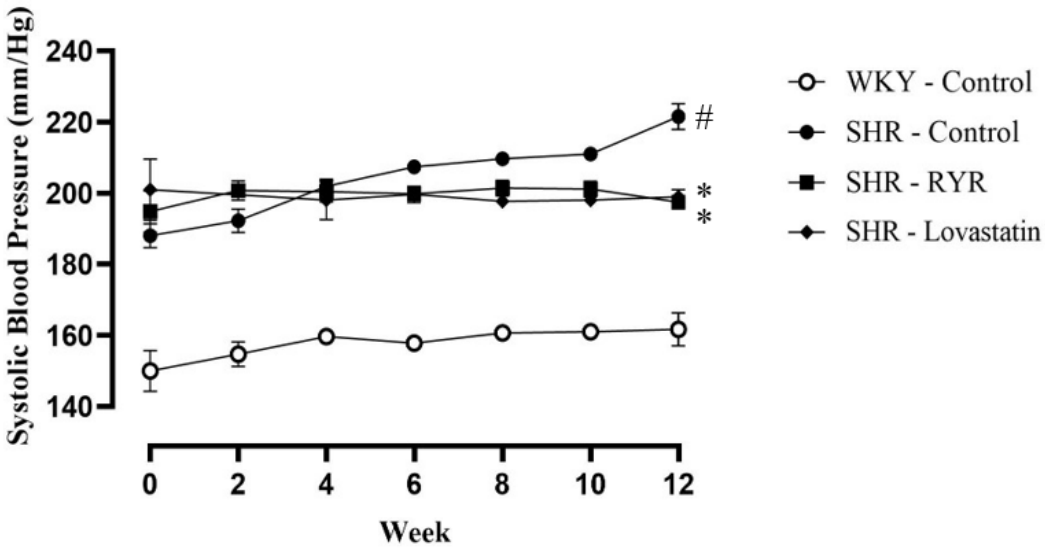

Figure 4.1: Measurement of average systolic blood pressure by tail-cuff method in Wistar-Kyoto Rats (WKY) Control and Spontaneously Hypertensive Rats (SHR) with and without *in-vivo* treatment with red yeast rice (RYR; 100 mg/kg/day) or lovastatin (10 mg/kg/day) for 12 weeks. Data is expressed as mean ± SEM (n=6-8). $^{\#}p < 0.05$ compared to WKY – Control; $^{*}p < 0.05$ compared to SHR – Control.

Table 4.1: Systolic blood pressure measurements throughout the treatment period recorded at 2-week intervals.

Week	WKY – Control	SHR – Control	SHR – RYR	SHR - Lovastatin
0	150.0 ± 5.8	188.0 ± 3.4 #	194.8 ± 5.8	201.0 ± 8.6
2	154.7 ± 3.5	192.3 ± 3.3 #	200.8 ± 2.7	200.8 ± 3.4
4	159.7 ± 2.1	201.2 ± 2.0 #	200.4 ± 2.8	198.0 ± 5.5
6	157.8 ± 1.4	207.4 ± 1.4 #	199.8 ± 0.8	199.6 ± 2.3
8	160.1 ± 1.0	209.7 ± 1.0 #	201.4 ± 2.2	197.6 ± 0.9
10	161.0 ± 1.9	211.0 ± 1.2 #	201.1 ± 2.1	198.0 ± 0.1
12	161.7 ± 4.7	221.5 ± 3.6 #	197.4 ± 1.7 *	199.0 ± 2.1 *

Data is expressed as mean ± SEM (n=6-8).

$^{\#}p < 0.05$ compared to WKY – Control; $^{*}p < 0.05$ compared to SHR – Control.

4.2 Modulatory effect of treatment with RYR extract on vascular function

4.2.1 Effect of treatment with RYR on ACh-induced relaxation in aortic rings in WKY and SHR

To determine the presence of endothelial dysfunction, ACh-induced endothelium-dependent relaxation was assesed in the aortic rings from WKY-Control and SHR-Control. Figure 4.2A and 4.2B shows that there was a significantly blunted response to ACh in SHR than WKY aortic rings (Table 4.2, R_{max} SHR-Control vs WKY-Control: 53.55 % ± 3.45 % vs 76.86 % ± 2.19 %).

In SHR treated with RYR extract for 12 weeks, there is a significant increase in ACh-induced endothelium-dependent relaxation compared to SHR-Control (Table 4.2, R_{max} SHR-RYR vs SHR-Control 69.21 % ± 2.18 % vs 53.55 % ± 3.45 %). Similarly, improvement in relaxation induced by ACh was also observed in SHR treated with lovastatin compared to SHR-Control (Table 4.2, R_{max} SHR-Lovastatin vs SHR-Control 71.86 % ± 0.79 % vs 53.55 % ± 3.45 %)

No significant differences between the potency (pEC_{50}) were observed across all experimental groups.

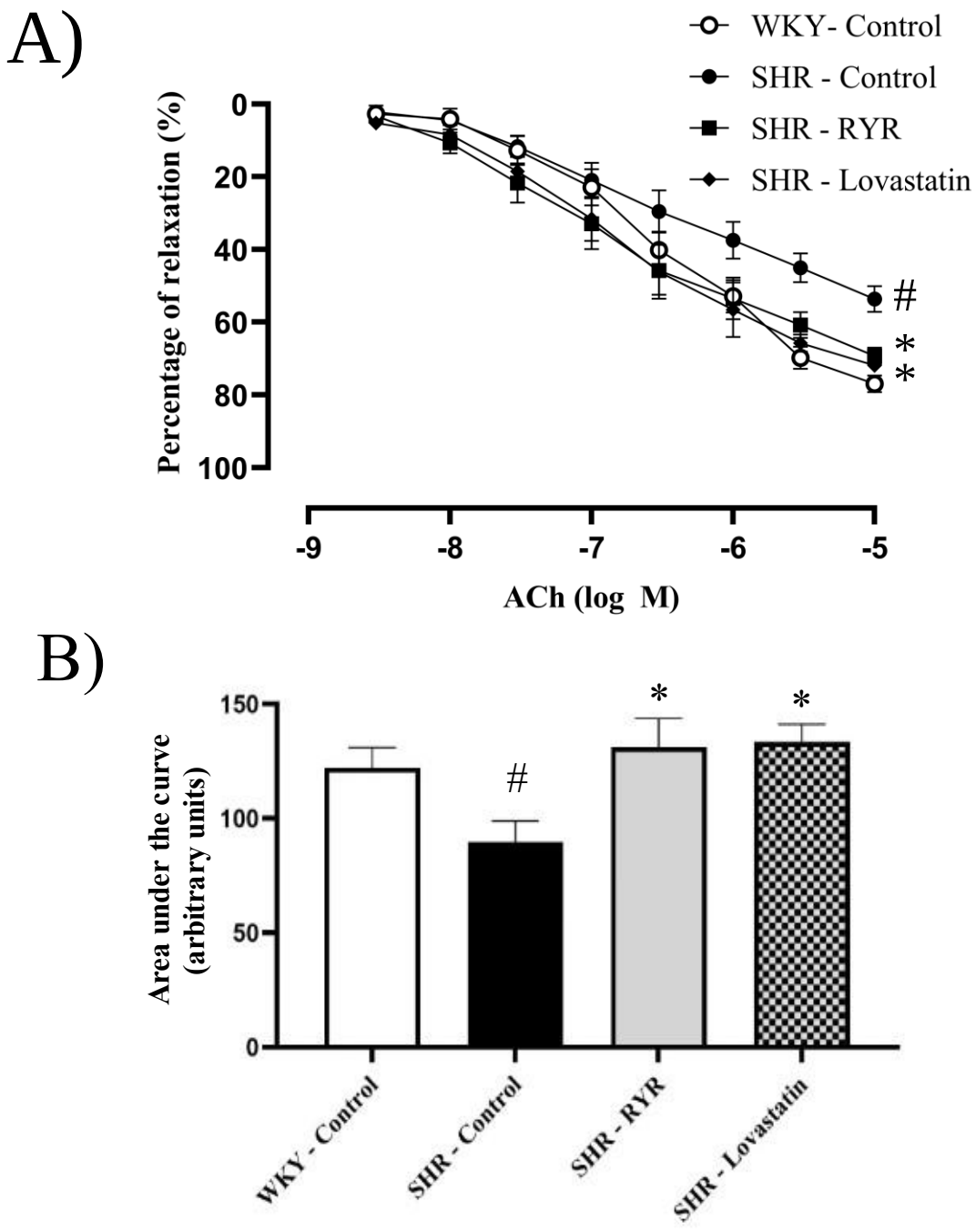

Figure 4.2: Concentration-response curves (A) and areas under the concentration-response curves (AUC) (B) to ACh-induced endothelium-dependent relaxation in Wistar-Kyoto Rats (WKY) Control and Spontaneously Hypertensive Rats (SHR) with and without *in-vivo* treatment with red yeast rice (RYR; 100 mg/kg/day) or lovastatin (10 mg/kg/day) for 12 weeks. Data is expressed as mean ± SEM (n=6-8). $^{\#}p < 0.05$ compared to WKY – Control; *p < 0.05 compared to SHR – Control.

4.2.2 Effect of treatment with RYR on SNP-induced relaxation in aortic rings in WKY and SHR

Figure 4.3 shows that there were no significant differences in both R_{max} and pEC_{50} of SNP-induced relaxation between aortic rings in WKY and SHR across all treatment groups.

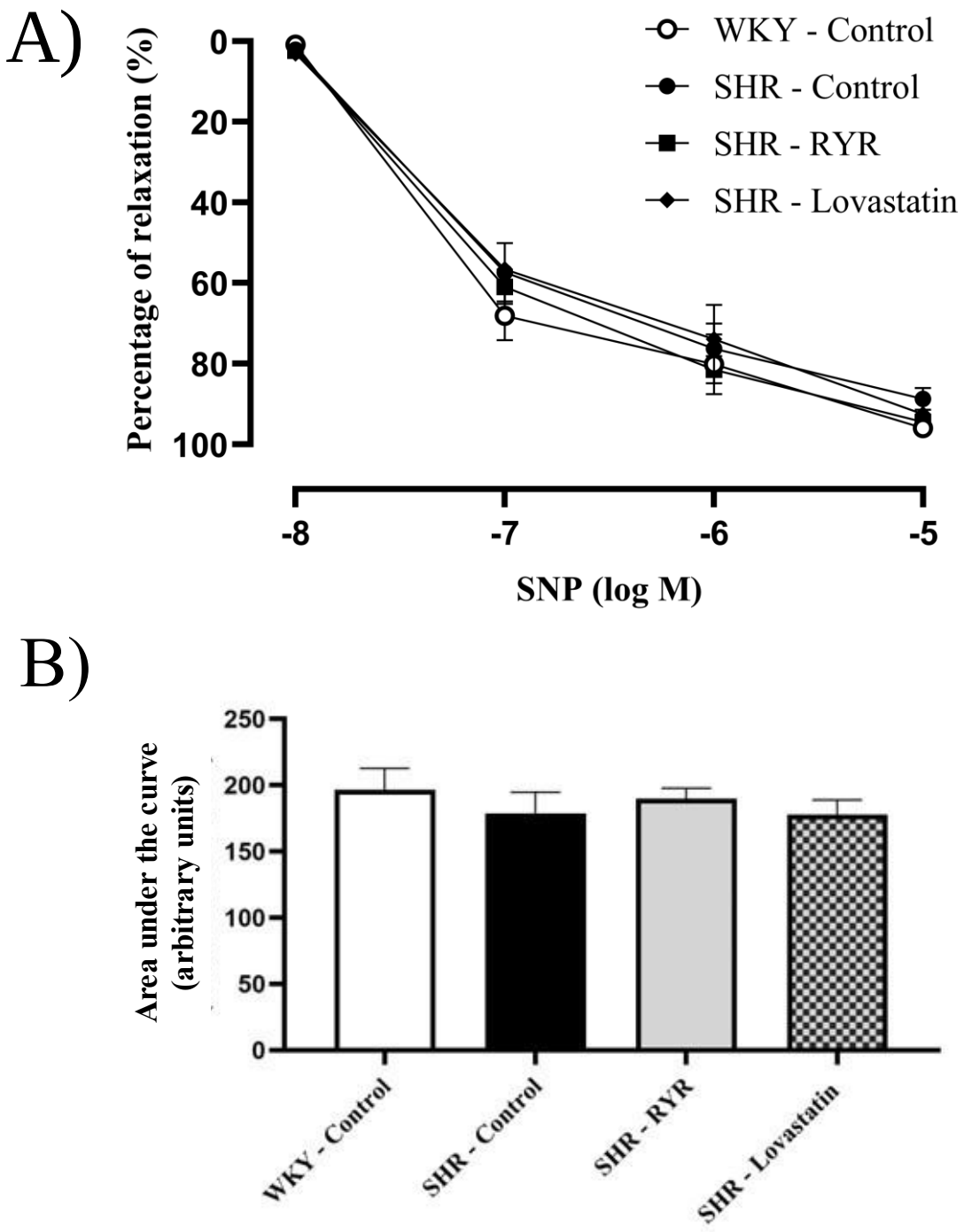

Figure 4.3: Concentration-response curves (A) and areas under the concentration-response curves (AUC) (B) to SNP-induced endothelium-dependent relaxation in Wistar-Kyoto Rats (WKY) Control and Spontaneously Hypertensive Rats (SHR) with and without *in-vivo* treatment with red yeast rice (RYR; 100 mg/kg/day) or lovastatin (10 mg/kg/day) for 12 weeks. Data is expressed as mean ± SEM (n=6-8).

Table 4.2: Potency (pEC_{50}) and maximal relaxation (R_{max}) to ACh and SNP-induced relaxation in aortae of WKY and SHR (n=6-8) with and without *in-vivo* treatment with red yeast rice (RYR; 100 mg/kg) or lovastatin (10 mg/kg/day) for 12 weeks.

	ACh		SNP	
	pEC_{50} (log M)	R_{max} (%)	pEC_{50} (log M)	R_{max} (%)
WKY - Control	6.10 ± 0.06	76.86 ± 2.19 #	7.19 ± 0.09	95.93 ± 1.01
SHR - Control	5.31 ± 0.11	53.55 ± 3.45	6.92 ± 0.12	88.64 ± 2.57
SHR - RYR	6.10 ± 0.09	69.21 ± 2.18 *	7.07 ± 0.08	94.44 ± 1.75
SHR - Lovastatin	6.18 ± 0.08	71.86 ± 0.79 *	6.90 ± 0.13	92.48 ± 3.81

Data is expressed as mean ± SEM (n=6-8)

$^{\#}p < 0.05$ compared to WKY – Control; $^{*}p < 0.05$ compared to SHR – Control.

4.3 Modulatory effects of RYR on ACh-induced relaxation in SHR

Ex-vivo study was carried out to determine the mechanism of action of RYR due to the improvement seen in ACh-induced relaxation from SHRs that were treated with RYR. SHR aortic rings demonstrated impaired relaxation to ACh (49.29 % ± 2.22 %). Figure 4.4A and Table 4.3 shows incubation with 100 μg/mL significantly improved ACh-induced relaxation (R_{max} RYR vs Control: 82.12 % ± 5.50 % vs 49.29 % ± 2.22 %) and sensitivity (Table 4.3; pEC_{50} RYR vs Control: 6.95 ± 0.24 vs 5.96 ± 0.81) when compared aortic rings from SHR with no pre-treatment.

To determine whether the beneficial effect observed from the treatment with RYR in SHR involved the COX pathway, SHR aortic rings were co-incubated with indomethacin (INDO;10 μM) and RYR (100 μg/mL). Figure 4.4A shows RYR incubated with INDO had significantly improved ACh-induced relaxation as compared to aortic rings from SHR with no pre-treatment (Table 4.3; INDO vs Control: R_{max}: 95.13 % ± 1.78 % vs 49.29 % ± 2.22 %).

To investigate if the improvement in relaxation after the treatment with RYR in SHR involved the role of eNOS, L-NAME (10 μM) was incubated in the presence of RYR (100 μg/mL) in SHR aortic rings. Figure 4.4 shows aortic rings incubated with L-NAME had significantly decreased ACh-induced relaxation compared to aortic rings from SHR with no pre-treatment and aortic rings incubated with RYR (Table 4.3; L-NAME vs Control, R_{max}: 9.09 % ± 4.38

% vs 49.29 % ± 2.22 %; L-NAME vs RYR: 9.09 % ± 4.38 % vs 82.12 % ± 5.50 %).

Lastly, aortic rings incubated with losartan (AT_1 receptor inhibitor, 10 μM) or lovastatin (HMG-CoA inhibitor, 10 μM) without the presence of RYR were used as positive control. Both inhibitors were observed to significantly increase ACh-induced relaxation in isolated SHR aortic rings when compared to aortic rings from SHR with no pre-treatment (Table 4.3; Losartan vs Control: 91.46 % ± 2.59 % vs 49.29 % ± 2.22 %; Lovastatin vs Control: 72.76 ± 1.94 vs 49.29 % ± 2.22 %).

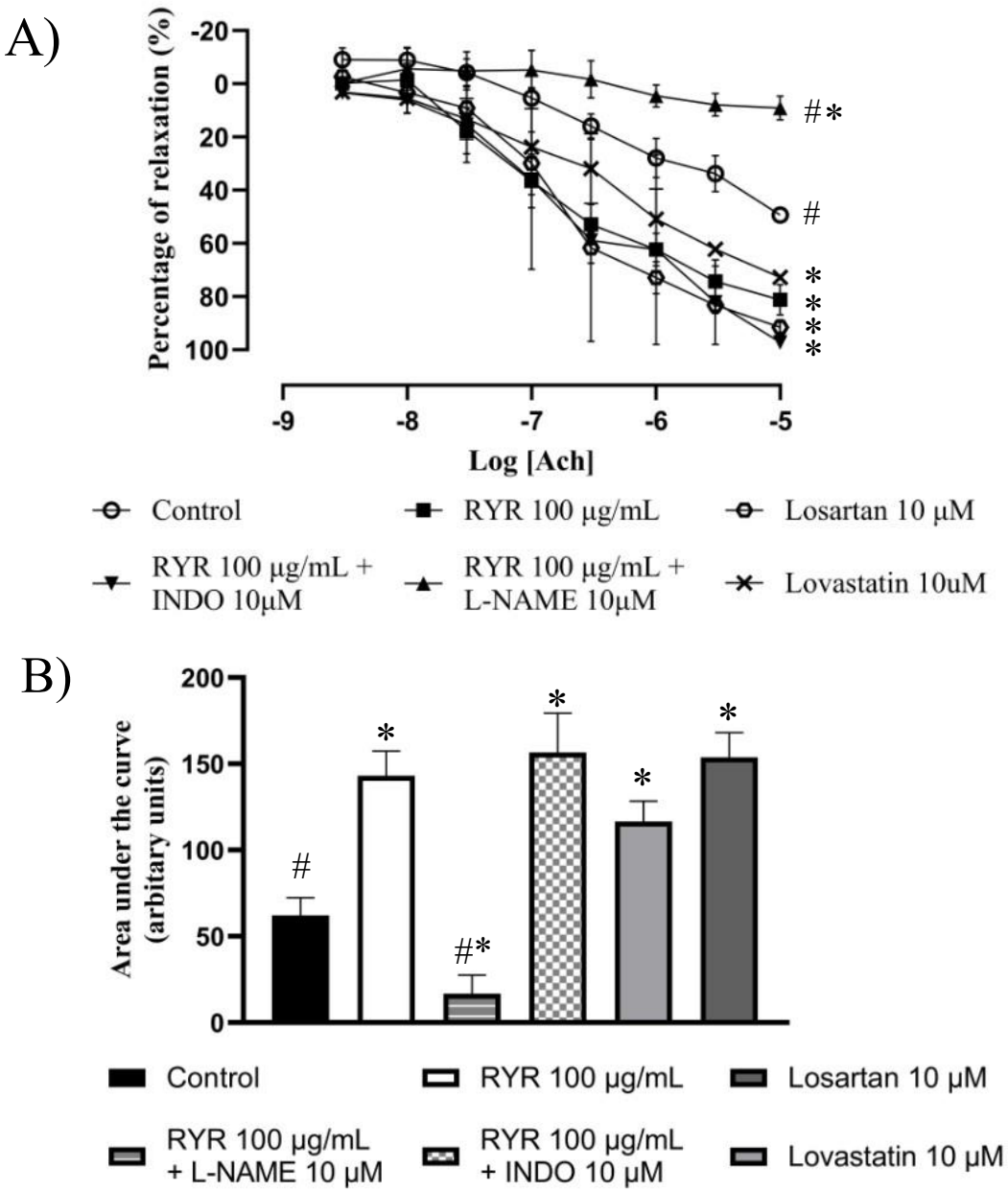

Figure 4.4: Concentration-response curves (A) and areas under the concentration-response curves (AUC) (B) to ACh-induced endothelium-dependent relaxation in SHR when incubated with or without different pre-treatments: RYR 100 µg/mL + L-NAME 10 µM, RYR 100 µg/mL + INDO 10 µM, RYR 100 µg/mL, losartan 10 µM and lovastatin 10 µM. Data is expressed as mean ± SEM (n=5). $^{\#}p < 0.05$ compared to RYR 100 ug/mL; *$p < 0.05$ compared to Control.

Table 4.3: Potency (pEC_{50}) and maximal relaxation (R_{max}) to ACh -induced relaxation in aortae of SHR (n=5) incubated with or without the following pre-treatments: RYR 100 μg/mL + L-NAME 10μM, RYR 100 μg/mL + INDO 10μM, RYR 100 μg/mL, losartan 10 μM and lovastatin 10 μM.

	ACh	
	pEC_{50} (log M)	R_{max} (%)
Control	5.96 ± 0.81 #	49.29 ± 2.22 #
RYR 100 μg/mL + L-NAME 10μM	6.15 ± 0.44	9.09 ± 4.38 # *
RYR 100 μg/mL + INDO 10μM	6.49 ± 1.12	95.13 ± 1.78 *
RYR 100 μg/mL	6.95 ± 0.24	82.12 ± 5.50 *
Losartan 10 μM	6.79 ± 0.13	91.46 ± 2.59 *
Lovastatin 10 μM	6.20 ± 0.54	72.76 ± 1.94 *

Data is expressed as mean ± SEM (n=6-8)

$^{\#}p < 0.05$ compared to RYR 100 ug/mL; $^{*}p < 0.05$ compared to Control.

4.4 Effect of RYR on vascular reactive oxygen species production in cryostat sections of aortas from WKY and SHR

Measurement of intracellular ROS formation by DHE fluorescence staining showed that aortas from SHR-Control had significantly higher level of fluorescence intensity, implying that an increase in the level of ROS compared to WKY-Control (Figure 4.5). The aortic rings of SHR treated with RYR showed significantly decreased intensity of DHE fluorescence compared to SHR-Control, implying the aortic rings in SHR treated with RYR has significantly decreased ROS levels. There was also a comparable decrease in DHE fluorescence intensity in the aortic rings of SHR treated with lovastatin compared to SHR Control, indicating a significant reduction in ROS production as well.

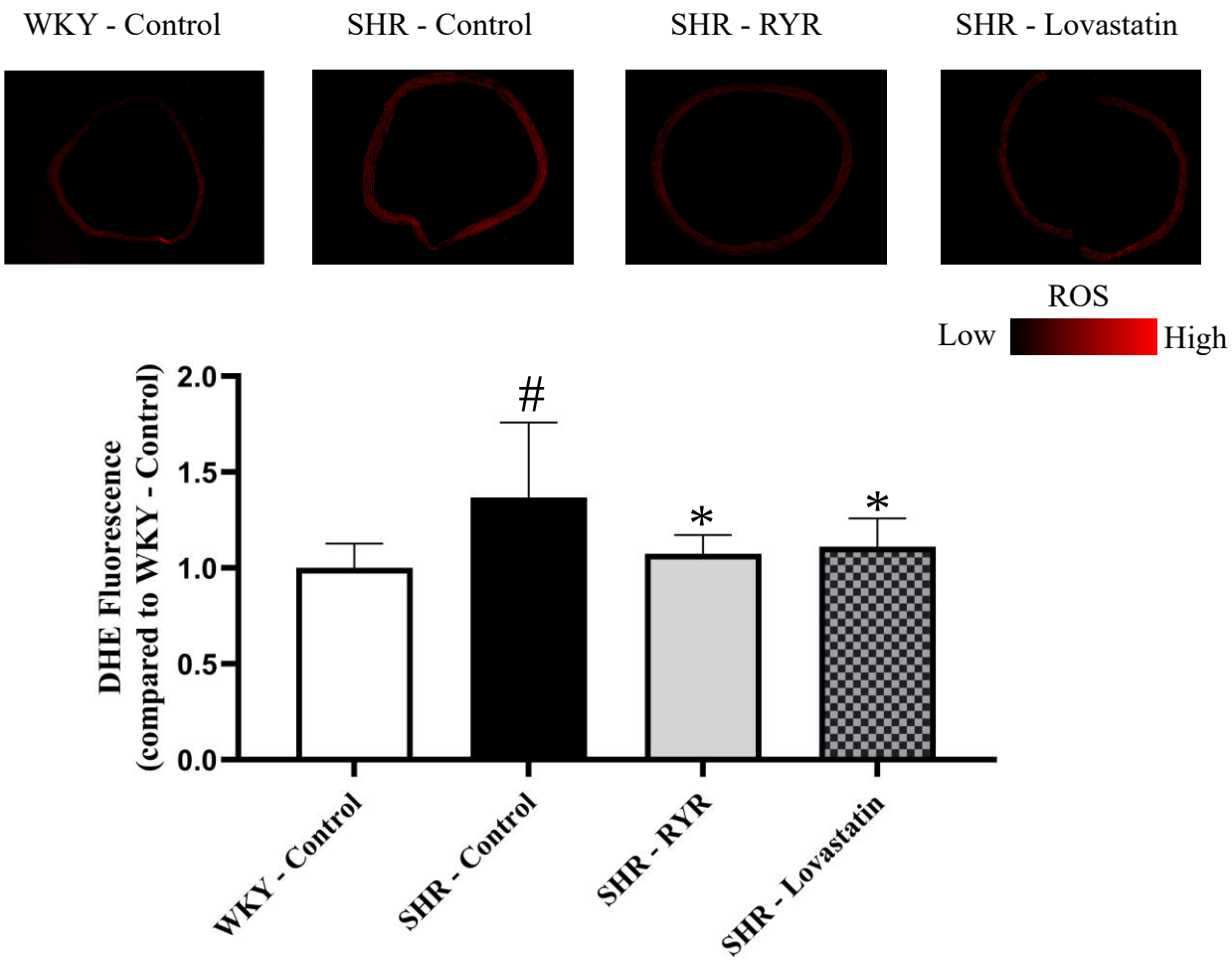

Figure 4.5: Relative fluorescence signal intensity of dihydroethidium (DHE) stained aortae of Wistar-Kyoto Rats (WKY) Control and Spontaneously Hypertensive Rats (SHR) with and without *in-vivo* treatment of red yeast rice (RYR; 100 mg/kg/day) or lovastatin (10 mg/kg/day) for 12 weeks. Data is expressed as mean ± SEM (n=6-8). #$p < 0.05$ compared to WKY – Control; *$p < 0.05$ compared to SHR – Control.

4.5 Effect of RYR on vascular nitric oxide production in cryostat sections of aortas from WKY and SHR

Measurement of intracellular NO formation by DAF-FM fluorescence staining showed that aortas from SHR-Control had significantly decreased level of fluorescence intensity, implying a decrease in NO production compared to WKY-Control (Figure 4.6). Treatment with RYR extract for 12 weeks had significantly increased fluorescence intensity of DAF-FM in aortic rings, indicating an improvement in NO production compared to SHR-Control. There was also a stronger DAF-FM fluorescence intensity in the aortic rings of SHR treated with lovastatin compared to SHR Control, indicating a significant increase in NO production as well.

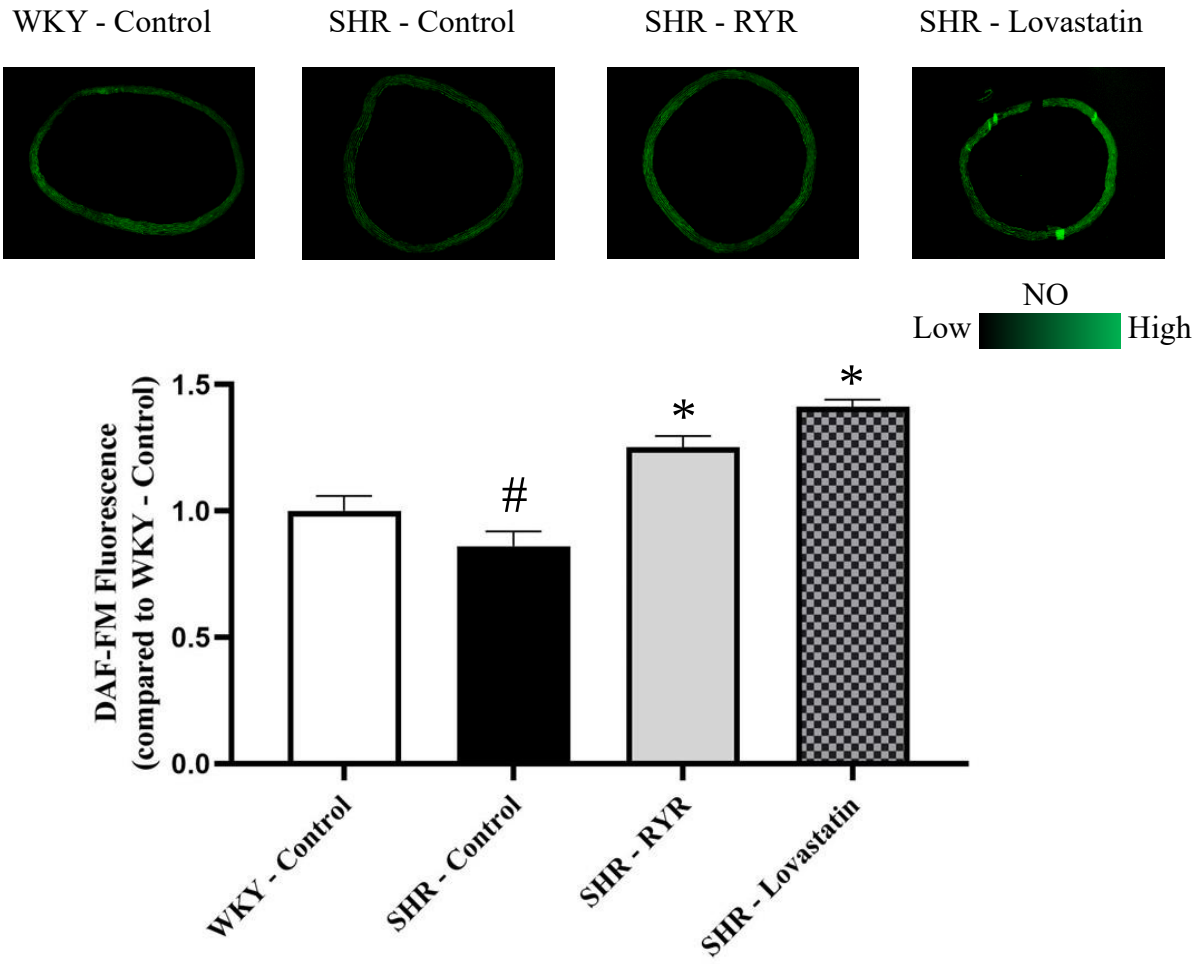

Figure 4.6: Relative fluorescence signal intensity of diaminofluorescein-FM (DAF-FM) stained aortae of Wistar-Kyoto Rats (WKY) Control and Spontaneously Hypertensive Rats (SHR) with and without *in-vivo* treatment of red yeast rice (RYR; 100 mg/kg/day) or lovastatin (10 mg/kg/day) for 12 weeks. Data is expressed as mean ± SEM (n=6-8). #$p < 0.05$ compared to WKY – Control; *$p < 0.05$ compared to SHR – Control.

4.6 Effect of RYR on vascular BH_4 level

Measurement of total vascular BH_4 level by ELISA showed that SHR-Control had significantly reduced the level of BH_4 compared to WKY-Control (Figure 4.7). SHR treated with RYR had significantly increased the BH_4 levels when compared to SHR-Control. Furthermore, SHR treated with lovastatin also showed similar increased levels of BH_4 compared to SHR-Control.

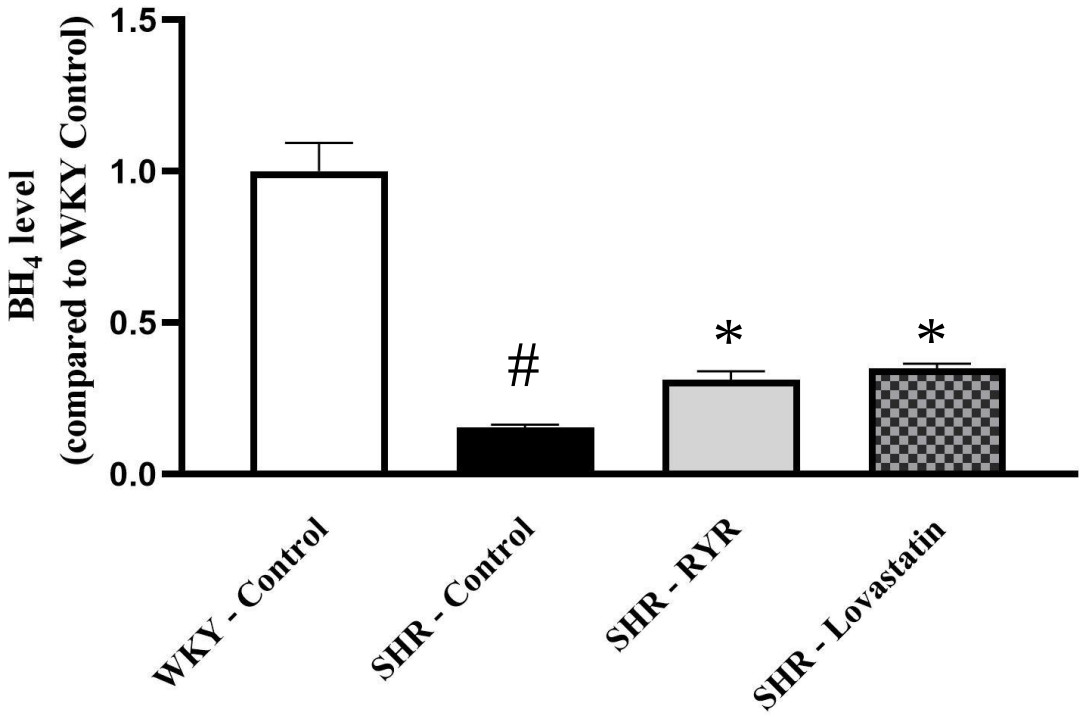

Figure 4.7: Relative BH_4 levels in aortae of Wistar-Kyoto Rats (WKY) Control and Spontaneously Hypertensive Rats (SHR) with and without *in-vivo* treatment of red yeast rice (RYR; 100 mg/kg/day) or lovastatin (10 mg/kg/day) for 12 weeks. Data is expressed as mean ± SEM (n=6-8). #$p < 0.05$ compared to WKY – Control; *$p < 0.05$ compared to SHR – Control.

4.7 Effect of RYR on vascular cGMP level

Measurement of the level of total vascular cGMP production by ELISA demonstrated that SHR-Control had markedly reduced level of cGMP compared to WKY-Control (Figure 4.8). Treatment with RYR had significantly increased the cGMP level in the aortic rings of the treated animals compared to SHR-Control. Furthermore, treatment with lovastatin had significantly increased vascular cGMP level when compared to SHR-Control.

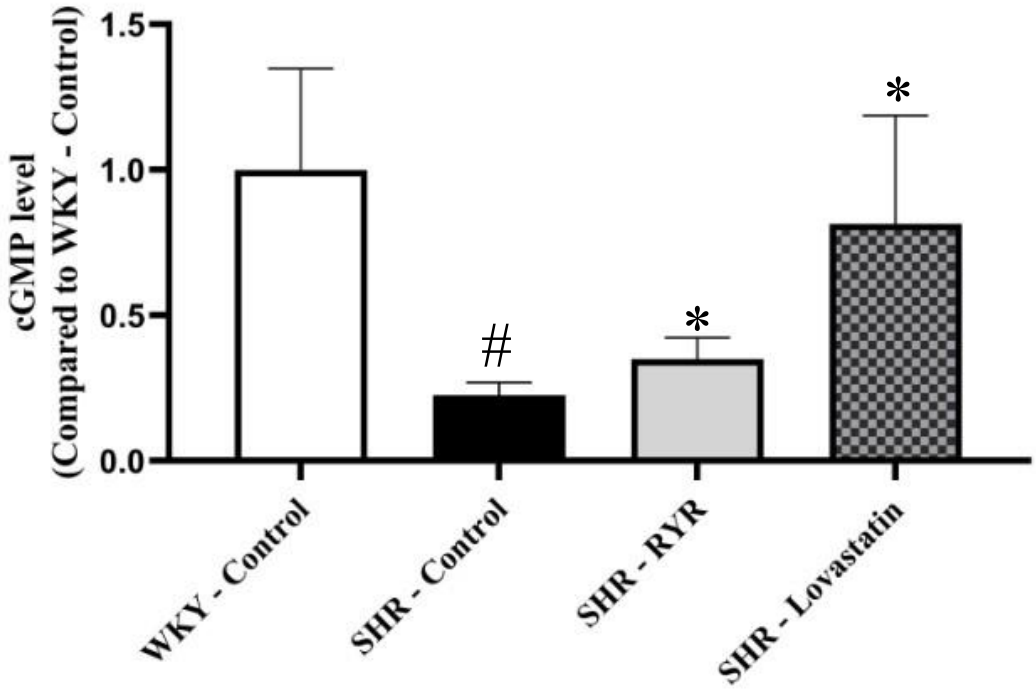

Figure 4.8: Relative cGMP levels in aortae of Wistar-Kyoto Rats (WKY) Control and Spontaneously Hypertensive Rats (SHR) with and without *in-vivo* treatment of red yeast rice (RYR; 100 mg/kg/day) or lovastatin (10 mg/kg/day) for 12 weeks. Data is expressed as mean ± SEM (n=6-8). #$p < 0.05$ compared to WKY – Control; *$p < 0.05$ compared to SHR – Control.

4.8 Effect of RYR on vascular AT_1 receptor expression

Western blot demonstrated that SHR-Control had a significant two-fold elevated AT_1 receptor expression in the aorta relative to WKY-Control as shown in Figure 4.9. Treatment with RYR had reduced the expressional elevation of AT_1 receptor expression by 54% in the aorta when compared to SHR-Control. Similarly, treatment with lovastatin had decreased AT_1 receptor expression in the aorta but was not statistically significant when compared to SHR-Control.

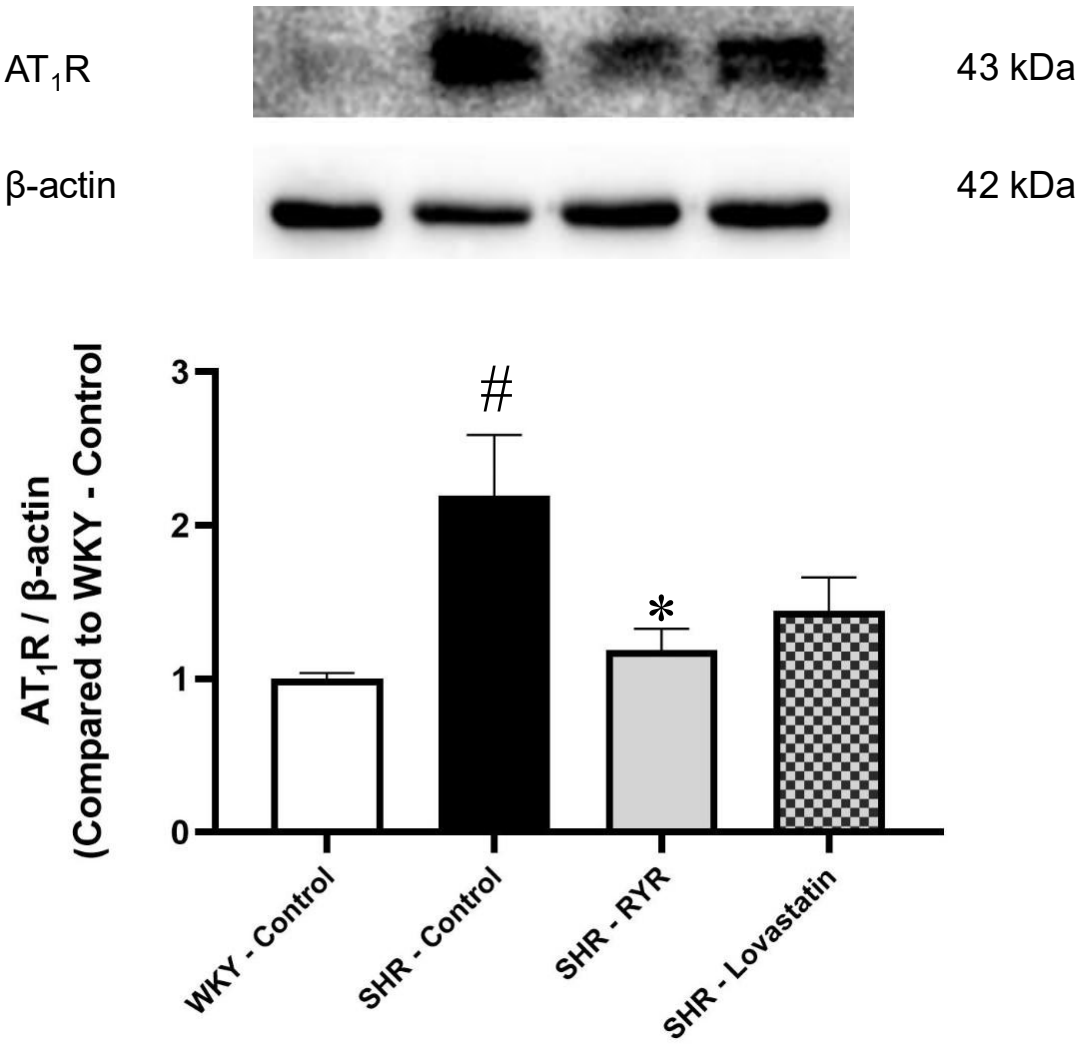

Figure 4.9: Total AT_1 receptor expression in aortae of Wistar-Kyoto Rats (WKY) Control and Spontaneously Hypertensive Rats (SHR) with and without *in-vivo* treatment with red yeast rice (RYR; 100 mg/kg/day) or lovastatin (10 mg/kg/day) for 12 weeks. The upper panel shows the representative Western blot and the bottom panel shows the ratio of AT_1 receptor to β-actin relative to WKY- Control. Data is expressed as mean ± SEM (n=6-8). #$p < 0.05$ compared to WKY – Control; *$p < 0.05$ compared to SHR – Control.

4.9 Effect of RYR on vascular AT_2 receptor expression

Western blot demonstrated that aortic AT_2 receptor expression in SHR-Control was not significantly different compared to WKY-Control as shown in Figure 4.10. Treatment with RYR had significantly increased AT_2 receptor expression in the aorta by more than 1.5-fold compared to SHR-Control. However, there was no significant difference in AT_2 receptor expression in SHR treated with lovastatin.

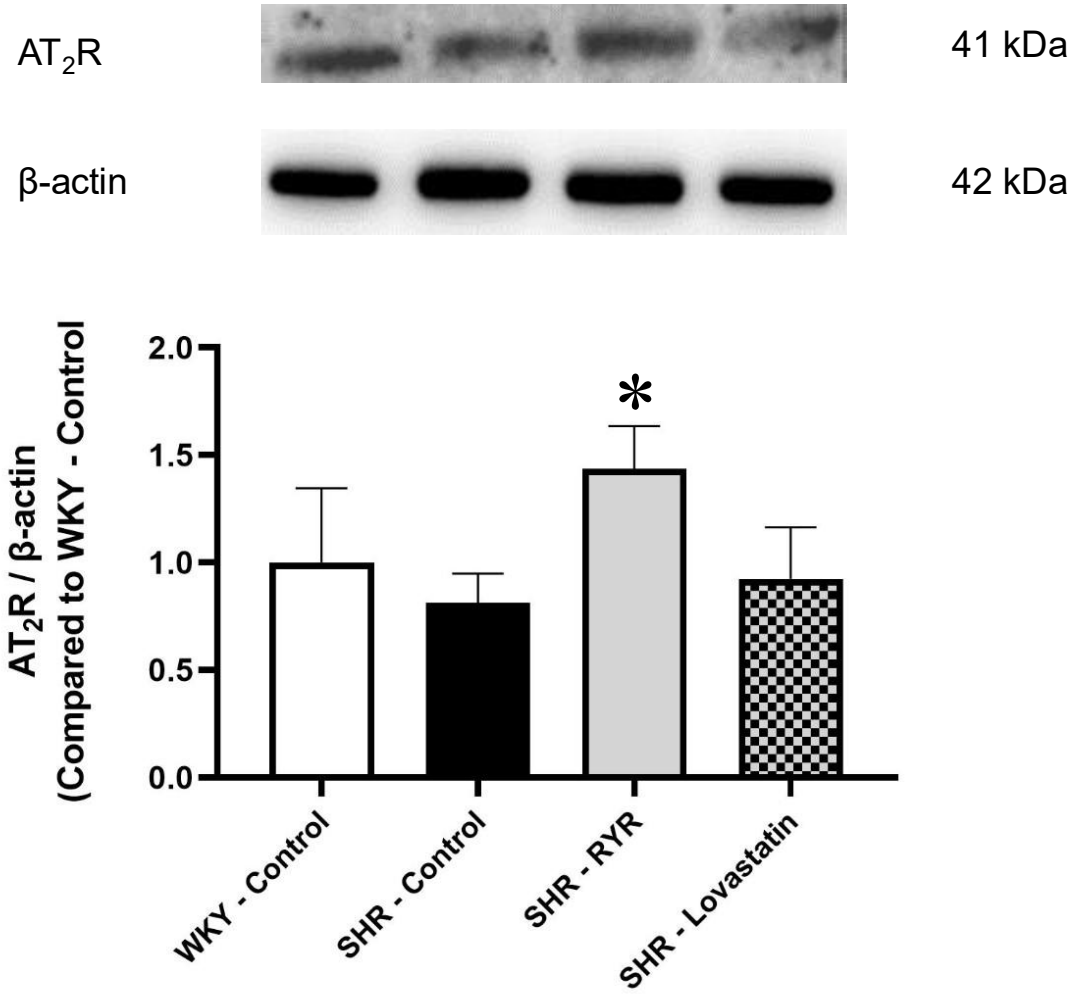

Figure 4.10: Presence of total AT_2 receptor expression in aortae of Wistar-Kyoto Rats (WKY) Control and Spontaneously Hypertensive Rats (SHR) with and without *in-vivo* treatment of red yeast rice (RYR; 100 mg/kg/day) or lovastatin (10 mg/kg/day) for 12 weeks. The upper panel shows the representative Western blot, and the bottom panel shows the ratio of AT_2 receptor to β-actin relative to WKY- Control. Data is expressed as mean ± SEM (n=6-8). *$p < 0.05$ compared to SHR – Control.

Chapter 5

Discussion

In the present study, it is demonstrated that *in-vivo* treatment with RYR for 12 weeks exhibits a blood pressure lowering effect on SHR. Treatment with RYR had also improved endothelium-dependent, NO-mediated vasorelaxation in the hypertensive animals. Align with these results, the vascular levels of NO, cGMP and BH_4 were also significantly increased. Furthermore, there was a decrease in vascular ROS levels that was accompanied by a decreased expression of AT_1 receptors and an increased expression of AT_2 receptors in the aorta of SHR. Collectively, these findings suggest that the blood pressure lowering effect of RYR is associated with an increase in endothelial-dependent vascular relaxation that is partly attributable to the inhibitory effect of RYR on AT_1 receptors, leading to decreased oxidative stress and an improvement in NO-cGMP signalling in SHR.

There was a persistent elevation of systolic blood pressure in the SHR – Control group, a characteristic of the selected disease model (Figure 4.1). The systolic blood pressure of SHR – Control group was also significantly higher when compared to the systolic blood pressure of WKY – Control group throughout the treatment period (Table 4.1; Week 12, SHR-Control vs WKY-Control: 221.5 ± 3.6 mmHg vs 161.7 ± 4.7 mmHg). Treatment with RYR for 12 weeks has progressively suppressed the elevation of systolic blood pressure seen in SHR-Control group from week 6 onwards (Figure 4.1), with statistically significant effects observed at the end of the treatment period (SHR-Control vs

SHR-RYR: 221.5 ± 3.6 mmHg vs 197.4 ± 1.7 mmHg). This observation implies that treatment with RYR in SHR has exerted blood pressure lowering effects in hypertensive animals. This is consistent with a previous *in-vivo* study performed by Wang and colleagues who reported that intravenous bolus administration of ethanolic extract of RYR for 120 minutes decreased systolic blood pressure through the inhibition of sympathetic activity in SHR (Wang et al., 2010).

The increase in systolic blood pressure in SHR-Control group was accompanied by a blunted response to acetylcholine when compared to WKY-Control group, consistent with previous studies performed on SHR (Khor et al., 2023, Loh et al., 2015). After treatment with RYR for 12 weeks, there is significant alleviation of endothelial dysfunction, as supported by the increased relaxation in ACh-induced relaxation curves when compared to SHR-Control (Figure 4.2; SHR-Control vs SHR-RYR: 53.55 % ± 3.45 % vs 69.21 % ± 2.18 %). This finding is in line with previous study done whereby treatment with RYR extract for 3 weeks was also shown to also attenuate vascular dysfunction in obese Wistar albino rats (Alkholifi et al., 2021). These results suggest the decrease in systolic blood pressure of SHR can be attributed to the improvements seen in ACh-induced relaxations of the aorta, suggesting an improvement in vasodilator response to ACh (Leal et al., 2020). This improvement in response to ACh could be due to an increased production in NO. The uniform relaxation of aortic rings of all animal groups to exogenous NO donor, sodium nitroprusside (Figure 4.3), suggests that the beneficial effect of RYR in terms of improving endothelial function is most likely due to the modulation of endothelial cells to

release NO and not by the increase in sensitivity of the vascular smooth muscle cells toward NO.

To further support the interpretation that RYR was able to modulate NO release by the endothelium, *ex-vivo* studies on aortic rings treated with various relaxants were carried out on SHR without any prior oral treatment. When the aortic rings were pre-treated with only RYR (100 µg/mL), an increase in ACh-induced relaxation was observed when compared to untreated aortic rings (Figure 4.4). However, when RYR was introduced in the presence of L-NAME, an eNOS inhibitor (10 µM), an almost complete loss of relaxation was observed. Besides, relaxation induced by RYR was not negatively affected by the non-specific COX inhibitor indomethacin (10 µM). These findings implies that the endothelium-dependent relaxation produced by RYR is mediated through the release of NO by eNOS and not through the PGI_2-cAMP vasodilatory pathway. The present findings agree with the work done by Rhyu et.al, whereby relaxation of aortic rings from Sprague-Dawley rats pre-treated with RYR produced from fermentation with *Monascus ruber* was significantly reduced when exposed the combination of L-NAME and RYR extract but not by the combination of indomethacin and RYR (Rhyu et al., 2000). Notably, isolated aortic rings of untreated SHR pre-treated with losartan, an AT_1 receptor inhibitor (10 µM), or lovastatin, an HMG-CoA reductase inhibitor (10 µM) had significantly increased relaxation to ACh compared to aortic rings treated with RYR (Table 4.3). This observation is consistent with a study conducted by Lunder and colleagues showing that the aortic rings of Wistar rats treated with atorvastatin or losartan had similar degree of maximal vasodilatory activity (Lunder et al., 2013). The current finding shows a comparable level of vasorelaxation among SHR treated

with RYR and lovastatin. With the available evidence regarding the potential AT_1 receptor blocking ability of statins (Wan and Chen, 2023, Costa et al., 2023), it can be suggested that RYR, which contains monacolin K that has similar structure to lovastatin, is probably able to modulate AT_1 receptor and restrict the activation of its downstream signalling pathway.

Increasing NO bioavailability in the vascular system has been viewed as an important therapeutic approach to address endothelial dysfunction. NO acts as a paracrine hormone on the underlying vascular smooth muscles by activating soluble guanylyl cyclase to produce cGMP and cGMP dependent protein kinases to ultimately cause vasorelaxation. To further validate that treatment with RYR was able to increase the bioavailability of NO, the levels of NO in the aorta of SHR were measured. The present findings show that treatment with RYR had significantly increased DAF-FM fluorescence signal in the aorta of SHR compared to SHR-Control (Figure 4.6), indicating the increase in NO levels. Furthermore, the increase in NO levels is also paralleled with an increase in aortic tissue cGMP levels (Figure 4.8). This provides further evidence that the beneficial effects of RYR is due to, at least in part, to the upstream activation of eNOS and thus the increased production of NO and cGMP, and thus the improved endothelial-dependent relaxation observed in SHR. This increase of vascular NO leading to increased cGMP production is consistent with previous studies done in SHR (Khor et al., 2023, Mohd Sabri et al., 2022). Adding on, a previous study by Zhu and colleagues also demonstrated that treatment with Xuezhikang, a traditional Chinese medicine containing RYR extract on

atherosclerotic rats was able to increase plasma levels of NO and cGMP (Zhu et al., 2013).

An optimal concentration of the cofactor BH_4 is required for functional eNOS. It is an important factor of NO synthesis and thus normal endothelial function (Stuehr and Haque, 2019). Previous studies have shown that during states of oxidative stress and hypertension, oxidative degradation of BH_4 by ROS occurs (Yuyun et al., 2018). As BH_4 becomes limiting, uncoupling of BH_4 and eNOS occurs, electron transfer from NADPH to molecular oxygen becomes uninhibited (Wu et al., 2021). This causes eNOS to produce superoxide (O_2^-) instead, further aggravating oxidative stress and endothelial dysfunction. In line with this, the present study has demonstrated that a decrease in BH_4 level (Figure 4.7) was accompanied by an increase in DHE fluorescence intensity (Figure 4.5) in the aorta of SHR-Control group, indicating an increase in oxidative stress. After SHR was treated with RYR for 12 weeks, it was observed that there was an increase in BH_4 concentration paired with a decrease in DHE fluorescence intensity. This implies that RYR may have increased the bioavailability of NO by increasing eNOS activity through improving BH_4 concentration thus reducing oxidative stress. This agrees with previous studies whereby monacolin K has been shown to suppress ROS production in human umbilical vein endothelial cells (HUVECs) (Wang et al., 2017) and promote BH_4-eNOS recoupling in streptozotocin (STZ)-induced hyperglycaemic Sprague-Dawley Rats (Wang et al., 2017).

Angiotensin II is the primary endocrine ligand in the RAAS, contributing to the development of cardiovascular disorders including hypertension (Karnik et al., 2015). Activation of AT_1 receptor through binding of angiotensin II to it leads to the downstream stimulation of NAPDH oxidase. As a mediator of oxidative stress, it represents one of the major sources of superoxide production within the vasculature. This was shown in a previous study by Rincón and colleagues that reported an up-regulation of NADPH oxidases and oxidative stress makers was accompanied by increased expression of AT_1 receptors in L-NAME induced hypertensive Sprague-Dawley rats (Rincón et al., 2015). This is consistent with the present results whereby SHR-Control was shown to have increased aortic DHE fluorescence along with elevated vascular AT_1 receptor expression when compared to WKY-Control. After treatment with RYR extract for 12 weeks, there was a significant decrease in AT_1 receptor expression compared to SHR-Control (Figure 4.9). While a study conducted by Wang and colleagues have shown that RYR was able to inhibit NOX activation through downregulation of its catalytic subunit, gp91phox (Wang et al., 2014), there have been no studies on the effect or RYR on the upstream of NOX activation. Thus, RYR may also partly improve endothelial function of SHR mediated by the inhibition of AT_1 receptor-dependent signalling pathway.

The angiotensin II type 2 (AT_2) receptor has been increasingly recognised as the protective arm of the RAAS. While it shares the same endogenous ligand (angiotensin II) with AT_1 receptor, it has been reported to have opposing effects to those of the AT_1 receptor by activating the NO-cGMP signalling cascade to induce vasodilation (Padia and Carey, 2013), either by increasing bradykinin

(BK) production or by direct activation of NO production independent of BK (Abadir et al., 2003). Additionally, Kemp and colleagues have shown that activation and upregulation of the renal AT_2 receptor by compound-21 was able to reduce systolic blood pressure in hypertensive angiotensin II infused Sprague-Dawley rats (Kemp et al., 2014). In this study, while statistically insignificant, there was a higher expression of AT_2 receptor in WKY-Control compared to SHR-Control, an inverse of AT_1 receptor expression between these 2 groups. After treatment for 12 weeks, SHR treated with RYR extract had increased the expression of AT_2 receptors (Figure 4.10), which was accompanied by a downregulation of AT_1 receptors. This reciprocal relationship is consistent with a previous study whereby inhibition of AT_1 receptor leads to an upregulation of AT_2 receptor in cardiac remodelled Sprague-Dawley Rats (Zheng et al., 2019). To the best of our knowledge, the current study is the first study that demonstrated the improvement of endothelial function and thus reduction of systolic blood pressure can be partly attributed to AT_2 receptor-induced vasodilation mediated by increased NO and cGMP production.

Lovastatin was chosen as the positive control for this study due to its analogous structure to monacolin K, the most abundant bioactive compound available in RYR. While mainly used as a lipid-lowering medication, pleotropic effects on cardiovascular diseases have been studied extensively. Lovastatin has been shown to reduce systolic blood pressure in cyclosporin A-induced hypertensive Sprague-Dawley rats (Wang et al., 2021), improve endothelial-dependent relaxations in SHR (Bravo et al., 1998) and enhance NO production by increasing eNOS activity in atherosclerotic C57BL/6 mice (Wijaya et al.,

2022). In terms of antioxidative properties, lovastatin was reported to reduce ROS production in the kidney of streptozotocin-induced diabetic rats (Ma et al., 2017). While there are limited studies regarding the inhibitory effects of lovastatin on the RAAS, other drugs in the statin family have been found to downregulate AT_1 receptor expression in hypertensive (Ichiki et al., 2001, Wassmann et al., 2001) and diabetic animal models (Tian et al., 2011). Based on our present findings, the effects observed in SHR treated with lovastatin was comparable to the effects of SHR treated with RYR. Thus, the beneficial effects of RYR can be partly attributed to monacolin K due to its abundance and structural similarity to lovastatin.

Till date, a number of supplements have been used to address hypertension. Magnesium is commonly consumed as it is a vital cofactor in enzymatic processes that regulates smooth muscle relaxation and reduce peripheral vascular resistance (Kostov and Halacheva, 2018). Omega-3 fatty acids are often supplemented for their ability to decrease arterial stiffness and vascular inflammation (Mason et al., 2020). Plant-based compounds such as epigallocatechin gallate (EGCG), a polyphenol in green tea, have also been suggested to have antihypertensive effects by improving endothelial function and increasing eNOS activity (Mahdavi-Roshan et al., 2020). Although there are a wide varieties of supplements that aim to alleviate the detrimental effects of cardiovascular diseases, the consumption of these supplements does present notable limitations and risks. Randomised controlled trials have reported that supplemental vitamins and minerals was not associated with reduced major adverse cardiovascular events such as myocardial infarction, stroke and cardiovascular death, with no consistent benefits in preventing or treating

cardiovascular diseases outcomes (Barawi et al., 2019, O'Connor et al., 2022). Another randomised controlled trial, which evaluated the effects of Omega-3 fatty acids, showed no significant impact in serious vascular events (ASCEND Study Collaborative Group, 2018), even though other studies have suggested that Omega-3 fatty acids may provide cardiovascular benefits, such as reducing triglyceride levels, lowering blood pressure and supporting heart rhythm stability (Wang et al., 2021). Additionally, the widespread use of ineffective supplements can lead to unnecessary healthcare costs, as individuals invest in products without proven benefits (Sultan et al., 2017). These challenges highlight the importance of targeted research, such as this study, to evaluate the benefits of RYR in cardiovascular health.

Chapter 6

Conclusion

In conclusion, the current work has shown that treatment with RYR extract for 12 weeks has reduced the expression of AT_1 receptor, leading to a decrease in oxidative stress thus decreasing eNOS uncoupling via increasing the level of BH_4 and therefore enhanced NO-cGMP signalling. These effects contribute to the improvement in vascular function and consequently the reduced systolic blood pressure observed in SHR. This study adds new information about the blood pressure lowering mechanisms of RYR and its potential use as a complementary treatment for hypertension.

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LIST OF PUBLICATION

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LIST OF CONFERENCE PRESENTATIONS

Tan, J.J., Kang, W.H., Lee, S.K., Murugan, D.D. and Ling, W.C., 2024. Treatment with Red Yeast Rice Improves Endothelial Dysfunction in Spontaneously Hypertensive Rats. International Conference on Drug Discovery and Translational Medicine 2023. [Oral Presentation] Abstract published in *Malaysian Journal of Medicine & Health Sciences*, *20*.

Tan, J.J., Kang, W.H., Lee, S.K., Murugan, D.D. and Ling, W.C., 2022. Vasoprotective Effects of Red Yeast Rice Supplementation in Spontanously Hypertensive Rats. The International Conference on Molecular Diagnostics & Biomarker Discovery 2022. [Poster Presentation]