

MODULATORY ACTIONS OF DES-ASPARTATE ANGIOTENSIN I  
ON VASCULAR REACTIVITY AND ENDOTHELIAL FUNCTION IN  
AORTA FROM SPONTANEOUSLY HYPERTENSIVE RATS

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ORIGINAL LITERARY WORK DECLARATION

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## ABSTRACT

Hypertension or chronic elevation of arterial blood pressure is associated with endothelial dysfunction. Imbalance of vasodilators and vasoconstrictors released from the endothelium contributes to the development of endothelial dysfunction in hypertension. Renin angiotensin system (RAS) is one of the major physiological systems contributing to the regulation of arterial blood pressure and angiotensin II (Ang II) is the most active component in RAS. Ang II exerts various physiological functions through its interaction with angiotensin type 1 and type 2 receptors (AT<sub>1</sub>R and AT<sub>2</sub>R, respectively). Stimulation of AT<sub>1</sub>R activates signaling cascades that causes vasoconstriction, oxidative stress, vascular remodeling and endothelial dysfunction contributing to the pathophysiology of hypertension. In contrast, Ang II causes vasodilatation, inhibits proliferation and induces apoptosis through activation of AT<sub>2</sub>R.

Des-aspartate angiotensin I (DAA-I), a nonapeptide of the RAS is known to counteract the various pathological effects attributed to Ang II. The nonapeptide showed anti-hyperplastic, anti-cardiac hypertrophy, anti-hyperglycemic, anti-inflammatory and anti-viral properties in *in vivo* and *in vitro* studies. Although earlier studies have demonstrated various beneficial effects, little is known on the modulatory effect of DAA-I on vascular and endothelial function in hypertensive rats. Thus, the objectives of this present study are 1) to determine the modulatory effect of DAA-I on vascular responses to endothelial –dependent vasoconstrictor (angiotensin II) and to endothelial –dependent [acetylcholine (ACh)] and –independent [sodium nitroprusside (SNP)] relaxation in isolated aorta from Wistar Kyoto rats (WKY) and Spontaneously Hypertensive rats (SHR), 2) to determine the effect of DAA-I on angiotensin II-induced oxidative stress in the vascular tissues from hypertensive rats; and 3) to investigate the molecular mechanisms underlying the protective effects of DAA-I.

Isometric tension experiments were carried out to study the vasomodulatory effects of DAA-I on vascular responses in SHR aorta. Several pharmacological inhibitors were tested to determine the mechanisms of modulatory action of DAA-I. Total nitrate and nitrite levels were assessed using a colorimetric method and reactive oxygen species (ROS) were measured by dihydroethidium (DHE) fluorescence and lucigenin-enhanced chemiluminescence in order to investigate the effect of DAA-I on oxidative stress.

The present study demonstrated that DAA-I has no effect on WKY aorta but it significantly attenuated Ang II-induced contraction in the isolated aortic rings from SHR. The attenuation of DAA-I on Ang II contraction was blunted in the rings without endothelium and in the presence of L-NAME (nitric oxide synthase inhibitor) and ODQ (soluble guanylate cyclase inhibitor), indicating the action of DAA-I is dependent of nitric oxide/ sGC pathway. The effect of DAA-I on Ang II-induced contraction was significantly reduced by losartan (AT<sub>1</sub> receptor antagonist) and partially reversed by PD123319 (AT<sub>2</sub> receptor antagonist), indicating both angiotensin receptors modulate the actions of DAA-I. In the presence of Ang II, total nitrate and nitrite levels were increased in DAA-I, losartan and tempol treated-SHR tissues while ROS level was reduced by DAA-I and the latter inhibitors. Treatment with DAA-I significantly enhanced ACh-induced relaxation in SHR aortas but not in WKY whilst SNP-induced relaxation remained unaltered in the WKY and SHR. Losartan and L-NAME reduced the DAA-I potentiated ACh-induced relaxations in the SHR aorta. Furthermore, the sensitivity to ACh in DAA-I -treated tissues was reduced in the presence of PD 123319.

In summary, the present study demonstrated that DAA-I exerts vasoprotective effect on the vascular and endothelium function in the aorta from SHR but not in WKY. The nonapeptide improves the endothelial function by attenuating Ang II-induced contraction and potentiating ACh-induced relaxation. Data obtained shows that the actions of DAA-I is endothelium-dependent. The protective effect of DAA-I is due to

the increase of NO bioavailability and reduced ROS by interfering with Ang II-mediated, NADPH oxidase derived oxidative stress. In conclusion, DAA-I improves endothelial function in SHR by counteracting AT<sub>1</sub>R –mediated effects.

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## ABSTRAK

Tekanan darah tinggi atau hipertensi adalah berkaitan dengan disfungsi endotelial. Ketidakseimbangan antara vasodilator dan vasokonstriktor daripada endotelium menyebabkan perkembangan disfungsi endotelial dalam hipertensi. Sementara itu, sistem “renin angiotensin” (RAS) adalah salah satu sistem fisiologi yang penting dalam pengawalan tekanan darah. Angiotensin II (Ang II) merupakan komponen yang paling aktif dalam sistem RAS di mana ianya merangsang pelbagai fungsi fisiologi melalui reseptor-reseptornya, iaitu jenis angiotensin reseptor 1 dan jenis angiotensin reseptor 2 (AT<sub>1</sub>R and AT<sub>2</sub>R). Interaksi antara Ang II/AT<sub>1</sub>R mengaktifkan isyarat lara seperti vasokonstriksi, stres oksidatif, pemodelan semula vaskular dan disfungsi endotelial yang menyumbang kepada patofisiologi hipertensi. Ang II menyebabkan vasodilatasi, merencat proliferasi sel-sel, menginduksi apoptosis melalui pengaktifan AT<sub>2</sub>R.

Des-aspartate angiotensin I (DAA-I), “nonapeptide” dalam sistem RAS telah dibuktikan dapat mengatasi kesan-kesan patologi yang melibatkan Ang II. “Nonapeptide” ini menunjukkan ciri-ciri seperti anti-hiperplastik, anti-hipertropi jantung, anti-hiperglisemia, anti-inflamasi dan anti-virus melalui kajian-kajian *in vivo* dan *in vitro*. Walaupun kajian-kajian ini dapat menunjukkan pelbagai kesan yang berfaedah, tetapi kesan modulatori DAA-I pada disfungsi endotelial dalam tikus hipertensi masih tidak diketahui. Oleh sebab itu, objektif kajian ini adalah, untuk 1) menentukan kesan modulatori DAA-I terhadap tindakbalas vaskular kepada agonis yang menghasilkan kontraksi yang bergantung pada endotelium (Ang II), agonis yang menghasilkan relaksasi bergantung endotelium [acetylcholine (ACh)] dan tidak bergantung endotelium [sodium nitroprusside (SNP)] pada aorta daripada tikus WKY dan SHR, 2) menentukan tindakan DAA-I pada kesan stres oksidatif yang disebabkan oleh Ang II pada tisu vaskular tikus hipertensi, 3) menentukan mekanisme-mekanisme yang mendasari tindakan modulatori DAA-I.

Eksperimen ketegangan isometrik telah dijalankan untuk mengkaji vasomodulasi DAA-I pada tindakbalas vaskular dalam aorta SHR. Beberapa perencat farmakologi telah diuji untuk menentukan mekanisme tindakan vasomodulasi DAA-I. Jumlah tahap nitrat dan nitrit dinilai dengan menggunakan kaedah kolorimetrik dan pembentukan spesies oksigen reaktif (ROS) dikaji dengan menggunakan pendaflour “dihydroethidium” (DHE) dan “lucigenin-enhanced chemiluminescence” untuk menyiasat kesan DAA-I pada stres oksidatif.

Kajian ini menunjukkan bahawa DAA-I tiada mempunyai kesan ke atas aorta tikus WKY tetapi menurunkan kontraksi yang disebabkan oleh Ang II pada aorta SHR. Penurunan ke atas kontraksi Ang II oleh DAA-I diberbalik pada cincin aorta SHR tanpa endotelium dan dalam cincin aorta yang telah dieram dengan L-NAME (perencat nitric oksida (NO) sintase) dan ODQ (perencat “soluble guanylate cyclase” (sGC)). Ini menunjukkan bahawa tindakan vasomodulasi DAA-I bergantung pada laluan NO/sGC. Kesan DAA-I ke atas kontraksi Ang II juga direncat oleh losartan (antagonis reseptor AT<sub>1</sub>R) dan direncat separa oleh PD 123319 (antagonis reseptor AT<sub>2</sub>R). Penemuan ini menunjukkan bahawa kedua-dua reseptor ini terlibat dalam kesan modulasi DAA-I. Dengan kehadiran Ang II, tahap nitrat and nitrit telah ditingkatkan dalam aorta SHR yang dieram dengan DAA-I, losartan dan tempol manakala tahap ROS telah dikurangkan oleh DAA-I dan penghadang-penghadang ROS.

Rawatan dengan DAA-I telah meningkatkan relaksasi terhadap ACh dalam aorta SHR manakala DAA-I tiada mempunyai kesan ke atas relaksasi terhadap SNP dalam kedua-dua spesies tikus. Losartan dan L-NAME telah mengurangkan kesan DAA-I ke atas relaksasi terhadap ACh dalam aorta SHR. Tambahan lagi, kepekaan (“sensitivity”) tisu SHR yang dieram dengan DAA-I terhadap ACh telah dikurangkan oleh PD 123319.

Secara ringkasnya, kajian ini menunjukkan bahawa DAA-I memberi perlindungan vaskular ke atas fungsi endotelium aorta SHR tetapi bukan pada aorta tikus WKY. DAA-I memperbaiki fungsi endotelium dengan menurunkan kontraksi Ang II dan meningkatkan relaksasi ACh. Data yang diperolehi menunjukkan bahawa tindakan vasomodulasi DAA-I bergantung kepada endotelium. Kesan perlindungan ini disebabkan oleh kenaikan bioavailabiliti NO dan pengurangan tahap ROS dengan mengganggu stres oksidatif yang disebabkan oleh NADPH oxidase yang diaktifkan oleh Ang II. Kesimpulannya, penemuan kajian ini mencadangkan bahawa DAA-I memperbaiki fungsi endotelial dalam aorta SHR dengan mengimbangi kesan-kesan tindakan AT<sub>1</sub>R.

## ACADEMIC AWARDS AND LIST OF COMMUNICATIONS

### **1. Academic Awards**

1. Best poster presenter in 24<sup>th</sup> Scientific Meeting of the Malaysian Society of Pharmacology and Physiology (MSPP), 2<sup>nd</sup> – 3<sup>rd</sup> June 2010, Universiti Teknologi Mara (UiTM), Malaysia

### **2. List of Publications**

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#### **Oral presentation**

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## **Poster Presentation**

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3. Loh WM, Dharmani DM, Achike FI, and Mustafa MR (2011). Improved endothelium-dependent relaxation by des-aspartate angiotensin I (DAA-I) in SHR isolated aorta is mediated by angiotensin receptors and nitric oxide. 25<sup>th</sup> Scientific Meeting of the Malaysian Society of Pharmacology and Physiology (MSPP), 25<sup>th</sup> – 26<sup>th</sup> May 2011, Universiti Putra Malaysia (UPM)

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## LIST OF SYMBOLS AND ABBREVIATIONS

$\mu\text{M}$	micromolar
$\bullet\text{OH}$	hydroxyl radical
a.u.	arbitrary unit
AA	arachidonic acid
ACE	angiotensin converting enzyme
ACh	acetylcholine chloride
Akt	protein kinase B
Ang (1-7)	Angiotensin (1-7)
Ang II	Angiotensin II
Ang III	Angiotensin III
Ang IV	Angiotensin IV
APA	aminopeptidase A
APN	aminopeptidase N
ARBs	angiotensin receptor blockers
AT <sub>1-7</sub> R	Ang (1-7) receptor
AT <sub>1</sub> R	angiotensin type 1 receptor
AT <sub>2</sub> R	angiotensin type 2 receptor
AUC	area under the curve
B <sub>2</sub> R	bradykinin receptor type 2
BH <sub>4</sub>	Tetrahydrobiopterin
Ca <sup>2+</sup>	calcium ion
CaM	Calmodulin
CCBs	calcium channel blockers

cGMP	cyclic guanosine 3',5'-monophosphate
CNP	C-natriuretic peptide
COX	cyclooxygenase
cpm	count per miligram
DAA-I	Des-Aspartate angiotensin I
DAG	diacylglycerol
DETCA	diethyldithiocarbamate
DHE	dihydroethidium
DMSO	dimethyl sulfoxide
DPI	diphenyliodonium
EDCFs	endothelium-derived constricting factors
EDHFs	endothelium-derived hyperpolarization factors
EDRFs	endothelium-derived relaxing factors
EETs	epoxyeicosatrienoic acids
EGF	epidermal growth factor
eNOS	endothelial nitric oxide synthase
ERK1/2	extracellular-regulated kinase 1 and 2
<i>et al.</i>	<i>et alia</i> (and other people)
ET-1	Endothelin-1
GK rat	Goto-Kakizaki rat
GLUT-4	glucose transporter-4
gT	gram tension
GTP	guanosine triphosphate
H <sub>2</sub> O <sub>2</sub>	hydrogen peroxide
ICAM-1	intercellular adhesion molecule-1

iNOS	inducible NOS
IP <sub>3</sub>	inositol triphosphate
IRS-1	insulin-related substrate-1
JNK	c-Jun NH <sub>2</sub> -terminal protein kinase
K <sup>+</sup>	potassium ion
L-NAME	N <sub>o</sub> -Nitro-L-Arginine Methyl Ester
M	Molar
MAPK	mitogen-activated protein kinase
Mg	Milligram
MKP-1	MAP kinase phosphatase
ml	Milliliter
MLCK	myosin light chain kinase
MLCP	myosine light chain phosphatase
Mm	Millimeter
mmHg	millimeter of mercury
Na <sub>2</sub> CO <sub>3</sub>	sodium carbonate
NAD(P)H	nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide
NEP	neutral endopeptidases
nM	Nanomolar
Nm	Nanometer
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
O <sub>2</sub> <sup>-</sup>	superoxide anion
OCT	optimal cutting temperature

ONOO <sup>-</sup>	peroxynitrite
PDGF	platelet-derived growth factor
PEP	prolylendopeptidases
PGF <sub>2α</sub>	prostaglandin F <sub>2α</sub>
PGI <sub>2</sub>	prostacyclin
Phe	phenylephrine
PI3K	phosphoinositide 3-kinase
PIP <sub>2</sub>	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PLA <sub>2</sub>	phospholipase A <sub>2</sub>
pM	picomolar
PP2	protein phosphatase 2
PRA	plasma renin activity
PRR	Pro-renin receptor
PYK2	proline-rich tyrosine kinase 2
RAS	renin-angiotensin system
ROS	reactive oxygen species
S.E.M	standard error of mean
SAPK	stress activated protein kinase
SD rat	Sprague-Dawley rat
sGC	soluble guanylate cyclase
SHR	Spontaneously Hypertensive Rats
SNP	sodium nitroprusside
TP receptor	thromboxane endoperoxide receptor
TxA <sub>2</sub>	thromboxane A <sub>2</sub>

U/ml	Unit/milliliter
VCAM-1	vascular cell adhesion molecule-1
vs.	<i>versus</i>
WKY	Wistar-Kyoto rat
X/XO	xanthine/xanthine oxidase
$\beta$ -NADH	$\beta$ -nicotinamide adenine dinucleotide phosphate

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# CHAPTER I

## INTRODUCTION

### 1.1 Hypertension and endothelial dysfunction

Hypertension is a multifactorial polygenic disease that involves a complex interaction between genetically determined homeostatic control mechanisms and environmental factors (Oparil *et al.*, 2003). It is also a major risk factor for vascular complications including elevated vascular tone and vessel wall remodelling (Oparil *et al.*, 2003; Zhou *et al.*, 2004). Chronic elevation of arterial blood pressure is associated with endothelial dysfunction. The latter follows an imbalance between the releases of endothelium-derived relaxing factors (EDRFs), including nitric oxide (NO) and prostacyclin, and endothelium-derived constricting factors (EDCFs) including vasoconstrictor prostaglandins, endothelin-1 and angiotensin II (Vanhoutte *et al.*, 2009). Overproduction of reactive oxygen species (ROS) essentially scavenges NO leading to reduced vasodilation (Forstermann, 2010), and increased cyclooxygenase products such as vasoconstrictors prostaglandin H<sub>2</sub> further contributes to the development of hypertension (Jameson *et al.*, 1993; Rodrigo *et al.*, 1997; Bauersachs *et al.*, 1998; Taddei *et al.*, 2001). The decrease in endothelium-dependent relaxation and increase in endothelium-dependent contraction results in elevated peripheral vascular resistance as observed in essential hypertension in both human and the Spontaneously Hypertensive Rats (SHRs) (Furchgott & Vanhoutte, 1989; Félétou *et al.*, 2009).

## 1.2 Renin-Angiotensin System and endothelial dysfunction

Renin-angiotensin system (RAS) first described by Trigerstedt and Bergman in the 1898, is involved in pathophysiology of several cardiovascular diseases (Basso & Terragno, 2001). Classically, this system is initiated by the increased production of renin by the kidney in response to lower blood volume in the circulation system. The enzyme, renin released in the kidney converts the precursor angiotensinogen released from the liver into angiotensin I. Angiotensin I is subsequently converted into angiotensin II by angiotensin converting enzyme (ACE). Angiotensin II is further cleaved into angiotensin III and then to angiotensin IV by aminopeptidases A and M, respectively (Schmieder *et al.*, 2007). RAS is one of the major physiological systems contributing to the regulation of arterial blood pressure. It does so primarily because of the vasoactive properties of angiotensin II and the sodium retaining properties of aldosterone. Angiotensin II, the major and most active component in the RAS, stimulates a variety of signalling pathways by binding to two distinct subtypes of specific G-protein coupled angiotensin receptors, which are angiotensin type 1 receptor (AT<sub>1</sub>R) and angiotensin type 2 receptor (AT<sub>2</sub>R) (Touyz & Schiffrin, 2000). AT<sub>1</sub>R coupling to G-protein activates phospholipase C to form second messengers including inositol triphosphate (IP<sub>3</sub>) and diacylglycerol (DAG) which eventually leads to the phosphorylation of the voltage-sensitive calcium channels, whereby calcium influx is enhanced, followed by an increased vascular tone (Touyz & Schiffrin, 2000). AT<sub>1</sub>R activation also stimulates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and enhances the production of ROS which contributes to vasoconstriction, vascular remodelling and vascular inflammation, resulting from the expression of adhesion molecules and the release of cytokines and chemokines (Kim *et al.*, 2008; Kim *et al.*, 2009). On the other hand, AT<sub>2</sub>R activation has the opposite functional effects as it enhances

bradykinin production which in turn stimulates NO release and promotes vascular relaxation.

Angiotensin II has been linked to pathophysiology of many cardiovascular diseases and is closely associated with endothelial dysfunction. The actions of AT<sub>1</sub>R antagonists and ACE inhibitors in correcting the altered structure and endothelial dysfunction of resistance arteries from hypertensive patients and animal models demonstrated the importances of angiotensin II in modulating endothelial function (Schiffrin *et al.*, 2000; Mukai *et al.*, 2002; Yung *et al.*, 2011). Besides that, oral administration of AT<sub>1</sub>R antagonists and ACE inhibitors similarly improved the endothelial function as demonstrated by enhanced acetylcholine-induced endothelium-dependent relaxations, attenuation of upregulated expression of AT<sub>1</sub>R and NAD(P)H oxidase subunits and reduced ROS formation in both diabetic patients and animal models of diabetes (Azizi & Menard, 2004; Wong *et al.*, 2010; Tian *et al.*, 2011; Tiyerili *et al.*, 2013). All these studies showed that angiotensin II/AT<sub>1</sub>R signalling pathways play an important role in modulating endothelial dysfunction.

Angiotensin II/AT<sub>1</sub>R signalling has also been demonstrated to stimulate the production of endothelin-1 (ET-1, a coagulation factor) (Lüscher, 2000). ET-1 itself is a potent vasoconstrictor and pro-inflammatory peptide and has been demonstrated to induce endothelial nitric oxide synthase (eNOS) uncoupling and thus increase ROS formation in endothelial cells and gradually cause endothelial dysfunction (Iglarz & Clozel, 2007). Furthermore, angiotensin II-induced ET-1 expression stimulates extracellular matrix and collagen and thus contributes to the vascular pathophysiology associated with oxidative stress and vascular remodelling (An *et al.*, 2007).

Angiotensin II has also been demonstrated to activate endothelial proline-rich tyrosine kinase 2 (PYK2) leading to phosphorylation of endothelial nitric oxide synthase (eNOS) at

the inhibitory site Tyr657 leading to attenuation of NO production and endothelium-dependent vasodilatation (Loot *et al.*, 2009). In addition, activation of Rho kinase-mediated p38 MAPK by angiotensin II enhances arginase activity in mice aorta resulting in increased ROS production and decreased NO production, which subsequently leads to vascular endothelial dysfunction (Shatanawi *et al.*, 2011). Inhibition of arginase activity and p38 MAPK activation had been reported to lower systolic blood pressure and enhance endothelial-dependent relaxation in aortas and cavernous segments from mice exposed to angiotensin II infusion (Toque *et al.*, 2010; Shatanawi *et al.*, 2011).

### 1.3 Des-aspartate angiotensin I (DAA-I)

Des-Aspartate angiotensin I (DAA-I) is an endogenous nonapeptide formed by an alternative degradation pathway of angiotensin I to form angiotensin III bypassing formation of angiotensin II (Sim, 1993; Sim & Qiu, 1994). This nonapeptide has been demonstrated to exert cardioprotective effects in pathologies involving angiotensin II (Min *et al.*, 2000; Chen & Sim, 2004; Dharmani *et al.*, 2005). Intracerebroventricular administration of DAA-I, attenuated the central pressor action of angiotensin II and III in Wistar-Kyoto (WKY) rats and Spontaneously Hypertensive Rats (SHRs) (Sim & Radhakrishnan, 1994). DAA-I also attenuated the hyperplastic effect of angiotensin II in cultured vascular smooth muscle cells (Min *et al.*, 2000), and reduced the age-related cardiac and vascular hypertrophy in SHR (Kwoon *et al.*, 2004). In the renal and mesenteric vasculatures of hypertensive rats, femtomolar concentrations of DAA-I attenuated the pressor effect of angiotensin II and III, an action mediated by AT<sub>1</sub>R (Mustafa *et al.*, 2004; Dharmani *et al.*, 2008). In addition, DAA-I improved glucose tolerance in high fat high sucrose diet-induced hyperglycaemic mice by altering insulin signalling and decreasing

ROS formation by inhibiting upregulated subunit gp91 of NAD(P)H oxidase (Wong *et al.*, 2011). The nonapeptide also reduced ROS formation and ROS-induced intercellular adhesion molecule-1 (ICAM-1) formation in rhinovirus-infected A549 cells (Ang *et al.*, 2012) and in soleus muscle of mice subjected to eccentric exercise (Sim *et al.*, 2014). The counteractive actions of DAA-I against angiotensin II on endothelial function remain unclear and require further investigations.

#### 1.4 Aims of the study

As reported earlier, angiotensin II plays an important pathological role in endothelial dysfunction associated with hypertension (Loot *et al.*, 2009; Gomolak & Didion, 2014). In contrast, DAA-I, a functional endogenous angiotensin peptide, was recently demonstrated to counteract the effects of angiotensin II in several cardiac related pathologies (Min *et al.*, 2000; Sim *et al.*, 2004; Wong *et al.*, 2011). However, there is little information about the modulation of vascular tone by DAA-I in hypertensive conditions. Using the SHR as an hypertensive animal model, the present study aims to (i) determine the modulatory effect of DAA-I on endothelial function of aortic vessels; (ii) determine the counteract effect of DAA-I on Ang II-induced oxidative stress, and (iii) investigate the underlying cellular mechanisms for the endothelial protective effects of DAA-I.

## CHAPTER II

### LITERATURE REVIEW

#### 2.1 Vascular endothelium

The endothelium is a thin monolayer of cells that lines the interior surface of blood vessels and acts as an endocrine organ that regulates vascular wall function via the release of several vasoactive chemicals (Figure 2.1). The adult vascular system comprises of arteries, arterioles, venules and veins which are rich in receptors that transduce a wide range of intracellular and extracellular signals that are vital for maintaining vascular homeostasis (Lüscher *et al.*, 1990; Félétou, 2011a). Under normal physiological condition, the endothelium releases various vasoactive factors in response to physical stimuli (for example changes in pH and shear stress) or chemical stimuli like circulating hormone, autocooids, cytokines and drugs.

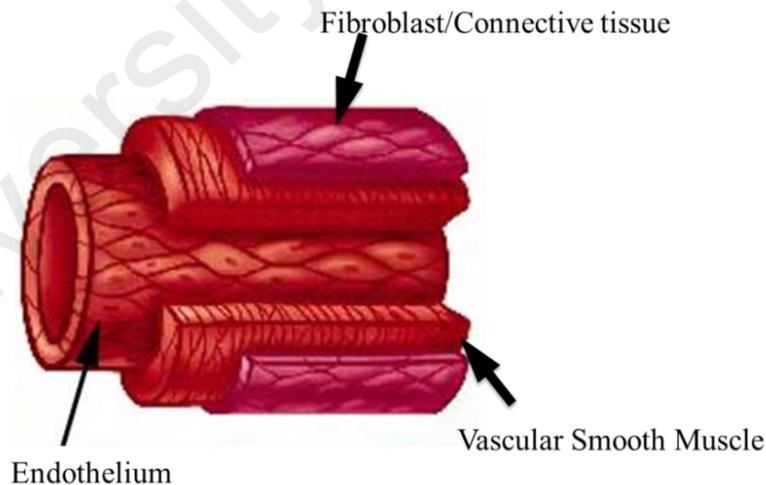


Figure 2.1: A general diagram illustrating the blood vessel structure (Reproduced from Ryan & Painter, 2012)

The endothelium is involved in the modulation of angiogenesis, inflammation and vascular tone and permeability through the release of endothelium-derived vasoactive factors. These mediators include vasodilators such as nitric oxide (NO), prostacyclin (PGI<sub>2</sub>), adenosine, epoxyeicosatrienoic acids (EETs) and C-natriuretic peptide (CNP); vasoconstrictors such as thromboxane A<sub>2</sub> (TxA<sub>2</sub>), isoprostanes, 20-hydroxyeicosatetraenoic acid, superoxide (O<sub>2</sub><sup>-</sup>) anions, endothelin-1, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), angiotensin II and uridine adenosine tetraphosphate (Furchgott & Zawadzki, 1980; Moncada & Higgs, 2006b). The endothelium also involves the myoendothelial gap junctions which allow endothelium-derived hyperpolarization-mediated responses such as the electrotonic tone and the transfer of ion and small molecules like calcium and cyclic nucleotides (Félétou, 2011b; Morgado *et al.*, 2012). Contraction or dilatation of the vessels within the body in response to the stimuli will affect total peripheral resistance and blood pressure.

### 2.1.1 Endothelium-derived relaxing factors (EDRFs)

Endothelial –derived relaxing factor (EDRF) was first identified in isolated rabbit aorta in response to acetylcholine and it was later identified as NO (Furchgott & Zawadzki, 1980; Ignarro *et al.*, 1987; Palmer *et al.*, 1987). The release of EDRFs is triggered in response to physical stimulation like shear stress, neurotransmitters, platelets products, and hormones which readily diffuses to adjacent vascular smooth muscle cells and causes relaxations (Loscalzo, 2001; Pyke & Tschakovsky, 2005). The endothelium produces at least three types of EDRFs which are NO, endothelium-derived hyperpolarization factors (EDHFs) and prostacyclin (Félétou *et al.*, 2011; Katusic *et al.*, 2012). Studies have demonstrated that EDRFs are differently released in various vascular beds. Larger conduit arteries like aorta

rely on NO and a smaller resistance artery like mesentery arteries depends on EDHF for relaxations (Hwa *et al.*, 1994; White *et al.*, 1996).

NO is the primary EDRF released in most vascular beds. The relaxant property of NO was first demonstrated in isolated strips of bovine coronary artery by delivering the mixture NO gaseous in nitrogen or argon into organ bath (Gruetter *et al.*, 1979). Then forward, NO has been associated with the physiological and pharmacological function in various vascular vessels (Moncada & Higgs, 2006a; Vanhoutte, 2009). NO is produced by nitric oxide synthase (NOS) that catalyst the conversion of L- arginine to L- citrulline. There are three isoforms of NOS found in mammalian cells namely neuronal NOS (nNOS / type I NOS), endothelial NOS (eNOS / type III NOS), and the inducible NOS (iNOS / type II NOS)(Balligand & Cannon, 1997). eNOS is mainly expressed in vascular endothelial cells, (Forstermann & Munzel, 2006) cardiac myocytes (Alderton *et al.*, 2001), and other myocardial cells. Up-regulation or activation of eNOS by stimuli stimulates NO production which in turn relaxes vascular smooth muscle via the activation of soluble guanylate cyclase that converts guanosine 5'- triphosphate (GTP) to cyclic guanosine 3',5'- monophosphate (cGMP). Imbalance production of the NO and reactive oxidant species leads to reduction of NO biological activity and this gradually results in endothelial dysfunction.

### 2.1.2 Endothelium-derived contracting factors (EDCFs)

Endothelium-derived contracting factors (EDCF) can increase the vascular tone and contributes to endothelial dysfunction when produced excessively. Among the known EDCFs are cyclooxygenase (COX)-derived prostanoids, ROS, endothelin-1 and angiotensin II (Furchgott & Vanhoutte, 1989; Lüscher *et al.*, 1992). Generation of EDCFs are involved in response to acetylcholine -induced vasoconstriction and leads to activation of cyclooxygenase-1, thromboxane endoperoxide (TP) receptors and ROS (Yang *et al.*, 2002; Vanhoutte *et al.*, 2005; Félétou *et al.*, 2011). Inhibition of acetylcholine- induced contraction by COX inhibitors in hypertensive rats supports the role of COX in EDCFs-mediated responses (Vanhoutte *et al.*, 2005). The two isoforms of COX, COX-1 and COX-2, metabolises arachidonic acid into endoperoxides which is further transformed into several prostanoids including prostacyclin (PGI<sub>2</sub>), thromboxane A<sub>2</sub> (TXA<sub>2</sub>), and other prostaglandins (Garavito & DeWitt, 1999; Tang & Vanhoutte, 2009). ROS is also formed as a by-product of arachidonic acid metabolism (Tang & Vanhoutte, 2009). COX-1 is constitutively expressed in most tissues and is involved in normal homeostasis while expression of COX-2 is inducible especially at the inflammation sites (Garavito & DeWitt, 1999) and also in the vascular endothelium, kidney and brain (Yamagata *et al.*, 1993; McAdam *et al.*, 1999; Therland *et al.*, 2004). COX-1-derived metabolites such as prostacyclin, TXA<sub>2</sub> and other endoperoxides contribute to endothelium-dependent contractions by activating thromboxane –endoperoxide (TP) receptors (Yang *et al.*, 2003; Gluais *et al.*, 2006; Gluais *et al.*, 2007). TP receptors are expressed in vascular endothelial and smooth muscle cells, platelets and circulating monocytes (Raychowdhury *et al.*, 1994; Miggin & Kinsella, 1998) and these receptors are involved in the regulation of vascular tone, platelets aggregation, cell proliferation in various cardiovascular diseases such as

atherogenesis, thrombosis or vascular inflammation (Kenagy *et al.*, 1997; Kobayashi *et al.*, 2004; Dogne *et al.*, 2005). Another EDCF that participate in regulating vascular tone is ET-1. ET-1 is a potent vasoconstrictor that binds to two subtypes of receptors namely ET<sub>A</sub> and ET<sub>B</sub> receptors. ET<sub>A</sub> receptors are present in smooth muscle cells and fibroblasts and triggers potent vasoconstriction, decrease NO bioavailability and inflammation, whereas ET<sub>B</sub> found in endothelial cells causes vasorelaxation via stimulation of NO and prostacyclin release (Bohm & Pernow, 2007; Sanchez *et al.*, 2014). In healthy human, endogenous ET-1 maintains normal physiology including mean arterial blood pressure, heart rate, stroke volume and cardiac output (Haynes & Webb, 1997). The net activity mediated by ET<sub>A</sub> receptor (vasoconstriction) is counterbalanced by ET<sub>B</sub> receptors which mediates NO release but the balance tend to shift towards more constriction, inflammation and oxidative stress under pathophysiological conditions such as endothelial dysfunction (Bohm & Pernow, 2007).

### 2.1.3 Endothelium dysfunction

The loss of normal endothelial function or impaired endothelium is increasingly recognised as a hallmark of several vascular diseases. Endothelial dysfunction is widely reported in patient with coronary artery diseases, diabetes mellitus, hypertension, hypercholesterolemia, as well as in smokers (Hadi *et al.*, 2005). A shift from vasodilatation to vasoconstriction due to imbalance between the release of EDRFs and EDCFs are observed with endothelial dysfunction (Hadi *et al.*, 2005; Vanhoutte *et al.*, 2009). Enhanced EDCFs subsequently augment the production of free radicals or ROS which reacts with NO to form peroxynitrite (ONOO<sup>-</sup>).

NO is synthesized by eNOS from L-arginine and molecular oxygen ( $O_2$ ), in a process involving NADPH, cofactor tetrahydrobiopterin (BH4) and calcium-calmodulin. Increased oxidative stress tends to oxidize the fragile cofactor, BH4 which is necessary for optimal eNOS activity. BH4 facilitates NADPH-derived electron transferring from the eNOS reductase to the oxygenase domain to convert L-arginine to NO and L-citrulline. When BH4 levels are inadequate, eNOS becomes unstable and uncoupled, leading to less NO production and more  $O_2^-$  generation. This phenomenon is referred as “eNOS uncoupling”. Moreover, the interaction between NO and  $O_2^-$  leads to the formation of  $ONOO^-$ , a potent oxidant, which further oxidizes BH4. The excess of ROS in vascular endothelium decrease the NO bioavailability and further aggravate the impairment of endothelial function (Figure 2.2) (Forstermann, 2010)

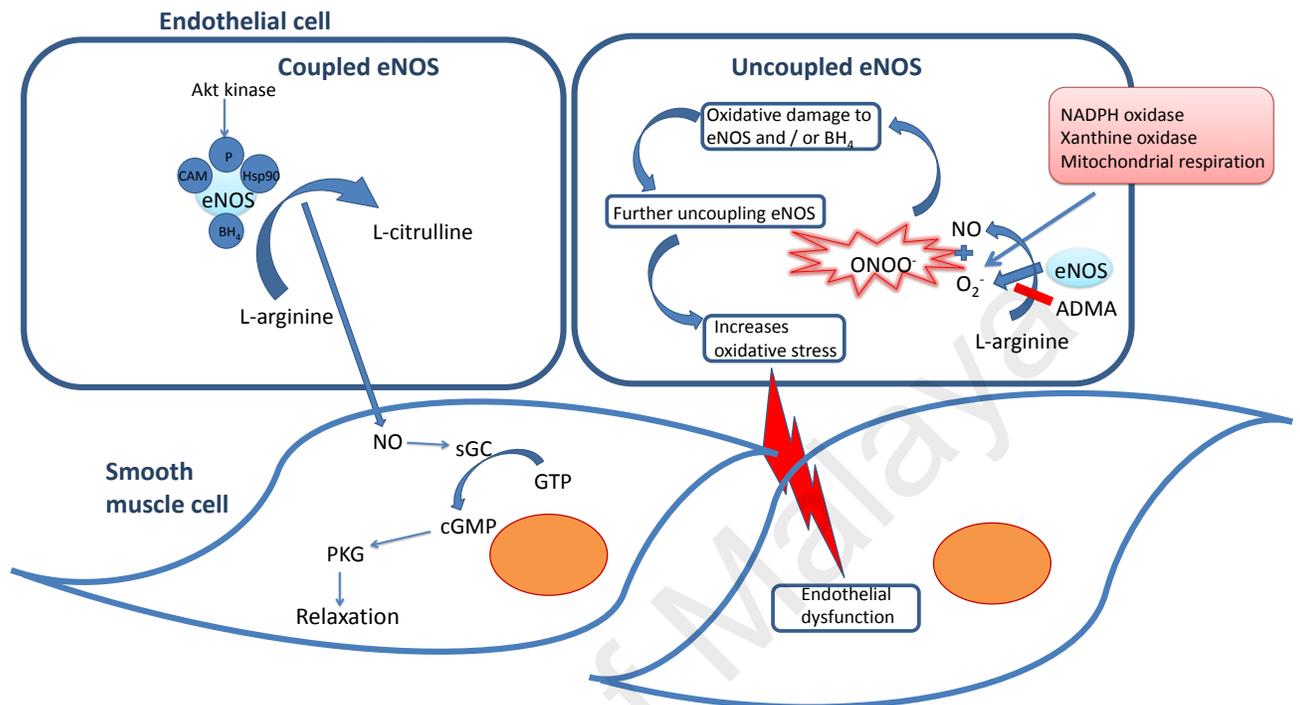


Figure 2.2 Schematic diagram showing general mechanisms in vascular endothelium and smooth muscle cells. The functional eNOS protein is a dimer (so-called coupled eNOS). Tetrahydrobiopterin (BH<sub>4</sub>), cofactor of eNOS facilitates NADPH-derived electron transferring from the eNOS reductase to the oxygenase domain to convert L-arginine to NO and L-citrulline. NO plays a major role in relaxation of smooth muscle through the cGMP-dependent downstream signaling cascade. Uncoupled eNOS is formed due to the deficiency of L-arginine or BH<sub>4</sub> level and leads to the reduction of NO and increases superoxide anion (O<sub>2</sub><sup>-</sup>) generation. Peroxynitrite (ONOO<sup>-</sup>), a potent oxidant is produced after interaction between NO and O<sub>2</sub><sup>-</sup>, also oxidizes BH<sub>4</sub> and resulting in eNOS uncoupling. Continuous impairment of NO activity and increased of oxidative stress attribute to the development of endothelial dysfunction (Reproduced from Kietadison *et al.*, 2012).

#### 2.1.4 Vascular endothelium and ROS

ROS-induced cardiovascular diseases involved activation of redox-signalling pathways related to myocardial growth, hypertrophic remodelling, vasoconstriction, inflammation and apoptosis in cardiovascular system (Dimmeler & Zeiher, 2000; Yang *et al.*, 2002; Miyajima *et al.*, 2007; Takimoto & Kass, 2007; Kvietys & Granger, 2012; Sarmiento *et al.*, 2015). The balance between ROS and antioxidant enzymes or ROS scavengers are maintained by the rate of ROS production and the rate of its scavenging (Droge, 2002). Increased production of vascular ROS leads to the imbalance in normal homeostasis and induces oxidative stress (Droge, 2002; Montezano & Touyz, 2012). Oxidative stress develops a condition where cellular metabolism and regulation are disturbed and cellular constituents are damaged, subsequently causes some pathophysiology diseases such as cardiovascular disease (Lushchak, 2014). The major types of ROS are superoxide ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), hydroxyl radical ( $\bullet OH$ ), and peroxynitrite ( $ONOO^-$ ). Sources of vascular ROS includes mitochondria, NAD(P)H oxidase, xanthine oxidase and prostaglandin H synthase (PHS) (Katsuyama *et al.*, 2002; Clempus & Griendling, 2006; Chandel & Budinger, 2007). Nevertheless, eNOS can become uncoupled and generate large amount of  $O_2^-$  in conditions of oxidative stress. The damage to eNOS and/or decreased bioavailability of BH<sub>4</sub> and also contributes in the generation of  $O_2^-$  (Taniyama & Griendling, 2003).

NAD(P)H oxidase is the major source of ROS formation in the vasculature (Pendyala *et al.*, 2009; Montezano & Touyz, 2012). It is a multiprotein complex formed from the subunits of Nox family which comprises of p47phox, p67phox, p40phox, p22phox, and gp91phox (Montezano & Touyz, 2012). All subunits are expressed in the endothelial cells and are up-regulated by shear stress, vasoactive factors (for example, angiotensin II, aldosterone and

ET-1), and growth factors (for example, epidermal growth factor (EGF) and platelet-derived growth factor (PDGF)). Upregulation of these subunits elevates the generation of ROS in the vasculature and subsequently leads to reduce of NO bioavailability (Jay *et al.*, 2008; Montezano *et al.*, 2010). Increased ROS formation has been observed in hypertensive patients and in animal models (for example angiotensin II- and aldosterone plus salt-induced hypertensive and SHR). The increased ROS leads to several pathological conditions such as apoptosis of endothelial cells, impaired endothelium-dependent vasorelaxation in vascular vessels, angiogenesis and atherosclerotic lesion, as well as vascular cell adhesion molecule-1 (VCAM-1) and intracellular cell adhesion molecule-1 (ICAM-1) formation in blood vessels (Romero & Reckelhoff, 1999; Dimmeler & Zeiher, 2000; Taniyama & Griendling, 2003). All these conditions promote the occurrence of hypertension associated endothelium dysfunction.

## 2.2 Renin Angiotensin System

Renin angiotensin system (RAS) is an important hormonal system that controls the electrolyte balance (water and sodium retention), renal and cardiovascular system (for example, aldosterone release and blood pressure regulation). Figure 2.3 shows the schematic illustration of RAS pathway in which the activation starts with the formation of angiotensin I (Ang I) from angiotensinogen by renin (Brunton *et al.*, 2011). Further on, Ang I is cleaved by angiotensin converting enzyme (ACE) and produces the active peptide, angiotensin II (Ang II), which modulates many physiological functions through its receptors, AT<sub>1</sub>R and AT<sub>2</sub>R (Kaschina & Unger, 2003). Alternatively, angiotensin I is degraded by aminopeptidase (AP) to form des-aspartate angiotensin I (Ang I [des-Asp<sup>1</sup>] or DAA-I). Angiotensin III (Ang III) is degraded by ACE and AP from DAA-I and Ang II,

respectively. Other important peptides formed are angiotensin (1-7) (Ang (1-7)) and angiotensin IV (Ang IV). Ang (1-7) is formed from Ang II by the action of angiotensin converting enzyme 2 (ACE2) or from another fragment, angiotensin (1-9) (Ang (1-9)). Ang (1-9) is formed from Ang I by ACE2 and further cleaved by ACE to form Ang (1-7). Ang IV is formed from the cleavage of Ang III by AP.

There are two types of RAS: plasma-localized and tissue-localized. Plasma-localized RAS regulates acute cardiovascular function whereas tissue-localized RAS regulates long-term changes (Kramkowski *et al.*, 2006). The activity of RAS depends on the type of angiotensin receptors it activates and the relative levels expressed on tissues. For example, AT<sub>1</sub>R and AT<sub>2</sub>R are differently distributed in kidney, where AT<sub>1</sub>R is found abundantly in vasculature throughout the kidney whereas AT<sub>2</sub>R is found in different segments of cortical and medullary vasculature such as the arcuate artery and afferent arterioles (Miyata *et al.*, 1999). Ang II mediates most of its effects via AT<sub>1</sub>R, however, binding of Ang II to AT<sub>2</sub>R evoke opposite effects which counteracts the effects of Ang II/AT<sub>1</sub>R interaction (Schmieder *et al.*, 2007).

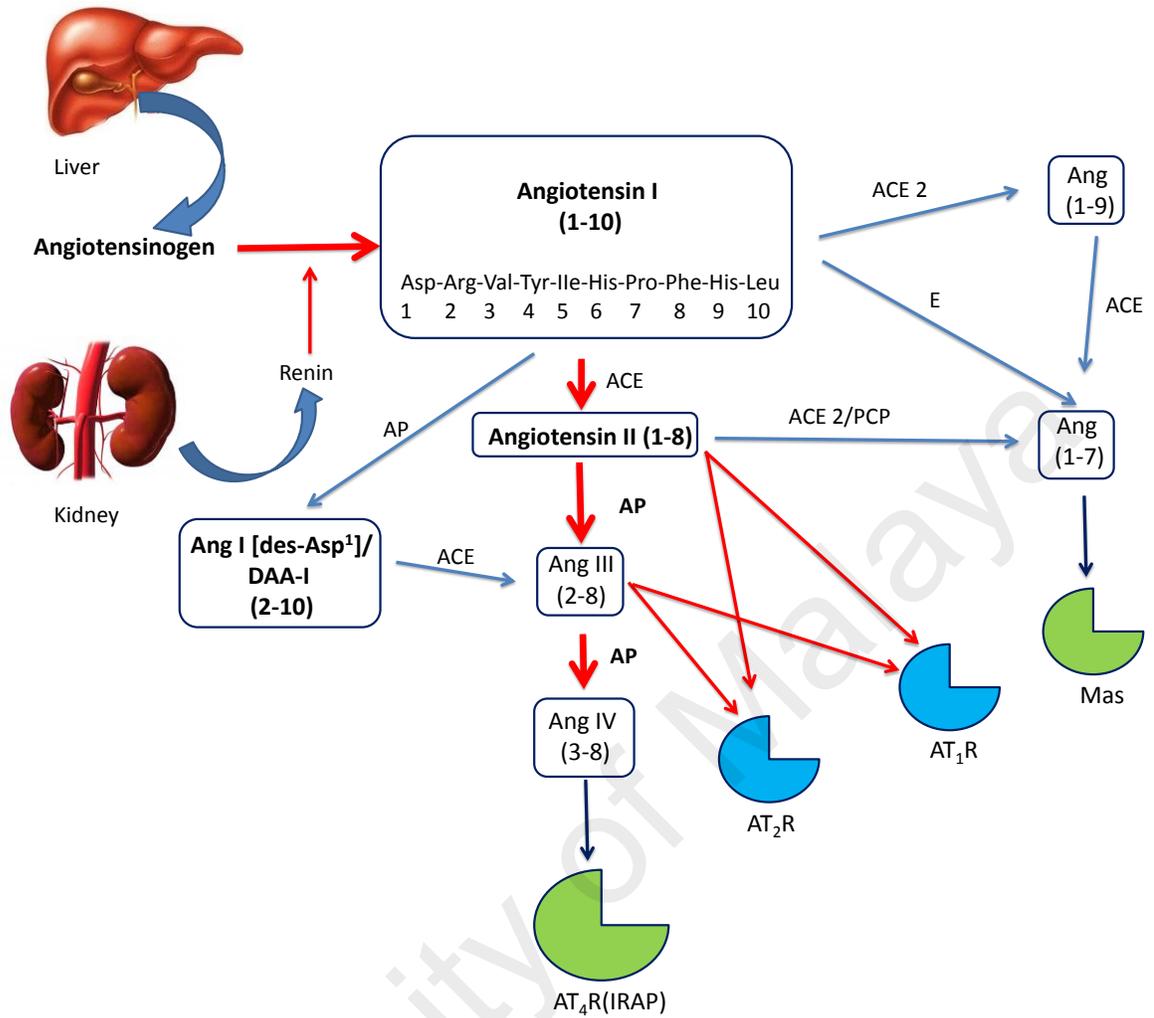


Figure 2.3 Schematic illustration of the components of the Renin-Angiotensin System (RAS). The heavy red arrows show the classical pathway, and the light blue arrows indicate alternative pathways. ACE, angiotensin –converting enzyme; Ang, angiotensin; AP, aminopeptidase; E, endopeptidase; IRAP, insulin –regulated amino peptidases; PCP, prolylcarboxylpeptidase. Receptors involved: AT<sub>1</sub>R, AT<sub>2</sub>R, Mas, and AT<sub>4</sub>R (Modified from Brunton *et al.*, 2011).

### 2.2.1 Angiotensin II

Angiotensin II (Ang II) is an octapeptide consists of eight amino acids and is the most active peptide of RAS. Ang II is produced from angiotensin I by ACE and ACE-independent enzymes (Figure 2.3). ACE cleaves the dipeptide (His-Leu) at the C-terminus of angiotensin I to form angiotensin II and also degrades the vasodilatory factor bradykinin (Murphey *et al.*, 2003; DeMello & Frohlich, 2009). *In vitro* experiments have shown that besides ACE, in the heart and during pathological conditions, at least 80% of angiotensin II is generated by the enzyme chymase (Urata *et al.*, 1990; Kinoshita *et al.*, 1991; Dell'Italia & Husain, 2002; Li *et al.*, 2004). Cathepsin G is another ACE-independent enzyme that forms Ang II directly from angiotensinogen (Owen & Campbell, 1998; Rykl *et al.*, 2006).

Ang II mediates a variety of actions including vasoconstriction, increases blood pressure, vascular remodelling and sodium retention (Figure 2.4) (de Gasparo *et al.*, 2000; Touyz & Schiffrin, 2000; Volpe *et al.*, 2002). The actions of Ang II are mediated by two major receptors subtypes, angiotensin II type 1 receptor (AT<sub>1</sub>R) and angiotensin II type 2 receptor (AT<sub>2</sub>R). Both AT<sub>1</sub>R and AT<sub>2</sub>R subtypes are seven transmembrane G protein coupled receptors that share 32–34% sequence homology (Nakajima *et al.*, 1995; Nouet & Nahmias, 2000; Porrello *et al.*, 2009) and differ by their biochemical and pharmacological properties, and the signalling pathways utilized. AT<sub>1</sub>R mediates most of the well-known pathophysiological effects of Ang II. In contrast, activation of AT<sub>2</sub>R causes vasodilatation by generating nitric oxide with subsequent stimulation of cGMP (Siragy & Carey, 1997; Arima & Ito, 2000), mediates differentiation in neural cells, inhibits proliferation, and even induces apoptosis in certain cells (Stoll *et al.*, 1995; Yamada *et al.*, 1996; Zimpelmann & Burns, 2001).

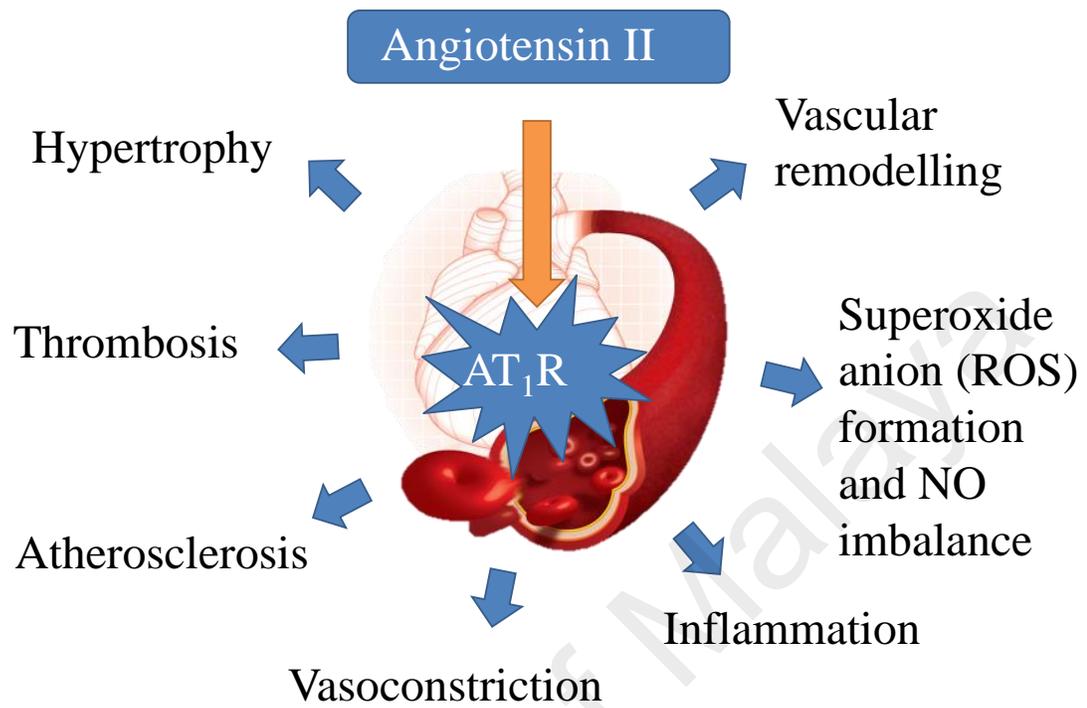


Figure 2.4 Diagram showing the major actions of angiotensin II and mediated by stimulation of AT<sub>1</sub>R (Modified from Metha & Griendling, 2007).

### 2.2.2 Angiotensin Receptors: Angiotensin type 1 receptor (AT<sub>1</sub>R)

AT<sub>1</sub>R mediates most of the known physiological actions of angiotensin II in cardiovascular, renal, neuronal, endocrine, hepatic and other target cells. These actions in turn affect the regulation of arterial blood pressure, electrolyte and water balance, thirst, hormone secretion and renal function (de Gasparo *et al.*, 2000). AT<sub>1</sub>Rs are found in the kidney, heart, vascular smooth muscle cells, endothelium, brain, adrenal glands, adipocytes and placenta (Timmermans *et al.*, 1993). Human AT<sub>1</sub>R gene is located at chromosome 3 and is only present in one isoform (Curnow *et al.*, 1992); while rat AT<sub>1</sub>R exist in two distinct subtype which are defined as AT<sub>1A</sub> and AT<sub>1B</sub> receptors and located at chromosome 17 and 2, respectively, with more than 95% homological amino acid sequence (Iwai *et al.*, 1991; Kakar *et al.*, 1992; Sandberg *et al.*, 1992). Binding of Ang II to AT<sub>1</sub>R activates G-protein to mediate several signal transduction pathways which occur within seconds and may last for many hours or days. Ang II induced phosphorylation of phospholipase C (PLC) and Src activation, oxidative stress induction (Ushio-Fukai *et al.*, 1996), activation of phospholipase A<sub>2</sub> (PLA<sub>2</sub>), tyrosine kinase and mitogen-activated protein kinases (MAPKs) occurs within minutes. Events that occur within hours or late signalling processes include proto-oncogene expression and protein synthesis (Touyz & Schiffrin, 2000). Angiotensin II-induced vasoconstriction occurs rapidly involving G-protein mediated activation of PLC and leads to formation of inositol triphosphate (IP<sub>3</sub>) and diacylglycerol (DAG) via hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>). DAG then activates protein kinase C (PKC) and the subsequent vasoconstriction (Peach & Dostal, 1990). Ang II may also activate phosphoinositide 3-kinase (PI3K)/ protein kinase B (Akt) pathway through AT<sub>1</sub>R and activates L-type calcium (Ca<sup>2+</sup>) channel to induce vasoconstriction (Seki *et al.*, 1999; Touyz & Schiffrin, 2000; Guo *et al.*, 2001). In addition, binding of angiotensin II

with AT<sub>1</sub>R also activates NAD(P)H oxidase via tyrosine kinases to increase formation of ROS which plays a role in vasoconstriction, nuclear signalling and thus induces vascular remodelling (Sohn *et al.*, 2000; Touyz & Schiffrin, 2000) (Figure 2.5).

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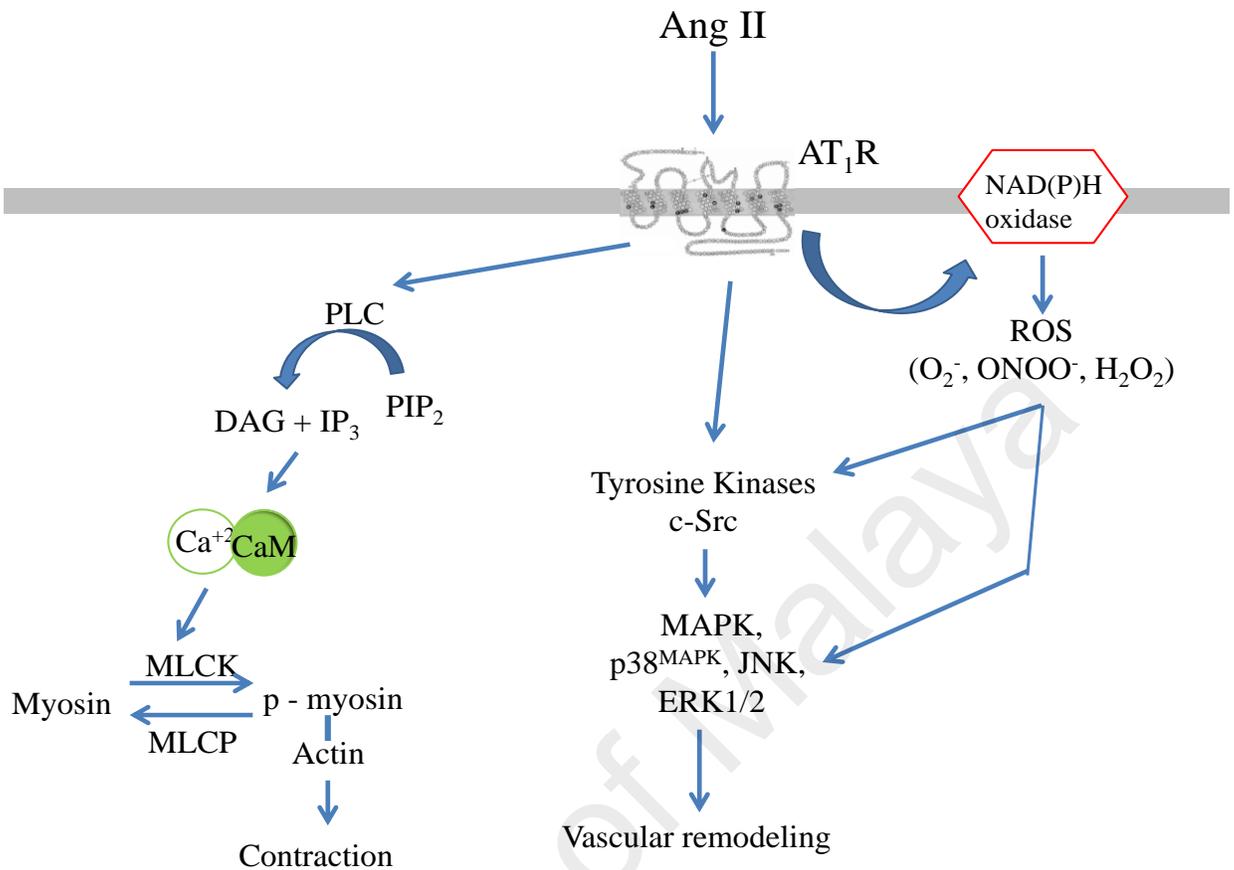


Figure 2.5 Signalling mechanisms of AT<sub>1</sub>R with Ang II in vasoconstriction and vascular remodeling. Ang II: angiotensin II; AT<sub>1</sub>R: angiotensin receptor type I; PLC: phospholipase C; DAG: diacylglycerol; IP<sub>3</sub>: inositol trisphosphate; PIP<sub>2</sub>: phosphatidylinositol 4,5-bisphosphate; CaM: calmodulin; MLCK: myosin light chain kinases; MLCP: myosin light chain phosphatases; ERK1/2: extracellular-regulated kinase 1 and 2; MAPK: mitogen-activated protein kinase; JNK: c-Jun NH<sub>2</sub>-terminal protein kinases; O<sub>2</sub><sup>-</sup>: superoxide anion, ONOO<sup>-</sup>: peroxynitrite; H<sub>2</sub>O<sub>2</sub>: hydrogen peroxide (Reproduced from Metha & Griendling, 2007).

### 2.2.3 Angiotensin Receptors: Angiotensin type 2 receptor (AT<sub>2</sub>R)

Activation of AT<sub>2</sub>R exerts opposing effects to AT<sub>1</sub>R activation (Steckelings *et al.*, 2005b; Jones *et al.*, 2008). The opposing effects includes vasodilatation, anti-proliferation, anti-hypertrophic and induced apoptosis (Allen *et al.*, 2000; Gallinat *et al.*, 2000; Siragy *et al.*, 2000; Touyz & Schiffrin, 2000). AT<sub>2</sub>R gene is mapped to only chromosome X (Koike *et al.*, 1994; Hein *et al.*, 1995). AT<sub>2</sub>R is highly expressed in the foetus tissues and reduced after birth (de Gasparo *et al.*, 2000; Gao *et al.*, 2012; Steckelings *et al.*, 2012). In adults, AT<sub>2</sub>R is found in the pancreas, heart, kidney, brain, myometrium, ovaries and vasculature (de Gasparo *et al.*, 2000; Kaschina & Unger, 2003; Steckelings *et al.*, 2012). An AT<sub>2</sub>R expression is shown to be up-regulated under pathophysiological conditions such as ischemia and injury (Gallinat *et al.*, 1998; Nouet & Nahmias, 2000; Steckelings *et al.*, 2005a). Activation of AT<sub>2</sub>R is also coupled to G proteins, but employs different signal transduction pathways such as activation of tyrosine and serine/threonine phosphatase (Carey *et al.*, 2000). There are three main signalling pathways activated by AT<sub>2</sub>R: (1) activation of protein phosphatases including mitogen-activated protein (MAP), kinase phosphatases (MKP-1), protein phosphatase 2 (PP2A) and SH2 domain-containing tyrosine phosphatases (SHP-1) which results in the inhibition of MAP kinases and NAD(P)H oxidase, which prevents cell proliferation and apoptosis, and oxidative stress, respectively (Huang *et al.*, 1995; Bedecs *et al.*, 1997; Mehta & Griendling, 2007; Faria-Costa *et al.*, 2014; Lu *et al.*, 2015); (2) stimulation of bradykinin and subsequent increase in synthesis of nitric oxide and cyclic guanosine monophosphate (NO/cGMP) level which leads to vasodilatation (Lemarie & Schiffrin, 2010) and (3) stimulation of phospholipase A2 (PLA<sub>2</sub>) with subsequent production of arachidonic acid which regulates potassium currents and cell hyperpolarization (Zhu *et al.*, 1998; Jöhren *et al.*, 2004) (Figure 2.6). Furthermore, studies

had shown that AT<sub>2</sub>R stimulation by exogenous angiotensin II increased cGMP levels through a mechanism involving bradykinin receptor (B<sub>2</sub>R) and NO release (Lemarie & Schiffrin, 2010; Faria-Costa *et al.*, 2014).

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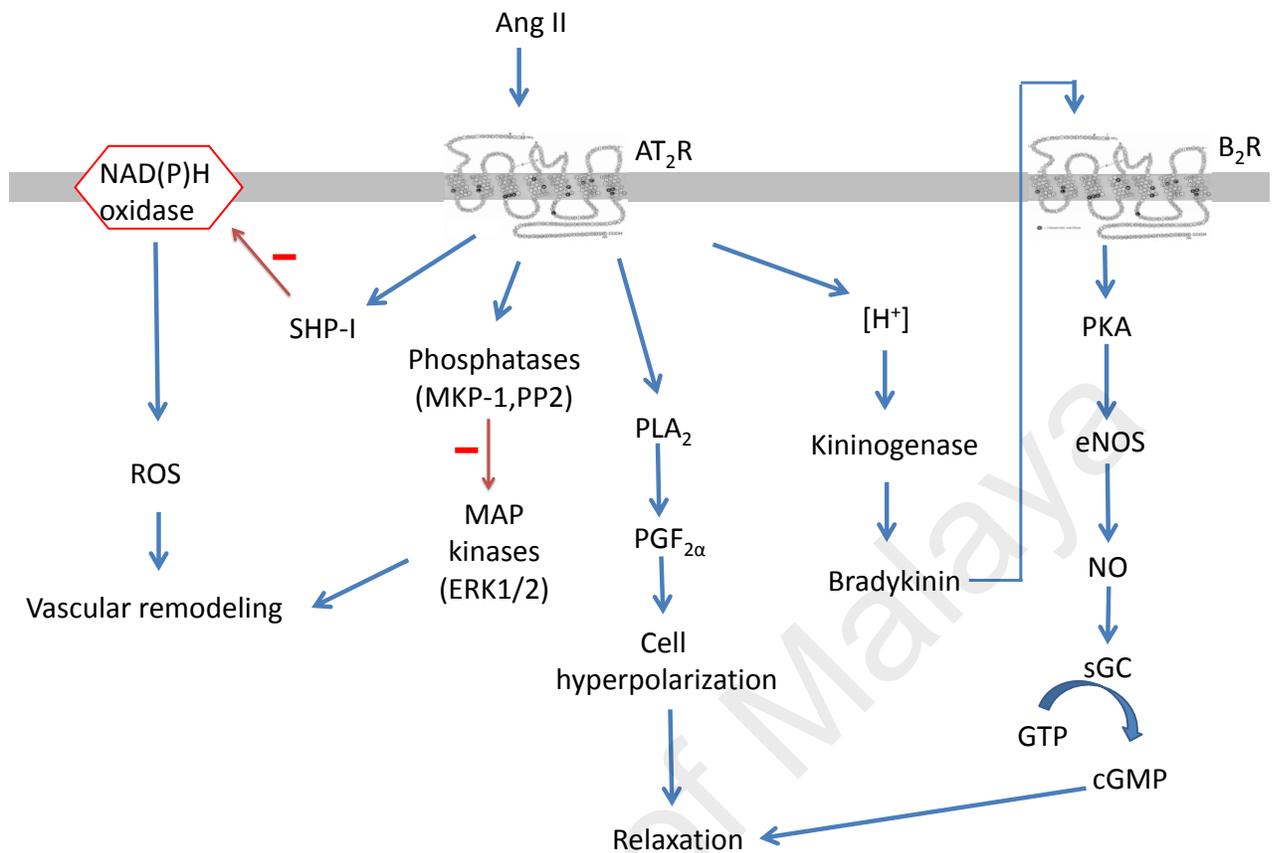


Figure 2.6 Signalling mechanisms of AT<sub>2</sub>R interaction with Ang II. Ang II: angiotensin II; AT<sub>2</sub>R: angiotensin receptor type 2; B<sub>2</sub>R: bradykinin receptor B2; cGMP: cyclic guanosine monophosphate; eNOS: endothelial nitric oxide synthase; ERK1/2: extracellular-regulated kinase 1 and 2; GTP: guanosine triphosphate; [H<sup>+</sup>]: hydrogen ion concentration; MKP-1: MAP kinase phosphatase; NO: nitric oxide; PKA: protein kinase A; PLA<sub>2</sub>: phospholipase A<sub>2</sub>; PP2: protein phosphatase 2; PGF<sub>2α</sub>: prostaglandin F<sub>2α</sub>; sGC: soluble guanylate cyclase. (-) indicates inhibition of the pathway. (Reproduced from Metha & Griendling, 2007; Faria-Costa *et al.*, 2014).

## 2.2.4 Other angiotensin peptides

### 2.2.4.1. Angiotensin III

Angiotensin III (Ang III) is metabolized from Ang II by aminopeptidase A (APA), and degraded to become Ang IV by aminopeptidase N (APN). In rabbit aorta, Ang III mediates contractile effects via AT<sub>1</sub>R similar to Ang II but the effects are less potent than Ang II (Li *et al.*, 1995). However, Ang III is equipotent to Ang II on blood pressure, renal function and aldosterone production through AT<sub>1</sub>R in conscious dog (Gammelgaard *et al.*, 2006). In another study the effect of Ang III on aldosterone release in rat is partially or independent on AT<sub>2</sub>R, but not via AT<sub>1</sub>R (Yatabe *et al.*, 2011). Furthermore, Ang III is shown to induce rat astrocyte growth through the activation of mitogen-activated protein (MAP) kinases ERK1/2 and stress activated protein kinase/Jun-terminal kinases (SAPK/JNK) phosphorylation involving AT<sub>1</sub>R (Clark *et al.*, 2013).

### 2.2.4.2. Angiotensin IV

Angiotensin IV is a hexapeptide formed from angiotensin III by aminopeptidase N, and it binds to the AT<sub>4</sub> receptor which is also known as insulin-regulated aminopeptidase (IRAP) enzyme (Albiston *et al.*, 2001). AT<sub>4</sub> receptor is not coupled with G-protein (Miller-Wing *et al.*, 1993; Bernier *et al.*, 1995) and is distributed in brain, coronary and aortic endothelial cells (Swanson *et al.*, 1992; Miller-Wing *et al.*, 1993; Kerins *et al.*, 1995). Binding of angiotensin IV to AT<sub>4</sub> receptor has been demonstrated to increase intracellular calcium release and eNOS activity and to induce vasodilatation in porcine pulmonary artery (Patel *et al.*, 1998; Chen *et al.*, 2000), Sprague-Dawley rat carotid artery (Kramar *et al.*, 1997) and mice aorta (Vinh *et al.*, 2008).

#### 2.2.4.3. Angiotensin 1-7

Angiotensin (1-7) (Ang (1-7)), a heptapeptide derived from angiotensin I and II by prolyl-endopeptidases (PEP)/ neutral endopeptidases (NEP) or ACE2, respectively is another angiotensin peptide that has been gaining importance. Many studies had showed that Ang (1-7) counteracts the effects of angiotensin II and inhibits the action of ACE (Roks *et al.*, 1999; Ueda *et al.*, 2000; Zhang *et al.*, 2010; Tassone *et al.*, 2013). Ang (1-7) induce vasodilatation via NO-dependent and –independent pathway (Sasaki *et al.*, 2001; Ueda *et al.*, 2001). Moreover, Ang (1-7) has anti-proliferative actions and exert modulatory effects on the sympathetic nervous system. The actions of this heptapeptide is modulated through Ang (1-7) (AT<sub>1-7</sub>) receptors or also known as *Mas* receptors (Freeman *et al.*, 1996; Schindler *et al.*, 2007). In contrast, Ang (1-7) has been demonstrated to exerts vasodepressor effect on mean arterial pressure (MAP) in SHR and WKY via AT<sub>2</sub>R as the effect was blocked by AT<sub>2</sub>R antagonist, PD 123319 but not by Ang (1-7) receptor antagonist, A-779 (Walters *et al.*, 2005). This study also showed that bradykinin and NO are involved in the vasodilation effects of as these effects by Ang (1-7) are attenuated by HOE 140, a bradykinin antagonist and the eNOS inhibitor, L-NAME (Walters *et al.*, 2005).

#### 2.2.4.4. Des-Aspartate angiotensin I

Des –aspartate Angiotensin I (DAA-I) is a nine amino acid peptide with the sequence Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu. It is formed from angiotensin I by the actions of aminopeptidase X with the removal of an amino acid at the N-terminus. DAA-I is then cleaved by ACE to form angiotensin III bypassing the formation of Ang II (Sim & Qiu, 1994; Brunton *et al.*, 2011). In humans, DAA-I remarkably caused increased blood pressure and plasma aldosterone production but decreased plasma renin activity (PRA). These pressor and steroidogenic actions of DAA-I were dependent on its conversion to Ang III as these effects were abolished by oral administration of captopril, an ACE inhibitor (Kono *et al.*, 1980). In the hypothalamic homogenates of rats, Sim and Qiu (1994) had demonstrated that exogenous Ang I was degraded into mainly DAA-I and its rate of formation was higher in SHR and deoxycorticosterone acetate/salt-induced hypertensive SD (DOCA-salt) rats compared to their normotensive animals (WKY and SD rat, respectively). DAA-I exerts specific action in which it is effective at concentrations below the enzymatic activity ( $K_m$ ) of ACE and other peptidases. For example, administration of DAA-I intravenously at a dose of 152 picomolar (pM) or equivalent to a concentration of 8.4 nanomolar (nM) in total blood volume attenuated cardiac hypertrophy in rats and this concentration is 1000 folds lower than that of most enzymes which normally is in the micromolar ( $\mu M$ ) (Sim & Min, 1998). Furthermore, DAA-I attenuated neointima growth and hypertrophy in rats at a lower dose of 12 pM in a 350g rats (Sim *et al.*, 2004). In another study, DAA-I showed biphasic effects on myocardial ischemia-reperfusion injury in rats (Wen & Sim, 2011). DAA-I significantly reduced the infarct injury at doses of 1524 nmol/kg/day for 14 days, however, doses higher than 1524 nmol/kg/day were less effectiveness. These finding suggests that actions of DAA-I is highly specific in the concentration used and down-

regulation of receptors involved may occur in higher doses of DAA-I. Besides that, DAA-I also has been demonstrated to prevent cardiac hypertrophy caused by coarctation of the abdominal aorta in Sprague-Dawley (SD) rats (Sim & Min, 1998).

DAA-I has been demonstrated to oppose the actions of Ang II. This nonapeptide reduced Ang II-induced incorporation of phenylalanine and thymidine, which are the markers for hypertrophy and hyperplasia, respectively in cardiomyocytes and its actions were mediated by indomethacin-sensitive AT<sub>1</sub>R (Min *et al.*, 2000). DAA-I attenuated pressor action of Ang II in renal and mesenteric vasculature in both WKY and SHR (Dharmani *et al.*, 2005) and this nonapeptide has been shown to reduce AT<sub>1</sub>R density in kidneys of WKY and SHR (Dharmani *et al.*, 2008). In type 2 diabetic Goto-Kakizaki (GK) rats and KKAY mice, oral administration of 600 nmol/kg DAA-I exerted hypoglycaemia actions by enhancing the insulin-related substrate-1 (IRS-1) and glucose transporter-4 (GLUT-4) translocation through AT<sub>1</sub>R and the release of prostaglandins (Sim *et al.*, 2007). Angiotensin II attenuates insulin-induced translocation of GLUT4 and further impairs tyrosine phosphorylation of insulin/IRS-1 pathway and activation of Akt by PI3K (Iwai *et al.*, 2006; Wei *et al.*, 2006; Mehta & Griendling, 2007). Studies show that the cardioprotective and hypoglycaemia effects of DAA-I was blocked by losartan and/or indomethacin and involved releases of prostaglandins (Sim & Chai, 1996; Ng *et al.*, 2011; Wen & Sim, 2011; Wong *et al.*, 2011) suggests that DAA-I acts on indomethacin-sensitive AT<sub>1</sub>R but preferentially activates the production of prostaglandins (Sim, 2015)

### 2.2.5 Renin angiotensin system and ROS

Oxidative stress plays a major role in the initiation and progression of endothelial dysfunction associated with hypertension. Overactivation of RAS leads to hypertension and also causes oxidative stress, particularly Ang II-induced ROS formation (Tsilimingas *et al.*, 2004; Kopkan & Cervenka, 2009). Ang II induces ROS generation by activating NAD(P)H oxidase through AT<sub>1</sub>R in vascular cells (Dikalov *et al.*, 2008; Pernomian *et al.*, 2012). In the endothelium, ROS acts as a signalling molecule in modulating vascular tone (causes vasoconstriction), vascular remodelling and thus contribute to endothelial dysfunction or injury. Furthermore, ROS stimulated by Ang II/NAD(P)H oxidase also triggers vascular inflammation which can be prevented by pretreatment with an antioxidant and ACE inhibitors (Cominacini *et al.*, 2002; Soehnlein *et al.*, 2005). Tempol, a superoxide dismutase mimetic reduces Ang II-induced contraction in isolated arteries from hypertensive patient further supports the involvement of ROS in Ang II-induced vasoconstriction (Puntmann *et al.*, 2005). Thus activation of the RAS and increased production of Ang II leads to elevation of ROS formation and endothelial dysfunction.

### 2.2.6 Renin Angiotensin System and hypertension

RAS plays pivotal role in regulating blood pressure and fluid balance in the body (Brunton *et al.*, 2011). When blood volume or blood pressure is low, cells in the kidney release renin which converts angiotensinogen from liver into Ang I. Then ACE in the lungs degrades Ang I to form Ang II. Ang II stimulates blood vessels to constrict and blood pressure to increase. Hypertension may results due to persistent overactivation of RAS leading to high level of Ang II in the circulation. Aliskiren, an inhibitor of renin, which blocks conversion

of angiotensinogen to Ang I significantly reduced high blood pressure, and reversed endothelial dysfunction in SHR rats (Dharmashankar & Widlansky, 2010). Besides that, treatment with ACE inhibitors and AT<sub>1</sub>R blockers significantly improved hyperpolarization and relaxation to acetylcholine via EDHF in mesenteric arteries from SHR in addition to lowering blood pressure (Goto *et al.*, 2000). Taken together, inhibition of RAS has been shown to prevent the abnormality of increased blood pressure and improve endothelial function.

### 2.3 Hypertension

Hypertension is characterised by elevation of systolic blood pressure with multifactorial inheritance, because both genetic and environment trigger its onset. Two types of hypertension have been categorized: i) primary or essential hypertension and ii) secondary hypertension. Essential hypertension can occur at any age and genetic factors play a role in this group of hypertension. Diet, sedentary lifestyle, obesity and stress increase the risk of getting essential hypertension (Messerli *et al.*, 2007). On the other hand, secondary hypertension is caused by other conditions or diseases such as kidney failure, adrenal gland disorder, sleep apnea and the others. Most of the hypertensive patients (95%) have essential hypertension and only 5% of the hypertensive patient accounts for the secondary hypertension (Mohan *et al.*, 2013).

### 2.3.1 Hypertension and endothelial dysfunction

Endothelium regulates vascular tone, cellular adhesion, inflammation and cell proliferation together with smooth muscle cells in response to stimuli such as physical and chemical signals (Deanfield *et al.*, 2007). Endothelial dysfunction is defined as impairment of endothelium integrity or vasomotor response, cell proliferation, platelet activation, vascular permeability and become pro-thrombotic, pro-inflammatory and pro-constrictive (Dharmashankar & Widlansky, 2010; Savoia *et al.*, 2011). Abnormalities of endothelial NO synthase has been demonstrated in blood vessels from hypertensive patients and increased blood pressure in youth is predicted to develop endothelial dysfunction in the future (Quyyumi & Patel, 2010). Hypertension related vascular phenotypes are also associated to the increased of ROS production and/or decreased in cellular antioxidant defence mechanism which leads to endothelial cell damage (Harvey *et al.*, 2015; Montezano *et al.*, 2015). In hypertension, higher ROS level induces increased production of prostanoids which lead to increased vasoconstriction and reduced endothelium-dependent relaxation (Montezano *et al.*, 2015).

## 2.4 Rationale of the study

Antihypertensive treatments with ACE inhibitors or angiotensin receptor blocker (ARBs) lowers blood pressure and reverses the endothelial dysfunctions associated with the actions of angiotensin II in human and animal models (Zhu *et al.*, 2007; Yung *et al.*, 2011). In addition, DAA-I had been shown to antagonise the effects of angiotensin II and angiotensin II-related pathophysiologic conditions such as cardiac hypertrophy and ischemic heart conditions. As this peptide has been shown to be protective at very low concentration, it may become a useful therapy for hypertension, whereby a low dosage can be utilized with minimal adverse effect. However, the modulation and the mechanism of actions of DAA-I in protecting endothelial functions in hypertension is yet to be fully elucidated. Thus, the objectives in the study are 1) to determine the modulatory effect of DAA-I on vascular responses to endothelial –dependent vasoconstrictor (angiotensin II) and to endothelial –dependent [acetylcholine (ACh)] and –independent [sodium nitroprusside (SNP)] relaxation in the thoracic aorta from normotensive (WKY) and hypertensive (SHR) rats, 2) to determine the effect of DAA-I on angiotensin II-induced oxidative stress in these vascular tissues from hypertensive rats; and 3) to investigate the molecular mechanisms underlying the protective effects of DAA-I.

## CHAPTER III

### MATERIALS & METHODOLOGY

#### 3.1 Animals

The animals used in this study were male Wistar-Kyoto rats (WKY) and Spontaneously Hypertensive rats (SHR) age between 18-20 weeks old. The animals were kept under controlled light (12h: 12h light-dark cycle) and temperature ( $23 \pm 1^\circ\text{C}$ ) conditions. The animals were fed with standard rat chow (Specialty Feeds Pty. Ltd., Glen Forrest, Australia) and had free access to tap water. Approval for the following studies was obtained from the Animal Care and Ethics Committee in the University of Malaya under the reference animal ethic number FAR/27/01/2010/0112/LYS(R). All experimental procedures were carried out according to the guidelines for the ethical care of experimental animal.

#### 3.2 Drugs and chemicals

Acetylcholine chloride (ACh), captopril, *N*<sub>ω</sub>-Nitro-L-arginine methyl ester hydrochloride (L-NAME), phenylephrine (Phe), indomethacin, sodium nitroprusside (SNP), angiotensin II (Ang II), diethyldithiocarbamate (DETCA), diphenyliodonium (DPI),  $\beta$ -nicotinamide adenine dinucleotide phosphate ( $\beta$ -NADH), allopurinol, xanthine, xanthine oxidase, lucigenin were purchased from Sigma-Aldrich Chemicals (St Louis, MO, USA). PD 123319 was purchased from Tocris Bioscience (Ellisville, UK). Losartan potassium was from Fluka Analytical (St Louis, MO, USA). Tempol, 1*H*-[1,2,4]-oxadiazolo [3,4-*a*]quinoxalin-1-one (ODQ) and apocynin were purchased from EMD Chemicals

(Calbiochem<sup>®</sup>, San Diego, CA, USA). Des-aspartate angiotensin I (DAA-I) was purchased from GL Bachem (Shanghai, China). Chemicals used for Krebs control solution preparation were purchased from BDH (VWR International, Pennsylvania, USA). Angiotensin II and DAA-I were prepared and diluted to the desired concentration in ice-cold distilled water on the day of experiment. Indomethacin was dissolved in 0.5% sodium carbonate ( $\text{Na}_2\text{CO}_3$ ). ODQ was dissolved in dimethyl sulfoxide (DMSO). Xanthine and allopurinol were dissolved in 0.01M sodium hydroxide (NaOH). All other drugs were freshly prepared in distilled water. The concentrations in the organ chamber solution are expressed in molar.

### 3.3 Preparation of Krebs physiological salt solution (control solution)

The control solution was prepared by dissolving the following salts composition (mM): NaCl, 118.9;  $\text{NaHCO}_3$ , 25.0;  $\text{MgSO}_4$ , 1.2; KCl, 4.7;  $\text{KH}_2\text{PO}_4$ , 1.0; Glucose, 11.1 and  $\text{CaCl}_2$ , 2.4 in one litre of distilled water. The solution was prepared before use and kept warmed at 37° C and oxygenated with mixture of 95% oxygen and 5% carbon dioxide. The pH of the solution was adjusted to pH 7.4.

### 3.4 Preparation of isolated thoracic aortic rings

The animals were sacrificed with carbon dioxide and the thoracic aorta was rapidly excised and placed into a beaker containing oxygenated control solution. The aorta was cleaned of perivascular tissues and care was taken not to dislodge the vascular endothelium. The aorta was sectioned into small rings (3 to 4 mm long). In some experiments, the endothelium was removed by gentle rubbing the intimal surface with the tip of a blunt forceps for determining the role of endothelium in responses.

### 3.5 Measurement of isometric tension in the thoracic aorta rings

In order to study the responses of the thoracic aortic rings to agonists, the rings were suspended between the L-shaped stainless steel holder and the tissue hook in the organ bath filled with 5 ml of control solution. The L-shaped stainless steel holder was fixed in position while the tissue hook was connected to a force displacement transducer (Grass Instrument Co., Quincy, MA, USA). The transducer outputs were amplified and recorded continuously using a PowerLab recording system (AD Instrument, Sydney, NSW, Australia) connected to a portable computer display monitor.

The organ bath was constantly aerated with mixture of 95% oxygen and 5% carbon dioxide, and maintained at 37<sup>0</sup> C. Change of Krebs solution was done by removing the solution and immediately refilling the organ bath with fresh control solution. Agonist – evoked relaxant and contractile responses in the aortic rings were monitored on the computer as decrease and increase of the isometric tension (gram tension, gT), respectively (Figure 3.1).

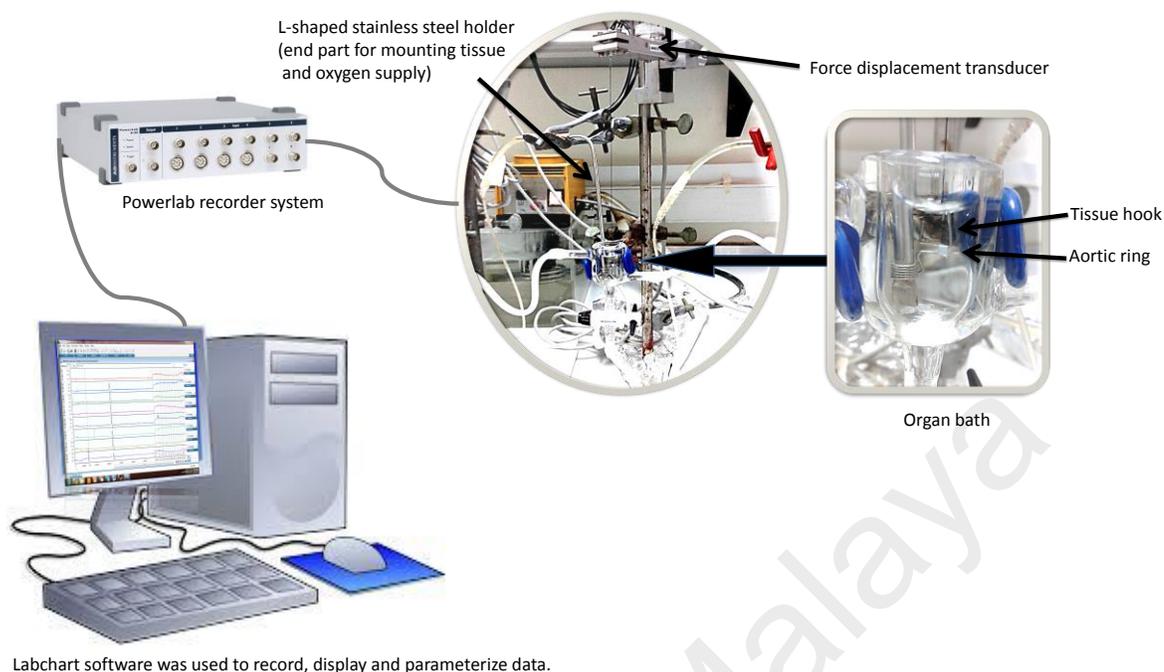


Figure 3.1 Diagram showing isometric tension experiment setup for functional test.

### 3.6 Experimental protocols for the functional tests

The isolated aortic rings were then mounted in the organ bath containing 5 ml of control solution as described in Section 3.3 and slowly stretched to 1 gT and left to be equilibrated for 45 minutes or until stabilized. During the equilibrium, the control solution was replaced every 15 minutes. After the equilibrium, the aortic rings were primed with an isotonic potassium solution (high  $K^+$ , 80 mM) to confirm the viability of the vascular tissue. Priming the tissues was done three times or until a consistent reference contraction was obtained, each time for a period of 4 minutes and after which a mean reading was recorded.

The presence or absence of functional endothelium was verified prior to the actual experiment by determining whether or not relaxation occurred upon exposure to acetylcholine ( $10^{-5}$  M), in phenylephrine ( $10^{-7}$  M) –contracted preparations. Rings with

more than 70% relaxation in response to acetylcholine were considered to have sufficient functional endothelium and while those which relaxed less than 5% were accepted as rings without endothelium.

Each aortic ring was exposed to only one of the pre-treatment test and each experiment was repeated at least 6 times in 6 individual tissue preparations from the animals. In all preparations, the rings were exposed to captopril ( $3 \times 10^{-5}$  M) for 20 minutes prior to the incubations of other treatments to inhibit the action of ACE in converting DAA-I into angiotensin III (Campbell *et al.*, 1977).

### 3.6.1 Modulation of DAA-I on Ang II-induced contraction

#### 3.6.1.1. Effect of DAA-I on Ang II-induced contraction in the isolated aortic rings from WKY and SHR

The range of concentrations of DAA-I that did not induce contraction in the rat aorta was chosen to study its modulation on Ang II contractility (Sim & Soh, 1995). The rings were exposed to the captopril prior to the incubation with DAA-I and followed by cumulative concentrations of Ang II ( $10^{-10}$  –  $10^{-5}$  M). An interval of 1 minute 30 seconds between successive concentrations was recorded. The effect of DAA-I on rings without endothelium also was tested in the responses to Ang II.

### 3.6.1.2 Modulatory effects of DAA-I on Ang II-induced contraction in aorta from SHR

The effects of various pharmacological inhibitors on the contractile responses induced by Ang II in the rings pre-treated with DAA-I ( $10^{-7}$  M) was determined only in the aortic ring from SHR. The chosen concentration of DAA-I ( $10^{-7}$  M) was determined based on its most effective concentration in the result on Ang II-induced contraction. Following incubation of the aortic rings with DAA-I, concentration-response curves to Ang II were constructed in the presence of the following pharmacological inhibitors: losartan ( $AT_1$  receptor antagonist,  $10^{-5}$  M), PD 123319 ( $AT_2$  receptor antagonist,  $10^{-5}$  M), L-NAME (eNOS inhibitor,  $10^{-4}$  M), indomethacin (non-selective COX inhibitor,  $10^{-5}$  M), ODQ (soluble guanylate cyclase (sGC) inhibitor,  $3 \times 10^{-6}$  M), tempol (a cell permeable superoxide dismutase mimetic,  $10^{-4}$  M) and apocynin (an inhibitor of NAD(P)H oxidase,  $10^{-4}$  M). Each inhibitor was added to the organ chamber at least 10 minutes before the incubation with DAA-I or captopril prior to concentration-response curve to Ang II.

### 3.6.2. Effect of DAA-I on nitric oxide level in the aortic rings from WKY and SHR

Nitric oxide (NO) breaks down rapidly into nitrate ( $NO_3^-$ ) and nitrite ( $NO_2^-$ ) (Kelm, 1999). In order to measure NO products (total nitrate and nitrite,  $NO_x$ ), isolated aortas were pre-incubated with captopril in the presence or absence of DAA-I ( $10^{-7}$  M) for 30 minutes, in control solution with continuous oxygen supply at  $37^\circ\text{C}$ . The isolated aortic rings were also incubated with or without inhibitors (losartan,  $10^{-5}$  M and tempol,  $10^{-4}$  M). Ang II ( $10^{-7}$  M) was added to all groups to mimic the experimental conditions of the organ chamber studies. The tissues were then homogenized in PBS (pH 7.4) using a glass tissue grinder (Wheaton<sup>TM</sup> Tenbroeck, New Jersey, USA) and centrifuged at  $10,000 \times g$  for 20 minutes.

The supernatants were aliquoted and used for NO<sub>x</sub> measurement. NO<sub>x</sub> were determined with a Nitrate/Nitrite Colorimetric Assay Kit from Cayman Chemical (Ann Arbor, MI, USA), following the manufacturer's instructions. The absorbance of the sample was read using Hidex microplate reader (CHAMELEON™ V; Hidex, Turku, Finland) at 540 nm. The measurement was based on the conversion of nitrate to nitrite, followed by the colorimetric determination of the total concentration of nitrite as a colored azo dye product of the Griess reaction that absorbs visible light at 540 nm. The amount of NO products is expressed in micromoles per milligram protein. Protein content of each sample was measured by the DC (Detergent Compatible) protein assay reagent kit (Bio-Rad Laboratories, Hercules, CA, USA).

### 3.6.3. Effect of DAA-I on Ang II-induced oxidative stress

#### 3.6.3.1 Effect of DAA-I on vascular superoxide anion production in isolated aortic rings from WKY and SHR in the presence or absence of Ang II.

Vascular superoxide anion production was detected using lucigenin-enhanced chemiluminescence method by modified from previous studies (Lau *et al.*, 2012; Lau *et al.*, 2013). Briefly, isolated aortic rings from WKY and SHR were pre-treated with 20 minutes of captopril and followed by DAA-I ( $10^{-7}$  M), losartan ( $10^{-5}$  M) and tempol ( $10^{-4}$  M) for 30 minutes before the addition of Ang II to mimic the experimental conditions of the organ chamber studies. All rings were incubated with the treatments in control solution with continuously oxygenated at 37°C. The rings were then incubated in a 24-wells plate (incubation plate) for 45 minutes at 37°C in 2 ml of Krebs-HEPES buffer (in mM: NaCl 99.0, NaHCO<sub>3</sub> 25, KCl 4.7, KH<sub>2</sub>PO<sub>4</sub> 1.0, MgSO<sub>4</sub>.7H<sub>2</sub>O 1.2, glucose 11.0, CaCl<sub>2</sub>.2H<sub>2</sub>O 2.5 and Na-HEPES 20.0) in the presence of diethylthiocarbamic acid

(DETCA, inactivation of superoxide dismutase,  $10^{-3}$  M) and  $\beta$ -nicotinamide adenine dinucleotide phosphate (NADPH, substrate for NADPH oxidase,  $10^{-4}$  M). Diphenyleneiodonium (DPI; NADPH oxidase inhibitor,  $5 \times 10^{-3}$  M) was added for the positive control. Another 24-wells plate (wash plate) was prepared in the same condition with incubation plate without DETCA. At the end of incubation, the aortic rings were transferred from incubation plate to wash plate and the plate was shaken gently to wash out the DETCA. Further, the rings were transferred into a 96-well Optiplate filled with 300 ml of Krebs-HEPES buffer containing lucigenin (a chemiluminescent superoxide probe,  $5 \times 10^{-3}$  M) and NADPH. The luminescence signal was measured using Hidex microplate reader CHAMELEON™ V; Hidex, Turku, Finland) in luminescent detection mode over 20 min. At the end of measurement, the rings were dried for 48 hours at  $65^{\circ}\text{C}$  and weighed. The data were expressed as average counts per mg of vessel dry weight.

#### 3.6.3.2 *In situ* detection of vascular superoxide anion production in cryostat section of rat aortas from WKY and SHR in the presence of Ang II

To measure *in situ* vascular superoxide anion formation in rat aortas, DHE fluorescence staining was performed as described (Lau *et al.*, 2013). Aortic rings from SHR were incubated with captopril for 20 minutes and followed by DAA-I, losartan and tempol for 30 minutes in control solution with continuously oxygenated at  $37^{\circ}\text{C}$ . Ang II ( $10^{-7}$  M) added to all groups of aortic rings to mimic the experimental conditions in the organ chambers. At the end of incubation, the aortic rings from all groups were transferred into a round mold made from aluminum foil contained OCT (optimal cutting temperature) compound (Sakura Finetek, Torrance CA, USA) and were frozen in liquid nitrogen. Cross sections of frozen aortic segments were obtained in  $10 \mu\text{m}$  thickness using a cryostat. The cross section was

placed on a slide and incubated in dark for 15 minutes in normal physiological saline solution [NPSS (mM): NaCl 140, KCl 5, CaCl<sub>2</sub> 1, MgCl<sub>2</sub> 1, glucose 10 and HEPES 5] containing 5 X 10<sup>-6</sup> M DHE. The fluorescence intensity was then measured with an Olympus FV1000 laser scanning confocal system (Olympus America, Inc., Melville, NY, USA) at excitation/emission wavelengths of 515/580 nm to visualize the signal. The images were analyzed using the Olympus Fluoview version 4 software.

#### 3.6.4. Effect of DAA-I on xanthine/xanthine oxidase (X/XO)-induced contraction

Reactive oxygen species (ROS) or oxygen-derived free radical are produced at low concentration and regulates vascular relaxation-contraction in normal condition (Touyz & Schiffrin, 1999). In pathophysiology condition, increased level of ROS leading to oxidative stress will causes negative effects such as vascular damage and increased vascular contractility (Virdis *et al.*, 2011). As xanthine/xanthine oxidase is also a source of ROS generation, besides investigating DAA-I effect on Ang II-induced oxidative stress, the effect of DAA-I on xanthine/xanthine oxidase-induced was evaluated.

The rings were exposed to oxygen-derived free radical from xanthine/xanthine oxidase (X/XO) in both WKY and SHR preparations as described (Yang *et al.*, 2002). Briefly, four concentrations of xanthine oxidase from 0.001 to 0.03 U/ml were used to generate superoxide anion in the presence of xanthine (10<sup>-4</sup> M). Xanthine was added 10 minutes before xanthine oxidase-induced contraction and the experiment was performed in the presence or absence of DAA-I, allopurinol (oxygen-derived free radical scavenger, 10<sup>-4</sup> M) and indomethacin (10<sup>-5</sup> M). DAA-I, allopurinol and indomethacin were incubated for 30 minutes after 20 minutes incubation of captopril as described above (section 3.6.1.1.).

3.6.5. Modulatory effect of DAA-I on the endothelium-dependent and –independent relaxation

3.6.5.1 Effect of DAA-I on ACh-induced endothelium–dependent relaxation in isolated aortic rings from WKY and SHR

In order to study the effect of DAA-I ( $10^{-15}$  to  $10^{-7}$  M) on the ACh-induced endothelium–dependent relaxation, the rings were exposed to a sustained contraction with phenylephrine ( $10^{-6}$  M) and exposure to increasing concentrations of ACh ( $10^{-10}$  –  $10^{-5}$  M) after incubated with captopril and DAA-I as described in section 3.6.1.1. Each concentration of DAA-I was tested on different individual aortic ring from both preparation.

3.6.5.2 Effect of DAA-I on SNP-induced endothelium–independent relaxation in isolated aortic rings from WKY and SHR

In order to study the effect of DAA-I ( $10^{-15}$  to  $10^{-7}$  M) on the SNP-induced endothelium – independent relaxation, the rings were exposed to the phenylephrine ( $10^{-6}$  M) to induce a sustained contraction and followed by addition of increasing concentrations of SNP ( $10^{-11}$  –  $10^{-6}$  M) following incubation of captopril and DAA-I as described above (section 3.6.1.1).

### 3.6.5.3 Mechanisms study on modulation effect of DAA-I on ACh-induced relaxation in aorta from SHR

The effects of various pharmacological inhibitors on the responses to ACh in the rings pre-treated with DAA-I ( $10^{-7}$  M) was determined in the aorta from SHR. The chosen concentration of DAA-I ( $10^{-7}$  M) was determined based on its most effective concentration in the result on ACh -induced relaxation. Following incubation of the aortic rings with DAA-I, concentration -response curves to ACh were generated in the presence of the following pharmacological inhibitors: losartan (an  $AT_1$  receptor antagonist,  $10^{-5}$  M), PD 123319, (an  $AT_2$  receptor antagonist,  $10^{-5}$  M), L-NAME (nitric oxide synthase inhibitor,  $10^{-4}$  M) and indomethacin (non-specific cyclooxygenase inhibitor,  $10^{-5}$  M), Each inhibitor was added to the organ chamber at least ten minutes before the incubation with DAA-I or captopril prior to obtain concentration -response curve to ACh.

### 3.7 Data evaluation and statistics

The concentrations given in the text or in the figures are the final ones achieved in the organ chamber solution. The response to ACh and SNP were calculated as percentage inhibition of phenylephrine ( $10^{-6}$  M)-induced contractions, while the responses to Ang II was calculated as percentage of the maximum reference contraction to KCl. Areas under the individual concentration –response curves (AUC) in arbitrary unit (a.u.) were calculated by nonlinear regression using Graphpad software. Data are reported as means  $\pm$  standard error of mean (S.E.M) and ‘n’ indicates number of animals used in each set of data. The statistical significance among the different experimental groups was analyzed using either Student’s t-test for differences between two groups and one -way ANOVA followed by Dunnett’s multiple comparison tests for comparison with more than two groups (Prism 5.0, Graphpad software, USA). P value less than 0.05 was considered to indicate statistically significant differences.

## CHAPTER IV

### RESULTS

#### 4.1 Role of DAA-I on Ang II-induced contraction in isolated aortic rings from WKY and SHR

##### 4.1.1 Effect of DAA-I on Ang II-induced contraction

Figure 4.1 shows that angiotensin II induced comparable contraction in aortic rings from both WKY and SHR. To examine whether DAA-I had effect on angiotensin II-induced contraction, different concentrations of DAA-I ( $10^{-15}$  M,  $10^{-9}$  M, and  $10^{-7}$  M) were pre-incubated prior to cumulative concentration-dependent curve to Ang II. In WKY aortic rings, DAA-I had no effect on Ang II-induced contraction compared to the control (Figure 4.2A), while it reduced Ang II contractility in SHR preparations (Figure 4.2B). The inhibition of Ang II induced contraction by the three DAA-I concentrations ( $10^{-15}$  M,  $10^{-9}$  M and  $10^{-7}$  M) were not concentration-dependent (at  $10^{-5}$  M of Ang II-induced contraction:  $39.73 \pm 6.42\%$ ,  $50.27 \pm 3.83\%$  and  $27.42 \pm 5.68\%$ , respectively) compared to control ( $82.28 \pm 3.66\%$ ).

#### 4.1.2 The role of endothelium in the action of DAA-I on Ang II-induced contraction

To test the role of endothelium in the action of DAA-I, the effect of DAA-I on Ang II-induced contraction was performed in the SHR aortic rings with removal the endothelium. Figure 4.3 shows the effect of DAA-I on Ang II-induced contraction in the SHR aortic rings with or without endothelium. There was no significant difference in the effect of DAA-I on Ang II contraction on rings from the control group with and without endothelium ( $82.28 \pm 3.66\%$  vs.  $80.91 \pm 6.99\%$ , respectively). DAA-I attenuated Ang II-induced contraction in the SHR aortic rings with endothelium but its effect was reverted in the rings with removal of endothelium ( $96.34 \pm 11.25\%$ ).

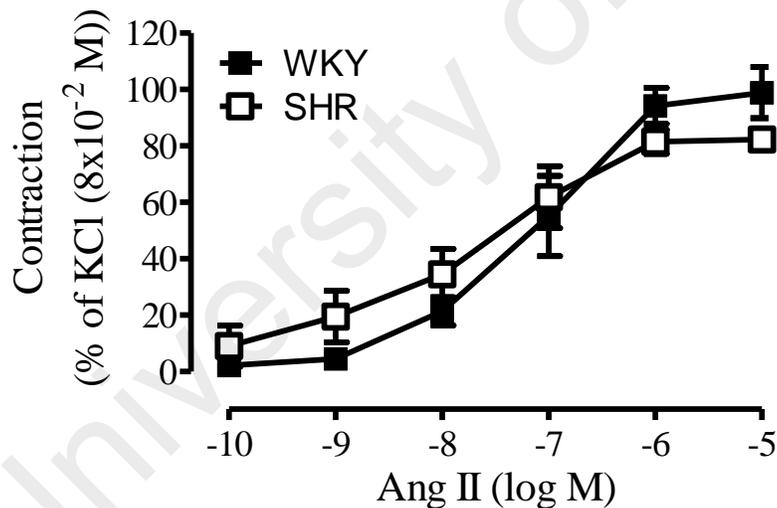


Figure 4.1. Concentration-response curves to Ang II in the aortic rings from WKY and SHR. Ang II-induced contraction was calculated as percentage of reference contraction to KCl ( $8 \times 10^{-2}$  M) obtained at the beginning of the experiment. Each data point represents mean  $\pm$  SEM of 6-7 rats.

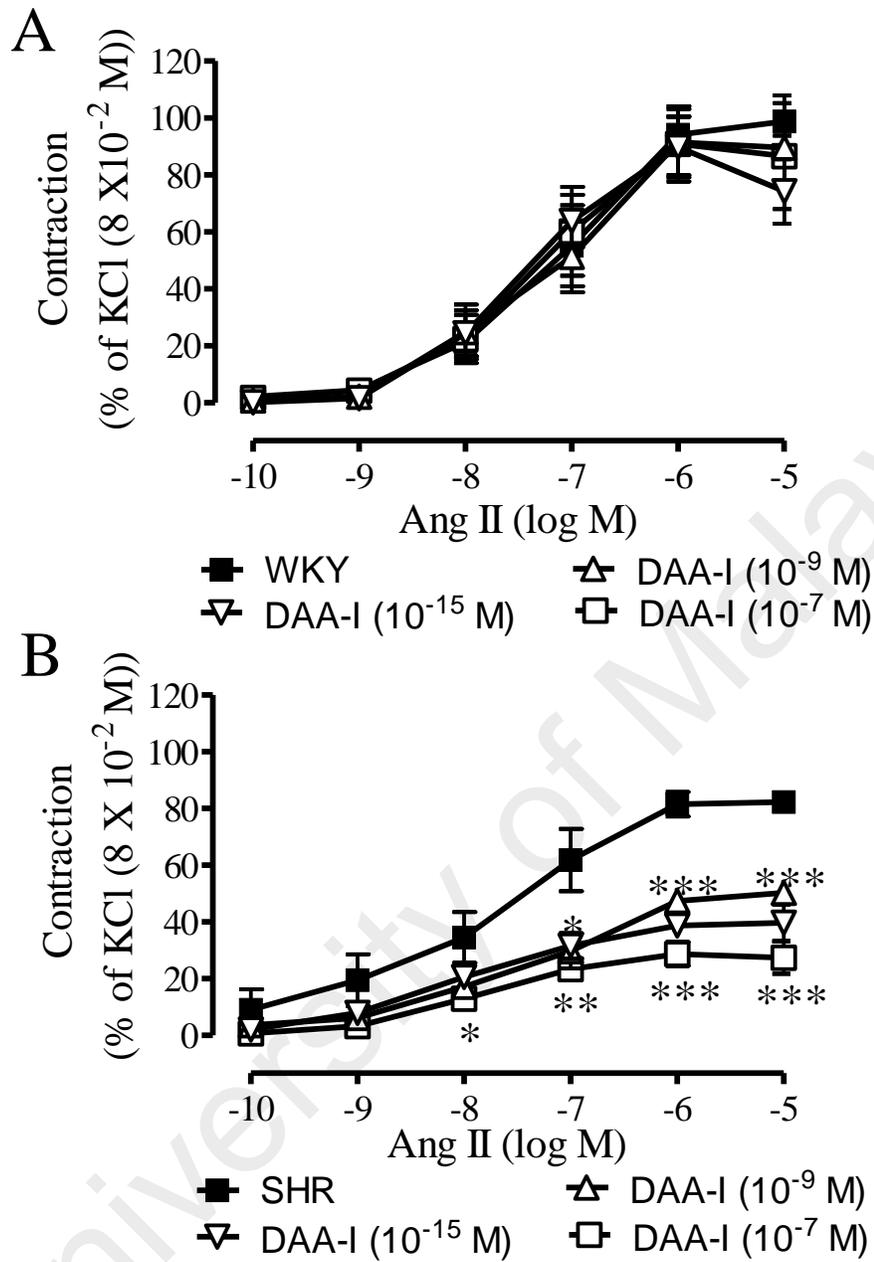


Figure 4.2. Concentration-response curves to Ang II in the aortic rings from (A) WKY and (B) SHR in the presence of different concentrations of DAA-I. Ang II-induced contraction was calculated as percentage of reference contraction to KCl ( $8 \times 10^{-2}$  M) obtained at the beginning of the experiment. Each data point represents mean  $\pm$  SEM of 6-7 rats. Significant difference from control is indicated by \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$

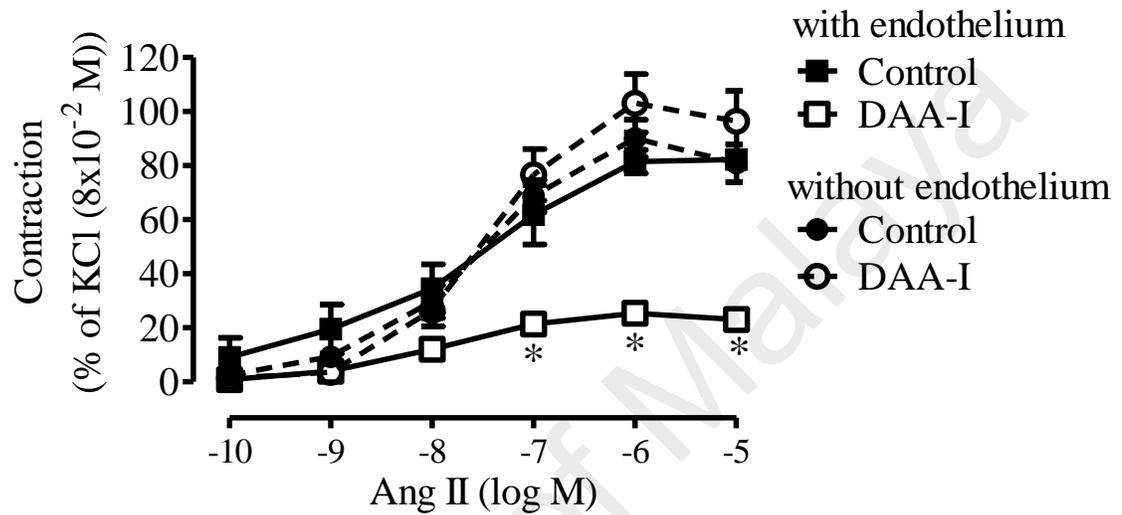


Figure 4.3. Concentration-response curves to Ang II in the aortic rings with and without endothelium from SHR in the absence or presence of DAA-I ( $10^{-7}$  M). Ang II-induced contraction was calculated as percentage of reference contraction to KCl ( $8 \times 10^{-2}$  M) obtained at the beginning of the experiment. Each data point represents mean  $\pm$  SEM of 6-7 rats. Significant difference from control with endothelium is indicated by  $*P < 0.05$

#### 4.1.3 Mechanisms underlying the reduction of Ang II-induced contraction

Ang II-induced contraction was attenuated by DAA-I in the aortic rings from SHR but not from WKY preparation. Thus, the following mechanism studies were carried out only in SHR preparation. In order to investigate the involvement of Ang II receptors in the action of DAA-I on Ang II-induced contraction, experiments were performed in the presence of DAA-I and angiotensin II type 1 (AT<sub>1</sub>R) and type II receptor (AT<sub>2</sub>R) antagonists.

Figure 4.4A shows the effect of DAA-I in the SHR aorta, in the absence or in the presence of losartan. Losartan, an AT<sub>1</sub>R antagonist ( $10^{-5}$  M), almost abolished the Ang II-induced contraction in SHR aorta compared to control ( $3.08 \pm 1.92\%$  vs.  $81.86 \pm 7.11\%$ , respectively). In the presence of losartan, the effect of DAA-I on Ang II-induced contraction was further inhibited compared to DAA-I alone ( $12.98 \pm 4.46\%$  vs.  $27.42 \pm 5.68\%$ , respectively). The data also showed that there is no differences between the groups treated with losartan and losartan plus DAA-I.

The data also shows that  $10^{-5}$  M of PD123319, an inhibitor of AT<sub>2</sub>R significantly reduced Ang II-induced contraction (Figure 4.4B). In the presence of PD 123319, the reduction effect of DAA-I on Ang II-induced contraction was reverted only at  $10^{-7}$  M and  $10^{-6}$  M.

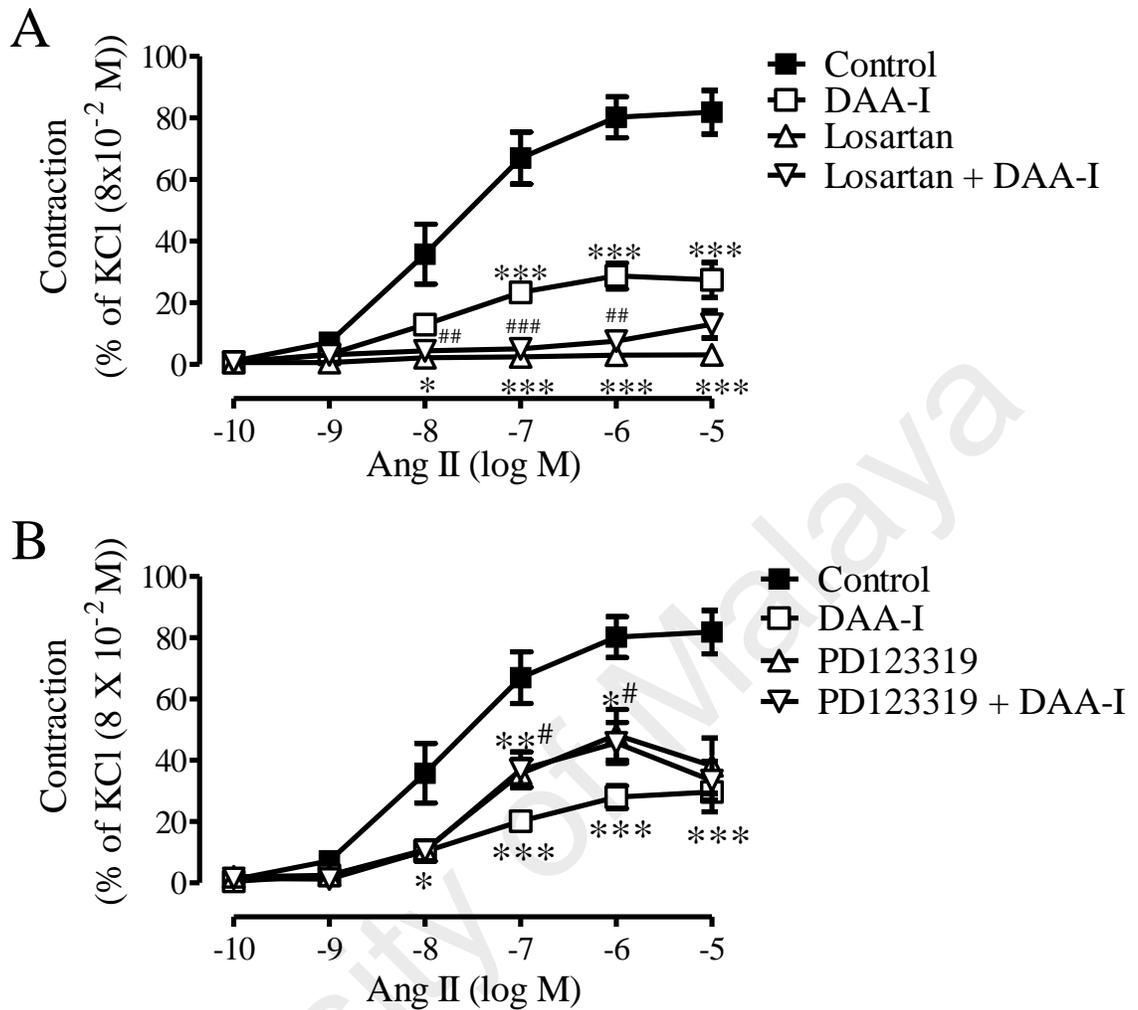


Figure 4.4. Concentration-response curves to Ang II in the aortic rings from SHR, pre-treatment with DAA-I ( $10^{-7}$  M) in the presence of (A) losartan ( $10^{-5}$  M) and (B) PD 123319 ( $10^{-5}$  M). Ang II-induced contraction was calculated as percentage of reference contraction to KCl ( $8 \times 10^{-2}$  M) obtained at the beginning of the experiment. Each data point represents mean  $\pm$  SEM of 6-7 rats. Significant difference from control is indicated by \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ . # $P < 0.05$ , ## $P < 0.01$  and ### $P < 0.001$  compared to the DAA-I ( $10^{-7}$  M) group

In order to determine whether reduction effect of DAA-I on Ang II-induced contraction was affected by inhibition of eNOS, DAA-I was pre-treated in the aortic rings from SHR in the absence or presence of L-NAME ( $10^{-4}$  M). Figure 4.5A shows that DAA-I reduced Ang contractility compared to the control group. eNOS inhibition by L-NAME significantly enhanced Ang II-induced contraction compared to control group and reversed the inhibitory effect of DAA-I on Ang II-induced contraction to the similar level as control group but lower than L-NAME alone (Figure 4.5A and 4.5B).

Furthermore, to test either the downstream of NO/sGC/cGMP pathway involvement in action of DAA-I, soluble guanylate cyclase (sGC) inhibitor, ODQ ( $3 \times 10^{-6}$  M) was pre-incubated in the absence or presence of DAA-I prior to Ang II-induced contraction. Figure 4.6 shows that ODQ had no effect on Ang II-induced contraction compared to control group but it significantly blocked the reduction effect of DAA-I.

To determine the involvement of COX pathway in the action of DAA-I on Ang II-induced contraction, indomethacin, a non-selective COX inhibitor was pre-treatment on the aortic rings in the absence or presence of DAA-I. Figure 4.7 shows that indomethacin itself significantly reduced the contractility to Ang II compared to control ( $15.43 \pm 2.65\%$  vs.  $83.12 \pm 6.50\%$ , respectively). In contrast, indomethacin had no effect on the inhibitory effect of DAA-I on Ang II-induced contraction.

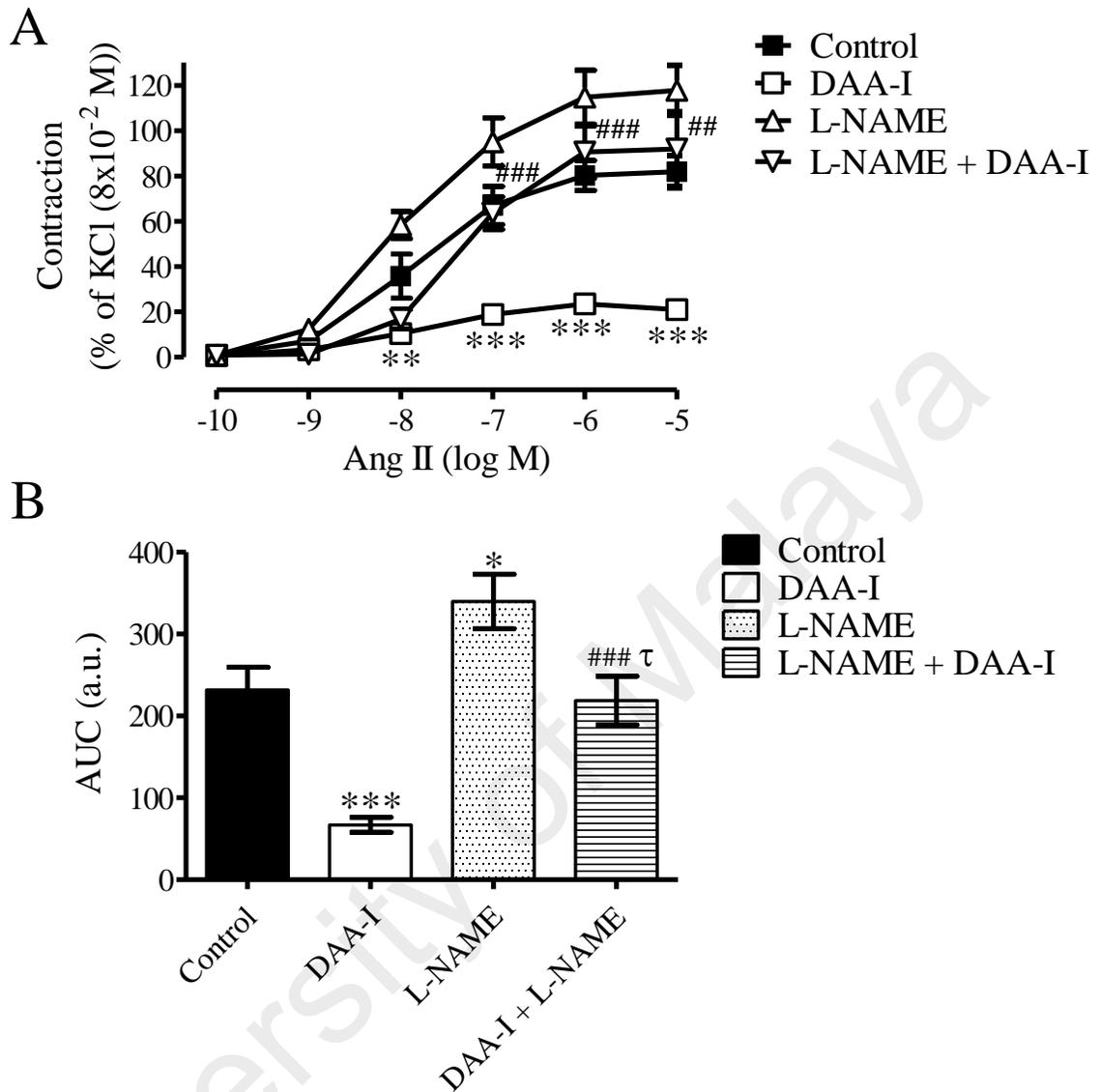


Figure 4.5. Concentration-response curves (A) and areas under the concentration-response curves (AUC) in arbitrary unit (a.u.) (B) to Ang II in the aortic rings from SHR, pretreatment with DAA-I ( $10^{-7}$  M) in the presence or absence of L-NAME ( $10^{-4}$  M). Each data point represents mean  $\pm$  SEM of 6-7 rats. Significant difference from control is indicated by \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ . # $P < 0.05$ , ## $P < 0.01$ ; ### $P < 0.001$  compared to DAA-I ( $10^{-7}$  M) group and  $^{\tau}P < 0.05$  compared to L-NAME group ( $10^{-4}$  M).

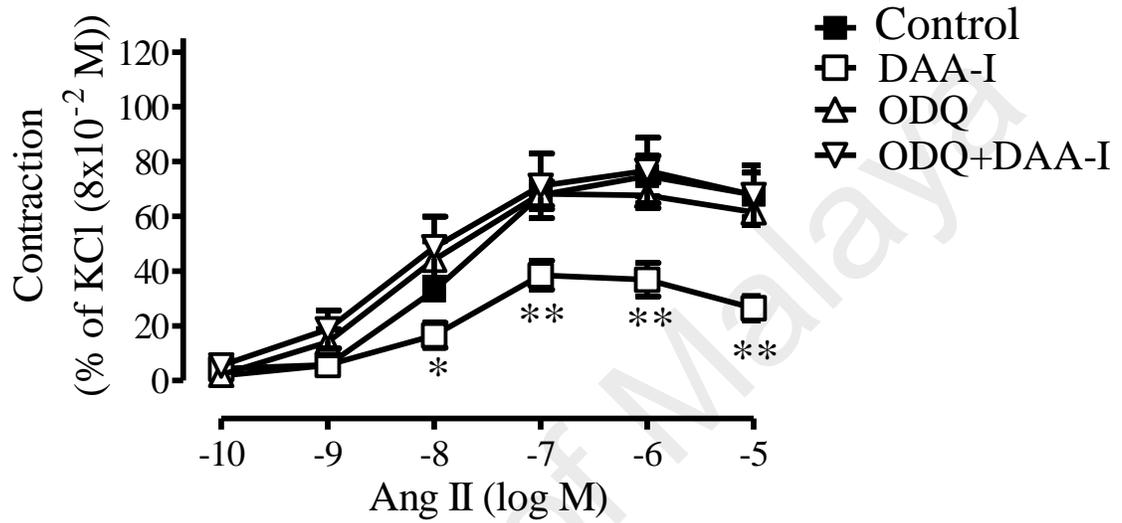


Figure 4.6. Concentration-response curves to Ang II in the aortic rings from SHR, pre-treatment with DAA-I ( $10^{-7}$  M) in the presence of ODQ ( $3 \times 10^{-6}$  M). Ang II-induced contraction was calculated as percentage of reference contraction to KCl ( $8 \times 10^{-2}$  M) obtained at the beginning of the experiment. Each data point represents mean  $\pm$  SEM of 6-7 rats. Significant difference from control is indicated by \* $P < 0.05$  and \*\* $P < 0.01$ .

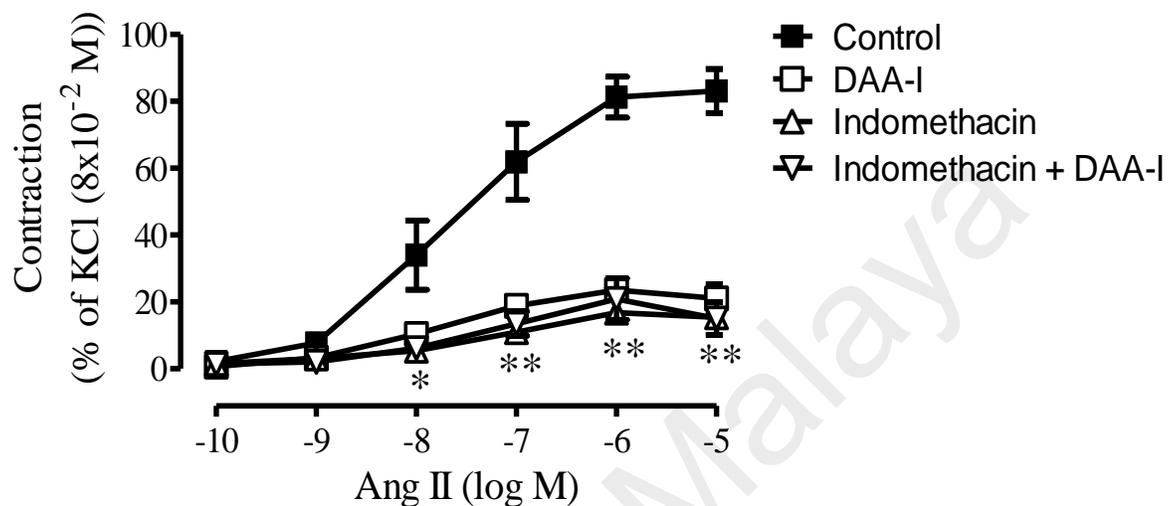


Figure 4.7. Concentration-response curves to Ang II in the aortic rings from SHR, pre-treatment with DAA-I ( $10^{-7}$  M) in the presence of indomethacin ( $10^{-5}$  M). Ang II-induced contraction was calculated as percentage of reference contraction to KCl ( $8 \times 10^{-2}$  M) obtained at the beginning of the experiment. Each data point represents mean  $\pm$  SEM of 6-7 rats. Significant difference from control is indicated by \* $P < 0.01$  and \*\* $P < 0.001$ .

#### 4.2 Effect of DAA-I on nitric oxide level in the aortic rings from WKY and SHR

In order to investigate the effect of DAA-I on nitric oxide production in the aortic rings, rings from WKY and SHR were pre-treated with DAA-I ( $10^{-7}$  M), losartan ( $10^{-5}$  M) and tempol ( $10^{-4}$  M) prior to addition of angiotensin II ( $10^{-7}$  M) to mimic the environment in the functional test. Figure 4.8 shows that total nitrate and nitrite ( $\text{NO}_x$ ) level were significantly lower in SHR aorta compared to that of WKY in the presence of angiotensin II ( $2.20 \pm 0.78$   $\mu\text{M}/\text{mg}$  vs.  $22.03 \pm 4.26$   $\mu\text{M}/\text{mg}$ , respectively). DAA-I pre-treatment had no effect on  $\text{NO}_x$  production in WKY aorta. In contrast, in the presence of angiotensin II, DAA-I significantly increased  $\text{NO}_x$  production ( $15.09 \pm 3.95$   $\mu\text{M}/\text{mg}$ ) in SHR compared to its control. Similarly, losartan and tempol also enhanced the  $\text{NO}_x$  production in the presence of angiotensin II ( $18.65 \pm 4.62$   $\mu\text{M}/\text{mg}$  and  $15.67 \pm 4.04$   $\mu\text{M}/\text{mg}$ , respectively).

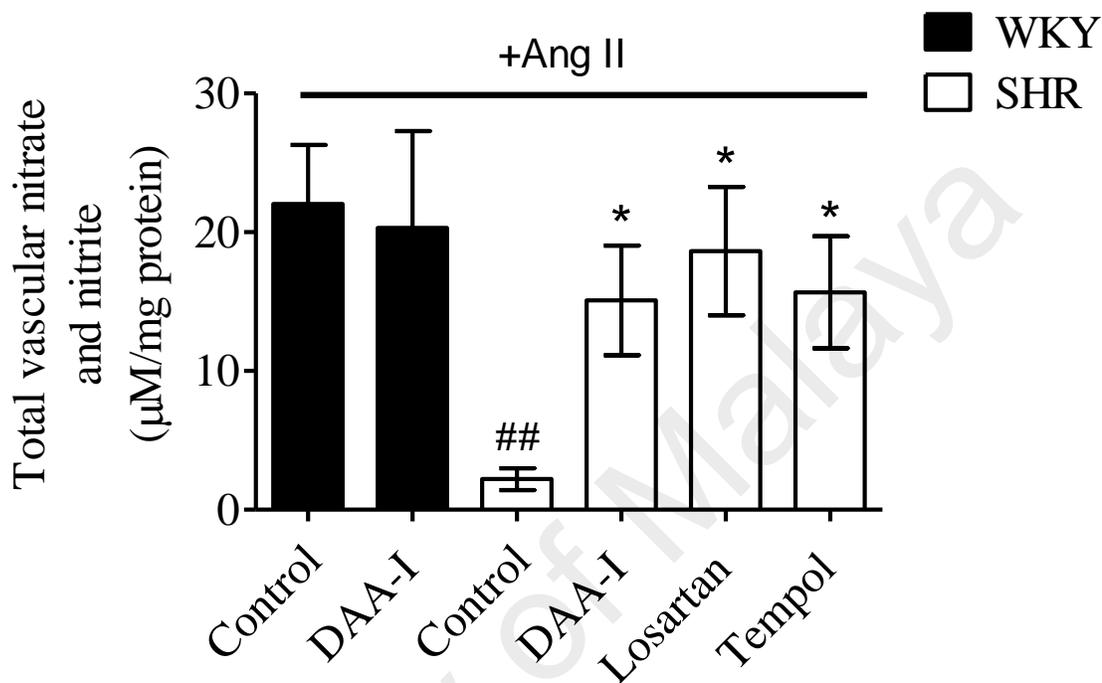


Figure 4.8. Measurement of total nitrite/ nitrate content in Ang II ( $10^{-7}$  M)-pretreated aortic rings from WKY and SHR. Results are shown as means  $\pm$  S.E.M (n= 6-7). Significant difference from WKY is indicated by ##  $p < 0.01$ ; \*  $p < 0.05$  compared to SHR.

#### 4.3 Effect of DAA-I on Ang II-induced oxidative stress in SHR aorta

To test the effect of DAA-I on Ang II-induced oxidative stress in SHR aorta, DAA-I was pre-treated prior to Ang II-induced contraction and the effect was compared to losartan, tempol ( $10^{-4}$  M, superoxide anion scavenger) and apocynin ( $10^{-4}$  M, a NAD(P)H oxidase inhibitor) which acts as positive control in reducing contraction to Ang II. Figure 4.9 show losartan, tempol and apocynin significantly reduced Ang II-induced contraction compared to the control. DAA-I reduced angiotensin II contraction to the similar extent as losartan and apocynin ( $27.42 \pm 5.68\%$ ,  $3.08 \pm 1.92\%$  and  $17.33 \pm 5.20\%$ , respectively at  $10^{-5}$  M of angiotensin II).

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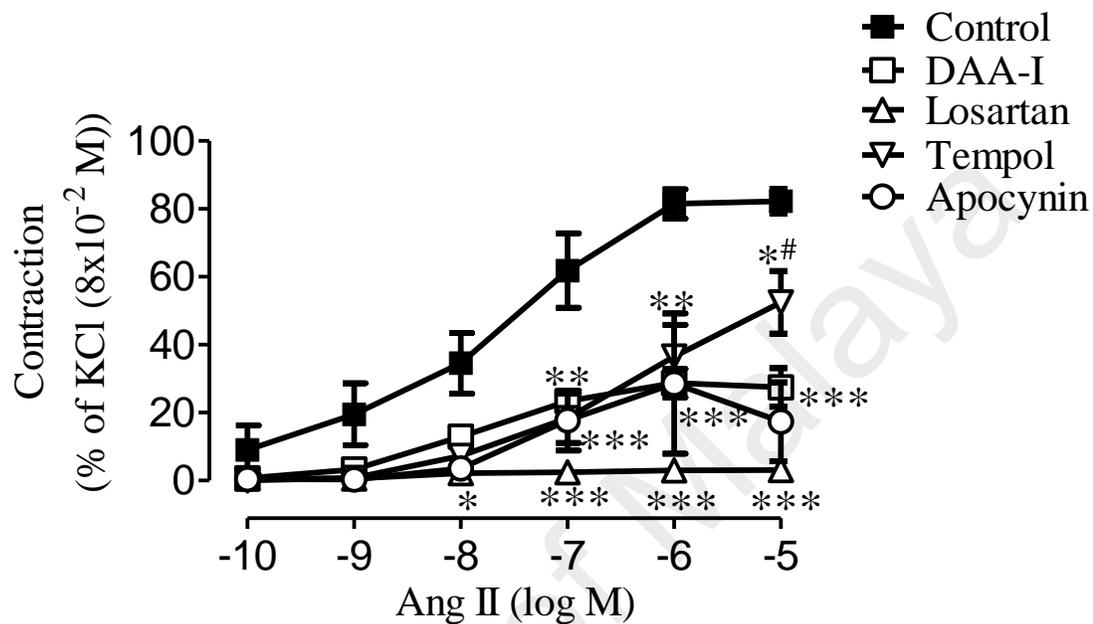


Figure 4.9. Concentration-contraction curves to Ang II in SHR aortas in the absence or presence of DAA-I ( $10^{-7}$  M), losartan ( $10^{-5}$  M), tempol ( $10^{-4}$  M) and apocynin ( $10^{-4}$  M). Each data point represents mean  $\pm$  SEM of 5-7 rats. Significant difference from control is indicated by \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ . # $P < 0.05$  compared to DAA-I ( $10^{-7}$  M).

#### 4.3.1 Effect of DAA-I on vascular superoxide anion production in isolated aortic rings from WKY and SHR

##### 4.3.3.1 Detection of vascular superoxide anion production in aortic rings from WKY and SHR using Lucigenin-enhanced chemiluminescence

In order to investigate the effect of DAA-I on vascular superoxide anion production in the aortic ring from WKY and SHR, DAA-I ( $10^{-7}$  M) were pre-incubated in the absence or presence of Ang II ( $10^{-7}$  M) and the vascular superoxide anion production was detected using lucigenin-enhanced chemiluminescence method. Figure 4.10 shows that superoxide anion production was higher in the aortic rings from SHR compared to WKY control ( $498.90 \pm 69.42$  cpm vs.  $99.37 \pm 43.70$  cpm, respectively). Pre-treatment of DPI ( $10^{-5}$  M, an inhibitor of NAD(P)H oxidase significantly reduced the production of superoxide anion in both WKY and SHR preparations. Pre-treatment of DAA-I had no effect on the superoxide anion production in both WKY and SHR aortas.

In the presence of Ang II, production of superoxide anion was slightly increased in WKY but significantly increased in SHR aortic ring ( $211.90 \pm 32.66$  cpm and  $735.50 \pm 98.18$  cpm, respectively). Pre-treatment of DPI significantly reduced the superoxide anion production in both preparations. In WKY aortic rings, pre-treatment of DAA-I had no effect on the superoxide anion production. However, DAA-I significantly reduced the production of superoxide anion ( $250.50 \pm 54.34$  cpm) compared to its respective control group in SHR aortic rings. Losartan and tempol also significantly attenuated the production of superoxide anion in the SHR aortic ring ( $226.40 \pm 51.32$  cpm and  $223.20 \pm 43.04$  cpm, respectively).

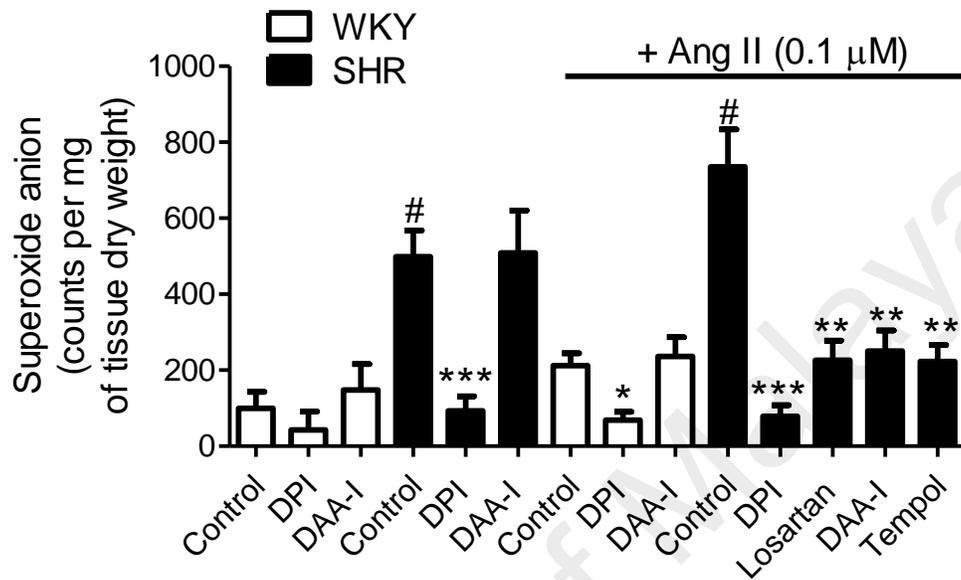


Figure 4.10. Detection of superoxide anions production in the aortas of WKY (open bars) and SHR (filled bars) in the absence or presence of Ang II ( $10^{-7}$  M) using Lucigenin-enhanced chemiluminescence method. The rings were treated with or without DAA-I ( $10^{-7}$  M), losartan ( $10^{-5}$  M) and tempol ( $10^{-4}$  M). DPI ( $10^{-5}$  M) was added as positive control group. Superoxide anion production was expressed as counts per mg (cpm) of tissue dry weight. Results are shown as means  $\pm$  S.E.M (n= 4-5). <sup>#</sup> $p < 0.05$  compare to WKY respective control group;  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$  compared with SHR respective control group.

#### 4.3.3.2 *In situ* detection of vascular superoxide anion production in cryostat section of aortas from WKY and SHR in the presence of Ang II

In a second set of experiments, dihydroethidium (DHE,  $5 \times 10^{-6}$  M) fluorescence staining was performed to determine the effect of DAA-I on superoxide anion production in the aortic rings from WKY and SHR in the presence of Ang II ( $10^{-7}$  M). Figure 4.11 shows higher production of superoxide anion (higher intensity level of the fluorescence) in the SHR aortic rings compared to the WKY preparations. Pre-treatment with DAA-I significantly reduced the superoxide anion productions in the SHR aortic rings in the presence of Ang II (Figure 4.11B) and the reduction level was comparable to losartan and tempol.

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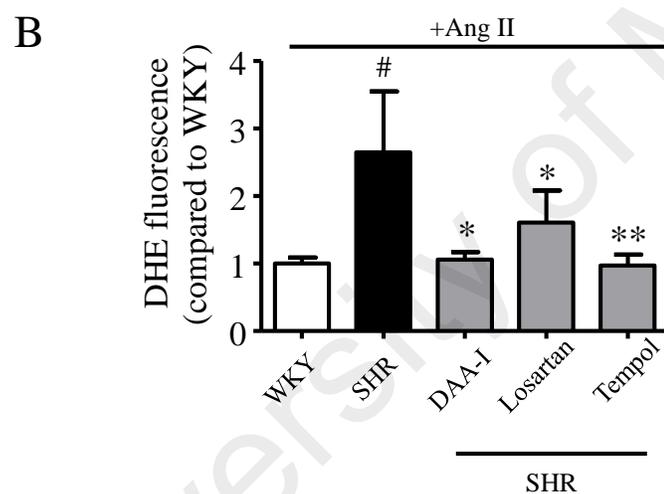
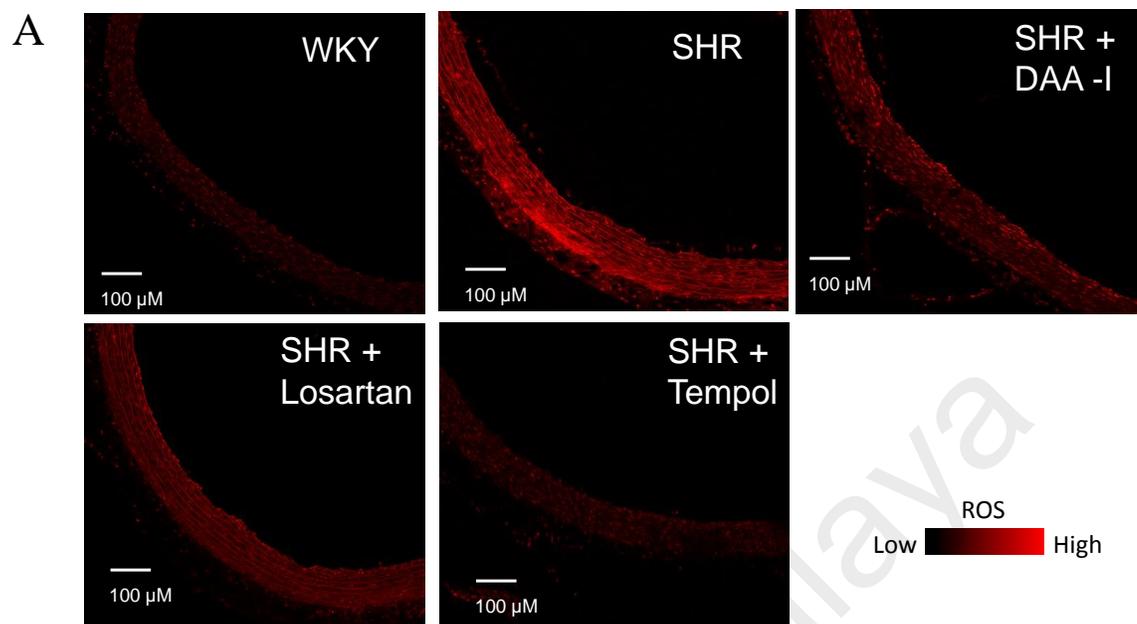


Figure 4.11. DHE fluorescence intensity indicating the level of the oxidative stress in WKY and SHR aortas. (A) Representative fluorescence image of rings from all groups which were pre-exposed to DAA-I ( $10^{-7}$  M), losartan ( $10^{-5}$  M) or tempol ( $10^{-4}$  M) followed by pre-incubated with Ang II ( $10^{-7}$  M). (B) Summarized DHE intensity for all groups. Results are shown as means  $\pm$  S.E.M (n= 4-6). Significant difference from WKY is indicated by # $p$ <0.01; \* $p$ <0.05, and \*\* $p$ <0.01 compared to SHR.

#### 4.4. Effect of DAA-I on xanthine/xanthine oxidase (X/XO)-induced contraction

In order to determine the effect of DAA-I on oxidative stress-induced contraction, experiments were performed in the presence of oxygen-derived free radical from X/XO in the WKY and SHR aortic rings. Figure 4.12 show that xanthine oxidase evoked contraction (0.001 U/ml to 0.03U/ml) in the presence of xanthine ( $10^{-4}$  M). X/XO-induced contraction was higher in SHR preparation compared to that of WKY ( $80.16 \pm 9.40\%$  vs.  $50.78 \pm 8.47\%$ , respectively). Allopurinol ( $10^{-4}$  M, an oxygen-derived free radical scavenger) and indomethacin ( $10^{-5}$  M) were significantly reduced X/XO-induced contraction in both WKY and SHR. However, DAA-I had no effect on X/XO-induced contraction in the aortic rings from both WKY and SHR.

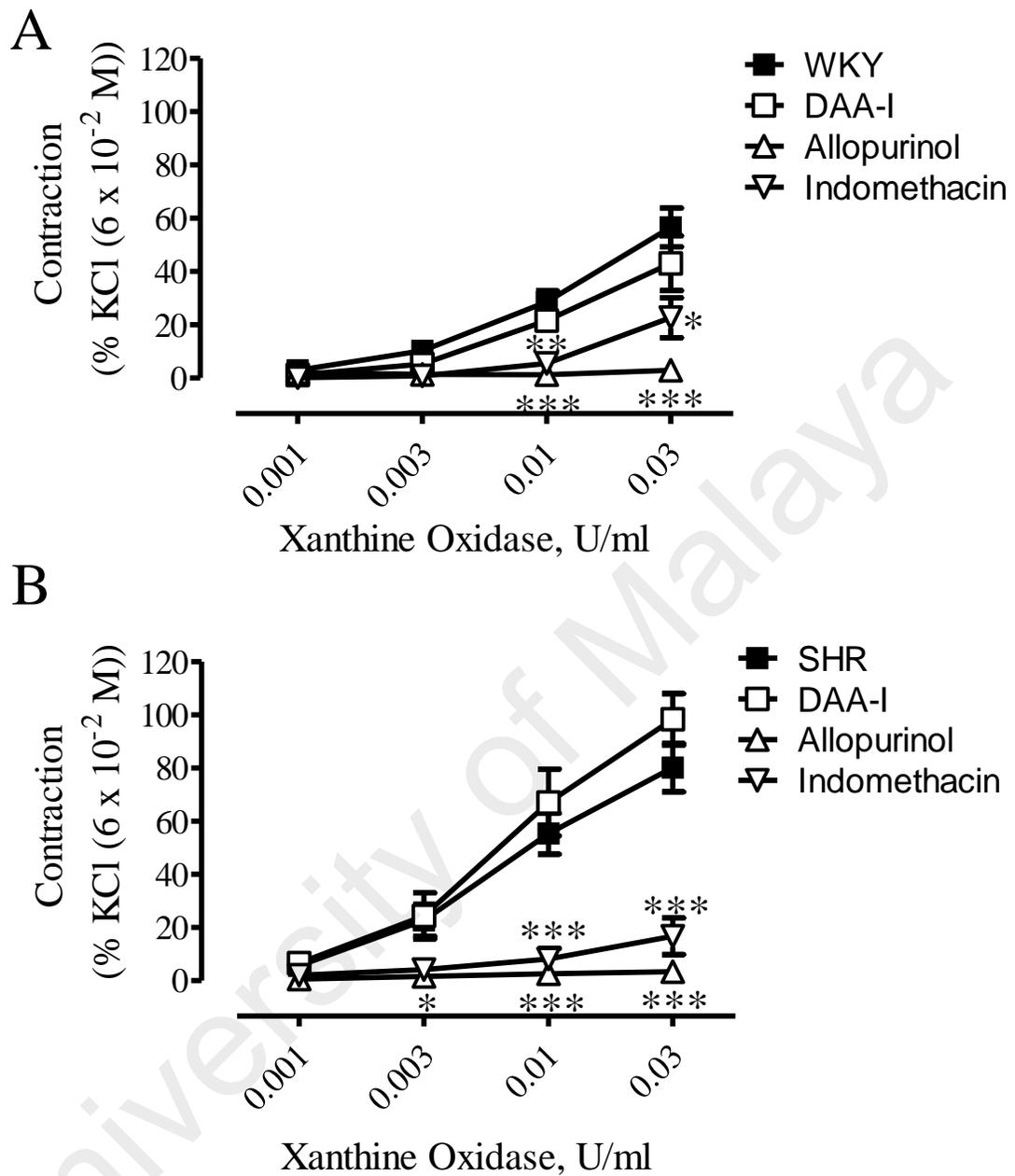


Figure 4.12. Concentration-contraction curves to xanthine/xanthine oxidase (X/XO)-induced in isolated aortas of (A) WKY and (B) SHR in the absence or presence of DAA-I ( $10^{-7}$  M), allopurinol ( $10^{-4}$  M) and indomethacin ( $10^{-5}$  M). Results are shown as means  $\pm$  S.E.M (n= 4-5). \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001 compared with WKY or SHR control.

## 4.5 Modulatory effect of DAA-I on endothelial-dependent and –independent relaxation

### 4.5.1 Effect of DAA-I on ACh-induced relaxation in aortic rings from WKY and SHR

To determine the endothelium dysfunction, ACh-induced endothelium dependent relaxation was assessed in the aortic rings from both WKY and SHR. Figure 4.13 shows that maximum relaxation response to ACh ( $10^{-5}$  M) in SHR was lower than WKY aortic rings ( $49.61 \pm 6.22\%$  vs.  $87.28 \pm 6.54\%$ , respectively).

In order to determine the modulation effect of DAA-I on ACh-induced endothelium-dependent relaxation, the aortic rings from both WKY and SHR were pre-treated by DAA-I with different concentrations within the range of  $10^{-15}$  M to  $10^{-7}$  M (concentrations that did not induce contraction). The aortic rings in all experiment were exposed to captopril ( $3 \times 10^{-5}$  M) for 20 minutes prior to the incubations of DAA-I to inhibit the action of ACE in converting DAA-I into angiotensin III (Campbell *et al.*, 1977).

Figure 4.14 shows the relaxation response to acetylcholine in aortic rings from WKY and SHR in the presence of DAA-I (different concentrations). Incubation with DAA-I did not affect the relaxation in WKY aortic rings (Figure 4.14A). However, incubation with increasing concentration of DAA-I ( $10^{-15}$ ,  $10^{-9}$ ,  $10^{-7}$  M) significantly enhanced the maximum relaxation response to ACh at  $10^{-5}$  M in SHR compared to the control (Figure 4.14B). There were no significant changes in  $pEC_{50}$  value between the control and DAA-I treated group for both WKY and SHR (Table 4.1).

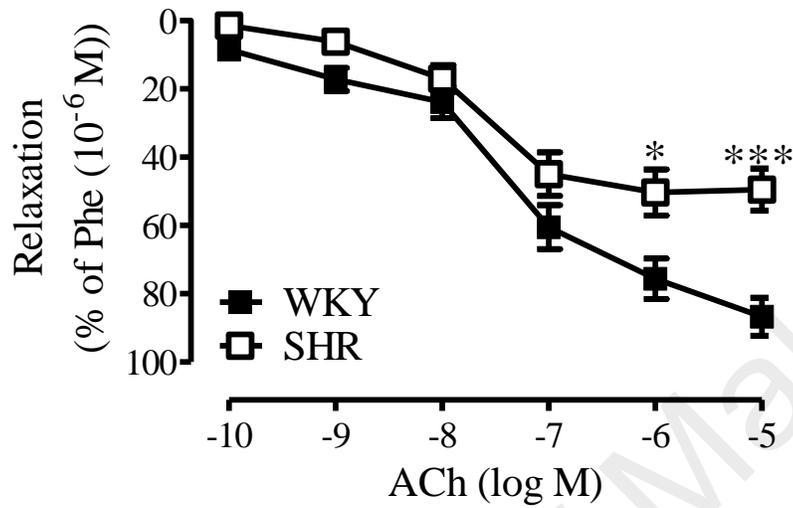


Figure 4.13. Concentration –response curve to acetylcholine in WKY and SHR aortic rings pre-contracted with phenylephrine. Each data point represents mean  $\pm$  SEM of 6-7rats. Significant difference from control is indicated by \* $P < 0.05$  and \*\*\* $P < 0.001$ .

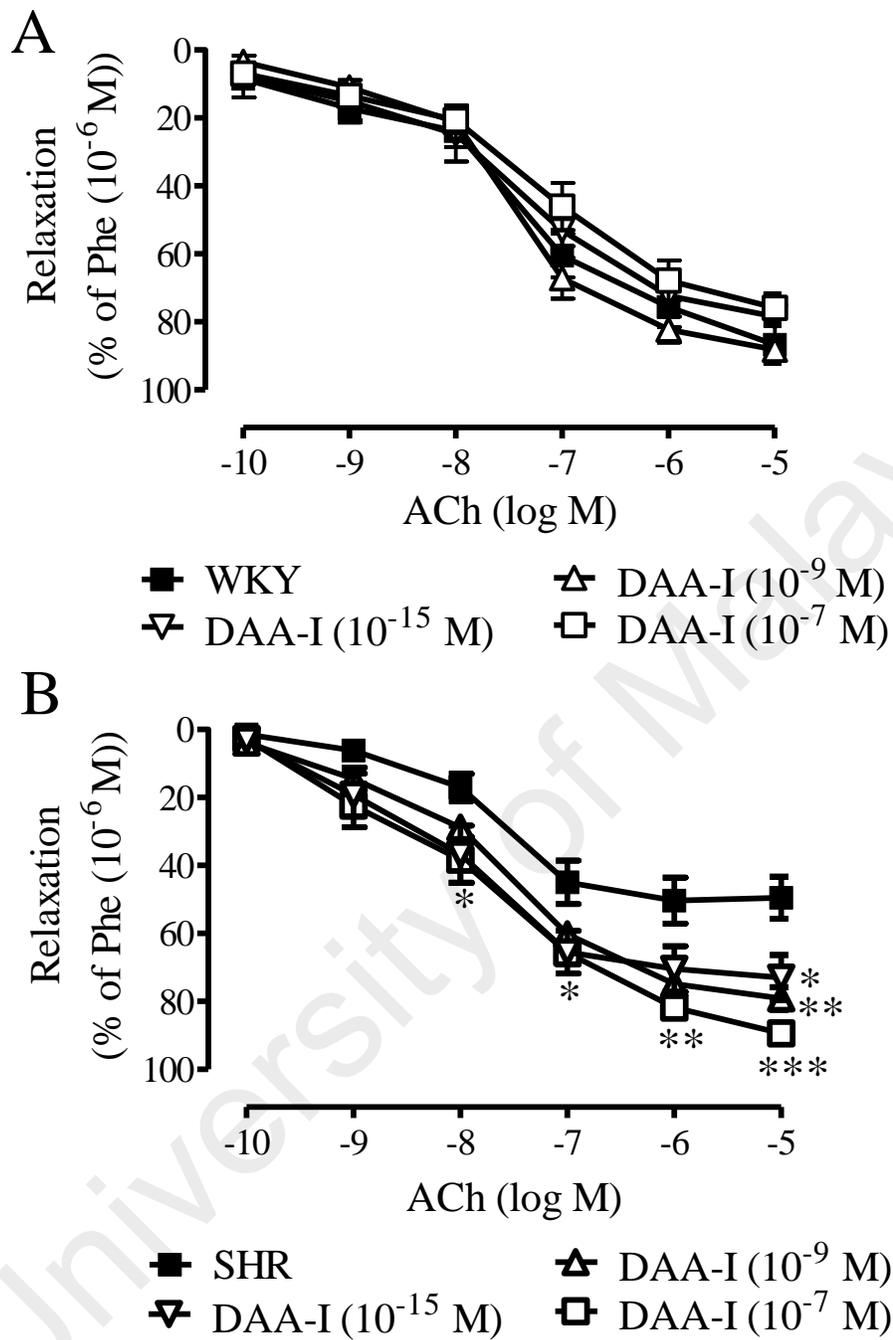


Figure 4.14. Concentration-response curves of aortic rings from (A) WKY and (B) SHR to ACh-induced endothelium-dependent relaxation in the presence of DAA-I ( $10^{-15}$ ,  $10^{-9}$ ,  $10^{-7}$  M). Results are mean  $\pm$  S.E.M (n= 6-7). Significant difference from control is indicated by \* $P$ <0.05, \*\* $P$ <0.01, and \*\*\* $P$ <0.001.

#### 4.5.2 Effect of DAA-I on SNP-induced relaxation in the aortic rings from WKY and SHR

Figure 4.15 shows that there were no significant differences in SNP-induced relaxation between both WKY and SHR preparations pre-contracted with phenylephrine. The maximum relaxation in WKY preparation was  $106.00 \pm 5.32\%$  compared to that of SHR ( $112.00 \pm 5.59\%$ ).

Figure 4.16 shows that incubation with difference concentrations of DAA-I had no effect on the SNP-induced relaxation in both WKY and SHR aorta. DAA-I pre-treatment also did not alter the sensitivity of the SNP-induced relaxation (Table 4.1).

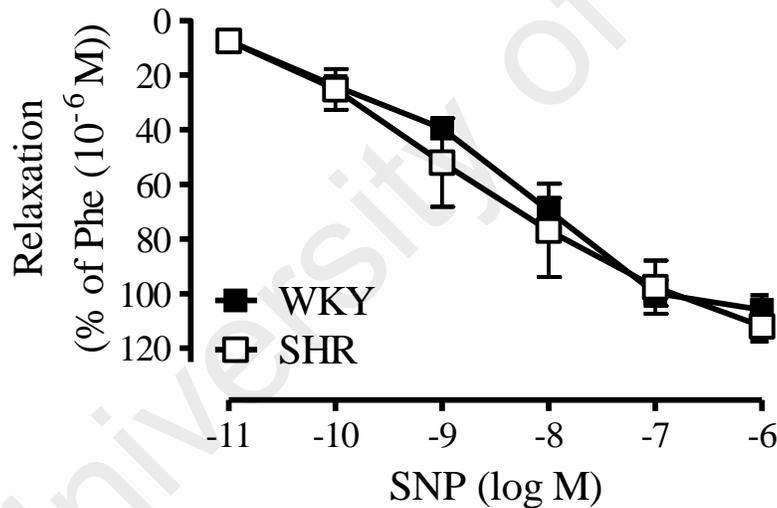


Figure 4.15. Concentration –response curves of aortic rings from WKY and SHR to SNP – induced endothelium –independent relaxation. Results are mean  $\pm$  S.E.M (n=5-7).

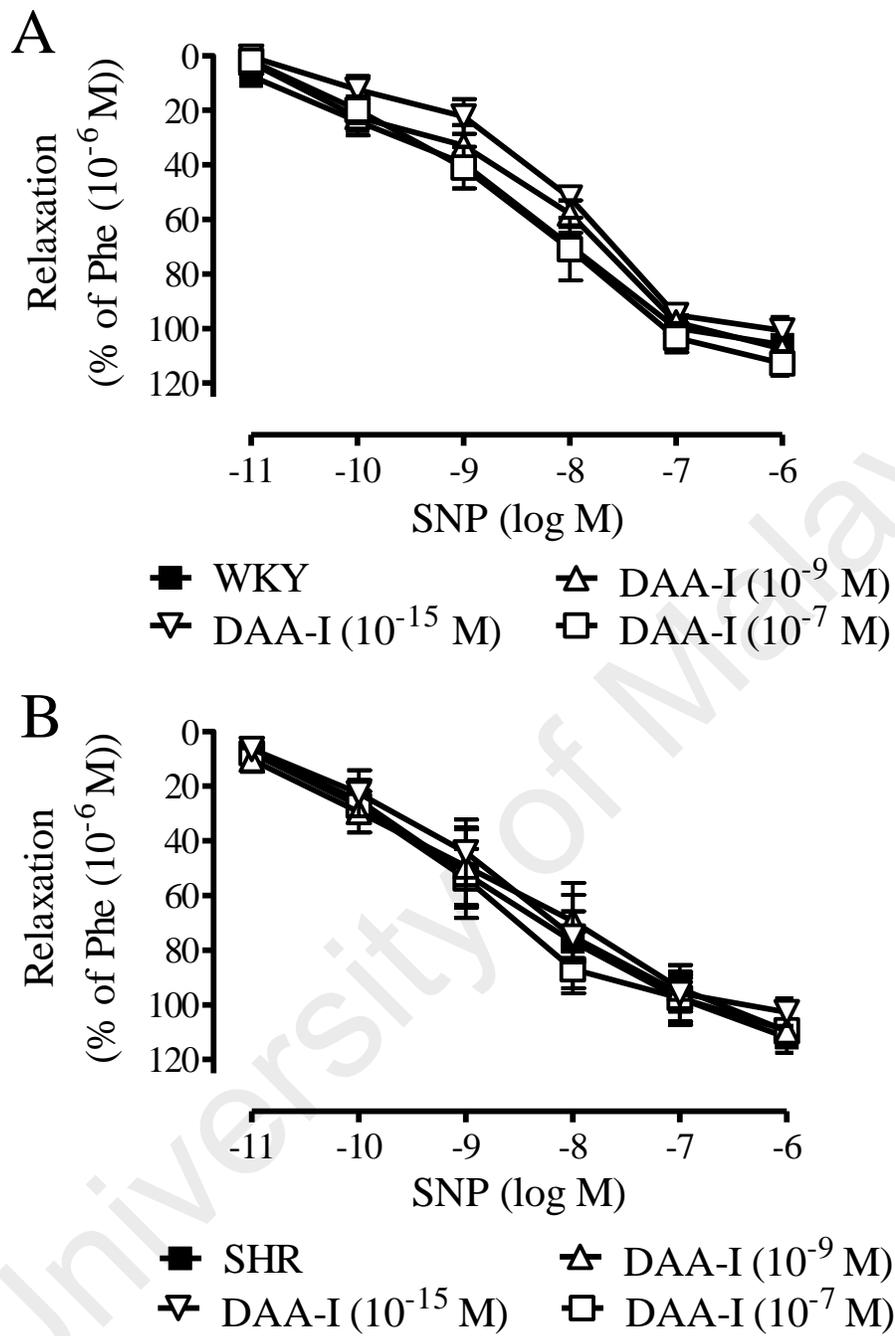


Figure 4.16. Concentration-response curves of aortic rings from (A) WKY and (B) SHR to SNP-induced endothelium-independent relaxation in the presence of DAA-I ( $10^{-15}$ ,  $10^{-9}$ ,  $10^{-7}$  M). Results are mean  $\pm$  S.E.M (n=5-7).

Table 4.1 shows the agonist sensitivity ( $pEC_{50}$ , M) and maximum relaxation ( $R_{max}$ , %) of vascular responses to ACh and SNP, respectively in isolated aortic rings from WKY and SHR.

Groups	Acetylcholine (ACh)				Sodium nitroprusside (SNP)			
	$R_{max}$ (%)		$pEC_{50}$ (M)		$R_{max}$ (%)		$pEC_{50}$ (M)	
	WKY	SHR	WKY	SHR	WKY	SHR	WKY	SHR
Control	87.28± 6.54	49.61± 6.22*	7.18± 0.12	7.72± 0.09	106.00± 5.32	112.00± 5.59	7.46± 0.17	7.38± 0.54
DAA-I ( $10^{-15}$ M)	78.57± 2.62	73.14± 6.66 <sup>#</sup>	7.50± 0.24	8.82± 0.88	100.80± 5.02	102.70± 4.97	7.17± 0.19	7.68± 0.30
DAA-I ( $10^{-9}$ M)	88.30± 2.93	79.24± 3.33 <sup>##</sup>	7.39± 0.12	7.66± 0.09	107.40± 2.77	109.70± 3.09	7.12± 0.25	7.48± 0.60
DAA-I ( $10^{-7}$ M)	75.95± 4.23	89.45± 1.94 <sup>###</sup>	7.07± 0.19	7.71± 0.31	112.80± 4.45	109.30± 2.79	7.39± 0.30	7.36± 0.80

All values are expressed as mean + SEM (n=5–7).  $R_{max}$  values are expressed as percentage of phenylephrine ( $10^{-6}$  M) contraction. Statistical comparison of  $pEC_{50}$  and  $R_{max}$  were performed using ANOVA analysis with Student's *t*-test where \* $P < 0.01$  vs control group of WKY and <sup>#</sup> $P < 0.05$ , <sup>##</sup> $P < 0.01$  and <sup>###</sup> $P < 0.001$  vs respective control group.

#### 4.5.3 Mechanisms underlying DAA-I modulation on the enhancement of ACh-induced relaxation in aortic rings from SHR

DAA-I improved the impaired ACh-induced relaxation in the aortic rings from SHR but had no effect on WKY preparation. Thus, the following mechanism studies were carried out only in SHR preparation. In order to investigate the involvement of angiotensin II receptors in the action of DAA-I on ACh-induced relaxation, the vasorelaxation was studied in the presence of angiotensin II type 1 (AT<sub>1</sub>R) and type II receptor (AT<sub>2</sub>R) antagonists.

Figure 4.17A shows the effect of DAA-I in the SHR aorta, in the absence or in the presence of losartan. Losartan, an AT<sub>1</sub>R antagonist ( $10^{-5}$  M), itself did not affect the relaxation induced by ACh in SHR aorta compared to control ( $46.70 \pm 7.05\%$  and  $53.99 \pm 5.40\%$ , respectively). However, the improvement of ACh-induced relaxation by DAA-I was reverted by losartan.

Similar with the losartan, the AT<sub>2</sub>R antagonist, PD 123319 ( $10^{-5}$  M) itself also had no effect ACh-induced relaxation. The maximum relaxation at  $10^{-5}$  M of ACh was not affected by PD123319 in the presence of DAA-I (DAA-I:  $89.45 \pm 1.94\%$  vs. PD123319:  $81.75 \pm 4.43\%$ , Figure 4.17B). However, the concentration-response curve to ACh was shifted to the right compared in the presence of PD123319 compared to the pre-treatment with DAA-I alone ( $pEC_{50}$ :  $6.81 \pm 0.37M$  vs.  $7.71 \pm 0.31M$ , respectively,  $p < 0.05$ ).

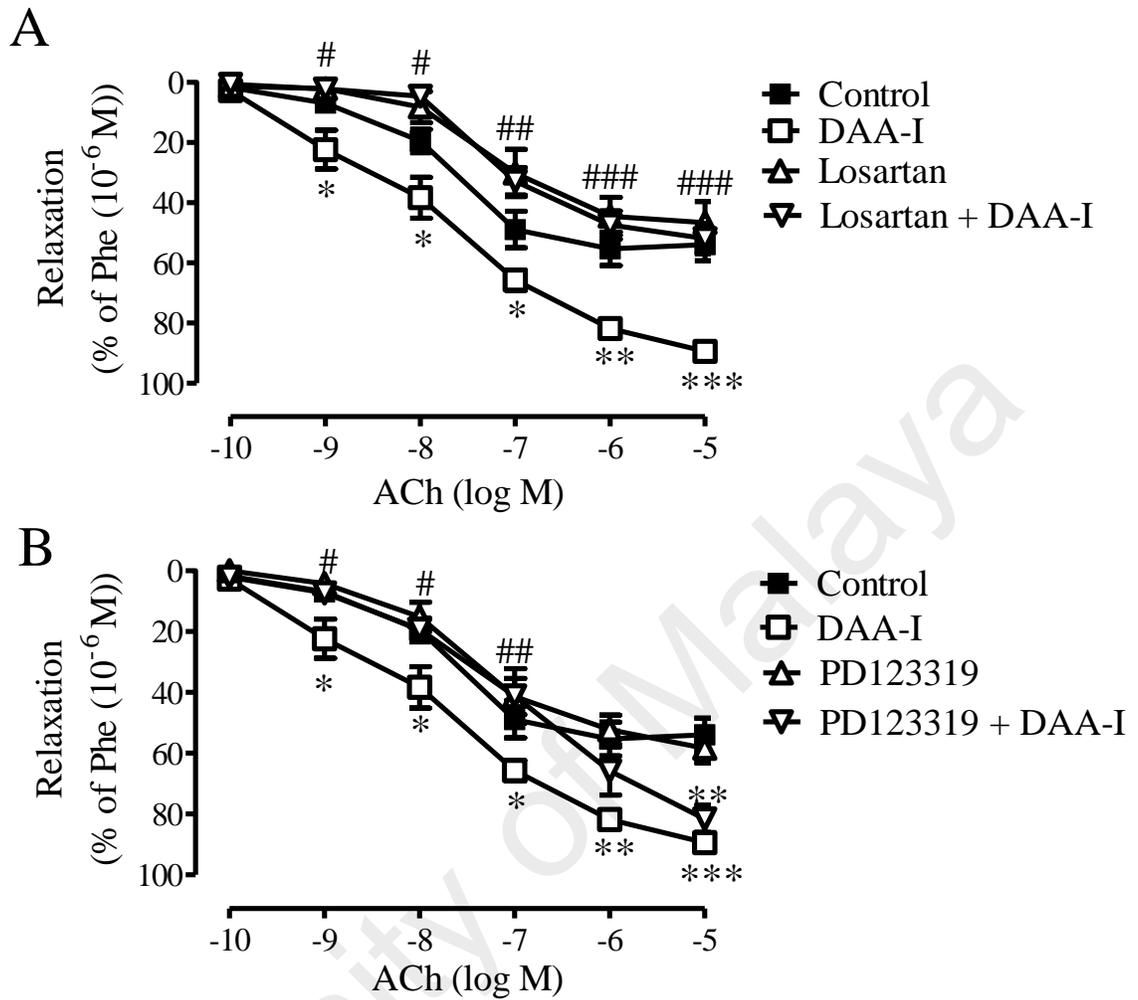


Figure 4.17. Concentration-relaxation curves to ACh in the aortic rings from SHR, pre-treatment with DAA-I ( $10^{-7}$  M) prior to the phenylephrine pre-contraction in the presence of (A) losartan ( $10^{-5}$  M) and (B) PD 123319 ( $10^{-5}$  M). Results are shown as means  $\pm$  S.E.M (n=5-7). Significant difference from control is indicated by \* $P$ <0.05, \*\* $P$ <0.01, and \*\*\* $P$ <0.001. # $P$ <0.05, ## $P$ <0.01 and ### $P$ <0.01 compared to the DAA-I ( $10^{-7}$ M) group.

In order to determine the role of eNOS in the action of DAA-I, L-NAME ( $10^{-4}$  M) was pre-incubated to inhibit eNOS in the aorta in the absence and presence of DAA-I prior to ACh-induced relaxation. Figure 4.18 shows that ACh-induced relaxation was abolished in the presence of L-NAME compared to control group. The potential effect of DAA-I on ACh-induced relaxation was totally blocked by L-NAME.

To confirm whether the action of DAA-I involves COX pathway, indomethacin ( $10^{-5}$  M) was pre-incubated in the absence or presence of DAA-I on ACh-induced relaxation in the SHR aortic ring. Figure 4.19 shows that indomethacin potentiated ACh-induced relaxation compared to control group (Rmax:  $85.07 \pm 6.97\%$  vs.  $53.99 \pm 5.40\%$ , respectively). There was no difference in ACh-induced relaxation in the presence of DAA-I with or without indomethacin (Rmax:  $96.16 \pm 5.40$  vs.  $89.45 \pm 1.94\%$ , respectively).

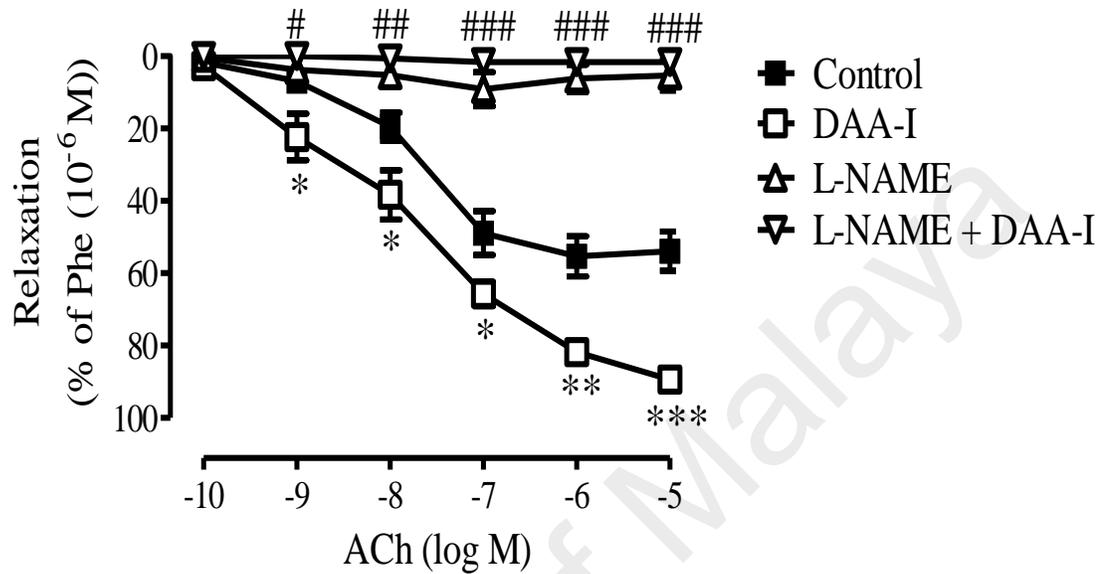


Figure 4.18. Concentration-relaxation curves to ACh in the aortic rings from SHR, pre-treatment with DAA-I ( $10^{-7}$  M) prior to the phenylephrine pre-contraction in the presence of L-NAME ( $10^{-4}$  M). Results are shown as means  $\pm$  S.E.M (n=5). Significant difference from control is indicated by \* $P$ <0.05, \*\* $P$ <0.01, and \*\*\* $P$ <0.001. # $P$ <0.05, ## $P$ <0.01 and ### $P$ <0.001 compared to the DAA-I ( $10^{-7}$  M) group.

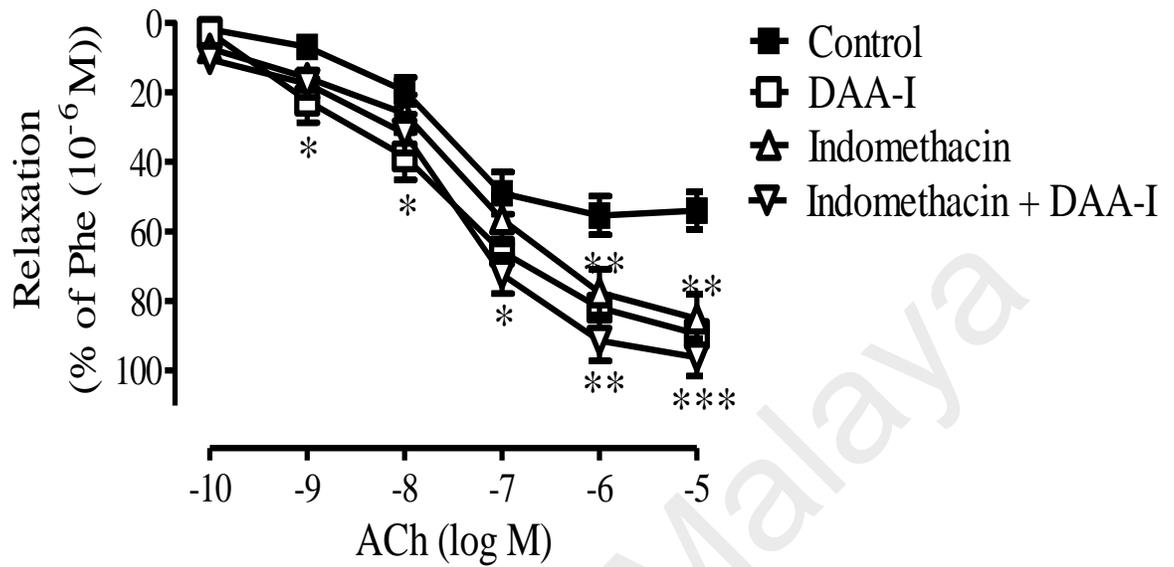


Figure 4.19. Concentration-relaxation curves to ACh in the aortic rings from SHR, pre-treatment with DAA-I ( $10^{-7}$  M) prior to the phenylephrine pre-contraction in the presence of Indomethacin ( $10^{-5}$  M). Results are shown as means  $\pm$  S.E.M (n=5-7). Significant difference from control is indicated by \* $P$ <0.05, \*\* $P$ <0.01, and \*\*\* $P$ <0.001.

## CHAPTER V

### DISCUSSION

#### 5.1 General discussion

Endothelial dysfunction is commonly associated with impaired endothelium-dependent relaxations as reported in arteries obtained from various animal models of hypertension (Rodrigo *et al.*, 1997; Colonna *et al.*, 2006; He *et al.*, 2011). The SHR represents a suitable animal model with similar pathological features to essential hypertension in human with elevated systolic blood pressure of greater than 180 mmHg. There are a number of reports showing that endothelium-dependent vasodilatation are impaired in the blood vessels of SHR compared to that of the normotensive, WKY rats (Sekiguchi *et al.*, 2001; Wind *et al.*, 2010; Lau *et al.*, 2012). Results from the present study showed ACh-induced relaxation was impaired in aorta from SHR compared to WKY supporting that endothelial dysfunction is associated with hypertension and is well developed at the age of 18-20 weeks old SHR.

Dysregulation of the RAS is closely related to hypertension as the hormonal system is important in the control of the blood pressure and electrolyte balance (Brunton *et al.*, 2011). The bioactive factors (Ang II, Ang III, Ang (1-7) and Ang IV) in RAS play important roles on the vascular system, either in inducing endothelial dysfunction or protecting it from further damage. The roles of the vasoactive components and their respective receptors or substrate are well-recognized (Schmieder *et al.*, 2007; Putnam *et al.*, 2012; Mallat, 2013). Ang II is the main effector substance in RAS and exerts its effects by binding with AT<sub>1</sub>R and AT<sub>2</sub>R (de Gasparo *et al.*, 2000). Ang II/ AT<sub>1</sub>R activation mediates vasoconstriction, cellular growth and proliferation while activation of Ang II/ AT<sub>2</sub>R leads

to vasodilatation, cell apoptosis and anti-cellular growth (de Gasparo *et al.*, 2000). Similarly, Ang (1-7) and Ang IV has exert effects that are opposite to the Ang II/ AT<sub>1</sub>R-mediated effects, by activating *Mas* receptors for Ang 1-7 and IRAP for Ang IV (Yoshida *et al.*, 1996; Ferreira & Santos, 2005; Silva *et al.*, 2011; Raffai *et al.*, 2014) . In addition to RAS inhibitors (renin inhibitors, angiotensin converting enzyme inhibitors (ACEs) and angiotensin receptor blockers (ARBs), the other active components or metabolites from Ang II have important role in counteracting the effect of Ang II. DAA-I, an endogenous peptide derived from Ang I, bypassing the formation of Ang II to form Ang III has been demonstrated to counter-regulate Ang II effects in age-related cardiac and vascular hypertrophy in SHR (Sim *et al.*, 2004) and reduced the infarct size of an ischemic-reperfusion injured rat heart by acting on AT<sub>1</sub>R (Wen *et al.*, 2004). It has also been demonstrated to improve glucose tolerance in diet-induced hyperglycemic mice via its action on the AT<sub>1</sub>R followed by the activation of insulin signaling (Wong *et al.*, 2011). In the present study, the protective effects of DAA-I is further demonstrated against Ang II-related endothelial dysfunction in the isolated thoracic aorta from SHR.

## 5.2 Effect of DAA-I on Ang II-induced constriction in isolated aortic rings from WKY and SHR

Ang II induced a comparable concentration-dependent increase in tension in both WKY and SHR aorta. Similar observation was observed in other studies where Ang II induced a contraction of aortic rings with endothelium from both strains of the animals (Zerrouk *et al.*, 1998; Stanke-Labesque *et al.*, 2001; Yu *et al.*, 2004). The present results showed that 30 minutes incubation of DAA-I in the presence of captopril, significantly reduced Ang II-

induced contraction in the isolated aortic rings with endothelium from SHR. However, this effect of DAA-I was not observed in the aortic rings from WKY. This result is in line with previous findings that DAA-I counteracts the central pressor action of angiotensin III in the SHR but not in the WKY aorta (Lim & Sim, 1998). The activity of aminopeptidase X, the enzyme which converts angiotensin I to DAA-I, is higher in both endothelium and plasma of the SHR than of the WKY (Sim & Lim, 1997). However, the plasma DAA-I level is lower in the former and inhibition of angiotensin converting enzyme significantly increased it (Sim & Qui, 2003). This may explain why reintroducing DAA-I *ex vivo* in the present study counteracted the effect of angiotensin II only in SHR preparations. The current finding also demonstrated that the action of DAA-I on Ang II-induced vasoconstriction is endothelium-dependent as the effect was completely blocked by the removal of endothelium.

### 5.3 Mechanism of modulatory actions DAA-I on the Ang II-induced contraction

Binding of Ang II to AT<sub>1</sub>R stimulates the mobilization of intracellular calcium ( $[Ca^{2+}]_i$ ) and activation of protein kinase C (PKC) causing vascular contraction. Losartan, an AT<sub>1</sub>R inhibitor completely blocked Ang II-induced contraction in the present study. This demonstrates that Ang II induced contraction is mainly due to the activation of Ang II/AT<sub>1</sub>R. Losartan is a selective and competitive antagonist to AT<sub>1</sub>R and is used widely to treat Ang II-related diseases (Aulakh *et al.*, 2007; Nemoto *et al.*, 2011; Mavroei *et al.*, 2013). Since losartan itself completely blocked Ang II-induced contraction, the result obtained could not show that DAA-I reduced Ang II-induced contraction by blocking

AT<sub>1</sub>R. However, it may be suggested that DAA-I is mimicking the effect of losartan by blocking AT<sub>1</sub>R and thus reduced the Ang II-induced contraction.

AT<sub>2</sub>R plays important role in Ang II-mediated effects which are opposite to the effects caused by the activation of AT<sub>1</sub>R. Although vasoconstriction induced by Ang II is mediated by AT<sub>1</sub> receptors, the present data demonstrated an attenuation of Ang II-induced contraction in the presence of the AT<sub>2</sub> receptor antagonist, PD 123319. Although PD123319 is said to be a selective inhibitor to AT<sub>2</sub> receptors, it has been shown to increase, decrease or not affecting Ang II- induced constriction in various other studies. Similar to the present findings, PD 123319 has been shown to reduce the Ang II-induced vasoconstriction in rat portal veins with the concentration at 10<sup>-5</sup> M (Pelet *et al.*, 1995), in the renal vasculature in mice following infusion of 100-1000 ng PD 123319 via the right jugular vein (Ruan *et al.*, 1999), and in the jejunum smooth muscle contraction in Wistar rat (PD 123319: 10<sup>-7</sup>M) (Hadzhibozheva *et al.*, 2012). However, in rat ileum and human jejunum (Ewert *et al.*, 2006), rat thoracic aorta (Watts *et al.*, 1998) and in the isolated perfused mesenteric beds (Loiola *et al.*, 2011), PD123319 did not affect concentration-curve response to Ang II. In contrast, PD123319 has been shown to increase Ang II-provoked stomach and ileum muscle contraction in Wistar rat (Hadzhibozheva *et al.*, 2012) and in the coronary microarteries in SD rats (Hannan *et al.*, 2003).

Furthermore, chronic blockade of AT<sub>2</sub>R by PD 123319 had no effect on the arterial pressure but inhibited Ang II-induced arterial hypertrophy and fibrosis in Wistar rats (Levy *et al.*, 1996). Daugherty *et al.* (2013) showed that PD123319 augments angiotensin II-induced abdominal aneurysms via an AT<sub>2</sub>R-independent mechanism. These may indicate the off-target effects of PD123319 and also the possible dual effects of AT<sub>2</sub>R stimulation. The present results obtained also showed that treatment with PD123319 alone decreased

angiotensin II-induced contractions in the SHR aorta. The inhibition by DAA-I of angiotensin II-induced contractions was partially suppressed by AT<sub>2</sub> receptor blockade with PD123319 at the concentration of 10<sup>-7</sup> -10<sup>-6</sup> M and decreased the sensitivity of ACh towards AT<sub>2</sub> receptors, suggesting that the reduction effect of DAA-I on Ang II-induced contraction may, in part through the stimulation of AT<sub>2</sub>R. However, off-target effects cannot be excluded and further experiments must be carried out to confirm the involvement of AT<sub>2</sub>R in the modulating action of the nonapeptide.

Inhibition of NO synthase with L-NAME further enhanced angiotensin II-induced contraction in the present study. This result is in agreement with Jerez *et al.* (2005) that inhibition of NO production augmented the contractile response to Ang II in rabbit aortic ring. Decreased NO production by eNOS following inhibition by L-NAME reduced the counterbalance ability in the preparations and the equilibrium shifted towards the enhancement of Ang II-induced contractions. However, to judge from the area under the concentration–response curves, DAA-I still reduced the angiotensin II–induced contraction even after incubation with L-NAME, and this indicates an effect independent of eNOS-derived NO in the action of DAA-I. eNOS -independent NO release has been demonstrated in SHR aorta (Zhao *et al.*, 2013). Otherwise, DAA-I is speculated to interfere with the activity of AT<sub>1</sub>R in reducing Ang II-induced contraction as explained in the earlier part of this study.

NO activates soluble guanylate cyclase (sGC) to convert GTP to cGMP which in turn relaxes vascular smooth muscle. ODQ, a potent and selective inhibitor of soluble guanylate cyclase (Feelisch *et al.*, 1999), was without effect on Ang II-induced contraction in the isolated aortic rings from SHR. However, preincubation of the aortic rings with the inhibitor blocked the vasodepressor effects of DAA-I on angiotensin II –induced

contraction, suggesting that the nonapeptide may act via the NO/ sGC /cGMP signaling cascade in counteracting the Ang II-induced contractions.

Prostanoids are the metabolites produced during the catalysis of arachidonic acid (AA) by cyclooxygenase (COX) and act on the thromboxane-prostanoid (TP) receptor to increase calcium ( $\text{Ca}^{2+}$ ) followed by an opening of voltage- and receptor-operated channels, which subsequent increase the cytosolic  $\text{Ca}^{2+}$  leading to constriction of the vascular smooth muscles (Gluais *et al.*, 2007). Involvement of vasoconstrictor prostanoids in Ang II-induced contraction are well documented in several studies, for example; indomethacin (the COX inhibitor) blocked the response to Ang II in rat (Lin & Nasjletti, 1991) and rabbit aortic rings (Jerez *et al.*, 2012) and in isolated uterine artery in rat (Pulgar *et al.*, 2014). In the present study, indomethacin also reduced Ang II-induced contraction in the isolated SHR aortic ring. This indicates that Ang II-induced contraction is modulated by the released vasoconstrictor prostanoids such as thromboxane  $\text{A}_2$ . Work by Wen *et al.*, (2004) demonstrated that DAA-I reduced the infarct size of an ischemic-reperfusion injured rat heart via indomethacin-sensitive receptors. However, in the SHR rat aorta, DAA-I-mediated decrease of Ang II contractility was not reversed in the presence of indomethacin, indicating that the action of DAA-I is not mediated by vasodilator prostaglandins.

#### 5.4 Effect of DAA-I on Ang II-induced oxidative stress in the aortic rings from SHR

Ang II increases the production of superoxide anions via the activation of  $\text{AT}_1\text{R}$  and this contributes to various pathological effects such as vascular remodeling, vascular contraction, inflammation and increased systolic arterial blood pressure (Zhang *et al.*, 1999; Welch, 2008). The present results showed that tempol, a superoxide dismutase (SOD)

mimetic significantly reduced the contractile response to Ang II, supporting the role of reactive oxygen species (i.e. superoxide anion) in the Ang II-induced contraction. Similarly tempol significantly inhibited the enhanced vascular response to Ang II in the diet-induced insulin resistant diabetic rat (Viswanad *et al.*, 2006) and the rat infused with Ang II hypertensive model (Wang *et al.*, 2010). The major source of ROS in the vascular system is NAD(P)H oxidase which importantly contributes to the angiotensin II-induced contraction (Touyz & Schiffrin, 2001; Hussain *et al.*, 2006; Welch, 2008). Apocynin, an antioxidant and inhibitor of NAD(P)H oxidase also blunted the contraction evoked by Ang II to a similar extent as DAA-I further supporting the role of NADPH-mediated, ROS-induced contraction in hypertensive animals.

In addition, increased superoxide anion production was observed in the isolated aortic rings from SHR compared to its respective control group. DAA-I had no effect on the superoxide anion production in the SHR control group. Addition of Ang II elevated the superoxide levels in the aorta of SHR. In addition, DAA-I attenuated the production of superoxide anion in angiotensin II stimulated (NAD(P)H oxidase activation) aortic rings from the SHR, indicating the nonapeptides interferes with oxidative stress generating action of Ang II. As shown in previous studies (Hussain *et al.*, 2006; Wang *et al.*, 2010), losartan and tempol also reduced superoxide anion production in SHR aortic rings in the presence of Ang II. Studies had demonstrated that DAA-I reduced the elevated pulmonary ROS levels in mice with 2-chloroethyl ethyl sulfide (CEES)-induced lung intoxication (Ng *et al.*, 2011), and the skeletal muscle content of gp91 protein (a major constituent of NADPH oxidase (Vignais, 2002; Nauseef, 2008)) in mice with diet-induced hyperglycaemia (Wong *et al.*, 2011). This finding was confirmed by *in situ* detection of vascular superoxide anion production using DHE fluorescence staining. In the presence of Ang II, high intensity level

of the fluorescence was observed in the aortic rings from SHR compared to that of WKY. Pre-treatment of SHR aortas with DAA-I significantly decreased the accumulation of vascular superoxide anion. Additionally, superoxide anion level in SHR aortas was normalized by losartan and tempol, a superoxide scavenger.

Furthermore, the present study shows that the total nitrite/nitrate level of SHR aortas is significantly lower compared to WKY in the presence of Ang II. Results obtained suggest that pre-treatment with DAA-I enhanced the level of NO products. Blocking AT<sub>1</sub>R and reducing the generation of superoxide anion (with losartan and tempol, respectively) also increased the level of NO products. These findings confirmed that Ang II-induced release of ROS counteracts the activity of NO and the level of NO was normalized by blocking AT<sub>1</sub>R and scavenging ROS in the vascular aortic ring. The actions of DAA-I can be speculated to mimic the effect of losartan and tempol in increasing the NO availability.

#### 5.5 Effect of DAA-I on Xanthine/Xanthine oxidase (X/XO)-induced oxidative stress in the aortic rings from SHR

In addition to NADPH oxidase, ROS are also produced by other sources that include mitochondria, xanthine oxidase and prostaglandin H synthase (PHS) (Katsuyama *et al.*, 2002; Clempus & Griendling, 2006; Chandel & Budinger, 2007). To verify whether DAA-I affects oxidative stress induced from other sources besides NADPH oxidase, the effect of the nonapeptide on the xanthine/xanthine oxidase (X/XO)-induced contraction was evaluated. X/XO-induced contraction was higher in SHR aortic preparations compared to that of WKY. Allopurinol (an oxygen-derived free radical scavenger) and indomethacin significantly reduced X/XO-induced contraction in both WKY and SHR. However, DAA-I

had no effect on X/XO-induced contraction in the aortic rings from both WKY and SHR. These data suggest that DAA-I exerts its endothelial protective effect mainly by preventing Ang II- NADPH oxidase mediated oxidative stress and itself does not possess ROS scavenging properties.

#### 5.6 Effect of DAA-I on endothelium-dependent and –independent relaxation in the isolated aortic rings from WKY and SHR

As discussed in section 5.1, the response of aortic rings to ACh-induced endothelium dependent relaxation was impaired in the SHR aortas compared to that of WKY. The present result shows that DAA-I did not alter the relaxation to acetylcholine in WKY aorta but enhanced it in SHR preparations with endothelium. This suggests that DAA-I potentiates acetylcholine-induced relaxations by increasing NO activity. This interpretation is supported by the observation that indeed the nonapeptide enhances the content of NO products in the SHR aorta. L-NAME, an eNOS inhibitor prevented the effect of DAA-I in enhancing acetylcholine-induced relaxation. This indicates that the improvement in relaxation in SHR preparation by DAA-I is eNOS-dependent.

DAA-I had no effect on the relaxation induced by the nitric oxide donor, sodium nitroprusside in either WKY or SHR aortic rings. NO whether released by endothelial cells or from sodium nitroprusside diffuses into the vascular smooth muscle cell, leading to activation of soluble guanylate cyclase with a resulting increase in intracellular cyclic guanosine monophosphate (cGMP) (Ignarro, 1990; Olson *et al.*, 1997; Feelisch *et al.*, 1999). This second messenger activates cGMP –dependent protein kinase thus causing relaxation of the vascular smooth muscle cells (Hansen & Nedergaard, 1999). The present

data thus suggest that the improvement by DAA-I of the acetylcholine –induced relaxation in SHR aortic rings must be due to modulation of endothelium-derived NO activity rather than to direct activation of the cGMP pathway in the effector cells. The absence of effect on responses to sodium nitroprusside also implies that the moderate direct relaxing effect exerted by DAA-I did not alter vascular responsiveness *per se*.

#### 5.7 Mechanisms of action of DAA-I on ACh-induced endothelium-dependent relaxation in the aortic rings of SHR

In the presence of losartan, an AT<sub>1</sub> receptor antagonist, the improvement of the relaxation to acetylcholine observed in SHR aortic rings with DAA-I was prevented, indicating that the action of DAA-I in reversing endothelial dysfunction in preparations of the hypertensive strain is mediated by activation of AT<sub>1</sub> angiotensin receptors. Indeed, AT<sub>1</sub>R has been demonstrated to mediate effects of DAA-I both *in vitro* and *in vivo* (Sim & Chai, 1996; Chen & Sim, 2004). Furthermore, the present finding showed that the potentiation by DAA-I of the response to lower concentration ( $10^{-9}$ -  $10^{-7}$  M) of acetylcholine was blocked also by PD 123319, an established selective AT<sub>2</sub>R antagonist (de Gasparo *et al.*, 1995; Touyz & Schiffrin, 2000). This suggests that DAA-I –induced enhancement of the response to the muscarinic agonist in the SHR aorta is partially mediated by AT<sub>2</sub>R and DAA-I increased the sensitivity of vascular tissue to ACh via interaction with AT<sub>2</sub>R. DAA-I has been shown to act as an agonist on both AT<sub>1</sub>R and non-AT<sub>1</sub>R subtypes (Min *et al.*, 2000).

Similarly to the findings with Ang II-induced contraction, relaxations to acetylcholine improved in the presence of indomethacin alone, as shown in previous studies (Lüscher & Vanhoutte, 1986; de Sotomayor *et al.*, 1999; Ulker *et al.*, 2003). The present observation

thus confirm that the release of vasoconstrictor cyclooxygenase product(s) in the SHR aorta counteract acetylcholine –induced relaxations and thus contributes to endothelial dysfunction (Vanhoutte, 2011). Previous finding had demonstrated that losartan and indomethacin blunt the cardioprotective effects of DAA-I in the animal model with hypertrophy and myocardial ischemic-reperfusion injury and suggesting that the effect of DAA-I is AT<sub>1</sub>R-mediated and involved prostaglandins (Sim, 2015). However, indomethacin was without obvious effect on the action of DAA-I in the present study. This indicates that the improvement in endothelium dependent relaxation in SHR aorta by DAA-I is mediated by both angiotensin receptors and involves increasing NO activity.

## CHAPTER VI

### CONCLUSION

The present study demonstrates that DAA-I improves the endothelial acetylcholine-induced relaxation and decreases angiotensin II-induced contraction in the SHR but not WKY aorta. The endothelial protective actions of DAA-I is due to higher NO bioavailability through increasing eNOS activity and reducing ROS production in the SHR, achieved at least in part by interfering with Ang II-mediated, NADPH derived oxidative stress. The activity of aminopeptidase X, the enzyme which degrades angiotensin I to DAA-I, is higher in both the endothelium and the plasma of the SHR than in the WKY (Sim and Lim, 1997). However, the plasma DAA-I level is lower in the hypertensive strain as in essential hypertensive patients (Sim and Qui, 2003). Inhibiting ACE pathways with ACE inhibitors while maintaining the activity of aminopeptidase X, increased plasma DAA-I level in the SHR (Sim and Qui, 2003). Thus, chronic lower level of DAA-I may cause a relative supersensitivity of its binding site on the AT<sub>1</sub>R which may explain why reintroducing the nonapeptide *ex vivo* (in the presence of captopril to prevent its conversion into angiotensin III), counteracted the effect of angiotensin II only in SHR preparations. In the Ang II-induced oxidative stress condition, the endothelial protective actions of DAA-I is due to higher NO bioavailability through increasing eNOS activity and reducing ROS production in the SHR, achieved at least in part by interfering with Ang II-mediated, NADPH derived oxidative stress. Lastly, taking these findings together, it is reasonable to suggest that DAA-I improves the endothelial function in SHR by counteracting AT<sub>1</sub>R –mediated effects. This finding opens up the possibility of DAA-I as a therapeutic avenue for treating hypertension. As this a peptide, it also may reduce the risk of adverse effect. However, the findings from the current study still require *in-vivo* validation.

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