VASOMODULATORY ACTIONS OF DES-ASP-ANGIOTENSIN I AND ANGIOTENSIN 1-7 IN RESISTANCE VESSELS OF HYPERTENSIVE AND DIABETIC RAT MODELS

THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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Abstract

A number of anatomical and functional disturbances of the vascular endothelium are observed in diabetes and hypertension. Angiotensin II (Ang II), a major effector peptide of the renin-angiotensin system (RAS), acts primarily on angiotensin AT\textsubscript{1} receptor. The oligopeptide plays a key role in the initiation and amplification of pathobiological events underlying several vascular diseases. The discovery of other angiotensin peptides i.e. des-Asp-angiotensin I (DAA-I) and angiotensin 1-7 (Ang 1-7) with contrasting vascular actions has led to further investigations to determine the roles of these peptides. The aim of this study was to evaluate the direct and modulatory actions of these angiotensin peptides in the isolated renal and mesenteric vascular bed preparations from Wistar-Kyoto (WKY) rats (as normotensive), Spontaneously Hypertensive rats (SHR) (as hypertensive) and streptozotocin (STZ)-induced diabetic rats (as diabetic model).

In the isolated renal vascular bed, Ang II pressor response was increased in SHR and reduced in STZ-induced diabetic rats. However, in the mesenteric vasculature, contractile action of Ang II was unaltered in diabetic but decreased in the hypertensive animals. DAA-I appears to have a smaller vasoconstrictor actions than Ang II in both vasculatures. In the mesenteric vasculature, the contractile actions of the nanopeptide were not significantly altered in hypertensive and diabetic conditions. In contrast, Ang 1-7 did not exhibit constrictor actions in either vascular beds.

DAA-I attenuated the Ang II pressor action in normotensive and hypertensive rat kidneys, however, this action was absent in the STZ-induced diabetic rat model. A similar pattern of DAA-I action was observed in the mesenteric vasculature. The vasodepressor action of DAA-I appeared not to involve AT\textsubscript{2} receptor or cyclooxygenase byproducts. Nitric oxide appeared to be involved in the modulatory actions of DAA-I in hypertensive animals. Similarly, Ang 1-7 attenuated Ang II-induced vasoconstriction in
both normotensive and hypertensive rat kidneys and this action was absent in the STZ-diabetic rat model. In the mesenteric vascular bed, Ang 1-7 attenuated Ang II-induced vasoconstrictions in all three animal groups. In the kidney, Ang 1-7 action was mediated via the Ang 1-7 receptor in WKY and SHR, and involved the release of prostaglandins and nitric oxide. In the mesentery, the vasodepressor action appeared to be mediated by Ang 1-7 receptor and involved vasodilator prostaglandins and nitric oxide in SHR, and only nitric oxide in the normotensive and diabetic rats.

The involvement of AT$_1$ receptor in the actions of DAA-I and Ang 1-7 actions was investigated in the renal vasculature. Receptor binding assay revealed that the rat kidney homogenate contained mainly the AT$_1$ receptor subtype. The AT$_1$ receptor density was found to be significantly increased in hypertensive rat kidney. This increase may explain the hyperresponsiveness to Ang II observed previously in the perfused kidney. RT-PCR and Western blot analysis were also in agreement with the results from the receptor binding assay. In contrast to SHR, the AT$_1$ receptor density was decreased in the diabetic rat kidney. This reduction is in tandem with the reduced responsiveness to Ang II observed in the perfused kidney. The results from RT-PCR and Western blot analysis were also in agreement with the binding and functional studies.

In kidneys perfused with $10^{-9}$ M DAA-I, receptor binding data demonstrated a reduced AT$_1$ receptor density. This suggests that the DAA-I vasodepressor actions in WKY and SHR, may be partly due to downregulation of AT$_1$ receptors. However, the findings from the RT-PCR and Western blot analysis showed no changes in the AT$_1$ receptor gene and protein expression. Alternatively, the reduced AT$_1$ receptor density may be due to receptor internalization of AT$_1$ receptors. On the other hand, in kidney homogenates perfused with lower concentrations of DAA-I, no significant changes in
AT\textsubscript{1} receptor were seen (density and expression). In diabetic rat model, no changes in AT\textsubscript{1} receptor density or expression were seen in kidneys perfused with DAA-I. This supports the earlier finding where DAA-I had no effect on Ang II-induced pressor action in STZ-induced diabetic rat kidney. A slightly lower receptor affinity was seen in STZ-induced diabetic untreated and DAA-I treated compared to WKY. This suggests that DAA-I could bind to other binding sites than AT\textsubscript{1} receptor and DAA-I binding ability may be altered in diabetes.

Receptor binding assay from kidney homogenate perfused with Ang 1-7 ($10^{-7}$M) demonstrated a reduced AT\textsubscript{1} receptor density in WKY and SHR. This showed that Ang 1-7 vasodepressor action is partially modulated via AT\textsubscript{1} receptor, possibly by receptor downregulation. However, RT-PCR and Western blot analysis ruled out receptor downregulation, suggesting that Ang 1-7 may internalize the renal AT\textsubscript{1} receptor similarly like the DAA-I. Pre-treatment of Ang 1-7 in STZ-induced diabetic kidney did not affect AT\textsubscript{1} receptor (density and expression). This finding agrees well with the isolated perfused kidney study where Ang 1-7 vasodepressor action was compromised in diabetic condition. Mas receptor has been shown to be the endogenous Ang 1-7 receptor and alteration on this receptor in diabetic condition may explain the diminished Ang 1-7 vasodepressor action.

Preliminary study of isolated perfused kidney from neonatal (n-STZ) induced diabetic rat as a model for type 2 diabetes mellitus demonstrated a similar pattern as type I diabetes mellitus rat model (STZ-induced) to the pressor responses to Ang II, DAA-I and Ang 1-7. Also similar are the actions of DAA-I and Ang 1-7 on Ang II-induced pressor response. This indicates that in both type of diabetic models, these peptides facilitate the ongoing renal vascular changes in a similar manner.
These findings provide further information on the actions of angiotensin peptides especially in resistance vasculatures and their roles in diseases such as hypertension and diabetes. The data also demonstrates the ability of both DAA-I and Ang 1-7 in reducing Ang II actions especially in hypertensive condition and this may enable targeted therapies to be developed. This study also acts as a first step toward understanding the compromised vasodepressor actions of DAA-I and Ang 1-7 in diabetes mellitus.
List of original communications


Conference Presentations


By God’s blessing this work is accomplished.

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This is for you, dad.
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<tr>
<td>AA</td>
<td>arachidonic acid</td>
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<td>A-779</td>
<td>D-Ala(^7)-Ang-(1-7)</td>
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<td>ACE</td>
<td>angiotensin converting enzyme</td>
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<td></td>
</tr>
<tr>
<td>AT(_{1B})</td>
<td>angiotensin subtype 1B</td>
<td></td>
</tr>
<tr>
<td>AT(_2)</td>
<td>angiotensin subtype 2</td>
<td></td>
</tr>
<tr>
<td>AT(_3)</td>
<td>angiotensin subtype 3</td>
<td></td>
</tr>
<tr>
<td>AT(_4)</td>
<td>angiotensin subtype 4</td>
<td></td>
</tr>
<tr>
<td>Ang 1-7</td>
<td>angiotensin subtype 1-7</td>
<td></td>
</tr>
<tr>
<td>AOGEN</td>
<td>angiotensinogen</td>
<td></td>
</tr>
<tr>
<td>APA</td>
<td>aminopeptidase A</td>
<td></td>
</tr>
<tr>
<td>APB</td>
<td>aminopeptidase B</td>
<td></td>
</tr>
<tr>
<td>APN</td>
<td>aminopeptidase N</td>
<td></td>
</tr>
<tr>
<td>Asp</td>
<td>aspartyl</td>
<td></td>
</tr>
<tr>
<td>B(_2)</td>
<td>bradykinin subtype 2</td>
<td></td>
</tr>
<tr>
<td>B(_{max})</td>
<td>maximal binding</td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
<td></td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>calcium ion</td>
<td></td>
</tr>
<tr>
<td>CAGE</td>
<td>chymostatin-sensitive angiotensin-generating enzyme</td>
<td></td>
</tr>
<tr>
<td>cDNA</td>
<td>copy Deoxyribonucleic acid</td>
<td></td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic Guanine MonoPhosphate</td>
<td></td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
<td></td>
</tr>
<tr>
<td>CO(_2)</td>
<td>carbon dioxide</td>
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</tr>
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<td>COX</td>
<td>cyclooxygenase</td>
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</tr>
<tr>
<td>CPA</td>
<td>carboxypeptidase A</td>
<td></td>
</tr>
<tr>
<td>cpm</td>
<td>count per minute</td>
<td></td>
</tr>
<tr>
<td>CPP</td>
<td>carboxypeptidase P</td>
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</tr>
<tr>
<td>DAA-I</td>
<td>des-aspartyl angiotensin I</td>
<td></td>
</tr>
<tr>
<td>DAG</td>
<td>diacetylglcerol</td>
<td></td>
</tr>
<tr>
<td>D-ALA</td>
<td>D-[Ala(^7)]-angiotensin 1-7</td>
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</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
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</tr>
<tr>
<td>des-Asp-Ang I</td>
<td>des-Aspartyl-angiotensin I</td>
<td></td>
</tr>
<tr>
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<td>deoxyribonucleic acid</td>
<td></td>
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<tr>
<td>dNTP</td>
<td>deoxynucleotidetriphosphates</td>
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</tr>
<tr>
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<td>dipeptidylaminopeptidase</td>
<td></td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
<td></td>
</tr>
<tr>
<td>EDHF</td>
<td>endothelium derived hyperpolarizing factor</td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetra-acetic acid</td>
<td></td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal regulated kinase</td>
<td></td>
</tr>
<tr>
<td>et al.</td>
<td>et alia (and other people)</td>
<td></td>
</tr>
<tr>
<td>e.g.</td>
<td>exempli gratia (for example)</td>
<td></td>
</tr>
<tr>
<td>fmol/ml</td>
<td>femtomol/ mililiter</td>
<td></td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
<td></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>GADPH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td></td>
</tr>
<tr>
<td>GDM</td>
<td>Gestational diabetes mellitus</td>
<td></td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
<td></td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein coupled receptor</td>
<td></td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
<td></td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
<td></td>
</tr>
<tr>
<td>i.e.</td>
<td>Id est (in other words)</td>
<td></td>
</tr>
<tr>
<td>i.c.v.</td>
<td>Intracerebroventricular</td>
<td></td>
</tr>
<tr>
<td>IDDM</td>
<td>Insulin dependent diabetes mellitus</td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin</td>
<td></td>
</tr>
<tr>
<td>InsP3</td>
<td>Inositol 1,4,5-triphosphate</td>
<td></td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneally</td>
<td></td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
<td></td>
</tr>
<tr>
<td>IRAP</td>
<td>Insulin-regulated aminopeptidase</td>
<td></td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinases</td>
<td></td>
</tr>
<tr>
<td>K+</td>
<td>Potassium ion</td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
<td></td>
</tr>
<tr>
<td>KH2PO4</td>
<td>Potassium dihydrogen orthophosphate</td>
<td></td>
</tr>
<tr>
<td>L-NAME</td>
<td>Nω-Nitro-L-Arginine Methyl Ester</td>
<td></td>
</tr>
<tr>
<td>ml</td>
<td>Mililiter</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
<td></td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
<td></td>
</tr>
<tr>
<td>MgSO4.7H2O</td>
<td>Magnesium sulphate heptahydrate</td>
<td></td>
</tr>
<tr>
<td>mM</td>
<td>Milimolar</td>
<td></td>
</tr>
<tr>
<td>Na+/H+</td>
<td>Sodium hydrogen ion</td>
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<tr>
<td>Na+</td>
<td>Sodium ion</td>
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</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
<td></td>
</tr>
<tr>
<td>Na2HCO3</td>
<td>Sodium bicarbonate</td>
<td></td>
</tr>
<tr>
<td>NEP</td>
<td>Neutral endopeptidase</td>
<td></td>
</tr>
<tr>
<td>NIDDM</td>
<td>Non-insulin dependent diabetes mellitus</td>
<td></td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
<td></td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
<td></td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
<td></td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
<td></td>
</tr>
<tr>
<td>NSB</td>
<td>Non specific binding</td>
<td></td>
</tr>
<tr>
<td>O2</td>
<td>Oxygen</td>
<td></td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
<td></td>
</tr>
<tr>
<td>PD 123319</td>
<td>1-[(4-(Dimethylamino)-3-methylphenyl)methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid difluoroacetate</td>
<td></td>
</tr>
<tr>
<td>PE</td>
<td>Phenylephrine</td>
<td></td>
</tr>
<tr>
<td>PEP</td>
<td>Propylendopeptidase</td>
<td></td>
</tr>
<tr>
<td>pg/ml</td>
<td>Picogram/milliliter</td>
<td></td>
</tr>
<tr>
<td>PGD2</td>
<td>Prostaglandin D2</td>
<td></td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
<td></td>
</tr>
<tr>
<td>PGF2α</td>
<td>Prostaglandin F2α</td>
<td></td>
</tr>
<tr>
<td>PGH2</td>
<td>Prostaglandin H2</td>
<td></td>
</tr>
<tr>
<td>PGI2</td>
<td>Prostaglandin I2 or prostacyclin</td>
<td></td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
<td></td>
</tr>
<tr>
<td>PLA2</td>
<td>Phospholipase A2</td>
<td></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
<td></td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
<td></td>
</tr>
<tr>
<td>PLD</td>
<td>Phospholipase D</td>
<td></td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulphonyl fluoride</td>
<td></td>
</tr>
<tr>
<td>PP2A</td>
<td>serine/threonine phosphatase 2A</td>
<td></td>
</tr>
<tr>
<td>PTP</td>
<td>protein synthesis phosphatase</td>
<td></td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
<td></td>
</tr>
<tr>
<td>RAS</td>
<td>renin-angiotensin system</td>
<td></td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
<td></td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
<td></td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
<td></td>
</tr>
<tr>
<td>s.e.m.</td>
<td>standard error of mean</td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>Spontaneous Hypertensive Rats</td>
<td></td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducers and activators of transcription</td>
<td></td>
</tr>
<tr>
<td>STZ</td>
<td>streptozotocin</td>
<td></td>
</tr>
<tr>
<td>TA</td>
<td>total binding</td>
<td></td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N', N'-tetraethylethylenediamine</td>
<td></td>
</tr>
<tr>
<td>TOP</td>
<td>thimet oligopeptidase</td>
<td></td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>Tris(hydroxymethyl)aminomethane hydrochloride</td>
<td></td>
</tr>
<tr>
<td>TXA</td>
<td>Thromboxane</td>
<td></td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
<td></td>
</tr>
<tr>
<td>µg/µl</td>
<td>microgram/microliter</td>
<td></td>
</tr>
<tr>
<td>µl</td>
<td>microliter</td>
<td></td>
</tr>
<tr>
<td>µM</td>
<td>micromolar</td>
<td></td>
</tr>
<tr>
<td>vs.</td>
<td>versus</td>
<td></td>
</tr>
<tr>
<td>w/v</td>
<td>weight/volume</td>
<td></td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
<td></td>
</tr>
<tr>
<td>WKY</td>
<td>Wistar Kyoto</td>
<td></td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
<td></td>
</tr>
<tr>
<td>2K1C</td>
<td>2 kidney 1 clamped</td>
<td></td>
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INTRODUCTION

1.1. Renin-angiotensin system

The renin-angiotensin system (RAS) is a bioenzymic cascade that plays an integral role in cardiovascular homeostasis by influencing the vascular tone, fluid and electrolyte balance and the sympathetic nervous system. Since Braun Menendez and associates (Braun-Menendez et al., 1940) first showed that angiotonin was the pressor substance formed in canine blood during renal artery clamping, the RAS has been the topic of intense research and the target of medical approaches to control high blood pressure. The biological actions of the RAS are mediated primarily by the highly active octapeptide, angiotensin II (Ang II). Traditionally, the RAS was viewed as a circulating endocrine system, whereby renin released from juxtaglomerular cells of the kidney cleaves the liver-derived macroglobulin precursor angiotensinogen to produce the inactive decapeptide angiotensin I, which is then converted to the active octapeptide Ang II by angiotensin-converting enzyme (ACE) within the pulmonary circulation (Figure 1.1) (Diem et al., 2001).

![Figure 1.1 Classical pathway of RAS.](image-url)
In addition to the systemic (circulating) RAS, there is also evidence to indicate that many tissues, including the vasculature, heart, kidney and brain, are capable of producing Ang II, which may thereby mediate autocrine, paracrine and intracrine effects (Diem et al., 2001; Johnston, 1992). Numerous studies have also shown that the requisite components of the RAS, such as angiotensinogen, renin, and ACE are present in such tissues (Vinson et al., 1995).

1.1.1. Angiotensin II

Ang II is derived from proteolytic cleavage of the hystidyl-leucine moiety from the Ang I by ACE. ACE, a proteolytic enzyme, is both generated by and resides in the lungs, the endothelial cells of the vasculature, and cell membranes of the kidneys, heart, and brain. Thus Ang II is produced in a number of organs via the systemic delivery of Ang I, the circulating component of the RAS (Perazella and Setaro., 2003). However, it is also important to recognize that non-renin and non-ACE pathways exist and are capable of producing Ang II either directly from angiotensinogen or from Ang I. This non-renin and non-ACE pathways included chymase, cathepsin G, chymostatin-sensitive Ang II–generating enzyme (‘CAGE’), tissue plasminogen activator and tonin (Diem et al., 2001; Zitnay and Siragy, 1998). These alternate pathways of Ang II formation are upregulated, especially in the vascular endothelium, by a diversity of stimuli including mechanical stretch, turbulence, and physical injury (Diem et al., 2001; Urata et al., 1995; Urata et al., 1996).

Many organs and tissues also innately possess angiotensinogen, ACE, and the alternate pathway enzymes noted above. The presence of these components in the individual organ permits the local generation of Ang I and Ang II to occur independently of
systemic Ang I synthesis or delivery. Thus, tissue RAS permits Ang II synthesis to continue in the absence of circulating Ang I and allows the autocrine or paracrine effects of Ang II to influence cells at the individual organ level (Johnston, 1992; Perazella and Setaro, 2003).

Ang II is a potent pressor agent, considerably more potent than noradrenaline. A large component of the pressor response to intravenous Ang II is due to direct contraction of arteriolar smooth muscle. Ang II has been demonstrated to exert direct vasoconstrictor actions via angiotensin receptor subtype I (AT$_1$) in various vascular beds, including renal vasculature, and in the coronary and mesenteric arteries. It also increases blood pressure through its actions on the brain and the autonomic nervous system. In particular, it acts centrally to increase sympathetic outflow and peripherally to facilitate sympathetic transmission. This is accomplished by increasing the release and reducing the reuptake of noradrenaline, at adrenergic nerve terminals (Chiu et al., 1991).

In addition to its vasoconstrictor effects, Ang II also has a less important direct positive inotropic action on the heart. The pressor response to angiotensin is usually accompanied by little or no reflex bradycardia because the peptide acts on the brain to reset the baroreceptor reflex control of heart rate to a higher pressure (Reid, 1998). Acting on the adrenal cortex, Ang II stimulates aldosterone biosynthesis, which in turn increases the renal sodium reabsorption. Ang II acts on the kidney to cause renal vasoconstriction, increase proximal tubular sodium reabsorption and inhibit the secretion of renin (Reid, 1998). Moreover, angiotensin II is mitogenic for vascular and cardiac muscle cells and may contribute to the development of cardiovascular hypertrophy (Dostal, 2000; Reid, 1998).
1.1.2. Other bioactive Angiotensin Peptides

With the identification and isolation of other bioactive angiotensin fragments, the classical concept of RAS has been transformed but with the common agreement of Ang II being the principal effector peptide (Moeller \textit{et al.}, 1998; Siragy, 2000). Four angiotensin fragments are of biological interest: angiotensin III, which is obtained by deletion of the N-terminal aspartic acid from angiotensin II; angiotensin IV, which is obtained by deletion of the N-terminal arginine from angiotensin III; angiotensin 1-7, which is obtained by deletion of the C-terminal phenylalanine from angiotensin II; and des-Asp-angiotensin I, which is obtained by deletion of the N-terminal aspartic acid from Ang I (Figure 1.2).
Angiotensinogen

\[ \text{Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Asn} \]

\[ \text{Angiotensin I} \]

\[ \text{Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu} \]

Des-Leu\(^0\)

\[ \text{Angiotensin I} \]

\[ \text{Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His} \]

Angiotensin I-7

\[ \text{Asp-Arg-Val-Tyr-Ile-His-Pro} \]

Angiotensin II (1-4)

\[ \text{Asp-Arg-Val-Tyr} \]

\[ \text{Angiotensin II} \]

\[ \text{Arg-Val-Tyr-Ile-His-Pro-Phe} \]

\[ \text{Angiotensin III} \]

\[ \text{Arg-Val-Tyr-Ile-His-Pro-Phe} \]

\[ \text{Angiotensin IV} \]

\[ \text{Val-Tyr-Ile-His-Pro-Phe} \]

Figure 1.2. Various enzymatic pathway of angiotensin I and angiotensin II degradation.

Abbreviations are: ACE, angiotensin converting enzyme; APA, aminopeptidase A; APN, aminopeptidase N; APB, aminopeptidase B; PEP, propylendopeptidase; NEP, neutral endopeptidase; DPA III, dipeptidylaminopeptidase III; TOP, thimet oligopeptidase; CPA, carboxypeptidase A (Ardaillou and Chansel, 1997).
1.1.2.1. Angiotensin III

Angiotensin III formation in vivo occurs by selective cleavage of the N-terminal aspartic acid from Ang II by the action of aminopeptidase A (APA) (Reaux et al., 1999; Zini et al., 1996). Alternatively, this heptapeptide could also be formed from the removal of N-terminal aspartyl moiety from Ang I to form des-Asp-angiotensin I first, which is then hydrolysed by ACE to produce Ang III, bypassing the formation of Ang II (Garcia et al., 1981). The presence of equal amount of Ang II and Ang III in the rat blood emphasized its importance in RAS (Garcia et al., 1981).

Whereas Ang II is generally considered more potent than Ang III, Wright et al., (1985) first reported that in the central nervous system (CNS), Ang III is equipotent to Ang II. Moreover, since the half-life of intracerebroventricular (i.c.v.) Ang III is 2–3-fold shorter than that of i.c.v. Ang II, it was suggested that Ang III is more potent than Ang II in the CNS (Song et al., 1997). Studies have shown that Ang III exerts some actions similar to Ang II, such as vasoconstriction and pressor response (Reaux et al., 2001; Wright et al., 1985), stimulation of aldosterone secretion (Blair-West et al., 1971) and dipsogenic activity (Wright et al., 1985).

In the peripheral target tissues, Ang III is generally less potent than Ang II because it is rapidly degraded due to the large amount of aminopeptidase N (APN), which metabolizes Ang III, but not Ang II, to angiotensin IV (Ahmad and Ward, 1990). Furthermore, Ang III has also been suggested as an important mediator of some of the central action of Ang II as studies revealed that Ang II-induced vasopressin secretion in rats depends on its conversion to Ang III (Zini et al., 1996). In addition, Ang III
functions as a product inhibitor of ACE activity (Tsai et al., 1975), to act as a negative feedback mechanism, conceivably to limit the formation of Ang II.

1.1.2.2. Angiotensin IV

Angiotensin IV (Ang IV) is formed from the action of APN on Ang III (Zini et al., 1996). Up to a time, Ang IV was thought to have no biological action as this peptide displays very poor affinities on the two classes of angiotensin receptor, AT\textsubscript{1} and AT\textsubscript{2} (Ardaillou and Chansel, 1997). However, further investigations have revealed that Ang IV acts as a weak agonist and that its specific properties were mediated via its own putative receptor (Chai et al., 2004; Hall et al., 1993). Ang IV is present in the blood at lower concentrations than Ang II (Cain et al., 1969). It is also formed locally in the brain and the peripheral tissues where it exerts pleiotropic effects (Ardaillou and Chansel, 1997).

Based on the study by Wright et al. (1993), Ang IV administered in the central ventricles activates neuronal pathways and is implicated in learning, memory and exploratory behaviour. Studies have shown that Ang IV causes endothelium-dependent vasodilatation in rabbit arterioles (Haberl et al., 1991), renal circulation (Harding et al., 1994) and cat mesenteric vascular bed (Champion and Kadowitz, 1997). However, it behaves as a vasoconstrictor in other vascular beds including the pulmonary circulation in the cat (Cheng et al., 1994) and in the rat (Nossaman et al., 1995) as well as the hindlimb vascular bed of the cat (Garrison et al., 1995). These opposite results are probably due to the fact that Ang IV may mimic some Ang II effects due to its low affinity towards AT\textsubscript{1} receptors. This conclusion is confirmed by the inhibitory effect of losartan on Ang IV-dependent vasoconstriction. Recently, work by Li et al., (2006) also
demonstrated that Ang IV induces renal cortical vasoconstriction by interacting with AT\textsubscript{1} receptor-activated signaling. In contrast, the vasodilatory effects of Ang IV are likely to be mediated by AT\textsubscript{4} receptor.

Several studies have concluded that Ang IV causes cell hypertrophy and hyperplasia either alone or in cooperation with other growth factors (Wang \textit{et al.}, 1995a; Wang \textit{et al.}, 1995b) by stimulating RNA and DNA synthesis in cardiac fibroblasts. In contrast, Ang IV antagonized Ang II-induced increase in RNA and protein synthesis in chick heart cells (Baker and Aceto, 1990). This peptide has a short half-life and is rapidly converted to inactive fragments by enzymes such as aminopeptidases, endopeptidases and carboxypeptidases.

1.1.2.3. Angiotensin 1-7

Angiotensin 1-7 (Ang 1-7) is synthesized from Ang II or directly from Ang I bypassing the synthesis of Ang II. The synthesis of Ang 1-7 from Ang I requires three tissue endopeptidases, namely neprilysin [EC 3.4.24.11], propyl endopeptidase [EC 3.4.24.26] and thimet oligopeptidase [EC 3.4.24.15] (Ferrario \textit{et al.}, 1997; Kucharewicz \textit{et al.}, 2002). Neprilysin is primarily responsible for Ang 1-7 production in the circulation and vascular endothelium; whereas the two other alternate enzymic pathways are more active in tissues such as kidney, brain and smooth muscles (Ferrario and Chappell, 2004). The conversion of Ang II to the Ang 1-7 is owing to postproline carboxypeptidase (Kucharewicz \textit{et al.}, 2002). The metabolism of Ang 1-7 involves ACE that cleaves it to Ang 1-5 and Ang 3-5, and aminopeptidases that are responsible for the degradation of the parent peptide to angiotensin 2-7 and angiotensin 3-7 (Kucharewicz \textit{et al.}, 2002; Santos \textit{et al.}, 2000).
Ang 1-7 was initially thought to be an inactive fragment since it did not reproduce the vasoconstrictor, dipsogenic and aldosterone-secreting effects of Ang II. However, works conducted since then have proven that Ang 1-7 is a biologically active peptide with properties that are distinct from those of Ang II and even often opposite to them. Ang 1-7 exerts effects in the kidney, the vessels and the brain. In contrast to vasoconstrictor effect of Ang II, it acts as a vasodilator agent in many vascular beds including porcine (Porsti et al., 1994), canine coronary (Broshinan et al., 1996) and feline mesenteric arteries (Osei et al., 1993). This heptapeptide has also been shown to act as an antiproliferative agent (Machado et al., 2001; Strawn et al., 1999; Tallant et al., 1999), antithrombotic peptide (Kucharewicz et al., 2000) and also increases diuresis and natriuresis (Handa et al., 1996). These biological effects are mediated by a specific non-A\textsubscript{T}1, non-A\textsubscript{T}2 receptor and are, in part, indirect since they often require the initial formation of prostaglandins and/or nitric oxide (Ambuhl et al., 1994; Tallant et al., 1997).

To date, Ang 1-7 receptor has not been cloned. However, its existence cannot be entirely excluded, since there are evidences that some of the effects of this peptide are reversed by Ang 1-7 receptor antagonist, A-779 (Ambuhl et al., 1994; Vallon et al., 1998). In a study by Tallant et al., (1997), a unique receptor, distinct from AT\textsubscript{1} and AT\textsubscript{2} that preferentially binds Ang 1-7 has been demonstrated in cultured bovine aortic endothelial cells. These characteristics of Ang (1-7) receptor have also been described in several other preparations (Broshinan et al., 1998; Neuss et al., 1994; Nickenig et al., 1997). On the other hand, the study by Ueda et al., (2000) has raised the possibility that Ang 1-7 acts as an endogenous antagonist of AT\textsubscript{1} receptor or it may modulate the effect of Ang II via AT\textsubscript{1} receptor. Also in human arteries, Ang 1-7 antagonized the
Ang–II evoked vasoconstriction by non-competitive blockade of AT₁ receptor (Roks et al., 1999).

In several studies, the actions of Ang 1-7 are shown to be mediated by kinins (Broshinan et al., 1996; Li et al., 1997). This peptide may potentiate the endogenous kinins or it may act by cross-talk mechanism involving bradykinin B₂ receptors (Fernandes et al., 2001). This interaction with kinins has been related to its antihypertensive properties and further illustrates its importances in the RAS.

1.1.2.4. des-Asp-angiotensin I

Des-Aspartate-angiotensin I (DAA-I) is an endogenous angiotensin peptide. It is formed by an alternative degradation pathway of Ang I to Ang III that bypasses the formation of Ang II (Blair-West et al., 1971). Homogenates of endothelium, vascular smooth muscle and hypothalamus was found to convert exogenous Ang I to DAA-I instead of Ang II (Sim, 1993; Sim and Qiu, 1994) and the enzyme responsible for this conversion was found to be a novel specific aminopeptidase (named aminopeptidase X) that is not inhibited by EDTA, bestatin or amastatin (Sim et al., 1994). The existence of this specific pathway and increasing functional evidence implicates DAA-I as a functional peptide of the RAS.

The activity of aminopeptidase X was found to be high in the hypothalamus of SHR indicating the involvement of DAA-I in the central regulation of blood pressure and a possible role in hypertension (Sim and Qiu, 1994). This speculation is further supported by the ability of DAA-I to attenuate the central pressor action of Ang II and III in the rats (Sim and Radhakrishnan, 1994), inhibit the electrically induced contraction of the
rabbit pulmonary artery (Sim and Soh, 1995), attenuate Ang III contractile responses in the rat aortic rings (Lim and Sim, 1998) and reduce cardiac hypertrophy in rats (Sim and Min, 1998). DAA-I has also been demonstrated to reduce infarct size in ischemic reperfused rat heart (Wen et al., 2004) and reduce the Ang II-induced phenylalanine incorporation in cultured rat cardiomyocytes (Min et al., 2000).

In in vitro studies, Sim and Chai, (1996) and Sim and Soh, (1995), demonstrated that the actions of DAA-I could be inhibited by losartan but not PD123319, indicating that its actions are mediated by the angiotensin AT$_1$ receptor. Further studies using radioimmunoassay in the rat cardiac ventricles revealed that DAA-I bind to angiotensin receptors (Sim and Min, 2005). It could also be postulated that DAA-I plays a role in mediating the therapeutic effects of ACE inhibitors as plasma level of DAA-I was markedly increased in subjects receiving ACE inhibitor therapy (Lawrence et al., 1990).

1.2. Angiotensin receptors

The vascular actions of angiotensin peptides are mediated by receptors found on the endothelial and vascular smooth muscle cells. These angiotensin receptors may bind to multiple receptor subtypes that may exhibit different and sometimes opposing actions. The main angiotensin peptide, Ang II mediates its effect via at least two high-affinity plasma membrane receptors, which are coupled to different effector systems and elicit different biological responses.

These receptor subtypes have been classified by the use of non-peptide Ang II antagonists; namely subtype 1 angiotensin receptor (AT$_1$), which is selectively blocked by biphenyylimidazoles such as losartan (Dup753) (Chui et al., 1988) and subtype 2
angiotensin receptor (AT\textsubscript{2}), which is blocked by tetrahydroimidazopyridines represented by PD 123319 (de Gasparo \textit{et al}., 1995). Both receptor subtypes have been cloned and pharmacologically characterized (Mukoyama \textit{et al}., 1993; Murphy \textit{et al}., 1991). As more and more works are been conducted in this area, other angiotensin receptors have been described, like AT\textsubscript{3}, AT\textsubscript{4} and AT\textsubscript{1-7} (de Gasparo \textit{et al}., 2000).

1.2.1. AT\textsubscript{1} receptor

AT\textsubscript{1} receptor is responsible for the majority of the effects of angiotensin II: vasoconstriction, sodium re-absorption, cell proliferation, extracellular matrix formation, inflammatory response and oxidative stress (de Gasparo \textit{et al}., 2000, de Gasparo, 2002). The gene for the AT\textsubscript{1} receptor was cloned from rat smooth muscle cells (Murphy \textit{et al}., 1991) and bovine adrenal gland (Sasaki \textit{et al}., 1991). The distribution of AT\textsubscript{1} receptor has been extensively studied in human and animals. AT\textsubscript{1} receptors are primarily found in the brain, adrenals, heart, vasculature and kidney and also been demonstrated in the central nervous system of the rat (Song \textit{et al}., 1992), rabbit (Aldred \textit{et al}., 1993) and human (MacGregor \textit{et al}., 1995). AT\textsubscript{1} receptors in the vasculature, including aorta, pulmonary and mesenteric arteries, are present in high density on smooth muscle cells and low levels in the adventitia (Zhou \textit{et al}., 1995).

The human AT\textsubscript{1} receptor contains 359 amino acids and gene coding for the AT\textsubscript{1} receptor is localized on chromosome 3 (Guo \textit{et al}., 1994). In contrast, there are two isoforms of the AT\textsubscript{1} receptor in rodents, termed AT\textsubscript{1A} and AT\textsubscript{1B} (Iwai and Inagami, 1992). In the rat, the AT\textsubscript{1A} gene is localized to chromosome 17 and the AT\textsubscript{1B} gene to chromosome 2 (Szpirer \textit{et al}., 1993). These isoforms share 95% homology in their
amino acid sequence, exhibit similar ligand binding and signal transduction properties but differ in their tissue distribution and transcriptional regulation.

AT$_{1A}$ receptors are found predominantly in kidney, lung, liver and vascular smooth muscle, whereas AT$_{1B}$ receptors are expressed mainly in the adrenal and anterior pituitary glands (Diem et al., 2001). Cloning of AT$_1$ receptors has revealed that they are members of the guanyl nucleotide-binding protein (G protein)-coupled receptor (GPCR) superfamily (Vaughan, 1998). AT$_1$ mediates its vascular action through several different second messengers. There are five classical signal transduction mechanisms for the AT$_1$ receptor. They are activation of phospholipase A$_2$, phospholipase C, phospholipase D and L-type Ca$^{2+}$ channels and inhibition of adenylate cyclase (Diem et al., 2001).

### 1.2.2. AT$_2$ receptor

The AT$_2$ receptor is characterized by its high affinity for PD 123319, PD 123177 and CGP42112, and very low affinity for losartan and candersertan (Timmermans et al., 1992). Ang II binds to AT$_2$ receptor with similar affinity as to the AT$_1$ receptor. In contrast to the AT$_1$ receptor, the AT$_2$ receptor is not inhibited by dithiothreitol (DTT) or GTP analogues (de Gasparo et al., 1995). The reducing agent DTT was used as a discriminator of the AT$_1$ and AT$_2$ receptors, before the isoform-specific antagonist became available. The DTT sensitive Ang II receptor turned out to be the AT$_1$ receptor, whereas DTT increased the ligand binding capability of the AT$_2$ receptor and allows binding to AT$_2$ receptor to remain stable for hours (de Gasparo et al., 2000). The gene for this receptor resides as a single copy on the X chromosome (Lazard et al., 1994). The AT$_2$ receptor is a seven-transmembrane – type G protein coupled receptor
containing 363 amino acids. It has a low amino acid sequence homology (34%) with AT₁ receptors (Mukoyama et al., 1993).

The AT₂ receptor is highly expressed during foetal development but rapidly declines at birth (de Gasparo et al., 2000). Recent studies have demonstrated that the AT₂ receptor is involved in the production of cGMP, nitric oxide (NO) and prostaglandin F₂α in the kidney, suggesting an important role in the renal function, including vasodilatation and blood pressure regulation. It has also been shown to mediate vascular repair, promote nerve generation and neuronal differentiation and act as an anti-proliferative agent (Diem et al., 2001). The expression of the AT₂ has been shown to be upregulated in pathological condition, such as renal failure (Chung and Unger, 1998), heart failure (Ohkubo et al., 1997), myocardial infarction (Nio et al., 1995), diabetes (Hakam et al., 2000) and wound healing (Kimura et al., 1992).

Various second messengers coupled to the AT₂ receptor has been described, including indirect negative coupling to guanylate cyclase (inhibition of cGMP production) (Bottari et al., 1992) and activation of potassium channels (Kang et al., 1993; Kang et al., 1994). Recently, there have been new insights into the AT₂ receptor signaling pathways, including activation of protein phosphates and protein dephosphorylation, the NO-cGMP system, and phospholipase A₂ (Nuoet and Nahmias, 2000) (Figure 1.3).
Figure 1.3. Signal transduction mechanisms and physiological effects mediated by the AT$_2$ receptor. Abbreviation: PLA$_2$, phospholipase A$_2$; PTP, protein synthesis phosphatase; PP2A, serine/threonine phosphatase 2A; ERK, extracellular signal regulated kinase (Reproduced from Nuot et and Nahmias, 2000).

1.2.3. AT$_3$ receptor

The AT$_3$ receptor subtype, initially described in the neuro2A neuroblastoma cell line (Chaki and Inagami, 1992) is peptide-specific, recognizing mainly Ang II or Ang III.
This subtype does not bind non-peptide ligand such as losartan and PD123319, and has only been observed in cell lines (Chaki and Inagami, 1992). However, this receptor has not been cloned and not much information is available.

### 1.2.4. AT₄ receptor

The AT₄ receptor binds Ang IV and its specific antagonist is divalinal Ang IV. Evidence of the AT₄ receptor existence has accumulated over recent years, based primarily on the affinity and specificity of radioligand binding to brain, heart and adrenal membranes (de Gasparo, 2000; Mustafa et al., 2001). Albiston et al., (2001) have demonstrated AT₄ receptor as insulin-regulated aminopeptidase (IRAP), an abundant protein that is found in specialized vesicles containing the insulin-sensitive glucose transporter, GLUT4 (Chai et al., 2004; Keller et al., 1995). This receptor is predominantly expressed in brain structures (neurones) and is concerned with learning and memory. High levels of IRAP/AT₄ are also expressed in the heart, placenta, skeletal muscle, kidney and small intestine, whilst lower expression was seen in the brain, liver, testes and spleen (Rogi et al., 1996; Thomas et al., 2003). Besides learning and memory, other putative physiological functions associated with AT₄ receptor includes regulating blood flow, inhibiting Na⁺ reabsorption in the kidney and blocking cardiac hypertrophy (de Gasparo et al., 2000; Hamilton et al., 2001; Thomas et al., 2003).

### 1.2.5. Ang 1-7 receptor

Although a specific receptor for Ang 1-7 has not been cloned yet, there are several pharmacological and physiological evidence for its existence. The existence of a different receptor and / or differential signal transduction mechanism for Ang 1-7 is
based on the opposing and/or differential actions of Ang II and Ang 1-7 (Chappell et al., 1998; Freeman et al., 1996; Gironacci et al., 2004; Moriguchi et al., 1995; Oliveira et al., 1996; Tallant et al., 1999). These opposing effects are shown to be mediated by an angiotensin receptor distinct from AT$_1$ and AT$_2$ receptors.

Physiological studies using the selective Ang 1-7 receptor antagonist, D-[Ala$^7$]-Ang 1-7 (D-ALA), was demonstrated to antagonize Ang 1-7 actions centrally (Fontes et al., 1994; Fontes et al., 1997; Santos et al., 1994) and peripherally (Gironacci et al., 2004; Hilchey et al., 1995; Santos et al., 1996; Vallon et al., 1998). In line with the functional studies, binding studies in bovine endothelial cell culture (Tallant et al., 1997), SHR aorta (Tallant et al., 1999), and rat mesenteric arteries (Neves et al., 2003) have provided further evidence for the existence of a specific Ang 1-7 receptor.

1.3. AT$_1$ Signalling pathways in the vasculature

AT$_1$ receptors are coupled to multiple, distinct signal transduction processes, leading to diverse biological actions. This complex and highly regulated cascade of intracellular transduction leads to short term vascular effects, such as contraction, and long-term biological effects, such as cell growth, migration, extracellular matrix deposition and inflammation (Touyz and Schiffrin, 2000). The signal transducers associated with AT$_1$ receptor include phospholipase A$_2$ (PLA$_2$), phospholipase C (PLC), phospholipase D (PLD) and adenylate cyclase. These signaling processes are multiphasic with distinct temporal characteristics. Immediate, early and late signaling events occur within seconds, minutes and hours, respectively (Figure 1.4).
One of the earliest detectable events resulting from Ang II stimulation of vascular smooth muscle cells is a rapid PLC–dependent hydrolysis of inositol phospholipids to generate water soluble inositol 1,4,5-triphosphate (InsP$_3$) and membrane bound diacylglycerol (DAG) (Diem et al., 2001; Griendling et al., 1988; Touyz and Schiffrin, 2000). There are at least three isoforms of PLC: PLC-β, PLC-γ and PLC-δ (Rhee and Choi, 1992). All three isoforms have been identified in vascular smooth muscle cells (VSMCs). PLC-β appears to be important in the rapid generation of InsP$_3$ (within 15s), whereas PLC-γ seems to play a role in the late phase of InsP$_3$ formation (Ushio-Fukai et al., 1998). InsP$_3$ binds to InsP$_3$ receptor on the sarcoplasmic reticulum in VSMCs and mediates the phasic release of intracellularly stored Ca$^{2+}$. Together with calmodulin, Ca$^{2+}$ activates myosin light chain kinase that catalyses the phosphorylation of myosin which results in cellular contraction (Adelstein and Eisenberg, 1980; Greindling et al., 1988). DAG exerts its effect by activating protein kinase C (PKC), which contributes to the tonic, sustained cellular contraction by activation of Na$^+$/H$^+$ exchanger and the resultant intracellular alkalinization (Bazan et al., 1992; Greindling et al., 1988; Touyz et al., 1999b).

In addition to the rapid signaling events described above, early signaling processes, such as activation of PLA$_2$ and arachidonic acid (AA) metabolism are stimulated by AT$_1$ activation within minutes (Bonventre, 1992; Rao et al., 1994). Released AA is processed by cyclooxygenases, lipoxygenases, or cytochrome P450 oxygenases to many different eisonoids in vascular and renal tissues and influences the blood pressure regulation (Nasjletti, 1998). Cyclooxygenase catalyzes the formation of prostaglandin H$_2$ (PGH$_2$), which is subsequently converted to thromboxane (TXA), prostacyclin (PGI$_2$), or prostaglandin E$_2$, D$_2$, F$_{2a}$ (PGE$_2$, PGD$_2$, PGF$_{2a}$) by different enzymes (Smith et al., 1991).
Phospholipase D (PLD), hydrolyzes phospholipids (mainly phosphotidylcholine) to
generate phosphatidic acid. This is followed by subsequent generation of DAG by
phosphatidic acid phosphohydrolase (Billah, 1993). DAG contributes to prolonged
activation of PKC. This pathway probably represents the major cascade by which Ang
II-induced activation of PKC remains sustained in VSMCs. Ang II-induced PLD
signaling has been implicated in cardiac hypertrophy, proliferation of VSMCs (Dhalla et
al., 1997; Morton et al., 1995) and in cardiac contraction (Xu et al., 1996). The long-
term signaling pathways associated with Ang II-stimulated growth and remodeling in
the cardiovascular system are dependent, in part, on PLD mediated responses.

Ang II-mediated stimulation of AT₁ receptor coupled to G protein family (G_{i/o}) can also
inhibit adenylate cyclase in several target tissues, including liver, kidney, and adrenal
glomerulosa, thereby attenuating the production of second messenger cAMP (Diem et
al., 2001). cAMP is a vasodilator and when its production is decreased due to AT₁
receptor activation, vasoconstriction ensues. Moreover, the AT₁ receptor is also
involved in the opening of Ca^{2+} channels and influx of extracellular Ca^{2+} into cells. This
mechanism has been linked to Ang II mediated vasoconstriction (Ruan and Arendshorst,
1996).

It has been reported that activation of AT₁ stimulates growth factor pathways, such as
tyrosine phosphorylation, leading to downstream proteins, including mitogen-activated
protein (MAP) kinases, Janus kinases (“JAK”), and the signal transducers and activators
of transcription (‘STAT’) proteins (Marrero et al., 1995; Schieffer et al., 1996). Activation of these protein kinases results in increased expression of early response
genes, such as c-fos, c-jun and c-myc, which control thymidine incorporation, cellular
proliferation and growth (Berk and Corson, 1997; Hefti et al., 1997). Amongst many of
the G-proteins, AT\textsubscript{1} receptor seems to be the most important in the VSMC regulation. The selective importance of Ang II actions via AT\textsubscript{1} may be due to its ability to amplify its vascular responses via other agonists. The end result, contraction, hypertrophy, or proliferation is determined by the selective activation of multiple signaling pathways.

![Diagram of signal transduction mechanisms and physiological effects mediated by AT\textsubscript{1} receptor](image)

**Figure 1.4.** Signal transduction mechanisms and physiological effects mediated by AT\textsubscript{1} receptor. Abbreviation: PLA, phospholipase A; PLC, phospholipase C; JAK, Janus kinase; STAT, signal transducers and activators of transcription; InsP\textsubscript{3}, inositol 1,4,5-triphosphate; DAG, diacylglycerol; MAP, mitogen activating protein; PKC, protein kinase C (Reproduced from Nuoet and Nahmias, 2000).
1.4. Diabetes mellitus

Diabetes is reaching epidemic proportion in developed countries. An estimated 30 million people world-wide had diabetes in 1985. By 1995, this number had shot up to 135 million. This is estimated to increase to at least 300 million by 2025. According to a World Health Organization (WHO) survey, in Malaysia alone about 942,000 had diabetes in year 2000 and is estimated to increase up to 2,479,000 by the 2030. Diabetes mellitus is a group of metabolic diseases characterized by high blood sugar (glucose) levels, which result from defects in insulin secretion, or action, or both. Any patient with two fasting plasma glucose levels of 126 mg/dL (7.0mmol/L) or greater is considered to have diabetes mellitus (Alberti and Zimmet, 1998; World Health Organization, 1999). Hyperglycemia and other related disturbances in the body’s metabolism can lead to serious damage to many of the body’s systems, especially the nerves and blood vessels. Loss of modulatory role of the endothelium may be a critical and initiating factor in the development of diabetes vascular disease.

There are 2 forms of diabetes: insulin dependent diabetes mellitus (IDDM, also called type I diabetes) and non-insulin dependent diabetes mellitus (NIDDM, also called type II diabetes). Type I diabetes is generally characterized by the sudden onset of severe symptoms. The type I diabetes patients depend on exogenous insulin to sustain life, are prone to ketosis, all of which are caused by absolute insulin deficiency. IDDM is the most prevalent type of diabetes among children and young adults; it was formerly termed ‘juvenile diabetes’. The etiology of IDDM is still unclear, but the disease is associated with various genetic and environmental factors (Harris and Zimmet, 1997).
Type II diabetes greatly outnumbers all other forms of diabetes. In type II diabetes, patients can still produce insulin, but the amount is relatively inadequate for their body’s needs, particularly in the face of insulin resistance. Type II diabetes occurs mostly in individuals over 30 years old and the incidence increases with age. There is a strong genetic component to developing this form of diabetes and also other risk factors - the most significant of which is obesity. There is a direct relationship between the degree of obesity and the risk of developing type II diabetes. It is estimated that the chance to develop diabetes doubles for every 20% increase over desirable body weight. A third type of diabetes, gestational diabetes mellitus (GDM), develops during some cases of pregnancy but usually disappears after pregnancy (Alberti and Zimmet, 1998).

In patients with diabetes, an increased blood glucose concentration (hyperglycemia) causes thirst, increased appetite and increased urine volume, but it is the chronic complications of diabetes that are the major health issues. These chronic complications are related to blood vessel diseases and are generally classified into small vessel disease, such as those involving the eyes, kidneys and nerves (microvascular disease), and large vessel disease involving the heart and blood vessels (macrovascular disease). Diabetes accelerates the hardening of the arteries (atherosclerosis) of larger blood vessels, leading to coronary heart disease (angina or heart attack), strokes, and pain in the lower extremities because of lack of blood supply (Wei et al., 2003).

1.5. Hypertension

Hypertension is a common disorder, affecting nearly 60 million individuals in the United States (Hajjar and Kotchen, 2003) and 972 million worldwide (Kearney et al., 2005). Two forms of hypertension have been described: - essential (or primary)
hypertension and secondary hypertension. Essential hypertension is a far more common condition and accounts for 95% of hypertension. Aetiologic factors for hypertension include environmental factors, such as high salt intake and lack of exercise. Obesity and genetic factors also play a role. In most cases, however, no clear cause can be identified, hence, essential hypertension, which represents a vast majority of the cases of hypertension, has been hypothesized to be caused by multiple factors acting in concert (Cohuet and Struijker-Boudier, 2006). In secondary hypertension, which accounts for about 5% of all cases of hypertension, the high blood pressure is secondary to (caused by) a specific abnormality in one of the organs or systems of the body.

Hypertension is characterized by a normal cardiac output and elevated arterial pressure (Izzard and Heagerty, 1995). The elevated arterial pressure of hypertension is usually caused by an increased total peripheral resistance (Bohr and Webb, 1988). This increase in total peripheral resistance could be the result of (1) structural alterations of the blood vessel, (2) increases in neural or humoral vasoconstrictor influences, or (3) intrinsic changes in reactivity of the vascular smooth muscle cells (Holloway and Bohr, 1973). Moreover, genetic heritability averaging ~30% has been shown and various genes have been linked to the pathogenesis of hypertension (Agarwal et al., 2005).

In many experimental models of hypertension, a decrease in the lumen diameter and an increase in the wall thickness to lumen diameter ratio are seen in resistance vessels (Hughes and Schachter, 1994). The narrowing of these small arteries and arterioles contribute to increased peripheral vascular resistance and consequently to elevated blood pressure. Regression of the abnormal structure of resistance vessels towards normal value with antihypertensive treatments further proves the role of structural alteration in hypertension (Mulvany et al., 1996; Schiffrin et al., 1994). Many studies
have demonstrated an alteration of vascular responsiveness which is connected to endothelial structural and functional damage in hypertension (De Artinano and Gonzalez, 1999; Li et al., 2007; Triggle and Laher, 1985).

1.6. Endothelial dysfunction

The vascular endothelium plays a central role in many aspects of vascular physiology. It responds to flow/shear stress, blood borne agent and cells, and is a source of growth factors, vasoconstrictor and vasodilator substances (Calles-Escandon and Cipolla, 2001; Endemann and Schiffrin, 2004; Hughes and Schachter, 1994). Since the actions of endothelial cells are multiple and involve several systems, alterations in endothelial function may affect one or more of these systems, either simultaneously or at distinct time periods.

Endothelial dysfunction may be defined as an imbalance between synthesis, release or effect of those endothelial factors capable of relaxing vascular smooth muscle, such as prostacyclin, nitric oxide or endothelium derived hyperpolarising factor (EDHF), and vasoconstrictor substances like endothelin-1, thromboxane A\textsubscript{2} (TXA\textsubscript{2}), angiotensin II and PGH\textsubscript{2}. The imbalance manifests itself as a reduction in endothelium dependent vasodilatory response, or as an increased vasoconstrictor response (De Artinano and Gonzalez, 1999).

A major result of endothelial dysfunction is the damage to underlying smooth muscle cell structures that include vascular smooth muscle cells in blood vessels, mesangial cells in renal glomeruli, and pericytes in retinal capillaries. Because of different milieu in large versus small vessels and in one organ versus another, endothelial cells respond
to stress differently and thus, impact differently on the neighboring vascular smooth muscle cells. In blood vessels and kidneys the pathological response of smooth muscle cells includes: hypertrophy and proliferation, enhanced endogenous expression of growth factors, altered contractile matrix material production and altered contractile protein gene expression (Hsueh and Anderson, 1992). The modified endothelial-smooth muscle cell interaction may contribute to the complication of diabetes and hypertension.

The endothelial cells are a target of the diabetic milieu and endothelial dysfunction is thought to play an important role in the vasculopathy of this disease. One of the major symptoms seen in diabetes is hyperglycemia. Hyperglycemia exacerbates the vascular disease associated with diabetes. At high concentration glucose has a direct, toxic effect on vascular endothelial cells. This toxic effect may lead to decreased endothelium-mediated vascular relaxation, increased vasoconstriction and other cellular changes (Sowers and Epstein, 1995). A large body of evidence in humans indicates that endothelial dysfunction is closely associated with microangiopathy and arteriosclerosis in both type I and II diabetes mellitus (Cosentino and Luscher, 1998). In addition, impaired responses to different endothelium-dependent agonists have been repeatedly and consistently demonstrated in different vascular beds of both chemically-induced and genetic models of type 1 diabetes (Fukou et al., 1997; Heygate et al., 1995; Kamata and Hosokawa, 1997; Majithiya and Balaraman, 2006; Tesfamariam et al., 1993). Similarly, impaired endothelium-dependent vasodilatation has also been demonstrated in patients with type I (Clarkson et al., 1996) and type II diabetes (Ting et al., 1996; Williams et al., 1996).

A change in endothelial function is common in patients with essential hypertension and in animals with hypertension, whether primary or induced by a salt-rich diet.
Hypertension affects blood vessels by altering shear stress, which is related to vascular flow, blood viscosity and other factors. Hypertension alters the production and activity of contractile substances, ions, and ion channels and decreases the endothelial response capacity (Hsueh and Anderson, 1992; Schmieder et al., 1997). In hypertensive patients, changes in the synthesis, or the effect of nitric oxide (Kelm et al., 1996; Panza et al., 1993) and an increase in vasoconstriction processes (De Artinano and Gonzalez, 1999) have been demonstrated. Besides this, some studies have demonstrated that descendents of hypertensive patients also display a certain degree of endothelial dysfunction (Lacy et al., 1998; Taddei et al., 1992).

1.6.1. Endothelial dysfunction, RAS and diabetes

Alteration in the endothelial cells affects the RAS system as the angiotensin peptides are synthesized in endothelial cells. Endothelial dysfunction leads to increase and/or decrease in vascular response to angiotensin peptides. In diabetes, in particular, the systemic and local RAS systems may be discordant. The finding of volume expansion and reduced plasma renin activity in diabetes has been interpreted as indicating that the RAS is suppressed in diabetes (Christlieb et al., 1976). Several studies in the 1980s have demonstrated a reduction in plasma renin activity (Funakawa et al., 1983; Ubeda et al., 1988) and plasma Ang II concentrations in STZ-treated rats (Kikkawa et al., 1986). However, as more and more research is being conducted in this area, conflicting results are seen. These conflicting results are attributed to time-dependent changes to activity of the RAS following diabetes induction. A study by Zimpelmann et al., (2000) has demonstrated an increase in plasma and intrarenal Ang II and enhanced mRNA renin level in STZ-induced diabetic rats after 2 weeks induction.
Altered RAS activity could affect regulatory control of the cardiovascular system in diabetes through changes in the direct pressor action of Ang II (Garcia et al., 2003; Kennefick et al., 1996; Muller et al., 1998). Reduced plasma renin activity in diabetes has been interpreted as an indication that the RAS is suppressed in diabetes (Ballermann et al., 1984; Ubeda et al., 1988). Whereas, when the circulating RAS is normal or suppressed, the renal tissue RAS seems to be activated (Anderson et al., 1993; Price et al., 1999). In rats with spontaneous or streptozotocin-induced diabetes, renin mRNA and protein expression have been demonstrated to be increased in both juxtaglomerular and proximal tubule cells, accompanied by a rise in Ang II production (Zimpelmann et al., 2000).

In pathological state, the body tries to adapt to prevent major changes in the body hemodynamics and this modification affects the vascular tone. The changes in vascular tone observed are closely linked to the pressor action of Ang II. Increased (Arun et al., 2005), unchanged (Head et al., 1987) and decreased (Sarubbi et al., 1989; Sharma et al., 1999) pressor action of Ang II have been observed in STZ-induced diabetic rats. This conflicting result depends on the type of vessels (either macro- or micro-) and duration of diabetes. In the kidney, one of the major organs of the RAS, decreased pressor action to Ang II has been observed, as early as 1 week post-STZ induction (Inman et al., 1994). Numerous other studies also have found that infusion of Ang II to the diabetic rat exhibits reduced pressor responsiveness in the kidney (Beenen et al., 1996; Hebden et al., 1987; Jackson and Carrier, 1983). Depressed angiotensin contractile responses in the isolated resistance vessels have also been observed in diabetic patients (McNally et al., 1994).
The ability of ACE and AT$_1$ receptor inhibitors to block and reduce Ang II activity further strengthen the role of RAS in diabetes. RAS inhibitors have been shown to prevent or delay the development of diabetes mellitus and provide better end-organ protection to the kidneys, blood vessels and heart (Ibrahim, 2006). Treatment with losartan has been shown to improve beta-cell function and glucose tolerance in mouse model of type 2 diabetes (Chu et al., 2006). The combined treatment with ACE and angiotensin receptor blocker has also been able to improve the cardiovascular outcome in individuals with diabetes (Abuissa et al., 2005; Kalantarinia and Okusa, 2006; Rosen, 2006).

1.6.2. Endothelial dysfunction, RAS and hypertension

Changes in the endothelial cells function affect the RAS system and vice-versa. Both systemic and local tissue RAS play an important role in regulation of blood pressure. Thus, changes in the RAS affect blood pressure, which subsequently lead to hypertension. Hyperactivity of the RAS has been implicated in the development of hypertension (Lever et al., 1992; Wolf et al., 1996). The study by Han and Sim, (1998), demonstrated that the brain of SHR has a hyperactive RAS compared to the normotensive Wistar-Kyoto rats. Moreover, over-expression of ACE in tissues is linked to various cardiovascular and renal pathological conditions (Ardaillou and Chansel, 1997).

Genetic studies in humans have also demonstrated that particular alleles of the angiotensinogen and ACE gene are linked to higher incidence of hypertension (Juenemaitre et al., 1992). Furthermore, increasing evidence support the notion that AT$_1$-mediated Ang II responses have an important pathological role in cardiovascular
CHAPTER 1

Introduction

and renal diseases. Ang II was found to increase heart, pulmonary and renal vascular
tone (Collis et al., 1980; Kost et al., 1994) of hypertensive rat models (Kim and Iwao,
2000). As more work is being conducted, the levels of other peptides of angiotensin,
such as angiotensin 1-7, were found to be increased in hypertensive condition (Ferrario
et al., 1998; Simoes e Silva et al., 2004). This heptapeptide has been documented to
reduce Ang II pressor action (Gironacci et al., 2004; Hocht et al., 2006; Stegbauer et al.,
2004) and may have antihypertensive property.

The efficacy of ACE inhibition as treatment in many studies further indicates that the
RAS is probably involved in many hypertensive states (Lewis et al., 2006, Yusuf et al.,
1999). Ang II receptor antagonists and renin inhibitors (CGP 44 099 and aliskiren) are
also noted as effective antihypertensive agents (Dendorfer et al., 2005; Gradman et al.,
2005, Staessan et al., 2006; Wood et al., 1990). Besides using drugs, gene therapy using
antisense inhibition of ACE, angiotensinogen or AT\textsubscript{1} receptor genes has been shown to
effectively reduce high blood pressure in animal models of hypertension. These include
a genetic model (SHR), a surgical model - 2 kidney 1 clipped (2KIC), and an
environmental model (cold-induced hypertension). In all the animal models, the authors
demonstrated a decreased blood pressure by about 25 mmHg with a single systemic
administration of antisenses to inhibit ACE and AT\textsubscript{1} receptor genes (Phillips and
Kimura, 2005). These findings indicate that both systemic and tissue RAS play an
important role in the regulation of vascular tone and consequently in the overall blood
pressure.
1.7. Animal models

1.7.1. Animal models of diabetes

Diabetes can be investigated either in animals made diabetic by chemical compounds or using diabetic strains. The diabetogenic chemical compounds may cause specific damage of insulin producing β-cells, a temporary inhibition of insulin release and/ or production or decreased efficacy of insulin in target tissues. Of all compounds, mainly streptozotocin (STZ) and alloxan are used to induce diabetes (Verspohl, 2002). The diabetogenic action of STZ destroys most islet β cells after a single injection and is more effective than alloxan in certain species like guinea pigs, and Syrian hamster (Shafrir, 1999).

<table>
<thead>
<tr>
<th>Chemically induced diabetes</th>
<th>Genetically diabetic animal</th>
<th>Normal diabetic animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Streptozotocin</td>
<td>Rats</td>
<td>Mouse</td>
</tr>
<tr>
<td>b) Alloxan</td>
<td>a) Zucker fa/fa</td>
<td>a) KK</td>
</tr>
<tr>
<td>c) Dithizone</td>
<td>b) BB</td>
<td>b) KK-Ay</td>
</tr>
<tr>
<td>d) Pancreatectimized dog</td>
<td>c) Cohen diabetic</td>
<td>c) NOD</td>
</tr>
<tr>
<td>e) Dexamethasone</td>
<td>d) Obese SHR</td>
<td>d) Ob/ob (Leptin Wellesly)</td>
</tr>
<tr>
<td>f) EMC-D Virus</td>
<td>e) Goto</td>
<td></td>
</tr>
<tr>
<td>g) Insulin antibodies</td>
<td>f) Kakizaki (GK)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>a) Chinese</td>
</tr>
</tbody>
</table>

Table 1.1. Models of diabetic animals (Verspohl, 2002).
STZ is a 2-deoxymethyl-nitrosourea-glycopyronose molecule that produces selective toxic effect in β-cells and induces diabetes mellitus in most adult laboratory animals (Rerup, 1970). An impressive body of knowledge on the mechanism of STZ diabetogenicity has been accumulated. The glucose moiety of STZ directs this agent to the pancreatic β-cells where it binds to a membrane protein, probably the glucose transporter GLUT2 and the highly reactive nitrosourea moiety is responsible for the β-cells toxicity (Shafrir, 1999; Thulesen et al., 1997; Verspohl, 2002).

Since STZ is a nitric oxide (NO) donor and NO was found to bring about the destruction of pancreatic islet cells, it was proposed that this molecule contributes to STZ-induced DNA damage (Kroncke et al., 1995, Morgan et al., 1994). During decomposition of STZ, highly reactive carbonium ions are formed, which causes alkylation of DNA bases. In the following phase of excision DNA repair, the nuclear enzyme poly (ADP-ribose) synthase becomes activated to such an extent that cellular levels of its substrates become critically depleted, leading to cell death (Bolzan and Bianchi, 2002). STZ was found to generate reactive oxygen species, which also contributed to DNA fragmentation and evoked other deleterious changes in the cells (Takasu et al., 1991, Bedoya et al., 1996) (Figure 1.5).
Figure 1.5. Mechanism of action of alloxan, streptozotocin, and other beta-cell toxins, leading to free radical formation, DNA strand breaks, poly(ADP)ribose synthase activation and nicotinamide adenine dinucleotide (NAD) depletion and resulting in irreversible damage to the insulin secretion apparatus (Shafrir, 1997).

STZ administered at different age of the animals will produce a slightly different characteristic distinguishing type I and type II diabetes. A single high dose of STZ (e.g. 90 mg/kg, i.v) given to 2 day old neonatal rats induces β-cells injuries followed by limited regeneration. This model represents type II diabetes mellitus (Arulmozhi et al., 2004). Injecting a high dose of STZ in adult (8-10 weeks old) rats will produce type I diabetes mellitus animal model. One advantage of this model is that it makes it possible to study diabetes mellitus per se, and not diabetes as a consequence of obesity (Shafrir, 1997).
1.7.2. Animal models of hypertension

Hypertension is a multifactorial, polygenic disease that involves complex interactions between genetically determined homeostatic control mechanisms and environmental factors, and its exploration thus requires the availability of experimentally manipulable animal models (Takahashi and Smithies, 2004). The ideal animal model for hypertension research should have human-like cardiovascular anatomy, hemodynamics, and physiology and develop human disease characteristics and complications in a timely or even accelerated fashion. However, no species can consistently answer all of these needs, and experimental design and other constraints often dictate the choice of animal models for specific research applications. Several genetic and non-genetic animal models are available for hypertensive studies as listed in Table 1.2 (Lerman et al., 2005).

The most commonly used hypertensive model is the spontaneously hypertensive rats (SHR). Since the development of this strain from a colony of normotensive Wistar-Kyoto rats (WKY) by Okamoto and Aoki, (1963), it has been widely accepted as the best experimental model there is to represent the human essential hypertension (Udenfriend and Spector, 1972). The major similarities between SHR and essential hypertension patients are that both showed no specific etiology and are genetically predisposed to hypertensive state (Frohlich, 1986). Both are also associated with increased total peripheral resistance, and increased vasoconstriction (Doggrell and Brown, 1998; Trippodo et al., 1978).
### Secondary Hypertension

<table>
<thead>
<tr>
<th>Renovascular</th>
<th>Genetic models</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two-kidneys, one-clip (2K1C)</td>
<td>Phenotype-driven</td>
</tr>
<tr>
<td>One-kidney, one-clip</td>
<td>SHR</td>
</tr>
<tr>
<td>Aortic coarctation</td>
<td>SHR-stroke prone</td>
</tr>
<tr>
<td>Total occlusion</td>
<td>Dahl salt-sensitive rat</td>
</tr>
<tr>
<td>Microembolization</td>
<td>Genetically</td>
</tr>
<tr>
<td></td>
<td>hypertensive rat</td>
</tr>
<tr>
<td></td>
<td>Sabra model</td>
</tr>
<tr>
<td>Renal injury</td>
<td>kidney Lyon</td>
</tr>
<tr>
<td>Page-kidney</td>
<td>hypertensive rat</td>
</tr>
<tr>
<td>Partial or total nephrectomy</td>
<td>Milan SHR</td>
</tr>
<tr>
<td>Injection-induced inflammation</td>
<td>Obesity-related</td>
</tr>
<tr>
<td>Irradiation</td>
<td>Postmenopause-related</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Vasoactive Intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renin-angiotensin-aldosterone</td>
</tr>
<tr>
<td>Nitric oxide inhibition</td>
</tr>
<tr>
<td>Noradrenaline</td>
</tr>
<tr>
<td>Pressor prostaglandins</td>
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<tr>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Endocrine and Dietary</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOCA-salt</td>
</tr>
<tr>
<td>Glucocorticoids</td>
</tr>
<tr>
<td>Adrenal regeneration</td>
</tr>
<tr>
<td>Sex-hormone induced</td>
</tr>
<tr>
<td>Dahl salt-sensitive</td>
</tr>
<tr>
<td>Pregnancy, pre-eclampsia</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Neurogenic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Psychological</td>
</tr>
<tr>
<td>Environmental</td>
</tr>
<tr>
<td>Central NS stimulation</td>
</tr>
<tr>
<td>Baroreceptor denervation</td>
</tr>
</tbody>
</table>

DOCA, deoxycorticosterone-acetate; NS, nervous system; SHR, spontaneously hypertensive rat.

Table 1.2. Various experimental models of hypertension (Lerman et al., 2005).
SHRs have, within each colony, uniform polygenetic disposition and excitatory factors which produce uniform changes in the indirect and direct effects on the cardiovascular system. This lack of inter-individual variation is one of the major advantages of the SHR (Lindpaintner et al., 1992). Another advantage of the SHR is that it follows the same progression of hypertension as human hypertension with pre-hypertensive, developing, and sustained hypertensive phases with each phase lasting at least several weeks (Folkow, 1993). SHR are also therapeutically responsive to ACE inhibitors, (Harrap et al., 1990), AT₁ receptor antagonists (Averill et al., 1994; Li et al., 1997), diuretics and vasodilators (Onaka et al., 1998) and other antihypertensive agents. Moreover, the life span of SHR is reduced by one third of the normal 2.5-3 years of the rat to 1.5-2.5 years, which resemble that in essential hypertension (Okamoto and Aoki, 1963).

However, SHR differs from essential hypertension in that; SHRs reproducibly develop hypertension in young adulthood rather than in middle age as in humans (Doggrell and Brown, 1998). Also, when age matched, the body weight of SHR is usually less than their normotensive control, WKY (Haddad and Garcia, 1996). This is in contrast with human essential hypertension, where the patients are heavier than the normotensive in the population (Frohlich et al., 1981). Despite these differences, SHR is by far the best model to study hypertension as it is a chronic stable model that produces symptoms which are predictable and controllable, thus avoiding difficult or life-threatening technical interventions.
1.8. Summary

The RAS is a highly complex system that generates pleiotropic effects in many target organs, including the brain, kidney, heart and the vasculature. The successful clinical use of ACE inhibitors and AT₁ receptor antagonists suggests the importance of the RAS in the pathophysiology of many cardiovascular diseases. Most of the known effects of the RAS are mediated through the AT₁ receptor. The recent demonstration that there are several pathways for degradation of Ang I and Ang II leading to different bioactive fragments with their own binding sites raises the question of the relative importance of these pathways in regulating angiotensin responses.

The RAS has been revealed to play an essential role in cardiovascular diseases like diabetes and hypertension. Research conducted with angiotensin fragments, especially angiotensin 1-7 has revealed its potential as an antihypertensive agent. Similarly, des-Asp-angiotensin I has been documented to possess vasodepressor action and may possibly help in controlling blood pressure. Understanding the role of the RAS in diseased states may help man to come up with better and safer drugs.
CHAPTER 1  

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1.9. Aims of the present study

Ang II contractile responses have been extensively studied in various vascular tissues in both \textit{in vivo} and \textit{in vitro} experimental conditions. However, data on the contractile responsiveness of the vascular tissues to Ang II has been conflicting, for both hypertensive and diabetes conditions. Given this discordant state, it is of interest to attempt to characterize and compare the vasoconstrictor effect of Ang II with those of other angiotensin peptides, des-Asp-angiotensin I and angiotensin 1-7. Much work has been conducted in conduit vessels (aorta), and a lesser amount was done in resistance vessels to understand the role of these peptides. It is important to look at resistance vessels as well, because small changes in these vessels may significantly affect regulation of the body hemodynamics. Ang (1-7) and DAA-I have been shown to have modulatory actions on angiotensin II and work have been done so far in hypertensive models especially in conduit vessels. The exact mechanism underlying these effects remains uncertain. These modulatory actions involve angiotensin receptors, local mediators released from endothelium or activation or inhibition of specific mechanisms in the signalling pathways. The aims of these studies therefore, are

1) To evaluate the \textit{in vitro} actions of Ang II, DAA-I and Ang 1-7 in renal and mesenteric arterial beds of hypertensive and diabetes rat models.

2) To evaluate the vasomodulatory action of DAA-I and Ang 1-7 on the action of Ang II in the renal and mesenteric arterial beds of hypertensive and diabetic rat models.

3) To explore the possible mechanism involved in the vasomodulatory actions of DAA-I and Ang 1-7 in the renal and mesenteric arterial beds.

4) To explore the involvement of the AT\textsubscript{1} receptor subtype in DAA-I and Ang 1-7 vasomodulatory actions.
CONTRACTILE ACTION OF ANGIOTENSIN PEPTIDES IN RENAL AND MESENTERIC VASCULATURE

2.1. Introduction

Vascular damage underlies many of the major complications of diabetes mellitus such as atherosclerosis, nephropathy and retinopathy (Sowers and Epstein, 1995; Zimmet and Defronzo, 1997). Hypertension accelerates all the vascular complications of diabetes and is by itself, in the absence of obesity or diabetes, associated with insulin resistance (Harris and Zimmet, 1997). RAS has been known to play an important role in both of these diseases. Ang II, the major peptide of RAS has been demonstrated to lead to cardiovascular diseases by changing the hemodynamics of the body i.e. increase in blood pressure and increase contractility (Campbell, 2001).

In SHR, a hypertensive animal model, increase in the vascular tone and contractility has been observed and this increase was mainly correlated with the changes in RAS peptides (Makarious et al., 1993). However, conflicting reports on the contractile responsiveness of vascular tissues to Ang II has been observed (Arun et al., 2005, Beenen et al., 1996; Farina et al., 1996; Campbell, 2001) in various animal models of diabetes.

As more and more research are carried out, the importance of other angiotensin peptides in modulating the RAS system is being revealed. Two of the peptides that are gaining importance in the cardiovascular system are DAA-I and Ang 1-7. DAA-I has been shown to possess contractile action while in contrast, Ang 1-7 has been reported to exert mainly vasodilatory action in many vascular beds. Although the vascular actions of the
peptides have been studied previously, there is not much work done to understand their role in resistance vessels. This lack of information is the basis of the present study, that is to evaluate the *in vitro* action (contractility) of angiotensin peptides (Ang II, DAA-I and Ang 1-7) in two resistant vessels; renal and mesenteric arterial beds. This study provides more conclusive information on the vascular reactivity of these peptides in the renal and mesenteric vasculature from normotensive, hypertensive and diabetic rat models.

### 2.2. Methods

#### 2.2.1. Preparation of normal Krebs

The physiological salt solution used in this study was prepared by dissolving the salts shown in Table 2.1. in 1 liter of distilled water. The solution was freshly prepared before use and kept warm at 37°C, and aerated with 95% O₂ and 5% CO₂. The pH of the Krebs solution was 7.4.

<table>
<thead>
<tr>
<th>Salt (BDH Analar, England)</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>120</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>20</td>
</tr>
<tr>
<td>KCl</td>
<td>4.7</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>1.2</td>
</tr>
<tr>
<td>Glucose</td>
<td>10</td>
</tr>
<tr>
<td>CaCl₂.2H₂O</td>
<td>2.4</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Table 2.1. Composition of the Krebs solution
2.2.2. Animals

Male Wistar-Kyoto (WKY) rats and Spontaneously Hypertensive Rats (SHRs) aged 10 weeks were obtained from the Animal House of the University of Malaya Medical Centre. The animals were then housed in the departmental animal holding room (at a constant temperature (24± 1°C) and 12 hour dark/light cycle) for two weeks adaptation period before initiation of any of the following protocols. The animals were fed standard rat chow (Gold Coin Sdn Bhd, Malaysia) and tap water ad libitum and diabetes was induced at 12 week of age. Approval for the studies was obtained from the Committee for Animal Care and Use at the Laboratory Animal Center of the Faculty of Medicine in the University of Malaya under the reference number FAR/22/10/03/MRM(R). All experimental procedures were carried out according to the guidelines for ethical care of experimental animals.

2.2.3. Induction of diabetes

WKY rats were made diabetic by administration of a single dose of streptozotocin (STZ) (Sigma-Aldrich) 75 mg/kg intraperitoneally (i.p) at 12 weeks old. Age matched controls received equal volume of vehicle. Body weight and blood glucose for each rat were measured three days later and monitored every 2 weeks until the 8th week (i.e. age 20 week). Animals were considered diabetic if their blood glucose levels were > 17 mM. Fasting blood glucose levels were assessed using the Advantage II blood glucose test strips (Roche Diagnostics, Germany). Blood was collected from the rat tail by pricking with a 26G needle at the tip of the tail.
2.2.4. Preparation of isolated perfused renal and mesenteric arterial bed

To isolate the right kidney, rats were anaesthetized with sodium pentobarbitone (60 mg/kg, i.p.). The right kidney was exposed by midline laparotomy. The renal artery was cannulated with a catheter (PET-50, internal diameter .023 inch) via the superior mesenteric artery and *in situ* perfusion was started and maintained until the end of the experiment. The right renal vein and ureter were cut. The right kidney was then ligated, excised and placed in a water jacketed chamber maintained at 37° C and perfused with an oxygenated (95% O₂ and 5% CO₂) Kreb’s solution by means of a peristaltic pump (Minipuls 3 Model 312, Gilson Villiers Le Bel, France) at a rate of 5ml/min.

In order to isolate the mesenteric arterial bed, the remaining length of the superior mesentery artery was cannulated according to the method of McGregor, (1965); a catheter (PET-50) was inserted into the superior mesenteric artery and secured with ligature. Before the perfusion was started, the pancreotico-duodenal, ileo-colic and colic branches of the superior mesenteric artery were ligated to ensure that the perfusion is to the branches supplying the small intestine. The mesentery was carefully excised from the intestine and placed in a water-jacketed chamber maintained at 37° C and perfused with an oxygenated Kreb’s solution by means of a peristaltic pump at a rate of 5ml/min.

Changes in perfusion pressure were measured by means of a pressure transducer (Model P23XL, Ohmeda Medical Devices Division Inc, USA) and recorded via a MacLab data acquisition system (AD Instruments, Australia).
2.2.5. Research protocol

After an equilibration period of 20 min, the preparation was constricted with phenyleprine (PE, $10^{-5}$ M) given by perfusion and the increase in perfusion pressure was recorded until a 5-min plateau was observed. PE ($10^{-5}$ M) was chosen to give a submaximal contraction and prime the tissues. This contractile response to phenylephrine was taken as a unity and responses to other pressor compounds were normalized against this unit. Another equilibration period of 20 minutes was allowed following which the integrity of the endothelium was tested by observing the relaxation to acetylcholine (ACh) ($10^{-4}$ M) in PE-precontracted tissues. The experimental protocols that followed are as described below.

2.2.5.1. Contractile responses to the angiotensin peptides in WKY, SHR and STZ-induced diabetic rats

After testing the endothelium integrity, the preparations were allowed to rest for 30 minutes. This was followed by perfusion with Kreb’s solution that contained 30 µM captopril for another 30 minutes. The presence of the ACE inhibitor, ([2S]-N-[3-Mercato-2-methylpropionyl]-l-proline) (captopril), was essential in evaluating DAA-I response, as it prevents the degradation of this peptide to Ang III (Lim and Sim, 1998). In other experiments, captopril was added to provide a uniform experimental condition. Following 1 hour of basal perfusion, various concentrations ($10^{-13}$ – $10^{-6}$M) of angiotensin peptides (Ang II, Ang 1-7 and DAA-I) were administered to produce concentration-dependent contractile responses for each peptide. In the case where contraction was not observed even at $10^{-6}$M, higher concentrations of the peptide were injected. Each concentration of peptide was administered as a single bolus injection of
20 µl (renal) and 50 µl (mesenteric arterial bed) into the perfusion system. The minimum time interval between successive bolus injections was 10 minutes or until the basal pressure was regained. Each experiment was repeated 5-6 times in individual tissue preparation (different animals).

2.2.6. Drugs

([2S]-N-[3-Mercato-2-methylpropionyl]-l-proline) (Captopril), Angiotensin II, streptozotocin, des-Asp-angiotensin I and Angiotensin 1-7 were purchased from Sigma Chemical Co., (Missouri, USA). Captopril was prepared in dH$_2$O as 0.02M stock. Angiotensin II and Angiotensin 1-7 were prepared in cold dH$_2$O freshly daily. Streptozotocin was prepared fresh and dissolved in normal saline just prior to use.

2.2.7. Statistical analysis

The perfusion pressure of the kidney and mesenteric artery upon drug administration was recorded and then normalized against the pressor response to PE (10$^{-5}$M) and expressed as percentage. Data are presented as mean ± s.e.m. Significant difference between means was evaluated using unpaired Student’s t-test when comparing two groups. Results with p<0.05 were considered statistically significant.
2.3. Results

2.3.1. Preparation characteristics.

The general characteristics of the preparation are summarized in Table 2.2. At a perfusion rate of 5 ml/min for all preparations, the SHR tissues had a higher basal perfusion pressure compared to WKY and STZ-induced diabetic animals in both the renal and mesenteric vasculature. A $10^{-5}$M concentration of PE caused an average perfusion pressure reading of $246.4 \pm 3.23$ mmHg in SHR, $195.3 \pm 1.82$ mmHg in the WKY and $198.2 \pm 1.85$ mmHg in the STZ-induced diabetic kidney. The responses to $10^{-5}$M PE in the mesenteric artery were $100.9 \pm 1.5$ mmHg for the SHR, $87.99 \pm 1.79$ mmHg for the WKY and $84.75 \pm 2.35$ mmHg for the STZ-induced diabetic animals. The limits of detection of the perfusion system were 5-350 mmHg.

In the kidney, WKY demonstrated the highest relaxation to ACh ($78.86 \pm 2.63$ %), followed by STZ-induced diabetic ($55.66 \pm 1.39$ %) and the lowest in the SHR ($40.47 \pm 2.02$ %). The ACh relaxation is shown as percentage drop of the PE contraction. There was no significant difference in ACh relaxation between all three groups in the mesenteric bed where all the groups showed good relaxation (>80 %).

2.3.2. Body weight and blood glucose

At 12 weeks old, the WKY rats and SHR had a body weight of $270 \pm 10$ g and $250 \pm 5$ g respectively. Both groups of rats gained weight consistently throughout the 8 weeks induction period with a significantly ($p<0.01$) higher body weight in WKY rats compared to SHR at 20 weeks of age ($330 \pm 10$ g vs. $280 \pm 10$ g). STZ-induced diabetic
rats had a significant loss in body weight during the 8 weeks period, with the final body weight of $220 \pm 20$ g (Figure 2.1). STZ-induced diabetic rats showed a significant increase in blood glucose 3 days after induction ($25 \pm 5$ mM) and this increase was maintained throughout the 8 weeks period. Blood glucose for WKY and SHR animals remained below 10 mM (Figure 2.2).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WKY</th>
<th>STZ</th>
<th>SHR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal PP in kidney (mmHg)</td>
<td>$43.9 \pm 0.51$</td>
<td>$39.31 \pm 0.76$ ***</td>
<td>$51.4 \pm 0.75$ ***</td>
</tr>
<tr>
<td>Basal PP in mesenteric (mmHg)</td>
<td>$24.38 \pm 0.69$</td>
<td>$22.28 \pm 1.07$</td>
<td>$28.6 \pm 0.65$ *</td>
</tr>
<tr>
<td>Response to 10µM PE in kidney (mmHg)</td>
<td>$195.3 \pm 1.82$</td>
<td>$198.2 \pm 1.85$</td>
<td>$246.4 \pm 3.23$ ***</td>
</tr>
<tr>
<td>Response to 10µM PE in mesentery (mmHg)</td>
<td>$87.99 \pm 1.79$</td>
<td>$84.75 \pm 2.35$</td>
<td>$100.9 \pm 1.5$ ***</td>
</tr>
<tr>
<td>ACh relaxation in kidney (%)</td>
<td>$78.86 \pm 2.63$</td>
<td>$55.66 \pm 1.39$ **</td>
<td>$40.47 \pm 2.02$ ***</td>
</tr>
<tr>
<td>ACh relaxation in mesentery (%)</td>
<td>$110.6 \pm 16.78$</td>
<td>$89.52 \pm 1.33$</td>
<td>$86.11 \pm 0.96$</td>
</tr>
</tbody>
</table>

Table 2.2. Characteristics of the perfused tissues for WKY, SHR and STZ-induced diabetic rats used in this study. PP, perfusion pressure. The table represents the mean ± s.e.m. of 40-60 rats. * Indicates significantly different from the WKY (* p< 0.05, ** p< 0.01, *** p< 0.001) using unpaired Student’s t-test.
Figure 2.1. The graph shows the body weight for WKY ( ), STZ-induced diabetic rats ( ) and SHR ( ) from the day of induction until the 8th week. The graph represents the mean ± s.e.m of 40-60 rats. * Indicates significant difference from the WKY value (* p< 0.01; ** p< 0.001).
Figure 2.2. The graph shows the blood glucose for WKY (●), STZ-induced diabetic rats (■) and SHR (▲) from the day of induction until the 8th week. The graph represents the mean ± s.e.m. of 40-60 rats. * Indicates significant difference from the WKY value (p< 0.05).
2.3.3. Contractile action of angiotensin peptides in the isolated perfused kidney

Figure 2.3 shows the dose-response increase in renal perfusion pressure curves of Ang II in WKY, SHR and STZ-induced diabetic animals. Response to bolus injections of Ang II in the SHR was significantly greater than those in the WKY (63.41 ± 3.25 vs. 51.62 ± 1.32, maximal contraction in SHR and WKY respectively). In contrast, Ang II pressor responses in the STZ-induced diabetic rats were significantly lower than those in the WKY (38.48 ± 1.25 vs. 51.62 ± 1.32, maximal contraction in STZ-induced diabetic and WKY respectively).

Figure 2.4 shows the dose-response (renal perfusion pressure) curves of DAA-I in WKY, SHR and STZ-induced diabetic rats. SHR had a slightly higher vasoconstriction than WKY (40.26 ± 2.50 vs. 32.00 ± 1.50, SHR and WKY respectively). There was no significant difference in DAA-I induced vasoconstriction between WKY and STZ-induced diabetic at lower concentration, however, a slight decrease in pressor action was observed in the STZ-induced diabetic at the highest concentration (19.88 ± 3.81 vs. 32.00 ± 1.50, STZ-induced diabetic and WKY, respectively). The renal perfusion pressure response to DAA-I was less than that of Ang II and was observed only at higher concentrations (> 10^{-6}M).

In contrast to Ang II and DAA-I, Ang 1-7 (10^{-13}M-10^{-6}M) did not induce any increase in renal perfusion pressure in the isolated perfused kidney of all the three groups of animals. Higher concentrations of Ang 1-7 (10^{-5}M -10^{-4}M) tested also did not induce vasoconstriction.
Figure 2.3. Angiotensin II induced pressure response in the renal arterial bed of WKY, SHR and STZ-induced diabetic rats. The graph represents the mean ± s.e.m. of 5-6 rats. * Indicates significant difference from the WKY value (* p< 0.05), (**) p<0.01) and (*** p<0.001).
Figure 2.4. Des-Asp-angiotein I (DAA-I) induced pressure response in the renal arterial bed of WKY, SHR and STZ-induced diabetic rats. The graph represents the mean ± s.e.m. of 5 rats. * Indicates significant difference from the WKY value (* p< 0.05).
2.3.4. Contractile action of angiotensin peptides in the isolated perfused mesenteric arterial bed.

Figure 2.5 shows the concentration-response (perfusion pressure) curves of mesenteric arterial bed to Ang II in WKY, SHR and STZ-induced diabetic. In contrast to the Ang II responses observed in the kidney, the responses in SHR were significantly smaller than those in WKY in the mesenteric. There were no significant differences in the contractile response between WKY and STZ-induced diabetic rats.

Figure 2.6 shows the mesenteric perfusion pressure responses to DAA-I in WKY, SHR and STZ-induced diabetic at the concentration of $10^{-5}$M. Injection of DAA-I (from $10^{-13}$M to $10^{-6}$M) did not induce increase in renal perfusion pressure. Thus, a higher DAA-I concentration ($10^{-5}$M) was injected and a slight pressor response was observed and this pressor response was much smaller than that observed with Ang II ($10^{-6}$M). Further administration of DAA-I (> $10^{-5}$M) did not evoke an increase in perfusion pressure. In effect DAA-I elicited an all-or-none type of contractile response at $10^{-5}$M. There was no significant difference in the responses between the groups of animals.

Similarly to the renal vasculature, Ang 1-7 did not induce any vasoconstriction in the mesenteric artery of all the three groups.
Figure 2.5. Angiotensin II-induced pressure response in the mesenteric arterial bed of WKY, SHR and STZ-induced diabetic rats. The graph represents the mean ± s.e.m. of 5-6 rats. * Indicates significant difference from the WKY value (** p<0.01) and (*** p<0.001).
Figure 2.6. Des-Asp-angiotensin I induced pressure response in the mesenteric arterial bed of WKY, SHR and STZ-induced diabetic rats. The graph represents the mean ± s.e.m. of 5 rats.
2.4. Discussion

2.4.1. Contractile action of angiotensin peptides in renal vasculature

The present study shows that Ang II produces a greater contractile response of the renal vasculature in SHR than WKY. Similar observation has also been reported by earlier investigators (Collis et al., 1980; Haddad and Garcia, 1996; Kost et al., 1994; Shibouta et al., 1979; Tuncer and Vanhoutte, 1991). The mechanisms responsible for increased vasoconstriction have been the subject of intense investigation. Studies by Haddad and Garcia, (1996) and Makarious et al., (1993) have demonstrated that the hyperresponsiveness observed in SHR correlates with increased Ang II receptor density. Various other mechanisms such as reduced offsetting activity of vasodilator prostaglandins (Chatziantoniou and Arendshorst, 1992), greater negative influence on phosphodiesterase induced increase in cAMP (Vyas et al., 1996), and defective interaction between receptor and G-protein activation (Chatziantoniou et al., 1990) by Ang II have been alluded to explain the increased responsiveness to Ang II in hypertensive vessels.

In contrast to SHR, Ang II pressor action was reduced in STZ-induced diabetic rats. This is in agreement with earlier observation by Sarubbi et al., (1989). They have demonstrated that Ang II induced vasoconstriction was significantly impaired in 2 and 8-12 weeks diabetic animal models. In addition to animal studies, attenuated renal and systemic responsiveness to Ang II had also been shown in type 1 diabetic patients (Fioretto et al., 1991). The reduced Ang II reactivity in diabetes has been associated with a downregulation of glomerular Ang II receptors (Ballermann et al., 1984; Brown et al., 1997). Sharma et al., (1999), however, did not find a downregulation of Ang II
receptor level but demonstrated that impaired Ang II response in diabetes is caused by decreased expression of the type 1 inositol 1,4,5-triphosphate (InsP$_3$) isoform receptor. Increased basal nitric oxide (NO) production in diabetes has also been reported (Bank & Aynedjian, 1993) and this has been suggested as a cause of the reduced Ang II pressor response in diabetes.

In this study, DAA-I induced a dose-dependent vasoconstriction in the isolated renal arterial bed, with a slightly greater responsiveness in the SHR. This nonapeptide has already been reported to induce concentration-dependent contraction of the rabbit aortic strips (Ackerly et al., 1977) and in rat aortic rings (Lim and Sim, 1998). The responsiveness was not altered in STZ-induced diabetic renal arterial bed, suggesting that DAA-I receptor is not markedly altered in diabetic states. However, the threshold for its contractile action was at 10$^{-6}$M, which lies outside the normal (picomolar) physiological range of DAA-I. Study by Garcia et al., (1981) has shown that plasma concentration of DAA-I was 7.6 fmol/ml using radioimmunoassay and chromatographic technique. Thus, it maybe concluded that DAA-I is at least not a potent vasoconstrictor. In agreement with this suggestion, a modulatory role for DAA-I in the RAS has been proposed (Sim and Radhakrishan, 1994; Sim and Yuan, 1995).

In contrast to Ang II and DAA-I, Ang 1-7 did not increase renal perfusion pressure in the renal arterial bed of WKY, SHR and STZ-induced diabetic rat. Ang 1-7 has been shown to act mostly as a vasodilator in many vascular beds (Ferrario et al., 1997; Feterik et al., 2000; Ren et al., 2002). Numerous studies have demonstrated that this peptide exerts biological actions that are both complementary to and distinct from those of Ang II (Ferrario et al., 1991) and exerts antihypertensive actions (Chappell et al.,
1998; Ferrario et al., 1998). It is likely that Ang 1-7 has a modulatory role in the RAS especially in conditions where the actions of Ang II are augmented.

### 2.4.2. Contractile action of angiotensin peptides in mesenteric vasculature

At $10^{-5}$M concentration, PE caused an increase in perfusion pressure between $84.75 \pm 2.35$mmHg and $100.9 \pm 1.5$ mmHg in perfused mesenteric arterial bed and this response was smaller than the increase recorded for the renal vasculature. The responses induced by Ang II in the mesenteric bed were also smaller than those recorded in the kidney, and the threshold dose was $10^{-10}$M for WKY and $10^{-9}$M for SHR and STZ-induced diabetic rats. Warner in 1990 demonstrated that Ang II to be a stronger venoconstrictor than an arterioconstrictor in albino Wistar rats.

Several studies have documented either no change (Sitzmann et al., 1990) or enhanced response (Endemann et al., 1999; Schlegel et al., 1985; Touyz et al., 1999a) of the mesenteric vasculature to Ang II induced vasoconstriction in SHR compared to WKY. This equivocal finding could be due to the use of the different sub-branches and sizes of the mesenteric vessel. In the present study, pressor response of the mesenteric vasculature to Ang II was found to be significantly less in the SHR than WKY. This could be due to decreased Ang II receptor density in the SHR mesenteric vasculature. This possibility may be a feedback mechanism to the increased response to Ang II observed in the SHR kidneys. However, this postulation requires further investigation.

The lack of differences in the vasopressor response to Ang II between the WKY and diabetic mesenteric vasculature is in contrast to that observed for the SHR. Although the vascular RAS has been known to be activated in diabetes and is a cause of vascular
remodeling, the sensitivity of mesenteric vessels to vasoconstrictors such as noradrenaline and serotonin remains controversial (Cooper et al., 2001). Four weeks after STZ treatment, significant increase in the vasoconstrictor responses induced by noradrenaline, endothelin-I and Ang II has been reported by Yousif et al., (2003), but longer treatment with STZ has been shown to increase blood flow in the superior mesenteric artery (Dias et al., 2004). It is likely that the lack of differences in the responses to Ang II between WKY and STZ-treated rats could be dependent on the time lapse since the induction of diabetes.

Not much is known about the biological action of DAA-I in the isolated perfused mesenteric arterial bed of rats. In fact, there is little data on DAA-I itself. In the present study, there were no significant differences in the contractile response to DAA-I in WKY, SHR and STZ-treated rats. The response is very small compared to the already low responses of Ang II and this confirms its lack of constrictor effect in resistance vessels. Similarly, Sexton et al., (1979) showed that intramesenteric infusion of DAA-I in the presence of ACE inhibitor did not produce any significant reduction to mesenteric blood flow of dogs and hence ruling it out as a vasoconstrictor in this vascular bed.

Similar to the renal vascular bed, Ang 1-7 did not have a contractile action in mesenteric vascular bed. Also, in this vascular bed, this heptapeptide has been shown to act as a vasodilator (Fernandes et al., 2001; Neves et al., 2003; Osei et al., 1993). It is possible that Ang 1-7 has a similar modulatory role on the responses of Ang II in this vascular bed.
2.5. Summary

This study demonstrates that Ang II is a potent vasoconstrictor in both the renal and mesenteric bed and its contractile action appears to be altered differently in pathological states—increased in hypertensive, and reduced in diabetic condition. As renal vasculature plays a major role in body homeostasis, it appears to be more affected than the mesenteric vasculature and is probably because the body tries to compensate the renal function in diseased state.

This is the first study to show that DAA-I has constrictor action in both the renal and mesenteric vasculatures, and it is 10 times less potent than Ang II and its effect is not significantly altered in the disease states of diabetes and hypertension. However, this nonapeptide has been previously demonstrated to attenuate Ang II contractile action in hypertensive animal model (Sim and Yuan, 1995). On the other hand, Ang 1-7 did not exhibit constrictor action in both vascular beds. Similar to DAA-I, this peptide has also been demonstrated to posses anti-angiotensin II action in hypertensive animal model (Stegbaur et al., 2005). Thus, it is likely that these peptides have modulatory roles on Ang II action in different pathological conditions.
VASODEPRESSOR ACTION OF DES-ASP-ANGIOTENSIN I (DAA-I) AND ANGIOTENSIN 1-7 (ANG 1-7) ON THE ACTION OF ANGIOTENSIN II IN RENAL AND MESENTERIC VASCULATURE OF HYPERTENSIVE AND STZ-INDUCED DIABETIC RATS

3.1. Introduction

From the previous study, it was demonstrated that DAA-I is a weak vasoconstrictor in both renal and mesenteric vasculature, and contractions were observed at concentration well above the physiological range, \( i.e. \) more than \( 10^{-6} \text{M} \). However, DAA-I has been shown to attenuate the action of Ang III in the aortic rings of the rabbit (Sim and Yuan, 1995) and the renal and mesenteric vasculature of SHR and WKY rats (Mustafa \textit{et al.}, 2004). Thus, in this part of the study, we investigated the effect of DAA-I on the responses of Ang II in the renal and mesenteric vasculature.

Vasoconstrictor responses in the earlier study showed that Ang 1-7 was without significant contractile actions in both the renal and mesenteric vasculature. This further supports the findings by other investigators who have demonstrated vasodilatory action with this peptide. More interestingly, this heptapeptide has been shown to have antihypertensive action. It has also been shown to block Ang II induced vasoconstriction in rat aorta (Le Tran and Forster, 1997). In this part of the study, we investigated the effects of Ang 1-7 on Ang II-induced contractions in renal and mesenteric vasculature of hypertensive and STZ-induced diabetic rats.
3.2. Methods

3.2.1. Animals

Male Wistar-Kyoto (WKY) rats and Spontaneously Hypertensive Rats (SHRs) age 10 weeks were obtained from the Animal House in the University of Malaya Medical Centre and kept two weeks for adaptation period before initiating any of the following protocols involving usage of the experimental animals. The animals were fed standard rat chow and tap water *ad libitum* for 2 weeks and diabetes was induced at age 12 weeks. Approval for the studies was obtained from the Committee for Animal Care and Use at the Laboratory Animal Center of Faculty of Medicine in the University of Malaya under the reference number FAR/22/10/03/MRM(R). All experimental procedures were carried out according to the guidelines for ethical care of experimental animals.

3.2.2. Induction of diabetes

As described in Section 2.2.3 (Chapter 2).

3.2.3. Preparation of isolated perfused renal and mesenteric arterial bed

As described in Section 2.2.4 (Chapter 2).
3.2.4. Research protocol

The first step of the experimental protocol is as mentioned in Section 2.2.5 (Chapter 2). In summary, after equilibration, the preparation was constricted with phenyleprine (PE, $10^{-5}$ M) and the recorded response was taken as a unity and responses to other pressor compounds were normalized against this unit. Another equilibration period of 20 minutes was allowed following which the integrity of the endothelium was tested by observing the relaxation to acetylcholine (ACh) ($10^{-4}$M) in PE-precontracted tissues. The experimental protocols that followed are as described below.

3.2.4.1. Effect of DAA-I and Ang 1-7 on the contractile action of angiotensin II in WKY, SHR and STZ-induced diabetic rats

The effects of various concentrations of DAA-I ($10^{-15}$ M - $10^{-9}$ M) and of Ang 1-7 ($10^{-15}$ M- $10^{-7}$ M) on the contractile responses of Ang II were studied according to the following protocol. The concentrations of DAA-I and Ang 1-7 chosen did not induce contraction directly on the vasculature. After testing the endothelium integrity, the preparation was perfused with Kreb’s solution containing 30 µM captopril and a concentration of DAA-I / Ang 1-7 for 60 min, prior to initiating a dose-dependent response to Ang II in the continuing presences of DAA-I or Ang 1-7. Ang II is given as bolus injection as mentioned in Section 2.2.5.1 (Chapter 2). Each concentration of DAA-I / Ang 1-7 was studied using a new set of the isolated vascular preparation.
3.2.4.2. Studies on the mechanism involved in the action of DAA-I and Ang 1-7

Similar protocol as of Section 3.2.4.1. was used to study the direct effect of indomethacin ($10^{-7}$ M), PD123319 ($10^{-5}$ M), L-NAME ($10^{-4}$ M) and D-ALA ($10^{-5}$ M) on the contractile responses to Ang II (Li et al., 1995, Mohan et al., 1994) with slight modification. A submaximal effective concentration of DAA-I or Ang 1-7 that reduced Ang II-induced vasoconstriction was chosen to study the effect of receptor antagonists and enzyme inhibitors on the modulatory actions of DAA-I and Ang 1-7.

For DAA-I, the vascular preparation was perfused with DAA-I ($10^{-9}$ M) in the presence of either 1-[4-(Dimethylamino)-3-methylphenyl] methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid ditrifluoroacetate (PD123319) (an AT$_2$ receptor antagonist), Nω-Nitro-L-Arginine Methyl Ester (L-NAME) (a nitric oxide synthase inhibitor) or indomethacin (cyclooxygenase inhibitor) for 30 min prior to a bolus injection of Ang II.

As for studying the action of Ang 1-7, the preparation was perfused with Ang 1-7 ($10^{-7}$ M) in the presence of either PD123319, L-NAME or indomethacin for 30 min prior to bolus injection of Ang II. Additionally, the effect of the Ang 1-7 receptor antagonist, (D-ALA$^7$)-Angiotensin I/II (1-7) (D-ALA) was also tested to investigate the involvement of Ang 1-7 receptor in the modulatory action of Ang 1-7.

The results from the mechanism studies for Ang 1-7 (incubation with indomethacin) have shown the involvement of prostaglandins. As it depresses the contractile action of Ang II, we investigated the possible involvement of the vasodilator prostaglandins, prostaglandin E$_2$ (PGE$_2$) and prostaglandin I$_2$ (PGI$_2$) in the relaxant effect of Ang 1-7.
This was done by assaying for these prostaglandins in the perfusate collected at 0 min (before perfusion), 30 minutes and 60 minutes from kidney and mesenteric beds perfused with Ang 1-7 (10^{-7}M). This concentration of Ang 1-7 was chosen as vasodepressor action was mostly observed at this concentration. Minimum animal sample required for this experiment was calculated using a statistical software (excel spreadsheet, SAMPLESZ.XLS) prepared by Dr. La Morte from the Boston University [http://www.hms.harvard.edu/orsp/coms/Statistics/Statistical_Explanation_from_Dr._La_Morte_of_BU.htm](http://www.hms.harvard.edu/orsp/coms/Statistics/Statistical_Explanation_from_Dr._La_Morte_of_BU.htm).

a) Prostaglandin E\(_2\)

Prostaglandin E\(_2\) was investigated using Prostaglandin E\(_2\) enzyme-immunoassay (EIA) Monoclonal kit from Cayman Chemicals, Michigan USA. The assay was conducted according to the manufacturer’s instructions: 50 µl sample / standard is added with 50 µl tracer and 50µl antibody in the 96 well plate. For detecting non-specific binding (NSB), 100 µl buffer and 50 µl tracer was added in 2 wells. One well was left empty for determining total activity (TA). The plate was incubated overnight at 4°C and then all wells were washed 5 to 6 times. 200 µl Ellman’s reagent was added to all wells. For TA, 5 µl tracer was added to Ellman’s reagent in the empty wells. The plate was incubated for 60 minutes at room temperature with gentle shaking and read at 412 nm. Standard curve was plotted and the level of PGE\(_2\) in the perfusate was then calculated.
b) Prostaglandin I$_2$

Prostaglandin I$_2$ was investigated using 6-keto Prostaglandin F$_{1\alpha}$EIA kit from Cayman Chemicals. As PGI$_2$ is unstable, its stable metabolite 6-keto Prostaglandin F$_{1\alpha}$ is used as an index for PGI$_2$. The assay was conducted according to the manufacturer’s instructions: 50 µl sample / standard is added with 50 µl tracer and 50 µl antibody in the 96 well plate. For detecting non-specific binding (NSB), 100 µl buffer and 50 µl tracer was added in 2 of the wells. One well was left empty for determination of total activity (TA). The plate was incubated overnight at 4°C and then all wells were washed 5 to 6 times. 200 µl Ellman’s reagent was added to all wells. For TA, 5 µl tracer was added to Ellman’s reagent in the empty wells. The plate was incubated for 60 minutes at room temperature with gentle shaking and read at 412 nm. Standard curve was plotted and the level of 6-keto PGF$_{1\alpha}$ in the perfusate was then calculated.

3.2.5. Drugs

Captopril, Ang II, DAA-I, Ang 1-7 and indomethacin were purchased from Sigma Chemical Co., Missouri, USA. D-ALA was purchased from BACHEM AC, Bubendorf, Switzerland. PD123319 was a generous gift from Parke-Davis Pharmaceutical Research, Michigan, USA. Captopril was prepared in dH$_2$O as 0.02M stock. Ang II, DAA-I, D-ALA and Ang 1-7 were prepared in cold dH$_2$O fresh daily. Indomethacin was dissolved in 0.5% Na$_2$CO$_3$ and the solution was kept at -4°C.
3.2.6. Statistical analysis

The perfusion pressure of the kidney and mesenteric artery upon drug administration was taken and the responses were normalized against the responses to PE and expressed as percentage. Data are presented as mean ± s.e.m. Significant difference (p<0.05) between means was evaluated using unpaired Student’s t-test when comparing two groups. When more than two groups are compared and for the comparison of the dose-response curves, data were evaluated by one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test (Prism version 4.0, GraphPad software, USA). Results with p<0.05 were considered statistically significant.

3.3. Results

3.3.1. Effect of DAA-I on the angiotensin II-induced vasoconstriction in the isolated perfused kidney.

Pressor responses to Ang II in the WKY and SHR were significantly attenuated by DAA-I. The attenuation was seen at the higher Ang II concentration (10^{-9}M-10^{-6}M) in both animal groups (Figure 3.1). In STZ-induced diabetic, DAA-I significantly potentiated the pressor action of lower concentrations of Ang II (10^{-13}M-10^{-11}M) and was without effect against the higher concentrations (10^{-10}M-10^{-7}M) of the octapeptide. However, at 10^{-6}M Ang II, DAA-I (10^{-9}M) decreased the contractile action of Ang II. DAA-I, by itself, had no affect on the basal perfusion pressure.

As the main interest was to investigate the vasodepressor action of DAA-I, further investigation for its mechanism of action was carried out in WKY and SHR rats.
Although the lack of DAA-I vasodepressor action in STZ-induced diabetic rats was not investigated, it is fully noted as part of a future investigation, together with the observed increase in contractile responses at lower concentrations of Ang II in the STZ-induced diabetic rats. The vasodepressor action of DAA-I was not affected by PD123319 and indomethacin in both WKY and SHR (Figure 3.2 & Figure 3.3). L-NAME reversed the attenuation by DAA-I in the WKY and SHR group (Figure 3.3).

3.3.2. Effect of DAA-I on the angiotensin II-induced vasoconstriction in the isolated perfused mesenteric arterial bed.

DAA-I ($10^{-15}$M-$10^{-9}$M) attenuated the responses to Ang II in WKY and SHR (Figure 3.4). Similar to the renal vasculature, attenuation by DAA-I was also not seen against the higher concentrations of Ang II in the STZ-induced diabetic mesenteric vasculature but was seen at $10^{-9}$M Ang II (Figure 3.4). As DAA-I vasodepressor effect was observed predominantly only in WKY and SHR, the mechanism of the vasodepressor effect was further studied in these groups. DAA-I showed a vasodepressor action at $10^{-9}$M Ang II in the STZ-induced diabetic mesenteric artery. The action of DAA-I on Ang II-induced vasoconstriction was not affected by PD123319 and indomethacin in WKY (Figure 3.5) and SHR (Figure 3.6). L-NAME attenuated the vasodepressor action of DAA-I on Ang II induced contractile action in SHR (Figure 3.6).
Figure 3.1. Effect of graded concentrations ($10^{-15}$M - $10^{-9}$M) of DAA-I on the angiotensin II ($10^{-13}$M - $10^{-6}$M) induced pressure responses in the perfused renal vasculature of WKY, SHR and STZ-induced diabetic rats. C, control (untreated). Each bar represents the mean ± s.e.m. of 5 - 7 separate preparations. * Indicates significant difference from the DAA-I vehicle-pretreated control (C) (p< 0.05).
Figure 3.2. Effect of cyclooxygenase inhibitor, indomethacin (A), AT₂ receptor antagonist, PD 123319 (B) and NOS inhibitor, L-NAME (C) on the inhibitory effects of DAA-I on the angiotensin II-induced pressure response in the renal vascular bed of the WKY. Alphabets inside histograms symbolise treatment given to the particular group: C, control (untreated); D, DAA-I (10⁻⁹ M); In, indomethacin (10⁻⁷ M); PD, PD 123319 (10⁻⁵ M); L-N, L-NAME (10⁻⁴ M). Each bar chart group represents the mean ± s.e.m. of 5-7 separate preparations. a, indicates significant difference from the control (p<0.05), b, indicates significant difference from DAA-I (p<0.05).
Figure 3.3. Effect of cyclooxygenase inhibitor, indomethacin (A), AT2 receptor antagonist, PD 123319 (B) and NOS inhibitor, L-NAME (C) on the inhibitory effects of DAA-I on the angiotensin II-induced pressure response in the renal vascular bed of the SHR. Alphabets inside histograms symbolise treatment given to the particular group: C, control (untreated); D, DAA-I (10^{-9} M); In, indomethacin (10^{-7} M); PD, PD 123319 (10^{-5} M); L-N, L-NAME (10^{-4} M). Each bar chart group represents the mean ± s.e.m. of 5-7 separate preparations. a, indicates significant difference from the control (p<0.05), b, indicates significant difference from DAA-I (p<0.05).
Figure 3.4. Effect of graded concentrations (10^{-15} M - 10^{-9} M) of DAA-I on the angiotensin II (10^{-13} M - 10^{-6} M) induced pressure responses in the perfused mesenteric vasculature of WKY, SHR and STZ-induced diabetic rats. C, control (untreated). Each bar represents the mean ± s.e.m. of 5 - 7 separate preparations. * Indicates significant difference from the DAA-I vehicle-pretreated control (C) (p< 0.05).
Figure 3.5. Effect of cyclooxygenase inhibitor, indomethacin (A), AT₂ receptor antagonist, PD 123319 (B) and NOS inhibitor, L-NAME (C) on the inhibitory effects of DAA-I on the angiotensin II-induced pressure response in the mesenteric vascular bed of the WKY. Alphabets inside histograms symbolise treatment given to the particular group: C, control (untreated); D, DAA-I (10⁻⁹ M); In, indomethacin (10⁻⁷ M); PD, PD 123319 (10⁻⁵ M); L-N, L-NAME (10⁻⁴ M). Each bar chart group represents the mean ± s.e.m. of 5-7 separate preparations. a, indicates significant difference from the control (p<0.05), b, indicates significant difference from DAA-I (p<0.05).
Figure 3.6. Effect of cyclooxygenase inhibitor, indomethacin (A), AT2 receptor antagonist, PD 123319 (B) and NOS inhibitor, L-NAME (C) on the inhibitory effects of DAA-I on the angiotensin II-induced pressure response in the mesenteric vascular bed of the SHR. Alphabets inside histograms symbolise treatment given to the particular group: C, control (untreated); D, DAA-I (10^{-9} M); In, indomethacin (10^{-7} M); PD, PD 123319 (10^{-5} M); L-N, L-NAME (10^{-4} M). Each bar chart group represents the mean ± s.e.m. of 5-7 separate preparations. a, indicates significant difference from the control (p<0.05), b, indicates significant difference from DAA-I (p<0.05).
3.3.3. Effect of Ang 1-7 on the angiotensin II induced vasoconstriction in the isolated perfused kidney.

The contractile responses to Ang II were attenuated by Ang 1-7 (10^{-7} M) in both WKY and SHR. The attenuation was observed at the higher concentrations of Ang II (10^{-9} M – 10^{-6} M) in SHR and from 10^{-10} M for WKY (Figure 3.7). Ang 1-7 did not affect the Ang II-induced vasoconstriction in STZ-induced diabetic rats. Ang 1-7 by itself did not affect the basal perfusion pressure. As the key interest was to investigate the vasodepressor action of Ang 1-7, and this was observed only in WKY and SHR, further investigation for its mechanism of action was carried out in WKY and SHR but not in the STZ-induced diabetic rats. The action of Ang 1-7 was not affected by PD 123319 in WKY and SHR. Indomethacin, L-NAME and D-ALA reversed the vasodepressor action of Ang 1-7 in WKY and SHR (Figure 3.8 and Figure 3.9, respectively).

3.3.4. Effect of Ang 1-7 on the angiotensin II induced vasoconstriction in the isolated perfused mesenteric arterial bed.

In contrast to responses seen in the kidney, Ang 1-7 attenuated the pressor responses to Ang II in mesenteric vascular bed of STZ-induced diabetic rats. For the SHR, the attenuation was observed only at the higher concentrations of Ang II (10^{-7} M-10^{-6} M) (Figure 3.10). As DAA-I depressor effect was observed in WKY, SHR and STZ-induced diabetic, all groups were studied for the mechanism of action. PD 123319 did not affect the action of Ang 1-7 in all the groups. L-NAME and D-ALA reduced the attenuation by Ang 1-7 in WKY (Figure 3.11(D)), SHR (Figure 3.12(D)) and STZ-induced diabetic rats (Figure 3.13(D)). Preincubation with indomethacin reduced the vasodepressor actions of Ang 1-7 only in hypertensive animals.
Figure 3.7. Effect of graded concentrations (10^-15 M - 10^-7 M) of angiotensin I-7 on the angiotensin II (10^-13 M - 10^-6 M) induced pressure responses in the perfused renal vasculature of WKY, SHR and STZ-induced diabetic rats. C, control (untreated). Each bar represents the mean ± s.e.m. of 5 - 6 separate preparations. * Indicates significant difference from the DAA-I vehicle-pretreated control (C) (p< 0.05).
Figure 3.8. Effect of cyclooxygenase inhibitor, indomethacin (A), AT\(_2\) receptor antagonist, PD 123319 (B), NOS inhibitor, L-NAME (C) and Ang 1-7 receptor antagonist, D-ALA (D) on the inhibitory effects of angiotensin 1-7 on the angiotensin II-induced pressure response in the renal vascular bed of the WKY. Alphabets inside histograms symbolise treatment given to the particular group: C, control (untreated); A 1-7, Angiotensin 1-7 (10\(^{-7}\) M); In, indomethacin (10\(^{-7}\) M); PD, PD 123319 (10\(^{-5}\) M); L-N, L-NAME (10\(^{-4}\) M); D-ALA, D-ALA (10\(^{-5}\) M). D-ALA was used in addition for Ang 1-7 as it is an Ang 1-7 receptor antagonist. Each bar chart group represents the mean ± s.e.m. of 5-6 separate preparations. a, indicates significant difference from the control (p<0.05), b, indicates significant difference from DAA-I (p<0.05).
Figure 3.9. Effect of cyclooxygenase inhibitor, indomethacin (A), AT2 receptor antagonist, PD 123319 (B), NOS inhibitor, L-NAME (C) and Ang 1-7 receptor antagonist, D-ALA (D) on the inhibitory effects of angiotensin 1-7 on the angiotensin II-induced pressure response in the renal vascular bed of the SHR. Alphabets inside histograms symbolise treatment given to the particular group: C, control (untreated); A 1-7, Angiotensin 1-7 (10^{-7} M); In, indomethacin (10^{-7} M); PD, PD 123319 (10^{-5} M); L-N, L-NAME (10^{-4} M); D-ALA, D-ALA (10^{-5} M). D-ALA was used in addition as it is an Ang 1-7 receptor antagonist. Each bar chart group represents the mean ± s.e.m. of 5-6 separate preparations. a, indicates significant difference from the control (p<0.05), b, indicates significant difference from DAA-I (p<0.05).
CHAPTER 3  
Vasodepressor action of DAA-I and Ang 1-7

Figure 3.10. Effect of graded concentrations (10^{-15}M - 10^{-7}M) of angiotensin 1-7 on the angiotensin II (10^{-13}M - 10^{-6}M) induced pressure responses in the perfused mesenteric vasculature of WKY, SHR and STZ-induced diabetic rats. C, control (untreated). Each bar represents the mean ± s.e.m. of 5 - 6 separate preparations. * Indicates significant difference from the DAA-I vehicle-pretreated control (C) (p< 0.05).
Figure 3.11. Effect of cyclooxygenase inhibitor, indomethacin (A), AT2 receptor antagonist, PD 123319 (B), NOS inhibitor, L-NAME (C) and Ang 1-7 receptor antagonist, D-ALA (D) on the inhibitory effects of angiotensin 1-7 on the angiotensin II-induced pressure response in the mesenteric vascular bed of the WKY. Alphabets inside histograms symbolise treatment given to the particular group: C, control (untreated); A 1-7, Angiotensin 1-7 (10^-7 M); In, indomethacin (10^-7 M); PD, PD 123319 (10^-5 M); L-N, L-NAME (10^-4 M); D-ALA, D-ALA (10^-5 M). D-ALA was used in addition for Ang 1-7 as it is an Ang 1-7 receptor antagonist. Each bar chart group represents the mean ± s.e.m. of 5-6 separate preparations. a, indicates significant difference from the control (p<0.05), b, indicates significant difference from DAA-I (p<0.05).
Figure 3.12. Effect of cyclooxygenase inhibitor, indomethacin (A), AT$_2$ receptor antagonist, PD 123319 (B), NOS inhibitor, L-NAME (C) and Ang 1-7 receptor antagonist, D-ALA (D) on the inhibitory effects of angiotensin 1-7 on the angiotensin II-induced pressure response in the mesenteric vascular bed of the SHR. Alphabets inside histograms symbolise treatment given to the particular group: C, control (untreated); A 1-7, Angiotensin 1-7 (10$^{-7}$ M); In, indomethacin (10$^{-7}$ M); PD, PD 123319 (10$^{-5}$ M); L-N, L-NAME (10$^{-4}$ M); D-ALA, D-ALA (10$^{-5}$ M). D-ALA was used in addition for Ang 1-7 as it is an Ang 1-7 receptor antagonist. Each bar chart group represents the mean ± s.e.m. of 5-6 separate preparations. a, indicates significant difference from the control (p<0.05), b, indicates significant difference from DAA-I (p<0.05).
Figure 3.13. Effect of cyclooxygenase inhibitor, indomethacin (A), AT\textsubscript{2} receptor antagonist, PD 123319 (B), NOS inhibitor, L-NAME (C) and Ang 1-7 receptor antagonist, D-ALA (D) on the inhibitory effects of angiotensin 1-7 on the angiotensin II-induced pressure response in the mesenteric vascular bed of the STZ-induced diabetic rats. Alphabets inside histograms symbolise treatment given to the particular group: C, control (untreated); A 1-7, Angiotensin 1-7 (10\textsuperscript{-7} M); In, indomethacin (10\textsuperscript{-7} M); PD, PD 123319 (10\textsuperscript{-5} M); L-N, L-NAME (10\textsuperscript{-4} M); D-ALA, D-ALA (10\textsuperscript{-5} M). D-ALA was used in addition for Ang 1-7 as it is an Ang 1-7 receptor antagonist. Each bar chart group represents the mean ± s.e.m. of 5-6 separate preparations. a, indicates significant difference from the control (p<0.05), b, indicates significant difference from DAA-I (p<0.05).
3.3.5. Prostaglandin Assay

In the WKY and SHR isolated kidneys perfused with Ang 1-7 (10^{-7} M), the PGE_2 levels were significantly increased after 30 and 60 minutes perfusion. The increase was about 30% in WKY and 20% in SHR when compared to the basal PGE_2 level. There was no change in the PGE_2 level from the perfusate collected from STZ diabetic kidney. Perfusate collected from mesenteric bed for all of the groups showed no significant changes in PGE_2 level (Figure 3.14).

The levels of 6-keto PGF_{1α} were increased in perfusate collected from WKY (~75% at 30 minute and 120% at 60 minutes) and SHR (~ 58%) perfused kidney compared to its basal. The levels of 6-keto PGF_{1α} were higher than PGE_2 level detected in the kidney. In the perfusate from STZ-diabetic kidney, there was no change in the 6-keto PGF_{1α}. Perfusate collected from mesenteric arterial bed of SHR showed an increase in 6-keto PGF_{1α} level (~30%). There was no difference in 6-keto PGF_{1α} level from WKY and STZ-induced diabetic mesentery perfusates. However, the basal level 6-keto PGF_{1α} from diabetic mesentery was much higher than in the WKY and SHR (Figure 3.15).

To ensure that the change in the prostaglandin levels were not attributed to temporal change, parallel studies were performed by perfusing the vascular preparations with normal Krebs solution. The result showed no changes in PGE_2 and 6-keto PGF_{1α} levels.
Figure 3.14. Prostaglandin E$_2$ level (pg/ml) measured in the perfusate collected from kidney and mesenteric arterial bed of WKY, SHR and STZ-induced diabetic rats before, 30 minutes and 60 minutes after perfusing with Ang 1-7 (10$^{-7}$M). Each bar represents the mean ± s.e.m. of 3 samples. * Indicates significant difference from the corresponding basal level (p< 0.05).
Figure 3.15. 6-keto Prostaglandin F$_{1\alpha}$ level (pg/ml) measured in the perfusate collected from kidney and mesenteric arterial bed of WKY, SHR and STZ-induced diabetic rats before, 30 minutes and 60 minutes after perfusing with Ang 1-7 (10$^{-7}$M). Each bar represents the mean ± s.e.m. of 3 samples. * Indicates significant difference from the corresponding basal level (p< 0.05).
3.4. Discussion

3.4.1. Effect of DAA-I on angiotensin-II induced vasoconstriction in the renal vasculature

DAA-I attenuated Ang II pressor action in the renal vasculature of both WKY and SHR. The attenuation by DAA-I was observed against the higher concentrations of Ang II (10^{-9} to 10^{-6} M) (Figure 3.1). Similar attenuation was also reported against the pressor actions of Ang III (Mustafa et al., 2004). However, in the present study, the effective dose of DAA-I was a hundred-fold lower (10^{-15} M) and the vasodepressor effect was seen in both the WKY and SHR. At this femtomolar level, the concentration of DAA-I is much below the reported circulating (pico-molar) level in vivo (Sim and Qui, 2003).

The renal vasculature is an ex vivo preparation devoid of circulating DAA-I, but the re-introduction of DAA-I by perfusion provides a model for measuring the effective physiological concentration of the peptide and the possible roles it may exert in normal and pathological conditions. The present findings suggest, at circulating level, DAA-I attenuates pressor action to Ang II particularly when the local concentration of the octapeptide rises above a certain level i.e. nano molar concentration. In this way, DAA-I may regulate the action of Ang II and prevent the latter from exerting excessive and damaging vascular effect. However, the attenuation in the SHR though equally significant, was not sufficient to match the absolute value seen in the WKY indicating that the circulating level of DAA-I was not able to ameliorate the hypertensive malady. At femtomolar concentrations, DAA-I is indeed the most specific angiotensin peptide that is known to attenuate the pressor actions of Ang II.
A different profile of DAA-I action was seen in the renal vasculature of STZ-induced diabetic rats. Unlike the WKY and SHR, DAA-I had no effect against the higher pressor concentrations of Ang II (Figure 3.1). Instead, DAA-I potentiated the pressor action of the lower concentrations ($10^{-13}$ to $10^{-11}$ M) of the octapeptide. Although the exact mechanism for such contrasting effect is not known, changes in levels of cellular diacylglycerol (DAG) and isoforms of protein kinase C (PKC) following induction of diabetes (Amiri and Garcia, 2000; Feener and King, 1997; Koya et al., 1997) could be contributory factors, as the contractile actions of different agonists are mediated by different isoforms of PKC (Ohanian et al., 1996; Tan et al., 2004), which are in turn regulated by different PKC-interacting proteins (Poole et al., 2004). Whatever the cellular mechanisms responsible, in the STZ-induced diabetic animals, it is tempting to speculate that DAA-I normalizes the pressor response to low concentrations of Ang II to near normal and, in this way, maintains the physiological vascular tone in the diabetic renal vasculature. The need to exert negative control on the higher concentrations of Ang II may not be critical in the diabetic renal vasculature as the response to the octapeptide is inherently diminished.

PD 123319 and indomethacin were without effect against the vasodepressor action of DAA-I in WKY and SHR, indicating that the action of the peptide (DAA-I) does not involve activation of AT$_2$ angiotensin receptors or mediated by the release of prostaglandins. This is in agreement with our earlier finding with Ang III (Mustafa et al., 2004), suggesting that DAA-I probably acts via the angiotensin AT$_1$ receptor as has been shown in other preparations (Sim and Chai, 1996; Sim and Min, 2005). L-NAME significantly reversed the vasodepressor action of DAA-I in WKY and SHR, suggesting nitric oxide plays a role in the vasomodulatory action of DAA-I in the renal vascular bed.
3.4.2. Effect of DAA-I on angiotensin-II induced vasoconstriction in mesenteric vasculature

Comparable vasodepressor action of DAA-I was observed between the renal and mesenteric vasculature. In both vasculatures, DAA-I was more effective in attenuating the pressor action of Ang II in the WKY and SHR than in the diabetic animals (Figure 3.4). The attenuating effect of DAA-I on WKY mesenteric bed appeared to be an all or none phenomenon. This is because the degree of attenuation caused by the various concentrations of DAA-I for each concentration of Ang II was statistically (ANOVA) similar. However, in the diseased state of hypertension (SHR), and diabetes (STZ-induced rats), the attenuating effect of DAA-I on Ang II contractile action assumes a dose-dependent characteristic. This was so for all the concentration of Ang II tested in SHR (10^{-10}M-10^{-6}M) and for the lower concentrations (10^{-10}M-10^{-9}M) tested in the diabetic rats (Figure 3.4). This finding suggests a modifying effect of the disease process (hypertension and diabetes) on the action on DAA-I, perhaps DAA-I function is a natural attempt at processes modifying the disease.

Resistance arteries (100–300 µm in diameter) respond to physiological and pathophysiological stimuli to maintain perfusion according to the metabolic needs of tissues. Vasomotor control (contraction/relaxation) is responsible for rapid adaptation of lumen diameter, whereas alterations in structural properties of the vascular wall constitute a dynamic process occurring in response to long-term haemodynamic modifications. Initially structural changes are adaptive, but subsequently become maladaptive resulting in changes in media thickness and lumen diameter. This process, called vascular remodelling, contributes to the pathophysiology of vascular diseases like hypertension (Mulvany et al., 1996).
In terms of the effects of DAA-I, PD123319 and indomethacin on Ang II contractions, a similar pattern to those of the renal vasculature were observed in both WKY and SHR (Figure 3.5 and Figure 3.6). This suggests that DAA-I action is via the angiotensin AT\textsubscript{1} receptors and is unlikely to be mediated by prostaglandins. L-NAME significantly reversed the vasodepressor action of DAA-I in the SHR only, suggesting a dependence on the nitric oxide synthase-nitric oxide (NOS-NO) mechanism. Perhaps, in hypertension pathophysiology, DAA-I triggers the NOS-NO path as compensatory mechanism.

3.4.3. Effect of Ang 1-7 on angiotensin-II induced vasoconstriction in renal vasculature

The present study demonstrated that Ang 1-7 significantly attenuates Ang II-induced vasoconstrictions in both the WKY and SHR (Figure 3.7). Stegbaur et al., (2005) and Wouden et al., (2006) have also demonstrated similar vasodepressor action in WKY rats with a higher concentration of the heptapeptide (10\textsuperscript{-5}M). In the SHR, the attenuation was observed against the higher concentrations of Ang II (10\textsuperscript{-9}M - 10\textsuperscript{-6}M). This finding supports the reported antihypertensive actions of Ang 1-7 in human (Roks et al., 1999; Ueda et al., 2000). Interestingly, Mohan et al., (1994) have shown that Ang 1-7 attenuates the pressor response of Ang II in rabbit aortic rings, and in anesthetized cats. Because this effect was observed specifically with Ang II (and not with other vasoconstrictors) and was blocked by losartan, the authors suggested that Ang 1-7 modulates the effect of Ang II via the AT\textsubscript{1} receptor.

Micromolar concentration of Ang 1-7 caused a modest downregulation of the AT\textsubscript{1} receptors in Chinese hamster ovary cells stably transferred with the AT\textsubscript{1A} receptor
Vasodepressor action of DAA-I and Ang 1-7 (Clark et al., 2001) and in kidney slices from SD rats (Clark et al., 2003). Ang 1-7 has also been proposed to bind to and activate the AT$_1$ receptor, which results in receptor internalization without coupling to G-proteins and activation of phospholipase C (Ueda et al., 2000). The downregulation and internalization of AT$_1$ receptor by Ang 1-7 could be a possible cause of the observed reduction in pressor response of the octapeptide in the present study. In addition, Ang 1-7 has been demonstrated to reduce Ang II-induced phosphorylation of protein kinase C-zeta and extracellular signal-regulated kinase (ERK) ½ (Zhi et al., 2002). The ability of Ang 1-7 to modulate the mechanisms of action of Ang II at both the receptor and cellular levels suggests that the heptapeptide plays important roles in modulating the vascular actions of Ang II.

In the present study, Ang 1-7 significantly reduced the response to Ang II at a dose of $10^{-7}$M, i.e. a concentration significantly lower than the IC$_{50}$ of the AT$_1$ receptor (>1µM) (Ueda et al., 2000). The finding implies that Ang 1-7 may act via non-AT$_1$ receptor/s. Similar magnitude of Ang 1-7 actions was observed in the WKY and SHR. In contrast, Kost et al., (1998) and Stegbauer et al., (2004) found the action of Ang 1-7 to be more potent in the SHR. Differences in periods of exposure to Ang 1-7 and protocol of drug administration (bolus injection) may have caused the observed differences. In the STZ-induced diabetic rats, Ang 1-7 had no effect on the pressor response to Ang II. Reduced sensitivity or changes in Ang 1-7 receptor may have led to the loss of its vasodepressor action.

PD 123319 was without effect on the action of Ang 1-7 in WKY and SHR, implying that the AT$_2$ angiotensin receptor was not involved in its action (Figure 3.8 and Figure 3.9). Recent studies have shown that mRNA expression of AT$_2$ expression was not altered in VSMCs pretreated with Ang 1-7 (Zhi et al., 2002). The heptapeptide has also
been shown to bind to type 1 angiotensin II receptor, which is predominantly present in the rat renal cortex (Gironacci et al., 1999). Ang 1-7 receptor has been postulated to exert its effect on its own functional receptors, which are distinct from AT\(_1\) and AT\(_2\) receptors (Santos et al., 1994; Tallant et al., 1997). Recently, the G-protein coupled receptor, Mas, was characterized as an endogenous Ang 1-7 receptor (Santos et al., 2003). Mas forms a constitutive hetero-oligomeric complex with the AT\(_1\) receptor and by so doing inhibits the action of Ang II (Kostenis et al., 2005). Hence, reduced level or absence of Mas may explain the loss of Ang 1-7 action in the STZ-induced diabetic kidney. However, this assumption requires further investigation.

Indomethacin and L-NAME inhibited the actions of Ang 1-7 in WKY and SHR, suggesting an involvement of prostaglandins, presumably vasodilator prostaglandins and nitric oxide in mediating the actions of the peptide (Figure 3.8 and Figure 3.9). In agreement with results from the current study, a number of studies clearly indicate that Ang 1-7 produces prostaglandins, stimulate nitric oxide, and activates a non-AT\(_1\) and AT\(_2\) receptor that is sensitive to [D-Ala\(^7\)]-Angiotensin 1-7 (Jaiswal et al., 1993; Le Tran & Forster, 1997). The involvement of prostaglandins, especially vasodilator prostaglandins (PGE\(_2\) and PGI\(_2\)) in WKY and SHR are further supported by our findings on measuring the prostaglandin levels. Studies by Hilchey and Bell-Quilley, (1995) have also demonstrated an increase in PGI\(_2\) release from the isolated kidney perfused with Ang 1-7. The study also showed no involvement of prostaglandins in STZ-induced diabetic rats perfused with Ang 1-7. Work by Brunkwall and Bergqvist, (1993) also demonstrated that there was no difference in release of 6-keto-PGF\(_{1\alpha}\) from aorta and renal tissue in diabetic animals compared to controls. Since nitric oxide and cyclooxygenase pathways are not completely independent, and nitric oxide decreases
AT₁ receptor mRNA levels (Ichiki et al., 1998), Ang 1-7 perhaps modulates effects of Ang II through this mechanism as well.

3.4.4. Effect of Ang 1-7 on angiotensin-II induced vasoconstriction in mesenteric vasculature

Differences exist between the effects of Ang 1-7 in the renal and mesenteric vascular beds. In the mesenteric vascular bed, Ang 1-7 attenuated Ang II-induced vasoconstriction in WKY, SHR and STZ-induced diabetic rats (Figure 3.10). The attenuation was seen at the lower concentrations (10⁻¹⁰M-10⁻⁸M) of Ang II in WKY and STZ-induced diabetic rats but not in the SHR rats. The present data show that Ang II-induced vasoconstriction is smaller in the SHR compared to the WKY and STZ-induced diabetic rats (Figure 3.10). Thus, it is possible that the absence of Ang 1-7 in attenuating the contractile effect of Ang II at these lower concentrations in the SHR mesenteric bed could be due to the lower contractile responses in these tissues. In effect, it may be postulated that a critical level of Ang II induced contraction is required to trigger the Ang II – Ang 1-7 crosstalk which produces the above mentioned attenuation.

In contrast to the kidney, vasodepressor action of angiotensin 1-7 was witnessed in all the groups (WKY, SHR and STZ-induced diabetic) (Figure 3.11, Figure 3.12 and Figure 3.13). In terms of the effects of Ang 1-7 on Ang II constrictor response, PD123319, D-ALA and L-NAME showed similar effects to those observed in the renal vasculature. In addition, indomethacin reversed Ang 1-7 action in the SHR and not in WKY and STZ-induced diabetic. This suggests that Ang 1-7 action is mediated via the Ang 1-7 receptor in both groups and involves vasodilator prostaglandins and nitric oxide in SHR, and only nitric oxide in WKY and STZ-induced diabetic. The
prostaglandin measurements showed no significant difference in the vasodilator prostaglandins levels in diabetic animals before and following perfusion with Ang 1-7. However, elevated level of 6-keto-PGF$_{1\alpha}$ was observed in diabetic animals even before perfusion with Ang 1-7. In diabetic mesenteric bed, increased prostaglandin especially PGI$_2$ has been demonstrated (Fujii et al., 1986 and Fujii et al., 1987). These authors have postulated that an increase in micro-circulation of PGI$_2$ may partially be protective against progression of angiopathy. As the vasodilator PGI$_2$ was already increased in diabetic mesentery, it is possible that Ang 1-7 does not further stimulate PGI$_2$. Accordingly, Oliveira et al., (1999) demonstrated that Ang 1-7 causes both vasodilatation and bradykinin potentiation in mesenteric arterioles, which was blocked by A-779, L-NAME, and indomethacin, suggesting an important participation of local prostanoids and nitric oxide in Ang 1-7 action. The same authors showed that Ang 1-7 potentiates the bradykinin vasodilatory effect in mesenteric arterioles of SHR via release of prostanoids and EDHF.

### 3.5. Summary

This is the primary finding to reiterates a regulatory role for DAA-I in vascular beds of the kidney and mesentery in the WKY and SHR. Foremost in this study, DAA-I is shown to be active at circulating concentrations. By being active at femtomolar concentration, DAA-I subserves a physiological role. This is the also the first study to show that the functional regulatory role of DAA-I appeared to be active in animal models of hypertension but compromised in diabetic model. The vasodepressor action of DAA-I in the WKY and SHR renal and mesenteric vascular beds appears not to involve AT$_2$ receptor or cyclooxygenase byproducts. However, nitric oxide appears to be involved, especially in hypertensive animals.
The current data also suggest that Ang 1-7 has a regulatory role in the kidney and mesenteric vasculatures which includes the attenuation of the contractile responses to Ang II. This finding is the first to show a regulatory role for Ang 1-7 in the mesenteric vasculature. This action is possibly modulated by Ang 1-7 receptor and involves a cyclooxygenase dependent pathway and nitric oxide release. Similar apparent vasculo-protective effect of Ang 1-7 also appears to be present in diseased state such as hypertension and diabetes. It is likely that DAA-I functions and Ang 1-7 receptors or its action are modified to accommodate the ongoing vascular remodeling in hypertension and diabetes.
EFFECT OF DES-ASP-ANGIOTENSIN I (DAA-I) AND ANGIOTENSIN 1-7 (ANG 1-7) ON RENAL AT$_1$ RECEPTORS

4.1. Introduction

In the RAS, it is apparent that angiotensin peptides may bind to multiple receptor subtypes that often exhibit different and sometimes opposing actions. For example, Ang II has been shown to bind to both AT$_1$ and AT$_2$ receptor subtypes which are coupled to specific effector systems and eliciting different biological responses. DAA-I has been shown to modulate both AT$_1$ and AT$_2$ receptor differently in cardiac hypertrophy after aortic coarctation (Chen and Sim, 2004). Studies with rabbit aortic ring (Sim and Yuan, 1995), rat aortic rings (Lim and Sim, 1998) and rabbit pulmonary artery (Sim and Min, 2005) demonstrated that DAA-I acts as an agonist on losartan–sensitive angiotensin receptor.

Receptor duality has been demonstrated for Ang 1-7. This heptapeptide exhibits several effects that are blocked by AT$_1$ antagonist, including the release of $^3$H-noradrenaline from rat atria (Gironacci et al., 1994) and increase fluid absorption in the proximal tubule (Garcia et al., 1994). In addition to the AT$_1$ receptors, Ang 1-7 recognizes its own receptors, which have been described in a number of preparations such as the dorsal medulla oblongata (Diz and Ferrario, 1996) and cultured astrocytes (Tallant et al., 1997).

In Chapter 2 (Figure 2.3), we have demonstrated increased contractile action of Ang II in the kidney of SHR rats and decreased contractile responses in the STZ-induced diabetic rat kidneys. Thus, the first objective of this part of the study is to investigate the correlation between renal angiotensin receptors especially AT$_1$ receptor with the
differences observed in the renal Ang II contractile action between SHR and STZ-induced diabetic rats.

The earlier results (in Chapter 3) demonstrated that DAA-I and Ang 1-7 have vasodepressor action on the contractile response to Ang II in WKY and SHR renal vasculature. The involvement of AT$_2$ receptor in these peptide vasodepressor actions was excluded (Chapter 3). Further work was therefore carried out to determine DAA-I and Ang 1-7 action on AT$_1$ receptor in the renal vasculature. In the earlier protocol (Chapter 3), DAA-I or Ang 1-7 was perfused into the kidney and the possibility was considered that these peptides acted on the angiotensin receptors present on the membrane of any part of the kidney. Thus, we used the whole kidney in the current study. The second objective therefore was to determine the involvement of AT$_1$ receptor in the vasodepressor action observed with DAA-I and Ang 1-7 on Ang II contractile action in the kidney.

Losartan is not suitable to be used in the *ex vivo* study to test whether AT$_1$ was involved as the antagonist blocks the vasoconstriction induced by Ang II. Even at the concentration as low as $10^{-8}$M, losartan was able to reduce Ang II-induced vasoconstriction by 50%. A different approach was therefore employed to investigate the receptor subtype involved in mediating Ang II modulation in the renal vasculature and determine the effect of DAA-I and Ang 1-7 on renal AT$_1$ receptors. Receptor binding assay, semi-quantitative reverse-transcriptase Polymerase Chain Reaction (RT-PCR) and Western Blotting was used to investigate the above objectives.
4.2. Methodology

4.2.1. Receptor Binding Assay

4.2.1.1. Preparation of crude kidney homogenates

The right kidneys from WKY, SHR and STZ-induced diabetic rats were isolated and perfused with Krebs and captopril (30 µM) (control) or with DAA-I (10⁻⁹M to 10⁻¹⁵M) / Ang 1-7 (10⁻⁷M) for 90 minutes prior to the homogenate preparation. The concentrations of DAA-I and Ang 1-7 chosen were those that reduced Ang II contraction in the kidney from the earlier part of the study (Chapter 3). The whole kidney membrane homogenate preparations were carried out as described by Brown et al., (1997) with slight modification. Minimum animal sample required for this analysis was calculated using statistical software, SAMPLESZ.XLS. Right kidneys were obtained from 3-4 individual animals per group and experiment was conducted on each independent kidney preparation.

The kidneys were minced into small pieces and suspended 1:10 (w/v) in Tris-HCl buffer (50 mM, pH 7.4) and homogenised with an ultraturax, followed by Glass-Gol homogenizer. The homogenate was centrifuged at 30,000 x g at 4°C for 40 minutes. The resultant pellet was resuspended and recentrifuged twice under the same conditions. The final pellet was resuspended in Tris-HCl (20 mM, pH 7.4), containing NaCl (135 mM), KCl (10 mM), glucose (5 mM) and MgCl₂ (10 mM). Protein concentration was determined using the Biuret assay as described in section 4.2.1.3. of this chapter. The membranes prepared were kept as aliquots at -80°C for not longer than 3 months.
4.2.1.2. Binding assay

The radioligand binding experiments were carried out using homologous competitive binding method as described previously by Brown et al., (1997) with slight modification.

Tissue suspension of 30 µl (40 µg) was added to 30 µl of $^{125}$I-Sar$^1$–Ile$^8$-Ang II with 2.3-2.5 $\times$ $10^4$ c.p.m and 30 µl of increasing concentration of non-radioactive Sar$^1$ – Ile$^8$- Ang II (0.0001-300 nM) in 50 mM Tris-HCl buffer (pH 7.4) containing 0.3 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 mM bacitracin and 0.2 % bovine serum albumin (BSA). The assay mixture was incubated at 22°C for 2 hours with continuous shaking. The bound and unbound ligand was separated by addition of cold bovine gamma globulin (0.4 % w/v) followed by 20 % polyethylene glycol. The tubes were centrifuged at 5,000 x g and 4°C for 40 minutes and the radioactivity trapped in the pellets was counted using a gamma counter (Wizard Gamma Counter, Perkins Elmer). Efficiency of the counter was 95 %. Receptor density was calculated using Graph Pad Prism software. The assay was conducted in duplicate and repeated 3-4 times per group.

4.2.1.3. Protein assay (Biuret assay)

Protein concentration of samples was determined using Biuret assay according to the manufacturer’s instructions (Biuret reagent, Sigma, USA). A series of BSA solutions were prepared as protein standards in Tris-HCl, 50 mM (pH 7.4). The linear range of the assay is 2 mg/ml to 200 mg/ml. 20 µl of each standard or the sample was added to 1 ml of Biuret reagent and mixed immediately. The mixture was incubated for 30 - 40 minutes at room temperature and the absorbance at 540 nm was measured.
4.2.1.4. Chemicals

Tris-HCl is purchased from BHD Analar, England. Phenylmethylsulphonyl fluoride, bacitracin, bovine serum albumin, bovine gamma globulin and polyethylene glycol were purchased from Sigma Chemical Co., (Missouri, USA).

4.2.2. Semi-quantitative Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

4.2.2.1. RNA isolation

The right kidneys from WKY, SHR and STZ-induced diabetic rats were isolated and perfused with Krebs and captopril (30 µM) (control) or with DAA-I (10\(^{-9}\)M to 10\(^{-15}\)M) / Ang 1-7 (10\(^{-7}\)M) for 90 minutes. The kidneys were frozen in liquid nitrogen and kept in -80\(^{\circ}\)C for RNA isolation. Minimum animal sample required was calculated using statistical software, SAMPLESZ.XLS. Right kidneys were obtained from 3-5 individual animals per group and experiment was conducted on each independent kidney preparation.

Total RNA was isolated from the kidneys using Reagent ® system according to the manufacturer’s instructions (Promega, USA). The tissue (100 mg) was homogenised using Glass-Gol homogeniser in 1.2 ml of denaturing solution provided in Reagent ® system. Then 120 µl of 2 M sodium Acetate (pH 4) was added, followed by 1.2 ml Phenol: Chloroform: Isamyl Alcohol. This mixture was then kept in ice for 15 minutes before centrifuging at 10,000 x g for 20 minutes at 4\(^{\circ}\)C. After centrifugation, the top aqueous phase containing the RNA was carefully removed and transferred to a new sterile tube. Equal volume of isopropanol was added to the aqueous phase and the
solution was incubated at -20°C for 30 minutes to allow precipitation of RNA. After the RNA was pelleted by ultracentrifugation, it was washed in ice-cold 75% ethanol, re-pelleted and dissolved in Nuclease-Free Water. The amount of RNA was quantified by absorbance at 260 nm and stored at -20°C for not more than 6 months.

4.2.2.2. Reverse Transcriptase

Reverse-Transcriptase was performed using Revertaid™ H Minus First Strand cDNA Synthesis Kit, according to the manufacturer’s protocols (Fermentas Life Sciences, Canada). One microliter (1 µl) of 0.5 µg/μl oligo (dT)$_{18}$ primer was added to 1 µg of total RNA. DEPC-treated water was added to make up a total volume of 12 µl. The mixture was mixed gently, spun down and incubated at 70°C for 5 minutes. Then, 4 µl of 5X reaction buffer, 1 µl of RiboLock™ Ribonuclease inhibitor (20 U/µl), and 2 µl of 10 mM dNTP mix were added to the mixture and spun down. The mixture was incubated at 37°C for 5 minutes. Lastly, 1 µl of RevertAid™ H Minus M-MuLV Reverse Transcriptase was added and the mixture was incubated at 42°C for 60 minutes. The reaction was stopped by heating at 70°C for 10 minutes. The synthesized cDNA was kept at -20°C.

4.2.2.3. Polymerase Chain Reaction (PCR)

One microliter (1 µl) of synthesized cDNA was added to the reaction mixture containing reaction buffer, 0.2 µM of each of the two primers, 2 mM MgCl$_2$, 0.2 mM of each dNTP and 1.25 U of Taq polymerase. The final volume was 25 µl. The mixture was spun down and placed in the thermal cycler (PTC-200, MJ Research). After denaturation at 95°C for 15 minutes, amplification cycles of 94°C, 60°C and 72°C for 30
seconds were performed. Final elongation was carried out at 72°C for 2 minutes. GADPH was chosen as the internal standard. The PCR products were electrophoresed using 1.5% agarose gel containing ethidium bromide 0.5 µg/ml. The gel was subjected to ultraviolet light, photographed (Kodak Digital Science) and analyzed.

<table>
<thead>
<tr>
<th>Primer (5’ to 3’)</th>
<th>PCR cycle</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT₁</td>
<td>Fwd : ATCTCGCCTTGGCTGACTTA</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Rvs : GACTTCATTGGGTGGACGAT</td>
<td></td>
</tr>
<tr>
<td>GADPH</td>
<td>Fwd : GGTGCTGAGTATGTCGTG</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Rvs : TTCAGCTCTGGGATGACC</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1 List of primers used in semi-quantitative RT-PCR

4.2.3. Western Blotting

4.2.3.1. Tissue preparation and blotting

The right kidneys from WKY, SHR and STZ-induced diabetic rats were isolated and perfused with Krebs and captopril (30 µM) (control) or with DAA-I (10⁻⁹M to 10⁻¹⁵M) / Ang 1-7 (10⁻⁷M) for 90 minutes prior to the homogenate preparation. Minimum animal sample required was calculated using statistical software, SAMPLESZ.XLS. Right kidneys were obtained from 3 individual animals per group and experiment was conducted on each independent kidney preparation.
Tissue homogenate was prepared according to the method described by Zelezna et al., (1992) with slight modification. Kidneys were homogenised in phosphate-buffered saline (PBS) containing protease inhibitors (30 µg PMSF, 300 µg EDTA, and 0.5 µg bacitracin/ml). Homogenates were centrifuged at 20,000 x g for 10 minutes, and pellets were re-suspended in PBS with protease inhibitors and rinsed twice by centrifugation. Final pellet were re-suspended in 1 ml buffer and protein concentration were determined using Biuret assay as described in section 4.2.1.3. of this chapter. The supernatant was heated to 95°C for 5 minutes in the presence of 2X treatment buffer (0.125 M Tris-HCl, 4 % SDS, 20 % glycerol, 0.2 M DTT, 0.02 % Bromophenol blue, pH 6.8).

The protein (80 µg) was resolved on a 12.5 % SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane. The blotted PVDF membrane was saturated with 3 % gelatin in PBS, pH 7.4 containing 0.1 % Tween-20 for 1 hour at room temperature to block non-specific binding. The membrane was then sequentially incubated with AT1 receptor antibody (Santa Cruz Biotechnology, 1:1100) overnight at 4°C, washed six to seven times with Tris-buffered saline containing 0.1 % Tween-20 and followed by incubation with goat anti-rabbit IgG –HRP conjugated (1: 1300) for 2 hour at room temperature. After thorough washing, the blot was developed by incubating with HRP-complex until the bands have adequate intensity. The blot was dried, scanned and analyzed.

4.2.3.2. Chemicals

Phosphate-buffered saline and SDS were purchased from Biorad Laboratories, Canada. Phenylmethysulphonyl fluoride (PMSF), bacitracin, glycerol, dithiothreitol (DTT),
Bromophenol blue, gelatin and Tween-20 were purchased from Sigma Chemical Co, Missouri, USA.

4.2.4. Statistical analysis

Data were expressed as mean ± s.e.m. One-way ANOVA and Dunnett post hoc test were employed to determine the significance differences, with an accepted level of significance of p< 0.05. \( B_{\text{max}} \) and \( K_d \) value for receptor binding were determined using the homologous competitive equation (GraphPad Prism). The band intensities for RT-PCR and Western Blot were measured using a software package (Scion image). The signals for RT-PCR were expressed relative to the intensity of the GADPH amplicon for each co-amplified sample.

4.3. Results

4.3.1. Receptor binding assay

The binding of \(^{125}\text{I}-\text{Sar}^1-\text{Ile}^8\)-Ang II to kidney membranes was displaced by losartan and Sar\(^1\)-Ile\(^8\)-Ang II in a concentration-dependent manner with an almost 100% displacement at \(10^{-7}\)M concentration (Figure 4.1). On the other hand, PD 123319, an AT\(_2\) receptor antagonist was not able to displace the \(^{125}\text{I}-\text{Sar}^1-\text{Ile}^8\)-Ang II from the kidney membrane homogenate. PD123319 at \(10^{-6}\)M concentration displaced less than 30% of the labeled ligand. This shows that the kidney membranes contain mainly AT\(_1\) receptors and a very small proportion of AT\(_2\) receptors. AT\(_1\) receptor density was significantly higher in SHR kidney (739.6 ± 44.0 fmol/mg) and reduced in STZ-induced diabetic (271.5 ± 34.94 fmol/mg) compared to WKY (430.0 ± 8.12 fmol/mg) (Figure
4.2. AT$_1$ binding affinity ($K_d$) between WKY, SHR and STZ-induced diabetic rats were not significantly altered (Table 4.2).

In the functional studies conducted earlier (Chapter 3), the involvement of AT$_2$ receptor in the vasomodulatory role of DAA-I and Ang 1-7 was excluded. Thus, the following study was focused on evaluating the involvement of AT$_1$ receptors. There was a significant reduction in the AT$_1$ receptor density in WKY and SHR kidney pre-treated with DAA-I ($10^{-9}$M) (Figure 4.3 (A) and (B) respectively). Pre-treatment with $10^{-12}$M and $10^{-15}$M DAA-I was without effect on the receptor density in the WKY and SHR kidneys, although a small but statistically insignificant reduction in AT$_1$ receptor density was observed in SHR with DAA-I $10^{-12}$M pre-treatment. There was no significant difference in AT$_1$ receptor density in STZ-induced diabetic rat kidney pre-treated with DAA-I (Figure 4.3 (C)). AT$_1$ receptor binding affinity between the respective untreated (control) and the DAA-I treated WKY, SHR and STZ-induced diabetic rats was not significantly different (Table 4.2).

As found in the functional studies and described in Chapter 3 (Figure 3.7), vasodepressor action on the contractile action of Ang II in the isolated perfused kidney was mostly observed in the presence of Ang 1-7 ($10^{-7}$M). Therefore, only this concentration was investigated in this part of the study. Ang 1-7 ($10^{-7}$M) significantly reduced AT$_1$ receptor density in both WKY and SHR kidneys (Figure 4.4 (A) and (B) respectively). There was no significant difference in AT$_1$ receptor density in STZ-induced diabetic rat kidney pre-treated with Ang 1-7 compared to its untreated control group (Figure 4.4 (C)). No significant changes were seen in the AT$_1$ receptor binding affinity between the respective untreated groups (control) with their respective Ang 1-7 treated groups (Table 4.2).
Figure 4.1. Displacement of $^{125}$I-Sar$^1$–Ile$^8$-Ang II by losartan (■), Sar-Ile-Ang II (▲) and PD123319 (▼) in kidney membrane preparations of untreated rats (control). Graph represents the mean value ± s.e.m. of at least 5 independent experiments performed in triplicates.
Figure 4.2. Maximal binding capacity of $[^{125}\text{I}]-\text{Sar}^1\text{-Ile}^8\text{-Ang II}$ ($B_{\text{max}}$) in kidney membranes of WKY, SHR and STZ-treated rats. Bars represent the mean value ± s.e.m. of 4 independent experiments performed in duplicates. * Indicates significant difference from WKY (p<0.05).
Figure 4.3. \(\text{AT}_1\) receptor density in kidney of WKY (A), SHR (B) and STZ-induced diabetic rats (C) pre-treated with DAA-I respectively. The bars represent the mean value ± s.e.m. of 4-5 independent experiments performed in triplicates. Empty bars represent control groups and the shaded bars represents groups treated with graded concentrations (\(10^{-9}\)M, \(10^{-12}\)M and \(10^{-15}\)M) of DAA-I. * Indicates significant difference from respective control group (p< 0.05).
Figure 4.4. AT$_1$ receptor density in kidneys from WKY (A), SHR (B) and STZ-induced diabetic rats (C) pre-treated with $10^{-7}$M Ang 1-7 (shaded bars) or the vehicle-treated control group (empty bar). The bars represent the mean ± s.e.m. of 3-4 independent experiments performed in triplicates. * Indicates significant difference from respective control group (p< 0.05).
Table 4.2. Homologuos competitive binding parameters. Values are expressed as mean ± s.e.m. of 3-4 independent binding experiments. \( B_{\text{max}} \) values are in fmol/mg protein; \( K_d \) values are in nM. \(^a\) Indicates significant difference from WKY. \(^b\) Indicates significant difference from respective controls (p< 0.05).
4.3.2. RT-PCR

The mRNA expression of AT\textsubscript{1} receptors in the kidneys of WKY, SHR and STZ-induced diabetic rats is shown in Figure 4.5. GADPH was used as an internal standard. The densitometry values of AT\textsubscript{1} were normalized against the GADPH values. There was an increase in the AT\textsubscript{1} mRNA expression level in SHR and a decrease was seen in the STZ-induced diabetic group compared to the control WKY.

The effects of DAA-I (10\textsuperscript{-9}M to 10\textsuperscript{-15}M) on the mRNA expression of AT\textsubscript{1} receptors in the kidneys of WKY, SHR and STZ-induced diabetic are shown in Figure 4.6 (A), (B) and (C) respectively. The densitometry values were normalized against the GADPH and expressed as percentages of the respective control groups. DAA-I did not alter the level of AT\textsubscript{1} mRNA in the kidney for WKY, SHR and STZ-induced diabetic rats.

The effects of Ang 1-7 (10\textsuperscript{-7}M) on the mRNA expression of AT\textsubscript{1} receptors in the kidney of WKY, SHR and STZ-induced diabetic rats are shown in Figure 4.7 (A), (B) and (C) respectively. The densitometry values were normalized against the GADPH values and expressed as percentages of the respective control groups. Similar to DAA-I, Ang 1-7 did not affect the level of AT\textsubscript{1} mRNA in the kidney for WKY, SHR and STZ-induced diabetic rats.
Figure 4.5. AT$_1$ mRNA expression level in the kidney of WKY, SHR and STZ-induced diabetic rats. A: Agarose gel electropherogram of AT$_1$ and GAPDH cDNA. B: Quantification of AT$_1$ mRNA (normalized against GADPH mRNA). The bars represent the mean ± s.e.m. of 3-4 independent experiments performed in triplicates. * Indicates significant difference from the WKY value (p< 0.05).
Figure 4.6. Effect of DAA-I on the expression of AT₁ mRNA in perfused kidney of WKY (A), SHR (B) and STZ-induced diabetic rats (C). Above each bar chart is the respective agarose gel electropherogram of AT₁ and GADPH cDNA. The bars represent the mean value ± s.e.m. of 4-5 independent experiments performed in triplicates. Empty bars represent control groups and the shaded bars represent groups treated with graded concentrations (10⁻⁹M, 10⁻¹²M and 10⁻¹⁵M) of DAA-I. Each bar is expressed as a percentage of the control. * Indicates significant difference from respective control group (p< 0.05).
Figure 4.7. Effect of $10^{-7}$M Ang 1-7 (shaded bar) or the vehicle-treated control group (empty bar) on the expression level of AT$_1$ mRNA in perfused kidney of WKY (A), SHR (B) and STZ-induced diabetic rats (C). The bars represent the mean ± s.e.m. of 3-4 independent experiments performed in triplicates. Above each bar is the respective agarose gel electropherogram of AT$_1$ and GADPH cDNA. Each bar is expressed as a percentage of the control. * Indicates significant difference from respective control group (p< 0.05).
4.3.3. Western Blotting

The expression of AT₁ receptors in the kidney of WKY, SHR and STZ-induced diabetic rats is shown in Figure 4.8 and is expressed in densitometric values. There was an increase in the AT₁ receptor expression level in SHR compared to the control WKY and a decrease was seen in the STZ-induced diabetic group.

The effects of DAA-I (10⁻⁹M to 10⁻¹⁵M) on the expression of AT₁ receptors in the kidney of WKY, SHR and STZ-induced diabetic rats are shown in Figure 4.9 (A), (B) and (C) respectively. Levels of AT₁ receptor expression are recorded as percentages of the respective control groups. DAA-I perfusion did not affect the level of AT₁ receptor expression in the kidney for WKY, SHR and STZ-induced diabetic.

The effects of Ang 1-7 (10⁻⁷M) on the expression of AT₁ receptors in the kidney of WKY, SHR and STZ-induced diabetic are shown in Figure 4.10 (A), (B) and (C) respectively and are expressed as percentages of the respective control group value. Similar to DAA-I, Ang 1-7 did not affect the expression level of AT₁ receptor in the kidney for WKY, SHR and STZ-induced diabetic rats.
Figure 4.8. AT_1 receptor expression levels in the kidney of WKY, SHR and STZ-induced diabetic rats. A: Western blot of AT_1 receptor; B: Densitometric quantification of AT_1 receptor level. The bars represent the mean ± s.e.m. of 3 independent experiments performed in triplicates. * Indicates significant difference from the WKY value (p< 0.05).
Figure 4.9. Effect of DAA-I on the expression of AT$_1$ receptor levels in perfused kidney of WKY (A), SHR (B) and STZ-induced diabetic rats (C). Above each bar is the respective blot for AT$_1$ receptor. Empty bars represent control groups and the shaded bars represent groups treated with graded concentrations (10$^{-9}$M, 10$^{-12}$M and 10$^{-15}$M) of DAA-I. Each bar represents the mean ± s.e.m. of 3 independent experiments performed in triplicates and is expressed as % of its control. * Indicates significantly different from its control value (p< 0.05).
Figure 4.10. The expression levels of AT$_1$ receptor in perfused kidney of WKY (A), SHR (B) and STZ-induced diabetic rats (C) pre-treated with $10^{-7}$M Ang 1-7 (shaded bars) or the vehicle-treated control group (empty bars). Above each bar is the respective blot of AT$_1$ receptor. Each bar represents the mean ± s.e.m. of 3 independent experiments performed in triplicates. * Indicates significant difference from respective control value (p< 0.05).
4.4. Discussion

4.4.1. Angiotensin II modulation by AT\textsubscript{1} receptor in the kidney

The RAS plays an important role in the regulation of blood pressure and fluid homeostasis and exerts this effect through the production of Ang II. Ang II increases the renal vascular resistance by constricting afferent and efferent arterioles including interlobular arteries (Carmines \textit{et al.}, 1986). The effects of Ang II are mediated through high-affinity membrane bound receptors, classified as AT\textsubscript{1} and AT\textsubscript{2} receptor subtypes. Most of the known effects of Ang II have been attributed to the AT\textsubscript{1} receptor. In the kidney, AT\textsubscript{1} receptors are present in preglomerular arteries and arterioles, glomeruli, vasa rectae and proximal convoluted tubules (Chatziantoniou and Arendshorst, 1993).

In the present study, homologous competitive binding assay performed with specific AT\textsubscript{1} receptor antagonist, losartan and the AT\textsubscript{2} receptor antagonist, PD123319 revealed that mainly AT\textsubscript{1}, and not AT\textsubscript{2}, receptor subtype was present in the kidney membrane homogenates (Figure 4.1). Several binding studies have also shown exclusive presence of AT\textsubscript{1} receptors in the renal glomeruli, tubular and outer medullary membranes of Sprague-Dawley rats (Edwards and Aiyar, 1993; Gauquelin and Garcia, 1992). Autoradiographic techniques have also localized a predominant expression of AT\textsubscript{1} receptor in the adult rat kidneys (Ciuffo \textit{et al.}, 1993). Other reports also have documented the predominance of the AT\textsubscript{1} receptor subtype in preglomerular microvessels of the rabbit and human kidney (Brown and Venuto, 1988; Grone \textit{et al.}, 1992). RT-PCR and Western blot analysis comparably showed a predominance of AT\textsubscript{1} receptor localization in the rat kidneys (Miyata \textit{et al.}, 1999; Paxton \textit{et al.}, 1993; Zelenza \textit{et al.}, 1992).
In this study, the AT$_1$ receptor density was found to be increased in SHR kidney compared to the WKY rats (Figure 4.2). Several other authors have also demonstrated an increase in Ang II receptor density in the SHR kidney (Brown et al., 1997; Gutkind et al., 1998; Haddad and Garcia, 1996). These authors have correlated the increase in AT$_1$ receptor density with the enhanced contractile responses to Ang II. Although the AT$_1$ receptor density was found to be increased, the receptor affinity ($K_d$) was not significantly different between WKY and SHR animals (Table 4.2). The lack of alteration in AT$_1$ receptor affinity between WKY rats and SHR has also been reported by other authors (Chatziantoniou and Arendhorst, 1991; Haddad and Garcia, 1996). RT-PCR and Western blot analysis is also in agreement with the result from the receptor binding assay (Figure 4.5 and Figure 4.8). Increase in AT$_1$ receptor mRNA in the SHR has been observed in brain (Raizada et al., 1993, Reja et al., 2006), aorta (Otsuka et al., 1998), heart (Makino et al., 1997), and glomeruli of Stroke-prone SHR (Obata et al., 2000).

In contrast to SHR, the AT$_1$ receptor density was much reduced in the kidney of STZ-treated rats (Figure 4.2). This reduction mirrors the reduced responsiveness to Ang II observed in the earlier perfused kidney study (Chapter 2, Figure 2.3). Downregulation of the AT$_1$ receptor density has also been demonstrated in the whole kidney homogenates (Brown et al., 1997) and in the glomerulus (Amiri and Garcia, 2000; Ballermann et al., 1984; Wilkes, 1978) of STZ-induced diabetic rats. No significant difference in AT$_1$ receptor affinity was observed between WKY and STZ-treated groups with receptor binding assay (Figure 4.2). Similar observation was also documented by other researchers (Ballermann et al., 1984; Amiri and Garcia, 2000).
RT-PCR results and Western blot analysis are in agreement with the binding and functional studies where a lower AT$_1$ mRNA level was observed in the STZ-induced diabetic rat kidneys (Figure 4.5 and Figure 4.8). Reduced renal expression of the AT$_1$ receptor has been reported in renal promixal tubules (Cheng H.F. et al., 1994), in diabetic SHR animals (Bonnet et al., 2002) and in diabetic patients (Wagner et al., 1999). However, a study by Sharma et al., (1999) found that kidneys from diabetic rats exhibit decreased expression of the type I inositol triphosphatase receptor (InsP$_3$R) isoform and speculated that the impaired Ang II-mediated vasoconstriction in diabetic animals may be caused by downregulation of InsP$_3$Rs. Sharma et al. however reported in 2003, that AT$_1$ receptor mRNA remains unchanged in aorta from STZ-induced diabetic rats. In contrast, Wehbi et al., (2001) showed no significant change of the AT$_1$ receptor but reduced AT$_2$ receptor expression in the kidney from rats of STZ-induced diabetic animals. Such anomaly may be attributed to the differences in the strain of the animals and induction period of the diabetes.

4.4.2. Effect of DAA-I on AT$_1$ receptor in the kidney

At 10$^{-9}$M DAA-I, AT$_1$ receptor density was significantly decreased in both the WKY and SHR kidney homogenates (Figure 4.3). This suggests that the vasodepressor action of DAA-I may in part be due to a down-regulation of the AT$_1$ receptor in the kidney of WKY rats and SHR. Changes in receptor protein synthesis have been identified as late signaling events, and take hours to occur (Touyz and Schiffrin, 2000). As DAA-I was perfused into the kidney for more than an hour; it is possible that an adequate concentration could down-regulate the AT$_1$ receptor. However, the RT-PCR and Western blot analysis did not show down-regulation of AT$_1$ receptor as there were no changes in gene and protein expression with pretreatment of 10$^{-9}$ M DAA-I
concentration (Figure 4.6). It is possible that the reduced receptor density observed at $10^{-9}$ M DAA-I may be due to receptor internalization, thus reflecting the reduced receptor density described above.

Receptor internalization reduces the number of receptors present on the membrane surface (Figure 4.11). This may explain the findings from our binding assay as DAA-I may have repositioned the AT$_1$ receptor into the membrane and lysis of the membrane for Western blot may have exposed the internalized receptors. Agonist-induced receptor internalization especially of AT$_1$ receptor has been extensively reported (Conchon et al., 1994; Hunyady et al., 1994; Hunyady et al., 2000). Studies on endogenous AT$_2$ receptors have demonstrated that it is an internalization-deficient receptor (Hunyady et al., 2000). Internalization of the AT$_1$ receptor into the membrane may explain the vasodepressor action observed earlier in the isolated perfused kidneys of WKY and SHR animals.

Pre-treatment with $10^{-12}$M and $10^{-15}$M DAA-I did not affect the AT$_1$ receptor density although reduction in contractility was observed in the *ex vivo* study (Chapter 3, Figure 3.1). Although not statistically significant, a slight reduction in receptor density was seen in $10^{-12}$ M and $10^{-15}$ M DAA-I pre-treated kidneys especially in the SHR. This pattern was also observed with RT-PCR and Western blot analysis. At these physiological concentrations, a small difference in receptor availability could still contribute to the observed attenuation of Ang II contraction. Other possible mechanisms including coupling to different second messenger pathway, which negatively affect IP$_3$ pathway could also be postulated. However, this possibility requires further investigation.
CHAPTER 4  
Receptor studies

DAA-I

AT₁ receptor

1

G protein

PLC

DAA-I

AT₁ receptor

2

G protein

PLC

* *

Intracellular vesicle

G protein

PLC

Recycling and/or degradation of receptor

Degradation of ligand

3

AT₁ receptor

Figure 4.11. Receptor Internalization. Interaction of the receptor and ligand results in the activation of PLC via the coupling of the receptor with G proteins (stage 1). The receptor becomes uncoupled very rapidly following ligand addition by PKC dependent or independent mechanism (perhaps via phosphorylation of the receptor (*, stage 2). This is followed by internalization of the receptor with the subsequent recycling and/or degradation of the receptor and the degradation of the ligand (stage 3). (Reproduced from Sasamura et al., (1994)).
Pre-treatment with DAA-I did not affect the AT$_1$ receptor density in STZ-treated rat kidney (Figure 4.3). This perhaps explains the earlier result (Chapter 3, Figure 3.1), where DAA-I had no effect on Ang II-induced vasoconstriction in STZ-induced diabetic rat kidney. As the receptor density of AT$_1$ receptor was already reduced in diabetic state, possibly DAA-I did not reduce the receptor density further. In agreement with the binding assay, no changes in AT$_1$ receptor gene and protein expression was seen in STZ-induced diabetic rat kidney (Figure 4.6 and Figure 4.9). As structural changes occur during diabetes, it is possible that the binding site for DAA-I at AT$_1$ receptor may be altered, thus reducing DAA-I ability to bind to AT$_1$ receptor. A slightly lower AT$_1$ receptor affinity was detected in STZ-induced diabetic untreated and DAA-I treated animals compared to WKY (Table 4.2). No significant difference in receptor affinity was observed between the DAA-I treated group with its own control further suggests that DAA-I binds to other binding site than Ang II to modulate the AT$_1$ receptors.

4.4.3. Effect of Angiotensin 1-7 on AT$_1$ receptor in the kidney

Ang 1-7 has been shown to display an important role in rat renal homeostasis, i.e. produces natriuretic and diuretic responses, increase urinary sodium concentration and increase in glomerular filtration rate (DellPizzi et al., 1994). In the present study, WKY and SHR kidney perfused with Ang 1-7 ($10^{-7}$M) lowered the angiotensin receptor (AT$_1$) density compared to the control (Figure 4.4). The reduction observed in SHR (33.3 %) was higher than that observed in the WKY (17.8 %). Receptor binding assay suggests that the vasodepressor action of Ang 1-7 is partly modulated via AT$_1$ receptor and is more apparent in SHR. We say partly because in earlier investigation with isolated perfused kidney, involvement of Ang 1-7 receptor was demonstrated and shown to activate both prostaglandins and nitric oxide (Chapter 3, Figure 3.8 and Figure 3.9).
Gironacci et al., (1999) demonstrated that Ang 1-7 binds to AT$_1$ receptor in rat cortex. The renal action of Ang 1-7 may result from a regulatory mechanism in which it competes with Ang II for AT$_1$ receptor binding sites and this has been hypothesized earlier (Ferrario et al., 1997). However, results of this study (Figure 4.7 and Figure 4.10) on the effect of Ang 1-7 on AT$_1$ gene and protein expression ruled out AT$_1$ receptor down-regulation. Similar to DAA-I, it is possible that binding of Ang 1-7 to AT$_1$ receptor leads to internalization of AT$_1$ receptor besides acting on its own receptor to elicit the vasodepressor actions observed in isolated perfused kidney (Chapter 3, Figure 3.7). Clark et al., (2001) has suggested the possibility of Ang 1-7 binding to and activating AT$_1$ receptor to produce internalization without coupling to G-proteins to activate Phospholipase C (PLC). No significant differences in AT$_1$ receptor affinity were observed between the Ang 1-7 treated groups and the control group. This suggests that Ang 1-7 may bind at a site different from that of Ang II on AT$_1$ receptor to modulate the receptor, possibly via internalization.

Similar to DAA-I, pre-treatment with Ang 1-7 in STZ-induced diabetic kidney, did not affect the angiotensin (AT$_1$) receptor density (Figure 4.4). Furthermore, no changes were seen in AT$_1$ receptor gene and protein expression in the STZ-induced diabetic rat kidney perfused with Ang 1-7 (Figure 4.7 and Figure 4.10). This shows that Ang 1-7 does not affect the AT$_1$ receptor density. This finding is in agreement with the earlier finding where the vasodepressor action of Ang 1-7 was lost in diabetic condition (Chapter 3, Figure 3.7). No significant differences in AT$_1$ receptor affinity was observed between STZ-induced control and Ang 1-7 treated group. Santos et al., (2003) have demonstrated the G protein-coupled receptor Mas as a functional Ang 1-7 receptor. Hence, reduced level or absence of Mas receptor may explain the loss of Ang 1-7 action.
in the STZ-induced diabetic kidney. However, this assumption requires further investigation.

4.5. Summary

This part of this study was conducted to determine the involvement of AT₁ receptor in the modulation of Ang II induced contraction as well as in the vasodepressor action of DAA-I and Ang 1-7 in both physiological and pathological conditions like hypertension and diabetes mellitus. Receptor binding assay revealed that the rat kidney contains mainly of AT₁ receptor subtype whilst AT₂ receptor subtype may be present to a lesser amount. Interestingly, receptor binding assay revealed that these AT₁ receptors are found to be increased in hypertensive rat kidney and decreased in STZ-induced diabetic rat kidneys. These results are further supported with the similar findings from RT-PCR and Western blot analysis.

The changes seen in AT₁ receptor gene and protein expression may be altered in pathological states to accommodate the ongoing vascular changes (arterial remodeling). The term ‘arterial remodelling’ can mean either an increase or a decrease in the arterial diameter compared to normal (Ward et al., 2000). Receptor binding assay showed that 10⁻⁹M DAA-I decreased AT₁ receptor density but findings from RT-PCR and Western blot analysis showed no difference in the expression level between DAA-I treated and untreated control. DAA-I appears to modulate AT₁ receptor in the renal vasculature via AT₁ receptor internalization. Similar to DAA-I, receptor binding assay showed a decrease in AT₁ receptor density with Ang 1-7 (10⁻⁷M) pre-treatment but this decrease was not observed with RT-PCR and Western blot analysis. This suggests that the action of Ang 1-7 also appears to involve the internalization of AT₁ receptor.
VASODPRESSOR ACTION OF DAA-I AND ANG 1-7 IN NEONATAL STREPTOZOTOCIN INDUCED DIABETIC (n-STZ) RATS

5.1. Introduction

Insulin insufficiency is a key feature in both type 1 and type 2 diabetes mellitus. However, type 2 diabetes is further characterized by several metabolic defects, among which β-cell secretory dysfunction and peripheral insulin resistance are considered as hallmarks of the disease (Masiello, 2006). Activation of the RAS by high glucose, mechanical stress, and proteinuria has been implicated in the major changes associated with diabetic nephropathy (Wolf, 2004). The majority of studies on RAS especially involving Ang II, have been conducted primarily in type 1 diabetic animal models (Haddad & Garcia, 1996; Kost et al., 1994), whilst work using type 2 diabetes model remains scarce. One of the animal models that is frequently used to represent type 2 diabetes is the neonatal streptozotocin- induced (n-STZ). It has been reported that the β-cell function in n-STZ exhibits similar insulin secretory characteristics found in type 2 diabetic patients (Dachicourt et al., 1997; Morin et al., 1997; Shinde et al., 2001). The pattern of insulin release found in n-STZ is also reported to be qualitatively similar to Goto-Kakizaki (GK) rats, which is a genetically diabetic non-obese animal model of human diabetes mellitus (Dachicourt et al., 1997).

In Chapter 2, reduced contractile response to angiotensin peptides, especially Ang II in type 1 diabetic animal model has been demonstrated (Figure 2.3). In Chapter 3, the vasodepressor actions of DAA-I and Ang 1-7 were also shown to be diminished in the STZ-induced diabetic rats especially in the kidney (Figure 3.1 and Figure 3.7). We were, therefore interested in comparing the vasomodulatory role of each of these two
peptides in the renal vasculature of type 1 (STZ-induced) and type 2 (neonatal STZ-induced) diabetes models.

5.2. Material and method

5.2.1. Development of n-STZ rat model.

Two day old Male WKY pups were injected with STZ (100 mg/kg) intraperitoneally (i.p). Age matched controls received equal volume of the vehicle. Body weight and blood glucose for each rat were taken three days later and monitored every 2 weeks until the 20th week-the same age as the STZ-induced diabetic rats which were used in the earlier part of the study (Chapter 2, section 2.2.3.). Approval for the following studies was obtained from the Committee for Animal Care and Use at the Animal Center of Faculty of Medicine in the University of Malaya. All experimental procedures were carried out according to the guidelines for ethical care of experimental animals.

Besides monitoring the blood glucose level, oral glucose tolerance test and insulin assay were also conducted when the rats were 12 weeks and 20 weeks old to confirm the validity of the n-STZ model.

5.2.2. Oral Glucose Tolerance test (OGTT)

OGTT was conducted between the 19th- 20th week of age in both STZ- and n-STZ-induced diabetic rats, prior to experimentation. The rats were fasted overnight (approximately 12 hours) prior to test. Each rat was given an oral glucose load, 3 g/kg body weight according to Al-Awadi et al., (1985). Blood glucose was checked at time 0
(prior to glucose load), 30, 60, 90 and 120 minutes after the glucose load. Blood glucose levels were assessed using the Advantage II blood glucose test strips (Roche Diagnostics, Germany). Blood (1 drop) was collected from the rat tail by pricking with a 26G needle at the tip of the tail.

5.2.3. Insulin assay

Blood (3 ml) was collected from the tail of STZ- and n-STZ-induced diabetic rats. The blood was then spun down at 3,500 x g for 10 minutes and plasma was separated and kept at -80°C for insulin assay.

Insulin assay was conducted using Mercodia Rat Insulin ELISA, Sweden. The assay was conducted as follows: - 25 µl calibrators/ samples were added into the plate provided, followed by 50 µl enzyme conjugate and then the plate was incubated for 2 hours at 22°C with continuous shaking. After incubation, the plate was washed at least 6 times with the buffer provided. Then 200 µl substrate was added and incubated for 15 minutes at 22°C with continuous shaking. To stop the reaction, 50 µl of the stop solution was added and shook for 5 seconds. The absorbance was measured at 450 nm. The detection limit of the assay is 0.07 µg/l. Calibration curve was then plotted and insulin concentration of the samples calculated.

5.2.4. Preparation of isolated perfused kidney

This was performed as described in Section 2.2.4 (Chapter 2).
5.2.4.1. Studies of the contractile responses to angiotensin peptides in n-STZ-induced diabetic rats

The isolated renal vasculature preparation was perfused with Kreb’s solution containing 30 µM captopril. Following 1 hour of perfusion, various concentrations (10^{-10} – 10^{-6} M) of angiotensin peptides (Ang II, Ang 1-7 and DAA-I) were used to produce a contractile response. The angiotensins were administered as a single bolus injection of 20 µl (renal) and 50 µl (mesenteric bed) into the perfusion system. The minimum time interval between successive bolus injections was 10 min or until the initial basal perfusion pressure was again recorded.

5.2.4.2. Effects of DAA-I and Ang 1-7 on the contractile action of angiotensin II in n-STZ induced diabetic rats

The first step of the experimental protocol is as described in Section 2.2.5 (Chapter 2). The effects of various concentrations of DAA-I (10^{-15} M - 10^{-9} M) and Ang 1-7 (10^{-7} M) on the contractile response of Ang II were studied with the following protocol. The concentrations chosen were the concentration that did not induce contraction directly on the vasculature. The preparation was first perfused with Kreb’s solution containing 30 µM captopril and a concentration of DAA-I or Ang 1-7 for 60 min, prior to initiating a contractile dose-dependent response to Ang II. Ang II was given as bolus injections as mentioned in Section 2.2.5.1 (Chapter 2). Each concentration of DAA-I or Ang 1-7 was studied using a new set of isolated vascular preparation.
5.2.5. Statistical analysis

The perfusion pressure of the kidney upon drug administration was taken and the pressor responses to these drugs were normalized against the pressor action of PE and expressed as percentage. Data are presented as mean ± s.e.m. Significant difference (p<0.05) between means was evaluated using unpaired Student’s t-test when comparing two groups. When more than two groups are compared and for the comparison of the dose-response curves, data were evaluated by one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. Results with p<0.05 were considered statistically significant.

5.3. Results

Body weight and blood glucose results for WKY (control) and STZ-induced diabetic rats are included for comparison and have been presented in Chapter 2 (Figure 2.1 and Figure 2.2). WKY (control) and n-STZ induced diabetic rats gained weight continuously until the 20th week. However, the weight gained by the control group rats was significantly more than that of the n-STZ induced diabetic rats. In contrast, the body weight of STZ-induced diabetic group significantly dropped after induction and continued to decrease throughout the 8 weeks induction period as shown in Chapter 2. The body weight for all the three groups at week 20 is shown in Table 5.1.

Blood glucose taken prior to isolation of the tissue for experiment is shown in Table 5.1. WKY rats displayed resting blood glucose of 6.09 ± 0.32 mM. The n-STZ induced diabetic group showed mild hyperglycemia (12.36 ± 0.91 mM), while the STZ-induced group showed a very significant elevation in blood glucose (29.54 ± 0.73 mM).
Plasma insulin measured for all the groups is also summarized in Table 5.1. The STZ-induced diabetic group showed significantly lower level of insulin compared to control (0.388 ± 0.027 µg/L vs. 0.866 ± 0.099 µg/L). The n-STZ showed a mild hypoinsulinemia compared to control (0.628 ± 0.073 µg/L vs. 0.866 ± 0.099 µg/L) but is significantly higher than the STZ-induced group.

<table>
<thead>
<tr>
<th></th>
<th>WKY</th>
<th>STZ-induced diabetic</th>
<th>n-STZ induced diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight (g)</strong></td>
<td>339.47 ± 5.05 (n=38)</td>
<td>227.76 ± 5.31* (n=38)</td>
<td>297.71 ± 4.49 * b (n=35)</td>
</tr>
<tr>
<td><strong>Blood glucose (mM)</strong></td>
<td>6.09 ± 0.32 (n=50)</td>
<td>29.54 ± 0.73 * (n=50)</td>
<td>12.36 ± 0.91 * b (n=35)</td>
</tr>
<tr>
<td><strong>Plasma Insulin (µg/L)</strong></td>
<td>0.886 ± 0.099 (n=8)</td>
<td>0.388 ± 0.027 * (n=10)</td>
<td>0.628 ± 0.073 * b (n=19)</td>
</tr>
</tbody>
</table>

Table 5.1. Body weight, blood glucose and plasma insulin levels measured for WKY, STZ- and n-STZ induced diabetic rats prior to tissue preparations. * Indicates significant difference from WKY; b indicates significant difference from STZ-induced diabetic (p<0.05).
5.3.1. Oral Glucose Tolerance Test (OGTT)

The OGTT results for WKY (control), STZ- and n-STZ induced diabetic rats are shown in Figure 5.1. After overnight fasting, STZ-induced diabetic rats had higher blood glucose than n-STZ-induced diabetic or the WKY (control) rats. 30 minutes after oral glucose load, the n-STZ induced diabetic group showed significantly higher blood glucose than WKY (12.99 ± 0.32 mM vs. 6.07 ± 0.23 mM) and took a longer time to return to basal level. The STZ-induced diabetic group also showed a significant increase compared to WKY rats after oral glucose (28.33 ± 3.43 mM vs. 6.07 ± 0.23 mM). However, compared to the n-STZ group, the blood glucose of the STZ did not return to basal level within the 2 hour period of experiment.
Figure 5.1. Oral Glucose Tolerance Test (OGTT) conducted on WKY (control), STZ- and n-STZ-induced diabetic rats after overnight fasting. Blood glucose taken before oral glucose load, and every 30 minutes after load for 2 hour. The graph represents the mean ± s.e.m. of 20-30 rats. * Indicates significant difference from the WKY value (* p< 0.05, ** p< 0.01, *** p< 0.001).
5.3.2. Vascular reactivity of angiotensin peptides

Figure 5.2 shows the dose-response of renal perfusion pressure to Ang II in WKY (control), STZ- and n-STZ-induced diabetic rats. The responses showed concentration dependent increase in contractions which were significantly less in the STZ- and n-STZ-induced rats for the highest four or three concentrations of Ang II, respectively.

Figure 5.3 shows the dose-response of renal perfusion of DAA-I in WKY (control), STZ- and n-STZ-induced diabetic rats. The responses to DAA-I was lesser than Ang II and was observed at higher concentrations (> $10^{-6}$M). There was no significant difference in DAA-I induced vasoconstriction between WKY, STZ- and n-STZ-induced diabetic rats at lower concentrations. In the n-STZ-diabetic group, an increase in DAA-I contractile action was observed at $10^{-7}$M compared to WKY. However, a slight decrease in pressor action was observed in both diabetic groups at the highest concentration.

In contrast to Ang II and DAA-I, Ang 1-7 did not induce any increase in renal perfusion pressure in the isolated perfused kidney of all the three groups of animals.
Figure 5.2. Angiotensin II induced pressure response in the renal arterial bed. Alphabet immediately below bar: C, control (WKY); S, STZ-induced diabetic; nS, n-STZ-induced diabetic. Each bar represents the mean ± s.e.m. of 4-5 separate preparations. * Indicates significant difference from the control (p< 0.05).
Figure 5.3. des-Asp-angiotensin I induced pressure response in the renal arterial bed. Alphabet immediately below bar: C, control (WKY); S, STZ-induced diabetic; nS, n-STZ-induced diabetic. Each bar represents the mean ± s.e.m. of 4 separate preparations.

* Indicates significant difference from the control (p< 0.05).
5.3.3. Effect of DAA-I on the angiotensin II-induced vasoconstriction in the isolated perfused kidney of n-STZ induced diabetic rat.

Pressor responses to Ang II in the WKY were significantly attenuated by DAA-I. The attenuation was observed at the higher Ang II concentrations (10\(^{-9}\)M-10\(^{-6}\) M) (Figure 5.4). In STZ-induced diabetic, DAA-I had no effect on Ang II pressor responses. At DAA-I (10\(^{-9}\)M), a slight decrease in contractile action was seen with 10\(^{-6}\)M Ang II. DAA-I, by itself, had no affect on the basal perfusion pressure. Similar to STZ-induced diabetic group, DAA-I also had no effect on the contractile responses to Ang II in the n-STZ induced diabetic kidney (Figure 5.4).

5.3.4. Effect of Ang 1-7 on the angiotensin II induced vasoconstriction in the isolated perfused kidney of n-STZ induced diabetic rat.

Earlier work conducted showed that the increase in renal perfusion pressure to Ang II was attenuated by Ang 1-7 (10\(^{-7}\)M) in WKY rat kidney (Figure 3.7, Chapter 3). The attenuation was observed from 10\(^{-10}\)M Ang II for WKY. Ang 1-7 did not affect the Ang II-induced vasoconstriction in STZ-induced diabetic rats. However, Ang 1-7 by itself did not affect the basal perfusion pressure. Similar to STZ-induced diabetic, Ang 1-7 did not affect Ang II induced vasoconstriction in n-STZ induced diabetic group (Figure 5.5).
Figure 5.4. Effect of graded concentrations (10^{-15}M - 10^{-9}M) of DAA-I on the angiotensin II (10^{-10}M - 10^{-6}M) induced pressure responses in the perfused renal vasculature of WKY, STZ- and n-STZ-induced diabetic rats. C, control (untreated). Each bar represents the mean ± s.e.m. of 4-5 separate preparations. * Indicates significant difference from the DAA-I vehicle-pretreated control (C) (p< 0.05).
Figure 5.5. Effect of $10^{-7}$ M Ang 1-7 (-7) or the vehicle-treated control group (C) on the angiotensin II ($10^{-10}$ M - $10^{-6}$ M) induced pressure responses in the perfused renal vasculature of WKY, STZ-induced and n-STZ induced diabetic rats. Each bar represents the mean ± s.e.m. of 4-5 separate preparations. * Indicates significant difference from the DAA-I vehicle-pretreated control (C) (p< 0.05).
5.4. Discussion

The neonatal WKY rats injected with STZ (2 days after birth) showed mild hyperglycemia after 3 days (12.73 ± 0.6150 mM) and then the blood glucose returned to normal (6.454 ± 0.3579 mM). After 8 weeks of age, mild hyperglycemia started to become evident again and blood glucose of 12.36 ± 0.9027 mM was detected at 20 weeks of age. This is in agreement with other authors who have demonstrated that neonatal Wistar rats treated with 90-100 mg/kg body weight STZ 2 days after birth develop transient hyperglycemia 3 days after injection (Junod et al., 1969; Masiello, 2006; Portha et al., 1989). After recovery from the initial acute hyperglycemia reaction to STZ, blood glucose tends to be normal or near-normal until after puberty when overt hyperglycemia becomes apparent. (Arulmozhi et al., 2004; Oliver et al., 1989). These authors also demonstrated that by 8 weeks of age and thereafter, the n-STZ induced diabetic rats shows mild hyperglycemia.

Together with the mild hyperglycemia, the n-STZ induced rats showed consistent increase in body weight, although at a much slower rate than the WKY rats. It has been shown that the moderate diabetic state of adult n-STZ does not affect body weight gain (Masiello, 2006). In the present study, the n-STZ animals showed slightly lower plasma insulin levels than the control. Fantus et al., (1987) also have shown minimal decrease in the plasma insulin at 4 and 8 week in n-STZ induced rats. Similarly, a mild hypoinsulinemia was observed 4 weeks after STZ injection by Kergoat et al., (1991). The n-STZ induced rats also showed abnormal response to glucose tolerance test. The blood glucose taken 30 minutes after oral glucose load was significantly higher than WKY and required a longer time to return to basal level. These findings fairly validate the characteristics of this type 2 diabetes mellitus model.
5.4.1. Vascular reactivity of angiotensin peptides in n-STZ induced rat kidney.

In Chapter 2, we have demonstrated that the Ang II contractile action was decreased in STZ-induced diabetic kidney. Similar responses were also seen in n-STZ induced diabetic kidney (Figure 5.2). Our finding is in agreement with the work conducted by Harker et al., (1993), who showed that contractile responses to Ang II were reduced in aorta of Zucker Obese rats, a genetic model of type 2 diabetes mellitus. Fliser et al., (1997), have shown that the Ang II-induced increase in mean blood flow was attenuated in type 2 diabetic patients compared to healthy volunteers. Furthermore, Wagner et al., (1999) have demonstrated decreased expression of AT₁ angiotensin receptor mRNA in type 2 diabetic nephropathy patients. The decreased expression of AT₁ angiotensin receptor mRNA may explain the reduced Ang II pressor response observed in our studies in both STZ- and n-STZ-induced diabetic rats. Changes in signaling pathway may also contribute to the reduced Ang II pressor response observed in both STZ- and n-STZ induced diabetic rats. Ang II, as activator of G-protein coupled receptors has been shown to exhibit cross-talk with phosphatidylinositol 3-kinase (PI3-K) activity in human vascular smooth muscle cells (Hafizi et al., 2004). This cross-talk regulation of PI3-K/Akt activity by Ang II could have an important role in the development of insulin resistance (Henriksen et al., 2001; Juan et al., 2005).

In contrast, Guo et al., (2005), demonstrated enhanced Ang II-induced contraction in endothelium-denuded aorta and mesenteric artery from db/db mice. Increased expressions of AT₁ angiotensin receptor expression in aorta and kidney have also been demonstrated (Yang et al., 2005). However, recent finding by Konoshita et al., (2006) showed unchanged expression of both renal AT₁ and AT₂ receptor expressions in
human diabetic nephropathy patients. Unaltered vasoconstritor response to Ang II has also been demonstrated in type 2 diabetic patients (Harada et al., 2002).

5.4.2. Effect of DAA-I on Ang II induced vasoconstriction in the renal vasculature of n-STZ induced diabetic rat.

DAA-I reduced Ang II-induced pressor responses in WKY rat renal vasculature. Unlike in WKY rats, DAA-I had no effect on the Ang II-induced pressor response in STZ-induced diabetic and n-STZ induced rat kidneys (Figure 5.5). However, in the STZ-induced diabetic rat kidneys, DAA-I (10^{-9}M) demonstrated a slight decrease in contractile action with 10^{-6}M Ang II. It is possible DAA-I at higher concentration is attempting to maintain Ang II contractile responses within a certain range. This suggests that the mechanisms involved in the vasodepressor action of DAA-I is compromised in diabetic state in either of the type 1 or type 2 diabetic model. As DAA-I has been demonstrated to act via AT_1 receptor (Sim and Chai, 1996), it may exhibit cross-talk with PI3-K activity similar to Ang II, and alteration in this cross-talk may affect the action of DAA-I. However, further work is required to ascertain this assumption with both type 1 and type 2 diabetic models.

Cellular mechanisms may be altered by Ang II to facilitate the vascular changes that occur in pathological states. These cellular changes have been demonstrated in hypertension states (Figure 5.6, Touyz, 2005). Similar cellular mechanism may operate in diabetes condition. From the present data, one can postulate that DAA-I modulates Ang II contractile effect settling it within a normal range, akin to a homeostatic regulatory mechanism. Thus, it is possible that the need to exert negative control on the
increase pressor response to Ang II may not be crucial in the diabetic kidney as the responses to Ang II were already diminished.

5.4.3. Effect of Ang 1-7 on Ang II induced vasoconstriction in the renal vasculature of n-STZ induced diabetic rat.

Similarly to DAA-I, Ang 1-7 also reduced Ang II-induced pressor response in WKY rat kidneys and this action was lost in both STZ- and n-STZ induced diabetic rat kidney. Many studies have shown the involvement of Ang 1-7 receptor, since some of its effects...
could be inhibited by the Ang 1-7 receptor antagonist, D-ALA (Ambuhl et al., 1994, Santos et al., 2000, Santos et al., 2001 and Vallon et al., 1998). Other studies have raised the possibility that Ang 1-7 may act as an endogenous antagonist of the AT1 receptor or it may modulate Ang II via AT1 receptor (Clark et al., 2001; Mohan et al., 1994). Santos et al., (2003) have demonstrated that Mas, a G coupled protein, acts as an endogenous Ang 1-7 receptor. Mas may form a complex with AT1 receptor to inhibit the action of Ang II (Kostenis et al., 2005). Therefore, reduced level or absence of Mas may explain the loss of Ang 1-7 action in both types of diabetic models. However, this assumption requires further investigation. Similar to DAA-I, it is also possible that the need to exert negative control by Ang 1-7 on the increase in pressor response of Ang II may not be critical in the diabetic kidney since the responses to Ang II are already diminished.

5.5. Summary

The present preliminary findings demonstrated that pressor responses to Ang II, DAA-I and Ang 1-7 in n-STZ-induced (type 2) diabetic rat kidneys are similar to STZ-induced (type 1) diabetes mellitus rat model. Also similar are the actions of DAA-I and Ang 1-7 on Ang II induced pressor response in type 2 diabetic rat kidney. This indicates that in both types of diabetic models, the receptors for or the actions of these peptides are modified in a similar manner, perhaps to facilitate the ongoing vascular changes.
GENERAL DISCUSSION & CONCLUSION

Angiotensin peptides play an essential role in controlling the functional and structural integrity of the arterial wall and are involved in the physiological processes regulating blood pressure. The majority of the effects in vascular responses are mediated by Ang II acting via the AT$_1$ receptor subtype (Touyz and Schiffrin, 2000). Recent evidence suggest that in addition to Ang II, other functionally active angiotensin fragments, e.g. des-Asp-angiotensin I (Min et al., 2000; Sim and Chai, 1996; Sim and Min, 1998; Wen et al., 2004), angiotensin 1-7 (Ferrario et al., 1997; Kucharewicz et al., 2002) and angiotensin IV (Wright et al., 1995) may be equally important. Besides existing AT$_1$ and AT$_2$ angiotensin receptors, putative binding sites for angiotensin IV (AT$_4$) and angiotensin 1-7 (Ang 1-7) have been demonstrated and pharmacologically characterized (Gironacci et al., 2004; Hamilton et al., 2001; Sampaio et al., 2007; Silva et al., 2007; Thomas et al., 2003). These findings have questioned the concept of a ‘single’ regulatory peptide and opened up avenues for an integrated role of the angiotensins in the RAS.

Ang II elicits a complex and highly regulated cascade of intercellular signal transduction that leads to short term vascular effects, such as contraction, and long term biological effects, such as cell growth, migration, extracellular matrix deposition and inflammation (Touyz et al., 2000). Because of their role in cardiovascular diseases like hypertension and diabetes, it is important to understand the actions of these angiotensin peptides in physiological and pathological conditions. Taking a step further, the present study looked at the role of these peptides in resistance vessels, as small changes in such vessels may significantly affect regulation of the body hemodynamics.
In the present study, the importance of Ang II is further established as it proves to be a potent vasoconstrictor in both the renal and mesenteric vasculature and its contractile action appears to be altered differently during pathological states (Figure 2.3 and Figure 2.5). In the renal vasculature, Ang II pressor response was increased in hypertensive and reduced in STZ-induced diabetic rats (type 1 diabetes rat model). The changes in renal angiotensin receptor density may account for the changes in contractile responses observed in hypertension (Haddad and Garcia, 1996) and diabetes (Ballermann et al., 1984; Brown et al., 1997). Alteration in signalling pathways like cAMP (Chatziantoniou et al., 1990) and vasodilator prostaglandin production (Chatziantoniou and Arendshorst, 1992) has also been associated with hyperresponsiveness of Ang II in SHR. In diabetes, decreased nitric oxide release (Bank and Aynedian, 1993) and changes in the receptors involved in intracellular signalling of Ang II, (Sharma et al., 1999) may reduce Ang II contractile action. In the present study, Ang II induced vasoconstriction in the mesenteric artery was much smaller than that seen in the kidney. An earlier study by Warner, (1990) has shown Ang II to be a strong venoconstrictor in the mesenteric artery. In this study, Ang II contractile action was unaltered in type 1 diabetes but decreased in hypertensive condition. The differences in Ang II contraction between hypertension and diabetes could be temporal dependent.

The present study is the first in the literature to demonstrate contractile action of DAA-I in the renal and mesenteric vascular beds. DAA-I appears to have constrictor action in both the renal and mesenteric vasculatures but about 10 times less potent than Ang II (Figure 2.4 and Figure 2.6). The constrictor action was almost negligible in the mesenteric vasculature and observed at higher concentration of DAA-I (>10^{-6}M). A modulatory role for DAA-I in the RAS was proposed by Sim and Yuan, (1995) and has been demonstrated by other studies (Mustafa et al., 2004; Wen et al., 2004). The
contractile action of this nanopeptide was not significantly altered in hypertensive and diabetic conditions, suggesting that the receptors mediating DAA-I are not markedly altered in pathological conditions. In contrast to the other angiotensin peptides, Ang 1-7 is devoid of constrictor action in both vascular beds examined in this study in all the conditions. Ang 1-7 has been shown to exhibit vasodilatation in many vascular beds (Feterik et al., 2000; Ren et al., 2002). Similar to DAA-I, this heptapeptide has been shown to have opposing actions to Ang II. Thus, it is proposed that both of these peptides (DAA-I and Ang 1-7) may have modulatory role on Ang II effect in most vasculatures.

The result of the present study is the first finding to demonstrate a regulatory role of DAA-I on Ang II contractile action in the vascular beds of the kidney and mesentery in normotensive, hypertensive and diabetic rat model. Treatment with DAA-I attenuated the Ang II pressor actions in normotensive and hypertensive rat kidney (Figure 3.1). Similar attenuation was also previously reported against the pressor actions of Ang III (Mustafa et al, 2004). In the present study, the effective concentration of DAA-I ranges from 10^{-9}M to 10^{-11}M, and this is well below the reported circulating (pico molar) level in vivo (Sim and Qui, 2003).

However, the vasodepressor action of DAA-I appeared to be compromised in the STZ-diabetic rat model. This may be explained by lack of necessity to exert negative control on the higher concentrations of Ang II in the diabetic renal vasculature because the responses to Ang II are already reduced in the diabetic animals. In effect, we postulate that a critical level of Ang II-induced contraction is required to trigger an Ang II-DAA-I crosstalk which produces attenuation. At the lower Ang II concentrations (10^{-13}M-10^{-11}M), DAA-I however, enhanced Ang II mediated contraction (Figure 3.1). It thus
appears, as if DAA-I regulates a homeostasis mechanism for maintaining Ang II induced renal artery contraction within a certain range. In the mesenteric vasculature, a similar pattern of DAA-I action was observed as in the renal vasculature.

The vasodepressor actions of DAA-I in both renal and mesenteric vasculature of WKY and SHR appeared not to involve AT$_2$ receptor stimulation or release of cyclooxygenase byproducts. However in hypertensive animals, the present findings is supportive of a role of nitric oxide as L-NAME pre-treatment attenuated the actions of DAA-I. Perhaps hypertension pathophysiology prompts DAA-I to trigger NOS-NO pathway as a compensatory mechanism.

The current data also suggest that Ang 1-7 has a similar regulatory role in the contractile response to Ang II of the kidney and mesenteric vasculatures (Figure 3.7 and Figure 3.10). Attenuation of Ang II pressor responses by Ang 1-7 has been demonstrated in the rabbit and rat aortic rings (Le Tran and Forster, 1997; Mohan et al., 1994). Similar to DAA-I, Ang 1-7 attenuated the Ang II-induced vasoconstrictions in both normotensive and hypertensive rat kidneys. This is the first study to report on the role of Ang 1-7 in the mesenteric vascular bed of the diabetic rat model.

In the mesenteric vascular bed, Ang 1-7 attenuated Ang II-induced vasoconstriction in the three different groups of animals-the WKY, SHR and STZ-induced diabetic (Figure 3.10). The attenuation in WKY and diabetic mesenteric beds was observed in the lower concentrations ($10^{-10}$M - $10^{-8}$M) of Ang II and the opposite was seen in SHR rats. It is possible that the failure of Ang 1-7 in attenuating the contractile effect of Ang II at these lower concentrations in the SHR could be due to the lower contractile responses.
It is also possible a crucial level of Ang II-induced contraction is required to trigger the Ang II-Ang 1-7 crosstalk which produced the above mentioned attenuation.

In the kidney, Ang 1-7 action appears to be mediated via the Ang 1-7 receptor in the WKY and SHR rats, and involves the release of vasodilator prostaglandins and nitric oxide. In the mesentery, this vasodepressor action appears to be modulated by Ang 1-7 receptor and involves vasodilator prostaglandins and nitric oxide in SHR. However, only nitric oxide appears to be involved in the vasodepressor actions of Ang 1-7 in the WKY and STZ-induced diabetic rats. Other researchers have also demonstrated the involvement of Ang 1-7 receptor, prostaglandins and / or nitric oxide (Jaiswal et al., 1993; Santos et al., 1994; Tallant et al., 1997) in SHR and Sprague-Dawley (SD) rats.

AT$_1$ receptor has been shown to mediate the actions of DAA-I in vitro (Sim and Chai, 1996; Sim and Soh, 1995). Similarly, Ang 1-7 has also been shown to act indirectly on AT$_1$ receptor either through downregulation (Clark et al., 2003) or internalization (Ueda et al., 2000) besides acting on putative Ang 1-7 receptor. Thus, the involvement of AT$_1$ receptor in DAA-I and Ang 1-7 action was not excluded entirely in the present study.

Given the more prominent findings observed in the kidney and due to its important association with the RAS, the relationship between AT$_1$ receptor and the vasodepressor action of these two peptides was further investigated in this vascular bed. Receptor binding study revealed that the rat kidney homogenate contains mainly AT$_1$ receptor subtype and with the AT$_2$ receptor in a lesser proportion (Figure 4.1). This finding is in agreement with work reported previously (Chatziantoniou et al., 1993; Gauqueli and Garcia, 1992).
Interestingly, the density of AT1 receptors was altered in both hypertensive and diabetic rats, albeit in different ways. The AT1 receptor density was found to be increased in the hypertensive rat kidney (Figure 4.2). The increased AT1 receptor density may explain the hyperresponsiveness to the vasopressor actions to Ang II observed in the SHR perfused kidney. RT-PCR and Western blot findings for SHR kidneys were in agreement with the results from the receptor binding study. Similar findings have also been reported previously in SHR kidneys (Haddad and Garcia, 1996), in the glomeruli of stroke-prone SHR (Obata et al., 2000) and in the SHR rat brains (Reja et al., 2006).

In contrast to the SHR, the AT1 receptor density was found to be significantly reduced in the STZ-induced diabetic rat kidney (Figure 4.2). This finding may explain the lower vasopressor responses to Ang II observed in the perfused kidney of the STZ-induced diabetic rats. Downregulation of AT1 receptors have been linked with the reduced Ang II effect in STZ-induced diabetic rat kidney (Amiri and Garcia, 2000; Brown et al., 1996). The results from RT-PCR and Western blot analysis of STZ-induced diabetic rat kidneys which showed no changes in AT1 receptor gene and protein expression are also in agreement with the receptor binding and functional studies.

In kidneys perfused with DAA-I, receptor binding data demonstrated a reduced kidney AT1 receptor density only at the 10^{-9} M (Figure 4.3). This data suggests that the DAA-I vasodepressor action in WKY rats and SHR at the highest concentration (10^{-9}M) used may partially be attributed to downregulation of AT1 receptor in the kidney. However, the RT-PCR and Western blot analysis failed to support such an assumption as no changes in the AT1 receptor gene and protein expression was evident (Figure 4.6 and Figure 4.9, respectively). Another explanation for the reduced AT1 receptor density may involve the process of receptor internalization. AT1 receptors have been shown to be
susceptible to agonist-induced internalization (Hunyady et al., 1994; Hunyady et al., 2000). The ability of DAA-I to promote internalization of the AT$_1$ receptor has been demonstrated previously by Chen and Sim, (2004) in hypertrophic rat hearts.

In the present study, kidneys perfused with $10^{-12}$M and $10^{-15}$M DAA-I and subsequently homogenised were without significant effect on AT$_1$ receptor density as well as the AT$_1$ receptor mRNA and protein expression. Although not significant, a slight reduction in AT$_1$ receptor density was seen at the lower concentrations DAA-I ($10^{-12}$M - $10^{-15}$M). At these physiological ranges, a small discrepancy in the receptor availability could still contribute to the attenuation of Ang II pressor responses seen in functional studies.

In diabetic rat model, no changes in AT$_1$ receptor density or gene and protein expression was seen in kidneys perfused with DAA-I. This finding support the earlier result that DAA-I was without effect on Ang II-induced pressor action in STZ-induced diabetic rat kidney. A slightly lower receptor affinity was seen in STZ-induced diabetic (untreated or treated with DAA-I) compared to WKY. There remains a possibility that DAA-I could bind to other binding sites on AT$_1$ receptor and diabetes may have altered DAA-I binding ability. Such an effect on the DAA-I binding site may affect the vasomodulatory effect of the nanopeptide (DAA-I) on the AT$_1$ receptor.

In functional studies, Ang 1-7 attenuated Ang II pressor actions in both WKY rats and SHR only at $10^{-7}$ M. Further work with receptor binding study at this concentration also demonstrated reduced AT$_1$ receptor density (Figure 4.4). This shows that Ang 1-7 vasodepressor action is in part modulated via AT$_1$ receptor possibly by receptor downregulation. Functional studies of Ang 1-7 have demonstrated that the action of the peptide may also involve the Ang 1-7 receptor, and/or release of prostaglandin and
nitric oxide. However, RT-PCR and Western blot analysis ruled out evidence of receptor downregulation of AT\textsubscript{1} receptor by Ang 1-7 (Figure 4.7 and Figure 4.10, respectively). It is possible that besides acting on its own receptor, binding of Ang 1-7 to the renal AT\textsubscript{1} receptor may lead to internalization of the receptor as observed with DAA-I. Ang 1-7 ability to bind to AT\textsubscript{1} receptor and bring about internalization of the AT\textsubscript{1} receptors has been demonstrated previously (Clark \textit{et al.}, 2001; Ueda \textit{et al.}, 2000). Pre-treatment with Ang 1-7 in STZ-induced diabetic kidney did not affect AT\textsubscript{1} receptor density and expression. This finding agrees well with the isolated perfused kidney study where Ang 1-7 vasodepressor action was unchanged in the diabetic rat. As Mas receptor is shown to be the endogenous Ang 1-7 receptor (Santos \textit{et al.}, 2003), modification of this receptor in diabetic conditions may offer a plausible explanation for the observed Ang 1-7 effect.

Similar pressor responses to Ang II, DAA-I and Ang 1-7 were observed in a preliminary study of the isolated perfused kidney from neonatal streptozotocin-induced (n-STZ) diabetic rat as a model for type 2 diabetes mellitus. DAA-I and Ang 1-7 also exhibited similar modulatory effect on the vasopressor actions of Ang II in the kidney of both n-STZ induced and STZ-induced diabetic rats. The findings suggest that in both types I and II diabetic animal models, these angiotensin peptides facilitate similar renal vascular changes.

The present findings provide important new information on the action of angiotensin peptides like DAA-I and Ang 1-7, especially in resistance vasculatures and their role in vascular diseases like hypertension and diabetes. The data presented also demonstrate the ability of one peptide to modulate the actions of another differentially in normal and diseased states. The present finding is also the first to demonstrate the ability of both
DAA-I and Ang 1-7 to reduce Ang II action, especially in hypertensive condition and may enable targeted therapies to be developed. These findings are also a first step in understanding the compromised vasodepressor actions of DAA-I and Ang 1-7 in diabetes mellitus.

The present data demonstrated in vitro actions of DAA-I and Ang 1-7. The role of DAA-I and Ang 1-7 should be evaluated in vivo to provide more information for developing targeted therapies. Pre-treatment with DAA-I and Ang 1-7 in vivo in hypertensive animal model may provide further information to the therapeutic antihypertensive potential of these peptides. Although this study was able to provide a relative information on the role of the AT$_1$ receptor on the vasodepressor actions of DAA-I and Ang 1-7, further work should be conducted with real-time PCR to detect minute changes in the AT$_1$ receptor expression over time. The present results were obtained from studies done on a type 1 diabetes mellitus (STZ-treated WKY rats). It would be interesting to test the possibility of observing similar results in a type 2 diabetes mellitus model such as the genetically diabetic rats, Goto-Kakizaki rats.


Fujii, K., Soma, M., Huang, Y.S., Manku, M.S. and Horrobin, D.F. (1986). Increased release of prostaglandins from the mesenteric vascular bed of diabetic animals: the effects of glucose and insulin. *Prostaglandins Leukotrienes and Medicine, 24(2-3)*, 151-161


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Sigary, H.M. (2000). AT(1) and AT(2) receptors in the kidney: role in disease and treatment. *Am. J. Kidney Dis.*, **36**(3 suppl 1), S4-S9


Appendix

Example 1 from SAMPLESZ.XLS

PGE$_2$ prostaglandin assay (WKY-K control vs WKY-K 60 minutes) Figure 3.14

I - Sample Size Calculations for Means

**Anticipated Values**

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean</th>
<th>Stan. Dev</th>
<th>Difference in means=</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>124</td>
<td>1.3</td>
<td>27.41935 %</td>
</tr>
<tr>
<td>Group 2</td>
<td>158</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

Table for (Z1-alpha/2+Z1-beta)squared

<table>
<thead>
<tr>
<th>Beta</th>
<th>0.05</th>
<th>0.1</th>
<th>0.2</th>
<th>0.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>10.8</td>
<td>8.6</td>
<td>6.2</td>
<td>2.7</td>
</tr>
<tr>
<td>0.05</td>
<td>13</td>
<td>10.5</td>
<td>7.8</td>
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<tr>
<td>0.02</td>
<td>15.8</td>
<td>13</td>
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<td>5.4</td>
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<tr>
<td>0.01</td>
<td>17.8</td>
<td>14.9</td>
<td>11.7</td>
<td>6.6</td>
</tr>
</tbody>
</table>

The cells in the table below show the estimated number of subjects needed in each group in order to demonstrate a statistically significant difference at "p" values ranging from 0.10 - 0.01 and at varying levels of "power". Power is the probability of finding a statistically significant difference at a given "P" value with the specified number of subjects in each group.

**Sample Size Needed in Each Group**

<table>
<thead>
<tr>
<th>Alpha level (&quot;p&quot; value)</th>
<th>Power</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>95%</td>
</tr>
<tr>
<td>0.10</td>
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</tr>
<tr>
<td>0.05</td>
<td>3</td>
</tr>
<tr>
<td>0.02</td>
<td>4</td>
</tr>
<tr>
<td>0.01</td>
<td>5</td>
</tr>
</tbody>
</table>

Example 2 (Figure 4.3)

Receptor binding assay (WKY control vs WKY pre-treated with DAA-I 10$^{-12}$M)

I - Sample Size Calculations for Means

**Anticipated Values**

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean</th>
<th>Stan. Dev</th>
<th>Difference in means=</th>
</tr>
</thead>
<tbody>
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<td>6.906977 %</td>
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<td>Group 2</td>
<td>400.3</td>
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</tbody>
</table>

Table for (Z1-alpha/2+Z1-beta)squared

<table>
<thead>
<tr>
<th>Beta</th>
<th>0.05</th>
<th>0.1</th>
<th>0.2</th>
<th>0.5</th>
</tr>
</thead>
<tbody>
<tr>
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<td>2.7</td>
</tr>
<tr>
<td>0.05</td>
<td>13</td>
<td>10.5</td>
<td>7.8</td>
<td>3.8</td>
</tr>
<tr>
<td>0.02</td>
<td>15.8</td>
<td>13</td>
<td>10</td>
<td>5.4</td>
</tr>
<tr>
<td>0.01</td>
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<td>6.6</td>
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**Sample Size Needed in Each Group**

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<tr>
<td>0.02</td>
<td>4</td>
</tr>
<tr>
<td>0.01</td>
<td>5</td>
</tr>
</tbody>
</table>
Standard curve for PGE$_2$ kit

% B/Bo = % bound / maximum bound

Standard curve for 6-keto PGF$_{1\alpha}$

% B/Bo = % bound / maximum bound
Appendix

Standard curve for protein estimation

\[ y = 0.005x \]
\[ r^2 = 0.9980 \]

Standard curve for plasma insulin estimation
Appendix

\[ y = 0.4714x - 0.0656 \]

\[ R^2 = 0.9964 \]

Insulin concentration (ug/l)

OD at 450nm
Effects of des-aspartate-angiotensin I on the actions of angiotensin III in the renal and mesenteric vasculature of normo- and hypertensive rats

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Received 16 July 2003; accepted 23 December 2003

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Abstract

An earlier study showed that des-aspartate-angiotensin I (DAA-I) attenuated the pressor action of angiotensin III in aortic rings of the spontaneously hypertensive rat (SHR) but not the normotensive Wistar Kyoto (WKY) rat. The present study investigated similar properties of DAA-I in isolated perfused kidneys and mesenteric beds of WKY and SHR. In the renal vasculature, angiotensin III induced a dose-dependent pressor response, which was more marked in the SHR than WKY in terms of significant greater magnitude of response and lower threshold. DAA-I attenuated the pressor action of angiotensin III in both the WKY and SHR. The attenuation in SHR was much more marked, occurring at doses as low as 10^{-15} M DAA-I, while effective attenuation was only seen with 10^{-9} M in WKY. The effects of DAA-I was not inhibited by PD123319 and indomethacin, indicating that its action was not mediated by angiotensin AT2 receptors and prostaglandins. However, the direct pressor action of angiotensin III in the SHR but not the WKY was attenuated by indomethacin suggesting that this notable difference could be due to known decreased response of renal vasculature to vasodilator prostaglandins in the SHR. Pressor responses to angiotensin III in the mesenteric vascular bed was also dose dependent, but smaller in magnitude compared to the renal response. The responses in the SHR, though generally smaller, were not significantly different from those of the WKY. This trend is in line with the similar observations with angiotensin III and II by other investigators. In terms of the effect of DAA-I, indomethacin and PD123319 on angiotensin III action, similar patterns to those of the renal vasculature were observed. This reaffirms that in the perfused kidney and mesenteric bed, where the majority of the vessels are contractile, femtomolar concentrations of DAA-I attenuates the pressor action of angiotensin III. The attenuation is not indomethacin sensitive and does not involve the angiotensin AT2 receptor. The findings suggest that DAA-I possesses protective vascular actions and is involved in the pathophysiology of hypertension.

Keywords: Des-aspartate-angiotensin I; Angiotensin III; Renal perfusion; Mesenteric; SHR; WKY

1. Introduction

Des-aspartate-angiotensin I (DAA-I), a nine-amino acid endogenous angiotensin peptide, has been shown to attenuate the actions of angiotensin III. It attenuated the contractile response to III in the aortic rings of the rabbit [1] and the spontaneously hypertensive rats (SHR) [2]. Intracerebroventricular administration of DAA-I attenuated dose-dependently the central pressor actions of angiotensin II and III in the SHR and Wistar Kyoto (WKY) rats [3,4]. Although attenuation of the actions of one angiotensin peptide by another is not a new phenomenon, such attenuations often occurs either by receptor antagonism, e.g., [Sar^{1},Ile^{8}]-angiotensin II and angiotensin-(1–7) block the angiotensin receptor and the actions of angiotensin II [5,6], or by acting on a different angiotensin receptor to produced opposite responses, e.g., angiotensin-(1–7) acts on the putative angiotensin-(1–7) receptor and counteracts most of the actions of angiotensin II in the vascular system [7]. Data obtained from electrically contracted endothelium-denuded rabbit pulmonary arteries showed that DAA-I acted as an agonist on the AT1 receptor in the pulmonary end of the artery to cause contraction, and via the same receptor at the cardiac end to cause relaxation [8,9]. The latter response was indomethacin sensitive and indicated that DAA-I accessed the AT1 receptor-coupled prostaglandin second messenger pathway to initiate the inhibitory action. This is a unique action and suggests that
the different second messenger pathways that are coupled to the AT1 receptor exhibit ligand specificity. The present study investigated further the effects of DAA-I on the pressor actions of angiotensin III in the renal and mesenteric vasculature of the SHR and WKY. The rationale for the study is the absence of information on the actions of angiotensin III in these two vascular beds and on the effects of DAA-I in contractile vessels. In the present study, angiotensin II was used as a positive control as it has been reported to cause enhanced pressor response in renal vasculature of the SHR [10–12].

2. Materials and methods

2.1. Animals

Male Wistar–Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs) weighing 250–300 g (11–13 weeks) were obtained from the Animal House in the University of Malaya Medical Centre. The animals were fed standard rat chow and tap water ad libitum.

2.2. Isolation of kidney and mesenteric vascular bed

Rats were anaesthetized with sodium pentobarbitone (60 mg/kg, i.p.). The right kidney was exposed by midline laparotomy. The renal artery was cannulated with a catheter (PET-50) via the superior mesenteric artery, and perfusion was started in situ. The right renal vein and ureter were cut. The right kidney was excised and placed in a water-jacketed chamber maintained at 37°C and perfused with an oxygenated (95% O2 and 5% CO2) Kreb’s solution by means of a peristaltic pump (Minipuls 3 Model 312, Gilson Villiers Le Bel, France) at a rate of 5 ml/min. In order to isolate the mesenteric arterial bed, the remaining length of the superior mesentery artery was cannulated with a catheter according to the method of McGregor [13]. The mesentery was carefully excised from the intestine and placed in a water-jacketed chamber maintained at 37°C and perfused with an oxygenated Kreb’s solution by means of a peristaltic pump at a rate of 5 ml/min. The composition of the Kreb’s solution was as follows (mmol/l): NaCl, 136.9; KCl, 5.4; CaCl2, 1.5; MgCl2, 1.0; NaHCO3, 23.8; EDTA, 0.01; and glucose, 5.5. Changes in perfusion pressure were measured by means of a pressure transducer (Model P23XL, Ohmeda Medical Devices Division, USA) and recorded via a MacLab data acquisition system (AD instruments, Australia).

2.3. Angiotensin and drug administration

After an equilibration period of 20 min, the preparation (renal or mesenteric) was preconstricted with phenylephrine (PE, 10^{-5} M), and the increase in perfusion pressure was recorded until a 5-min plateau was observed. This contractile response to phenylephrine was taken as unity, and responses to other pressor compounds were normalized against this unit. The preparation was then perfused with a Kreb’s solution that contained 30 µM captopril. Following 30 min of perfusion, various concentrations (10^{-10}–10^{-6} M) of angiotensin III were used to produce a dose response. Angiotensin III was administered as a single bolus injection of 20 µl into the perfusion system. The minimum time interval between successive bolus injections was 10 min, or the time until the basal pressure was again recorded. For the renal preparation, a dose response to angiotensin II was similarly obtained as a positive control.

The effects of various concentrations (10^{-15}–10^{-9} M) of DAA-I on the response to angiotensin III were studied with the following protocol. This concentration range was based on the findings of an earlier study showing that DAA-I attenuated the pressor action of angiotensin III at a concentration as low as 10^{-11} M [2]. The preparation was first perfused with Kreb’s solution containing 30 µM captopril and a concentration of DAA-I for 30 min, prior to initiating a dose response to angiotensin III. Each concentration of DAA-I was studied using a different preparation. The same protocol was used to study the direct effect of indomethacin or PD123319 on the responses to two doses of angiotensin III. The effect of PD123319 or indomethacin on the attenuation of angiotensin III pressor response by DAA-I was studied by perfusing the preparation with DAA-I and PD123319 (or indomethacin) for 30 min prior to bolus injections of angiotensin III.

2.4. Drugs

Captopril, angiotensin II, angiotensin III and indomethacin were purchased from Sigma. Des-aspartate-angiotensin

![Fig. 1. Perfusion pressure response to angiotensin II in the kidney of the WKY (■) and SHR (▲). Each point is the mean ± S.E.M. of four to six individual determinations. *Indicates significant difference from the WKY.](image-url)
I was purchased from BACHEM, Bubendorf, Switzerland. PD123319 was a generous gift from Parke-Davis Pharmaceutical Research, MI, USA. All other reagents used were of analytical grade.

2.5. Statistical analysis

Data are presented as mean ± S.E.M. Significant difference \((p<0.05)\) between means was evaluated using Stu-
Student's $t$-test when comparing two groups. When more than two groups were compared and for the comparison of the dose–response curves, data were evaluated by two-factor analysis of variance (ANOVA) followed by Newmann–Keul’s post hoc test. Results with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Renal vasculature

A $10^{-5}$ M concentration of PE caused an average increase in perfusion pressure of $250 \pm 20$ mm Hg in
SHR and 210 ± 25 mm Hg in WKY. The limits of detection of the system were 5–350 mm Hg. Fig. 1 shows the dose response of renal perfusion pressure to angiotensin II in WKY and SHR. The responses to the eight increasing doses of angiotensin II were significantly greater in the SHR than WKY. Fig. 2 shows the dose response of renal perfusion pressure to angiotensin III in the WKY and SHR. Similar greater responses to the lower doses (10^{-9} and 10^{-8} M) of angiotensin III were seen with the SHR (10.1, 25.6% vs. 1.2, 25.6%, p < 0.05). SHR also exhibited a lower threshold
response to angiotensin III, i.e., $10^{-10}$ M as compared to $10^{-9}$ M for the WKY.

The responses to angiotensin III were attenuated by DAA-I. Responses in the SHR were more markedly attenuated, especially with the lower doses of angiotensin III (see Fig. 2). DAA-I, by itself, did not affect the basal perfusion pressure when used up to a dose of $10^{-9}$ M (data not shown). Figs. 3 and 4 show that the actions of DAA-I on the response to angiotensin III in the renal vasculature of the WKY and SHR, respectively, were not affected by PD123319 and indomethacin. However, the direct contractile action to angiotensin III in the renal vasculature of the SHR, but not WKY, was attenuated by indomethacin (Fig. 4).

3.2. Mesenteric vasculature

A $10^{-5}$ M concentration of PE caused an average increase in perfusion pressure of $100 \pm 25$ mm Hg. This was smaller than the increase recorded with the renal vasculature. Similarly, the response to angiotensin III was also smaller, and the threshold dose was $10^{-5}$ M. Fig. 5 shows the dose response of mesenteric perfusion pressure to angiotensin III in WKY and SHR. Except for the maximum dose, the responses in SHR (though generally smaller) were not significantly different from those of WKY. DAA-I attenuated the angiotensin III response in both the WKY and SHR. The attenuation was seen with all doses of angiotensin III and was more marked in SHR than WKY and seen . In addition, $10^{-15}$ M DAA-I was effective in attenuating the response to the maximum dose of angiotensin III. The action of DAA-I on angiotensin III-induced increase in perfusion pressure was not affected by either PD123319 or indomethacin (Figs. 6 and 7). However, as seen with the renal vasculature, the direct contractile action of angiotensin III in the mesenteric vasculature of the SHR, but not WKY, was attenuated by indomethacin (Fig. 7).

4. Discussion

4.1. Renal vasculature

Angiotensin II produced a greater pressor response in the renal vasculature of SHR than WKY. This finding is in line with similar findings reported by earlier investigators [10–12] and served as a good positive control for the present study. Angiotensin III, the immediate metabolite of angiotensin II, induced similar dose–response increases in renal perfusion pressure in both the WKY and SHR. The response was enhanced in the SHR in terms of a lower threshold dose and greater magnitudes of pressor action at lower doses. Plasma levels of angiotensin III in the WKY and SHR are less than $10^{-10}$ M [14], and the lower threshold seen in the SHR would suggest that its renal vasculature is under the pressor action of the heptapeptide in vivo. The importance of this constant pressor action in the development of hypertension in SHR remains to be investigated. Of related interest are the findings by Healy and Song [15] showing that aminopeptidase A, the principal enzyme that hydrolyzes angiotensin II to angiotensin III, was significantly higher in the kidneys of SHR than WKY. Under this scenario, angiotensin III could have contributed to the observed enhanced response to angiotensin II in the SHR. Such a possibility requires further study with aminopeptidase A inhibitors as has been carried in the brain to establish that angiotensin III is the active central angiotensin peptide [16]. The SHR is not a high renin hypertension model, and plasma renin activity and plasma renin substrate were not significantly different from the WKY [17]. Plasma ACE activity [18], angiotensin II level [14,19] and angiotensin III level [14] were also found not to be significantly different from the WKY. These findings rule out changes in circulating level of the heptapeptide and its precursors as causes of hypertension and hyperresponsiveness to angiotensin III in the SHR. Various mechanisms such as deficiency in the action of endogenous vasodilator prostaglandins [10] or their release [20], genetically determined enhanced responsiveness [11], greater negative influence on phosphodiesterase-induced increase in cAMP [21], upregulation of angiotensin II receptors [22] and augmented cross-talk in the renal microcirculation between the $G_i$ signal transduction pathway and the signal transduction pathway used by angiotensin II [23] have been alluded to cause hyperresponsiveness to angiotensin II. These open up avenues for further investigation on the mechanisms of angiotensin III hyperresponsiveness and the likelihood of sharing similar mechanisms with angiotensin II.

DAA-I attenuated the pressor actions of angiotensin III in the WKY at a dose of $10^{-9}$ M. In the SHR, the enhanced responses to lower doses of angiotensin III ($10^{-8}$–$10^{-10}$ M) were highly susceptible to attenuation by much lower doses of DAA-I. Remarkably, a dose as low as $10^{-15}$ M DAA-I significantly attenuated the pressor action of angiotensin III. Contractile responses to femtomolar concentrations of angiotensin II has been reported in the saphenous vein of the dog [24] and endothelium-intact rabbit aortic rings [25]. However, the present finding is the first demonstration of a specific attenuation by femtomolar DAA-I that is seen only in the renal vasculature of SHR but not WKY. SHR has significantly lower plasma level of DAA-I than WKY [14]. In an ex vivo preparation like the perfused isolated kidney, the circulating solution was free of DAA-I. Reintroducing DAA-I at concentrations ranging the circulating level attenuated the pressor action of angiotensin III. Hence, in the in vivo situation, circulating DAA-I modulates the action of angiotensin III in the kidneys. Noting that circulating DAA-I was lower in the SHR and the renal vasculature was more responsive to angiotensin III, the modulation would probably be compromised in the SHR. Both indomethacin and PD123319 were without effect on the action of DAA-I, indicating that the $AT_2$ angiotensin receptors and prostaglandins were not involved in its
actions. In our earlier findings, indomethacin was also found to have no effect on the actions of DAA-I [2]. A recent study by Badzynska et al. [26] showed that AT2 receptors were also not involved in the vasoconstriction and vasodilatation induced by angiotensin II in kidneys of the rat.

The direct pressor action of angiotensin III in the SHR but not WKY was attenuated by indomethacin. The exact mechanism for this difference is unknown. However, vasodilator prostaglandins are less effective in reducing the pressor response of constricting agents in renal vasculature of the SHR than WKY. U-46619, the stable thromboxane A2 agonist, has been shown to increase renal perfusion of the SHR than WKY. U-46619, the stable thromboxane pressor response of constricting agents in renal vasculature mechanism for this difference is unknown. However, vasodilator prostaglandins are less effective in reducing the pressor response of constricting agents in renal vasculature of the SHR than WKY. However, the differences (except for the maximum dose of 10−5 M) were not significantly different. Angiotensin III has also been reported to produce similar magnitude of pressor response in perirterial nerve stimulated-perfused mesenteric vascular bed of the WKY and SHR [29]. Pressor responses to angiotensin II in perfused mesenteric bed of the SHR were also not different from those of the WKY [30,31]. The absence of enhanced response to angiotensin III and II in the SHR contrasted markedly to the enhanced response observed in the renal vascular bed and is a notable difference worthy of further investigation especially where angiotensin II is known to produce differential effect in renal circulation [26]. In terms of the effects of DAA-I, indomethacin and PD123319 on angiotensin III action, similar patterns to those of the renal vasculature were observed. This reaffirms that in the perfused kidney and mesenteric bed, where the majority of the vessels are contractile, femtomolar concentrations of DAA-I attenuates the pressor action of angiotensin III. The attenuation is not indomethacin sensitive and does not involve the angiotensin AT2 receptor.

The roles of DAA-I in hypertension are not known. Earlier studies on aminopeptidase X, the specific enzyme that converts angiotensin I to DAA-I, showed that the hypothalamus [32], plasma and endothelium [33] of SHR contained higher level of the enzyme. Based on these findings, it was theorized that degradation of angiotensin I in certain critical tissues of the SHR is shunted in favor of DAA-I. In a such a scenario, the formation of pressor angiotensin II and III would be curtailed, and the formed DAA-I would further attenuate the action of pressor angiotensins. However, despite the increase in activity of plasma and endothelial aminopeptidase X in the SHR, its plasma DAA-I level is significantly lower than WKY [14]. These findings support the contention that the causes and responses to hypertension are multifactorial.

References


4.2. Mesenteric vasculature

Angiotensin III induced smaller increases in perfusion pressure in the SHR than WKY. However, the differences (except for the maximum dose of 10−5 M) were not significantly different. Angiotensin III has also been reported to produce similar magnitude of pressor response in perirterial nerve stimulated-perfused mesenteric vascular bed of the WKY and SHR [29]. Pressor responses to angiotensin II in perfused mesenteric bed of the SHR were also not different from those of the WKY [30,31]. The absence of enhanced response to angiotensin III and II in the SHR contrasted markedly to the enhanced response observed in the renal vascular bed and is a notable difference worthy of further investigation especially where angiotensin II is known to produce differential effect in renal circulation [26]. In terms of the effects of DAA-I, indomethacin and PD123319 on angiotensin III action, similar patterns to those of the renal vasculature were observed. This reaffirms that in the perfused kidney and mesenteric bed, where the majority of the vessels are contractile, femtomolar concentrations of DAA-I attenuates the pressor action of angiotensin III. The attenuation is not indomethacin sensitive and does not involve the angiotensin AT2 receptor.

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Effect of des-aspartate-angiotensin I on the actions of angiotensin II in the isolated renal and mesenteric vasculature of hypertensive and STZ-induced diabetic rats

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Abstract

The present study investigated the action of des-aspartate-angiotensin I (DAA-I) on the pressor action of angiotensin II in the renal and mesenteric vasculature of WKY, SHR and streptozotocin (STZ)-induced diabetic rats. Angiotensin II-induced a dose-dependent pressor response in the renal vasculature. Compared to the WKY, the pressor response was enhanced in the SHR and reduced in the STZ-induced diabetic rat. DAA-I attenuated the angiotensin II pressor action in renal vasculature of WKY and SHR. The attenuation was observed for DAA-I concentration as low as $10^{-18}$ M and was more prominent in SHR. However, the ability of DAA-I to reduce angiotensin II response was lost in the STZ-induced diabetic kidney. Instead, enhancement of angiotensin II pressor response was seen at the lower doses of the octapeptide. The effect of DAA-I was not inhibited by PD123319, an AT2 receptor antagonist, and indomethacin, a cyclo-oxygenase inhibitor in both WKY and SHR, indicating that its action was not mediated by angiotensin AT2 receptor and prostaglandins. The pressor responses to angiotensin II in mesenteric vascular bed were also dose-dependent but smaller in magnitude compared to the renal vasculature. The responses were significantly smaller in SHR but no significant difference was observed between STZ-induced diabetic and WKY rat. Similarly, PD123319 and indomethacin had no effect on the action of DAA-I. The findings reiterate a regulatory role for DAA-I in vascular bed of the kidney and mesentery. By being active at circulating level, DAA-I subserves a physiological role. This function appears to be present in animals with diseased state of hypertension and diabetes. It is likely that DAA-I functions are modified to accommodate the ongoing vascular remodeling.

Keywords: Des-aspartate-angiotensin I; Renal; Mesenteric; Vasculature; Angiotensin II; Streptozotocin

1. Introduction

The renin–angiotensin system (RAS) plays a major role in the regulation of blood pressure as well as sodium and water balance. Most of the modulating effects are mediated via angiotensin II, acting at the AT1 subtype of the angiotensin receptor [1]. Changes in the renin–angiotensin system have been implicated in the pathophysiology of cardiovascular diseases [2]. Des-aspartate-angiotensin I (DAA-I), a nine amino acid peptide has been shown to attenuate the action of angiotensin III in the aortic rings of the rabbit [3] and the SHR [4], and in the renal and mesenteric vasculature of SHR and Wistar–Kyoto (WKY) rats [5]. Intracerebroventricular administration of DAA-I attenuated the central pressor actions of angiotensin II and III in the SHR and WKY rats [6,7]. Data obtained from electrically contracted endothelium-denuded rabbit pulmonary arteries showed that DAA-I...
acted as an agonist on the AT$_1$ receptor [8]. In the renal and mesenteric vasculature, DAA-I action was not blocked by PD123319, a specific AT$_2$ receptor agonist, which suggests that DAA-I may act through the AT$_1$ receptor [5]. The present study was designed to investigate the effects of DAA-I on the pressor action of angiotensin II in the renal and mesenteric vasculature of SHR, WKY and STZ-induced diabetic rats. Angiotensin II is implicated in the pathophysiology of these two disease states, and the extent its pressor action could be modulated by DAA-I would determine the likely roles of the nonapeptide.

2. Materials and methods

2.1. Animals

Male Wistar–Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs) age 10 weeks were obtained from Animal House in the University of Malaya Medical Centre. The animals were fed standard rat chow and tap water ad libitum for 2 weeks and diabetes was induced at age 12 week.

2.2. Induction of diabetes

WKY was made diabetic by administration of streptozotocin (STZ) 75 mg/kg intraperitoneally. Age matched controls received equal volume of vehicle. Body weight and blood glucose levels of each rat were taken every 2 weeks until the 8th week. Animals were considered diabetic if their blood glucose level was >17 mM.

2.3. Isolated kidney and mesenteric vascular bed

To isolate the right kidney, rats were anaesthetized with sodium pentobarbitone (60 mg/kg, i.p.). The right kidney was exposed by midline laparotomy. The renal artery was cannulated with a catheter (PET-50) via the superior mesenteric artery and perfusion was started in situ. The right renal vein and ureter were cut. The right kidney was ligated, excised and placed in a water-jacketed chamber maintained at 37 °C and perfused with an oxygenated (95% O$_2$ and 5% CO$_2$) Kreb’s solution by means of a peristaltic pump (Minipuls 3 Model 312, Gilson Villiers Le Bel, France) at a rate of 5 ml/min. In order to isolate the mesenteric arterial bed, the remaining length of the superior mesenteric artery was cannulated with a catheter according to the method of McGreggor [9]. The mesentery was carefully excised from the intestine and placed in a water-jacketed chamber maintained at 37 °C and perfused with an oxygenated Kreb’s solution by means of a peristaltic pump at a rate of 5 ml/min. The composition of the Kreb’s solution was as follows (mmol/l): NaCl, 120; KCl, 4.7; CaCl$_2$, 2.4; MgCl$_2$, 1.2; NaHCO$_3$, 20; EDTA, 0.06; and glucose, 10. Changes in perfusion pressure were measured by means of a pressure transducer (Model P23XL, Ohmeda Medical Devices Division Inc, USA) and recorded via a MacLab data acquisition system (AD Instruments, Australia).

2.4. Experimental protocol

After an equilibration period of 20 min, the preparation was preconstricted with phenylephrine (PE, 10$^{-5}$ M) and the increase in perfusion pressure was recorded until a 5-min plateau was observed. This contractile response to phenylephrine was taken as a unity and responses to other pressor compounds were normalized against this unit. The preparation was then perfused with a Kreb’s solution that contained 30 μM captopril. Following 1 h of perfusion, various concentrations (10$^{-12}$–10$^{-8}$ M) of angiotensin II was used to produce a contractile response. The angiotensin was administered as a single bolus injection of 20 μl (renal) and 50 μl (mesenterial bed) into the perfusion system. The minimum time interval between successive bolus injections was 10 min or the time till the basal pressure was again recorded.

The effects of various concentrations (10$^{-18}$–10$^{-9}$ M) of DAA-I on the response to angiotensin II were studied with the following protocol. The preparation was first perfused with Kreb’s solution containing 30 μM captopril and a concentration of DAA-I for 30 min, prior to initiating a concentration response to angiotensin II. Each concentration of DAA-I was studied using a new set of preparation. A similar protocol was used to study the direct effect of indomethacin (10$^{-7}$ M) or PD123319 (10$^{-5}$ M) on the concentration response to angiotensin II. The effect of PD123319 or indomethacin on the actions of DAA-I on the concentration responses to angiotensin II was studied by perfusing the preparation with DAA-I and PD123319 (or indomethacin) for 30 min prior to bolus injection of angiotensin II.

2.5. Drugs

Captopril, angiotensin II, streptozotocin and indomethacin were purchased from Sigma. Des-aspartate-angiotensin I was purchased from Bachem AC, Bubendorf, Switzerland. PD123319 was a generous gift from Parke-Davis Pharmaceutical Research, Michigan, USA.

2.6. Statistical analysis

Data are presented as mean ± SEM. Significant difference ($p<0.05$) between means was evaluated using Student’s $t$-test when comparing two groups. When more than two groups were compared, and for the comparison of the dose–response curves, data were evaluated by one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. Results with $p<0.05$ were considered statistically significant.
3. Results

3.1. Renal vasculature

A $10^{-5}$ M concentration of PE caused an average increase in perfusion pressure of 250 ± 20 mm Hg in SHR, and 210 ± 25 mm Hg in the WKY and STZ-induced diabetic rats. The lower and higher limits of detection of the system were 5 and 350 mm Hg, respectively. Fig. 1 shows the dose–response of renal perfusion pressure to angiotensin II in WKY, SHR and STZ-induced diabetic rats. The response to the higher five of the eight increasing doses of angiotensin II in the SHR was significantly greater than those in the WKY. Angiotensin II pressor responses in the STZ-induced diabetic rats were significantly lower than those in the WKY.

Pressor responses to angiotensin II in the WKY and SHR were attenuated by DAA-I. The attenuation was seen at the higher angiotensin II concentration ($10^{-9}$–$10^{-6}$ M) in both animal groups (Fig. 2). In STZ-induced diabetic rats, DAA-I significantly potentiated the pressor action of lower concentrations of angiotensin II ($10^{-13}$–$10^{-11}$ M) and had no effect on the high concentrations of the octapeptide. DAA-I, by itself, had no effect on the basal perfusion pressure (data not shown). The action of DAA-I was not affected by PD123319 and indomethacin in both WKY (Fig. 3, upper set of histograms) and SHR (data not shown).

3.2. Mesenteric vasculature

A $10^{-5}$ M concentration of PE caused an average increase in perfusion pressure of 100 ± 25 mm Hg. This response was smaller than the increase recorded for the renal vasculature. The responses for angiotensin II recorded for mesenteric bed were also smaller, and the threshold dose was $10^{-10}$ M for WKY and STZ-induced diabetic rats, and $10^{-9}$ M for SHR. Fig. 4 shows the dose–response of mesenteric perfusion pressure to angiotensin II in WKY, SHR and STZ-induced diabetic rats. The responses in SHR were significantly smaller than those in WKY. There were no significant differences in angiotensin II contractile responses between WKY and STZ-induced diabetic rats. DAA-I ($10^{-18}$–$10^{-9}$ M) attenuated the angiotensin II response in WKY and SHR (Fig. 5). Similar to the renal vasculature, attenuation by DAA-I was also not seen with the higher concentrations of angiotensin II in the STZ-induced diabetic mesenteric vasculature (Fig. 5).
II-induced vasoconstriction was not affected by PD123319 and indomethacin in WKY (Fig. 3, lower set of histograms) and SHR (data not shown).

4. Discussion

4.1. Renal vasculature

Angiotensin II produced a greater pressor response in the SHR than WKY. A similar observation has also been reported by earlier investigators [10–12]. The mechanisms
responsible for increased vascular resistance have been the subject of intense investigation. Studies by Makarios et al. [13] and Haddad and Garcia [14] have demonstrated that the hyperresponsiveness observed in SHR correlates with increased angiotensin II receptor density. Various other mechanisms such as reduced offsetting activity of vasodilator prostaglandins [15], greater negative influence on phosphodiesterase induced increase in cAMP [16], defective interaction between receptor and G-protein activation [17] by angiotensin II have been alluded to cause hyperresponsiveness to angiotensin II in the SHR.

In contrast to SHR, angiotensin II pressor action was reduced in STZ-induced diabetic rats. This is in line with earlier work done by Sarubbi et al. [18]. They have demonstrated that angiotensin II-induced vasoconstriction was significantly impaired at 2 and 8–12 weeks diabetes. In addition to animal studies, attenuated renal and systemic responsiveness to angiotensin II has been shown in type 1 diabetic patient [19]. The reduced angiotensin II reactivity in diabetes has been associated with a downregulation of glomerular angiotensin II receptors [20,21]. Study by Sharma et al. [22], however, did not find a downregulation in angiotensin II receptor level but demonstrated that impaired angiotensin II response in diabetes is caused by decreased expression of the type I inositol 1,4,5-triphosphate (InsP₃) isoform receptor. Increased basal nitric oxide (NO) production in diabetes has also been reported [23] and has been suggested as a cause of reduced angiotensin II pressor response in diabetes.

DAA-I attenuated angiotensin II pressor action in both WKY and SHR. The attenuation was observed at the higher concentration range of angiotensin II (10⁻⁹–10⁻⁶ M). Similar attenuation has also been reported for the pressor action of angiotensin III [5]. However in the present study, the effective dose of DAA-I was a thousand-fold lower (10⁻¹³–10⁻¹¹ M) and the effect was seen in both the WKY and SHR. At this atto concentration, the concentration of DAA-I was below the reported circulation (pico molar) level [24].

The renal vasculature is an ex vivo preparation devoid of circulating DAA-I and re-introduction of DAA-I provides a means for measuring the effective physiological concentration of the peptide and possible roles it exerts in normal and pathological conditions. The present findings may indicate that, at circulating level, DAA-I attenuates pressor action to angiotensin II only when the local concentration of the octapeptide rises above a certain level i.e. nano molar concentration. In this way, DAA-I regulates the action of angiotensin II and prevent the latter from exerting excessive and damaging pressor effect. However, the attenuation in the SHR though equally significant was not sufficient to match the absolute value seen in the WKY indicating that the circulating level of DAA-I was not able to ameliorate the hypertensive malady. Effective at atto concentration, DAA-I is indeed the most specific angiotensin peptide that is known to attenuate the pressor action of angiotensin II.

A different profile of DAA-I action was seen in the renal vasculature of STZ-induced diabetic rats. Unlike in the WKY and SHR, DAA-I had no effect on the higher pressor concentrations of angiotensin II. Instead, DAA-I potentiated the pressor action of the lower concentrations (10⁻¹³–10⁻¹¹ M) of the octapeptide. Although the exact mechanism for this reversal is not known, changes in levels of cellular diacylglycerol (DAG) and isoforms of protein kinase C (PKC) following induction of diabetes [25–27] could be contributory factors, as the contractile actions of different agonists are mediated by different isoforms of PKC [28,29], which are in turn regulated by different PKC-interacting proteins [30]. Whatever the cellular mechanisms responsible, it is tempting to speculate that DAA-I normalizes the STZ-induced lower pressor response to angiotensin II to near normal and, in this way, maintains the physiological vascular tone in the diabetic renal vasculature. The need to exert negative control on the higher concentrations of angiotensin II may not be critical in the diabetic renal vasculature as the response to the octapeptide is below normal.

PD 123319 and indomethacin were without effect on the action of DAA-I in WKY and SHR, indicating that the AT₂ angiotensin receptors and prostaglandins were not involved in its action. This is in agreement with our earlier finding with angiotensin III [5]. These findings suggest that DAA-I probably acts via the angiotensin AT₁ receptor as has been shown in other preparations.

4.2. Mesenteric vasculature

Several studies have documented enhanced contractile response as well as no change in SHR compared to WKY [31–34]. This equivocal finding could be due to the use of the different sub-branches and sizes of the mesenteric vessel. In the present study, pressor response of the mesenteric vasculature to angiotensin II has been found to be significantly smaller in SHR than WKY. Noting the in situ preparation includes the venules, the decrease in response of the venular circulation could have contributed to the observation in the SHR as angiotensin II has been shown to be a stronger vasoconstrictor than arterioconstrictor [35].

The absence of differences in response to angiotensin II between the WKY and diabetic mesenteric vasculature is a contrast to that observed for the SHR. Although the vascular renin–angiotensin system (RAS) has been known to be activated in diabetes and is a cause of vascular remodeling, sensitivity of mesenteric vessels to vasoconstrictors such as noradrenaline and serotonin remains controversial [36]. With 4 weeks of STZ treatment, the vasoconstrictor responses induced by noradrenaline, endothelin-I and angiotensin II were significantly increased [37], but longer treatment with STZ has been shown to increase blood flow in the superior mesenteric artery [38]. It is likely that the observation of no significant difference
from WKY for the STZ-treated rats could be temporal dependent.

There are subtle differences between the effects of DAA-I on the renal and mesenteric vascular bed. In the latter, DAA-I was more effective in attenuating the pressor action of angiotensin II in the WKY and less so in the diabetic animals. It is difficult to speculate on the underlying mechanisms for the differences. One possibility could be the ongoing remodeling caused by an activated RAS and the different roles the blood vessels play in the kidneys and intestines. In terms of the effects of DAA-I, PD123319 and indomethacin on angiotensin II contraction, similar patterns to those of the renal vasculature were observed. This suggests that DAA-I action is via the angiotensin AT1 receptors and is unlikely to be mediated by prostaglandins.

The present findings reiterate a regulatory role for DAA-I in vascular bed of the kidney and mesentery. By being active at circulating level, DAA-I subserves a physiological role. This function appears to be present in animals with diseased state of hypertension and diabetes. The vascular RAS is activated and angiotensin II contributes to remodeling in vascular tissues of hypertensive and diabetic rats. It is likely that DAA-I functions are modified to accommodate the ongoing vascular remodeling.

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Effects of angiotensin 1-7 on the actions of angiotensin II in the renal and mesenteric vasculature of hypertensive and streptozotocin-induced diabetic rats

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Abstract

Angiotensin 1-7, a heptapeptide derived from metabolism of either angiotensin I or angiotensin II, is a biologically active peptide of the renin–angiotensin system. The present study investigated the effect of angiotensin 1-7 on the vasopressor action of angiotensin II in the renal and mesenteric vasculature of Wistar-Kyoto (WKY) rats, spontaneously hypertensive rats (SHR) and streptozotocin-induced diabetic rats. Angiotensin II-induced dose-dependent vasoconstrictions in the renal vasculature. The pressor response was enhanced in the SHR and reduced in the streptozotocin-diabetic rat compared to WKY rats. Angiotensin 1-7 attenuated the angiotensin II pressor responses in the renal vasculature of WKY and SHR rats. However, the ability to reduce angiotensin II response was diminished in diabetic-induced rat kidneys. The effect of angiotensin 1-7 was not inhibited by 1-[(4-(Dimethylamino)-3-methylphenyl)methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid ditrifluoroacetate (PD123319), an angiotensin AT2 receptor antagonist. (D-ALA7)-Angiotensin I/II (1-7) (D-ALA) (an angiotensin 1-7 receptor antagonist), indomethacin (a cyclo-oxygenase inhibitor), and Nω-Nitro-L-Arginine Methyl Ester (L-NAME)(a nitric oxide synthetase inhibitor) abolished the attenuation by angiotensin 1-7 in both WKY rats and SHR, indicating that its action is mediated by angiotensin 1-7 receptor that is either coupled to the release of prostaglandins and/or nitric oxide. The vasopressor responses to angiotensin II in mesenteric vasculature bed was also dose-dependent but smaller in magnitude compared to the renal vasculature. The responses to angiotensin II were relatively smaller in SHR but no significant difference was observed between WKY and streptozotocin-induced diabetic rats. Angiotensin 1-7 attenuated the angiotensin II pressor responses in WKY, SHR and diabetic-induced mesenteric bed. The attenuation was observed at the lower concentrations of angiotensin II in WKY and diabetic-induced rats but at higher concentrations in SHR. Similar observation as in the renal vasculature was seen with PD123319, D-ALA, and L-NAME. Indomethacin reversed the attenuation by angiotensin 1-7 only in the SHR mesenteric vascular bed. The present findings support the regulatory role of angiotensin 1-7 in the renal and mesenteric vasculature, which is differentially altered in hypertension and diabetes.

Keywords: Angiotensin 1-7; Angiotensin II; Isolated perfused kidney; Mesenterial arterial bed; Diabetic rat

1. Introduction

Angiotensin II is believed to be the major effector peptide of the renin–angiotensin system (Ardaillou and Chansel, 1997). However, recent evidence shows that angiotensin II is not the only active peptide of renin–angiotensin system (Kucharewicz et al., 2002). Three endopeptidases that convert angiotensin I and II directly to angiotensin 1-7 have been reported (Ferrario et al., 1991; Kucharewicz et al., 2002). Angiotensin 1-7 was found to be present in central and peripheral tissues of rats, dogs and humans and is therefore thought to be an active component of renin–angiotensin system (Ferrario et al., 1991; Santos et al., 2000). The heptapeptide has been shown to activate several subtypes of angiotensin receptors in neural, endothelial, and vascular smooth muscle cell (VSMC) preparations and to exert biological actions that are both complementary to and distinct from those of angiotensin II.
(Ferrario et al., 1991). It acts as a vasodilating agent in many vascular beds (Ferrario et al., 1997), inhibits vascular smooth muscle growth (Freeman et al., 1996), and blocks the angiotensin II-induced vasoconstriction in rat aorta (Le Tran and Forster, 1997; Loot et al., 2005), and human arteries (Roks et al., 1999). Moreover, accumulating studies demonstrated that angiotensin 1-7 stimulates the synthesis of vasodilator prostaglandins (Ferrario et al., 1991; Jaiswal et al., 1993) and nitric oxide (Brosnihan, 1998), and potentiates the hypotensive action of bradykinin (Santos et al., 2001). This explains why angiotensin 1-7 exerts antihypertensive actions (Chappell et al., 1998; Ferrario, 1998), particularly in situations of increased angiotensin II activity. Angiotensin 1-7 has been demonstrated to act through its putative angiotensin 1-7 receptor or via other known angiotensin receptors. Although the angiotensin 1-7 receptor has not been cloned, its existence cannot be entirely excluded, since some of its effects could be inhibited by the angiotensin 1-7 receptor antagonist, D-ALA (Ambuhl et al., 1994; Santos et al., 2000, 2001; Vallon et al., 1998). Other studies have raised the possibility that angiotensin 1-7 may act as an endogenous antagonist of the AT1 receptor or it may modulate angiotensin II via AT1 receptor (Clark et al., 2001b; Mohan et al., 1994). With regard to these possibilities, the present study investigated the effects of angiotensin 1-7 on the angiotensin II-induced vasoconstriction in the renal and mesenteric vasculature of normal, hypertensive and experimentally-induced diabetic rats.

2. Material and methods

2.1. Animals

Male Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs) weighing 250–300 g (11–12 weeks) were obtained from the Animal House in the University of Malaya Medical Centre. Approval for the following studies was obtained from the Animal Care and Use Committee at the Laboratory Animal Center of Faculty of Medicine in the

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Fig. 1. Effects of angiotensin 1-7 on the angiotensin II-induced pressure response in the renal vasculature of WKY (A), SHR (B) and STZ-induced diabetic (C) rats. Alphabet and numbers immediately below histogram: C, control (not treated); -7, pretreated with 10^{-7} M angiotensin 1-7; -11, pretreated with 10^{-11} M angiotensin 1-7, -15, pretreated with 10^{-15} M angiotensin 1-7. Each histogram and bar represents the mean±S.E.M. of 5–6 separate preparations. *Indicates significant difference from the control.

Fig. 2. Effects of PD123319 (A), indomethacin (B), L-NAME (C) and D-ALA (D) on the inhibitory actions of angiotensin 1-7 on the angiotensin II-induced pressure response in the renal vasculature of the WKY. Alphabets inside histograms: C, control (not treated); PD, pretreated with 10^{-5} M PD123319; A1-7, pretreated with 10^{-7} M angiotensin II; A1-7+PD, pretreated with angiotensin 1-7 and PD123319; In, pretreated with 10^{-7} M indomethacin; A1-7+In, pretreated with angiotensin 1-7 and indomethacin; L–N, pretreated with 10^{-7} M L-NAME; A1-7+L–N, pretreated with angiotensin 1-7 and L-NAME; D-ALA, pretreated with 10^{-5} M D-ALA; A1-7+D-ALA, pretreated with angiotensin 1-7 and D-ALA. Each histogram and bar represents the mean±S.E.M. of 5–6 separate preparations. *Indicates significant difference from the control.
University of Malaya and procedures were carried out according to the guidelines for ethical care of experimental animals. The animals were fed standard rat chow and tap water ad libitum.

2.2. Induction of diabetes

WKY rats were made diabetic by administration of streptozotocin 75 mg/kg intraperitoneally. Age matched controls received equal volume. Prior to injection, animal weights and blood glucose levels were recorded. Body weights and blood glucose were taken every 2 weeks until the 8-week. Animals were considered diabetic if their blood glucose concentration was >17 mM.

2.3. Isolated kidney and mesenteric vascular bed

Rats were anaesthetized with sodium pentobarbitone (60 mg/kg, i.p.) and the renal and mesenteric vasculature beds were prepared as described previously (Dharmani et al., 2005). Briefly, the right kidney was exposed by midline laparotomy and the renal artery was cannulated with a catheter (PET-50) via the superior mesenteric artery. Perfusion was started in situ and the right renal vein and ureter were cut. The right kidney was excised and placed in a water-jacketed chamber maintained at 37 °C and perfused with an oxygenated Kreb’s solution by means of a peristaltic pump (Minipuls 3 Model 312, Gilson Villiers Le Bel, France) at a rate of 5 ml/min. For isolation of the mesenteric arterial bed, the remaining length of the superior mesentery artery was cannulated with a catheter. The mesentery was carefully excised from the intestine and placed in a water-jacketed chamber maintained at 37 °C and perfused with an oxygenated Kreb’s solution by means of a peristaltic pump at a rate of 5 ml/min. The composition of the Kreb’s solution was as follows (mmol/L): NaCl, 120; KCl, 4.7; CaCl2, 2.4; MgCl2, 1.2; NaHCO3, 20; KH2PO4, 1; EDTA, 0.06; and glucose, 10).

Fig. 3. Effects of PD123319 (A), indomethacin (B), L-NAME (C) and D-ALA (D) on the inhibitory actions of angiotensin I-7 on the angiotensin II-induced pressure response in the renal vasculature of the SHR. Alphabets inside histograms: C, control (not treated); PD, pretreated with $10^{-7}$ M PD123319; A1-7, pretreated with $10^{-7}$ M angiotensin II; A1-7+PD, pretreated with angiotensin I-7 and PD123319; In, pretreated with $10^{-7}$ M indomethacin; A1-7+In, pretreated with angiotensin I-7 and indomethacin; L-N, pretreated with $10^{-4}$ M L-NAME; A1-7+L–N, pretreated with angiotensin I-7 and L-NAME; D-ALA, pretreated with $10^{-5}$ M D-ALA; A1-7+D-ALA, pretreated with angiotensin I-7 and D-ALA. Each histogram and bar represents the mean±S.E.M. of 5–6 separate preparations. *Indicates significant difference from the control.

Fig. 4. Effects of angiotensin 1-7 on the angiotensin II-induced pressure response in the mesenteric vascular bed of WKY (A), SHR (B) and STZ-induced diabetic (C) rats. Alphabet and numbers immediately below histogram: C, control (not treated); -7, pretreated with $10^{-7}$ M angiotensin I-7; -11, pretreated with $10^{-11}$ M angiotensin 1-7; -15, pretreated with $10^{-15}$ M angiotensin I-7. Each histogram and bar represents the mean±S.E.M. of 5–6 separate preparations. *Indicates significant difference from the control.
Changes in perfusion pressure were measured by means of a pressure transducer (Model P23XL, Ohmeda Medical Devices Division Inc, USA) and recorded via a MacLab data acquisition system (AD Instruments, Australia).

2.4. Experimental protocol

After an equilibration period of 20 min, the preparation was preconstricted with phenylephrine (PE, $10^{-5}$ M) and the increase in perfusion pressure was recorded until a 5-min plateau was observed. This contractile response to phenylephrine was taken as a unity and responses to other pressor compounds were normalized against this unit. Following 1 h of perfusion, various concentrations ($10^{-13}$–$10^{-7}$ M) of either angiotensin II or angiotensin 1-7 were used to produce a concentration response. The angiotensins were administered as a single bolus injection of 20 μl (renal) and 50 μl (mesenteric bed) into the perfusion system. The minimum time interval between successive bolus injections was 10 min or the time till the basal pressure was again recorded.

The effects of various concentrations ($10^{-15}$–$10^{-7}$ M) of angiotensin 1-7 on the response to angiotensin II were studied with the following protocol. The preparation was first perfused with Kreb’s solution containing a concentration of angiotensin 1-7 for 30 min, prior to initiating a concentration response to angiotensin II. Each concentration of angiotensin 1-7 was studied using a different preparation. The same protocol was used to study the direct effect of indomethacin ($10^{-7}$ M), PD123319 ($10^{-5}$ M), L-NAME ($10^{-4}$ M) and D-ALA ($10^{-5}$ M) on the concentration response to angiotensin II. The effects of PD123319, indomethacin, L-NAME and D-ALA on the actions of angiotensin 1-7 on the concentration responses to angiotensin II were studied by perfusing the preparation with a effective concentration of angiotensin 1-7 and PD123319 (or indomethacin, L-NAME, D-ALA) for 30 min prior to bolus injection of angiotensin II.

2.5. Drugs

Angiotensin II, angiotensin 1-7, L-NAME and indomethacin were purchased from Sigma. D-ALA was purchased from BACHEM AC, Bubendorf, Switzerland. PD123319 was a generous gift from Parke-Davis Pharmaceutical Research, Michigan, USA.

2.6. Statistical analysis

Data are presented as mean±S.E.M. Significant difference ($p<0.05$) between means was evaluated using Student’s $t$-test when comparing two groups. When more than two groups were compared and for the comparison of the dose-response curves, data were evaluated by two-factor analysis of variance (ANOVA) followed by Bonferroni post hoc test. Results with $p<0.05$ were considered statistically significant.
3. Results

SHR rats in the experiments were age matched and have higher blood pressure than the control (173 ± 4 mm Hg vs. 124 ± 2 mm Hg). The streptozotocin-induced diabetic rats have a significantly higher blood glucose level than WKY rats (25 ± 5 mM vs. 5 ± 3 mM).

3.1. Renal vasculature

10^{-5} M PE caused an average increase in perfusion pressure of 250 ± 20 mm Hg in SHR and 210 ± 25 mm Hg in WKY and diabetic-induced rats. The limit of detection of the system was 350 ± 50 mm Hg. Bolus injections of angiotensin 1-7 did not induce contraction in all the three groups of animal. We have reported previously that the responses to the five increasing doses of angiotensin II in SHR were significantly greater than WKY rats (p<0.05) whilst the responses in the diabetic-induced rats were significantly lower than those in the WKY rats (Dharmani et al., 2005). No tachyphylaxis was observed with subsequent bolus addition of angiotensin II.

The contractile responses to angiotensin II were attenuated by angiotensin 1-7 (10^{-7} M) in both WKY and SHR rats. The attenuation was observed at the higher concentrations of angiotensin II (10^{-9} M – 10^{-6} M) in SHR and from 10^{-10} M for WKY rats (Fig. 1). Angiotensin 1-7 did not affect the angiotensin II-induced vasoconstriction in streptozotocin-induced diabetic rats. The action of angiotensin 1-7 was not affected by PD123319 in WKY rats (Fig. 2A) and SHR (Fig. 3A). Indomethacin, L-NAME and D-ALA reversed the vasodepressor action of angiotensin 1-7 in WKY rats (Fig. 2) and SHR (Fig. 3).

3.2. Mesenteric vasculature

10^{-5} M PE caused an average increase in perfusion pressure of 100 ± 25 mm Hg in all the three groups. As reported earlier (Dharmani et al., 2005), this was much smaller than the response recorded with the renal vasculature. Angiotensin 1-7 did not induce vasopressor responses in all the three groups of animals. In contrast to responses seen in the kidney, angiotensin 1-7 attenuated the pressor responses to angiotensin II in mesenteric vascular bed of streptozotocin-induced diabetic rats. For the SHR, the attenuation was observed only at the higher concentrations of angiotensin II (10^{-7} M – 10^{-6} M) (Fig. 4). PD123319 did not affect the action of angiotensin 1-7 in WKY rats, SHR and streptozotocin-induced diabetic rats (Figs. 5A, 6A and 7A respectively). L-NAME and D-ALA also reduced the attenuation by angiotensin 1-7 in all the three groups (Figs. 5, 6 and 7(C)). Pre-incubation with indomethacin reduced the vasodepressor actions of angiotensin 1-7 only in hypertensive animals.

4. Discussion

4.1. Renal vasculature

The present study demonstrated that angiotensin 1-7 significantly attenuated angiotensin II-induced vasoconstrictions in both the WKY rats and SHR. Stegbauer et al. (2005) and van der Wouden et al. (2006) have also demonstrated similar vasodepressor action in WKY rats with a higher concentration of the heptapeptide (10^{-5} M). In the SHR, the attenuation was observed at higher concentrations of angiotensin II (10^{-9} M – 10^{-6} M). This action supports the reported antihypertensive actions of angiotensin 1-7 in human (Roks et al., 1999; Ueda et al., 2000). Interestingly, Mohan et al. (1994) have shown that angiotensin 1-7 attenuates the pressor response of angiotensin II in rabbit aortic rings, and in anesthetized cats. Because this effect was observed specifically with angiotensin II (and not with other vasoconstrictors) and was blocked by losartan, the authors suggested that angiotensin 1-7 modulates the effect of angiotensin II via the angiotensin AT_{1} receptor. Micromolar concentration of angiotensin 1-7 caused a modest downregulation of the angiotensin AT_{1} receptors in Chinese hamster ovary cells stably transfected with the angiotensin AT_{1A} receptor (Clark et al., 2001a) and in kidney slices from SD rats (Clark et al., 2003). Angiotensin 1-7 has also been proposed to bind to and activate angiotensin AT_{1} receptor, which results in receptor internalization without coupling to G-proteins and activation of phospholipase C (Ueda et al., 2000). The downregulation and internalization of angiotensin II receptor by angiotensin 1-7 could be a possible cause of the observed reduction in pressor response of the
octapeptide in the present study. In addition, angiotensin 1-7 has been demonstrated to reduce angiotensin II-induced phosphorylation of protein kinase C-zena and extracellular signal-regulated kinase (ERK) 1/2 (Zhu et al., 2002). The ability of angiotensin 1-7 to modulate the mechanisms of action of angiotensin II at both the receptor and cellular levels suggests that the heptapeptide plays important regulatory roles in the vascular system.

In the present study, angiotensin 1-7 significantly reduced the response to angiotensin II at a dose of $10^{-7}$ M, i.e. a concentration significantly lower than the IC$_{50}$ of the heptapeptide for the angiotensin AT$_1$ receptor ($>1$ μM) (Ueda et al., 2000). The finding implies that angiotensin 1-7 may act via non-AT$_{1}$ receptor. Similar magnitude of angiotensin 1-7 actions was reported to decrease angiotensin AT$_{1}$ receptor mRNA levels (Tran and Forster, 1997). In addition, nitric oxide has also been shown to decrease angiotensin AT$_{1}$ receptor mRNA levels (Ichiki et al., 1998), and angiotensin 1-7 may also modulate the effects of angiotensin II through this mechanism.

### 4.2. Mesentery

There are differences between the effects of angiotensin 1-7 on the renal and mesenteric vascular bed. In the mesenteric vascular bed, angiotensin 1-7 attenuated angiotensin II-induced vasoconstriction in WKY rats, SHR and streptozotocin-induced diabetic rats. The attenuation was seen at the lower concentrations of angiotensin II in WKY rats. In contrast to the kidney, angiotensin 1-7 reduced angiotensin II pressor action in streptozotocin-induced diabetic rats. Ongoing remodeling caused by activated RAS and the different roles that blood vessels play in the kidney and intestines may explain these differences. In SHR, the reduction in the pressor response by angiotensin 1-7 was seen at much higher concentrations of angiotensin II ($10^{-5}-10^{-6}$ M). Angiotensin II-induced vasoconstriction has been shown to be less in the SHR (Dharmani et al., 2005).

In contrast to the kidney, vasodepressor action of angiotensin 1-7 was witnessed in all the groups. In terms of the effects of angiotensin 1-7 on angiotensin II response, PD123319, D-ALA and L-NAME showed a similar pattern to those observed in the renal vasculature. Indomethacin reversed angiotensin 1-7 action in the SHR and not in WKY and streptozotocin-induced diabetic rats. This suggests that angiotensin 1-7 action is mediated via the angiotensin 1-7 receptor coupled with release of vasodilator prostaglandins and nitric oxide in SHR and only nitric oxide in WKY and streptozotocin-induced diabetic rats. Accordingly, Oliveira et al. (1999) demonstrated that angiotensin 1-7 causes both vasodilation and bradykinin potentiation in mesenteric arterioles, which was blocked by A-779, N$^{ω}$-nitro-L-arginine methyl ester, and indomethacin, suggesting an important participation of local prostanoids and nitric oxide in the actions of angiotensin 1-7. The same authors showed that angiotensin 1-7 potentiates the bradykinin vasodilatory effect in mesenteric arterioles of SHR via release of prostanooids and endothelium derived hyperpolarization factor (EDHF) (Oliveira et al., 1999).

In diabetic mesenteric bed, increased prostaglandin especially prostaglandin I$_2$ (PGI$_2$) has been demonstrated (Fujii et al., 1986, 1987). These authors have postulated that an increase in micro-circulation of PGI$_2$ may partially be protective against progression of angiopathy. As the vasodilator PGI$_2$ was already increased in diabetic mesentery, it is possible that angiotensin 1-7 does not further stimulate PGI$_2$.

The current data suggest that angiotensin 1-7 has a regulatory role in the kidney and mesenteric vasculature which includes the attenuation of the contractile effect of angiotensin II. This action is possibly modulated by angiotensin 1-7 receptor and involves a cyclo-oxygenase dependent pathway and nitric oxide release. This apparent protective effect of angiotensin 1-7 appears to be compromised in diseased state such as...
hypertension and diabetes. It is likely that angiotensin 1-7 receptors or its action is altered to accommodate the ongoing vascular remodeling.

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References


