# Pharmacological Evaluation of Dual Acting Peptide Targeting MasR and pGC-A Receptor in Hypertension

**THESIS**

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by

**Trupti Santosh Ghatage**

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Under the Supervision of

**Prof. Arti Dhar**


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# CERTIFICATE

This is to certify that the thesis titled **Pharmacological Evaluation of Dual Acting Peptide Targeting MasR and pGC-A Receptor in Hypertension** submitted by **Trupti Santosh Ghatage** ID No **2019PHXF0450H** for award of Ph.D. of the Institute embodies original work done by him/her under my supervision.

**Signature of the Supervisor:**
**Name in capital letters: ARTI DHAR**
**Designation**: Professor
**Date**: 19/4/24

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**-Trupti Ghatage**

# *Abstract*

Hypertension is marked by elevated blood pressure and poses significant risks to the health of vital organs, standing as a prominent contributor to mortality and morbidity. Diagnosis of hypertensive patients and treatment choices revolve around assessing systolic and diastolic pressure. The American College of Cardiology (ACC) and American Heart Association (AHA) guidelines define hypertension as blood pressure consistently exceeding 130/80 mm Hg. These guidelines suggest a comprehensive approach to accepting lifestyle adjustments with appropriate medications, addressing all aspects of diagnosis, evaluation, monitoring, and identification of secondary causes, and implementing both pharmacological and non-pharmacological interventions for effective hypertension management.

The World Health Organization's 2023 report reveals a global prevalence of hypertension, affecting 1.28 billion adults aged 30 to 79 years. Surprisingly, 46 % of these individuals remain unaware of their hypertensive status, primarily due to its asymptomatic nature. In some cases, hypertension may exhibit symptoms such as severe headaches, chest pain, dizziness, difficulty breathing, nausea, vomiting, blurred vision, or abnormal heart rhythm. The remaining patients receive a diagnosis and undergo appropriate treatment. Moreover, merely 1 in 5 adults (approximately 21 %) successfully manage to control their hypertension despite therapeutic interventions. This lack of control underscores the seriousness of hypertension as a prominent risk factor for cardiovascular events and premature death on a global scale. Furthermore, undiagnosed or neglected hypertension serves as a potential contributor to co-morbidities like diabetes, chronic kidney diseases, and strokes, which elevates the complexity of managing hypertension.

The progression of hypertension is influenced by various factors such as unhealthy dietary habits, stress, sympathetic nervous system (SNS) activity, insulin resistance, less bioavailability of nitric oxide (NO) and over-activity of the renin-angiotensin-aldosterone system (RAAS). Non-modifiable risk factors, including endothelial dysfunction, renal inflammation, and aging, also play a role in the pathogenesis of hypertension. Treatment strategies have emerged addressing these complexities, primarily targeting the SNS and RAAS. Among the first-line therapies are Angiotensin-converting enzyme (ACE) inhibitors, Angiotensin-2 receptor blockers (ARBs), Calcium channel blockers (CCB), and Diuretics. While these interventions effectively reduce blood pressure, they are often insufficient in controlling end-organ damage and are associated with off-target side effects. Recognizing the

limitations of traditional therapies, researchers are exploring alternative approaches with multivalent properties.

In the early 20$^{th}$ century, discovering the intricate relationship between cardiovascular diseases and peptides created the platform for peptide-based innovative therapeutic approaches. A pivotal breakthrough occurred with the discovery of natriuretic peptides and Angiotensin1-7 (Ang1-7), leading to a new era in the healthcare system. The receptors for these peptides, particulate guanylyl cyclase A (pGCA) and Mas receptor, showcased remarkable cardioprotective properties, respectively. The natriuretic peptide system, mainly through pGCA, plays a crucial role in maintaining cardiorenal homeostasis, exhibiting diuretic and vasodilatory effects. Brain natriuretic peptide (BNP), one of the natriuretic peptides, has emerged as a key player in diagnosing and treating cardiovascular diseases, demonstrating protective properties against hypertrophy, hypertension, and inflammation effects in animal models of hypertension. After extensive exploration over the last three decades, natriuretic peptides have made their way into clinical practice as angiotensin receptor-neprilysin inhibitors (ARNi). Neprilysin inhibition, while accumulating various vasoactive peptides, has raised concerns over hypotension and potential long-term adverse effects.

Conversely, Ang1-7, acting through the Mas receptor, counter-regulates the detrimental effects of central peptide Angiotensin II (AngII), maintaining blood pressure through vasodilation. Studies on Ang1-7 have shown protective effects against oxidative stress, vascular remodeling, renal vasoconstriction, and hypertension in animal models. The development of the Ang1-7 agonist AVE0091 has demonstrated improved cardiac function in models of cardiac hypertrophy. Despite these advancements, the clinical application of peptides remains restricted due to debates on their bioavailability and suitability across diverse clinical settings. However, development in peptide engineering by structural modification has overcome these limitations. Additionally, these investigations lay the groundwork for the potential development of natural peptide-based strategies as novel therapeutic interventions for hypertension.

The primary objective is to establish advanced and safer anti-hypertensive treatments that effectively manage blood pressure and mitigate the risk of associated complications. Traditional approaches to cardiovascular disease treatment have focused on targeting single signalling pathways. While investigating novel peptides and their therapeutic potential, scientists have made significant inventions in developing superior therapies than existing ones. However, recent breakthroughs in targeting multiple pathways have laid the groundwork for

pioneering innovative therapeutic strategies. This concept is supported by the remarkable success of LCZ696 sacubitril/valsartan, a dual-inhibition therapy targeting both neprilysin and Angiotensin type I receptors in treating heart failure. Inspired by these groundbreaking results, our approach involves the design of a novel dual-acting peptide. This innovative peptide is specifically designed to target both Mas and pGCA receptors simultaneously, presenting a promising and effective treatment strategy for hypertension and cardiovascular health.

Therefore, considering all previously reported evidence, our study adopted two distinct approaches to delve into the therapeutic potential of the dual activation of Mas and pGCA receptors in hypertension. The first avenue involved detailed *in-vitro* investigations utilizing renal tubular epithelial cells (NRK-52E), primary vascular smooth muscle cells (VSMCs), and primary endothelial cells (EC). This phase sought to validate the protective effects of the dual-acting peptide against oxidative stress, vascular remodeling, and vascular damage, particularly under hydrogen peroxide ($H_2O_2$) conditions.

The second approach extended our exploration into *in-vivo* studies, employing a DOCA-salt animal model featuring male Wistar rats. This model was selected as it closely mimics the characteristics of low-renin hypertension observed in a human population. Excessive salt consumption can lead to the development of hypertension. Extensive clinical trials have found a strong link between high dietary salt intake and elevated blood pressure. In a healthy environment, mineralocorticoid receptors and ENaC sodium channels regulate sodium homeostasis. Elevated sodium intake causes overexpression of these channels, resulting in sodium retention and an increase in blood pressure. This study investigates the therapeutic potential of simultaneously activating Mas and pGCA receptors to influence the expression of apical and basolateral sodium channels. The goal is to maintain a balanced sodium level and regulate blood pressure. Thus, the results of this study highlight the crucial role of co-activating Mas and pGCA in modulating ENaC expression for enhanced sodium excretion.

Further extending this study, DOCA-salt-induced hypertension in experimental animals manifests as impaired vascular tone, endothelium-dependent relaxation, elevated blood pressure, inflammation, and concurrent cardiorenal and vascular fibrosis. Additionally, it is associated with intimal thickening and structural changes in the aortic vessel wall. This hypertensive model induced by DOCA-salt administration correlates with the overactivation of the RAAS, production of superoxide, and reactive oxygen species. These biochemical changes further diminish NO availability, contributing to the intricate cascade of events leading

to hypertension. By employing both *in-vitro* and *in-vivo* approaches, our study aims to comprehensively elucidate the therapeutic efficacy of the dual-acting peptide in addressing the multifaceted aspects of hypertension, from molecular and cellular levels to the complex interactions observed in living organisms.

Therefore, this study is designed to examine: (I) Determine the efficacy of dual activation of Mas and pGCA receptors *in-vitro* in vascular smooth muscle cells, endothelial cells and renal tubular cells, (II) Determine the cardiorenal protective properties of dual activation of Mas and pGCA receptors in an animal model of hypertension and associated kidney disease and (III) To investigate the effect of dual activation of Mas and pGCA receptors on markers of endothelial dysfunction in hypertension.

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# Appendix I

# *List of Abbreviations*

(3-(4,5-DIMETHYLTHIAZOL-2-YL)-2,5-DIPHENYLTETRAZOLIUM BROMIDE) (MTT)

2,7'-dicholorofluorescein acetate (CM-H2DCFDA)

6-diamidino-2-phenylindole (DAPI)

Acetylcholine (Ach)

Akt serine/threonine kinase (AKT)

Aldosterone (ALDO)

Alizarin red staining (ARS)

Alpha smooth muscle actin (α-SMA)

American College of Cardiology (ACC)

American Heart Association (AHA)

Analysis of variance (ANOVA)

Angiotensin 1-9 (Ang 1-9)

Angiotensin Converting Enzyme (ACE)

Angiotensin II receptor blockers (ARB)

Angiotensin receptor neprilysin inhibitors (ARNi)

Angiotensin type 1 receptor (AT1R)

Angiotensin type II receptor (AT2R)

Angiotensin1-7 (Ang1-7)

Angiotensin-converting enzyme (ACE)

Angiotensin-converting enzyme 2 (ACE2)

Angiotensin-II (AngII)

Antidiuretic hormone (ADH)

Arbitrary unit (A.U.)

Atrial natriuretic peptide (ANP)

Base pair (bp)

B-cell lymphoma 2 (Bcl-2)

Bcl-2-associated X protein (BAX)

Beats per minute (BPM)

Bicinchoninic acid (BCA)

Blood pressure (BP)

Blood urea nitrogen (BUN)

Bovine serum albumin (BSA)

B-type natriuretic peptide (BNP)

Calcium channel blockers (CCB)

Calmodulin (CaM)

Cardiac Output (CO)

Cardiovascular diseases (CVD)

Cenderitide (CD-NP)

Chronic kidney disease (CKD)

Collecting duct (CT)

Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA)

Complementary DNA (cDNA)

Coronary artery disease (CAD)

Creatine kinase-myocardial band (CkMB)

C-type natriuretic peptide (CNP)

Cumulative concentration-response curve (CRC)

Cyclic adenosine monophosphate (cAMP)

Cyclic Guanosine Monophosphate (cGMP)

Dendroaspis NP (DNP)

Deoxycorticosterone acetate (DOCA)

Diaminofluorescein-FM diacetate (DAF-FM)

Diastolic blood pressure (DBP)

Diethyl pyrocarbonate (DEPC)

Distal convoluted Tubule (DCT)

Deoxyribonucleic acid (DNA)

Dual-acting peptide (DAP)

Dulbecco's Modified Eagle's Medium (DMEM)

Endothelial cells (ECs)

Endothelial nitric oxide synthase (eNOS)

Endothelin 1 (ET1)

Epithelial $Na^+$ channel (ENaC)

Ethylene diamine tetra acetic acid (EDTA)

Extracellular matrix (ECM)

Fetal bovine serum (FBS)

Fluorescein isothiocyanate (FITC)

Glomerular filtration rate (GFR)

G-protein coupled receptor (GPCR)

Gram (gm)

Green fluorescent protein (GFP)

Guanosine triphosphate (GTP)

Guanylyl cyclase (GC)

Hanks balanced salt solution (HBSS)

High-density lipoprotein (HDL)

Horseradish peroxidase (HRP)

Human epidermal growth factor (hEGF)

Human fibroblast growth factor (hFGF-B)

Human umbilical vein endothelial cells (HUVECs)

Hydrogen peroxide ($H_2O_2$)

Hydroxyl radical ($OH^-$)

Immunocytochemistry (ICC)

Immunohistochemistry (IHC)

Inducible nitric oxide synthase (iNOS)

Inositol triphosphate ($IP_3$)

Institutional Animal Ethics Committee (IAEC)

Insulin-regulated aminopeptidase (IRAP)

Interleukin-6 (IL-6)

Jun N-terminal kinase (JNK)

Kidney Injury Molecule-1 (KIM-1)

Kilo base pair (kb)

Lipopolysaccharide (LPS)

Losartan (Lst)

Low-density lipoprotein (LDL)

Lumen (L)

Mean blood pressure (MBP)

Melting temperature of primer (Tm)

Messenger ribonucleic acid (mRNA)

Micrometre (µm)

Millilitre (ml)

Millimetres of mercury (mm Hg)

Millimolar (mmol/l or mM)

Mineralocorticoid receptor (MR)

Mitogen-activated protein kinase (MAPK)

Myosin light chain (MLC)

Myosin light chain kinase (MLCK)

Myosin light chain phosphatase (MLCP)

N(gamma)-nitro-L-arginine methyl ester (L-NAME)

N-acetyl cysteine (NAC)

Na-Cl cotransporter (NCC)

Nano gram (ng)

National Centre for Cell Sciences (NCCS)

Natriuretic peptide receptors (NPRs)

Neprilysin (NEP)

Neprilysin inhibitors (NEPi)

Neutrophil gelatinase-associated lipocalin (NGAL)

Nicotinamide adenine dinucleotide phosphate (NADPH)

Nitric oxide (NO)

Novel peptide (NP)

Optical density (OD)

Paraformaldehyde (PFA)

Particulate guanylyl cyclase B (pGCB)

Particulate guanylyl cyclase-A receptor (pGCAR)

Peripheral artery disease (PAD)

Peroxynitrite ($ONOO^-$)

Phenylephrine (PhE)

Phosphate-buffered saline (PBS)

Phosphate-buffered saline with 0.1 % Tween 20 (PBS-T)

Phosphoinositide-3-kinase (PI3K)

Plasma renin activity (PRA)

Polyvinylidene difluoride (PVDF)

Proliferating Cell Nuclear Antigen (PCNA)

Propidium Iodide (PI)

Protein kinase G (PKG)

Proximal convoluted Tubule (PCT)

Quantitative Polymerase Chain Reaction (qPCR)

Radioimmunoprecipitation assay (RIPA)

Rat proximal tubular epithelial cell line (NRK-52E)

Reactive oxygen species (ROS)

Renin-angiotensin-aldosterone system (RAAS)

Revolutions per minute (RPM)

Ribonucleic acid (RNA)

Room temperature (RT)

Second mitochondria-derived activator of caspase (Smac)

Smooth muscle myosin heavy chain (SM-MHC)

Sodium chloride (NaCl)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium hydrogen exchanger (NHE)

Sodium nitroprusside (SNP)

Sodium-potassium ATPase ($Na^+K^+$ ATPase)

Soluble guanylate cyclase (sGC)

Spontaneously hypertensive rats (SHR)

Standard error of the mean (SEM)

Subcutaneously (SQ)

Sympathetic nervous system (SNS)

Systolic Blood pressure (SBP)

Total cholesterol (TC)

Total Peripheral Resistance (TPR)

Transforming growth factor-β (TGF-β)

Triglycerides (TGs)

Tumor necrosis factor-alpha (TNF-α)

Tunica adventitia (TA)

Vascular endothelial growth factor (VEGF)

Vascular smooth muscle cells (VSMCs)

Volt (V)

Volume by volume (v/v)

Wall-to-lumen ratio (W/L)

Water intake (WI)

Weight by volume (w/v)

World Health Organization (WHO)

β-Mercaptoethanol (β-ME)

# *Chapter 1*
# *Literature review*

## *1.1 Hypertension*

Hypertension, or high blood pressure (BP), stands as a major risk factor for cardiovascular disease (CVD) and has a significant impact on global health (1). Untreated hypertension initiates a cascade that affects vital organs such as the heart, brain, kidneys, eyes, and arteries, contributing to end-organ damage (2). Clinically, this results in severe consequences such as myocardial infarction, stroke (3), vascular dementia (4), renal failure (5), retinopathy (6), and peripheral artery disease (2). Prolonged uncontrolled hypertension has the potential to cause chronic arterial and renal damage, eventually leading to the development of a treatment-resistant hypertensive state (7).

Hypertension is a chronic health issue that has earned its notoriety as the "silent killer" because it is often asymptomatic, silently damaging blood vessels, heart, brain and kidney-like vital organs over time (8). Generally, it is primarily identified by blood pressure screening (9). Blood pressure can be managed effectively through lifestyle changes, awareness and proper medication (10). Moreover, early detection can mitigate the possibilities of co-morbidities and improve the overall health and well-being of hypertensive patients (11).

## *1.2 Hypertension stages*

The American College of Cardiology (ACC) and American Heart Association (AHA) guidelines for hypertension consider blood pressure a vital sign and biomarker. The new guidelines define hypertension as blood pressure consistently exceeding 30/80 mm Hg (12). Guidelines suggest one should treat high blood pressure with lifestyle changes and medications at 130/80 mm Hg before it exceeds to avoid complications of hypertension. The revised guidelines are expected to significantly increase the global prevalence of high blood pressure, affecting nearly half of the adult population. This impact is expected to be especially noticeable among younger people. According to Whelton et al., the author of the guidelines, the prevalence of high blood pressure is expected to triple in men under 45 and double in women in the same age group (13).

Individuals are classified as either normotensive or hypertensive based on their blood pressure status. Hypertension is classified into four stages, ranging from early to advanced (Table 1.1). The range of blood pressure levels identifies each stage. The blood pressure above 180/120 mm Hg is a definitive milestone, marking the hypertensive crisis, an advanced stage of hypertension. In the absence of other warning signs, patients require immediate medication changes, which may impose multidrug therapy. If there are clear signs of organ damage,

immediate hospitalization is required. Scientifically, this classification emphasizes the importance of considering traditional CV health blood pressure metrics to fully understand (12,13).

*Table 1. 1: Stages of hypertension*

| Sr No. | Type | Systolic (mm Hg) | Diastolic (mm Hg) |
|---|---|---|---|
| **1.** | Normal blood pressure | ≤ 120 | ≤ 80 |
| **2.** | Elevated blood pressure | 120 to 129 | ≤ 80 |
| **3.** | Stage 1 hypertension | 130 to 139 | 80 and 89 |
| **4.** | Stage 2 hypertension | ≥ 140 | ≥ 90 |
| **4.** | Hypertension crisis | ≥ 180 | ≥ 120 |

## *1.3 Classification of hypertension*

Hypertension can be classified into several types, each with its characteristics and contributing factors. The worldwide prevalence of different types of hypertension in mentioned in Table 1.2.

### 1.3.1 Essential hypertension

Primary hypertension, also known as essential hypertension, is the most common type, accounting for roughly 90-95 % of all cases (2). This type of hypertension develops gradually over time and is frequently linked to aging, genetics, and lifestyle factors such as diet, physical inactivity, and obesity. The exact cause of primary hypertension is not always known, making it a complicated multifactorial condition. Primary hypertension is characterized by complex interactions between the heart, blood vessels, and the body's regulatory systems. Over time, persistently elevated blood pressure can cause damage to blood vessels, the heart, and other organs, increasing the risk of cardiovascular disease, stroke, and other complications.

Lifestyle changes are typically used to manage and treat primary hypertension, as well as pharmacological interventions if indicated. Adopting a heart-healthy diet, exercising regularly, limiting alcohol consumption, and managing stress are all examples of lifestyle changes (14).

### 1.3.2 Secondary hypertension

Secondary hypertension is uncommon and is caused by a known underlying medical condition or cause. Kidney diseases, hormonal disorders, sleep apnoea, and certain medications are some of the conditions that can cause secondary hypertension. Managing the underlying cause is critical for effectively controlling blood pressure in secondary hypertension (14).

### 1.3.3 Resistant hypertension

Resistant hypertension is a challenging and clinically significant subtype of high blood pressure that makes it difficult to achieve adequate blood pressure control despite the use of multiple antihypertensive medications (15). The definition of resistant hypertension has changed over time, but it is typically defined as persistently elevated blood pressure levels despite adherence to an appropriate three-drug antihypertensive regimen that includes a diuretic. Several investigations are focused on the management of resistant hypertension. Recent research has highlighted the importance of thiazide diuretics (16), mineralocorticoid receptor (MR) antagonists (17), angiotensin receptor blockers (ARB) and neprilysin inhibitors (18,19) in resistant hypertension management.

### 1.3.4 Salt-sensitive hypertension

Salt-sensitive hypertension is a subtype of essential hypertension in which salt intake significantly influences blood pressure levels (20). Individuals with salt-sensitive hypertension notice a significant increase in blood pressure in response to increased salt consumption. Genetics, age, and certain health conditions can all influence salt sensitivity. Diagnosing and treating salt-sensitive hypertension frequently requires lifestyle changes, such as limiting salt intake, as well as appropriate medical intervention (21).

High blood pressure is caused by a combination of genetic predisposition and environmental factors, with high salt intake being identified as a major environmental contributor. Individuals vary in their sensitivity to sodium, with some experiencing significant increases in blood pressure in response to increased sodium consumption, while others experience only minor changes. These differences can be attributed to genetic variability in sodium regulation and excretion pathways. Nakamura et al. proposed that aldosterone and its analogues act on MR, influencing sodium absorption and causing changes in the expression of epithelial sodium channels (22). Diets high in salt and DOCA activate various sodium transporters, such as the epithelial Na+ channel (ENaC), the Na-Cl cotransporter (NCC), and the sodium hydrogen exchanger (NHE) on the apical membrane, and the sodium-potassium ATPase ($Na^+K^+$ ATPase) on the basolateral membrane in the kidney (23).

Approximately 20 % of sodium reabsorption from tubules to blood occurs through the basolateral membrane in the thick ascending loop, with the remaining 75 % occurring through the apical membrane via the proximal convoluted tubule, distal convoluted tubule, and collecting duct from urine to tubules (24). ENaC, an epithelial sodium channel, is critical for

sodium reabsorption in the distal nephron, regulating extracellular fluid volume and, as a result, blood pressure (25).

*Table 1. 2: Classification of hypertension*

| Classification | Worldwide Prevalence Share (%) |
|---|---|
| **Primary Hypertension (Essential)** | 90-95 % (2) |
| **Secondary Hypertension** | 5-10 % (14) |
| **Resistant hypertension** | 12 % to 18 % of the hypertensive population (15) |
| **Salt-Sensitive Hypertension** | Approximately 30 % of healthy people are salt-sensitive. Prevalence rises with age 12 and with comorbidities that impair kidney and vascular function (20) |

## *1.4 Prevalence*

Hypertension is a highly prevalent and preventable risk factor of CVDs (26). Hypertension poses a significant global financial burden, accounting for a major portion of total medical spending. Globally, an estimated 1.28 billion adults between the ages of 30 and 79 are hypertensive (Figure 1.1) (27). However, genetics and older age can increase the risk of hypertension (28). Despite this, 46 % of individuals with hypertension are unaware of their condition, highlighting the silent and often asymptomatic nature of this disease. Even after diagnosis, less than half of those affected (42 %) receive the necessary diagnosis, care and treatment, and only about one in five adults with hypertension (21 %) effectively manage their blood pressure (27). This prevalence of uncontrolled hypertension is a major contributor to premature deaths worldwide. In response to this critical public health issue, one of the global targets for non-communicable diseases is reducing hypertension prevalence by 33 % between 2010 and 2030, emphasizing the urgent need for awareness, diagnosis, and effective management strategies (29).

However, hypertension prevalence is not uniform globally, and a significant majority reside in low and middle-income countries (30). However, high-income countries experienced a reduced prevalence of hypertension (26). Variations in the levels of risk factors for hypertension, such as high sodium intake, low potassium intake, obesity, alcohol consumption, physical inactivity, and an unhealthy diet, may account for some of the regional disparities in hypertension prevalence (31).

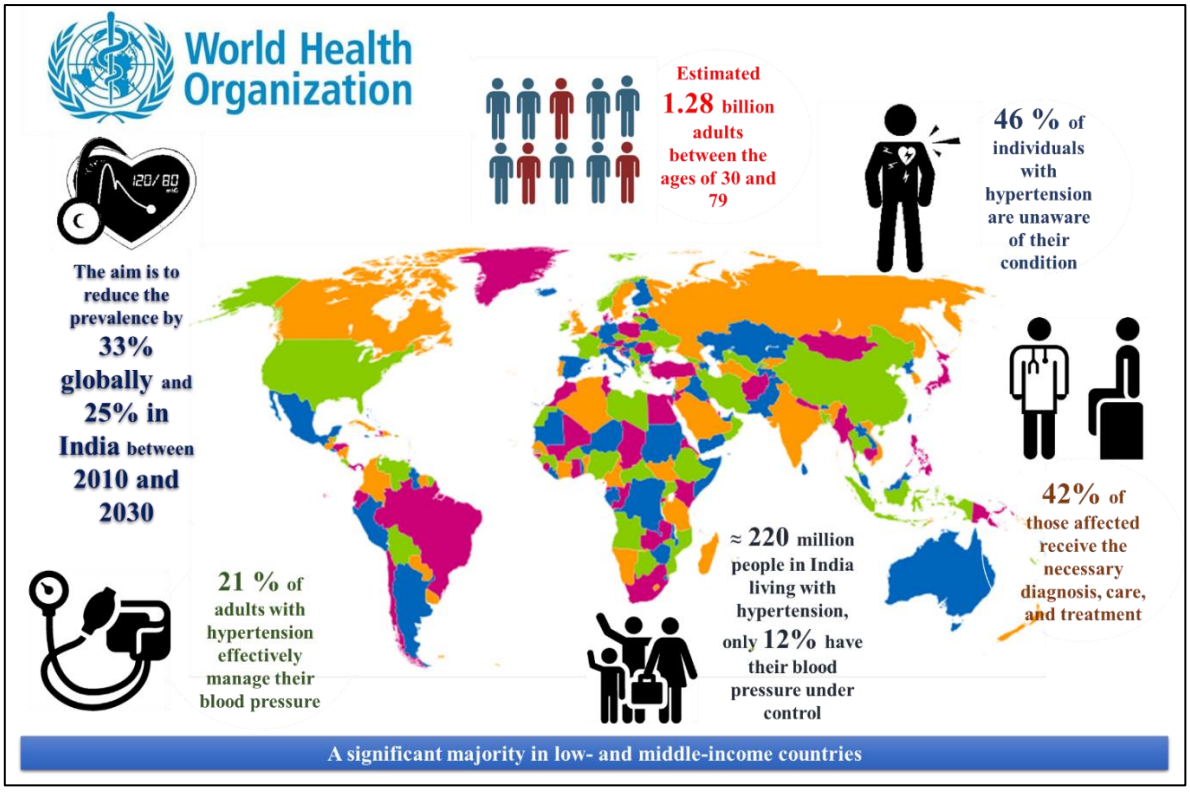



*Figure 1. 1: Prevalence of hypertension*

The figure is produced from reference (27)

### *1.5 Blood pressure regulation*

Blood pressure regulation in the body is a complex process involving multiple organs, systems, and feedback mechanisms. The primary goal of this regulation is to maintain adequate blood flow to all body tissues while preventing excessive pressure that could damage blood vessels. Blood pressure is usually measured in millimeters of mercury (mm Hg) and is expressed asystolic and diastolic pressure (32). The heart is the central player in blood pressure regulation. It functions as a pump that contracts and relaxes to circulate blood throughout the body. During each cardiac cycle, blood is ejected into the arteries, causing a temporary increase in systolic pressure. When the heart is at rest between beats, the pressure in the arteries decreases, and this is referred to as diastolic pressure.

The body maintains constant blood pressure through several mechanisms, ensuring a stable internal environment. Mean Arterial Pressure (MAP) is an important parameter in this regulation, representing the average blood pressure experienced by an individual throughout one cardiac cycle. It is determined by the combined influence of two main variables: Cardiac Output (amount of blood pumped by the heart per minute) and Total Peripheral Resistance (TPR). These variables must work in coordination to efficiently manage blood pressure (32).

The balance of arterial tone, a delicate balance influenced by intravascular volume and neuro-humoral systems, plays a pivotal role. On the other hand, the renin-angiotensin-aldosterone system (RAAS) attracts attention, affecting BP levels by maintaining fluid and electrolyte balance and vessel constriction (33). However, newly highlighted natriuretic peptides and the peptides of the protective axis of RAAS are the unsung heroes in regulating fluid balance and vessel dilation, complementing this system. We will discuss these systems and their role in blood pressure regulation.

### 1.5.1 The autonomic nervous system

It comprises the sympathetic (fight or flight) and parasympathetic (rest and digest) branches and plays a central role in the immediate, short-term adjustment of blood pressure. When needed, the sympathetic system stimulates the heart to beat faster and constricts arterioles with more force, increasing TPR and elevating blood pressure (34). Conversely, the parasympathetic system can slow the heart rate and relax blood vessels, lowering blood pressure (35).

The human heart typically receives predominant parasympathetic neural input in a healthy, resting state. This parasympathetic activity reduces the heart rate below its intrinsic rate by influencing the sinoatrial node. To facilitate an increase in heart rate, the initial response is to diminish the parasympathetic input to the sinoatrial node, enabling the heart rate to rise passively toward its intrinsic rate (36). This withdrawal of parasympathetic activity is crucial during heart rate oscillations during respiration and the initial phase of increased heart rate in response to factors like low blood pressure upon standing, low-grade exercise, and other minor perturbations. The effects of parasympathetic input on the sinoatrial node occur through the release of the neurotransmitter acetylcholine from post-ganglionic parasympathetic fibers onto the target tissue (37).

When an increase in heart rate is needed, the activation of sympathetic inputs surpasses the intrinsic rate to meet increased demands. Sympathetic nerve activity enhances blood pressure through various mechanisms (38):

- Increasing heart rate, thereby boosting cardiac output
- Increasing stroke volume promotes contractility and increases cardiac output
- Constricting arterioles, elevating systemic vascular resistance
- Constricting veins raises central venous pressure

- Stimulating the release of renin from the kidney, resulting in the production of angiotensin II and the release of aldosterone. Aldosterone promotes sodium retention by the kidneys, increasing blood volume and venous return

The sympathetic nervous system brings about these changes in the body through the release of the neurotransmitter norepinephrine from post-ganglionic sympathetic fibers onto the target tissue. A similar effect on blood pressure can be achieved by stimulating sympathetic preganglionic fibers to activate chromaffin cells in the adrenal medulla, releasing epinephrine (and norepinephrine) into the bloodstream. Circulating epinephrine (and norepinephrine) can activate receptors on target tissues, influencing blood pressure. Notably, there are two receptor types for norepinephrine and epinephrine (37):

- ß1 (beta 1) receptors, found on the heart and juxtaglomerular cells in the renal vasculature, are involved in increased heart rate, contractility, renin release, blood volume, and vasoconstriction. Blocking ß1 receptors with drugs like beta blockers can lower blood pressure
- α1 (alpha 1) receptors, located on arteriolar smooth muscle, induce contraction and arteriolar constriction. Inhibiting α1 receptors with drugs such as alpha blockers can reduce blood pressure

### 1.5.2 Renin-angiotensin-aldosterone system

The RAAS is a complex hormonal system that plays a crucial role in the regulation of blood pressure, fluid and electrolyte balance, and the overall homeostasis of the body. It is primarily responsible for maintaining blood pressure within a narrow range to ensure adequate blood flow to various organs and tissues (39). The RAAS, for instance, responds to low blood pressure or reduced blood flow to the kidneys, leading to the retention of sodium and water, which increases blood volume and pressure (40). Hormones like aldosterone and antidiuretic hormone (ADH) are involved in this process. Additionally, neural and hormonal signalling can impact blood pressure by adjusting TPR through the release of vasoconstrictor and vasodilator substances.

The classical RAAS pathway is activated when blood pressure drops, or blood flow to the kidneys decreases. Juxtaglomerular cells in the kidney detect these changes and respond by releasing an enzyme known as renin into the bloodstream: renin targets angiotensinogen, a precursor protein in the blood produced by the liver. Renin degrades angiotensinogen into an

inactive peptide called angiotensin I. Angiotensin-converting enzyme (ACE) then converts angiotensin I into an active peptide known as angiotensin II, as explained in Figure 1.2 (AngII) (41). ACE is primarily found in the lungs but also in other tissues. Angiotensin II is a potent vasoconstrictor which narrows blood vessels. This vasoconstriction causes an increase in TPR, which raises blood pressure (42). Furthermore, angiotensin II stimulates the release of aldosterone and ADH, both of which play important roles in blood pressure control. The adrenal glands, which are located on top of each kidney, produce the hormone aldosterone. It acts on renal tubules in the kidneys, promoting sodium and water reabsorption and potassium excretion. This process raises blood volume and, thus, blood pressure (43).

The hypothalamus produces ADH, or vasopressin, which is then released by the pituitary gland. ADH stimulates water reabsorption in the kidneys, concentrating urine and conserving water in the body. This action increases blood volume and elevates blood pressure (44). The RAAS's overall effect is to raise blood pressure by constricting the blood vessels and retaining sodium and water. This is an important response to situations in which blood pressure has dropped, such as dehydration or excessive salt loss. However, if the RAAS is overactive or dysregulated, it can contribute to chronic conditions such as hypertension, increasing the risk of cardiovascular disease.

### 1.5.3 Protective axis of RAAS

The Protective Axis of RAAS, Angiotensin-converting enzyme 2 (ACE2)/Angiotensin1-7 (Ang1-7)/Mas receptor plays an important role in maintaining blood pressure under physiological conditions (45). Unlike the classical RAAS pathway, characterized by the vasoconstrictive effects of AngII resulting in elevated blood pressure, the Mas receptor axis counter-regulates the AngII actions (For stimulus and location, refer to Table 1.3) (46). Ang1-7 (Refer to Table 3) plays a critical role in the blood pressure regulatory process. The ACE2 is a carboxypeptidase that is closely related to the well-known ACE. ACE2 regulates the RAAS and its complex interactions with various peptides (47). ACE2 is distinguished by its strong affinity for Ang-II and its ability to convert it to Ang1-7. Furthermore, ACE2 can convert Ang-I to angiotensin 1-9 (Ang 1-9), which is then converted into Ang1-7 by the enzyme ACE (Figure 1.2) (48). These peptide transformations are essential to the ACE2/Ang1-7/MasR axis, which provides protection by counteracting the actions of the classical ACE/AngII/AT1R axis.

The activation of the Mas receptor causes vasodilation, which is critical for blood pressure regulation (49). Furthermore, the Mas receptor axis promotes natriuresis (46), and has

significant anti-proliferative (50) and anti-fibrotic properties (51). In terms of blood pressure regulation, the Mas receptor axis' vasodilatory effects play an important role in maintaining blood vessel tone (52). By promoting vasodilation and counteracting some of AngII's vasoconstrictive actions, the Mas receptor axis effectively balances the hypertensive effects of the classical RAAS pathway. In summary, the Mas receptor axis, which is activated by Ang1-7, not only helps to maintain blood pressure through vasodilation, but it also serves as an important counter-regulatory and protective component of the RAAS system.

*Table 1. 3: Location of target receptors and its stimulus in the human body*

| Receptor/Cell/Secretion | Location | Stimulus |
|---|---|---|
| **Mas Receptor** | Renal, cardiac, adipose, arterial wall and cerebral level | Ang1–7 (46) |
| **pGCA** | kidney, lung, adipose, adrenal, brain, heart, testis, and vascular smooth muscle tissue | ANP and BNP (53) |
| **Angiotensin type I Receptors** | Various tissues, including the heart, smooth muscles, testes, lungs, and kidneys | AngII (41) |

### 1.5.4 Natriuretic peptides

Natriuretic peptides emerge as critical contributors to the intricate regulation of blood pressure in individuals in good cardiovascular health (53). Natriuretic peptides are a group of hormones that are released in response to the stretching of the atria and ventricles. The human heart secretes several NPs, including atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), and C-type natriuretic peptide (CNP). These natriuretic peptides interact with natriuretic peptide receptors (NPRs), which are classified as NPR-A, NPR-B, and NPR-C (54). Particulate Guanylyl Cyclase Receptor A (pGC-A) is an important natriuretic peptide membrane receptor that regulates cardiovascular, renal, and endocrine functions (For stimulus and location refer Table 1.4). Its critical role is demonstrated by the interaction of the extracellular domain with natriuretic peptides, which triggers the conversion of Guanosine triphosphate (GTP) to Cyclic Guanosine Monophosphate (cGMP) within the intracellular guanylyl cyclase (GC) domain (Figure 1.2) (55).

ANP is primarily released from the atria, whereas BNP is released from the ventricles (Refer Table 1.4). ANP and BNP exert their physiological effects via GC-A/NPR-A receptors, which trigger intracellular signalling pathways (56). Activation of GC-A causes an increase in the secondary messenger cyclic guanosine monophosphate (cGMP), which contributes to vasodilation (Figure 2) (57). This potent effect of NPs via the GC-A receptor pathway holds

promise as a potential therapeutic approach in the treatment of hypertension by inhibiting P38 mitogen-activated protein kinase (MAPK) and protecting against glomerular damage (58).

Natriuretic peptides influence the kidneys, causing natriuresis and diuresis. By facilitating the elimination of sodium and water, these peptides actively contribute to the reduction of blood volume, thereby lowering blood pressure (59). Furthermore, natriuretic peptides cause vasodilation in the intricate network of blood vessels. This vasodilatory effect relaxes smooth muscle within arterial walls, reducing systemic vascular resistance. The resulting decrease in resistance adds another layer to the coordinated effort to lower blood pressure (60). Notably, ANP and BNP play a regulatory role in inhibiting RAAS (61). This inhibition reduces the secretion of aldosterone, a hormone that promotes sodium and water retention and contribute to the overall regulation of blood pressure (62).

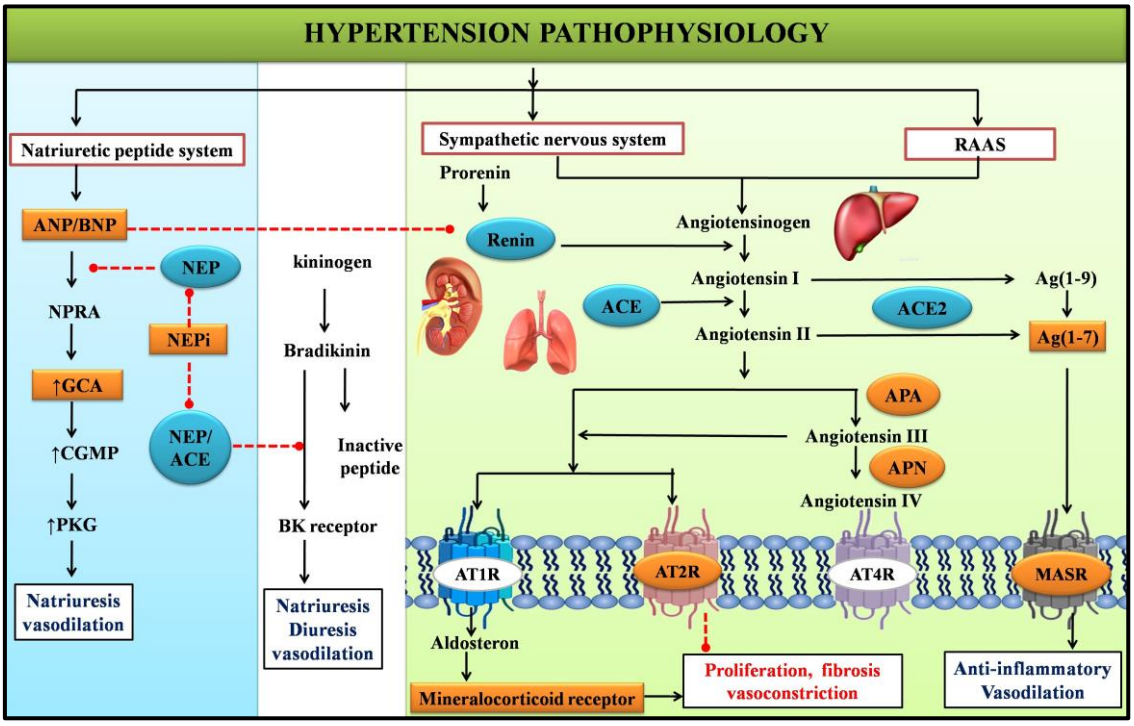



*Figure 1. 2: RAAS Components Involved in Hypertension Pathophysiology*

This Figure describes the various elements and interactions within the RAAS implicated in hypertension and the potential therapeutic targets for managing blood pressure. New emerging targets with vasodilator properties are highlighted in yellow, while enzymes are denoted in blue. The dotted red line indicates inhibition.

Figure is reproduced from references in (63–65)

Furthermore, these peptides have a counteractive effect on the SNS, which is associated with increased heart rate and vasoconstriction (60). The counteraction provided by natriuretic

peptides helps to maintain a delicate balance in cardiovascular function, preventing excessive sympathetic activity that can lead to high blood pressure. The multivalent actions of natriuretic peptides orchestrate a symphony of responses that are critical for keeping blood pressure within normal limits. These mechanisms, which range from promoting natriuresis and inducing vasodilation to inhibiting RAAS and counteracting SNS activity, work together to keep blood pressure stable in people who are in good cardiovascular health.

*Table 1. 4: Targeted endogenous peptide, respective secreting cells and its stimulus*

| Secretion | Cell Type | Stimulus |
|---|---|---|
| **BNP** | Cardiomyocytes (mainly ventricles of the heart) | Increased ventricular pressure and stretch (56) |
| **Ang1-7** | Various cells, including endothelial cells | ACE2 and Ang1–7 (46) |
| **AngII** | Various tissues, including smooth muscles, heart, and kidneys | ACE converts Angiotensin I to Angiotensin II (66) |

## *1.6 Hypertension complications*

Hypertension is a silent threat that can lead to several serious complications. This persistent elevation in blood pressure has an impact not only on the heart but also on the functioning of vital organs. Elevated blood pressure and damaged blood vessels increase the risk of heart disease, stroke, and heart failure. Under constant high pressure, the arteries may harden and narrow, resulting in vascular diseases, kidney damage, heart attacks, and strokes (67). Chronic high blood pressure can damage the intricate vessels within our eyes, resulting in vision impairments and, in severe cases, blindness (68). In addition, hypertension increases the risk of peripheral arterial disease, aneurysms, cognitive decline, and dementia (64). Hypertension is predictable and reversible, but the frightening truth is that it frequently causes no symptoms until complications arise. Awareness, regular check-ups, a healthy lifestyle, exercise, and proper medical care are all effective tools in the fight against hypertension.

### 1.6.1 Complications arising from oxidative stress in hypertension

Oxidative stress in hypertension a major mediator in complex mechanisms that drive disease progression and associated complications (69). Essentially, oxidative stress is an imbalance between free radicals and the body's ability to neutralize or detoxify these harmful molecules. In hypertension, this delicate balance is disrupted, resulting in an excess of reactive oxygen species (ROS) generation that can weaken the cardiovascular system. Overproduction of ROS in hypertension is frequently caused by a combination of factors, including increased activity of enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase

(70), mitochondrial dysfunction, and low levels of antioxidant defences (71). These free radicals, in turn, can harm cells, tissues, and essential biomolecules such as lipids, proteins, and DNA in the cardiovascular system (72).

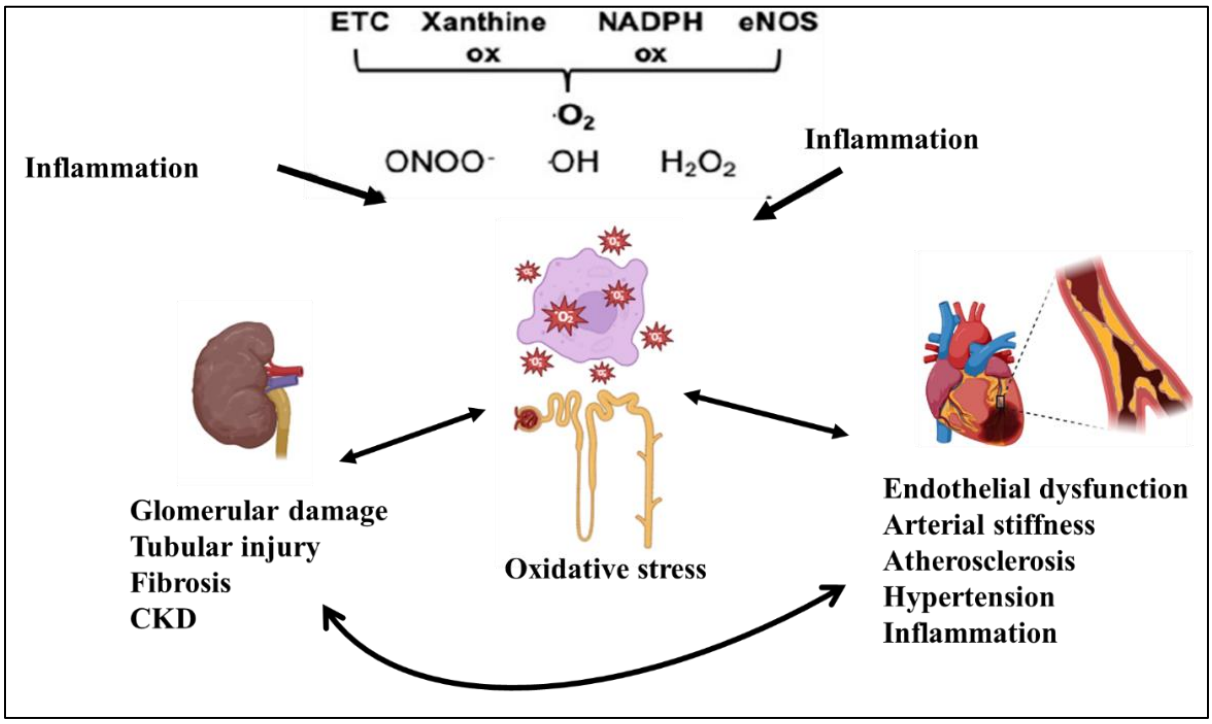



*Figure 1. 3: Oxidative stress mechanisms is main mediator in cardiovascular and renal disorders*

Physiologically relevant reactive ROS in the vascular endothelium include ONOO-, OH, $O_2$, and $H_2O_2$. Processes associated with oxidative stress are highlighted in bold black. Oxidative stress mechanisms instigate inflammation, initiating a feedback loop that enhances ROS production.

Figure reproduced from reference (73)

The effects of oxidative stress extend beyond cellular damage. It plays a significant role in the progression of hypertension by promoting inflammation, impairing vascular function, and initiating vascular remodelling (74). This oxidative stress on blood vessels causes endothelial dysfunction (75), which occurs when the delicate endothelial cell lining of blood vessels loses its ability to regulate vascular tone and maintain normal blood pressure (76). Furthermore, oxidative stress promotes atherosclerosis, which exacerbates the effects of hypertension (77). Damaged vessels lead to impaired vascular tone and more likely to constrict, raising blood pressure and increasing the risk of cardiovascular events such as heart attacks and strokes (78). Unravelling the relationship between oxidative stress and hypertension opens

up new treatment options and emphasizes the importance of lifestyle changes in reducing the impact of oxidative stress on cardiovascular health.

### 1.6.2 Kidney complication

As we all know, the kidney regulates blood pressure. Continuous high blood pressure can harm the kidneys' delicate structures, particularly the nephrons, which filter waste and regulate fluid and electrolyte balance. Over time, the increased pressure causes damage to the glomeruli and tubules, impairing their ability to filter waste and maintain electrolyte balance. Persistent high blood pressure can cause a decrease in glomerular filtration rate (GFR), indicating failure of kidney function. This decline in function may lead to chronic kidney disease (CKD) or kidney failure (79). Proteinuria can occur when blood vessels in the kidneys are damaged and proteins leak into the urine (80). Excess protein in urine indicates kidney damage and is a common feature of hypertensive nephropathy. Renal fibrosis can further exacerbate kidney damage and compromise kidney health (64,81). Kidney damage due to hypertension not only affects the kidney but also raises the risk of cardiovascular complications (82).

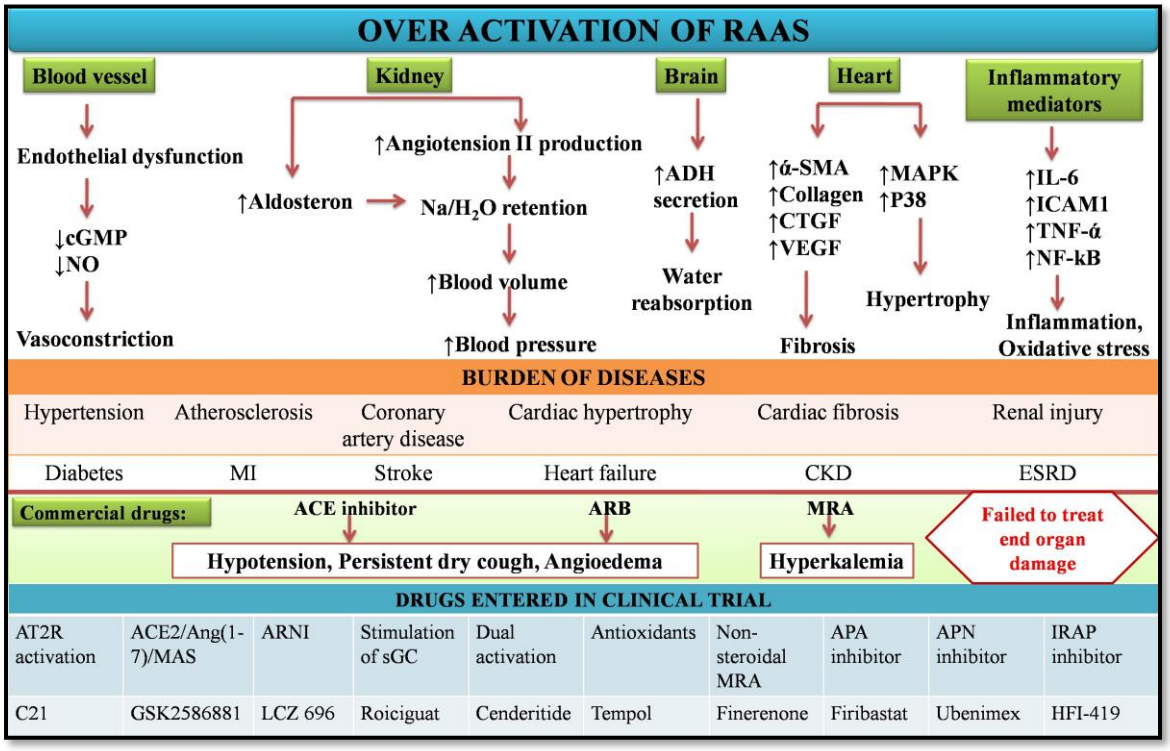



*Figure 1. 4: Overview of RAAS Over activation*

Excessive activation of the RAAS impacts numerous cellular processes within vital organs, contributing to the development of hypertension-related diseases. Existing commercially

available drugs often exhibit insufficient efficacy and intolerable side effects. Consequently, this scenario has spurred the exploration of novel emerging therapeutic targets.
Figure is reproduced from reference (83)

Furthermore, chronic hypertension can cause structural changes in the kidneys, such as thickening of blood vessel walls and scarring of kidney tissues, which impairs their ability to regulate sodium balance and blood pressure. One of the key mechanisms is the RAAS. When blood pressure is high, the RAAS can become overactive (Figure 1.3), causing the kidneys to retain more sodium and water. As a result, blood volume increases, and blood pressure rises. Excess dietary sodium can exacerbate hypertension, so managing sodium intake is critical for blood pressure control (84). Understanding the intricate relationship between sodium balance, kidney function, and hypertension underscores the importance of comprehensive approaches to managing blood pressure.

### 1.6.3 Endothelial dysfunction

Endothelial dysfunction emerges as a major player in the complex nature of hypertension, playing a critical role in the condition's onset, progression, and subsequent complications. The endothelium, an important inner lining of blood vessels, plays a critical role in regulating vascular tone, blood flow, and cardiovascular health. However, in the context of hypertension, the endothelial layer undergoes various changes (85).

Under normal, healthy conditions, the endothelium expertly maintains a delicate balance by releasing substances that promote either vasorelaxation or constriction, tailoring its responses to the body's physiological requirements (86). Unfortunately, in hypertension, persistently elevated blood pressure has a negative effect on the endothelium, disrupting its normal function. This disruption is characterized by a decrease in the bioavailability of nitric oxide, a key vasodilator produced (87).

The resulting imbalance between vasoconstrictor and vasodilator factors promotes increased vascular resistance, inflammation, and oxidative stress (88). Furthermore, endothelial dysfunction not only promotes thrombosis but also contributes to the development of atherosclerosis, compromising the cardiovascular health (89). Understanding and addressing the severity of endothelial dysfunction in hypertension is critical for effectively managing associated cardiovascular complications and improving overall vascular health.

### 1.6.4 Cardiovascular complications

Hypertension is a leading cause of a wide range of serious cardiovascular events. A prolonged increase in blood pressure on the delicate arterial walls triggers a chain reaction of negative effects on the cardiovascular system. This chronic condition contributes to life-altering complications, most notably coronary artery disease (CAD), in which the arteries that supply blood to the heart narrow, resulting in conditions such as angina or even heart attacks (90). Furthermore, hypertension can lead to heart failure events, reducing the heart's ability to efficiently pump blood and increasing the risk of strokes due to compromised blood vessel integrity in the brain (91). Furthermore, it can cause peripheral artery disease (PAD), kidney damage, aneurysms, abnormal heart rhythms, and cognitive decline (92).

Hypertension also has a significant impact on the structural integrity of the heart, contributing to the development of left ventricular hypertrophy. The constant strain on the heart muscle to pump blood against high pressure causes thickening of the left ventricle wall. If left untreated, this hypertrophy can impair heart function and eventually lead to heart failure (93). Furthermore, hypertension plays a significant role in the development and progression of cerebrovascular complication. The increased pressure within the arteries that supply blood to the brain increases the risk of stroke. Hypertension can cause blood clots or the rupture of blood vessels in the brain, resulting in potentially severe consequences (94).

In fact, hypertension associated cardiovascular complications cover a wide range of interconnected issues that affect not only the heart but also vital arteries throughout the body. Managing and treating hypertension is critical not only for blood pressure control, but also for avoiding and mitigating these potentially serious cardiovascular consequences.

## *1.7 Animal models of hypertension*

Animal models have been extremely useful in understanding hypertension, providing insights into its mechanisms, progression, and potential treatment strategies. Several animal models have been developed to simulate hypertension, giving researchers useful tools for studying the condition (95). Rodents' models have been widely used to advance our understanding of cardiovascular research areas. Rats are an important animal model for cardiovascular research (96). Several animal models routinely used for hypertension studies include spontaneously hypertensive rats, renovascular hypertension models, diet-induced models, genetically modified models, stress-induced models, spontaneously hypertensive stroke-prone rats (97). Here, for this study we used DOCA-salt-induced hypertension model.

Furthermore, isolated rat cardiac cells such as vascular smooth muscle cells and primary endothelial cells are frequently used for *in-vitro* studies of the underlying vascular mechanisms in the pathophysiology of cardiovascular disease progression (98). These vascular changes can lead to cardiac hypertrophy, hypertension, fibrosis, and atherosclerosis (99). We will discuss this in next heading in detail.

These animal models allow researchers to study various aspects of hypertension, such as its molecular mechanisms, cardiovascular complications, the impact of lifestyle factors, and the efficacy of potential treatments. However, while these models provide valuable insights, they may not fully replicate the complexity of human hypertension, and findings should be extrapolated with caution to clinical settings.

### 1.7.1 DOCA-salt hypertension model

The DOCA (Deoxy-corticosterone acetate) salt hypertension model is a well-known experimental method for inducing hypertension in animals, particularly rodents. The DOCA salt hypertension model is used to investigate various aspects of hypertension, such as its pathophysiology, cardiovascular complications, and potential treatment options. This model is used by researchers to evaluate the efficacy of anti-hypertensive drugs, better understand the mechanisms underlying salt-sensitive hypertension, and investigate the effects of hypertension on target organs such as the heart, kidneys, and blood vessels as described in Figure 1.5. This model uses deoxy-corticosterone acetate, a mineralocorticoid hormone, and a high-salt diet to induce hypertension in animals (100). In this hypertension model, animals are given DOCA in varying doses, usually ranges from 20 to 150 mg/kg in rats, depending on the study parameters (101).

The DOCA administration causes an imbalance in renal sodium handling, resulting in increased reabsorption of sodium and water by the kidneys, which eventually leads to hypervolemia (102). Along with DOCA, rat also receives high-salt diet, with 0.6-1 % NaCl in drinking water, which is combined with or without uninephrectomy depending on the extent of hypertension (103,104). It causes hypertension via mineralocorticoid excess, like conditions in which renin activity is reduced. Increased sodium intake exacerbates the effects of DOCA by increasing blood volume via water and sodium retention (96).

Salt sensitivity, a well-recognized feature of the DOCA-salt model, impacts the development and progression of human essential hypertension. Salt sensitivity was found in 26 % of normotensive people and 51 % of hypertensive people, indicating that it could be a key

indicator of hypertension risk (105). In addition to this, renin, an enzyme that converts angiotensinogen to angiotensin Ang-I, has been found to be low in salt-sensitive populations. This observation is important because a similar low-renin hypertension is observed in animals treated with DOCA-salt, mirroring the conditions seen in the human population (106). The DOCA-salt model induces a neurogenic form of hypertension with low renin levels, making it ideal for testing hypotheses about neurocentric factors or the effects of a high salt diet (101).

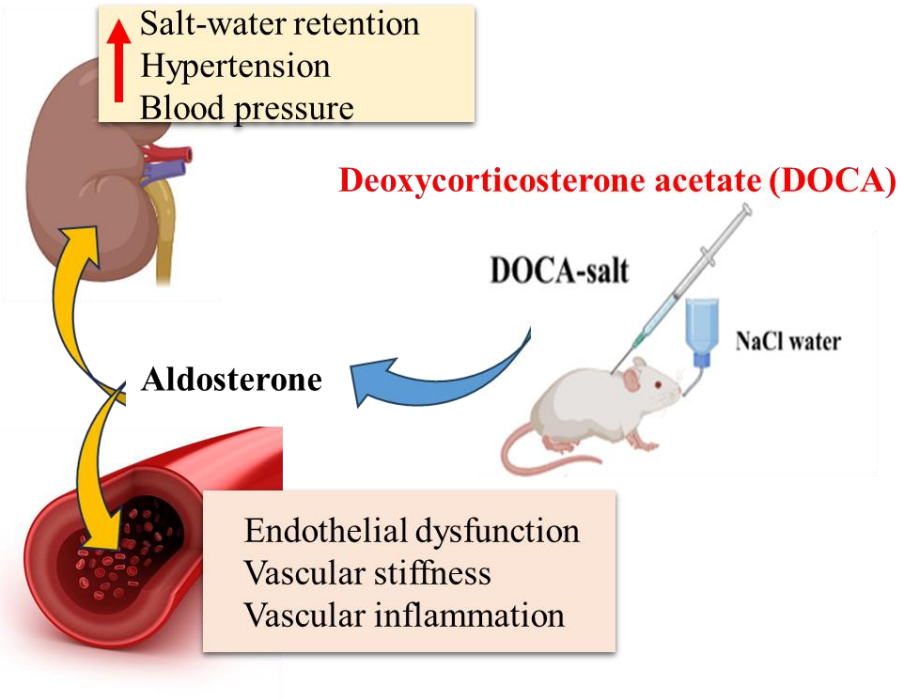



*Figure 1. 5: Mechanism of DOCA-salt induced hypertension.*
DOCA-salt is precursor of aldosterone which affects both kidney and aorta.
Figure is reproduced from the reference (101,107)

### *1.7.2 In-vitro* studies on primary cells

The isolation of primary rat aortic cells plays a pivotal role in unravelling the intricate mechanisms underlying hypertension. By specifically targeting specific cell types, researchers gain invaluable insights into the intricate mechanism to maintain blood pressure, shedding light on better understanding of vascular biology and their contributions to the pathophysiology of hypertension (108). Earlier Human Umbilical Vein Endothelial Cells (HUVEC) were widely used for investigation into EC for CVD (109). They are easy to handle and also generate reproducible data. However, problem with HUVEC is that they are from veins not from the arteries. Hence most of the research focus is shifted toward the isolation of primary arterial

cells such as vascular smooth muscle cells (VSMCs) and endothelial cells (ECs). The advantages of primary cells are it offers a close to *in-vivo* environment for studies (110).

Anatomically, the arterial wall is divided in three separate layers i.e., the inner layer-tunica intima, middle layer-tunica media and outer layer-tunica externa (Figure 1.4). The tunica intima contains a protecting covering of endothelial cells, Tunica media comprises elastic fibres and smooth muscle cells whereas the tunica externa holds connective tissue, predominantly consisting of collagen and elastin fibres (111). The endothelial cells from tunica interna provide various paracrine mediators to the underlying VSMCs that maintain vascular tone (112).

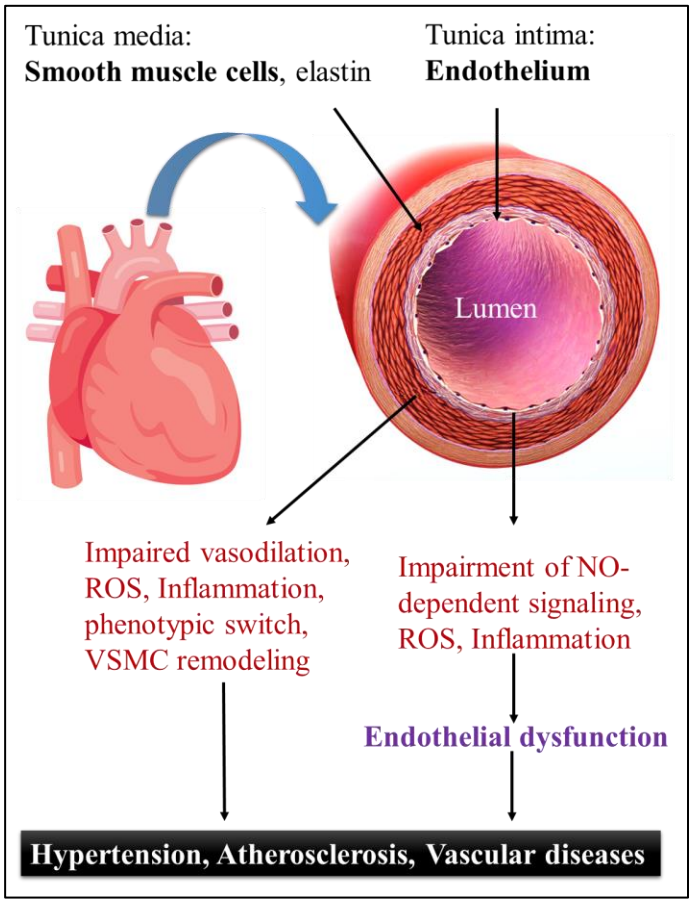



*Figure 1. 6: Schematic representation illustrating the distribution of Vascular Smooth Muscle Cells (VSMC) and Endothelial Cells (EC) within the heart.*

Figure is reproduced from reference (111)

In healthy individuals, the VSMCs are in "contractile" form, after injury or in pathological conditions like endothelial dysfunction switches contractile VSMCs to a "synthetic" phenotype (113). In hypertension condition, initially structural adaptations are observed, which, in acute conditions, might be beneficial. However, in chronic states, these structural changes can lead to vascular remodelling, stiffness, disturbs vascular tone and increases rigidity of vessels, a characteristic feature of chronic hypertension (114). These

structural alterations are strongly linked to elevated blood pressure and serves as an independent predictor of cardiovascular disease (115). Additionally, endothelial damage and stiffening plays a role in reducing nitric oxide availability, subsequently impacting vasorelaxation, potentially initiating the onset sequence of worsening of hypertension condition.

Isolated VSMCs provide a unique window into vascular remodelling, increased vascular resistance, and arterial stiffness—fundamental aspects contributing to elevated blood pressure (98). These vascular changes can be responsible for cardiac hypertrophy, hypertension, fibrosis and atherosclerosis (99). Simultaneously, studying ECs unveils critical factors like endothelial dysfunction, oxidative stress, and inflammation, all of which significantly impact vascular health and blood pressure regulation. These isolated primary cells serve as a bridge between theoretical knowledge and practical understanding, allowing for in-depth investigations into the molecular pathways and cellular responses implicated in hypertensive conditions. Moreover, these cellular models offer a controlled environment for drug testing, facilitating the evaluation of potential therapeutic agents that target either VSMCs or ECs (116). Ultimately, the isolation of primary rat aortic VSMCs and ECs stands as a cornerstone in hypertension research, offering a platform to decipher the complexities of cellular interactions, disease mechanisms, and potential avenues for innovative therapeutic interventions aiming to mitigate the burdens of hypertension-associated complications.

### *1.8 Classical signalling pathways in vascular function*

The signalling pathways that control vascular function and hypertension are complex. It is maintained by three classical mechanisms: calcium signalling pathway, the NO/NO/sGC (nitric oxide-soluble guanylate cyclase)-cGMP pathway, and vascular remodelling. Notably, the calcium and NO-sGC-cGMP signalling pathways are reversible, allowing for modulation, whereas vascular remodelling is defined as a pathological change with limited reversibility (117).

The primary mechanisms governing VSMC contractility are variations in cytosolic calcium concentration ($Ca^{2+}$). In response to vasoconstrictive stimuli, $Ca^{2+}$ is mobilized from intracellular stores or the extracellular space, increasing cytosolic ($Ca^{2+}$) in VSMC (118). This increased ($Ca^{2+}$) interacts with calmodulin (CaM), forming a complex that activates myosin light-chain kinase (MLCK) (119). MLCK phosphorylates myosin light-chain (MLC), which causes contraction. In contrast, myosin light chain phosphatase (MLCP) can dephosphorylate

phosphorylated MLC, resulting in vasodilation (120). Calcium signalling pathways involve the influx of extracellular calcium via voltage-gated $Ca^{2+}$ channels, with L-type and T-type channels serving different functions. The functional regulation of $Ca^{2+}$ channels is influenced by various stimuli and involves phosphorylation processes, which contribute to the intricate control of vascular tone (121).

The NO/sGC/cGMP pathway intricately regulates VSMC contractility, which is critical to the development of hypertension. Nitric oxide (NO) production in endothelial cells initiates this pathway, which is influenced by chemical factors such as L-arginine, nitrate, and nitrite, as well as various stimuli such as fluid shear stress. The main source of blood NO is the eNOS pathway, which is primarily initiated by L-arginine. Knockout of eNOS causes vascular dysfunction and hypertension, emphasizing its critical role (122). Interestingly, recent studies show that eNOS regulates blood pressure in both ECs and red blood cells (RBCs), providing a new perspective on NO sources. NO synthesized in ECs or RBCs diffuses into VSMCs and activates sGC, resulting in cGMP production (123). cGMP modulates cellular functions via cGMP-modulated cation channels and cGMP-dependent protein kinases. Protein kinases, including cGKIα, phosphorylate myosin-binding subunits and regulate calcium signalling, resulting in vasorelaxation (124). This comprehensive understanding reveals the intricate interplay between the NO/sGC/cGMP pathway and calcium signalling, providing insight into potential hypertension therapeutic targets (125).

However, third-factor vascular remodeling causes lumen narrowing, wall thickening, and elasticity loss, similar to arterial stiffness, which is reflected in increased pulse wave velocity (117). Pathological changes in the extracellular matrix (ECM) contribute to hypertension (126). Currently, there is no way to reverse altered vascular remodelling. Targeting these pathways offers a promising approach to managing vascular diseases.

### *1.9 Hypertension therapies and limitations*

Traditional therapies for hypertension aim to lower blood pressure and modulate RAAS pathways as described in Table 1.5 (66). Several classes of medications (127) and lifestyle modifications (128) are used. Unfortunately, first-line therapies come along with serious side effects (63–65,127).

### 1.9.1 Lifestyle modifications

**Dietary Changes:** Adopting a low-sodium diet, rich in fruits, vegetables, and whole grains, can help lower blood pressure. Also encouraging healthy eating habits can reduce hypertension risk.

**Exercise:** Regular physical activity aids in weight management and contributes to better blood pressure control.

**Stress Management**: Techniques like meditation, yoga, or mindfulness can help reduce stress, which can impact blood pressure.

### 1.9.2 Medications

**Diuretics:** Help the kidneys eliminate sodium and water, reducing blood volume. Thiazide diuretics are commonly used but may have side effects like potassium loss or increased blood sugar levels.

**ACE Inhibitors and ARBs:** These medications relax blood vessels, reducing blood pressure. They are often prescribed as first-line treatments but can cause side effects like cough (ACE inhibitors) or hyperkalaemia (ARBs).

**Calcium Channel Blockers:** Relax blood vessels by preventing calcium from entering cells of the heart and blood vessels. They can cause side effects like ankle swelling or constipation.

**Beta-Blockers:** Reduce the workload on the heart by slowing the heart rate. They might cause fatigue or worsen asthma symptoms in some individuals.

**Alpha-Blockers:** Relax certain muscles and help lower blood pressure. Side effects may include dizziness, drowsiness, or weakness.

### 1.9.3 Limitations

**Side Effects:** As discussed above many medications used for hypertension control can cause side effects, impacting patient compliance or necessitating changes in medication (Table 1.5).

**Adherence:** Long-term adherence to medications and lifestyle changes can be challenging for some individuals.

**Individual Variability:** Different people may respond differently to medications, and finding the right medication or combination for each person can be a trial-and-error process.

**Cost and Accessibility:** Availability and affordability of medications, as well as access to healthcare, can influence a person's ability to manage hypertension effectively.

*Table 1. 5: Available therapies for hypertension and its side effects*

| **Type of Hypertension** | **First-Line Treatment** (127) | **Multidrug Regimen** (129) | **Long-Term Side Effects** (63–65) |
|---|---|---|---|
| **Essential Hypertension** | Thiazide Diuretics | Thiazide Diuretics + ACE Inhibitors or Calcium Channel Blockers | Electrolyte imbalance (e.g., hypokalaemia), Persistent dry cough (ACE inhibitors), Dizziness, flushing (calcium channel blockers) |
| **Secondary Hypertension** | Treat the underlying cause, e.g., renal artery stenosis | Combination based on the underlying cause (e.g., beta-blockers, diuretics, ACE inhibitors, calcium channel blockers) | Bradycardia and hypotension (β-blocker) |
| **Resistant Hypertension** | Diuretics, ACE Inhibitors, Calcium Channel Blockers, Beta-Blockers | Triple therapy: Diuretics + ACE Inhibitors + Calcium Channel Blockers or Beta- Blockers | Similar to first line and multidrug regimens with potential cumulative side effects |

## *1.10 Emerging therapies*

Researchers are exploring new drug classes, multivalent therapies based and innovative non-pharmacological interventions such as renal denervation or device-based therapies. Additionally, designed and synthesized peptide analogues of endogenous peptides provide a safe natural peptide-based strategy for hypertension management (Figure 1.5) (130). Effective hypertension management frequently demands a combination of lifestyle changes and medications designed to an individual's unique needs and tolerances. Regular monitoring and communication with healthcare providers are essential for addressing limitations and improving hypertension management.

Previous research focused on novel therapeutic strategies for hypertension that target the protective axis of RAAS (131). The US FDA has approved several agents that are currently undergoing preclinical and clinical trials. These include ACE2/Ang1–7/MasR axis activators, neprilysin inhibitors, sGC stimulators (132), bispecific peptides, antioxidants (133), nonsteroidal mineralocorticoid receptor antagonists, therapeutic vaccines (30) and APA inhibitors (64,65,134). Current research, with a focus on the angiotensin II receptor (AT2R), investigates its function in various organs (135). AT2 receptor agonists such as Compound 21 (C21), β-Tyr4-AngII, β-Ile5-AngII, and LP2-3 are being studied for their potential use in hypertension management, including their impact on heart function and renal damage (65,136).

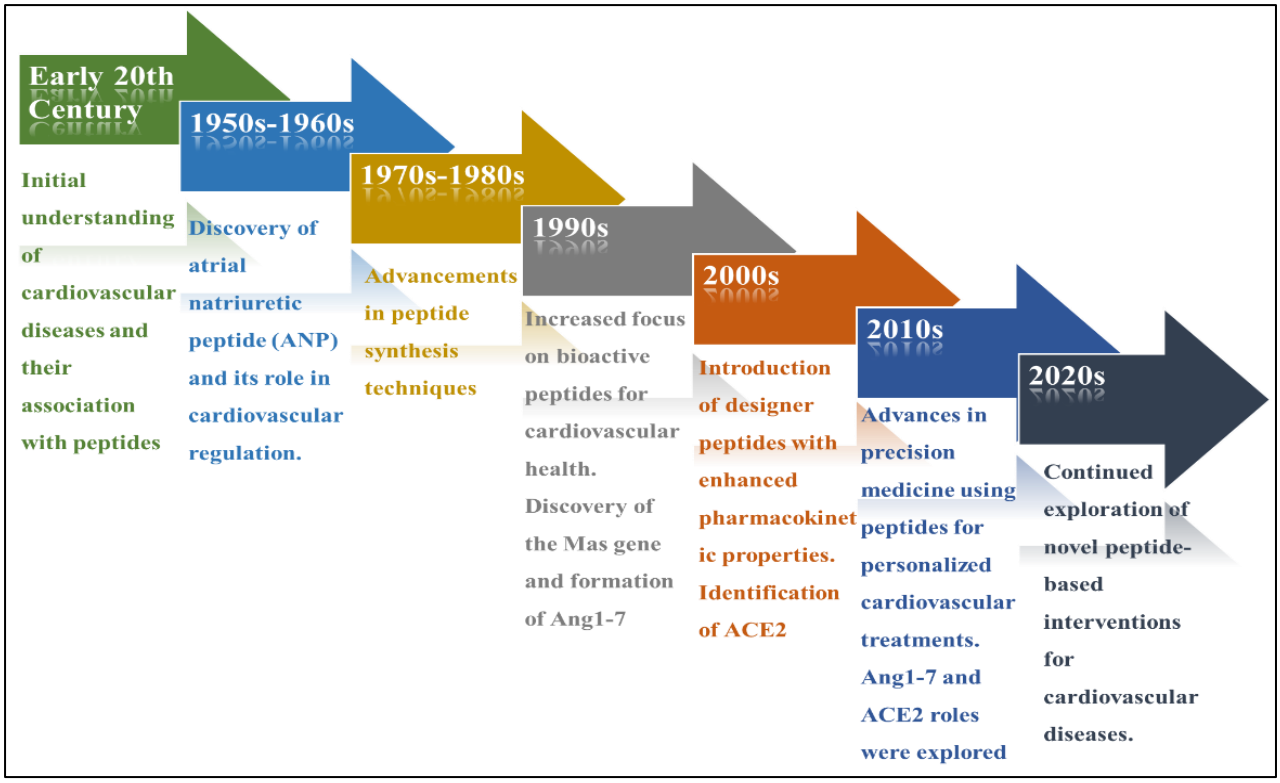



*Figure 1. 7: Timeline of peptide discovery for the management of cardiovascular diseases*
Figure is produced from reference in (137)

As previously discussed, the ACE2/Ang1-7/MasR protective axis has received attention for revealing ACE2's protective role in cardiac and renal function regulation. ACE2 activators such as diminazene aceturate (DIZE) and recombinant human ACE2 (rhACE2) are being studied for their potential applications in cardiovascular disease, diabetic nephropathy, and chronic kidney disease (138). Natriuretic peptides and neprilysin inhibitors have emerged as an alternative therapeutic avenue, demonstrating effects on blood pressure regulation and renal function. In fact, they are capable to providing additional benefits in cardiovascular and renal complication treatment due to their natriuretic and diuretic properties (139). Firibastat and other aminopeptidase inhibitors have been shown to lower blood pressure by modulating brain RAAS (140). Nonsteroidal mineralocorticoid receptor antagonists (finerenone, esaxerenone, and apararenone) are becoming increasingly important due to their efficacy and safety profiles (141,142). Furthermore, animal studies investigate the roles of antioxidant therapy, soluble guanylyl cyclase A stimulators, bispecific peptides, and insulin-regulated aminopeptidase (IRAP) inhibitors in managing hypertension (65). Previous research has revealed a variety of approaches to hypertension treatment, demonstrating the potential of these

novel therapeutic agents in alleviating the complications associated with hypertension and its associated conditions.

### 1.10.1 Mas receptor agonists

As we discussed earlier, ACE2/Ang1-7/Mas axis has attracted the attention. Generation and metabolism of Ang-1-7, the endothelium is an important target for its actions. Extensive evidence supports the endothelium-dependent vasodilatory activity of Ang1-7. This activity results in the release of vasodilator active products such as vasodilatory prostanoids, nitric oxide, and endothelial-derived hyperpolarizing factor (EDHF) (143). The peptide Ang1-7, acting through the MasR, has demonstrated its capacity to suppress inflammation, hypertrophy, fibrosis, and elevated blood pressure in hypertensive animal models (144). Additionally, it exhibits various promising effects in cardiovascular diseases, including anti-hypertensive and anti-arrhythmic properties, as well as the inhibition of cardiac remodelling (145). The non-peptide agonist of the MasR, AVE-0991, mirrors the action of endogenous Ang1-7 by promoting vasodilation and natriuresis in various tissues and offers potential therapeutic benefits in cardio-renal diseases, glucose and lipid metabolism, and other conditions (146). These findings highlight Ang-1-7's diverse and potent protective and vasodilatory actions in cardiovascular health.

### 1.10.2 Natriuretic peptide receptor agonists

Compounds such as carperitide (a synthetic human ANP), vastiras (a recombinant pro ANP), and nesiritide (a human recombinant BNP) have shown promise in heart disease treatment. Vastiras demonstrated positive effects such as vasodilation, natriuresis, and diuresis (147). Perioperative low dose carperitide (hANP) infusion demonstrated renal protective effects in patients with chronic kidney disease who did not require dialysis (148). Despite their therapeutic potential, NPs are rapidly degraded by tissue proteases, necessitating continuous intravenous infusion throughout treatment (149). To address this issue, modifications to native NPs have been made with the goal of improving their stability and overall effectiveness in clinical applications.

Recent studies have focused on improving pGC-A stimulators and developing new pGC-A activators, such as CRRL269 and MANP (ZD100). These agents exhibit vasorelaxation, antihypertensive properties, and significant renal protective functions. Neprilysin (NEP), an enzyme found in a variety of organs, helps to break down vasoactive

peptides such as bradykinin and natriuretic peptides (19,150). Inhibiting neprilysin (NEPi) inhibits peptide degradation, resulting in vasodilation, increased diuretic action, and decreased sympathetic activity (151). The combination of NEPi and RAAS blockers shows promise in treating cardiorenal diseases. Interestingly, angiotensin receptor neprilysin inhibitors (ARNi), such as LCZ696, have cardioprotective effects and improve outcomes in cardiac dysfunction, hypertrophy, fibrosis, and vasculopathy (152).

Animal studies have provided important insights into the role of natriuretic peptides in cardiovascular disease, revealing dysregulation in conditions such as heart failure, hypertension, and various cardiac disorders (153). Exogenous natriuretic peptide administration in animal models has resulted in positive outcomes such as blood pressure reduction, improved heart function, and prevention of cardiovascular disease progression (154). These findings have sparked interest in developing pharmacological interventions that target natriuretic peptides for the treatment and management of cardiovascular conditions in humans. Natriuretic peptide-based therapies offer novel approaches to improving cardiovascular health and managing blood pressure (155). In essence, the heart's endocrine function, specifically natriuretic peptide production, is critical for blood pressure regulation and overall cardiovascular health. Ongoing research and innovative compound development offer promising therapeutic avenues for managing cardiovascular diseases.

### 1.10.3 Dual-activation of receptors

A dual activation of receptors refers to a therapeutic agent or drug that possesses the ability to target two or more biological pathways or receptors simultaneously, typically through a single molecular structure or compound. This innovation gaining attraction in drug development, aiming to improve treatment efficacy and safety by addressing multiple disease mechanisms with a single medication. In addition to this, it can modulate different pathways involved in a disease process, potentially offering enhanced therapeutic effects compared to single-target medications or this combined action might lead to improved efficacy or reduced side effects. In conditions where multiple factors contribute to the disease's progression or symptoms (such as certain cardiovascular diseases or metabolic disorders), a dual activation of receptors could provide a more comprehensive treatment approach (156).

In recent years, this approach has revolutionized disease management across cardiovascular, metabolic, and neurological disorders. An example of validating this strategy is LCZ696, an ARNi. Combining the ARB valsartan with the NEPi prodrug sacubitril, LCZ696

showcased its efficacy without severe angioedema and any other side effects by modulating bradykinin metabolism (157). It outperformed the ARB Enalapril in cardio protection, significantly improving outcomes in cardiac dysfunction while curtailing hypertrophy, fibrosis, and vasculopathy (152). Cenderitide, another ARNi, activates NP receptors pGC-A and pGC-B, demonstrating natriuretic, vasodilatory, and anti-fibrotic effects in models of early cardiac fibrosis (158,159). Additionally, NPA7, a novel dual-acting peptide, activates both pGC-A and MasR receptors. Stimulating pGC-A triggers the cGMP pathway, leading to natriuresis, diuresis, and anti-hypertensive effects. Simultaneously, MasR activation delivers antiapoptotic, anti-inflammatory, and vasodilatory benefits through the cAMP pathway (156). In healthy canines, subcutaneous administration of NPA7 displayed cardiac unloading, diuretic, natriuretic, and RAAS-suppressive actions (160). In heart failure models, NPA7 exhibited superior hemodynamic effects, natriuresis, diuresis, and cardio-Reno protective effects. The synergistic action of bispecific peptide NPA7 with furosemide showed promise in experimental heart failure (161). Moreover, NPA7 demonstrated dose-dependent blood pressure-lowering effects and organ protection in hypertensive models (162).

While these findings are promising, further studies are warranted to fully explore the therapeutic potential of dual-acting bispecific peptides in managing hypertension and preventing target organ damage. Dual-acting peptides represent an innovative approach to drug development, offering the potential for more effective and targeted therapies for complex diseases. Research and development in this field continue to explore ways to optimize these compounds for clinical use, potentially revolutionizing treatment strategies in various medical domains.

## *1.11 Gaps in existing research*

### 1.11.1 Already known facts

Traditional treatments for hypertension have proven ineffective in addressing the associated end-organ damage. Moreover, conventional approaches, such as ACE inhibitors, ARBs, and calcium channel blockers, come with notable side effects (163). This has prompted an urgent exploration of natural multivalent strategies capable of not only reducing blood pressure but also safeguarding vital organs. In the 20$^{th}$ century, the association of peptides with cardiovascular diseases opened avenues for novel therapeutic interventions (137). Peptide research in cardiovascular diseases led to the identification of life-saving endogenous peptides BNP and Ang1-7, along with a comprehensive exploration of their respective molecular

pathways and protective roles (61). Despite these breakthroughs, the clinical application of peptides remains limited, primarily due to ongoing debates about their bioavailability and suitability across diverse clinical scenarios (164). In recent years, departing from the traditional single-pathway researchers have shifted focus towards targeting two or more pathways simultaneously to address this limitation (165). A few years ago, scientists discovered a promising molecule, LCZ969, as a notable example of inhibiting two molecular pathways simultaneously (166). This development has opened possibilities for dual-acting therapeutic strategies with multivalent properties.

### 1.11.2 Gaps

The existing research on hypertension therapies highlights several gaps that warrant further investigation. Firstly, while traditional treatments like ACE inhibitors, ARBs, and calcium channel blockers are commonly used, they are associated with notable side effects, which can impact patient health and necessitate changes in medication. However, there is a issues with the long-term effects of these therapies, especially in terms of their impact on end-organ damage and overall patient outcomes. Additionally, the effectiveness of lifestyle modifications, such as dietary changes, exercise, and stress management, may vary among individuals, and further research is needed to identify safe and natural optimal strategies for implementing and sustaining these lifestyle changes in real-world settings.

Furthermore, there is a need for more personalized approaches to hypertension management, considering individual variability in treatment response. Current research primarily focuses on identifying novel therapeutic targets and developing new drug classes, but there is limited understanding of how these interventions may affect different patient populations. Additionally, the cost and accessibility of medications remain significant barriers to effective hypertension management, particularly in underserved communities.

Moreover, while emerging therapies show promise in targeting various pathways involved in hypertension, such as the ACE2/Ang1-7/MasR axis and natriuretic peptide receptor agonists, there is a lack of robust clinical evidence supporting their efficacy and safety. Preclinical and clinical trials are needed to evaluate the long-term effects and comparative effectiveness of these therapies, as well as their potential to reduce cardiovascular morbidity and mortality.

Lastly, the concept of dual activation of receptors represents an innovative approach to hypertension treatment, but there is limited research exploring the therapeutic potential of bispecific peptides in real-world clinical settings. Further studies are warranted to elucidate the

mechanisms of action, optimal dosing regimens, and potential synergistic effects of these compounds, with the ultimate goal of improving outcomes for patients with hypertension and reducing the burden of cardiovascular disease worldwide.

### 1.11.2 Significance of this study

As previously highlighted, BNP and Ang1-7 have garnered attention in researchers due to their promising effects in cardio protection and vasodilation. However, their clinical use is limited, as discussed earlier. In response to these challenges, scientists have sought to overcome these limitations by structural modification and exploring the simultaneous co-activation of two pathways. Notably, a researcher in our laboratory has successfully engineered a dual-acting peptide with the ability to target Mas and pGCA receptors concurrently. This innovative peptide was then investigated for its therapeutic potential in addressing hypertension, renal damage, and endothelial dysfunction.

The exploration of co-activating Mas and pGCA receptors revealed significant effectiveness in reversing various adverse conditions, including oxidative stress, vascular damage, vascular remodelling, fibrosis, and hypertrophy. Furthermore, in hypertensive rats subjected to DOCA-salt treatment, the dual-acting peptide (DAP) demonstrated the restoration of vascular tone in the aorta. Intriguingly, DAP exhibited promising outcomes in preserving the structural and functional integrity of vital organs, such as the heart and kidneys.

Motivated by these findings, the present study encourages us to delve deeper into the therapeutic role of DAP in the context of hypertension. Additionally, our hypothesis suggests that the dual activation of Mas and pGCA receptors could represent a potential therapeutic strategy for treating hypertension and associated cardiovascular diseases, offering the vision of efficacy without introducing undesirable side effects.

### 1.11.3 Clinical significance

In recent years, the objective of developing hypertensive therapies has extended beyond blood pressure reduction, aiming to incorporate multivalent effects against associated complications. Traditional treatments, while addressing blood pressure concerns, often come with significant long-term side effects. The goal of our hypothesis suggests that achieving dual activation of receptors may present a synergistic and promising strategy for the treatment of hypertension. The dual-acting peptide, being a natural peptide-based therapeutic approach,

holds potential in concerned with the safety. However, a more in-depth exploration of the molecular and therapeutic pathways of dual activation is necessary.

Moreover, there is a critical need to unravel the specific effects of the simultaneous activation of Mas and pGCA receptors in various cardiac and renal conditions. The ever-increasing prevalence of hypertension underscores the urgency for the development of therapeutic interventions that are not only effective but also safe for long-term use. This underscores the importance of our ongoing investigation into the challenges and therapeutic efficacy of dual activation, offering a potential breakthrough in addressing the growing challenges associated with hypertension.

# *Chapter 2*

# *Rationale*

## *2.1 Rationale*

The rationale for this study is based on the urgent need to address the global health challenges posed by hypertension, a common condition characterized by elevated blood pressure that is intricately linked to complications in the cardiovascular and renal systems. The alarming prevalence of hypertension among over a billion adults worldwide (31), combined with a large proportion of people being unaware of their hypertensive status, emphasizes the critical need to advance awareness and antihypertensive treatments that go beyond simply managing blood pressure (167). Hypertension, with its intricate complexities and potential to cause vital organ damage, necessitates a paradigm shift towards multivalent approaches (165). While existing conventional therapeutic options aim to treat high blood pressure, they are limited in efficacy and have the potential to cause unwanted side effects (65). To address these shortcomings, there is an urgent need to investigate and develop novel strategies with protective effects against multifaceted nature of hypertension.

Furthermore, traditional therapies primarily focused on single signalling pathways, which has frequently proven ineffective in controlling end-organ damage and unintentionally inducing off-target side effects (43). Inspired by recent breakthroughs in dual-inhibition therapies, such as LCZ696 sacubitril/valsartan, which target multiple pathways at the same time (168), our study seeks to investigate the groundbreaking potential of a dual-acting peptide. This peptide, which are specifically designed by fusing amino acid sequences of endogenous peptides of Mas and pGCA receptors i.e. Ang1-7 and BNP respectively.

Mas and pGC-A receptors were chosen as targets for this study based on their complementary roles in regulating cardiovascular and renal functions. Mas receptors, part of the RAAS, are activated by ang1-7, a peptide known for its vasodilatory, anti-inflammatory, and anti-fibrotic effects. Activation of Mas receptors counteracts the detrimental actions of angiotensin II, promoting vasodilation, reducing oxidative stress, and preventing fibrosis, all of which are critical in managing hypertension and protecting end-organ function.

On the other hand, pGC-A receptors are activated by natriuretic peptides such as BNP. Activation of these receptors leads to vasodilation, natriuresis, and diuresis, contributing to the reduction of blood pressure and blood volume. Additionally, BNP has anti-fibrotic and anti-proliferative effects, further supporting cardiovascular and renal health.

The rationale for targeting both Mas and pGC-A receptors lies in their ability to provide a synergistic approach to managing hypertension. By concurrently activating these pathways, we aim to achieve a more robust reduction in blood pressure and enhance protection against

inflammation, fibrosis, and oxidative stress. This dual-targeting strategy is expected to offer superior therapeutic benefits compared to monotherapies, addressing the multifaceted nature of hypertension and its associated renal dysfunction.

Recognizing the limitations of current therapies and the complex nature of hypertension with its associated comorbidities, our study takes a comprehensive approach that includes both *in-vitro* and *in-vivo* methodologies. *In-vitro* studies aim to validate the protective effects of dual activation of Mas and pGCA receptors against oxidative stress as it is a main mediator in most cardiovascular and renal complications. Additionally, we have also evaluated the therapeutic efficacy of dual activation of Mas and pGCA receptors against oxidative stress induced vascular remodeling, phenotypic switch and vascular damage in primary aortic cells.

In contrast, *in-vivo* studies use a DOCA-salt animal model that closely resembles human low-renin hypertension to assess the efficacy of dual activation of Mas and pGCA receptors in treating impaired vascular tone, endothelial dysfunction, elevated blood pressure, inflammation, and cardiorenal and vascular damage. This study focuses on endothelial dysfunction and sodium retention, a critical factor in hypertension. The investigation aims to illuminate the impact of dual activation of Mas and pGCA receptors on endothelial dysfunction markers through chronic administration, contributing to a better understanding of its therapeutic potential. Additionally, this study delves into the impact of dual activation of Mas and pGCA receptors on sodium channels and sodium excretion.

In essence, the study aims to close existing gaps in hypertension management by investigating a novel therapeutic approach that addresses all aspects of the condition, from the molecular and cellular levels to complex interactions in living organisms. Through this comprehensive approach, the goal goes beyond simply controlling blood pressure to reducing the risk of associated complications, resulting in a more advanced and safer antihypertensive treatment strategy.

## *2.2 Hypothesis*

Considering previous research findings, growing emphasis on investigating natural peptide-based approaches to hypertension management. Peptides' inherent properties like biocompatibility and suitability make them promising therapy. Furthermore, the evolving concept of co-targeting two pathways has gained attention in the cardiovascular research community. This novel approach will help to address complex nature of various physiological

pathways and seeks to apply this knowledge to more comprehensive and synergistic therapeutic interventions in hypertension management.

We hypothesize that the dual activation of Mas and pGCA receptors plays a crucial role in maintaining blood pressure, vascular tone and protects kidney and aorta in in-vitro and in-vivo hypertension models

Also, based on all gaps and literature survey,

- We envisage that dual activation of Mas and pGCA receptors protects renal and aortic primary cells against oxidative stress and may provide superior effects to its endogenous peptides Ang1-7 and BNP
- We hypothesize that dual activation of Mas and pGCA receptors protects vital organs like kidneys, heart and blood vessels from DOCA-salt-induced complications
- Also, we hypothesize that dual activation of Mas and pGCA receptors reverses endothelial dysfunction and vascular remodeling in DOCA-salt induced hypertensive rats

### *2.3 Objectives and experimental approach*

The main objectives of our study are as follows:

- Determine the Efficacy of Dual Activation of Mas and pGCA Receptors *In-Vitro* in Vascular Smooth Muscle Cells, Endothelial cells and Renal Tubular Cells Against Oxidative Stress
- Determine the Cardiorenal Protective Properties of Dual Activation of Mas and pGCA Receptors in An Animal Model of Hypertension and Associated Kidney Disease
- To Investigate the Effect of Dual Activation of Mas and pGCA Receptors on Markers of Endothelial Dysfunction in Hypertension

# *Chapter 3*

# *Material and Methods*

## *3.1 Chemicals*

All reagents were used of molecular grade.

*Table 3. 1: Chemicals used during study*

| Sr. NO. | Chemical Name | Abbreviation | Make | CatLog Number |
|---|---|---|---|---|
| **1.** | Hydrogen Peroxide solution, 30 % | $H_2O_2$ | Thermo Fisher Scientific, USA | Q18755 |
| **2.** | Dulbecco′s Modified Eagle′s Medium | DMEM | Himedia, India | AL219A |
| **3.** | Collagenase type II | ColII | Himedia, India | TC212 |
| **4.** | Elastase | Elastase | Himedia, India | TC311 |
| **5.** | Bovine serum albumin | BSA | Himedia, India | TC348 |
| **6.** | Trypsin | Tryp | Himedia, India | TCL007 |
| **7.** | Antibiotic | Ab | Himedia, India | A002 |
| **8.** | Collagen peptide | Col | Himedia, India | TC3431 |
| **9.** | 3-(4,5-DIMETHYLTHIAZOL-2-YL)-2,5-DIPHENYLTETRAZOLIUM BROMIDE) | MTT | Himedia, India | TC191 |
| **10.** | Fetal bovine serum | FBS | Gibco, United States | 10270−106 |
| **11.** | 2′,7′-dichlorofluorescin diacetate | CM-H2DCFDA | Sigma-Aldrich, United States | D6883 |
| **12.** | Alizarin red staining | ARS | Sigma-Aldrich, United States | A5533 |
| **13.** | N-acetyl cysteine | NAC | Sigma-Aldrich, United States | A9165 |
| **14.** | Cotton seed oil | Cotton seed oil | Sigma-Aldrich, United States | C7767 |
| **15.** | Acetylcholine chloride and | Ach | Sigma-Aldrich, United States | A6625 |
| **16.** | (R)-(−)-Phenylephrine hydrochloride | PhE | Sigma-Aldrich, United States | P6126 |
| **17.** | Nω-Nitro-L-arginine methyl ester hydrochloride | L-NAME | Sigma-Aldrich, United States | N5751 |
| **18.** | 4′,6-diamidino-2-phenylindole | DAPI | Sigma-Aldrich, United States | |
| **19.** | A-779 trifluoroacetate salt | A779 | Sigma-Aldrich, United States | SML1370 |
| **20.** | Aldosterone | ALDO | Sigma-Aldrich, United States | A9477 |
| **21.** | Trypsin inhibitor | TrypI | Sisco Research Laboratories, India | 42657 |
| **22.** | PrimeScript RT reagent | - | Takara Bio USA | RR037A |
| **23.** | TB Green Premix Ex TaqII | - | Takara Bio USA | RR820A |
| **24.** | RNAiso plus | - | Takara Bio USA | 9109 |

| | | | | |
|---|---|---|---|---|
| **25.** | TACS Annexin V-FITC Apoptosis Detection Kit | - | R&D Systems | 4830-250-K |
| **26.** | Selective protein kinase G inhibitor | KT5823 | R&D Systems | 1289 |
| **27.** | Flura-2 AM | - | Invitrogen, United States | F1201 |
| **28.** | Deoxycorticosterone Acetate | DOCA salt | Tokyo Chemical Industry, India | D0047 |
| **29.** | Losartan | Lst | Tokyo Chemical Industry, India | L0232 |
| **30.** | Endothelial Cell Growth Medium-2 BulletKitTM | EGM2 | Lonza | CC-3162 |
| **31.** | Diaminofluorescein-FM diacetate | DAF-FM DA | Medchem Express, United States | HY-D0717 |
| **32.** | Sodium nitroprusside | SNP | SDFCL, India | 40190UR |
| **33.** | Bicinchoninic acid | BCA | Bio-Rad Laboratories India | |

## *3.2 Primer sequence used for mRNA expression in qPCR study*

*Table 3. 2: Primer Sequence Used For mRNA Expression in qPCR Study*

| Sr. No. | Gene | Symbol | Sequences 5' to 3' of forward and reverse primer pair |
|---|---|---|---|
| **1.** | Mas receptor | MasR | GGGCGGTCATCATCTTCATAG |
| | | | CCCATGTGTTCTTCCGTATCTT |
| **2.** | Particulate guanylyl cyclase A receptor | pGCAR | GGTGTGGTAGGGCTAAAGATG |
| | | | CTTGAGGGCTTCTCCATTAGAC |
| **3.** | Angiotensin II type 1 receptor | AT1R | TGTCATGATCCCTACCCTCTAC |
| | | | GCCACAGTCTTCAGCTTCAT |
| **4.** | Renin | Renin | CTATGACTCCTCGGAATCCTCTA |
| | | | CACCCACAGTTACCACATCTT |
| **4.** | Atrial natriuretic hormone | ANP | TCCGATAGATCTGCCCTCTT |
| | | | CTCCAATCCTGTCAATCCTACC |
| **5.** | Alpha-Smooth muscle actin | αSMA | AGGGAGTGATGGTTGGAATG |
| | | | GGTGATGATGCCGTGTTCTA |
| **6.** | Transforming growth factor-β | TGF-β | CTTTAGGAAGGACCTGGGTTG |
| | | | GTGTCCAGGCTCCAAATGTA |
| **7.** | Tumour Necrosis Factor alpha | TNF-α | GGAGAAGTTAGAGTCACAGAAGG |
| | | | CACTAGGTTTGCCGAGTAGAC |
| **8.** | Interleukin 6 | IL-6 | GCTTTCGGAACTCACTGGAT |
| | | | AGACATCTTCAGCAGCCTTG |
| **9.** | Nuclear factor kappa-light-chain-enhancer of activated B cells | NF-kβ | GGCTTCCTTTCTTGGCTCT |
| | | | AAGCTCAAGCCACCATACC |
| **10.** | Endothelial nitric oxide synthase | Caspase-3 | TGGAAAGCATCCAGCAATAGG |
| | | | GACTCAGCACCTCCATGATTAAG |
| **11.** | Protein kinase G | PKG | GGGAAGGTCGAAGTCACAAA |
| | | | CTGTCCGGGTACAGTTGTAAAG |

| | | | |
|---|---|---|---|
| **12.** | Soluble Guanylate Cyclase | sGC | AGGAGAAGAAGAAGACGGTAGA |
| | | | AGAGCATGGTGACCTCATTG |
| **13.** | Myosin Light-Chain Phosphatase | MLCP | TCCTCAGTCAGCCTATCCTT |
| | | | GAGGAAAGCCACCGTATTCA |
| **14.** | Inducible nitric oxide synthase | iNOS | TGGAGCGAGTTGTGGATTG |
| | | | GGGAAGCCTCTTGTCTTTGA |
| **15.** | Angiotensin II type 2 receptor | AT2 | TGGCTTGTCTGTCCTCAT |
| | | | AGACTTGGTCACGGGTAA |
| **16.** | Angiotensin-converting enzyme 2 | ACE2 | GACCAAAGCATTAAAGTGAGGATAAG |
| | | | AGGCAACAGATGATCGGAATAG |
| **17.** | Angiotensin-converting enzyme | ACE | TGGAGGGTCTTTGACGGAAG |
| | | | TCGTGGAACTGGAACTGGATG |
| **18.** | NADPH oxidase 4 | NOX | GTACAACCAAGGGCCAGAATA |
| | | | CAGTTGAGGTTCAGGACAGATG |
| **29.** | Endothelin 1 | ET1 | ACCAAGGGAACAGATGCCAG |
| | | | GGTACTTTGGGCTCGGAGTT |
| **20.** | Thromboxane A2 | TA2 | GGCTCATGTTTGCTCTCCT |
| | | | GAACCATCATCTCCACCTCAC |
| **21.** | Prostacyclin | PGI2 | CTCCTCCTCTTCCTCCTCAA |
| | | | .GGAGTGCGGTCATCTGTAAA |
| **22.** | $P2Y_{12}R$: Purinergic receptors for adenosine diphosphate | $P2Y_{12}R$ | CCATTGACCGATACCTGAAGAC |
| | | | CCCAGATGGCAACAGAAAGA |
| **23.** | Sodium Chloride Cotransporter | NCC | TCACCCTCCTCATCCCTTATC |
| | | | GCCTTTCTCTCTTCATCCATCC |
| **24.** | Epithelial sodium channels | ENaC | CGTCTCTGTCTCCATCAAAGTC |
| | | | CAAGTCAGTCAGAAGGTCACTC |
| **25.** | Mineralocorticoid receptor | MR | CCTTCCAACAACACCAACAATAG |
| | | | CCTGAAGTGGCATAGCTGAA |
| **26.** | $Na^+$-$H^+$ exchanger-1 | NHE-1 | GCCGTCTCAACTGTCTCTATG |
| | | | ATCTCCTCCTCCTTGTCCTT |
| **27.** | Sodium-Potassium-Adenosine triphosphatase | $Na^+K^+$ATPase | TCCCTACAGTCTCCTCATCTTC |
| | | | TCAGTAGTACGTCTCCTTCTCC |
| **28.** | Kidney Injury Molecule-1 | KIM1 | GCCATTTCCACTCCACTTCT |
| | | | CCTGCTCTCTCTCCTTTCTTTC |
| **29.** | Neutrophil gelatinase-associated lipocalin | NGAL | CCCTCAGATACAGAGCTACGA |
| | | | CTTCCGTACAGGGTGACTTTG |
| **30.** | 18S ribosomal RNA | 18s | CACGGACAGGATTGACAGATT |
| | | | GCCAGAGTCTCGTTCGTTATC |

### *3.3 Antibodies*

*Table 3. 3: Antibodies used during study*

| Sr. No. | Antibody | Abbreviation | Make | CatLog no. |
|---|---|---|---|---|
| **1.** | Renin Antibody | Renin | | sc-133145 |
| **2.** | α-Actin Antibody | α-SMA | | sc-32251 |

| | | | | |
|---|---|---|---|---|
| **3.** | Endothelial nitric oxide synthase antibody | eNOS | Santa Cruz Biotechnology, U.S.A. | sc-376751 |
| **4.** | cGKIα Antibody | PKG | | sc-393987 |
| **5.** | c-Jun N-terminal kinases 1 antibody | JNK | | sc-137018 |
| **6.** | p-JNK Antibody | p-JNK | | sc-6254 |
| **7.** | Angiotensin Receptor/AT1/AGTR 1 Antibody | AT1R | | sc-515884 |
| **8.** | MAS1 Antibody | Mas | | sc-390453 |
| **9.** | NPR-A Antibody | pGCA | | sc-137041 |
| **10.** | NF-κB Antibody | NF-kB | | |
| **11.** | Antinitrotyrosine | | | |
| **12.** | Vascular Endothelial Growth Factor A Rabbit pAb | VEGF | Abclonal, US | A12303 |
| **13.** | COL1A Antibody (COL-1) | | Santa Cruz Biotechnology, U.S.A. | sc-59772 |
| **14.** | β-actin antibody | β-actin | | sc-47778 |
| **15.** | Mouse anti-rabbit IgG-HRP | | | sc-2357 |
| **16.** | Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488 | | Invitrogen, USA. | A-11001 |

### *3.4 Elisa kit*

*Table 3. 4: Elisa kits used during study*

| Sr. No. | Elisa kit | Abbreviation | Make | CatLog No. |
|---|---|---|---|---|
| **1.** | Cyclic adenosine monophosphate Elisa Kit | cAMP | Elabsceince, USA | E-EL-0056 |
| **2.** | Cyclic guanosine monophosphate | cGMP | Elabsceince, USA | E-EL-0083 |
| **3.** | Rat Tumor Necrosis Factor Alpha ELISA Kit | TNF-α | Elabsceince, USA | E-EL-R2856 |

### *3.5 Equipements and softwares used*

*Table 3. 5: Equipements and sofwares used*

| Sr. No. | Equipemet | Make |
|---|---|---|
| 1. | Multimode reader | Spectramax M4, Molecular Devices, LLC |
| 2. | Microscope | ZEISS AXIOLAB 5 |

| | | |
|---|---|---|
| 3. | Confocal laser-scanning microscope | Leica DMi8, Leica Microsystems, Germany |
| 4. | Flow cytometer | BD FACSAria III |
| 5. | Tail-cuff method BP measurement system | MRBP system, IITC Life Science, Woodland Hills, CA |
| 6. | Centrifuge | Eppendrof US |
| 7. | $CO_2$ incubator | Thermo Fisher US |
| 8. | Prism 8 Software | GraphPad |
| 9. | Image J | National Institute of Health USA |
| 10. | Powerlab | AD Instruments |

### *3.6 Peptides*

Ang1-7, BNP and DAP peptides were purchased from S Biochem (Kerala, India) and Dr. Kalyaneswar Mandal's lab (TIFR, Hyderabad, India). The process of synthesis and purity data is explained in the annexure. All peptides and losartan are dissolved in a sterile phosphate-buffered saline (PBS). Stocks were stored at -20 $^{O}$C.

### *3.7 Peptide synthesizing approach*

The peptide segments Ang (1-7), BNP and DAP were synthesized using an automated peptide synthesizer (Tribute-UV/IR from Protein Technologies, USA). Fmoc solid phase peptide synthesis (SPPS) was carried out in 0.1 mmol 2-Cl-Trt-resin using amino acids (5 equiv., 0.25 M), DIC (0.25 M) as a coupling reagent and Oxyma (0.25 M) with DIEA (0.025 M) as additives. Cysteine residues were coupled for 15 min at room temperature followed by 15 min at 50 $^{O}$C. All other amino acid couplings were performed for 15 min at 50 $^{O}$C under N2 atmosphere with vortex mixing. After the substitution with first residue on the 2-Cl-Trt-resin, the unreacted functional group was capped using 5 % MeOH (vol/vol) in DMF. Fmoc deprotection after every coupling cycle was carried out by 20 % piperidine treatment at 50 °C. After synthesis, the peptides were cleaved from the resin using TFA (85 %), Phenol (5 %), TIPS (2.5 %), Water (5 %) and DODT (2.5 %) as a cleavage cocktail. After cleavage, the TFA was evaporated under N2 flow inside a well-ventilated fume hood. The cleaved peptide was precipitated and washed with diethyl ether. After lyophilization, dry crude peptide was dissolved in water followed by the filtration with 0.2 µm filter and loaded in preparative HPLC column for purification. Purified peptides were incubated at RT with 0.1 M Tris buffer for

disulfide formation under air oxidation having peptide concentration 1mg/ml and 0.5 M Gu. HCl at pH 8.5 followed by the HPLC purification.

### *3.8 Cell lines used*

*Table 3. 6: Cell lines used in study*

| Sr. No. | Cell line | Procured/isolated from | Media used |
|---|---|---|---|
| **1.** | NRK-52E cell line | National Centre for Cell Sciences (NCCS), Pune, India | DMEM + 10 % FBS + 1 % Antibiotic |
| **2.** | Primary VSMC cells | Rat thoracic aorta | DMEM + 20 % heat inactivated FBS + 1 % Antibiotic |
| **3.** | Primary endothelial cells | Rat thoracic aorta | EGM™-2 Endothelial Cell Growth Medium-2 + Growth factors + 20 % heat-inactivated FBS + 1 % Antibiotic |

All cells were cultured in their respective growth media in a 5 % $CO_2$ incubator at 37 $^{O}$C.

### *3.9 Experimental animals*

The animals utilized in the experimental research were approved by the Institutional Animal Ethics Committee (IAEC) under protocol number BITS-Hyd-IAEC-2022-08. Vyas Labs, a certified breeder accredited by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), provided thirty male Wistar rats weighing between 180 and 220 grams. The rats were housed in a controlled animal facility under barrier-sustained conditions, with a 12-hour light/dark cycle under standard environmental conditions (temperature: 23 $^{o}$C, relative humidity: 40 % to 80 %).

The rats were housed in autoclaved corn hub bedding from Hetron Biosciences and fed a commercially available rat pellet diet ad libitum (VRK Nutritional Solutions, India). Before being included in the study, the rats were acclimatized in the animal facility for at least one week.

After completion of the experimental timeline, humane euthanasia was performed on all using cervical dislocation conducted under xylazine–ketamine anaesthesia (60 mg/6 mg/kg i.p). The depth of anaesthesia was confirmed by the absence of a toe pinch response.

### *3.10 Isolation and characterization of primary rat aortic smooth muscle cells*

Rat VSMCs was isolated from the thoracic aorta utilizing an enzymatic digestion method (98). The thoracic aorta was carefully excised, and excess connective tissue was removed. Subsequently, the aortic tissue was minced into small pieces to facilitate enzymatic penetration. For the enzymatic digestion, a specialized enzyme solution was prepared by dissolving collagenase, elastase, and soybean trypsin inhibitor in Hanks balanced salt solution (HBSS). This enzyme solution was freshly prepared for each isolation procedure to ensure maximum enzymatic activity. The thorough mixing of these enzymes in the HBSS created an effective solution for breaking down the extracellular matrix and dissociating the cells.

The aortic tissue fragments were then incubated in the enzyme solution, allowing for enzymatic digestion to take place. The incubation period of 4 hours was decided to balance effective cell dissociation while minimizing damage to the isolated cells. Gentle agitation or rocking was employed every 1 hour to enhance the even distribution of the enzyme solution. Following the digestion process, the enzymatically dissociated cells were collected and centrifuged to separate them from the remaining undigested tissue fragments and enzyme solution. The resulting cell pellet was resuspended in an appropriate growth medium to support the survival and proliferation of the isolated smooth muscle cells.

The growth medium employed in this study consisted of DMEM supplemented with 20 % heat inactivated FBS. This medium provided the necessary nutrients and growth factors to sustain the viability and growth of the isolated cells. The cells were then cultured in a controlled environment, maintained at 37 $^{O}$C with 5 % $CO_2$, to mimic physiological conditions conducive to VSMC growth. For further experimentation, confluent VSMCs were subculture by splitting them among 6 well plates. Cells within passages 3–8 were utilized for subsequent experiments, ensuring the preservation of cell characteristics and minimizing potential alterations due to extensive passaging. The identification of VSMCs was confirmed through morphological assessment and immunocytochemical staining, employing a smooth muscle-specific α-actin antibody (α-SMA) (169). This meticulous isolation methodology provided a foundation for obtaining pure and viable rat aortic smooth muscle cells for subsequent analyses and investigations.

### *3.11 Isolation of primary rat aortic endothelial cells*

Rat aortic ECs were isolated using a carefully designed methodology to ensure the purity and viability of the endothelial cell population. The thoracic aorta was excised from rats, and careful

dissection was performed to remove surrounding connective tissue. The isolated aortic segments were then briefly washed in a sterile phosphate-buffered saline (PBS) solution to remove any residual blood.

For enzymatic digestion, the aortic segments were incubated in a specialized enzyme solution (170,171). The enzyme solution was prepared by dissolving collagenase in HBSS. This enzyme solution was freshly prepared for each isolation procedure to maintain its enzymatic activity. The digestion process was carried out for 45 min and closely monitored to balance efficient cell dissociation with the preservation of endothelial cell integrity. Following enzymatic digestion, the aortic segments were mechanically disrupted to release the cells. The cell suspension was collected, and the enzymatic activity was neutralized by adding an appropriate volume of culture medium containing EGM2 supplemented with 20 % heat-inactivated FBS and endothelial growth factors (0.2 µg/ml hydrocortisone, 0.5 ng/ml VEGF, 10 ng/ml of hFGF-b, 1 µg/ml of ascorbic acid, 5 ng/ml hEGF) (172).

The cell suspension was then centrifuged to separate endothelial cells from undigested tissue fragments and other cell types. The resulting cell pellet was resuspended in a fresh culture medium. The isolated ECs were cultured in a humidified incubator at 37 $^{O}$C with 5 % $CO_2$. The culture medium was changed after the initial seeding to remove non-adherent cells and debris, and subsequent medium changes were performed regularly to provide optimal growth conditions for the endothelial cells.

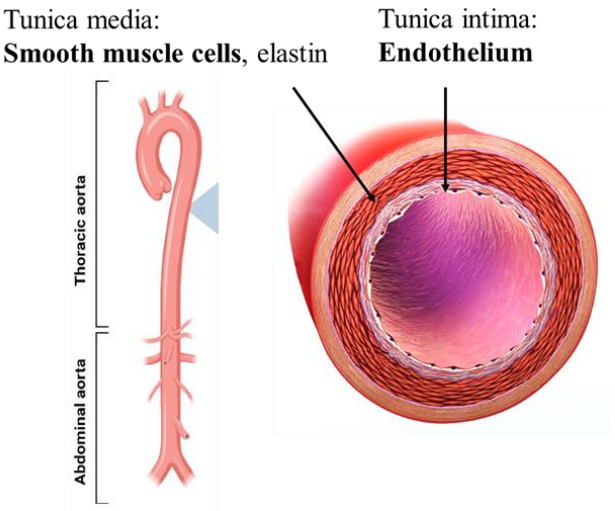



*Figure 3. 1: Location of VSMC and EC in aorta wall*

Figure is produced from reference in (111)

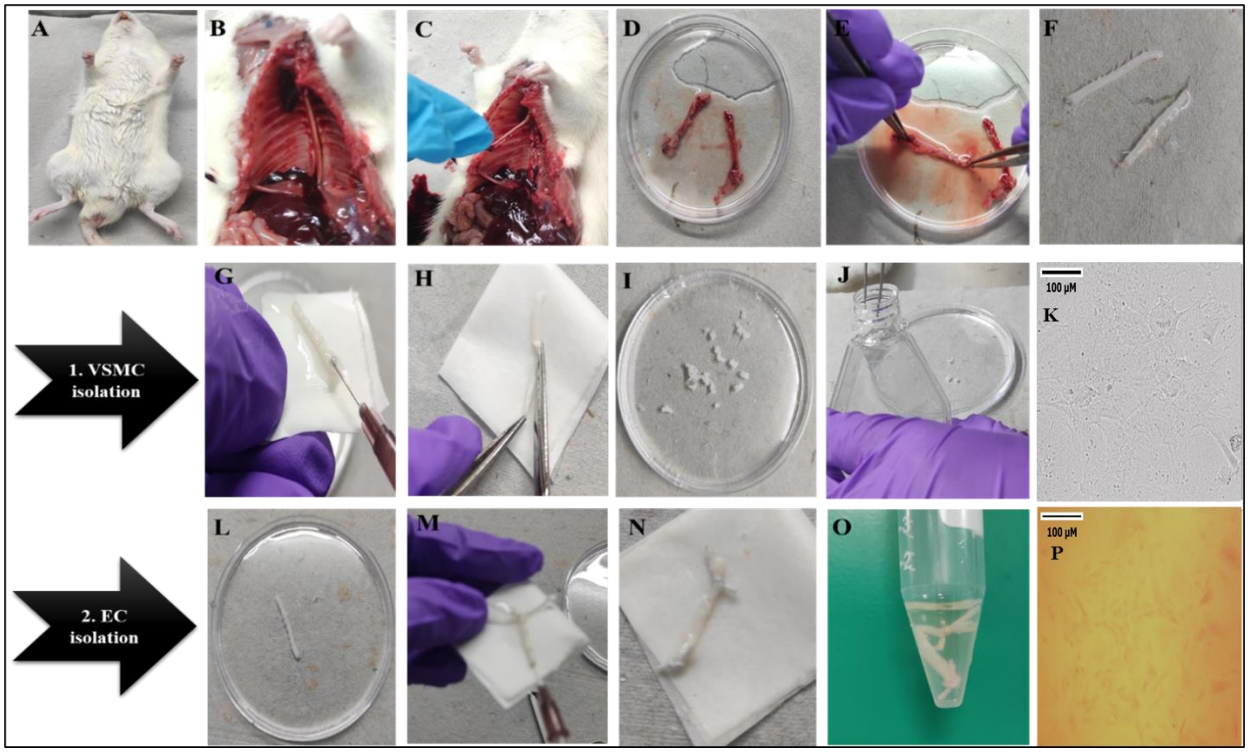



*Figure 3. 2: Isolation of rat aortic primary VSMC and EC*

Steps A to F are common for both VSMC and EC isolation. (A) Wistar rats were anesthetized and sacrificed. Then placed in a supine position. (B) The thoracic cavity was opened, and the rat aorta was carefully isolated. (C) The isolated aorta was transferred to ice-cold HBSS. (D-F) Surgical removal of blood stains and fat tissues was performed. (G) The aorta, prepared up to step F, was placed on Whatman filter paper and flushed with HBSS. (H) Longitudinal cutting of the aorta was done. (I) The aorta was further cut into small pieces. (J) These pieces were transferred into a T25 flask. After 4 hours of incubation, the aorta pieces were seeded into 48-well plates. (K) Brightfield images of VSMC after 1 week of incubation were captured. (L) For EC isolation, the aorta prepared up to step F was utilized. (M) One end of the aorta was tied with sterile thread, and a collagenase solution was injected inside. (N) The other end of the aorta was tied with sterile thread. (O) The aorta was placed in an enzymatic solution and incubated at 37 °C. After 45 min of incubation, EC were seeded into 48-well plates. (P) Brightfield images of EC after 1 week of seeding were obtained.

Endothelial cell identity was confirmed through immunocytochemical staining with specific endothelial markers, such as endothelial nitric oxide synthase (eNOS) (116). This thorough methodology aimed to ensure the isolation of a highly pure and viable population of rat aortic endothelial cells, providing a robust foundation for subsequent experimental investigations in the field of vascular biology.

### *3.12 Oxidative stress model for hypertension in in-vitro cells*

To simulate hypertension in an *in-vitro* setting, an oxidative stress model was implemented using $H_2O_2$. Cells were exposed to varying concentrations of hydrogen peroxide (100 μM) at different time intervals, as documented in previous studies (173,174). The impact on cell viability was assessed through MTT assays conducted at 1, 3, 6, 12, and 24 hours. The results indicated a gradual decrease in VSMC viability with prolonged exposure to $H_2O_2$. Notably, after 24 hours, a significant reduction was observed, with only 85 % of cell are viable under the influence of 100 μM $H_2O_2$.

### *3.13 Hypertension model using aldosterone in endothelial cells*

For the establishment of a hypertension model, primary ECs were cultivated in collagen-coated 12-well plates. Upon reaching confluency, the cells underwent an overnight fast before being subjected to aldosterone (ALDO) at a concentration of 1 nM for a duration of 24 hours, as described in a study (175). This model allowed for the investigation of the effects of aldosterone on endothelial cell behaviour under conditions simulating hypertension.

### *3.14 Peptide treatment in cells*

Cells were then treated with or without peptides at a concentration of Ang (1-7) (1 μM), BNP (1 μM), DAP (1 μM), Lst (1 μM) for 24 hr (99,176). After treatment, cells were isolated for qPCR, western blot analysis, immunocytochemistry and flow cytometry.

### *3.15 MTT assay*

NRK52E and VSMC were grown in complete media at a concentration of $1 \times 10^6$ cells in each well of a 96-well plate. After 24 hr, these cells were fasted with 1 % FBS overnight and treated with $H_2O_2$ (100 μM) for different time points in incomplete media. The second plate was treated with alone peptides 1 μM Ang1-7, BNP and DAP for different time points (156). After the treatment schedule, the supernatant was removed, and cells were incubated with 100 μL of MTT (0.5 mg/mL) solution in PBS for 4 hr. The resultant formazan crystals were solubilized in DMSO, and absorbance was read at 570 nm against blank with a multimode reader (Spectramax M4, Molecular Devices, LLC).

### *3.16 Crystal violet staining*

Cells were cultured on the sterile coverslip. After confluency, hydrogen peroxide and peptide-treated cells were fixed with ice-cold methanol for 10 min and stained with 0.2 % (w/v) crystal violet solution (in 20−25 % methanol) for 10 min. Further, cells were washed with sterile PBS. Images were captured using a ZEISS AXIOLAB 5 Microscope.

### *3.17 Griess reagent assay*

The accumulation of nitrite in the supernatant, an indicator of nitric oxide production, was determined by Griess reagent colorimetric assay. Cells were seeded in a 96-well plate till confluency. After $H_2O_2$ and peptide treatment 50 µl supernatant were collected. Further, we added 50 µl of Griess reagent. After 10 mins of incubation, absorbance was read at 342 nm against blank with a spetramax multimode reader. The nitrite concentration in the supernatant was determined from the sodium nitrite standard curve.

### *3.18 Annexin V/fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining*

Annexin V and PI double staining was carried out to differentiate the normal cells from apoptotic cells. Cultured NRK52E cells were incubated with $H_2O_2$ with or without peptide. After treatment, cells were trypsinized and harvested under cool conditions. The pellet was resuspended in 95 µL of annexin binding buffer. 5 µL of FITC-annexin V and 10 µL of the 100 µg/mL PI working solution were added to each 95 µL of cell suspension and incubated them at RT for 30 min. The final sample mixtures were immediately transferred onto the ice. Apoptotic cell percentage was analysed by using BD imaging flow cytometer (BD FACSAria III). For gating strategy refer Annexure I.II.VI.

### *3.19 Measurement of reactive oxygen species*

Flow cytometry analysis was used to determine the accumulation of intracellular peroxynitrite, hydrogen peroxide and free radicals. VSMC cells were incubated with 2,7'-dicholorofluorescein acetate (CM-H2DCFDA) (5 µmol/L) and kept in the incubator for 30 min maintained at 37 °C. The cells were trypsinized, washed twice with PBS, and resuspended in PBS. The fluorescent intensity of DCF was measured using by using a flow cytometer (BD FACSAria™ III). For gating strategy refer Annexure I.II.IV.

### *3.20 Quantification of nitric oxide*

Primary EC cells were seeded in each 35 mm confocal dish and cultured till 70–80 % confluency. Following the treatment, the cells were washed 3 times with HBSS, stained with DAF-FM (10 µM, 30 min), and washed with 1x PBS. Subsequently, cells were observed by confocal laser-scanning microscopy (Leica DMi8, Leica Microsystems, Germany) at an excitation of 488 nm and emission of 525 nm. To reconfirm the results quantitatively, we performed flow cytometry of nitric oxide. The cells were stained with DAF-FM (10 µM, 30 min) and washed with 1x PBS. Nitric oxide-positive cells were determined by using a flow cytometer (BD FACSAria™ III). Here, we used L-NAME and SNP-treated cells as a standard for comparing the effect of DAP. For gating strategy refer Annexure I.II.V.

### *3.21 Immunofluorescence and confocal microscopy*

The cells were cultured in confocal dishes. Following treatment with 100 µM $H_2O_2$ for 24 hours and subsequent exposure to the peptide for 30 min, the cells were fixed with 4 % paraformaldehyde at RT for 10 min. Subsequently, permeabilization was achieved with 0.1 % Triton X-100 for 5 min, followed by blocking with 3% BSA for 1 hour. Following these steps, the cells underwent an overnight incubation with the primary antibody (diluted at 1:200) at 4°C. This was succeeded by incubation with the secondary antibody, conjugated with Alexa Fluor (diluted at 1:500), for 1 hour at RT. Counterstaining with DAPI was performed, and the cells were observed using a confocal laser-scanning microscope (Leica DMi8 confocal microscope, Germany).

### *3.22 Measuring intracellular calcium levels*

Intracellular Ca2+ concentrations in VSMC cells were estimated by using Flura 2 AM. VSMC cells were grown on coverslips in six-well plates. Cells were then washed with HBSS two times. Thereafter cell was washed with HBSS+ BSA (1mg/mL). These cells were loaded with 1 µM Fura-2 AM in media and incubated at 37°C. Thereafter, the cells were washed twice with HBSS to remove the residual medium. The intracellular fluorescence intensity at 510nm emission was estimated by live cell imaging microscopy (Leica DMi8 confocal microscope) at 340-nm and 380-nm excitation wavelengths in the presence or absence of H2O2, Ang1-7, BNP, and NP. Intracellular fluorescence intensities were estimated, and their differences were statistically compared.

### *3.23 Actions of dual acting peptide with Masr and pGCAR antagonism*

Cultured renal tubular epithelial cells were treated with specific inhibitors for MasR, A779 (1 µM) and pGCAR, KT5823 (1 µM) before peptide treatment for 30 min in $H_2O_2$ stimulated cells. After treatment we performed the qPCR analysis for Mas, pGCA and AT1 receptor mRNA expression.

### *3.24 Mas and pGCA receptor activation by DAP*

Without any treatment, control rats received varying concentrations of DAP (57.5, 115, and 230 µg/kg/day). The production of cGMP (pGC-A second messenger) and cAMP (MasR second messenger) was measured using ELISA assays at three separate time points (15, 30, and 60 min) after subcutaneous DAP injections. Furthermore, in rats given Ang1-7 (100 µg/kg/day) or BNP (15 µg/kg/day) or with DAP (115 µg/kg/day) at the same intervals, the production of cAMP and cGMP was evaluated. For ELISA assay, blood samples were taken from the rat's by retroorbital method and serum was separated. The levels of cGMP and cAMP in serum were measured using an immunoassay kit in accordance with the manufacturer's instructions (E-EL-0083 for cGMP and E-EL-0056 for cAMP, Elabsceince, USA). As part of the test protocol, cGMP and cAMP compete in the serum with a fixed amount of HRP-labelled cGMP and cAMP for binding sites on a rabbit polyclonal antibody. Absorbance readings were measured at 450 nm using a multimode reader (Spectramax M4, Molecular Devices, LLC). Results were averaged from duplicate determinations and expressed accordingly.

### *3.25 DOCA-salt hypertensive rats experimental timeline*

Hypertension Induced by Injecting Deoxycorticosterone Acetate (DOCA) salt, 25 mg/kg of body weight subcutaneously (SQ) twice a week for 4 weeks. DOCA dissolved in cottonseed oil (solubility: 15 mg/ml). Simultaneously, drinking water was replaced by a high-salt drinking water containing 1 % sodium chloride (NaCl) solution (177). The sham or control rats received subcutaneous cottonseed oil and were given tap water. After 4 weeks, body weight, water intake and BP were measured. After 4 weeks of disease induction, the animals were randomly grouped to receive SQ Ang1-7 (100 µg/Kg/day, SQ, N = 4) (178), BNP (15 µg/Kg/day, SQ, N = 4) (179), DAP, (115 µg/Kg/day, SQ, N = 4), Losartan (Lst) (10 mg/Kg/day, Oral route with normal drinking water, N = 4) (180). After end of experiment, water intake, body weight and BP were measured. After 2 weeks of peptide treatment, blood and urine were collected from each animal. The serum was separated for the estimation of biochemical parameters. Further,

immunohistochemistry and qPCR were performed to explore underlying molecular mechanisms.

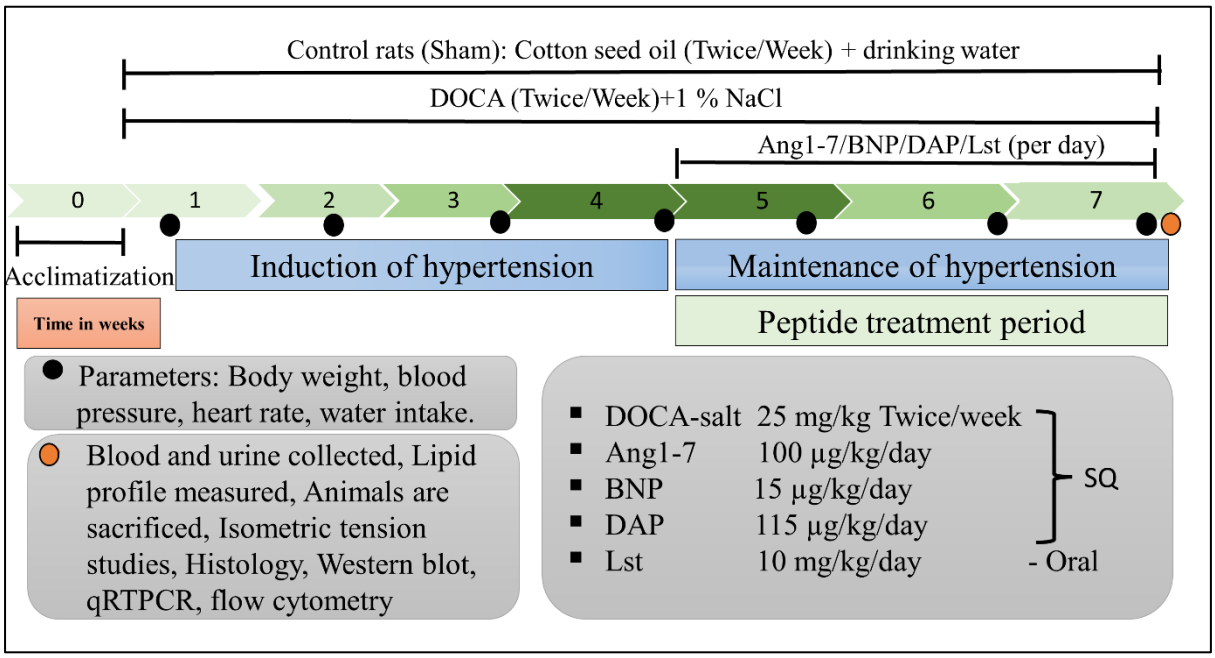



*Figure 3. 3: Experimental timeline of animal studies*

Male Wistar rats were used in a four-week study to establish hypertension using the DOCA-salt hypertension model. Throughout this time, blood pressure, body weight, and water intake were closely monitored to confirm the onset of hypertension. Following that, the rats were given either Ang1-7/BNP/DAP/Lst or no treatment for two weeks.

### *3.26 Blood pressure measurement*

Systolic (SBP), diastolic (DBP) and mean (MBP) BP were recorded weekly by tail-cuff method (MRBP system, IITC Life Science, Woodland Hills, CA). Before every reading, the instrument was calibrated as described by the manufacturer. The animals were acclimatized and trained one week prior to the experiment. Animals were conscious and placed in a restrainer for 15 min at a temperature of 32 $^{O}$C, 5-6 BP values were recorded. Computerized data acquisition systems and software were used for recordings and data analysis (181). All rats according to their blood pressure values considered hypertensive (>140/100 mmHg) and non-hypertensive (<140/100 mmHg).

### *3.27 Biochemical parameters of hypertension*

After 7 weeks, blood was removed from the rats and 15-30 min allowed to clot at room temperature (RT). After 30 min, serum was separated using a centrifuge at 2500 RPM for 15 min. Serum samples were stored at -20 $^{O}$C. Serum low-density lipoprotein (LDL), creatine kinase-myocardial band (CkMB) levels, total cholesterol (TC), high-density lipoprotein (HDL), triglycerides (TGs), albumin, creatinine and urinary creatinine, blood urea nitrogen (BUN), total protein were estimated by enzymatic colorimetric methods using assay kits. Absorbance was measured using a multimode reader (Spectramax M4, Molecular Devices, LLC).

### *3.28 Determination of serum and urinary electrolyte concentrations*

The electrolyte levels in the serum and urine were measured using a colorimetric technique using assay kits. The amounts of sodium, potassium, and chloride in serum and urine samples can be precisely measured using these techniques. Total sodium intake was measured by the formula (food weight × food [Na]) + (water intake × water [Na]) (182).

Further, to evaluate Sodium balance we used formula sodium intake - urinary sodium excretion. Understanding the balance between sodium intake and excretion is important for evaluating electrolyte balance and any variations that can point to physiological issues (182,183).

### *3.29 Plasma cyclic guanosine monophosphate and cyclic adenosine monophosphate level*

Blood was collected from the rat thoracic aorta at the end of the experimental timeline. As described earlier, serum was separated from blood. According to manufacturer protocol, cGMP and cAMP were determined in serum (N = 4/group) using an immunoassay kit (Elabscience, United States). Also, in primary endothelial cell lysate, cGMP levels were determined. Protocol suggests that cGMP and cAMP in the serum compete with a fixed amount of HRP-labelled cGMP and cAMP for sites on a rabbit polyclonal antibody. Absorbance was determined using the multimode reader at 450 nm (Spectramax M4, Molecular Devices, LLC). The results of duplicate determinations were averaged and expressed.

### *3.30 Isometric tension studies on rat aorta*

Rat aortic rings of control, diseases and treatment groups were used in isometric tension studies. After peptide treatments, the thoracic aorta was isolated from rats and removed adherent fat without damaging endothelial cells. The 10 ml of Kreb's buffer was filled in organ chambers (Composition of Kreb's buffer: NaCl 118 mM, KCl 4.7 mM, $MgSO_4$ 1.2 mM, $KH_2PO_4$ 1.2 mM, $NaHCO_3$ 22 mM, Glucose 5 mM, $CaCl_2$ 2.5 mM) (184). Aortic rings of 3-4 mm size were cut and without stretching mounted on triangular stirrups in organ chambers. The aortic rings were preserved at 37 °C and pH 7.4 with 95 % $O_2$/5 % $CO_2$. The tension (preload) on the aortic ring was maintained at an optimal tension of 2 gm for 1 hour. After resting, the rings were contracted with gradually increasing concentrations of Phenylephrine (PhE). After the plateau phase of contraction, the acetylcholine (Ach) cumulative concentration-response curve (CRC) was recorded. Endothelium was kept functionally intact which was assured by the Ach-induced relaxation at 1 μM concentration (185). Four rings were mounted simultaneously from a single rat. Isometric tension responses were recorded using isometric force transducers connected to 'Labchart' software and Power Lab equipment of AD Instruments Pvt. Ltd., India. Transducers and powerlab were operated according to the lab manual. Data are reported as percentages of maximum contraction achieved by PE and the percentage relaxation of PE tone.

### *3.31 Histopathological examination of tissues*

Paraffin-embedded tissues from all experimental groups were used for histological studies. Slides with 4-5 μM tissue sections were kept in the hot air oven for 2-4 hrs at 60 $^{O}$C temperature. Tissue slides were deparaffinized and rehydrated with xylene two changes for 10 min and 100, 90, 80, and 70 % alcohols for 5 min, then hydrated with distilled water for 2-3 min. After rehydration, section slides were stained with haematoxylin for 10 min and washed with tap water. After this, the tissue sections were stained with eosin for 30 seconds and again dehydrated with 70-100 % alcohol followed by xylene for 5 min each. Later the sections were mounted on coverslips using mounting media (Anti-Fade Fluorescence Mounting Medium, Abcam, US) and captured images by ZEISS AXIOLAB 5 Microscope. Three sections were considered for measuring aortic thickness and lumen diameter, and the result was averaged. Lumen thickness was measured by Zeiss software and aortic diameter was measured using Image J software. At this point, the wall-to-lumen ratio (W/L) was calculated. Brightfield

images were taken using a confocal laser-scanning microscope (Leica DMi8, Leica Microsystems, Germany).

### *3.32 Pathological evaluation of rat kidneys*

The severity of glomerular damage was assessed on a scale of 0 to 4 through a semiquantitative scoring scale from 0 to 4 (which indicates 0 for normal, 1 for mesangial expansion/sclerosis involving less than 25 % of the tuft, 2 for moderate damage (25–50 %), 3 for severe damage (50–75 %), and 4 for diffuse glomerular involving more than 75 % of the glomerular tuft (186). The Glomerular damage for each rat was computed as the average of all glomerular scores obtained from 10 different fields of H and E-stained slides. Tubulointerstitial damage was determined using a similar semiquantitative scoring system. Ten fields per kidney were analysed, and lesions were graded from 0 to 4 (0 for no change, 1 for changes affecting less than 25 % of the section, 2 for changes affecting 25–50 % of the section, 3 for changes affecting 50–75 % and 4 for changes affecting 75-100 % of the section) based on the area with tubulointerstitial damage (including tubular atrophy, casts, interstitial inflammation, and fibrosis) (187). The score index for each rat was expressed as the mean value of all obtained scores.

### *3.33 Sirius red staining*

Rehydration is performed as described earlier. Then, tissue sections were stained for 1 hour with Sirius red stain, followed by washing with acidified water. Then, stained tissues were dehydrated and mounted on coverslips with mounting media. Images were captured using the ZEISS AXIOLAB 5 microscope. The fold change in collagen deposition was measured by Image J software. Three different regions of each aorta were calculated, and the results were averaged.

### *3.34 Alizarin red staining*

Rehydrated tissue slides were stained with alizarin red for 10 min; later, slides were washed with distilled water. Then cross-stained with DAPI for 15 min. Followed by three washes with PBS and then slides were mounted with mounting media. Slides were observed under a confocal microscope (Leica DMi8 confocal microscope, Germany) at an excitation of 502 nm and an emission of 586 nm. The alizarin red fluorescence intensity was measured using Image

J software (Image J 1.49V). Three different aortas were considered for measuring fluorescence intensity, and the results were averaged.

### *3.35 Immunohistochemistry*

Rehydrated tissue slides were initially immersed in preheated sodium citrate buffer (pH 6.0, temperature: 95-100 $^{O}$C) for 10 min. Following this, the slides were allowed to cool for 15-20 min. Subsequently, the slides were washed twice with PBS buffer for 5 min each. The slides were then incubated with 0.5 % Triton X-100 for 5 min, followed by two additional washes with PBS. Blocking was performed by incubating the slides with PBS buffer supplemented with 1 % BSA for 1 hour. Next, the slides were incubated with the primary antibody anti-collagen (diluted 1:100) overnight at 4 °C. After washing the slides with PBST, they were incubated with a secondary antibody (Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor™ 488, diluted 1:100). The slides were washed three times with PBST before being counterstained with DAPI for 15 min and rinsed with PBS three times. Finally, the slides were mounted with coverslips using a mounting medium and images were acquired using confocal laser-scanning microscopy (Leica DMi8, Leica Microsystems, Germany).

### *3.36 Quantitative polymerase chain reaction*

RNA was extracted from the aortas, kidney and cells for gene expression studies using RNAiso Plus (Takara, Japan), as described by the manufacturers. Isolated RNA was quantified spectrophotometrically and stored at −80 °C. Isolated RNA is converted to cDNA using a prime Script RT Reagent Kit (Takara, Japan). An SYBR Premix Ex Taq kit (Takara, Japan) was used at 95 °C for 3 min, followed by amplification for 40 cycles (15 s at 95 °C, 30 s at 64 °C and 30 s at 72 °C). The Ct values of the amplified cDNA were calculated, and the transcript levels were analysed using the 2-ΔΔCt method (Bio-Rad CFX connect real-time machine). Samples were run in duplicates and the mean was used to calculate the fold change in the gene expression. The 18s is used as an internal control for calculating relative mRNA expression. The primer sequences used for the experiment are discussed in Annexure 1.

### *3.37 Western blotting*

Protein was isolated from the kidney, aorta and endothelial cells using radioimmunoprecipitation assay (RIPA) lysis buffer. A protein sample of 40 µg was loaded on an 8 %–12 % SDS page and allowed to transfer on a polyvinylidene difluoride (PVDF)

membrane (Bio-Rad). Then, the PVDF membrane was blocked with 3 % bovine serum albumin (BSA) prepared in phosphate-buffered saline with 0.1 % Tween 20 (PBS-T) for 1 hr at RT. After 1 hr, the same PVDF membrane was incubated with primary antibody for overnight at 4 °C. The following morning, the membrane was washed 3 times with PBS-T for 15 min each and incubated with horseradish peroxidase (HRP) conjugated secondary antibody for 2 hr at RT. After incubation, the membrane was washed three times. The immunoreactivity to the desired protein was detected with the help of an Enhanced Chemiluminescence Reagent. The Eppendorf fusion solo chemidoc machine was used to identify and analyse the target protein. The Antibody concentrations used for western blot analysis were primary antibody (1:1000) and secondary antibody (1: 3000).

### *3.38 Statistical analysis*

Data obtained from separate experiments are expressed as mean ± SEM, N denotes the number of animals in each group. The statistical analysis for experiments was performed using one-way ANOVA followed by post hoc Bonferroni's test. All analyses were performed using GraphPad Prism 8.0.2 (263) (GraphPad Software Inc, San Diego, CA, USA). A p-value $<0.05$ was considered statistically significant.

### *3.39 Sample size calculation statement*

Sample size calculations were conducted to ensure adequate power to detect statistically significant differences between groups. For in vivo experiments, a sample size of n=4 per group was determined based on preliminary data, aiming for a power of 80% and an alpha level of 0.05 to detect a significant effect size. For in vitro experiments, a sample size of n=6 per group was chosen to account for variability and ensure robust statistical analysis, similarly targeting a power of 80% and an alpha level of 0.05. These sample sizes were deemed sufficient to achieve reliable and reproducible results in our study

# *Chapter 4*

# *Activation of Mas and pGCA Receptor Pathways Protects Renal Epithelial Cell Damage Against Oxidative-Stress*

## *4.1 Graphical abstract*

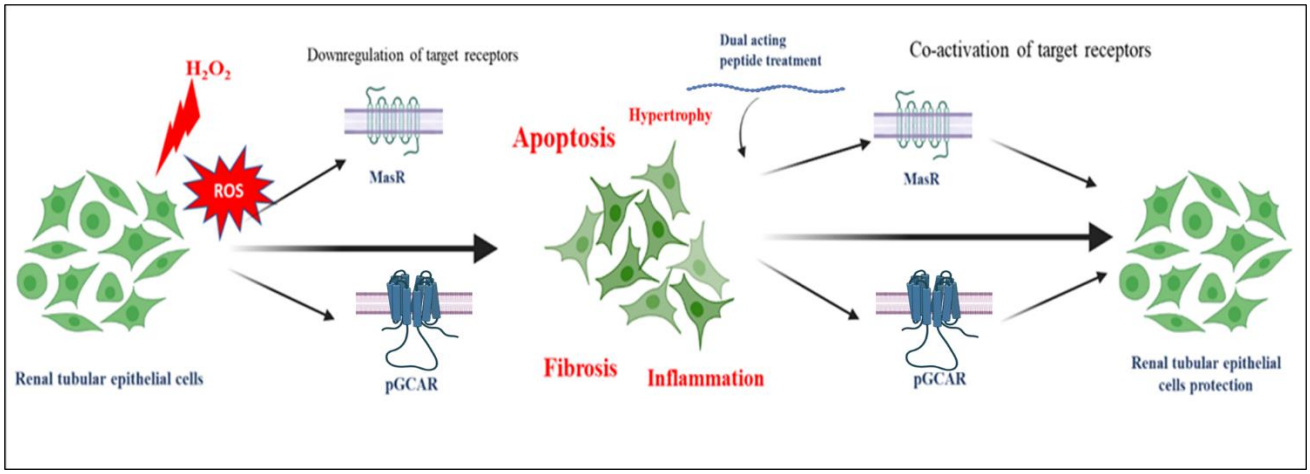



*Figure 4. 1: Graphical abstract of chapter 4*

## *4.2 Introduction*

The intricate relationship between the RAAS and oxidative stress has been extensively explored in understanding the pathogenesis of cardio-renal complications. As we know, activation of the RAAS initiates the production of central peptide AngII, and consequently, the classical ACE/AngII/AT1 pathway may initiate the progression of hypertension in conditions marked by abnormal RAAS activation (2). Furthermore, this activation is involved in harmful secondary complications such as hypertrophy, inflammation, fibrosis, angiogenesis, atherosclerosis, apoptosis, and vascular aging (188).

The connection between oxidative stress and cardio-renal diseases is established through RAAS activation. Multiple studies have reported that both RAAS and oxidative stress contribute to elevated blood pressure. In the kidney, RAAS overactivation leads to the production of reactive oxygen species, with NADPH oxidase, a major ROS generator expressed in most kidney cells, playing a pivotal role (189). The peptide AngII activates NADPH oxidase in the proximal tubule, further worsening the progression of cardiovascular complications (190). Importantly, the generated ROS acts as triggers for apoptosis, inflammation, hypertrophy, and increased deposition of extracellular matrix in cardiovascular diseases (74).

The ROS family comprises free oxygen radicals such as superoxide anion hydroxyl radical and hydrogen peroxide ($H_2O_2$). Growing evidence suggests that $H_2O_2$ serves as a paracrine mediator of oxidative stress and a major promoter of cardio-renal damage (191). The hypertensive effects attributed to $H_2O_2$ encompass vasoconstriction (192), hypertrophy (193),

inflammation (173,194), hyperplasia, fibrosis, and apoptosis (195). This comprehensive interplay between RAAS, oxidative stress, and their downstream effects underscores the multifaceted role they collectively play in the development and progression of cardio-renal complications.

Presently, commercially available inhibitors of the RAAS, namely angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers (ARB), are recommended as the first-line pharmacotherapy for hypertension (128,190). Nevertheless, these medications exhibit limitations in effectively ameliorating the progression of the disease and are associated with potential adverse effects (196,197). Consequently, there exists a critical demand for therapeutics with novel mechanisms of action for treating cardiovascular diseases and renal complications.

Recent scientific projects have focused on novel therapeutic approaches that simultaneously target multiple pathways. In supporting this trend, the emergence of dual-acting peptides represents a novel strategy in natural peptide-based strategies aimed at enhancing the efficacy of two distinct peptides by targeting multiple pathways simultaneously (65). This concept gained importance after the successful outcomes of LCZ696, an angiotensin receptor-neprilysin inhibitor that achieves dual inhibition and improves cardiac function in heart failure (18).

In the present investigation, our attention is directed towards the co-activation of the particulate guanylyl cyclase-A receptor (pGCAR) and MasR pathway. Past research has demonstrated that the individual activation of these pathways reveals cardiac and renal protective properties (198,199).

The protective axis of RAAS, the ACE2/Ang1–7/Mas receptor axis, emerging and extensively investigated target in scientific investigations (45). This axis acts as a counterregulatory to the classical ACE/AngII/AT1R axis. Previous research has demonstrated that the infusion of Ang1-7 mitigates the development of hypertension, renal vasoconstriction, and induces natriuresis in rats subjected to AngII infusion (200). The beneficial effects attributed to the Ang1-7 peptide encompass the regulation of blood pressure, enhanced endothelial function, modulation of vascular remodelling, alleviation of oxidative stress, and regulation of cardiovascular and renal functions (201). In addition to Ang1-7, the non-peptide agonist AVE 0991, targeting the Mas receptor, has demonstrated the mitigation of hypertrophy, oxidative stress, and improvement in cardiac function in a model of cardiac hypertrophy (202).

Another promising target for CVD is the pGC-A receptor pathway (55). Its endogenous ligands, ANP and BNP play crucial roles in cardiovascular function, arterial pressure regulation, and homeostasis. These natriuretic peptides bind to the membrane-bound pGC, which consists of a ligand-binding domain and a cytoplasmic guanylyl cyclase domain. Activation of the pGC receptor stimulates the production of the second messenger cGMP, further activating PKG (154). The activation of this pathway is considered an efficient strategy for cardiac remodelling and heart failure (203). After extensive research, the pGC-A activator, CRRL269, designed for natriuretic peptide signalling, has been reported to show renal and cardiac protective activity in animal models of ischemia-induced acute kidney injury (204). This underscores the potential therapeutic relevance of targeting the ACE2/Ang1–7/Mas receptor axis and the pGC-A pathway in addressing cardiovascular and renal complications.

As evidenced in earlier reports, the pathogenesis of cardiovascular disease (CVD) and renal disorders is complicated, involving the activation of multiple pathways, the synthesis and design of dual-acting peptides that simultaneously stimulate various pathways presents a promising strategy. This approach holds promise for developing novel therapeutic options with improved efficacy and safety in halting disease progression. Innovative natural peptide-based therapeutics employing multivalent strategies represent a breakthrough in addressing the complexities of worsening cardiovascular complications.

In line with this concept, we have synthesized a novel dual-acting peptide (DAP) by fusing amino acid sequences of B-type natriuretic peptide (BNP) and Angiotensin1-7 (Ang1-7) (For amino acid sequence refer Figure 4.2). The grounds behind this dual-acting peptide design are to explore its efficacy in addressing hydrogen peroxide-induced oxidative stress *in-vitro* conditions, specifically in renal epithelial NRK-52E cells. Our investigation aims to assess and compare the effectiveness of the dual-acting peptide, which simultaneously activates both the Mas receptor and the particulate guanylyl cyclase receptor A (pGCA), with the individual peptides Ang1-7 and BNP. This study seeks to provide insights into the potential benefits of a dual-acting peptide approach in attenuating oxidative stress-induced injury, creating the way for the development of novel therapeutic interventions for safeguarding vital organs against renal and cardiovascular complications.


*Figure 4. 2: Amino acid sequence of Ang1-7, BNP and DAP*

Scheme 1. Amino acid sequences of peptides (Ang1–7, BNP and DAP). Here, capital letter = L-amino acid residues, amino acid residues are either underlined or italicized to depict that the design of DAP peptide sequence is inspired from Ang1–7 and BNP peptide. Lines connecting two cysteine residues indicate disulphide linkage.

## *4.3 Material and methods*

In this study, we used NRK52E cells and cultured them in T25 flasks with DMEM supplemented with 10 % FBS and 1 % penicillin-streptomycin antibiotic solution. The induction of an *in-vitro* hypertension model involved treating cells with 100 μM $H_2O_2$ in the presence or absence of Ang1-7, BNP, and DAP (1 μM). Time points for further studies were selected based on MTT assays. Subsequently, various assays and analyses were performed, including MTT assay, qPCR, crystal violet staining, immunofluorescence and confocal microscopy, measurement of ROS, Griess reagent assay, Annexin V/FITC/PI staining, and western blotting. Detailed protocols for each procedure can be found in Chapter 3, Method Section. Statistical analysis of the data was conducted using ANOVA followed by post hoc Bonferroni's test in GraphPad Prism 8.0.2, with a significance level set at $P < 0.04$.

## *4.4 Results*

### 4.4.1 Results of peptide synthesis

Peptides were synthesized using machine-assisted SPPS at elevated temperature. Global deprotection using TFA cocktail and preparative HPLC purification yielded 45 mg (50.1 μmol, 50 %) of Ang (1-7), 97 mg (28 μmol, 28 %) of BNP and 74 mg (28.9 μmol, 29 %) of DAP. Disulfide bond formation of the purified BNP and DAP polypeptides were carried out under air oxidation condition. The Observed mass of the desired peptides was as follows; Ang (1-7): 898.462593.33 ± 0.01 Da (deconvoluted most abundant mono isotope); calculated mass: 898.472593.34 Da (Figure 4.3A), folded BNP: 34634.749 ± 0.023 Da (deconvoluted most abundant isotope); calculated mass: 34634.76 Da (Figure 4.3B), DAP: 34724.78 ± 0.01 Da (deconvoluted most abundant isotope); calculated mass: 34724.79 Da. (Figure 4.3C).

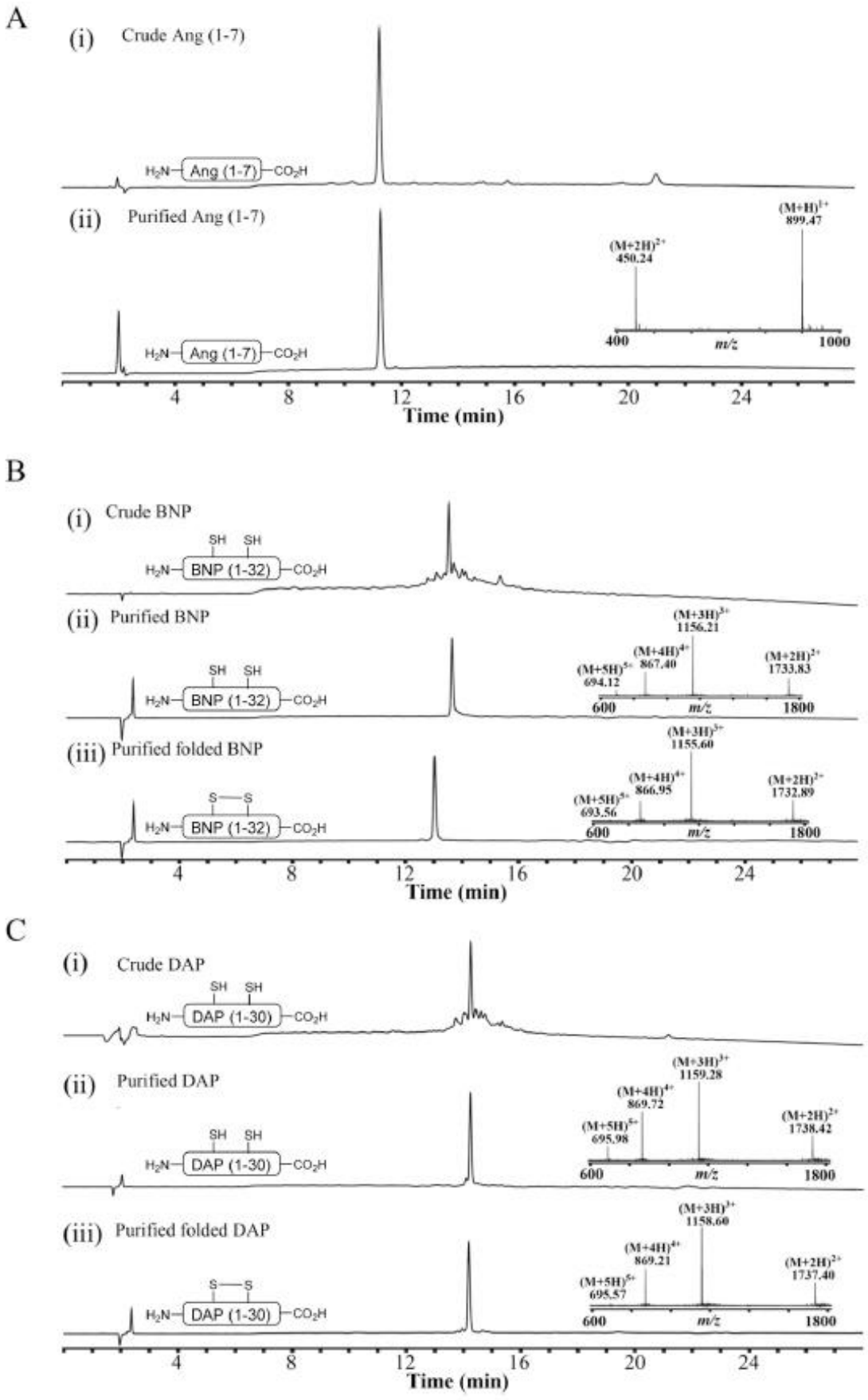



*Figure 4. 3: Analytical RP-HPLC profile of peptides*

(A). Analytical RP-HPLC profile (λ = 214 nm) together with ESI-MS data (inset) of Ang (1-7) peptide NPA7 seg2. Linear gradient, 10-54 % of B over 22 min including 4 min equilibration time using Agilent Zorbax SB-C3, 5 µm 4.6 □ x150 mm, LC column with 0.9 mL/min flow rate, was used for the chromatographic separation. Purification was performed using a linear gradient 05 %-35 % buffer B in buffer A over 30 min with a flow rate of 5 mL/min at 40 $^{O}$C (buffer A = 0.1 % AcOH in water; buffer B = 0.08 % AcOH in acetonitrile) using a C18, 10 □ x 250 mm, preparative HPLC column (Waters X select peptide CSH, 130 Å, 5 µm). B. Analytical RP-HPLC profile (λ = 214 nm) together with ESI-MS data (inset) of purified BNP peptide NPA7 seg2. Linear gradient, 10-54 % of B over 22 min including 4 min equilibration time using Agilent Zorbax SB-C3, 5 µm 4.6 □ x150 mm, LC column with 0.9 mL/min flow rate, was used for the chromatographic separation. Purification of the crude NPA7 seg2BNP polypeptide was performed using a linear gradient of 05 %-35 % buffer B in buffer A over 90 min with a flow rate of 5 mL/min at 40 °C (buffer A = 0.1 % TFA in water; buffer B = 0.08 % TFA in acetonitrile) using a C4, 9.4 □x 250 mm, preparative HPLC column (Agilent zorbax, 300 Å, 5 µm). While purification of folded NPA7 seg2BNP polypeptide was performed using a linear gradient 01%-31% buffer B in buffer A over 30 min with a flow rate of 5 mL/min at 40 °C (buffer A = 0.1% AcOH in water; buffer B = 0.08 % AcOH in acetonitrile) using a C4, 9.4 □ x 250 mm, preparative HPLC column (Agilent zorbax, 300 Å, 5 µm). C. Analytical RP-HPLC profile (λ = 214 nm) together with ESI-MS data (inset) of purified peptide BNP32DAP. Linear gradient, 10-54 % of B over 22 min including 4 min equilibration time using Agilent Zorbax SB-C3, 5 µm 4.6 □ x150 mm, LC column with 0.9 mL/min flow rate, was used for the chromatographic separation. Purification of crude BNP32 DAP polypeptide was performed using a linear gradient 051 %-351 % buffer B in buffer A over 90 min with a flow rate of 5 mL/min at 40 °C (buffer A = 0.1 % TFA in water; buffer B = 0.08 % TFA in acetonitrile) using a C4, 10 □x 250 mm, preparative HPLC column (Waters Xbridge protein BEH, 300 Å, 5 µm). While, purification of folded BNP32 DAP polypeptide was performed using a linear gradient 01 %-31 % buffer B in buffer A over 30 min with a flow rate of 5 mL/min at 40 °C (buffer A = 0.1 % AcOH in water; buffer B = 0.08 % AcOH in acetonitrile) using a C4, 9.4 □ x 250 mm, preparative HPLC column (Agilent zorbax, 300 Å, 5 µm).

### 4.4.2 Effect of $H_2O_2$ on cell viability *in-vitro* NRK52E cells

To establish the $H_2O_2$-induced oxidative stress model we performed an MTT assay at a concentration of 100 µM, NRK52E renal epithelial cells were subjected to $H_2O_2$ treatment at various time intervals. The MTT assay was employed to determine cell viability at 1, 3, 6, 12, and 24 hours. The results revealed a gradual decrease in NRK52E cell viability with increasing exposure to $H_2O_2$. Specifically, when exposed to 100 µM $H_2O_2$ for 24 hours, a visible reduction to 85 % of cell viability was observed as shown in Fig. 4.4A.

### 4.4.3 Effect of $H_2O_2$ on the mRNA expression of CVD markers and target receptor genes

To elucidate the optimal duration of $H_2O_2$ exposure in our *in-vitro* cell model, we analysed the mRNA expression of the CVD marker AT1R, along with the target receptor genes MasR and pGCAR in the presence of $H_2O_2$. Figure 4.4B indicates a significant increase in AT1R mRNA expression at 24 hour compared to the control. Additionally, Figures 4.4C and 4.4D illustrate a noteworthy reduction in mRNA expression of MasR and pGCAR with prolonged exposure to $H_2O_2$. Based on these findings, we selected the concentration of 100 µM $H_2O_2$ for 24 hours for subsequent experiments.

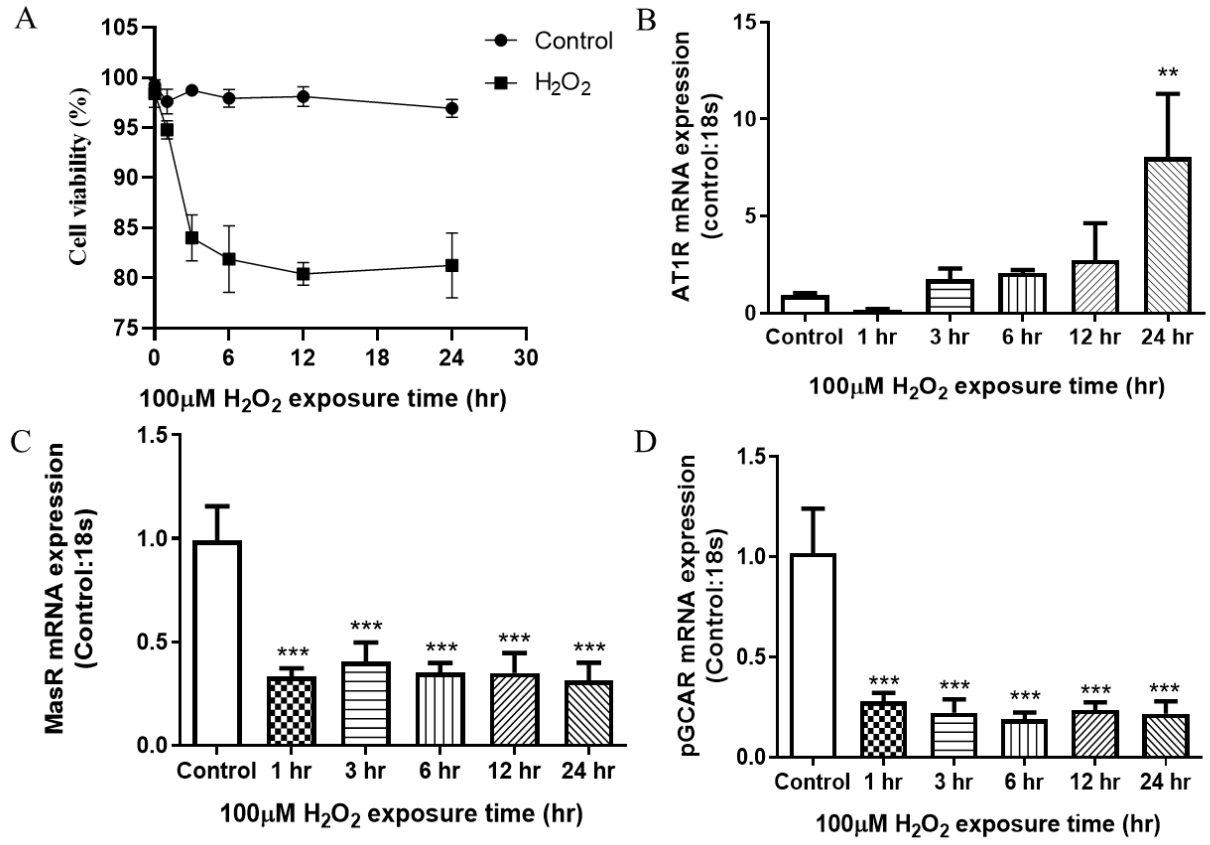



*Figure 4. 4: $H_2O_2$-induced oxidative stress model establishment*

NRK52E cells were subjected to $H_2O_2$ treatment. (A) Cell viability was assessed using MTT assay in cultured rat NRK52E cells incubated with normal culture medium (control, Con) or medium containing $H_2O_2$ (100 μM) for 1, 3, 6, 12, and 24 hour. N = 6. (B) Time-dependent mRNA expression of the CVD marker AT1R in $H_2O_2$-stimulated NRK52E cells. N = 4. (C) Time-dependent effect of $H_2O_2$ on MasR mRNA. N = 4. (D) Time-dependent effect of $H_2O_2$ on pGCA mRNA. N = 5. Data were expressed as mean ± SEM. Data were analysed using ordinary one-way ANOVA followed by Bonferroni multiple comparison test, where, **p < 0.01, ***p < 0.001 *vs* control.

### 4.4.4 Effect of peptides on NRK52E cell viability

Furthermore, we explored the effect of peptides Ang1-7, BNP, and the DAP at a concentration of 1 μM on cell viability at different time points. The MTT assay was employed to assess cell viability at 15, 30, 60, and 180 min. Notably, 100 % cell viability was observed at all time points when treated with 1 μM DAP, suggesting its potential as a promising peptide for cellular health (Fig. 4.5A).

### 4.4.5 Receptor characterization in NRK52E cells treated with peptides (Without $H_2O_2$)

To assess the effect of Ang1-7, BNP, and DAP (1 μM) on target receptor mRNA levels, we conducted qPCR experiments. As indicated in Figure 4.5B, the data revealed that a 30-minute exposure to DAP significantly upregulates MasR mRNA compared to the control. Similarly, pGCAR mRNA was significantly elevated with DAP at 30 min compared to the control (Fig. 4.5C). These findings guided the selection of parameters for future experiments, with a preference for a 30-minute exposure time and a 1 μM concentration of Ang1-7, BNP, and DAP.

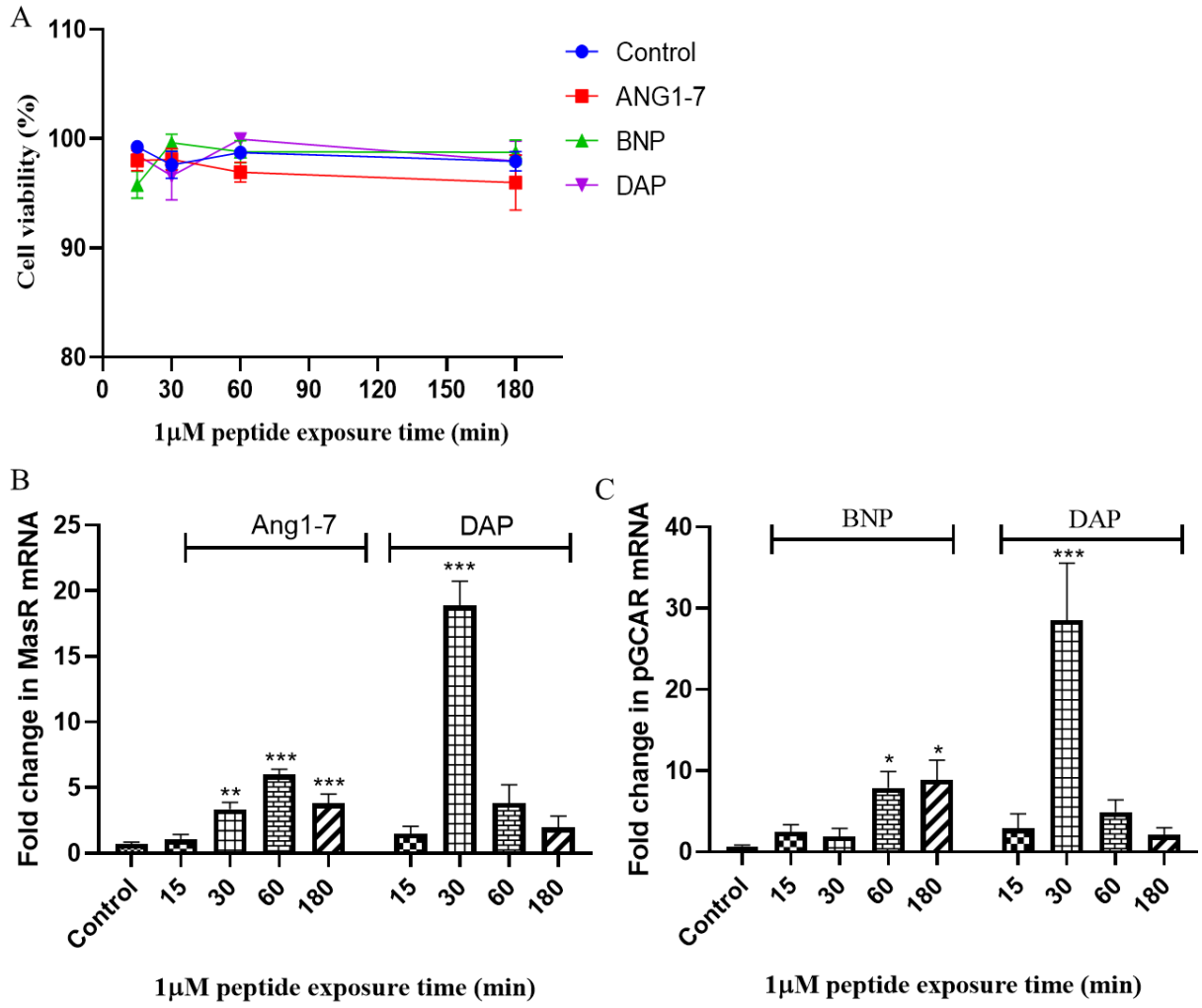


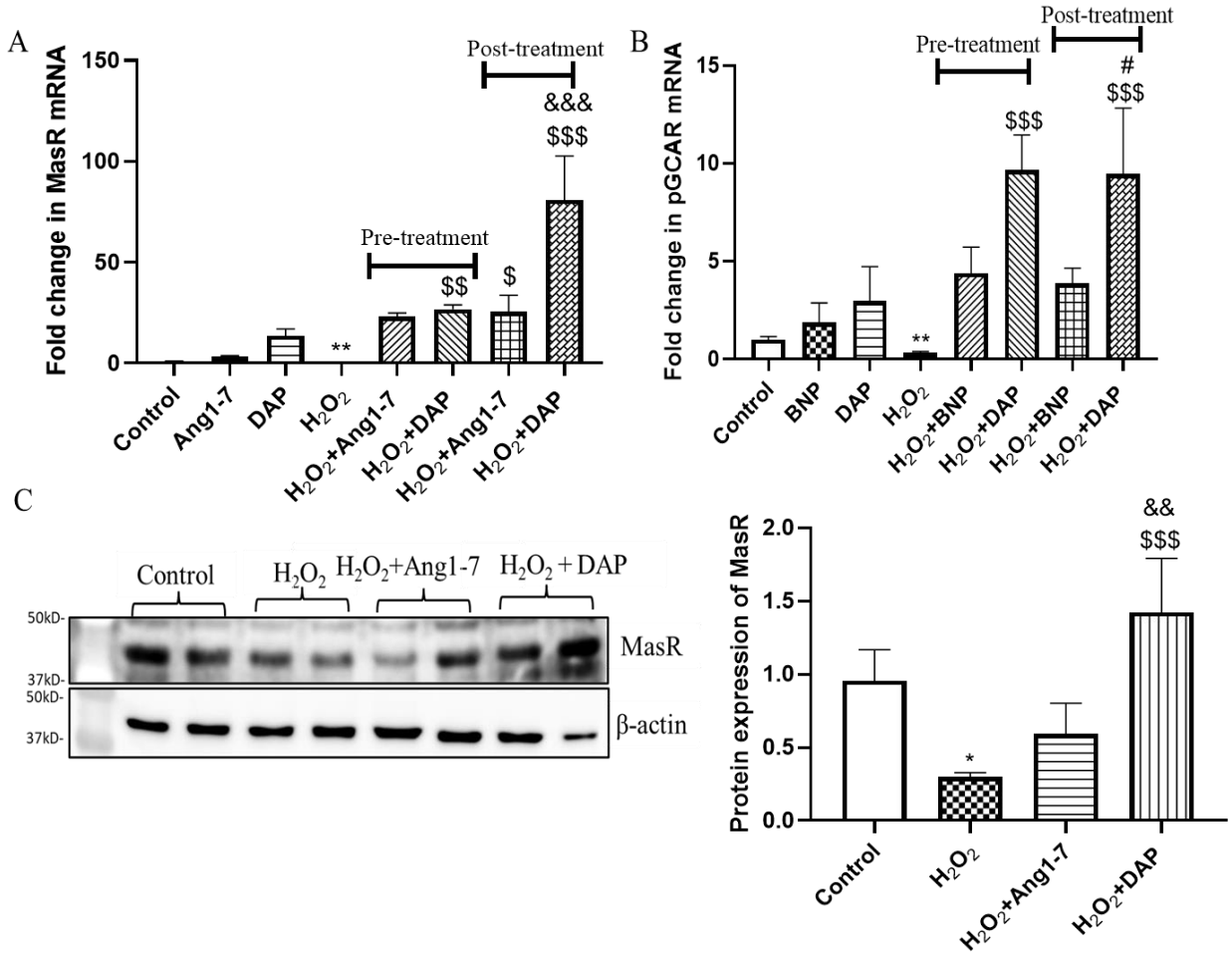

*Figure 4. 5: Effect peptides on cell viability and target receptor mRNA levels in NRK52E cells without $H_2O_2$*

NRK52E cells were individually treated with the peptides Ang1-7, BNP, and DAP. (A) Cell viability was assessed through MTT assay in cultured rat NRK52E cells incubated with normal culture medium (control) or medium containing Ang1-7, BNP, and DAP (1 µM, without $H_2O_2$) for 15, 30, 60, and 180 min. N = 6. (B) qPCR analysis of MasR mRNA in NRK52E cells treated with Ang1-7 and DAP. N = 4. (C) qPCR analysis of pGCAR mRNA in NRK52E cells treated with BNP and DAP. Data were expressed as mean ± SEM. Data were analysed using ordinary one-way ANOVA followed by Bonferroni multiple comparison test, Where *p < 0.05, **p < 0.01, ***p < 0.001 *vs* control.

### 4.4.6 Effect of dual activation, on target receptor mRNA and protein levels

In previous reports, the protective target receptors MasR and pGCAR demonstrated efficacy in experimental studies. We explored the effect of DAP both pre- and post-treatment on target

receptor mRNA levels through qPCR. As depicted in Figures 4.6A and 4.6B, both 30-minute pre- and post-treatments with the peptide led to an upregulation of MasR and pGCAR mRNA levels in $H_2O_2$-treated cells. Upon comparing the efficacy of pre- and post-treatment, we observed that post-treatment was more effective. Consequently, post-treatment with peptides was adopted for future experiments. To corroborate our findings from the post-treatment approach, we conducted a western blot analysis: Figures 4.6C and 4.6D revealed consistent results in the western blot analysis. Overall, our observations indicate that post-treatment with DAP is more effective than pre-treatment in $H_2O_2$-treated NRK52E cells.



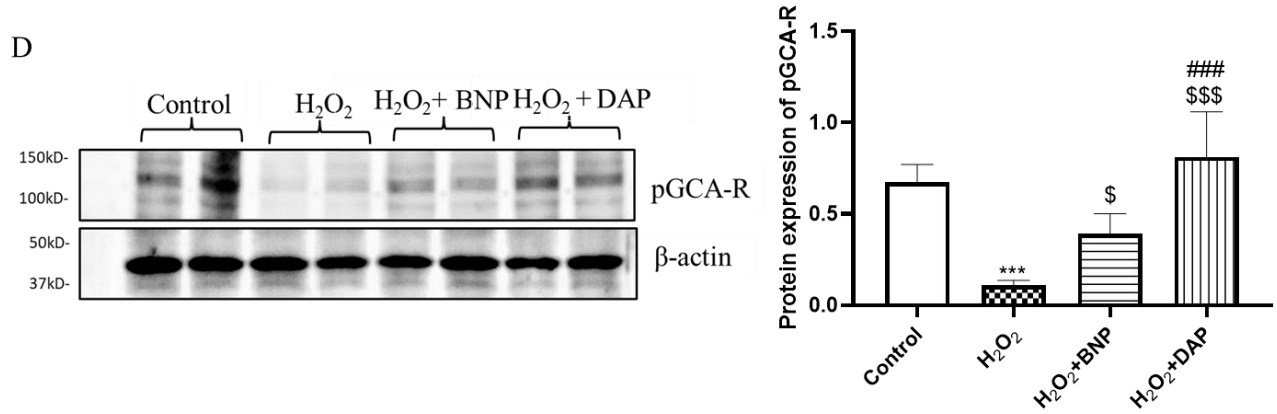


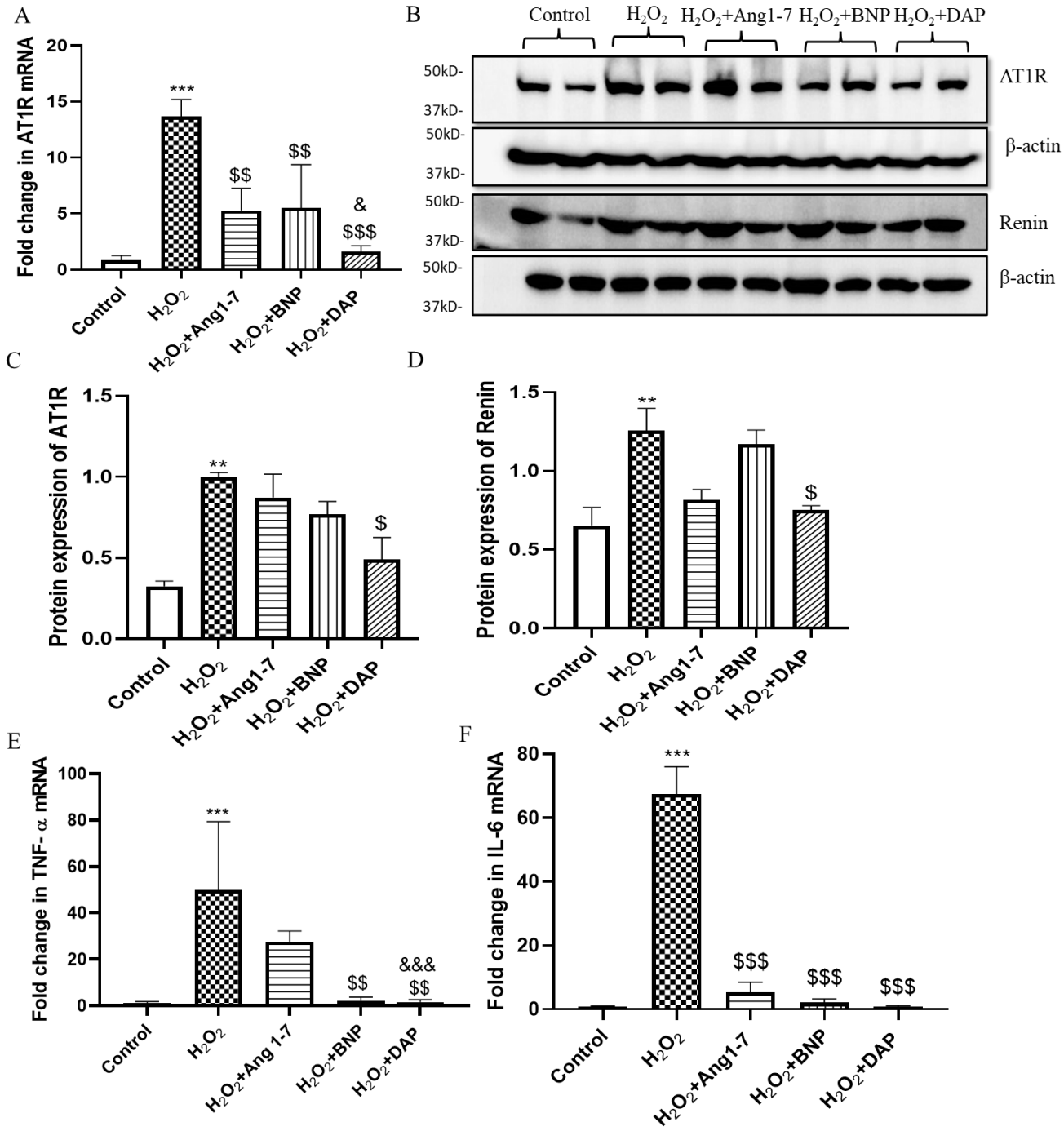

*Figure 4. 6: Effect of Dual Activation, on Target Receptor mRNA and protein Levels*

NRK52E cells were exposed to $H_2O_2$ with or without peptides Ang1-7, BNP, and DAP. In the pre-treatment group, cells were treated with peptides for 30 min followed by $H_2O_2$ for 24 hours. Conversely, in the post-treatment group, cells were incubated with $H_2O_2$ for 24 hour followed by peptide treatment for 30 min. (A) MasR mRNA levels in pre- and post-treated NRK52E cells. N = 4. (B) pGCAR mRNA levels in pre- and post-treated NRK52E cells. N = 4. (C) MasR protein expression by western blotting in $H_2O_2$-treated NRK52E cells followed by post-treatment with peptides. N = 4. (D) pGCAR protein expression by western blotting in $H_2O_2$-stimulated NRK52E cells followed by post-treatment with peptides. Data were expressed as mean ± SEM. Data were analysed using ordinary one-way ANOVA followed by Bonferroni multiple comparison test, where, N = 4, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *vs* control, $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ *vs* $H_2O_2$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ *vs* $H_2O_2$ + Ang1-7, $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ *vs* $H_2O_2$ + BNP.

### 4.4.7 Effect of dual activation on RAAS and inflammatory markers

Oxidative stress prompts the release of RAAS and inflammatory markers, contributing to the worsening of cardiovascular complications. Consequently, we examined the effect on renal health through the evaluation of AT1R and renin mRNA gene expression. Our findings indicate that dual activation with DAP effectively mitigates the $H_2O_2$-induced expression of RAAS markers, AT1R, and renin (Fig. 4.7A-D). To further elucidate the influence of dual activation on the inflammatory process, we conducted qPCR and western blotting for specific markers, TNFα, IL-6, and NF-kβ (Fig. 4.7E-H). Notably, our data underscores the anti-inflammatory efficacy of DAP; the simultaneous activation of receptors shows promise in mitigating the $H_2O_2$-induced progression of inflammation. In summary, DAP demonstrated effectiveness in reducing the pathogenesis of $H_2O_2$-induced over-activation of RAAS and inflammation.

Additionally, DAP proved more effective than individual pathway activation with alone peptides.



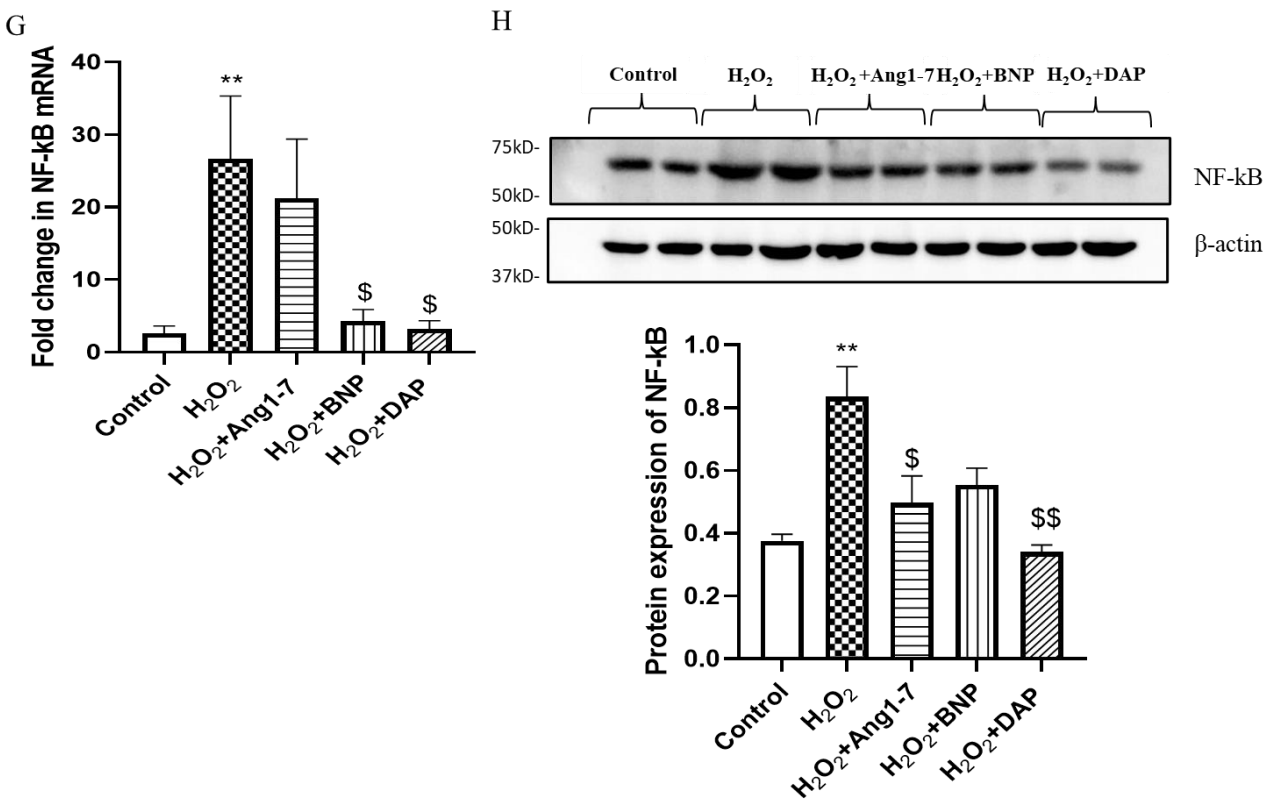



*Figure 4. 7: Effect of Dual Activation on RAAS and Inflammatory Markers*

RAAS Upregulation Markers in NRK52E Cells Treated with $H_2O_2$, with or without Peptides Ang1-7, BNP, and DAP. (A) mRNA expression of AT1R assessed by qPCR. N = 5, vs control. (B) Western blot analysis for RAAS upregulation markers AT1R and Renin. N = 4 (C) Quantification of protein expression for AT1R and (D) Renin. N = 4. The mRNA expression of inflammatory markers assessed by qPCR includes (E) TNF-α, (F) IL-6, and (G) NF-kβ. (H) Western blot analysis for NF-kβ protein. Data were expressed as mean ± SEM. Data were analysed using ordinary one-way ANOVA followed by Bonferroni multiple comparison test, where, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* control, $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ *vs* $H_2O_2$, $^{\&}p < 0.05$, $^{\&\&\&}p < 0.001$ *vs* $H_2O_2$ + Ang1-7.

### 4.4.8 Dual activation protects against $H_2O_2$-induced renal hypertrophy

Tubular hypertrophy and fibrosis are largely controlled by ROS regulation. Our study on hypertrophy in $H_2O_2$-induced NRK-52E renal epithelial cells, as shown in Figure 4.8A-4.8B, used crystal violet staining to detect an increased hypertrophic region in the $H_2O_2$-treated cells relative to the control. Interestingly, peptide treatment considerably reduced the hypertrophic area compared to the group exposed to $H_2O_2$. To verify the effect of dual activation of Mas and pGCA receptors on hypertrophy, we analysed the expression of markers associated with

hypertrophy, ANP. Dual activation with DAP showed a significant decrease in ANP mRNA induced by $H_2O_2$ exposure, as seen in Figure 4.8C.

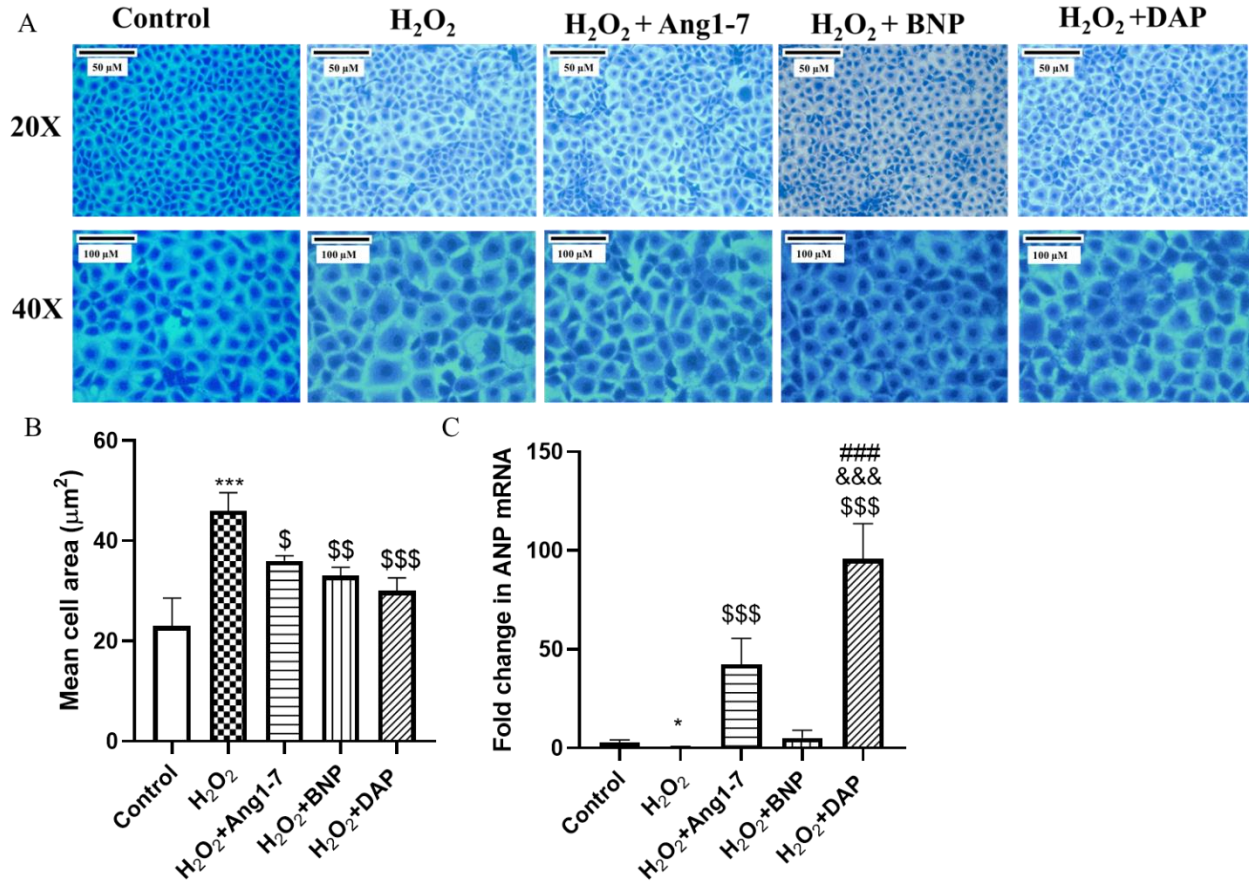



*Figure 4. 8: Protective Effects of Dual Receptor Activation against $H_2O_2$-Induced Hypertrophy Renal Epithelial Tubular NRK52E Cells*

NRK52E cells in culture were exposed to $H_2O_2$ (100 µM) for 24 hours, followed by incubation with peptides Ang1-7, BNP, and DAP (1 µM) for 30 min. (A) Hypertrophy was assessed using crystal violet staining, N = 3. (B) Quantification of mean surface area from the image in A (C) ANP mRNA expression, a hypertrophic marker, was analysed, N = 4. Data were expressed as mean ± SEM. Data were analysed using ordinary one-way ANOVA followed by Bonferroni multiple comparison test, where *$p < 0.05$, ***$p < 0.001$ *vs* control, $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ *vs* $H_2O_2$, $^{\&\&\&}p < 0.001$ *vs* $H_2O_2$ + Ang1-7, $^{\#\#\#}p < 0.001$ *vs* $H_2O_2$ + BNP.

### 4.4.9 Dual activation protects against $H_2O_2$-induced renal fibrosis

Moreover, we also examined the effect of dual activation on fibrosis. Both confocal pictures and western blot studies of the fibrosis marker α-SMA showed a dual activation-mediated reduction of $H_2O_2$-induced fibrosis, as shown in Figure 4.9A-C. Notably, DAP outperformed BNP and Ang1-7 in terms of effectiveness. To evaluate the expression of the fibrosis marker

TGFβ, we also performed qPCR. The peptide therapy significantly decreased TGFβ expression, as shown in Figure 4.9D. All of these *in-vitro* results collectively indicate the efficient reduction of $H_2O_2$-induced hypertrophy and fibrosis in renal tubular epithelial cells through dual activation with DAP.

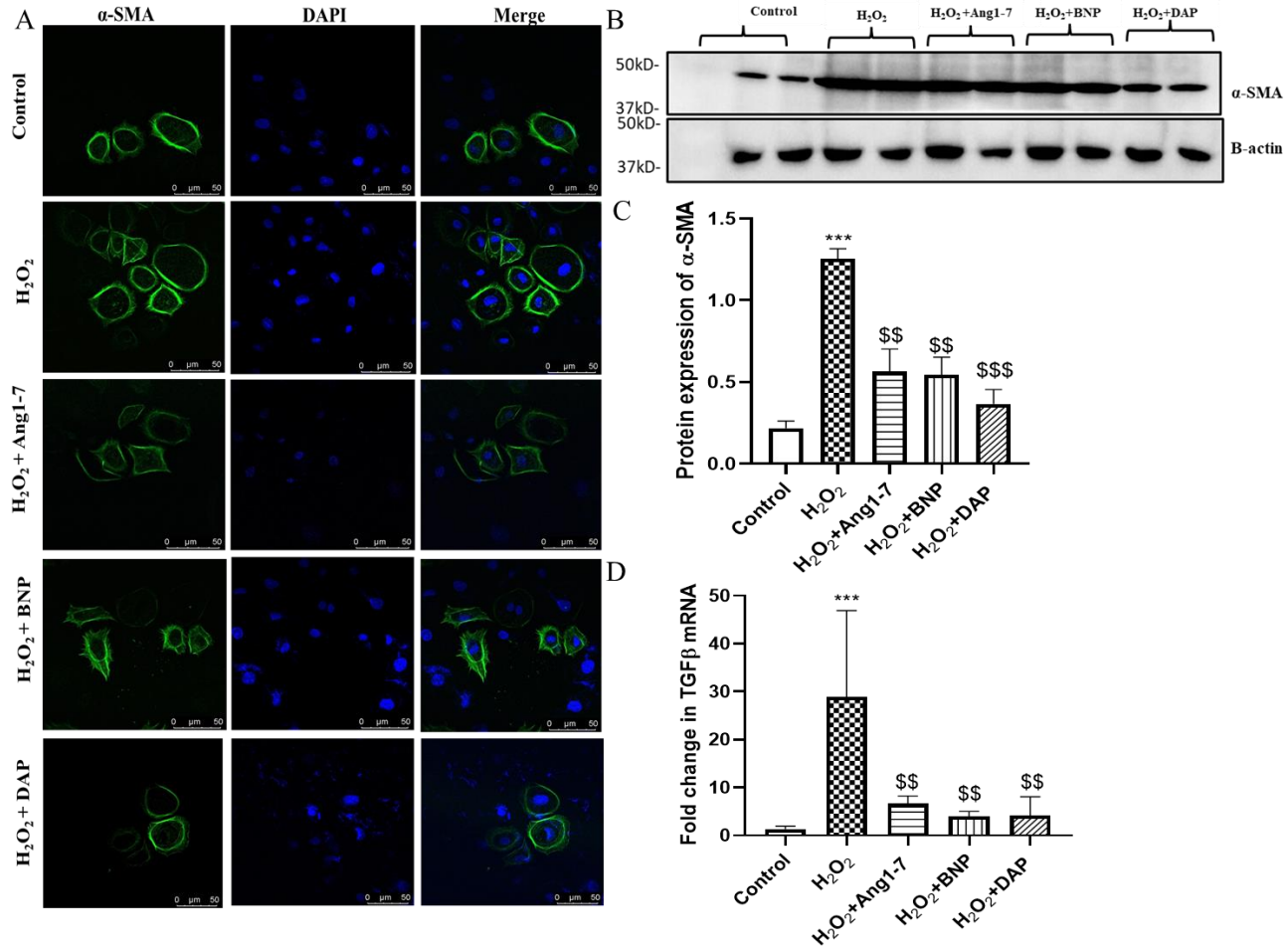



*Figure 4. 9: Protective Effects of Dual Receptor Activation against $H_2O_2$-Induced Fibrosis Renal Epithelial Tubular NRK52E Cells*

NRK52E cells in culture were exposed to $H_2O_2$ (100 µM) for 24 hours, followed by incubation with peptides Ang1-7, BNP, and DAP (1 µM) for 30 min. (A) Representative confocal microscopy images of the fibrosis marker α-SMA, scale bar 50 µm N = 3. (B) Representative western blot images of α-SMA. (C) Quantification of α-SMA protein expression from images in B, N = 4. (D) TGF-β mRNA expression, indicative of fibrosis, was measured. N = 5. Data were expressed as mean ± SEM. Data were analysed using ordinary one-way ANOVA followed by Bonferroni multiple comparison test, where ***$p < 0.001$ *vs* control, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ *vs* $H_2O_2$.

### 4.4.10 Dual activation shields renal tubular epithelial cells from $H_2O_2$-induced oxidative stress

In both physiological and pathological conditions, the interplay of ROS and apoptosis holds crucial importance. To discover the impact of dual receptor activation on ROS generation and apoptosis in disease progression, we employed confocal microscopy and flow cytometric analysis. Our observations revealed that $H_2O_2$ significantly upregulated ROS levels compared to the control group. However, as shown in Figure 4.10A-4.10C, treatment with peptides Ang1-7, BNP, and DAP substantially mitigated ROS generation in NRK-52E cells induced by $H_2O_2$. As a standard, we used N-acetyl cysteine (NAC), a well-known antioxidant, for a better understanding of antioxidant properties. The effect of DAP on ROS reduction is similar to that of the standard molecule NAC in $H_2O_2$-induced NRK-52E renal epithelial cells, confirming the antioxidant efficacy of DAP. These findings underscore the promise of dual receptor activation in attenuating $H_2O_2$-induced ROS.

### 4.4.11 Dual activation exerted a mitigating effect by reduction in nitrite and nitrotyrosine levels in NRK52E

The increased production of ROS in renal tubular epithelial cells contributes to an increase in nitric oxide levels. Nitric oxide, recognized as a reactive nitrogen species, gives rise to the metabolite nitrite, which can be quantified using the Griess assay in biological solutions. As illustrated in Figure 4.10D, both pre- and post-treatment with peptides resulted in a reduction of elevated nitrite levels in cells stimulated by $H_2O_2$.

$H_2O_2$ and peroxynitrite examples of non-radical ROS production, in turn, modify protein tyrosine residues, forming 3-nitrotyrosine a biomarker indicative of peroxynitrite presence in tissues and blood. Using confocal microscopy to investigate the nitrotyrosine metabolite marker, Figure 4.10E illustrates that dual activation significantly attenuates nitrotyrosine levels in the post-treated peptide group. These findings underscore the antioxidant properties of DAP in modulating ROS levels.

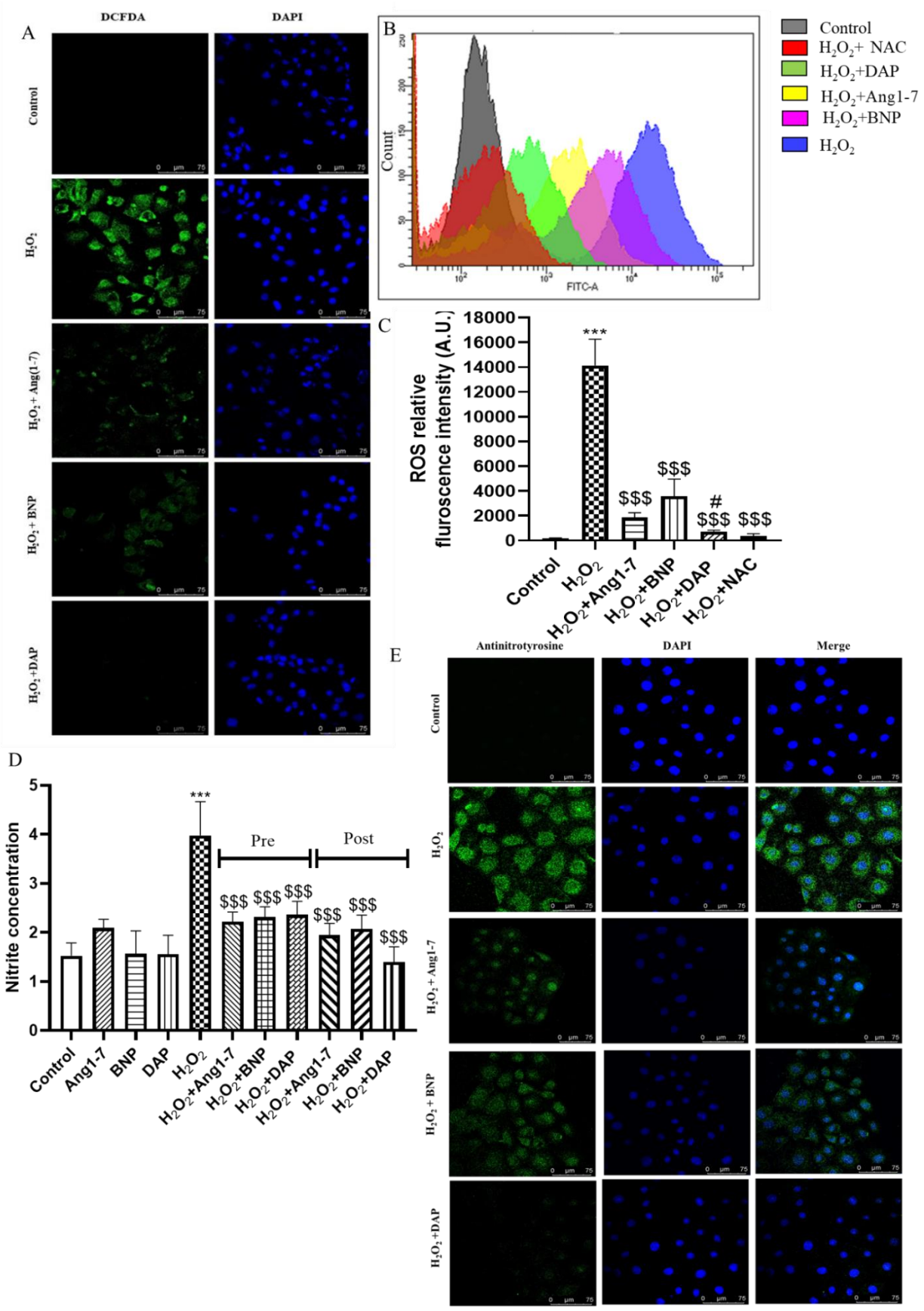



Figure 4. 10: Dual activation of the target receptor confers protection against $H_2O_2$-induced oxidative stress in rat NRK52E cells

(A) Representative confocal images illustrate ROS production using the DCFDA assay, scale bar 75 µm. (B) Additionally, ROS levels were analysed by FACS analysis. Cells were pre-treated with the ROS scavenger NAC (600 µM) for 24 hours before exposure to $H_2O_2$. (C) Quantification of ROS relative fluorescence intensity from image B. N = 3. (D) Griess reagent assay was conducted on both pre-and post-treated NRK52E cells to determine nitrite levels. N = 6. (E) Representative confocal images show Antinitrotyrosin staining in post-treated cells, scale bar 75 µm. Data were expressed as mean ± SEM. Data were analysed using ordinary one-way ANOVA followed by Bonferroni multiple comparison test, where ***$p < 0.001$ *vs* control, $^{\$\$\$}p < 0.001$ *vs* $H_2O_2$, $^{\#}P < 0.05$ *vs* $H_2O_2$ + BNP.

### 4.4.12 Dual activation confers protection to renal tubular epithelial cells against $H_2O_2$-induced apoptosis

We used FITC conjugated annexin labelling to recognize $H_2O_2$-induced apoptotic cells and FITC conjugated Annexin identified apoptotic cells after evaluating the effect of dual activation on $H_2O_2$-induced apoptosis in NRK-52E cells. Notably, in comparison to the control group, $H_2O_2$ stimulation led to a marked increase in apoptotic cells. On the other hand, co-treatment with 1 µM of DAP, BNP, and Ang1–7 peptides showed a significant decrease in the number of FITC-positive apoptotic cells in $H_2O_2$-induced NRK52E cells (Fig. 4.11A and 4.11B). To corroborate the anti-apoptotic impact of peptides on $H_2O_2$-induced renal epithelial cell apoptosis, we used qPCR to analyse the caspase-3 gene expression. Peptide therapy reduced the $H_2O_2$-induced production of caspase-3, as shown in Figure 4.11C. Furthermore, we found that DAP was noticeably more effective than Ang1-7 and BNP. These results imply that simultaneous activation with DAP outperforms the protective effects of Ang1-7 and BNP in reducing the damage caused by $H_2O_2$-induced apoptosis in NRK-52E renal epithelial cells.

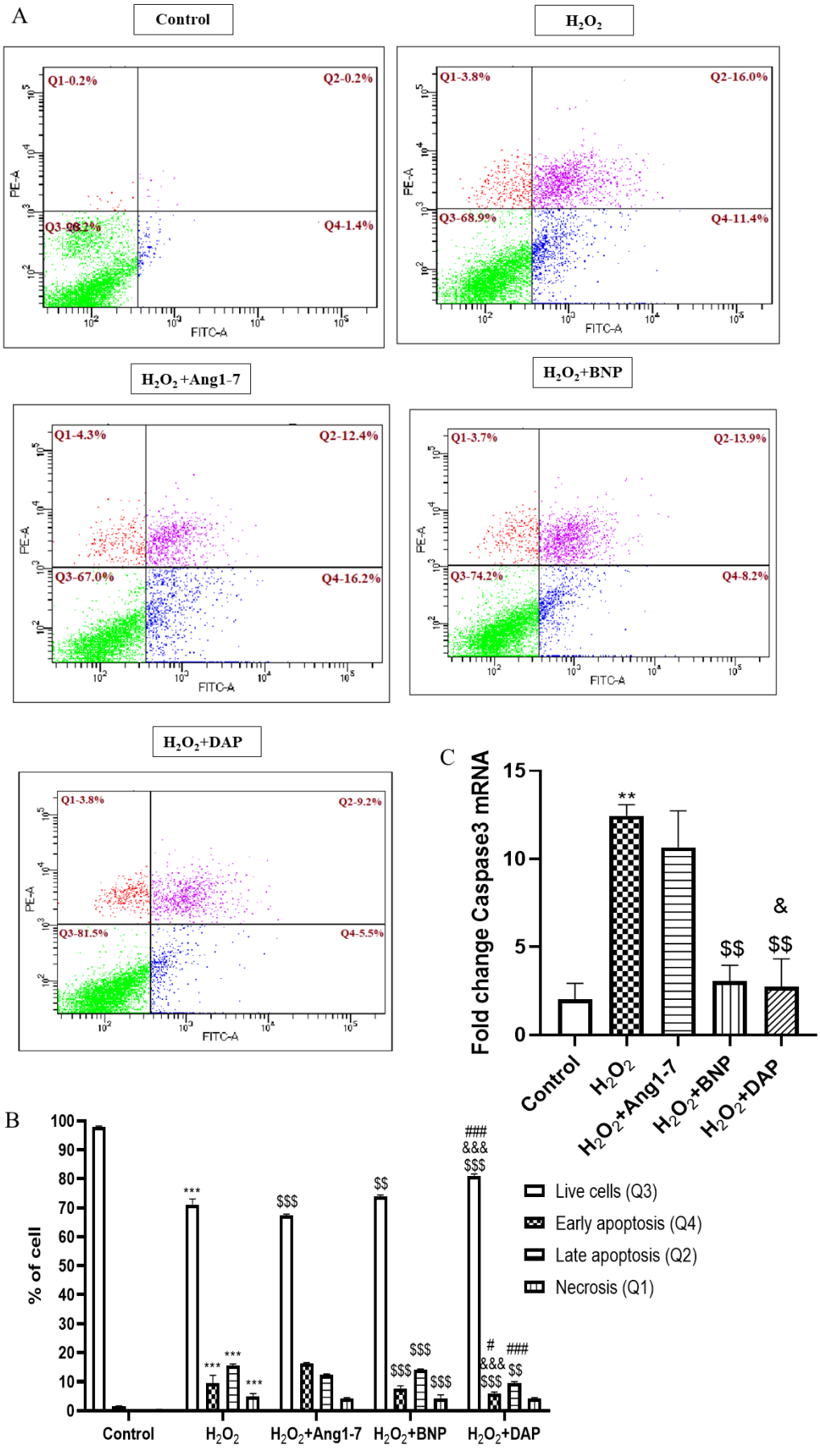

A
Control
Q1-0.2%
Q2-0.2%
Q3-98.2%
Q4-1.4%
H2O2
Q1-3.8%
Q2-16.0%
Q3-68.9%
Q4-11.4%
H2O2+Ang1-7
Q1-4.3%
Q2-12.4%
Q3-67.0%
Q4-16.2%
H2O2+BNP
Q1-3.7%
Q2-13.9%
Q3-74.2%
Q4-8.2%
H2O2+DAP
Q1-3.8%
Q2-9.2%
Q3-81.5%
Q4-5.5%
PE-A
FITC-A
C
Fold change Caspase3 mRNA
Control
H2O2
H2O2+Ang1-7
H2O2+BNP
H2O2+DAP
B
% of cell
Live cells (Q3)
Early apoptosis (Q4)
Late apoptosis (Q2)
Necrosis (Q1)

*Figure 4. 11: Dual activation of target receptor protects $H_2O_2$ induced apoptosis in rat NRK52E cells*

Cultured NRK52E cells were treated with $H_2O_2$ (100 μM) for 24 h. After that cell were incubated with peptides Ang1-7, BNP, DAP (1 μM) for 30min. (A) Apoptosis was measured by FACS analysis using Annexin-IV assay kit N = 3 for each group. (B) Quantification of apoptosis from images in A (C) Apoptotic marker caspase-3 mRNA expression N = 4. Data were expressed as mean ± SEM. Data were analysed using ordinary one-way ANOVA followed by Bonferroni multiple comparison test, where **p < 0.01, ***p < 0.001 *vs* control, $^{\$\$}$p < 0.01, $^{\$\$\$}$p < 0.001 *vs* $H_2O_2$, $^{\&}$p < 0.05 *vs* $H_2O_2$ + Ang1-7, $^{\#}$p < 0.05, $^{\#\#\#}$p < 0.001 *vs* $H_2O_2$ + BNP.

### 4.4.13 Inhibition of Mas and pGCA mitigates the impact of dual activation

Cultured renal tubular epithelial cells were subjected to specific inhibitors for MasR (A779) and pGCAR (KT5823) before peptide treatment for 30 min in $H_2O_2$-stimulated cells. In the presence of A779, both BNP and DAP significantly increased the mRNA expression of pGCAR. Conversely, in the presence of KT5823, no change in the mRNA expression of pGCAR was observed (Figure 4.12A). Figure 4.12B illustrates that there was no change in the mRNA expression of MasR in the presence of A779, but a significant increase in MasR mRNA expression was observed with Ang1-7 and DAP in the presence of KT5823. Additionally, there was no significant change in AT1R mRNA expression in $H_2O_2$-treated cells with inhibitors of MasR and pGCAR receptors (Figure 4.12C and 4.12D).

Collectively, this data suggests that blocking these receptors diminishes the effect of DAP, indicating that the protective effect of DAP may be mediated via MasR and pGCAR. Interestingly, the effect of DAP may also be involved in AT1R blockade. Further molecular studies are necessary to elucidate the detailed mechanism of action of DAP.

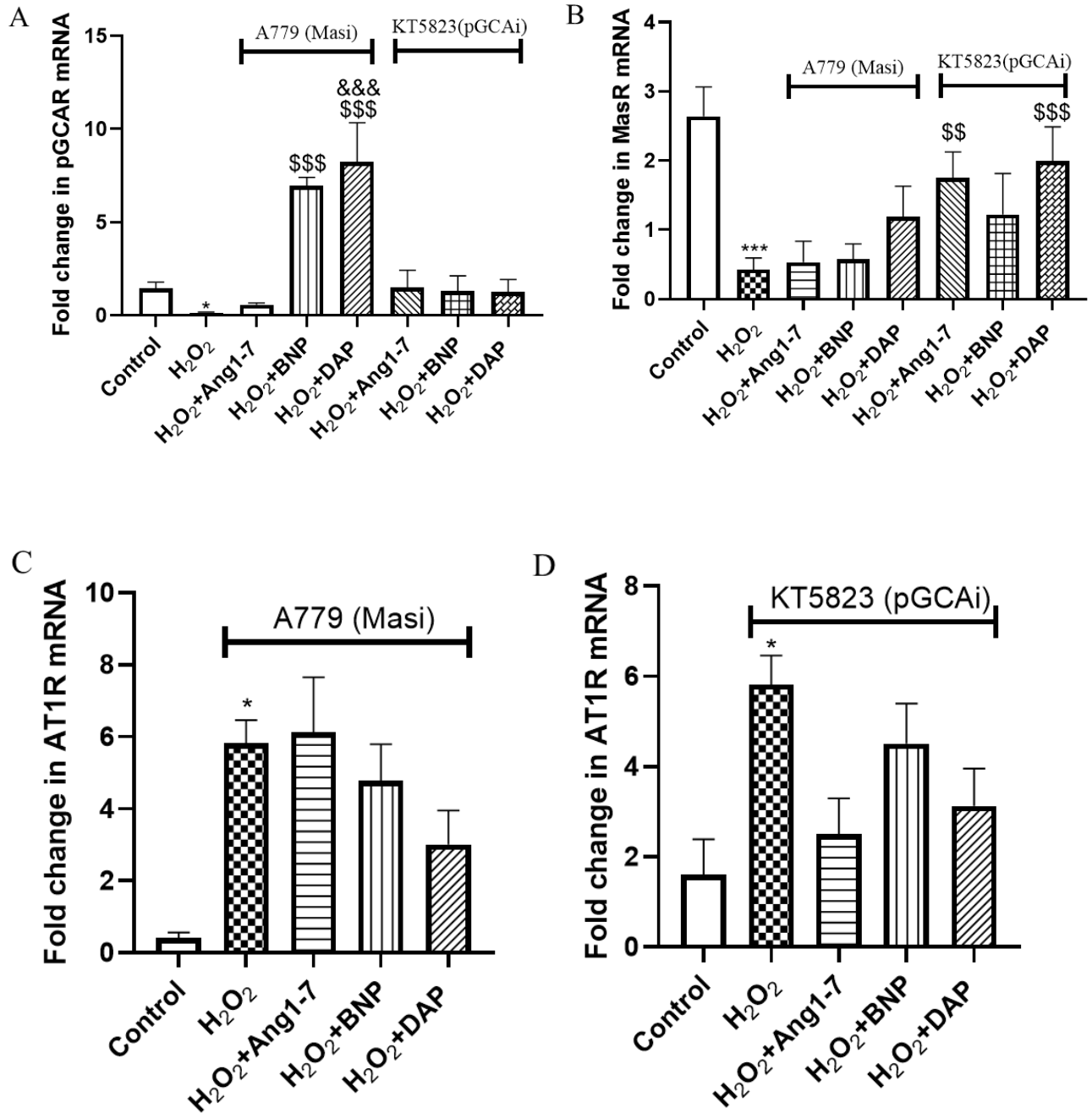



*Figure 4. 12: Inhibition of Mas and pGCA receptors blocks the DAP activity*

(A) pGCAR mRNA expression when MasR and pGCAR inhibitors are present. N = 4. (B) MasR's mRNA expression when pGCAR antagonist and MasR are present. N = 4. (C) AT1R mRNA expression when MasR antagonist is present, N = 4. (D) AT1R mRNA expression when pGCAR antagonist is present. N = 4. Data were expressed as mean ± SEM. Data were analysed using ordinary one-way ANOVA followed by Bonferroni multiple comparison test, where *p < 0.05, ***p < 0.001 *vs* control, $^{\$\$}$p < 0.01 and $^{\$\$\$}$p < 0.001 *vs* $H_2O_2$, $^{\&\&}$p < 0.001 *vs* Ang1-7.

### *4.5 Discussion*

In this work, using cultured renal tubular NRK-52E epithelial cells, we investigated the protective properties of DAP via the co-activation of Mas and pGCA receptors. Surprisingly, we found that DAP demonstrated significant protective effects in addition to activating Mas and pGCA receptors separately. Interestingly, the effects of dual activation by DAP were reduced when specific inhibitors were used to blockade Mas and pGCA receptor. Furthermore, we found that AT1 receptor inhibition is a necessary component of DAP therapeutic efficacy.

DAP, with a purity of 95 %, along with its parent peptides BNP and Ang1-7, served as control molecules in this study. At first, we established an *in-vitro* model of oxidative stress by exposing renal NRK-52E epithelial cells to 100 µM $H_2O_2$ for various time points, we observed a significant upregulation of AT1 receptor and a decrease in Mas and pGC-A receptor mRNA expression at 24 hours. Additionally, we found 100 % cell viability in cells exposed to individual peptides, Ang1-7, BNP, and DAP. Peptides demonstrated a significant upregulation of Mas and pGCA receptors at 30 min compared to other time points. Subsequently, we studied the effects of DAP on pGC-A and Mas receptor expression *in-vitro* in renal tubular epithelial cells in the presence of $H_2O_2$, revealing significant upregulation of both protein and mRNA expression after DAP treatment, surpassing the effects of Ang1-7 and BNP alone (Fig. 4.5). Surprisingly, DAP treatment is promising in reducing the RAAS mediator and inflammatory markers mRNA and protein levels.

The octapeptide AngII is the main cause of RAAS-mediated cardiovascular diseases. According to Arndse et al., (165). AngII actions in the classical axis are mainly mediated by AT1R, which causes elevated oxidative stress, vasoconstriction, increased aldosterone secretion, and activation of the inflammatory and apoptotic pathways. On the other hand, AT1R actions are neutralized by the protective axis of RAAS, ACE2/Ang1-7/MasR. The homolog of the ACE enzyme, ACE2, cleaves AngII to form Ang1–7, which causes vasodilation and has been shown to have anti-inflammatory, anti-fibrotic, and anti-proliferative properties through its proto-oncogene Mas receptor (205,206). Recent research suggests that Ang1-7 may function as a biased agonist to AT1R via β-arrestin to provide protective effects, but more research is required in this area (207). Experimental models of CVD have been the target of extensive studies on MasR agonists, including AVE-0991, CGEN-856, and CGEN-857 (208). Approaches based on the natriuretic peptide that target pGC-A and the resulting downstream cGMP signalling have been shown to have a protective effect on the kidneys. PKG is stimulated by native ligands such as ANP and BNP, which act through membrane-bound G protein-

coupled pGC-A receptors to activate the second messenger, cGMP. Using NPs to target pGC-A appears to be a promising treatment approach for cardio protection (209). Additionally, NP/pGCAR/cGMP has demonstrated a promising protective effect in experimental animal models of cardiovascular diseases (210). Novel pGC-A activators, such as C53, have been shown to have anti-fibrotic properties *in-vivo* (211). However, current therapies primarily target the classical RAAS axis. To address the complexities of RAAS-mediated CVDs, emerging therapies have become more focused on activating the ACE2/Ang1-7/MasR and NP/NO/cGMP pathways.

When renal epithelial cells received stimulation with $H_2O_2$, DAP showed superior anti-hypertrophic, anti-fibrotic, and anti-apoptotic effects. Particulate guanylyl cyclase receptors (pGC-A and pGC-B) are the targets of ANP and BNP. BNP treatment has previously been shown to reduce mortality and hospitalization duration in patients with chronic heart failure and impaired left ventricular systolic function (212). BNP treatment protects cardiomyocytes from apoptosis in the context of ischemia/reperfusion injury by inhibiting the opening of the mitochondrial calcium uniporter. BNP was also found to increase anti-apoptotic Bcl-2 protein levels while decreasing pro-apoptotic Bax and Smac expression. BNP acted via mitochondrial pathways, which were partially dependent on PI3K/Akt (213). Exogenous BNP treatment was also found to reduce TGFβ pro-fibrotic effects on mechanically stretched fibroblasts (214). Long-term BNP administration in rats has been shown to improve cardiac hypertrophy by decreasing TGFβ and increasing Smad7 (198).

Angiotensin1-7, a vasoactive peptide within the RAAS, is primarily produced by ACE2 and exerts its effects via the Mas receptor. Ang1-7 affects both the cardiovascular and renal systems. Ang1-7 treatment has been shown to significantly reduce ischemia/reperfusion-induced cardiac arrhythmias in isolated rat hearts (215). Numerous studies have found that Ang1-7 and other Mas receptor agonists, such as AVE0991 and CGEN-856S43, have anti-remodeling properties (216). Furthermore, Ang1-7 treatment has been shown to improve AngII-induced cardiac hypertrophy (217). In contrast, in the DOCA-salt hypertension model, Ang1-7 treatment reduced cardiac fibrosis without affecting blood pressure or hypertrophy (218,219). The Ang1-7 /MAS axis has also been shown to inhibit apoptosis in alveolar epithelial cells by increasing the activity of MAP kinase phosphatase 2, which dephosphorylates JNK and prevents apoptosis (219). Furthermore, in an acute lung injury model in mice, Ang1-7 has been shown to reduce lung fibrosis via the Mas receptor (220). While both BNP and Ang1-7 have protective effects in various disease models, their impact is

limited in cardiorenal disease due to limited bioavailability. Interestingly, the therapeutic efficacy of synthesized DAP outperforms the effects of individual receptor activation by Ang1-7 and BNP. This emphasizes the importance of combining two protective receptor activations and suggests that designer peptides may avoid degradation, promoting synergistic action through fusion.

To investigate the underlying molecular mechanism, $H_2O_2$-induced NRK-52E renal epithelial cells were incubated with the Mas receptor inhibitor A779 and the pGC-A receptor inhibitor KT5823. In the presence of KT5823, there was no change in the mRNA expression of the pGC-A receptor in $H_2O_2$-induced renal epithelial cells. In the presence of A779, however, there was a significant upregulation of pGC-A receptor mRNA in $H_2O_2$-induced renal epithelial cells. In contrast, A779 did not affect the mRNA expression of the Mas receptor in $H_2O_2$-induced renal epithelial cells. However, when KT5823-treated cells were exposed to $H_2O_2$, receptor expression increased. These findings indicate that DAP activity was reduced in the presence of both receptor inhibition. In addition, we explored AT1R mRNA expression to learn more about the molecular mechanism of DAP. The outcomes displayed that in the presence of inhibitors, there was a partial decrease in AT1R mRNA expression compared to the $H_2O_2$-induced group. This implies that DAP activity may involve the blocking of AT1R in part. However, additional research is needed to fully understand the role of other downstream markers.

$H_2O_2$ caused a significant increase in oxidative stress in renal epithelial cells. However, when $H_2O_2$-induced cells were co-treated with Ang1-7, BNP, and DAP, the levels of ROS were significantly reduced. Notably, the reduction was significantly greater in the DAP group compared to Ang1-7 and BNP alone. The noteworthy effect on nitrate and nitrotyrosine levels highlights DAP pleiotropic action on the NO/cGMP pathway. This critical pathway is emerging as a novel therapeutic target for cardiovascular and renal complications. The activation of membrane-bound pGCAR is thought to be responsible for DAP protective activity. However, a more in-depth investigation is required to fully comprehend the mechanism of action.

Current cardiorenal disease treatment approaches, which primarily rely on single-pathway activation, fall short of addressing associated secondary complications. Sacubitril/valsartan LCZ696, a combination of a neprilysin inhibitor and an angiotensin receptor blocker, has opened the way for new drugs that target multiple pathways and receptors. By simultaneously inhibiting both pathways, LCZ696 has shown promising effects in conditions such as hypertension, myocardial infarction, and heart failure (152,221).

Cenderitide (CD-NP) is another known example of a peptide that targets multiple pathways. CD-NP, which was created to combine the benefits of CNP and DNP, co-activates both pGC-A and pGC-B receptors (222). CD-NP, a dual-activating peptide, outperformed CNP in terms of efficacy by increasing the secondary messenger cGMP (159). As a result, ongoing research is looking into therapeutic strategies that target pGC-A receptors, increase cGMP activity, and activate MasR, which counteracts AT1R effects.

An imbalance in natriuretic peptides and the RAAS are implicated in cardio-renal diseases. Therapies that target pGCAR and Mas are critical in restoring the balance between these two classic targets. While previous research has shown that activating individual pathways improves cardiac and renal function, our study focuses on the protective effects of a dual-acting peptide that co-targets both MasR and pGCAR in cultured renal epithelial tubular cells stimulated with $H_2O_2$. The DAP significantly increased the expression of both Mas and pGCA receptors. Notably, DAP outperformed native peptides that activate Mas and pGCAR individually, demonstrating anti-hypertrophic, anti-fibrotic, anti-hypertensive, and anti-apoptotic properties. Our findings suggest that the synthesized dual-acting peptide has greater efficacy and protective properties than peptides targeting individual pathways. While these findings are encouraging, more research is needed to determine the molecular mechanism underlying DAP action. This research lays the groundwork for future research into the role of AT1R and its downstream effects in DAP protective actions.

## *4.6 Conclusion*

Dual activation emerges as a novel therapeutic approach for dealing with cardio-renal complications. Endogenous parent peptides of the Ang1-7 and BNP, show promise for alleviating the severe secondary complications associated with oxidative stress in cardiovascular disease. However, the results of this study strongly suggest that DAP outperforms individual peptide treatments. Our findings will be useful in future research aimed at designing and developing dual-acting therapeutics for the treatment of cardiovascular diseases. DAP's superior performance highlights its potential as a more effective option for dealing with the complex challenges posed by cardio-renal complications.

# *Chapter 5*

# *Mas And pGCA Receptor Activation Protects Primary Vascular Smooth Muscle Cells and Endothelial Cells Against Oxidative Stress via Inhibition of Intracellular Calcium*

## *5.1 Graphical abstract*

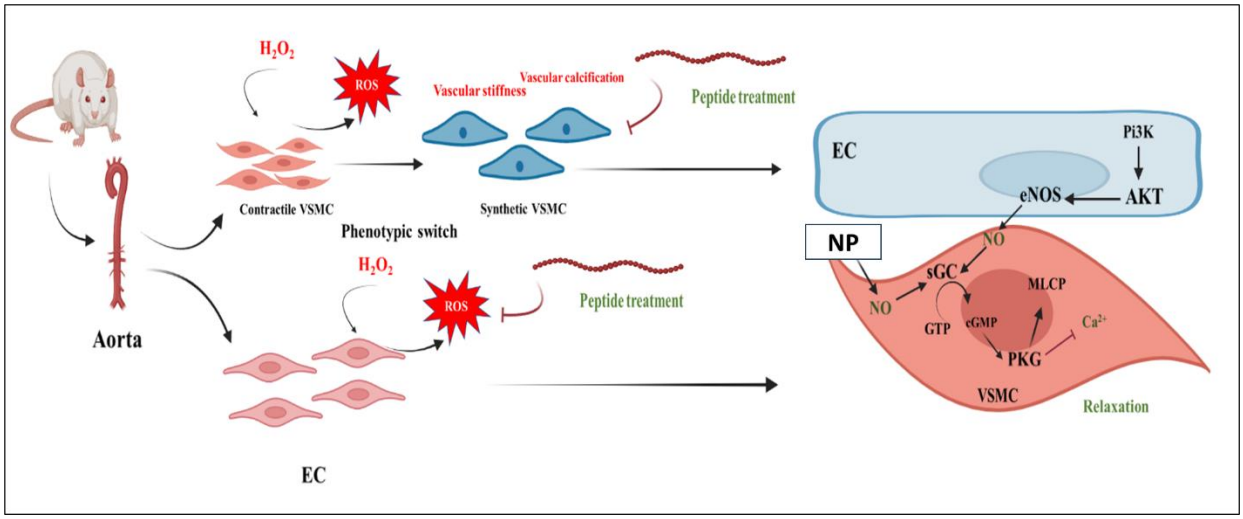



*Figure 5. 1: Graphical abstract of chapter 5*

Were, EC: Endothelial cells, VSMC: Vascular smooth muscle cells.

## *5.2 Introduction*

According to the World Health Organization, CVDs are the leading cause of premature death worldwide. In 2010, 31.1 % of adults, or approximately 1.39 billion people, were reported to have CVDs. By 2019, an estimated 17.9 million people were suffering from CVDs, accounting for approximately 32 % of all global mortality. The rise in CVD prevalence has been attributed to widespread lifestyle changes and poor dietary habits (223). CVDs are most associated with vascular injury and phenotypic switching, both of which contribute to vascular dysfunction and arterial remodeling. As a result, significant chronic complications affecting the heart, kidneys, and blood vessels development (224). Overactivation of the RAAS and the production of oxidative stress are critical in modulating vascular processes, disrupting vascular function, and triggering complications such as vascular hypertrophy, fibrosis, inflammation, calcification, and vasoconstriction (225,226).

Vascular smooth muscle cells and endothelial cells are major constituents of the arterial wall (111), and they play critical roles in regulating vascular tone via vasoconstriction and vasodilation. VSMCs undergo phenotypic changes under severe pathological conditions, transitioning from a contractile to a synthetic state (227). Endothelial cells in the vascular wall release vasoactive agents and produce ROS that regulate the contraction and relaxation processes of VSMCs by influencing nitric oxide bioavailability (228,229). The formation of an actin-myosin cross-bridge is promoted by an increase in intracellular free calcium concentration ($Ca^{2+}$) during the initiation of vascular smooth muscle contraction. However,

previous research has shown that intracellular $Ca^{2+}$ not only regulates contraction but also influences the stiffness and adhesion of VSMCs to the extracellular matrix (ECM) (230).

The prevalence of cardiovascular diseases is growing rapidly, and they are now the leading cause of morbidity and mortality (231). Frontline treatments for CVD include angiotensin-converting enzyme inhibitors and angiotensin receptor blockers that target the RAAS pathway (232). These RAAS blockers cause an increase in plasma renin activity (PRA). An elevated PRA in hypertensive individuals is associated with an increased susceptibility to CVD-related mortality (233). Existing research, however, suggests that these traditional therapies have limited efficacy in preventing the progression of cardiovascular complications. In contrast, ACE and AT1R receptor inhibition may be insufficient in some cases (197). As a result, there is an urgent need for novel therapeutic approaches to effectively manage cardiovascular complications.

The recently discovered ACE2/Ang1-7/Mas pathway is promising for its cardiovascular and renal protective effects (234). The enzyme ACE2 is essential in the synthesis of the Ang1-7 peptide (235). ACE2 is a membrane-bound zinc metalloprotease expressed in a variety of human tissues, including the heart, smooth muscles, testes, lungs, and kidneys (236). Tallant et al.'s research also confirmed the presence of Ang1-7 receptors in bovine aortic endothelial cells, which promote the production of nitric oxide (237,238). Recent research has shown that brief activation of the Mas receptor by Ang1-7 improves endothelial function in normotensive rats (239). ACE2 activation, on the other hand, has been shown to reduce inflammation and improve vascular dysfunction caused by AngII (240).

Conversely, the Natriuretic Peptide/pGCAR/cGMP pathway appears to be a promising target for treating cardiovascular complications. Natriuretic peptides are hormones secreted by the heart that are essential for cardiovascular homeostasis and function. Endogenous ligands for the pGC-A receptor include ANP and BNP (241). The widespread expression of pGC-A in tissues such as the kidney, vasculature, endothelial cells, lung, brain, adrenal, and adipose tissues emphasize its importance (242). Previous investigation has shown that activating the Natriuretic Peptides/pGC-A pathway increases the production of the secondary messenger cGMP while decreasing cAMP, calcium, and inositol triphosphate (IP3) levels in target cells (243). Surprisingly, the cGMP/PKG signalling pathway promotes cell proliferation and increases permeability in arterial endothelial cells (ECs). In vascular smooth muscle cells, on the other hand, the cGMP/PKG pathway inhibits cell proliferation while mediating vasorelaxation (244). This complex interplay within the Natriuretic Peptide/pGC-A pathway

provides a diverse landscape for investigating therapeutic strategies in the field of cardiovascular health.

In this study, synthesized novel peptide (NP) by fusing BNP and Ang1-7 amino acid sequences. NP is intended to activate two or more pathways at the same time, outperforming the impact of individual pathway activation. This breakthrough paves the way for the development of novel peptide-based therapeutics that are both effective and safe (21). This approach was inspired by the notable success of LCZ696 in treating heart failure. LCZ696, a dual inhibitor that combines an ACE inhibitor (sacubitril) and an ARB (valsartan), has shown promising therapeutic potential (245). Furthermore, the CRRL269 peptide has been shown to activate both pGCA and pGCB receptors (204). In this study, we propose that dual receptor activation could improve vascular injury repair by targeting both the Mas and pGCA receptor pathways in primary VSMCs. In addition, we perform a comparison of NP with its parent peptides. Primary rat aortic endothelial cells were isolated and used in our study to investigate the underlying molecular mechanisms. This study aims to shed light on the potential therapeutic benefits of NP while also contributing to our understanding of dual receptor activation in vascular health.

### *5.3 Material and methods*

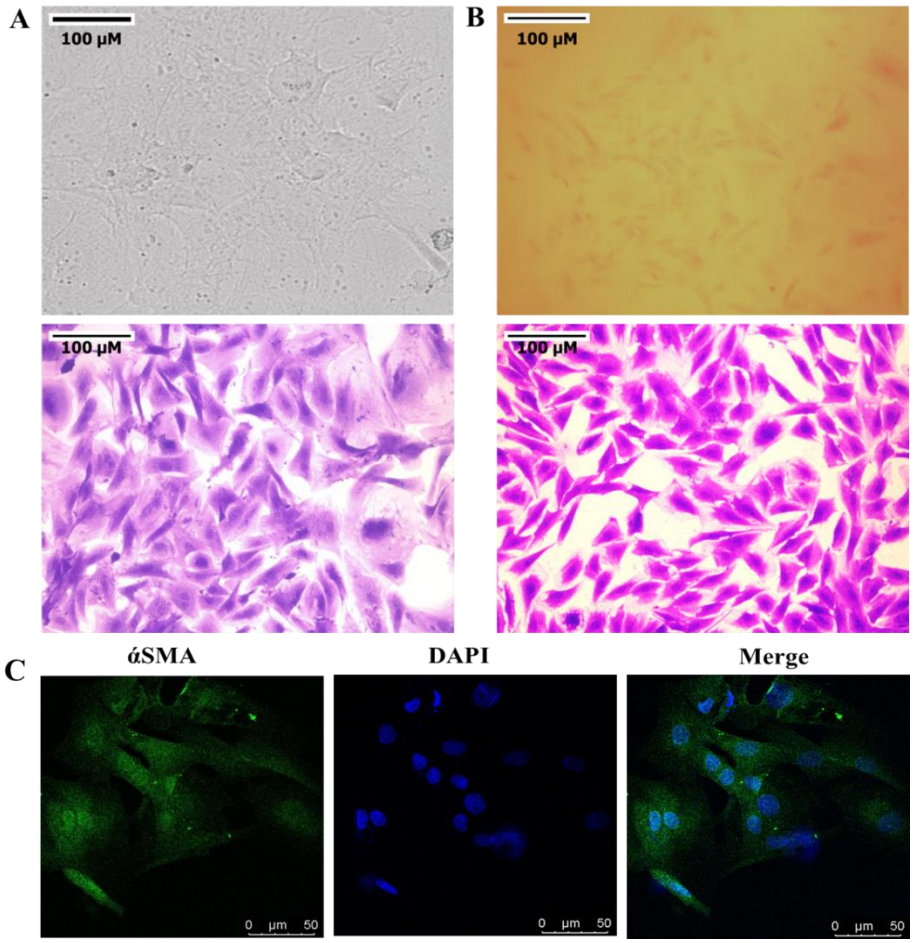
In the experimental studies we used Wistar rats, aged eight weeks and weighing 180-220 gm. Primary aortic VSMCs and EC were isolated and characterized through immunocytochemistry staining with α-SMA and eNOS antibody respectively. The research protocol for using the animals was ethically approved by the IAEC under protocol no BITS-Hyd-IAEC-2022-08. Confluent VSMCs and ECs up to passages 3–8 were utilized for further experiments. Subsequently, an oxidative stress model was induced in primary cells through exposure to $H_2O_2$ in the presence or absence of Ang1-7, BNP, and NP. Detailed protocols for each procedure can be found in Chapter 3, Method Section. The experiments included cell viability assessments, qPCR, Western blotting, measurement of ROS, Griess reagent assay, immunofluorescence and confocal microscopy, crystal violet staining, vascular calcification analysis, and intracellular calcium estimation. Statistical analysis was conducted using ANOVA followed by post hoc Bonferroni's test, with a significance level set at $p < 0.04$.

## *5.4 Results*

### 5.4.1 Isolated primary rat VSMCs and ECs characterization and identification

The primary goal of isolating primary VSMCs and ECs from Wistar rats was to characterize these cell types. The presence of essential contractile markers such as α-SMA, calponin, and smooth muscle myosin heavy chains (SM-1 and SM-2) was used to assess the contractile phenotype of primary VSMCs. These markers are critical in regulating blood vessel contraction and relaxation, allowing for changes in luminal diameter and the maintenance of normal blood pressure. The immunofluorescence staining in Figure 5.2C shows the expression of the contractile marker α-SMA, highlighting the isolated VSMCs' spindle-shaped and elongated morphology (Figure 5.2A).

The isolated primary ECs were then characterized. Figure 5.2D shows representative immunofluorescence staining images that show the distinctive cobblestone shape of ECs (Figure 5.2B) as well as the expression of the endothelial nitric oxide synthase (eNOS) marker. eNOS is a key player in influencing vascular health and disease because it is intricately involved in modulating blood flow and vascular remodeling.



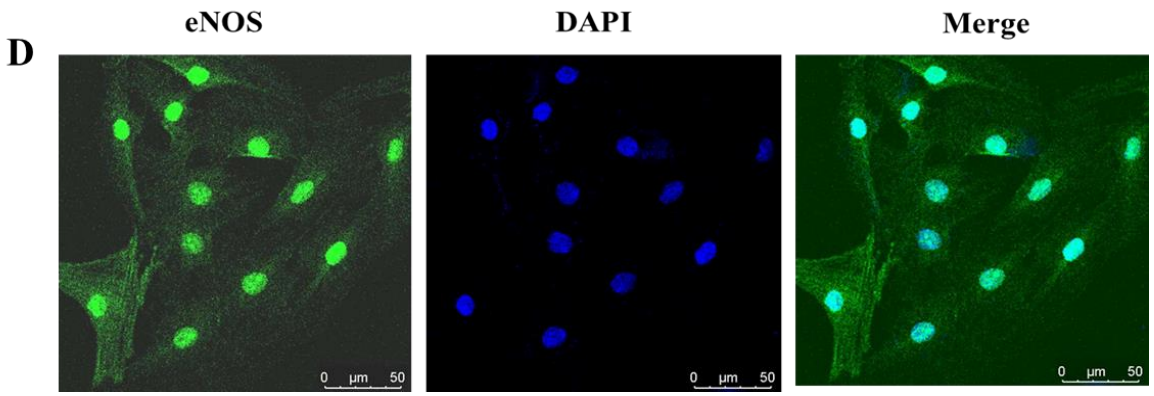



*Figure 5. 2: Characterization of VSMC and EC by immunocytochemistry of specific markers (A) Upper panel represents brightfield photographs of VSMC and lower panel represent the crystal violet staining for morphology (B) Upper panel represents brightfield photographs of EC and lower panel represent the crystal violet staining for morphology (C) Representative confocal images of contractile marker α-SMA in VSMC, Scale bar: 50 µm (D) Representative confocal images of contractile marker eNOS in EC, Scale bar: 50 µm.*

### 5.4.2 Effect of $H_2O_2$'s on viability of VSMC

Primary VSMCs were subjected to $H_2O_2$ (100 µM) treatment at various time intervals to assess the effect on cell viability. The MTT assay was used to assess cell viability at 1, 3, 6, 12, and 24 hours after treatment. The viability of VSMCs decreased noticeably as $H_2O_2$ exposure increased. Remarkably, cell viability was reduced to 85 % after 24 hours of exposure to 100 µM $H_2O_2$, as shown in Figure 5.3A.

### 5.4.2 Target receptor expression analysis in $H_2O_2$-treated cells

In our study of the *in-vitro* cell model, we looked at the mRNA expression of AT1R, MasR, and pGCAR in response to $H_2O_2$ to assess target receptor expression and identify markers associated with CVD progression. Figure 5.3B shows a significant increase in the mRNA expression of the CVD marker AT1 receptor after 24 hour of $H_2O_2$ exposure versus the control. Furthermore, Figures 5.3C and 5.3D demonstrated that prolonged $H_2O_2$ exposure reduces mRNA expression of MasR and pGCAR. As a result, all subsequent experiments were carried out with $H_2O_2$ at a concentration of 100 µM for 24 hours.

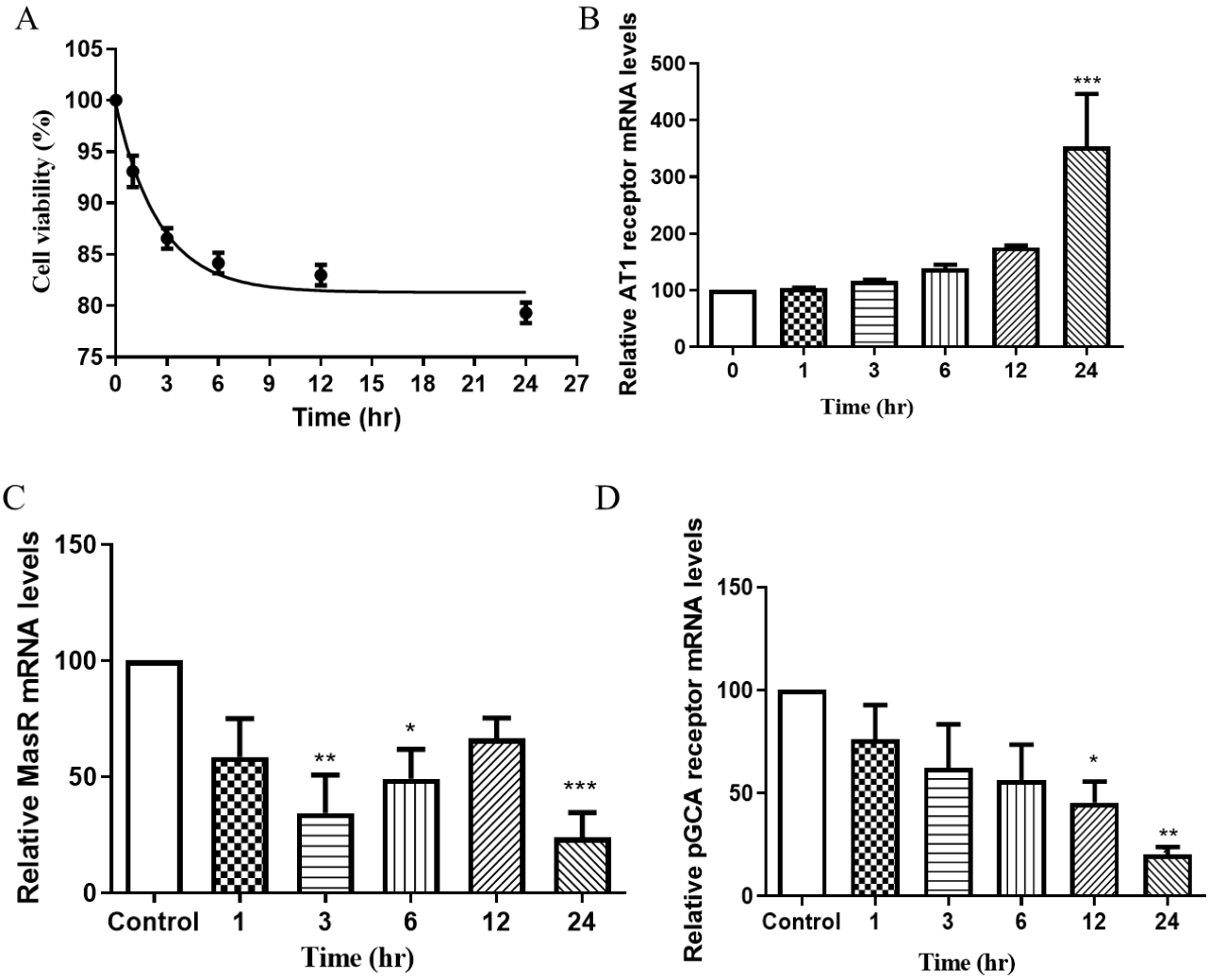



*Figure 5. 3: Establishment of $H_2O_2$ induced oxidative stress model in VSMC cells*

(A) MTT assay for cell viability in cultured rat aortic VSMC incubated with normal culture medium (control) or medium containing $H_2O_2$ (100 µM) for 1, 3, 6, 12 and 24 hr. N = 6 (B) Time-dependent mRNA expression of CVD marker AT1R in $H_2O_2$ stimulated VSMC. N = 5 (C) Time-dependent mRNA expression of MasR (D) Time-dependent mRNA expression of pGCAR. N = 5, Data are presented as mean ± SEM. Statistical analysis was performed via one-way ANOVA paired with Bonferroni's comparison. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. control.

### 5.4.3 Dual activation improves target receptor expression

Previous research has shown that individually activating the target receptors MasR and pGCAR can protect the heart and kidneys. Using qPCR, we investigated the effect of peptide treatment on target receptor expression in $H_2O_2$-treated VSMCs. Figures 5.4A and 5.4B show that treatment with the peptide increased the mRNA expression of MasR and pGCAR mRNA in

cells exposed to $H_2O_2$. We performed western blot analysis to reconfirm these findings, as shown in Figures 5.4C and 5.4D and found that dual activation increased the protein expression of Mas and pGCA receptors in $H_2O_2$-treated cells. Our collective observations suggest that NP treatment is responsible for the upregulation of protective pathways, proving to be more effective than the individual receptor activation.

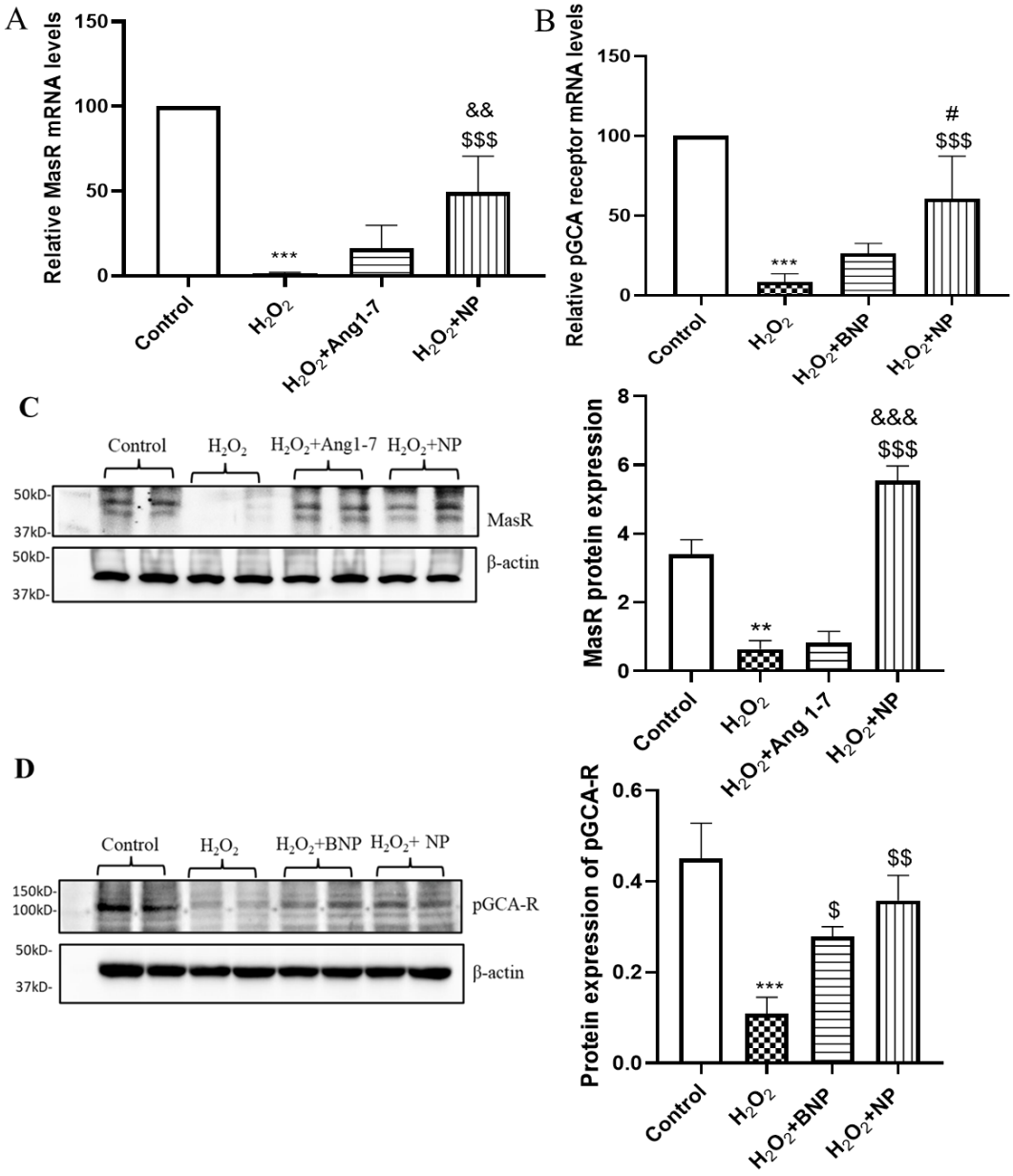



*Figure 5. 4: Dual Activation Improves Mas and pGCA Receptor mRNA Expression*

VSMC cells were treated with $H_2O_2$ for 24 hr followed by with or without peptide Ang1-7, BNP and NP 30 min incubation. (A) mRNA expression of MasR (B) mRNA expression of pGCA-R in VSMC cells. N = 4. (C) Protein expression of MasR by western blotting (D) Protein expression of pGCA-R by western blotting in $H_2O_2$ treated VSMC cells followed by peptide treatment. All error bars represent ± SEM of four experimental replicates. Statistical analysis

was performed via one-way ANOVA paired with Bonferroni's comparison. **p < 0.01, ***p < 0.001 vs control, $^{\$}$p < 0.05, $^{\$\$}$P < 0.01, $^{\$\$\$}$p < 0.001 vs $H_2O_2$, $^{\&\&}$p < 0.01, $^{\&\&\&}$p < 0.001 vs $H_2O_2$+Ang1-7, $^{\#}$p < 0.05 vs $H_2O_2$+BNP.

### 5.4.4 Dual activation confers protection to VSMCs against $H_2O_2$-induced ROS

The role of ROS in the progression of vascular diseases is critical. As a result, we used FACS analysis to see if simultaneous receptor activation could reduce ROS generation during disease progression. In comparison to the control, $H_2O_2$ significantly increased ROS generation, according to our findings. Notably, peptide treatment with Ang1-7, BNP, and NP reduced ROS generation in $H_2O_2$-induced VSMCs significantly. Furthermore, when compared to the individual treatments with Ang1-7 and BNP, the dual-acting peptide NP showed a more pronounced attenuation of ROS generation as illustrated in figure Fig. 5.5A and 5.5B. For comparison, we included NAC, an antioxidant, as a standard. Surprisingly, the effect of NP on reducing ROS production was nearly equivalent to that of standard NAC in $H_2O_2$-induced VSMCs, highlighting the importance of the antioxidant efficacy of NP. These results suggest that the dual activation of receptors holds promise in mitigating $H_2O_2$-induced ROS.

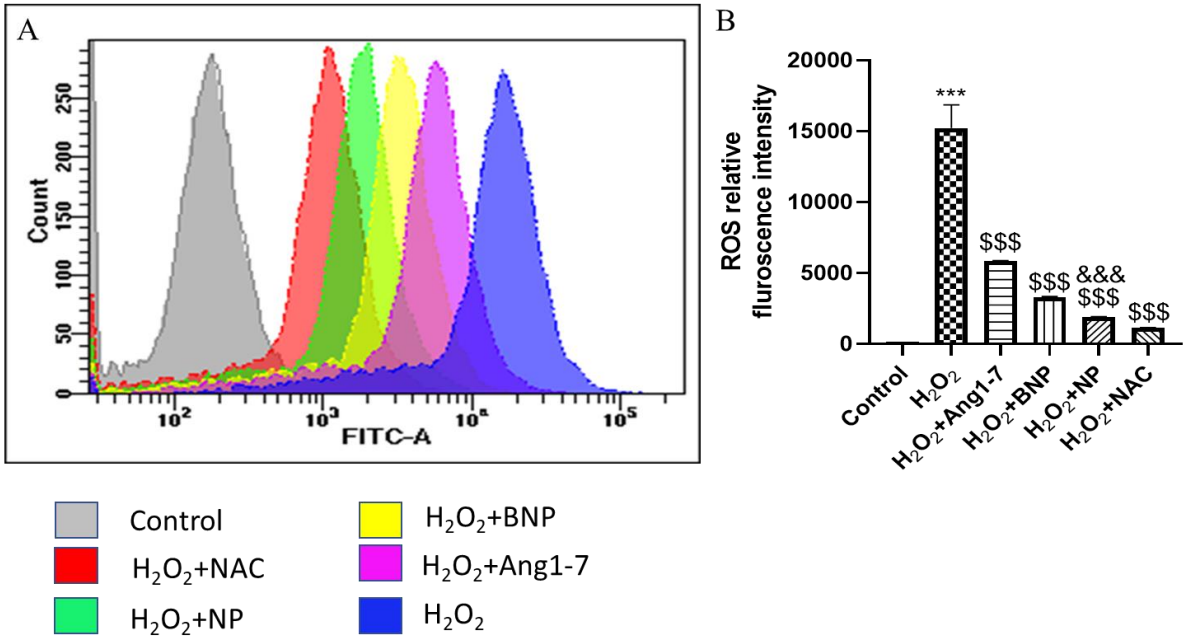



*Figure 5. 5: Dual Activation Confers Protection to VSMCs Against $H_2O_2$-Induced ROS*

(A) Representative images ROS production by FACS analysis (B) Quantification of ROS production by FACS analysis from A. N = 3. Data are presented as mean ± SEM. Statistical analysis was performed via one-way ANOVA paired with Bonferroni's comparison. ***p < 0.001 vs control, $^{\$\$\$}$p < 0.001 vs $H_2O_2$, $^{\&\&\&}$p < 0.001 vs $H_2O_2$+Ang1-7.

### 5.4.5 Dual activation mitigates the impact of $H_2O_2$ on cardiovascular disease markers and inflammatory indicators

Previous research has shown that oxidative stress plays an important role in the initiation of CVD and inflammation pathways. Considering this, we examined the expression of AT1 receptor mRNA and protein to see how CVD progressed. Dual activation with NP effectively suppresses the $H_2O_2$-induced expression of the cardiovascular marker AT1R as illustrated in Fig. 5.6A. To investigate the impact of dual activation on the inflammatory process further, we used qPCR to detect specific inflammatory markers such as TNF-α and IL-6. Remarkably, the data show that NP downregulates the mRNA levels of TNF-α and IL-6 as shown in Fig. 5.6B and 5.6C, implying that simultaneous receptor activation holds promise in reducing the $H_2O_2$-induced progression of inflammation. In conclusion, NP successfully diminishes the $H_2O_2$-induced expression of AT1 receptors and inflammatory markers. Additionally, NP demonstrates greater efficacy compared to the activation of individual pathways with Ang1-7 and BNP peptides.

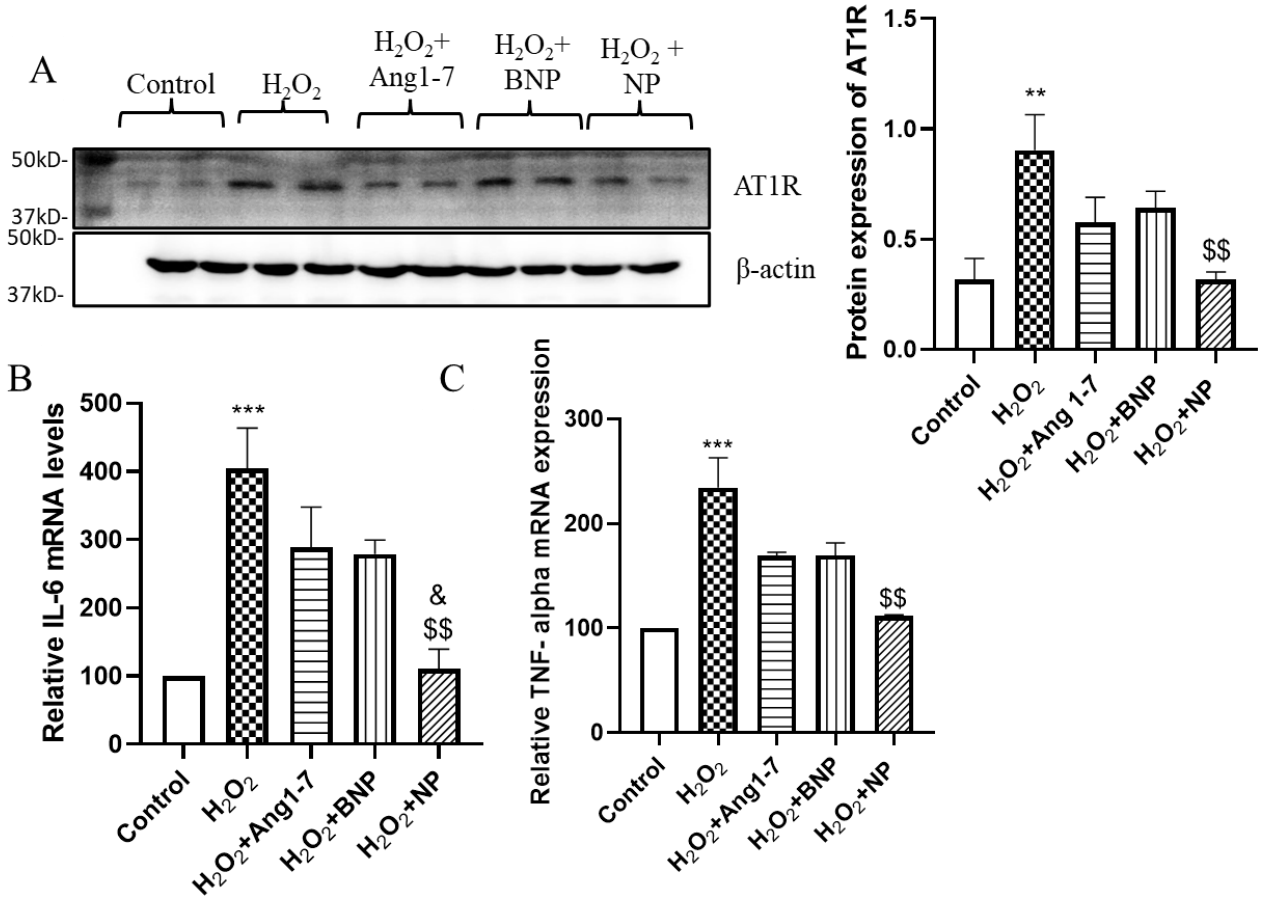



*Figure 5. 6: Dual Activation Mitigates the Impact of $H_2O_2$ on Cardiovascular Disease Markers and Inflammatory Indicators*

(A) Representative western blot and quantification of CVD markers AT1R. (B) mRNA expression of inflammatory markers IL-6 (N = 4) (F) mRNA expression of TNF-α by qPCR. N = 4. Data are presented as mean ± SEM. Statistical analysis was performed via one-way ANOVA paired with Bonferroni's comparison. **p < 0.01, ***p < 0.001 vs control, $^{\$\$}$p < 0.01 vs $H_2O_2$, $^{\&}$p < 0.05 vs $H_2O_2$+Ang1-7.

### 5.4.6 Dual activation mitigates the vascular phenotypic changes induced by $H_2O_2$

Vascular smooth muscle cells undergo a phenotypic transition from the contractile to the synthetic phenotype in response to various cellular stimuli. This transition is characterized by increased proliferation, as indicated by PCNA expression, and decreased expression of α-SMA, TGF-β, and SM-MHC (246). The data demonstrated in Figs. 5.7A and 5.7B show that NP significantly increases α-SMA expression, which is downregulated by $H_2O_2$, as measured by immunofluorescence and qPCR. TGF-β mRNA expression is also reduced when $H_2O_2$-stimulated cells are co-incubated with NP as illustrated in Fig. 5.7C. These findings suggest that NP is important in reversing the phenotypic changes caused by $H_2O_2$.

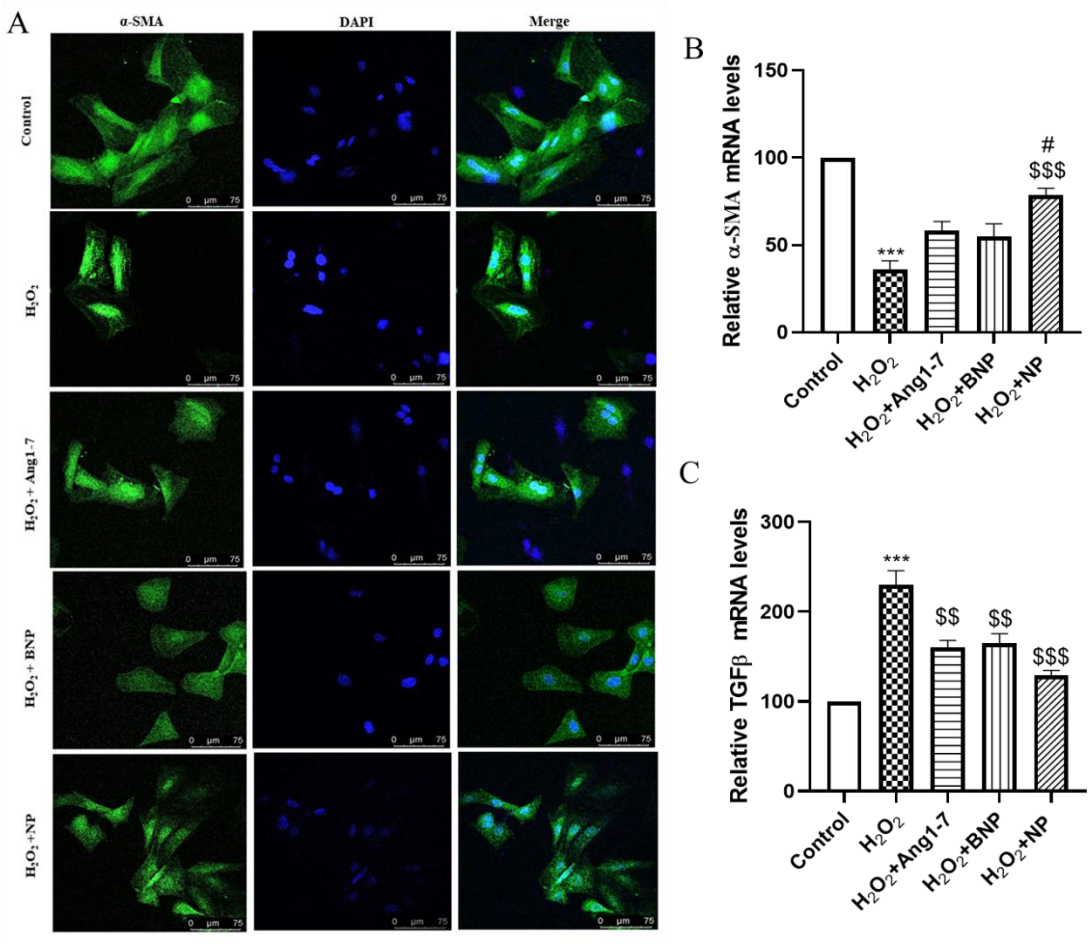



*Figure 5. 7: Dual Activation Mitigates the Vascular Phenotypic Changes Induced by $H_2O_2$*

(A) Representative images of confocal microscopy, Scale bar 75 µm and (B) mRNA expression of contractile marker α-SMA. N = 4. (C) mRNA expression of TGF-β. N = 4. Data are presented as mean ± SEM. Statistical analysis was performed via one-way ANOVA paired with Bonferroni's comparison. ***$p < 0.001$ vs control, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ vs $H_2O_2$, $^{\#}p < 0.05$ vs $H_2O_2$+BNP.

### 5.4.7 Dual activation inhibits vascular hypertrophy induced by $H_2O_2$

The production of ROS regulates hypertrophy. We used crystal violet staining to assess hypertrophy in $H_2O_2$-induced VSMCs, as shown in Fig. 5.8A. In comparison to the control, cells treated with $H_2O_2$ had a greater hypertrophic area. In comparison to $H_2O_2$-treated cells, peptide treatment significantly reduced hypertrophic area. We looked at the expression of hypertrophy markers to confirm the effect of dual activation on hypertrophy. ANP and BNP are well-known hypertrophy markers. Dual activation with peptides reduced the $H_2O_2$-induced expression of ANP mRNA in Fig. 5.8C, emphasizing the preventive effect of dual activation on vascular hypertrophy.

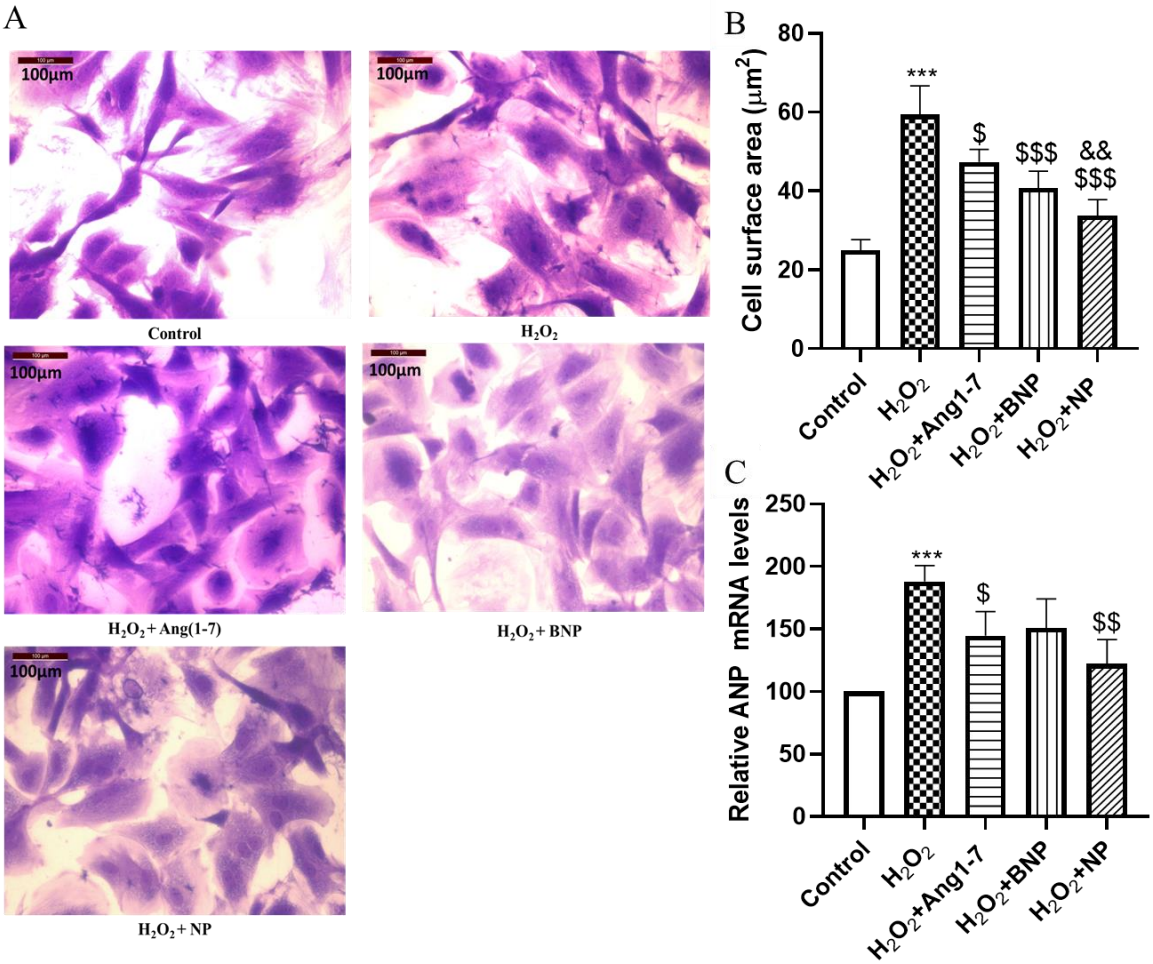
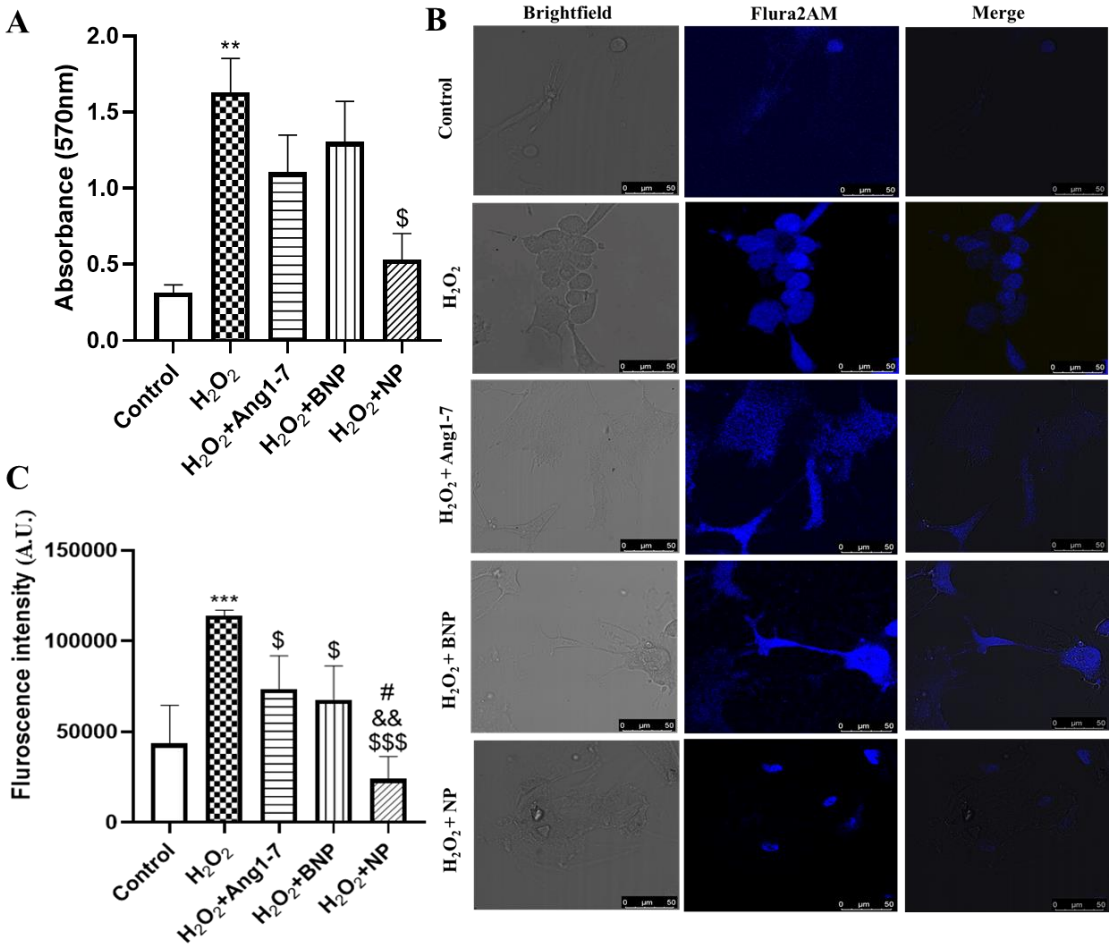



*Figure 5. 8: Dual Activation Inhibits Vascular Hypertrophy Induced by $H_2O_2$*

(A) Hypertrophy was measured by crystal violet staining, Scale bar 100 µm. N = 3. (B) Quantification of cell surface area for hypertrophy from image in A. (C) mRNA expression of hypertrophic marker ANP, N = 4. Data are presented as mean ± SEM. Statistical analysis was performed via one-way ANOVA paired with Bonferroni's comparison. ***$p < 0.001$ vs control, $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ vs $H_2O_2$, $^{\&\&}p < 0.01$ vs $H_2O_2$+Ang1-7.

### 5.4.8 Dual activation stimulates the sGC/PKG pathway and reduce intracellular calcium levels in VSMCs

$H_2O_2$ treatment resulted in significant vascular calcification (Fig. 5.9A) and elevated intracellular calcium levels (Figs. 5.9B and 5.9C) in VSMCs. Peptide treatment resulted in a significant reduction in calcification and a decrease in intracellular calcium levels in $H_2O_2$-exposed cells. In VSMCs incubated with $H_2O_2$, we found a significant decrease in the expression of sGC, PKG, and MLCP compared to the control. In contrast, co-incubating $H_2O_2$-treated cells with peptides resulted in upregulation as demonstrated in Fig. 5.9D-5.9F. This suggests that dual activation is critical for activating the sGC/PKG pathway and lowering intracellular calcium levels in VSMCs.



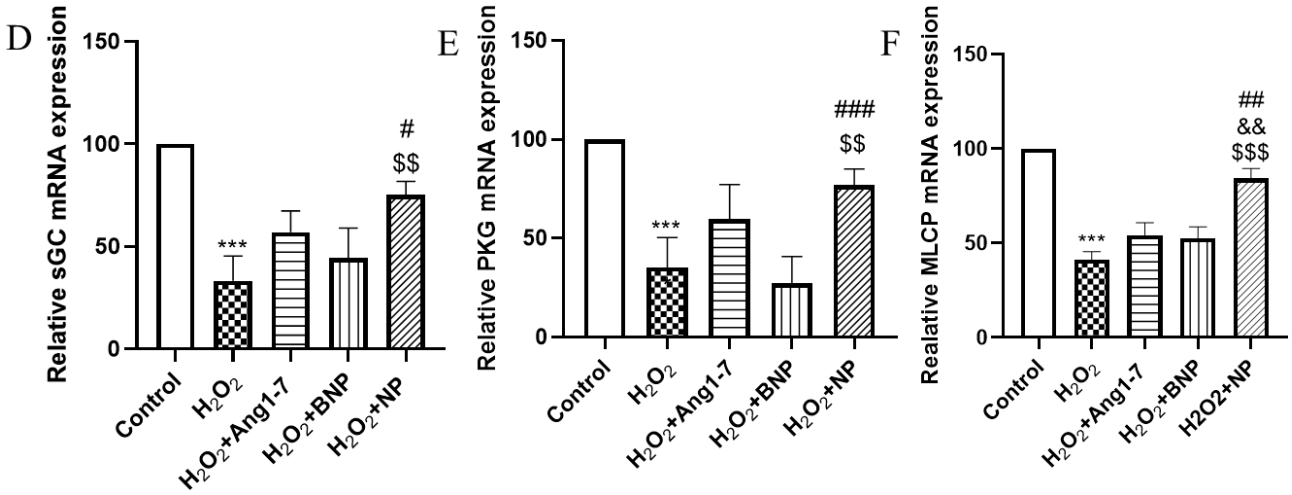



*Figure 5. 9: Dual Activation Stimulates the sGC/PKG Pathway and Mitigates Intracellular Calcium Levels in VSMCs*

(A) Quantification of absorbance of ARS at 570 nm, N = 5. (B) Representative images of confocal images of intracellular calcium imaging by flora-2 AM. Scale bar 50 μm. N = 3. (C) Quantification of fluorescence intensity of intracellular calcium by flura-2 AM from images in B. N = 3. (D) mRNA expression of sGC (E) mRNA expression of PKG (F) mRNA expression of MLCP by qPCR. N = 4, Data are presented as mean ± SEM. Statistical analysis was performed via one-way ANOVA paired with Bonferroni's comparison. $^{**}p < 0.01$, $^{***}p < 0.001$ vs control, $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ vs $H_2O_2$, $^{\&\&}p < 0.01$ vs $H_2O_2$+Ang1-7, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs $H_2O_2$+BNP.

### 5.4.9 Dual activation activates the PI3K/AKT/eNOS pathway in endothelial cells

Reduced expression of endothelial nitric oxide synthase (eNOS) is a notable feature of endothelial damage in the pathology of cardiovascular disease. $H_2O_2$ significantly reduced eNOS expression in ECs treated with $H_2O_2$ in this study. However, peptide treatment in $H_2O_2$-exposed cells resulted in a significant increase in eNOS expression as shown in Fig. 6A. In $H_2O_2$-treated cells, however, gene expression of iNOS was found to be downregulated after peptide treatment as illustrated in Fig. 6C. To better understand the mechanism, we explored at the gene expression of PI3K and AKT, both of which were significantly downregulated in $H_2O_2$-treated ECs compared to controls (Fig. 6D, 6E). In contrast, co-incubating $H_2O_2$-treated cells with peptides resulted in upregulation. This confirms that dual activation plays a pivotal role in activating the PI3K/AKT/eNOS pathway in ECs.

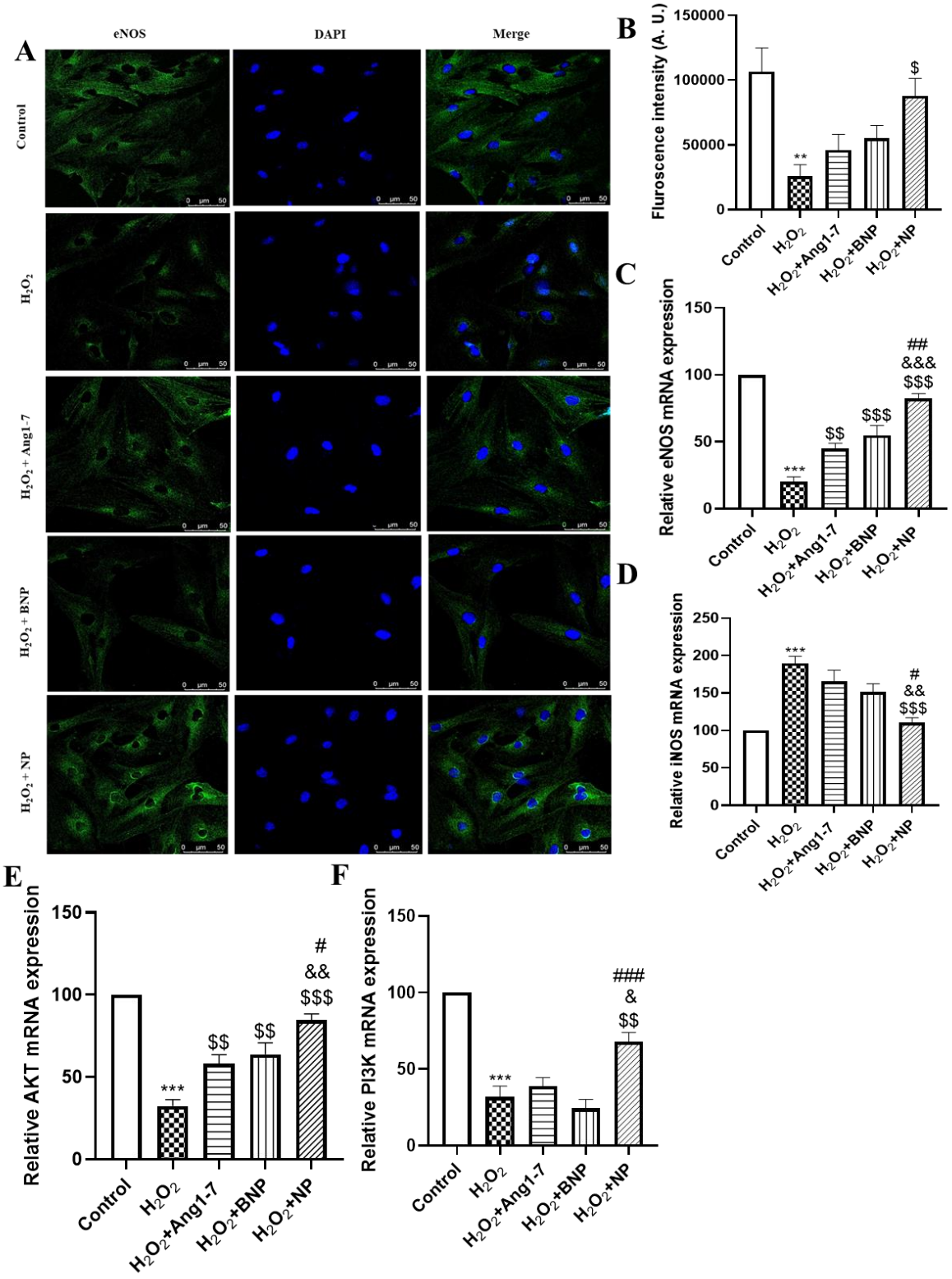



*Figure 5. 10: Dual Activation activates the PI3K/AKT/eNOS Pathway in Endothelial Cell*

(A) Representative confocal images of eNOS, Scale bar 50 µm. (B) Quantification of fluorescent intensity of eNOS (C) mRNA expression of eNOS by qPCR. N = 4. (D) mRNA expression of iNOS (E) mRNA expression of AKT (F) mRNA expression of PI3K by qPCR.

N = 4. Data are presented as mean ± SEM. Statistical analysis was performed via one-way ANOVA paired with Bonferroni's comparison. $^{**}p < 0.01$, $^{***}p < 0.001$ vs control, $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ vs $H_2O_2$, $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ vs $H_2O_2$+Ang1-7, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ $H_2O_2$+BNP.

### *5.5 Discussion*

In this study, we explored the protective properties of a novel peptide by simultaneously activating MasR and pGCAR receptors in cultured primary VSMCs and ECs treated with $H_2O_2$. Remarkably, NP demonstrated promising protective effects that outperformed the results obtained with individual activation of MasR and pGCAR receptors. Furthermore, we demonstrated that the therapeutic efficacy of NP is through the activation of multiple intracellular pathways. The protective effect of NP on ECs was linked to the activation of the PI3K/AKT/eNOS pathway. The detectable increase in eNOS expression in $H_2O_2$-exposed ECs after NP treatment suggests a possible avenue for mitigating endothelial damage in the context of cardiovascular diseases. Similarly, in VSMCs, the activation of the sGC/PKG pathway was attributed to NP's protective effects, emphasizing its ability to modulate vascular smooth muscle function and counteract the effects of oxidative stress.

BNP and Ang1-7 were combined to produce NP with a purity of 95 % (Annexure). Wistar rats were used to isolate and characterize primary VSMCs and ECs. Following that, oxidative stress was induced in VSMCs by incubating them with $H_2O_2$ at various time points to establish the disease model. Remarkably, in oxidative stress-induced VSMCs, there was a significant upregulation of the RAAS activation marker AT1 receptor and a decrease in MasR and pGC-A mRNA expression as demonstrated. Adding to this, CVDs are frequently associated with RAAS and an imbalance in NO/cGMP. In overactivated RAAS-mediated dysfunctions, AngII is critical. The harmful actions of AngII in the classical axis are primarily mediated by the AT1 receptor (2). Previous studies have accumulated evidence suggesting that the AT1 receptor triggers various intracellular signalling pathways that influence the functions of VSMCs and ECs in the cardiovascular system (247). Pre-hypertensive stimuli, on the other hand, activate NADPH oxidase, resulting in ROS generation, which is associated with RAAS overactivation and decreased endothelial vasorelaxation, thereby increasing risk factors for CVDs (248).

Following that, we investigated the effect of NP on pGCAR and MasR expression in primary VSMCs in the presence of $H_2O_2$. Surprisingly, both mRNA and protein expression of

MasR and pGCAR increased significantly after NP treatment, outperforming the effects of Ang1-7 and BNP alone. This evidence highlights the remarkable efficacy of the synthesized NP. Previous studies suggest that MasR stimulation with Ang1-7 has been shown to improve vascular remodeling and increase nitric oxide (NO) bioavailability in spontaneously hypertensive rats (143). Notably, the well-known biological actions of pGCAR, vasodilation and natriuresis, are mediated by the production of the secondary messenger cGMP (192). The RAAS protective axis involving ACE2/Ang1-7/MasR counteracts the AT1 receptor's actions. The ACE2 enzyme, a homolog of ACE, cleaves AngII to produce Ang1-7, which acts via the proto-oncogene Mas receptor (193). Apart from this several investigations concluded that Ang1-7 activation via MasR promotes vasodilation and has been linked to anti-fibrotic, antiangiogenic, antiproliferative, and anti-inflammatory effects (148). Experimental studies on MasR agonists such as AVE-0991, CGEN-856, and CGEN-857 are effective in animal models of cardiovascular disease (154).

Endothelial dysfunction, a critical event in cardiovascular disease, is characterized by increased ROS and inflammation, as well as decreased NO bioavailability. Endothelial dysfunction in patients with coronary heart disease associated with hypertension is characterized by decreased endothelial NO production by eNOS and increased systemic NO production via improved iNOS activity (194). Natriuretic peptide-based strategies that target the NO/cGMP/PKG signalling pathway help to protect against these cardiovascular complications. ANP and BNP act via the membrane-bound GPCR, pGCAR. When the pGCAR receptor is activated, the second messenger cGMP is released, which activates PKG (55). As a result, activating the pGCAR receptor appears to be an appealing strategy for treating cardiovascular complications. NP/pGCAR/cGMP is beneficial in experimental animal models for cardiovascular disease (195). C53, a novel pGCAR activator, has anti-fibrotic properties *in-vivo* (159). To address the complexities of cardiovascular diseases, current research strategies are strongly oriented toward activating the ACE2/Ang1-7/MasR and NP/NO/cGMP pathways.

Subsequently, we investigated the effect of co-activation/dual activation on oxidative stress in $H_2O_2$-stimulated primary VSMCs. NP treatment significantly improved anti-oxidative properties, and its efficacy was comparable to that of the standard ROS scavenger NAC. Furthermore, NP effectively reduced nitrite levels in primary VSMCs stimulated with $H_2O_2$. Given the importance of oxidative stress in the progression of vascular diseases, the induction of oxidative stress in animal models contributes significantly to the progression of various pathological events such as cardiac hypertrophy, apoptosis, atherosclerosis, and ischemia-

reperfusion, ultimately leading to severe complications in cardiovascular diseases (196). Previous evidence concludes that Ang1-7 protects human umbilical vein endothelial cells (HUVECs) from oxidative stress damage and to increase antioxidant capacity (197). Furthermore, BNP peptide treatment significantly reduced AngII-induced intracellular ROS and NADPH oxidase activity in rat VSMCs (198).

For the first time, we discovered that oxidative stress induced by $H_2O_2$ in VSMCs resulted in the upregulation of CVD markers, inflammation, and phenotypic switching, as well as the downregulation of Mas and pGCAR receptors. In $H_2O_2$-stimulated VSMCs, NP treatment reduced the expression of the CVD marker AT1R protein. Notably, NP outperformed Ang1-7 and BNP alone in terms of activity. In contrast, we investigated the effect of dual activation on inflammatory markers in $H_2O_2$-stimulated VSMCs. Surprisingly, NP significantly reduced $H_2O_2$-induced inflammation by downregulating IL-6 and TNF-α mRNA expression. Furthermore, dual activation of these receptors with NP reversed phenotypic switching by increasing the contractile marker α-SMA and decreasing the phenotypic switching markers TGF-β.

In response to pathological stimuli, VSMCs transition from the contractile to the synthetic phenotype, which is characterized by increased cell proliferation as indicated by PCNA expression and the synthesis of extracellular matrix components, resulting in vascular wall thickening. Furthermore, histologically, phenotypic switching is characterized by decreased α-SMA expression and increased TGF-β expression (199). Previous research indicates that subcutaneous BNP therapy in heart failure patients is a novel, effective, and safe peptide strategy that suppresses RAAS and improves left ventricular remodeling (200). Earlier research, on the other hand, has highlighted the importance of the ACE2/Ang1-7 system in maintaining vascular tone in cardiovascular biology (201).

Mas and pGCAR co-activation were found to have anti-hypertrophic effects in VSMCs stimulated by $H_2O_2$. Furthermore, NP treatment resulted in a significant downregulation of the hypertrophy marker ANP in VSMCs. Ang1-7 has been shown to have antifibrotic, antihypertrophic, and antiproliferative effects in both *in-vitro* and animal models of pulmonary hypertension, in addition to its vasodilatory properties (202). The natriuretic peptides ANP and BNP, on the other hand, target particulate guanylyl cyclase receptors (pGCA and pGCB). In adult rat cardiomyocytes, activation of pGCAR via ANP has been shown to have antihypertrophic effects (203). Previous research has shown that blocking the opening of the mitochondrial calcium uniporter protects cardiomyocytes from apoptosis during ischemia-

reperfusion injury. Furthermore, BNP treatment has been linked to higher levels of the anti-apoptotic Bcl-2 protein, as well as lower levels of the pro-apoptotic Bax and Smac proteins. BNP acts via the mitochondrial pathway, which is partially dependent on the PI3K/Akt signalling pathway (161).

The NO/sGC/cGMP/PKG-dependent pathway is a well-known vasodilatory mediator. Myosin light chain phosphorylation and dephosphorylation are intricately linked to the contraction and relaxation of VSMCs. This involves the phosphorylation of myosin light chain (MLC) by MLCK, which causes contraction, and the dephosphorylation of MLC by MLCP, which causes relaxation. Both the MLCK and MLCP mechanisms connect intracellular calcium concentration to smooth muscle contraction (204). Interestingly, Amberg et al. have highlighted the importance of $Ca^{2+}$-regulating pathways as key regulators of vascular function, particularly in the phenotypic switch where VSMCs initiate vascular calcification (205). Our findings suggest that NP treatment increased mRNA expression of sGC, PKG, and MLCP in $H_2O_2$-stimulated VSMCs. Furthermore, as shown in Figure 5.9A and 5.9B, our findings showed that NP effectively reduced intracellular calcium levels and calcification when compared to $H_2O_2$-stimulated VSMCs. Significantly, our current findings show that NP activation of sGC/PKG/MLCP contributes to a decrease in intracellular calcium in $H_2O_2$-induced VSMCs.

To gain a better understanding of the molecular mechanisms underlying the activation of the sGC/PKG/MLCP pathway in VSMCs, we isolated and characterized primary ECs. The functionality of arterial ECs is critical in regulating VSMC contraction and relaxation. Endothelial nitric oxide synthase is a major isoform within ECs that regulates vascular function. Shiojimo et al. demonstrated that the PI3K-Akt signalling axis controls the downstream target eNOS in arterial endothelial cells, potentially contributing to vascular homeostasis and angiogenesis (206). We found a significant downregulation of eNOS expression in $H_2O_2$-treated primary ECs that was effectively reversed by co-incubation with NP. We also investigated the effect of dual activation on iNOS mRNA expression. Figure 5.10D shows that NP treatment reduced iNOS mRNA expression in $H_2O_2$-treated primary ECs. Following that, we investigated the upstream markers of eNOS and discovered a surprising increase in the mRNA expression of PI3K and AKT following the co-incubation of $H_2O_2$-induced primary ECs with peptides. In contrast, NP treatment reduced the upregulated nitrite concentration in $H_2O_2$-treated primary ECs. These data suggest a mitigation of ROS generation in ECs. Based on this evidence, it can be inferred that the activation of the sGC/PKG/MLCP

pathway and the maintenance of intracellular calcium levels in VSMCs are dependent on the activation of PI3K/AKT/eNOS in ECs.

## *5.6 Conclusion*

In conclusion, our findings demonstrated that the downregulation of Mas and pGCA receptors is critical in $H_2O_2$-induced vascular phenotypic changes, vascular damage, and endothelial injury. This involvement could be due to an imbalance between the NO/sGC/cGMP/PKG and eNOS pathways. Notably, the dual activation of Mas and pGCA receptors with NP effectively counteracts the dysfunction caused by $H_2O_2$. As a result, our findings highlight the important roles of Mas and pGCA receptors in the development of vascular pathology. Targeting these receptors in vascular pathology appears to be a promising therapeutic approach for reducing the negative consequences of such conditions.

# *Chapter 6*

# *Dual Activation of Mas and pGCA Receptors Promotes Natriuresis in DOCA-Salt-Treated Rats*

## *6.1 Graphical abstract*

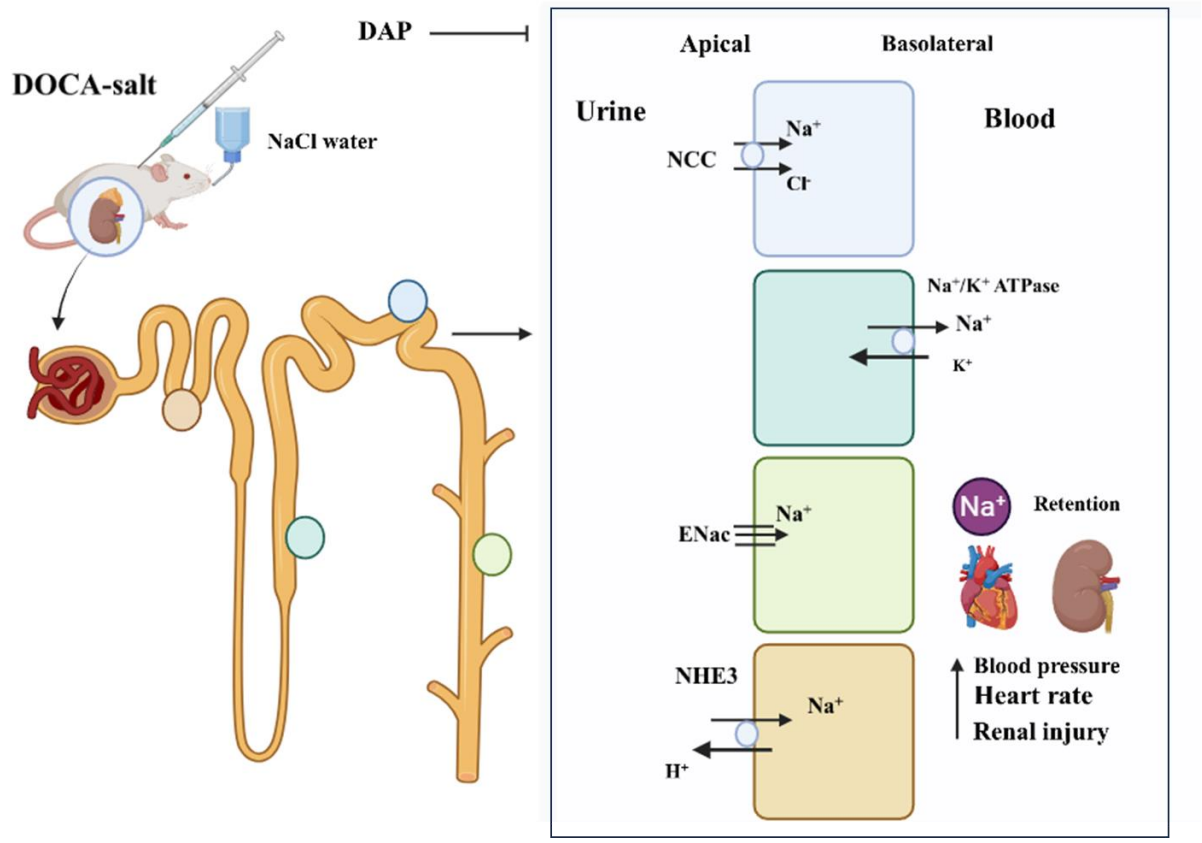



*Figure 6. 1: Graphical abstract for Chapter 6*

DOCA-salt influence sodium absorption by acting on MR, resulting in changes in epithelial sodium channel expression. A high-salt and DOCA diet activates epithelial $Na^+$ channels (ENaC), Na-Cl cotransporters (NCC), and sodium hydrogen exchangers (NHE) on the apical membrane, while $Na^+K^+$ ATPase is activated on the basolateral membrane of the kidney. Only 20 % of sodium reabsorption from tubules to blood goes through the basolateral membrane in a thick ascending loop, while the remaining 75 % goes through the apical membrane via the proximal convoluted tubule, distal convoluted tubule, and collecting duct from urine to tubules.

## *6.2 Introduction*

Salt-sensitive hypertension is a widespread and major global health concern. It is estimated to affect approximately 50 % of individuals with hypertension and 25 % of those with normal blood pressure (BP) (20). This condition is characterized by an increased blood pressure response to unrestricted salt intake, and it has been identified as an independent risk factor for cardiovascular deaths (263). This statement is supported by the fact that excessive salt consumption is responsible for an estimated 5 million fatalities annually. In recognition of the public health impact of high salt intake, the World Health Organization (WHO) has

recommended reducing daily sodium intake to less than 2 grams for adults, emphasizing the importance of salt reduction strategies to mitigate the global burden of salt-sensitive hypertension and its associated health risks (264).

The kidney plays an important role in sodium-sensitive hypertension. Impaired sodium balance in the body by increasing water and sodium reabsorption leads to renal injury and hypertension. In response to activation of the renin-angiotensin-aldosterone system (RAAS), aldosterone is synthesized and secreted. Where aldosterone through mineralocorticoid receptor (MR) regulates sodium reabsorption, it is highly expressed in the collecting ducts of the kidney (265). The use of an aldosterone precursor DOCA with a high-salt diet is a well-known model for inducing hypertension. Previous reports suggested that DOCA-salt hypertension is facilitated by sodium retention which contributes to disturbances in kidney function (266). Similar studies suggested that activated t-cell infiltrate and release of inflammation cytokines, DOCA-salt promotes sodium retention, hypertrophy and oxidative stress. However, after a long administration, it leads to the depletion of sodium and potassium balance (267).

In past years first-line therapies of hypertension like angiotensin-converting–enzyme (ACE) inhibitors, Angiotensin receptor blockers, calcium-channel blockers, beta-receptor blockers and diuretics have failed to treat this complex nature and salt sensitivity (268). Hence, it became necessary to investigate a novel antihypertensive strategy to better handle the sodium balance. Understanding the interplay between the kidney, sodium retention, and salt-sensitive hypertension is crucial for developing effective strategies to manage and treat this condition.

An emerging area of research in the field of salt-sensitive hypertension is the investigation of the Angiotensin-converting enzyme 2/Angiotensin1-7/Mas (ACE2/Ang1-7/Mas) and Natriuretic peptide/particulate guanylate cyclase-A (NP/pGCA) pathways, which have garnered notable attention for their potential contributions to blood pressure regulation and cardiovascular health (64). The ACE2/Ang1-7/Mas system has counter-regulatory effects on Angiotensin II (AngII) (50). Exogenous infusion of Ang1-7 exerts vasodilation, antifibrotic, antihypertrophic and reduction in blood pressure effects (269). However, infusion of Ang1-7 into rats shows natriuretic and diuretic direct tubular effects. Also, Ang1-7 shows protective cardiac and renal effects in rats with congestive heart failure (270). Similarly, the NP/pGCA pathway involves NPs and their receptor, pGCA. Activation of this pathway results in increased production of the secondary messenger cyclic guanosine monophosphate (cGMP), leading to vasodilation and natriuresis (53). Brain natriuretic peptide (BNP) is NP released from ventricular myocyte, associated with favourable diuretic, natriuretic and vasodilation effects

(54). Administration of exogenous BNP in the canine model of CHF improved cardiovascular function (271). Subcutaneous administration of BNP resulted in increased diuretic and natriuretic responses and maintained glomerular filtration rate in asymptomatic systolic heart failure subjects (272).

However, the Ang1-7 and BNP treatment is less effective and limited due to rapid metabolism and short half-life, resulting in less bioavailability (63). Scientists are planning research with animals to find ways to overcome these limitations. They are exploring innovative approaches like creating new designer peptides and activating multiple receptors (65). We have designed a novel dual-acting peptide (DAP) that aims to activate both the protective Mas and pGCA receptor pathways. Our research goal is to understand how these peptides impact salt balance and kidney function. We are expecting that activating both receptors simultaneously will have a stronger effect compared to activating Mas or pGCA receptors individually.

## *6.3 Material and methods*

In this experimental study, we used Wistar rats weighing between 180-220 gm were procured adhering to IAEC guidelines under protocol number BITS-Hyd-IAEC-2022-08. Hypertension was induced in rats by administering DOCA salt subcutaneously twice a week for 4 weeks, along with high-salt drinking water. After successful induction of hypertension, rats were randomly assigned to various treatment groups receiving Ang1-7, BNP, DAP, Losartan, or serving as controls. Weekly assessments included measurements of body weight, water intake, and blood pressure. Blood and urine were collected after two weeks of treatment for biochemical analyses. Molecular investigations involved qPCR, western blotting, and histopathological examinations of kidney tissues. Statistical analyses were conducted using one-way ANOVA with post hoc Bonferroni's test, and detailed methodologies are outlined in Chapter 3 for reference.

## *6.4 Results*

### 6.4.1 DAP activates secondary messengers of Mas and pGCA receptors

The ELISA assay was conducted at various concentrations (57.5, 115, and 230 µg/kg/day) and time points following subcutaneous injections of DAP, specifically at 15, 30, and 60 min. As illustrated in Figures 6.2A and 6.2B, suggest a significant increase in the levels of secondary messengers of Mas and pGCA receptors at doses of 115 and 230 µg/kg/day. Subsequently, we

selected to proceed with the lower concentration of 115 µg/kg/day for further investigation, considering its notable effects on the secondary messenger levels.

Furthermore, ELISA assays were conducted at a selected dose of DAP (115 µg/kg/day) along with Ang1-7 (100 µg/kg/day), BNP (15 µg/kg/day), at 15-, 30-, and 60-min post-administration. This analysis strengthens that DAP's activity involves the Mas and pGCA receptors activation. Additionally, as shown in Figures 6.2C and 6.2D, the results indicate a better efficacy of DAP at the 60-minute time point compared to Ang1-7 and BNP.

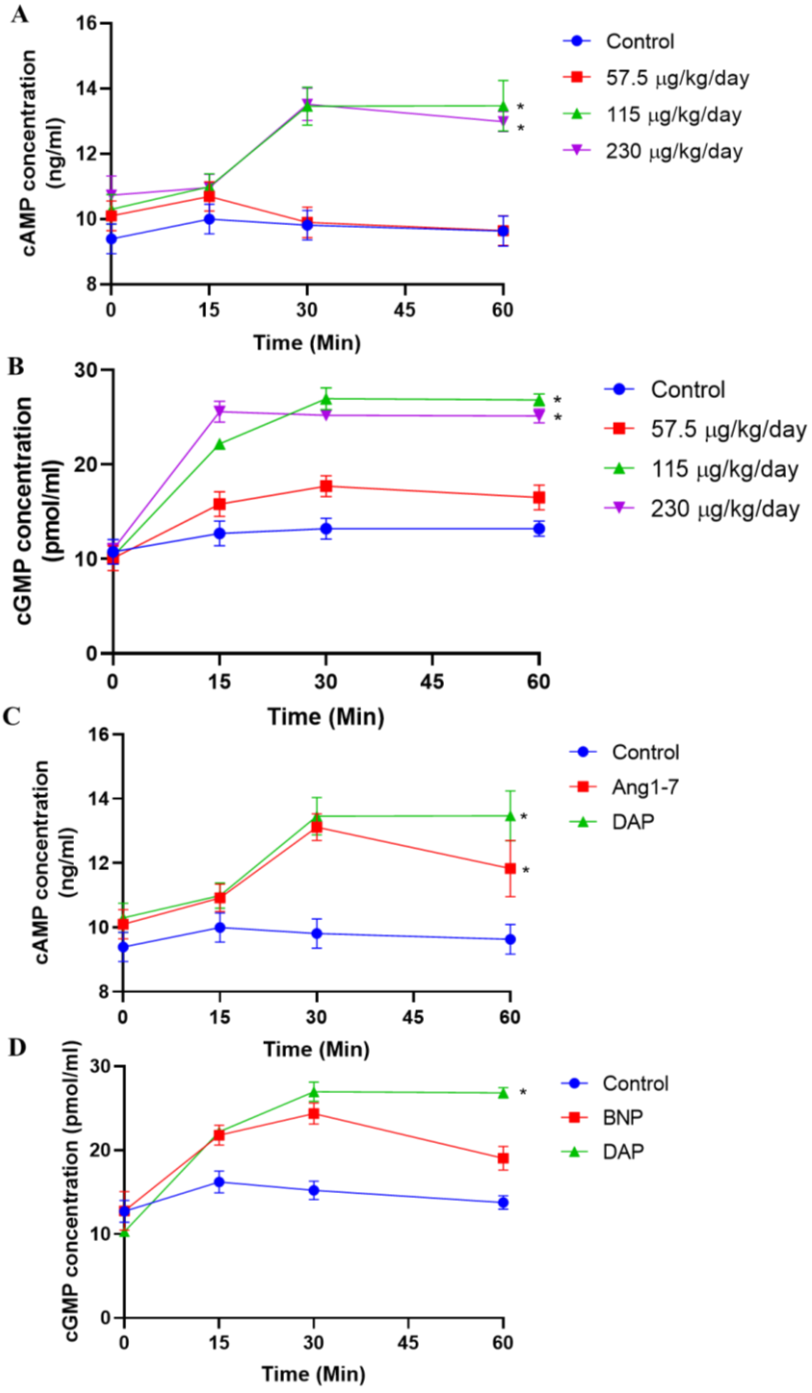
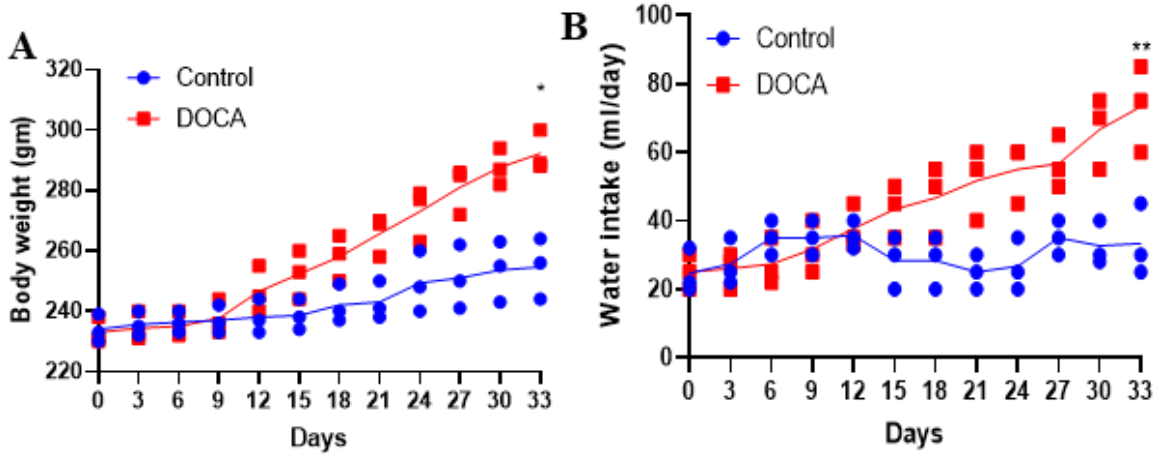



*Figure 6. 2: DAP activates secondary messengers of Mas and pGCA receptors.*

ELISA assay is performed at different concentrations as 57.5, 115 and 230 µg/kg/day and different time points after subcutaneous injections, i.e. after 15 min, 30 min, 60 min of DAP treatment. (A) Serum cAMP levels were measured with ELISA assay kit. (B) Serum cGMP levels were measured with ELISA assay kit. We have also performed Elisa for Ang1-7 (100 µg/kg/day), BNP (15 µg/kg/day) along with DAP (115 µg/kg/day) at 15, 30 and 60 min. (C) Serum cAMP levels were measured with ELISA assay kit. (D) Serum cGMP levels were measured with ELISA assay kit. Data were expressed as mean ± SEM (where N = 4). Data were analysed using ordinary one-way ANOVA followed by Bonferroni multiple comparison test, where *p < 0.05 vs. respective control.

### 6.4.2 Administration of DOCA-Salt resulted in increased blood pressure and heart rate

Following the acclimatization phase, all groups had similar baseline blood pressure values. We monitored key parameters every three days according to the experimental schedule to track the progression of hypertension. DOCA-salt treatment resulted in a significant increase in body weight compared to the control group (Figure 6.3A). Furthermore, the water intake of the control rats was lower than that of the DOCA-treated rats (Figure 6.3B). As shown in Figure 6.3C-6.3F, the combination of DOCA-salt and NaCl water intake resulted in a gradual increase in systolic, diastolic, mean blood pressure, and heart rate. Interestingly, blood pressure readings increased significantly between days 27-33 after DOCA administration. Following observations, experiments were carried out at the 28-day mark, corresponding to 4 weeks of DOCA-salt treatment with 1 % NaCl.



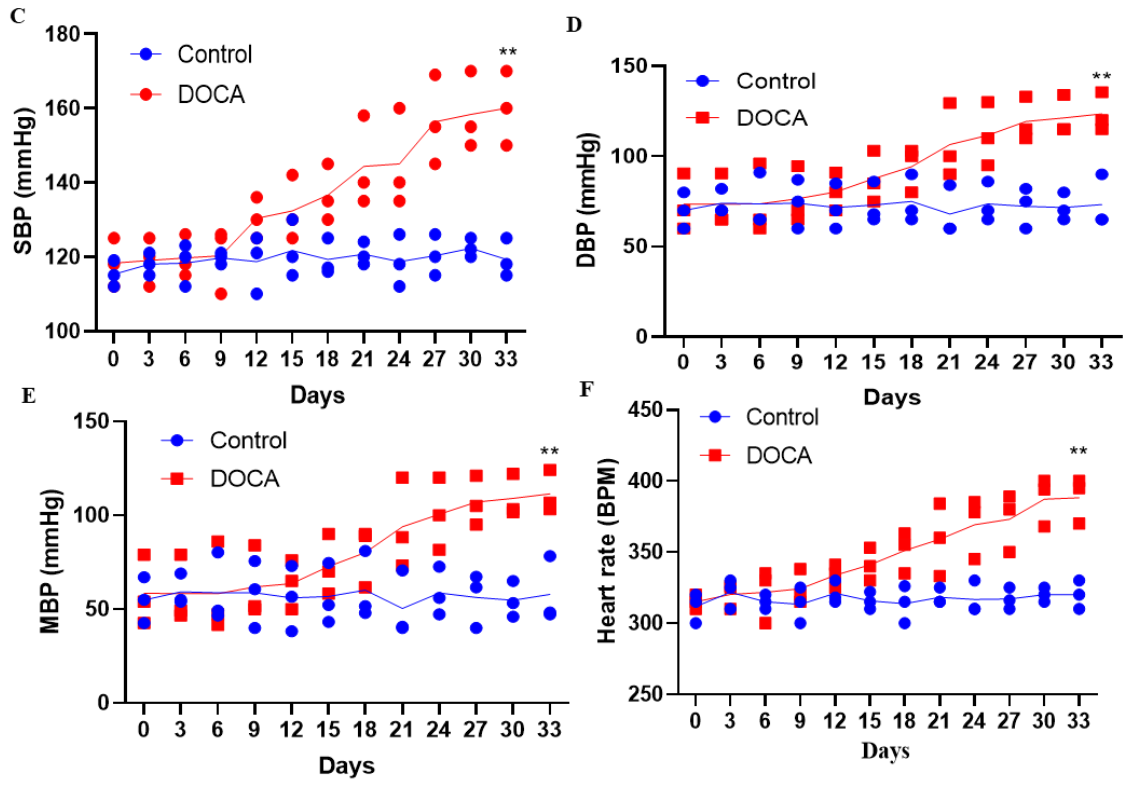



*Figure 6. 3: DOCA-salt administration increases blood pressure and heart rate*

Wistar rats were treated with DOCA-salt 25 mg/kg/day twice a week and after every 3 days we measured (A) Body weight (B) Water intake (C) SBP (D) DBP (E) MBP (F) Heart rate. Data were expressed as mean ± SEM (where N = 4). Data were analysed using an unpaired t-test (two-tailed). where *p < 0.05, **p < 0.01 vs. respective control.

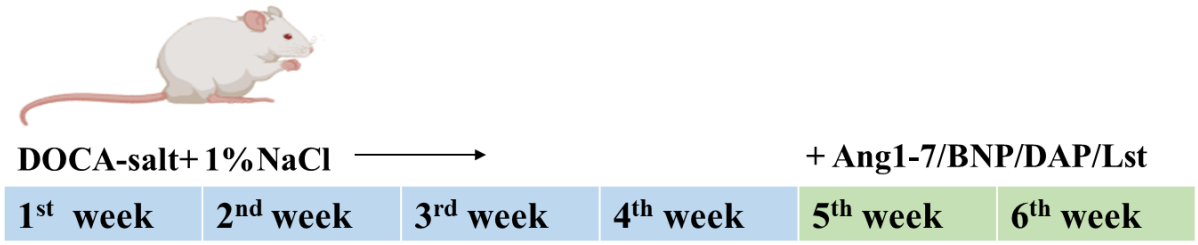



*Figure 6. 4: Timeline of DOCA-salt and peptide treatment*

Male Wistar rats were used in a four-week study to establish hypertension using the DOCA-salt hypertension model. Throughout this time, blood pressure, body weight, and water intake were closely monitored to confirm the onset of hypertension. Following that, the rats were given either Ang1-7/BNP/DAP/Lst or no treatment for two weeks.

### 6.4.3 Dual activation of Mas and pGCA receptors attenuates BP in DOCA-salt in rats

DOCA-salt administration elevated BP, heart rate, body weight and water intake. However, over the 2 weeks of DAP administration produced a significant decrease in systolic, diastolic and mean BP relative to DOCA-salt. Additionally, DAP also significantly reduced the heart rate, body weight, kidney weight, heart weight and water intake compared to DOCA-salt rats. Moreover, as illustrated in Table 6.1 effects of DAP are more significant than individual receptor activation with Ang1-7, BNP and losartan in opposing DOCA-salt effects.

*Table 6. 1: Effect of DAP treatment on blood pressure*

| Parameter | Control | DOCA | DOCA+Ang1-7 | DOCA+BNP | DOCA+DAP | DOCA+Lst |
|---|---|---|---|---|---|---|
| **SBP (mm Hg)** | 118.00 ± 2.86 | 182.33 ± 3.60$^{***}$ | 157.67 ± 2.59 | 155 ± 2.05 | 134.33 ± 2.59$^{\$\$\$}$ | 154.00 ± 2.62 |
| **DBP (mm Hg)** | 77.33 ± 3.31 | 129 ± 5.31$^{***}$ | 117.33 ± 7.30 | 112.00 ± 2.82 | 91 ± 3.55$^{\$\$}$ | 113.00 ± 1.25 |
| **MBP (mm Hg)** | 63.77 ± 5.32 | 11.22 ± 6.34$^{**}$ | 103.88 ± 8.95 | 97.66 ± 3.15 | 76.55 ± 3.88$^{\$}$ | 99.33 ± 2.32 |
| **HR (BPM)** | 339.33 ± 6.60 | 435.66 ± 4.38$^{***}$ | 398.33 ± 1.18 | 396.67 ± 4.83 | 356.66 ± 5.68$^{\$\$\$}$ | 392.67 ± 7.07 |
| **HW (gm)** | 0.93 ± 1.03 | 1.87 ± 1.03$^{***}$ | 1.35 ± 1.07 | 1.42 ± 1.07 | 1.22 ± 1.02$^{\$\$\$}$ | 0.92 ± 2.01 |
| **BW (gm)** | 258.67 ± 3.81 | 308.33 ± 3.60$^{***}$ | 298.33 ± 5.04 | 299.33 ± 1.44 | 270.00 ± 2.35$^{\$\$\$}$ | 306.33 ± 2.59 |
| **KW (gm)** | 0.94 ± 0.05 | 1.66 ± 0.06$^{***}$ | 1.46 ± 0.03 | 1.34 ± 0.04 | 0.99 ± 0.03$^{\$\$}$ | 1.33 ± 0.14 |
| **WI (ml)** | 28.33333 ± 3.60 | 87.67 ± 1.18$^{***}$ | 71.67 ± 1.37 | 68.33 ± 1.37 | 46.67 ± 2.72$^{\$\$\$}$ | 61.67 ± 1.37 |

All rats were administered DOCA-salt and 1 % NaCl for 4 weeks and received subcutaneous injections of Ang1-7, BNP and DAP. Losartan was administered by oral route. Blood pressure was measured using tail cuff method. We measured systolic, diastolic, mean blood pressure and heart rate. After scheduled treatment water intake, body weight, heart weight and kidney weight were measured. Data were expressed as mean ± SEM (where N = 4). Data were analysed using ordinary one-way ANOVA followed by Bonferroni multiple comparison test, where ***$p < 0.001$ vs. respective control, $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ vs. DOCA.

### 6.4.4 Dual activation of Mas and pGCA receptors maintains renal functional parameters

DOCA-salt administration elevated albumin, creatinine, BUN and protein excretion in urine levels as shown in Figure 6.5. These renal functional parameters indicate renal damage and loss of kidney functions. Interestingly, we found that DAP was capable of maintaining the balance between these important renal functional parameters as described in Figure 6.5.

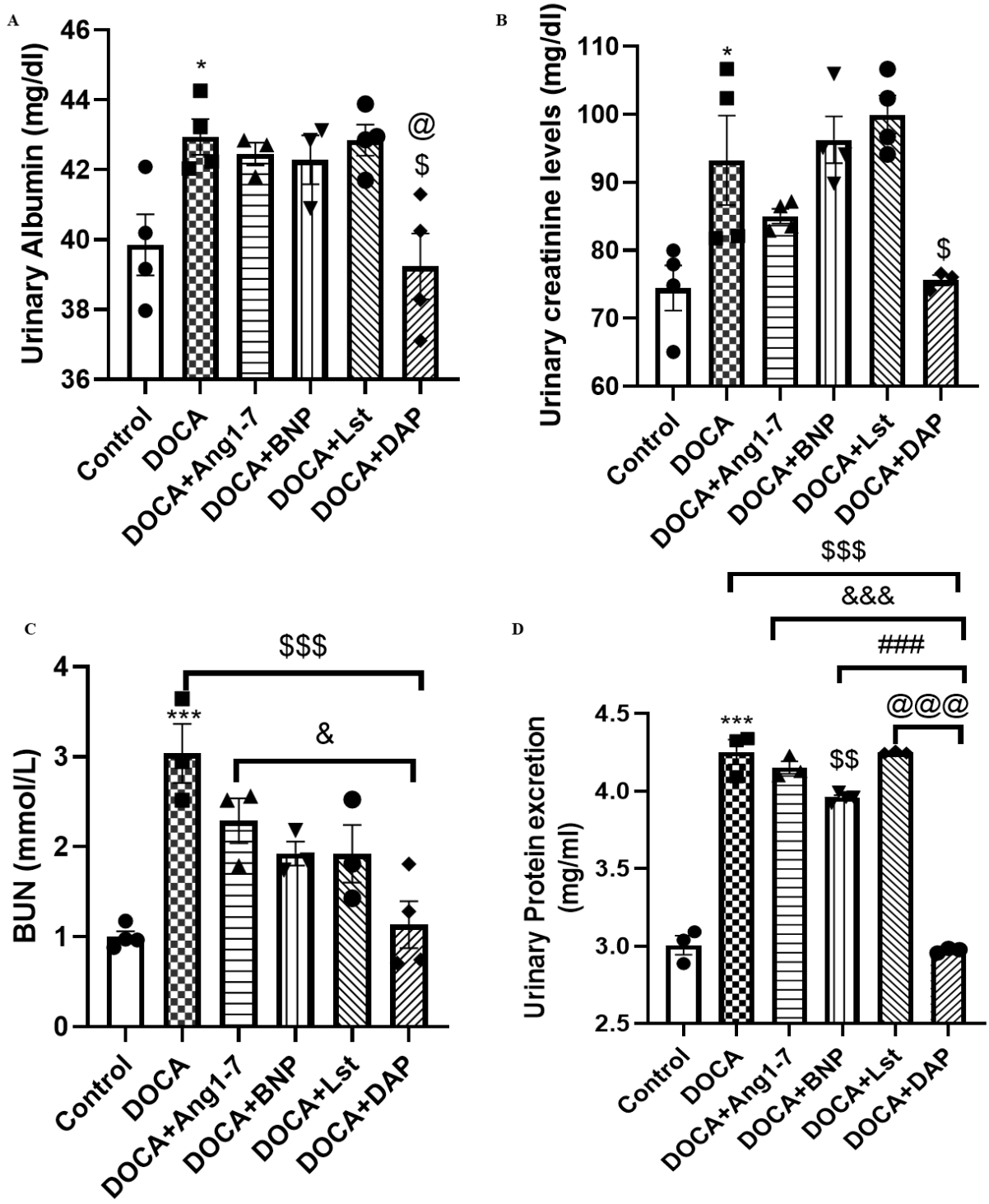



*Figure 6. 5: Effect of DAP on functional biochemical parameters*

(A) Schematic representing the timeline of DOC-salt exposure for hypertension in rats in a high-salt drinking water regimen over a 4-week experiment. Later hypertensive rats were treated with Ang1-7/BNP/DAP/losartan for 2 weeks. (B) Serum albumin (C) Serum creatinine (D) Urinary creatinine (E) Blood urea nitrogen (BUN) (F) Urinary protein excretion. Data were expressed as mean ± SEM (where N = 4). Data were analysed using ordinary one-way ANOVA

followed by Bonferroni multiple comparison test, where **p < 0.01, ***p < 0.001 vs. respective control, $^{\$\$}$p < 0.01, $^{\$\$\$}$p < 0.001 vs. DOCA, $^{\&}$p < 0.05, $^{\&\&\&}$p < 0.001 vs. DOCA + Ang1-7, $^{\#}$p < 0.05, $^{\#\#\#}$p < 0.001 vs. DOCA + BNP, $^{@@@}$p < 0.001 vs. DOCA + Lst.

### 6.4.5 Dual activation of Mas and pGCA receptors attenuates renal hypertrophy in DOCA-salt rats

As described in Table 6.1 increased kidney and heart weight indicated organ swelling and hypertrophy. Moreover, DAP is effective in reducing kidney weight relative to DOCA-salt. Also, Figure 6.6 A suggests that the kidney-to-body weight ratio, an indicator of organ hypertrophy was also significantly reduced in DAP-treated rats compared to DOCA-salt. Further, it is confirmed by the gene expression of hypertrophic marker TGFβ, which was significantly downregulated after DAP treatment relative to DOCA salt, Ang1-7, BNP and losartan treatment as shown in Figure 6.6B. Results suggest that DAP is more efficacious than the Ang1-7, BNP and Lst.

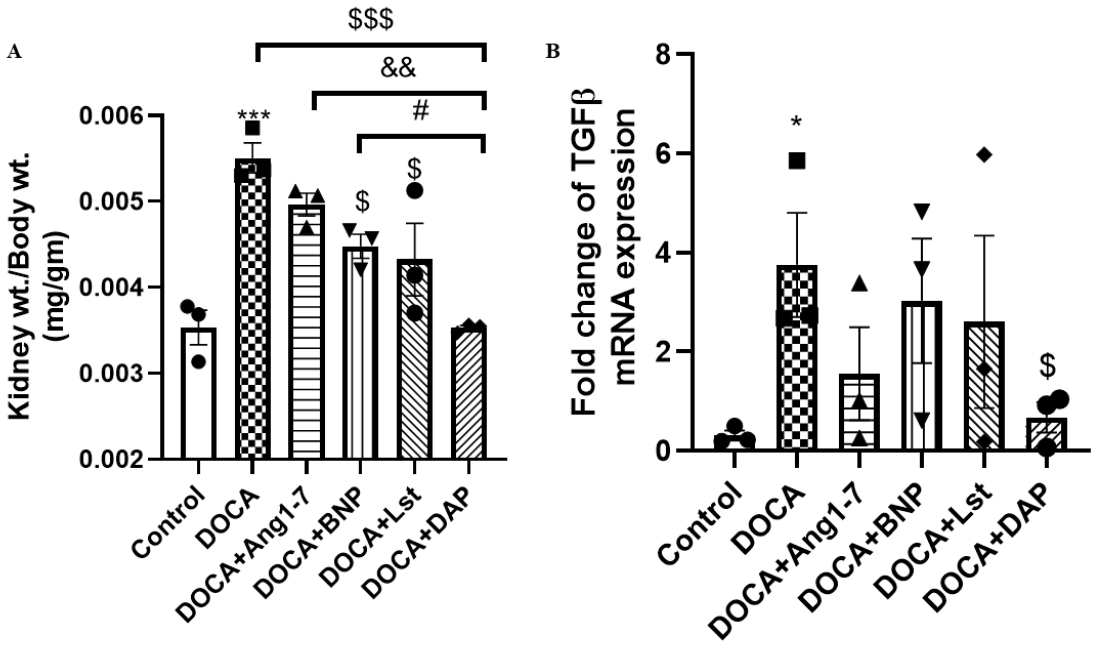
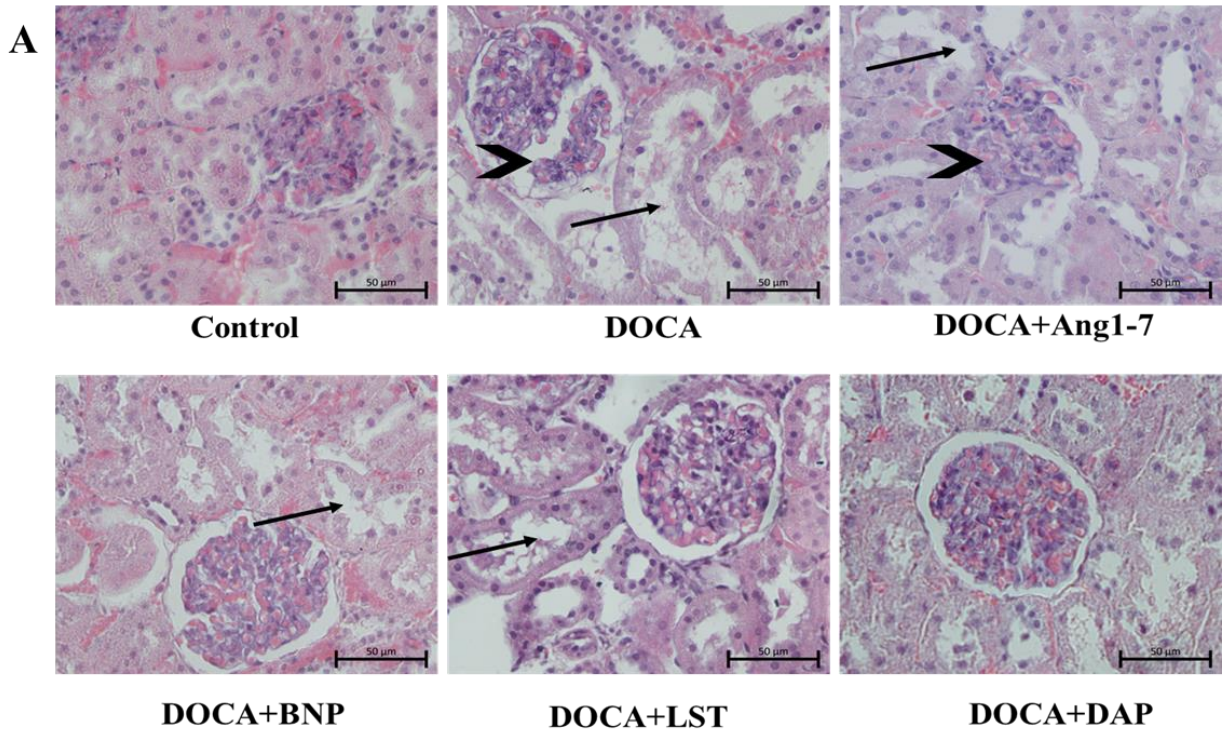



*Figure 6. 6: Effect of Dual activation on renal hypertrophy in DOCA-salt rats*

(A) Kidney weight/Body weight ratio (B) mRNA expression analysis of TGFβ. Data were expressed as mean ± SEM (where N = 4). Data were analysed using ordinary one-way ANOVA followed by Bonferroni multiple comparison test, where *p < 0.05, ***p < 0.001 vs. respective control, $^{\$}$p < 0.05, $^{\$\$\$}$p < 0.001 vs. DOCA, $^{\&\&}$p < 0.01 vs. DOCA + Ang1-7, $^{\#}$p < 0.05 vs. DOCA + BNP.

### 6.4.6 Dual activation of Mas and pGCA receptors attenuated tubular damage in DOCA-salt rats

As shown in Figure 6.7A and 6.7B, Haematoxylin-eosin staining revealed significant glomerular damage in DOCA-salt rats. Tubulointerstitial damage was also prominent in DOCA-salt rats which is crucial in renal injury, which was significantly attenuated after DAP treatment (Figures 6.7A and 6.7C). We also confirmed proximal and distal tubular damage by upregulation of gene expression of KIM1 and NGAL in DOCA-salt rats as described in Figures 6.7D and 6.7E. However, these unwanted changes in the glomerulus and tubules were significantly reversed with the downregulation of KIM1 and NGAL after DAP treatment.



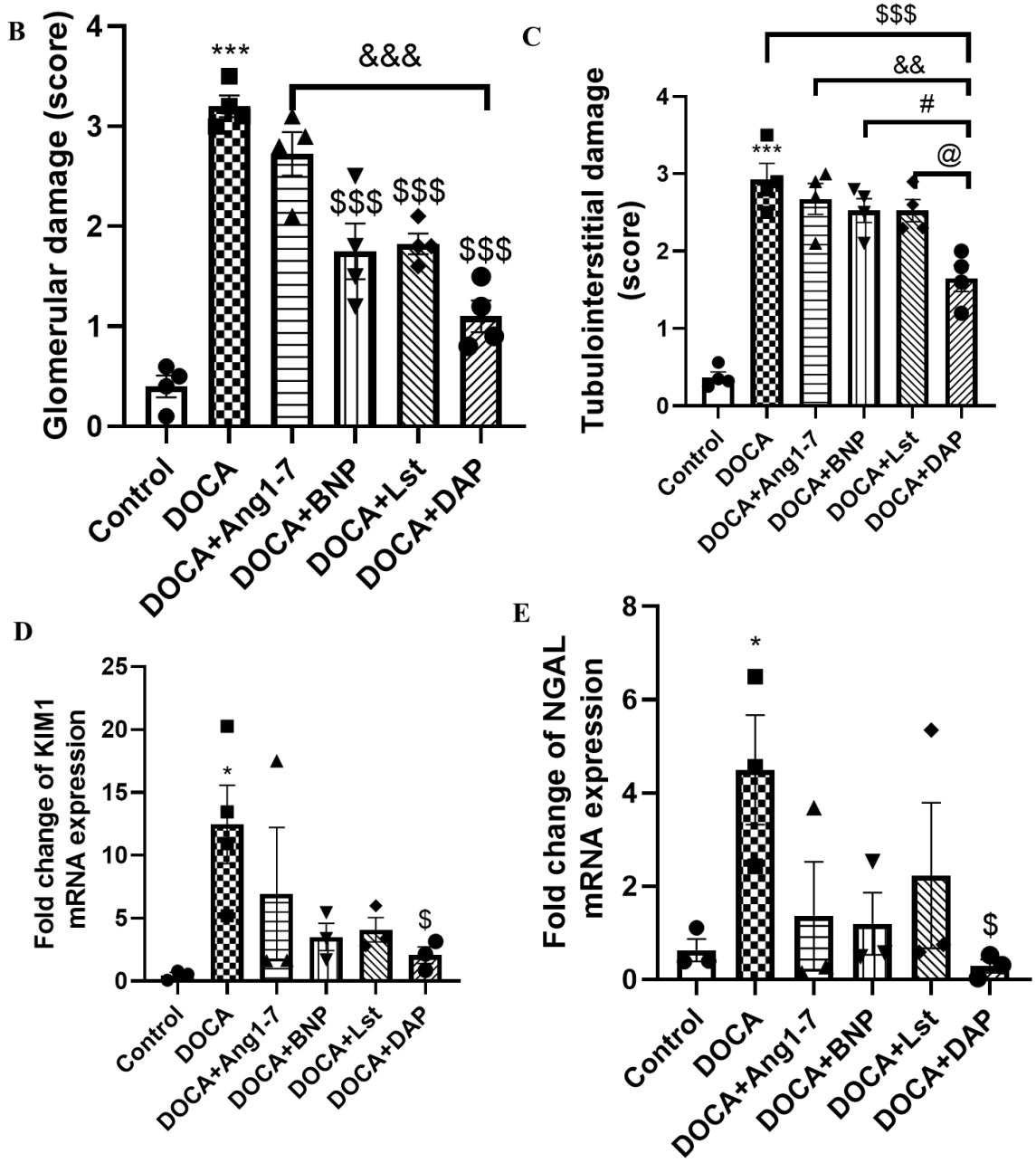


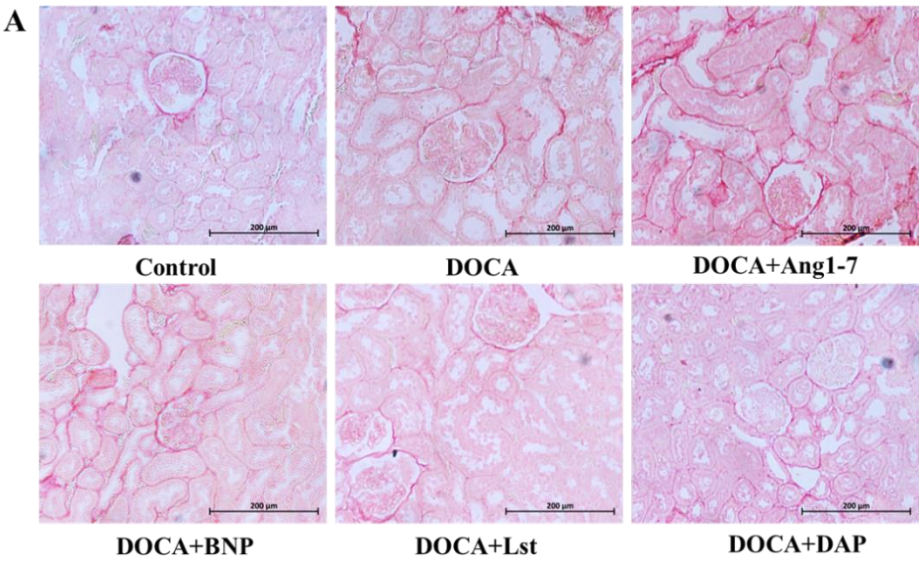

*Figure 6. 7: Dual activation of receptors effectively reversed the pathological changes observed in DOCA-salt rats*

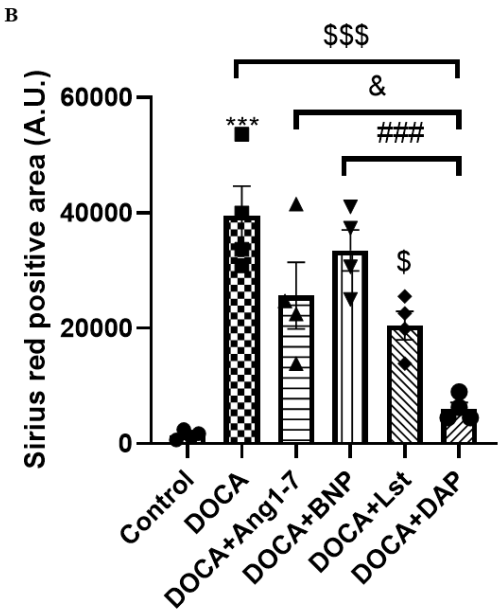
(A) Representative images for whole kidneys (Transverse sections) stained with H&E, Scale bar 50 µm (B) Quantification of glomerular damage by semiquantitative scoring method from 0-4 (C) Quantification of tubulointerstitial damage by semiquantitative scoring method from 0-4 (D) mRNA expression analysis of NGAL (E) mRNA expression analysis of KIM1. Data were expressed as mean ± SEM (where N = 4). Data were analysed using ordinary one-way ANOVA followed by Bonferroni multiple comparison test, where *$p < 0.05$, ***$p < 0.001$ vs.

respective control, $^{\$}p < 0.05$, $^{\$\$\$}p < 0.001$ vs. DOCA, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ vs. DOCA + Ang1-7, $^{\#}p < 0.05$ vs. DOCA + BNP, $^{@}p < 0.05$ vs. DOCA + Lst.

### 6.4.7 Dual activation of Mas and pGCA receptors mitigates renal fibrosis in DOCA-salt rats

DOCA-salt rapidly develops renal hypertrophy, inflammation and fibrosis. As shown in Figure 6.8A and 6.8B, Sirius red staining revealed that collagen deposition was significant in DOCA-salt rats. In addition to this, in DOCA-salt animal models collagen expression was upregulated as shown in Figure 6.8C and 6.8D. Interestingly, DAP is effective in reducing the expression of collagen and deposition compared to DOCA-salt rats as described in Figure 6.8. In addition to this, compared to Ang1-7, BNP and losartan treatment DAP was more promising.



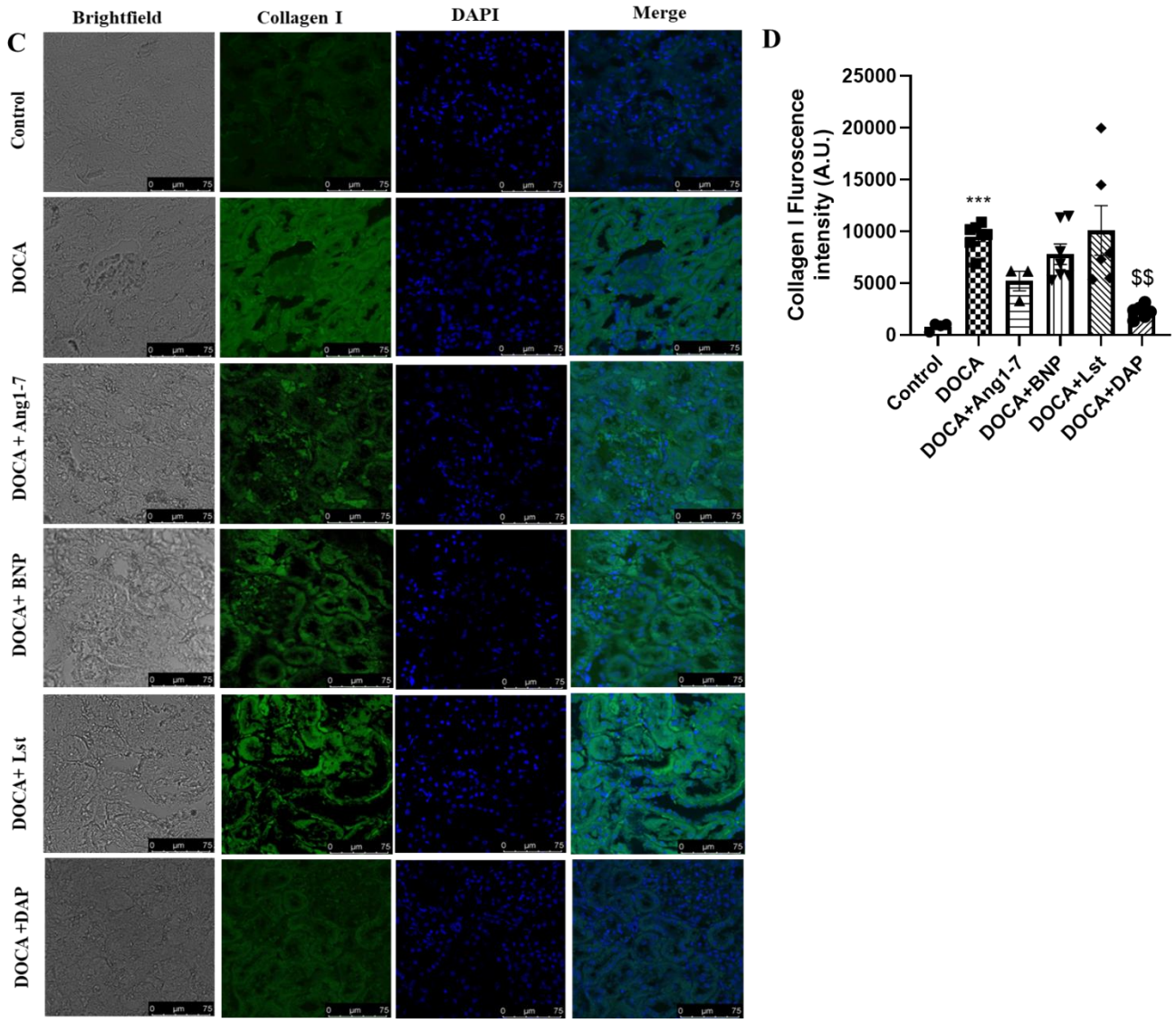



*Figure 6. 8: Dual activation of receptors attenuates renal fibrosis in DOCA-salt rats*

(A) Representative images for whole kidneys (Transverse sections) stained with Picrosirius red, Scale bar 50 µm. (B) Quantification of Sirius red positive area indicates fibrosis. (C) Immunohistochemistry (IHC) of collagen in kidney transverse sections, Scale bar 75 µm (D) Quantification of collagen fluorescence intensity. Data were expressed as mean ± SEM (where N = 4). Data were analysed using ordinary one-way ANOVA followed by Bonferroni multiple comparison test, where ***$p < 0.001$ vs. respective control, $^{\$}p < 0.05$, $^{\$\$\$}p < 0.001$ vs. DOCA, $^{\&}p < 0.05$ vs. DOCA + Ang1-7, $^{\#\#\#}p < 0.001$ vs. DOCA + BNP.

### 6.4.8 Dual activation of Mas and pGCA receptors reduces inflammatory cytokines in DOCA-salt rats

As discussed, earlier DOCA-salt showed prominent hypertrophy and fibrosis in the kidney. DOCA-salt is renin renin-dependent model that mimics human hypertension. Aligning with

this fact we found downregulated renin mRNA levels in DOCA-salt rats as described in Figure 6.9A. Also, as shown in Figure 6.9B, ELISA assay for TNF-α showed that DOCA- salt increased the release of inflammatory cytokines. Surprisingly, DAP is prominent in reducing the TNF-α levels and upregulated renin mRNA expression as shown in Figure 6.9. In addition to this, data suggests that DAP activity is superior to that of Ang1-7 and BNP.

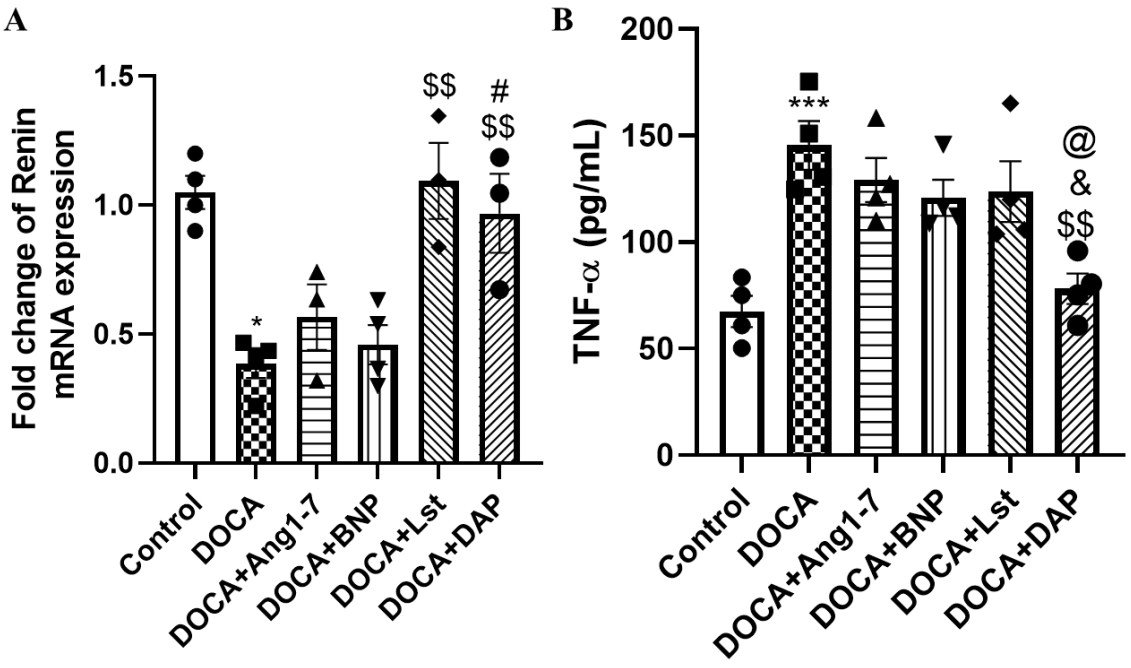



*Figure 6. 9: Dual activation of Mas and pGCA receptors reduces inflammatory cytokines in DOCA-salt rats*

(A) Renin mRNA expression (B) Serum TNF-α levels by ELISA assay. Data were analysed using ordinary one-way ANOVA followed by Bonferroni multiple comparison test, where *$p < 0.05$, ***$p < 0.001$ vs. respective control, $^{\$\$}p < 0.01$ vs. DOCA, $^{\&}p < 0.05$ vs. DOCA + Ang1-7, $^{\#}p < 0.05$ vs. DOCA + BNP, $^{@}p < 0.05$ vs. DOCA + Lst.

### 6.4.9 Dual activation of Mas and pGCA receptors modulates serum and urinary electrolytes

Figures 6.10A and 6.10B, suggest DOCA administered in the rats led to an increased sodium intake and sodium balance. Higher levels of sodium and lower levels of potassium intake are associated with elevated blood pressure. However, administration of DAP significantly reduced the sodium intake and sodium balance in DOCA salt rats.

Table 6.2 shows, that after DOCA-salt treatment, serum sodium levels are 145 % higher, however, there is no significant difference in sodium excretion compared to control rats.

Whereas, in DOCA-salt serum potassium levels are 35 % lower and urinary potassium excretion is 125 % higher than in control rats. Additionally, in DOCA-salt treatment, serum chloride levels are 191 % higher, however, chloride excretion is not significantly affected than in control rats. There is not any significant difference in urinary sodium/potassium ration in DOCA-salt-treated rats compared to control rats. In the DAP treated group serum levels of sodium and chloride were reduced by 82.15 % and 56 % respectively, and potassium levels were increased by 237.77 %. While urinary excretion of sodium, and chloride was significantly increased by 224 % and 107.68 % where the excretion of potassium was reduced by 88 %. Addition to this sodium/potassium ratio was also significantly increased by 239 % in DAP treatment rats compared to DOCA-salt rats. Collectively, these data suggest that DAP modulates serum and urinary electrolytes to maintain sodium balance by promoting sodium excretion.

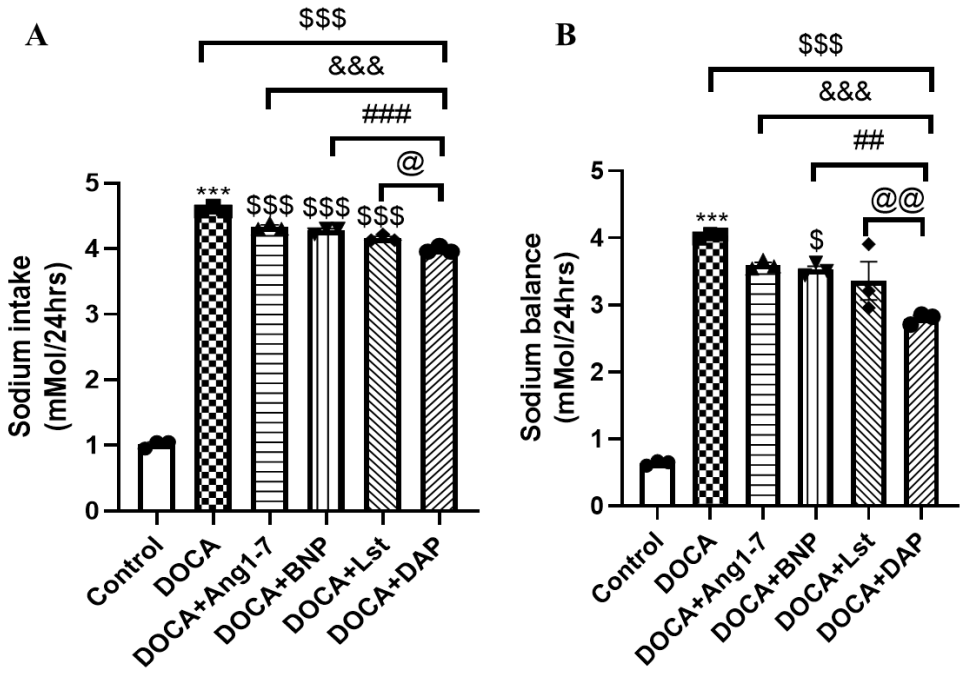



*Figure 6. 10: Dual activation improves sodium balance in DOCA-salt rats*
*(A) Sodium intake (B) Sodium balance. Data were expressed as mean ± SEM (where N = 4). Data were analysed using ordinary one-way ANOVA followed by Bonferroni multiple comparison test, where ***$p < 0.001$ vs. respective control, $^{\$}p < 0.05$, $^{\$\$\$}p < 0.001$ vs. DOCA, $^{\&}p < 0.05$ vs. DOCA + Ang1-7, $^{\#\#\#}p < 0.001$ vs. DOCA + BNP.*

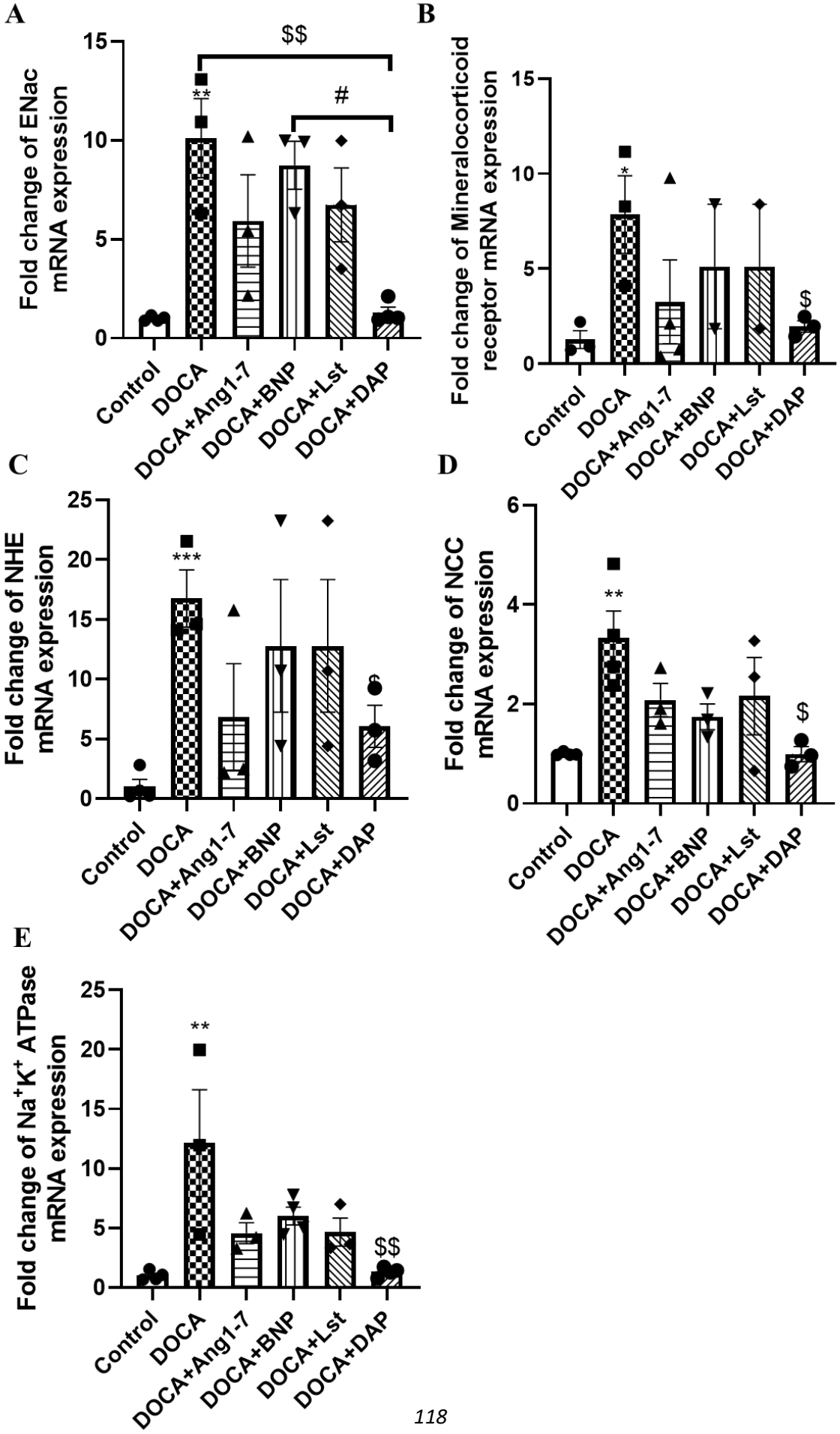
*Table 6. 2: Dual activation modulates serum and urinary electrolytes and promotes sodium excretion*

| Parameter (mMol/L) | Control | DOCA | DOCA+Ang1-7 | DOCA+BNP | DOCA+DAP | DOCA+Lst |
|---|---|---|---|---|---|---|
| Serum sodium concentration | 100 ± 7.39 | 145.79 ± 7.50*** | 137.92 ± 6.28 | 143.61 ± 9.82 | 119.48 ± 13.24$$ | 142.04± 9.84 |
| Urinary sodium excretion | 0.50 ± 0.01 | 0.54 ± 0.02 | 0.74 ± 0.01 | 0.75 ± 0.03 | 1.21 ± 0.04$$$ | 0.75 ± 0.04 |
| Serum potassium | 9.09 ± 0.25 | 3.23 ± 0.25*** | 6.71 ± 0.14 | 6.51 ± 0.6 | 7.68 ± 0.21$$$ | 5.45 ± 0.53 |
| Urinary potassium excretion | 0.27 ± 0.04 | 0.34 ± 0.01*** | 0.32 ± 0.06 | 0.32 ± 0.02 | 0.30 ± 0.05$$$ | 0.33 ± 0.09 |
| Serum chloride | 10.98 ± 0.19 | 20.99 ± 0.26*** | 19.01 ± 0.21 | 17.96 ± 0.29 | 11.83 ± 0.16$$$ | 19.72 ± 0.05 |
| Urinary chloride excretion | 24.05 ± 0.39 | 24.34 ± 0.44 | 25.74 ± 0.60 | 25.45 ± 0.39 | 26.21 ± 0.89$ | 25.78 ± 1.22 |
| Sodium/potassium excretion ratio | 1.38 ± 0.04 | 1.67 ± 0.06 | 2.27 ± 0.02 | 2.38 ± 0.08 | 4.00 ± 0.16$$$ | 2.27 ± 0.12 |

Serum and urinary electrolytes include sodium, potassium, chloride and urinary sodium to potassium ratio. Data were expressed as mean ± SEM (where N = 4). Data were analysed using ordinary one-way ANOVA followed by Bonferroni multiple comparison test, where *$p < 0.05$, ***$p < 0.001$ vs. respective control, $^{\$}p < 0.05$, $^{\$\$\$}p < 0.001$ vs. DOCA.

### 6.4.10 Dual activation of Mas and pGCA receptors downregulates mRNA expression of sodium transporters in DOCA-salt rats

Nakamura et al suggested that aldosterone and its analogue through mineralocorticoid receptors (MR) influence sodium absorption and correlate with changes in epithelial sodium channel expression. High salt and DOCA diet activate epithelial $Na^+$ channel (ENaC), Na-Cl cotransporter (NCC) and Sodium hydrogen exchanger (NHE) on the apical membrane whereas $Na^+$-$K^+$ ATPase on the basolateral membrane in the kidney. As shown in Figure 6.11, we found that gene expression of MR, ENac, NCC, NHE, $Na^+$-$K^+$ ATPase was upregulated in the DOCA salt group. Conversely, DAP is effective in downregulation of mRNA expression of these receptors. Only 20 % of sodium reabsorption from tubules to blood through the basolateral membrane occurs in a thick ascending loop other 75 % occurs in the apical membrane through the proximal convoluted tubule, distal convoluted tubule, and collecting duct from urine to tubules. Collectively, the inhibition of sodium channels promotes sodium excretion and prevents sodium reabsorption.


A
Fold change of ENac mRNA expression
$$
#
**
Control
DOCA
DOCA+Ang1-7
DOCA+BNP
DOCA+Lst
DOCA+DAP
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Fold change of Mineralocorticoid receptor mRNA expression
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C
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D
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Fold change of Na+K+ ATPase mRNA expression
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*Figure 6. 11: Dual activation of Mas and pGCA receptors suppresses the mRNA expression of sodium transporters in DOCA-salt rats*

(A) mRNA expression of the epithelial sodium channel (ENaC) (B) mRNA expression of mineralocorticoid receptor (C) mRNA expression of sodium hydrogen exchanger (NHE) (D) mRNA expression of Sodium-chloride co-transporters (NCC) (E) mRNA expression of Sodium-potassium ATPase ($Na^+k^+$ATPase). Data were expressed as mean ± SEM (where N = 4). Data were analysed using ordinary one-way ANOVA followed by Bonferroni multiple comparison test, where *p < 0.05, **p < 0.01, ***p < 0.001 vs. respective control, $^{\$}$p < 0.05, $^{\$\$}$p < 0.01 vs. DOCA, $^{\#}$p < 0.05 vs. DOCA + BNP.

### 6.4.11 Dual activation of Mas and pGCA receptors inhibits ENaC and promotes eNOS activation in DOCA-salt rats

ENaC in endothelial cells influences vascular tone by increasing intracellular $Na^+$, stabilizing f-actin, and inhibiting endothelial nitric oxide synthase (eNOS), leading to endothelial stiffening, and reduced nitric oxide production. Therefore, we evaluated the influence of ENaC inhibition with dual activation of Mas and pGCA receptors on eNOS activation in the kidney. As discussed earlier, In DOCA-salt ENaC activation was significant relative to the control group. Conversely, eNOS protein expression was downregulated as shown in Figure 6.12. However, the Co-administration of DAP showed upregulated eNOS expression compared to the DOCA-salt group. Simultaneously DAP downregulated the ENac mRNA expression. These results suggest the role of Dual activation of Mas and pGCA receptors on ENac inhibition and eNOS upregulation.

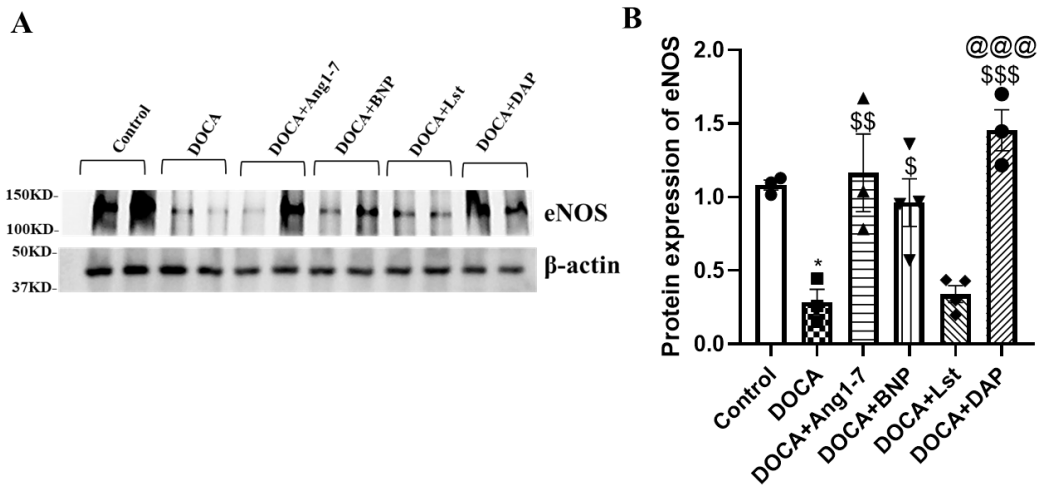



*Figure 6. 12: Dual Activation of Mas and pGCA Receptors Inhibits ENaC and Promotes eNOS Activation in DOCA-salt Rats*

(A) eNOS protein expression were measured by western blot analysis using rat kidney protein samples. (B) Quantification data on protein expression of eNOS from images in A. Data were expressed as mean ± SEM, where N = 4. Data were analysed using ordinary one-way ANOVA followed by Bonferroni multiple comparison test, where *$p < 0.05$ vs. respective control, $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ vs. DOCA, $^{@@@}p < 0.001$ vs. DOCA+Lst.

### *6.5 Discussion*

Dietary high salt intake is a risk factor in the pathogenesis of hypertension (273). Clinical trial studies established a strong relation between dietary salt intake and increased blood pressure; and in animal studies, it compromised vascular function (274). In a healthy environment, mineralocorticoid receptors and ENac sodium channels regulate sodium homeostasis. High sodium intake results in overexpression of these channels which leads to sodium retention and blood pressure increase in the body (23,275). First, in this study, we have investigated the therapeutic role of dual activation of Mas and pGCA receptors on altering apical and basolateral sodium channel gene expression to maintain sodium balance and blood pressure. Thus, the findings of the current study underscore the importance of the co-activation of Mas and pGCA in modulating ENaC and eNOS expression to increase sodium excretion.

The findings from this study provide a comprehensive understanding of the detailed actions of DAP in opposing the unwanted effects of DOCA-salt administration in rats. In our investigation, we first examined the impact of DAP on Mas and pGCA receptors by assessing the levels of their respective secondary messengers in serum. Our findings suggest that DAP functions as a co-activator of Mas and pGCA receptors by increasing the levels of their secondary messengers. This suggests a mechanism by which DAP exerts its effects through the upregulation of these signalling pathways, highlighting its potential therapeutic relevance in modulating the activity of these receptors. . In this study, we used an *in-vivo* model of DOCA-salt-induced hypertension in Wistar rats. In this study, we found that DOCA-salt significantly increased body weight, water intake, blood pressure, and heart rate. In particular, DAP's impact on blood pressure regulation is noteworthy. It is revealed that DAP, compared to Ang1-7, BNP, and losartan, effectively counteracts the actions of DOCA-salt, leading to significant reductions in systolic, diastolic, and mean blood pressure. This reduction in blood pressure is complemented by several other promising alterations, such as decreased heart rate, body weight, kidney weight, heart weight, and water intake. Notably, DAP emerges as a more significant regulator of DOCA-salt effects compared to the other peptides, emphasizing its potential as a potent therapeutic candidate for reducing blood pressure. This study based on the

concept of a first-in-class dual inhibitor, LCZ696 was more effective than angiotensin receptor blocker in blood pressure control (276).

Furthermore, data suggests that DAP is promising in maintaining renal functional parameters to its normal levels. We investigated the impact of DAP on organ hypertrophy and tubular damage. Researchers widely use the DOCA-salt hypertension model in rats to better understand the mechanisms underlying the development of hypertension-induced renal hypertrophy and fibrosis (277). DAP's ability to reduce kidney weight and kidney-to-body weight ratio, both indicators of organ swelling and hypertrophy, underscores its potential to prevent the pathological growth of vital organs. This is supported by the downregulation of TGFβ, a hypertrophic marker, after DAP treatment. The sensitive proximal tubular injury marker Kim-1, in acute kidney injury, is highly up-regulated (278). Additionally, acute and chronic injury marker NGAL was evaluated first time with DAP treatment (275). Notably, results suggest, the reversal of glomerular and tubular injuries in DOCA-salt rats following DAP treatment, as evidenced by haematoxylin-eosin staining. Additionally, the downregulation of gene expression of KIM1 and NGAL, highlights the therapeutic potential of DAP in preventing tubular damage associated with hypertension. Aligning with this, previous studies suggested that independently Ang1-7 and BNP protected against hypertrophy in animal models (198,279). Moreover, the effect of DAP is more noteworthy than the independent peptides administration.

DOCA-salt hypertension induces renal hypertrophy, fibrosis and inflammations. This study also emphasizes DAP's role in combating renal fibrosis, a common outcome of DOCA-salt exposure. As shown in Figure 6.8, the reduction in collagen deposition and downregulation of collagen gene expression in DAP-treated rats compared to DOCA-salt rats demonstrates its efficacy in mitigating fibrosis. Moreover, DAP's superiority to Ang1-7, BNP, and losartan in this aspect suggests that dual activation of Mas and pGCA receptors holds a distinct advantage in reducing fibrosis in the DOCA-salt animal model. In addition to this DAP is also promising in reducing the inflammatory cytokines levels and upregulating the renin mRNA expression in DOCA-salt-treated rats.

Sodium balance disruption is a characteristic consequence of DOCA-salt administration, leading to imbalances in serum and urinary electrolyte concentrations (266,273). DAP, effectively restores sodium balance by controlling sodium intake and promoting sodium excretion. As shown in Table 6.2, the normalization of sodium and chloride levels in both serum and urine, along with increased urinary excretion of sodium and

potassium, demonstrates DAP's ability to maintain healthy electrolyte balance in the body. Moreover, increased sodium excretion suggests decreased sodium retention in renal tubules. In addition to this, the increase in aldosterone levels in response to DOCA administration leads to enhanced sodium reabsorption and potassium excretion (280). Conversely, the reabsorption of sodium is often linked to chloride reabsorption, contributing to salt retention in these experimental settings (281). Understanding these mechanisms is crucial for investigating the effects of DAP on blood pressure regulation and electrolyte balance in the DOCA salt hypertension model.

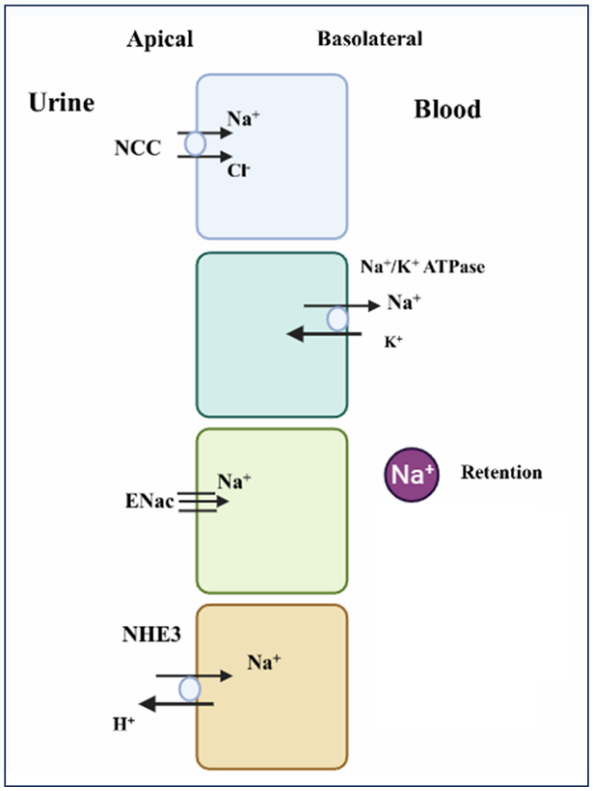



*Figure 6. 13: Regulation of sodium channels in maintaining sodium balance*

DOCA salt-induced hypertension frequently affects multiple sodium channels and transporter expression in the kidney (23). These changes are frequently triggered by the actions of aldosterone on the MR and can result in increased sodium reabsorption in various nephron segments, contributing to hypertension (23). Understanding the regulatory mechanisms of sodium channels and transporters is critical for developing strategies to treat salt-sensitive hypertension. Renal tubule sodium absorption is a critical physiological process controlled by membrane-transport proteins that manage the movement of sodium ions across the plasma membranes of renal epithelial cells. High salt and DOCA diet activate ENaC, NCC and NHE on the apical membrane whereas $Na^+$-$K^+$ ATPase on the basolateral membrane in the kidney. Only 20 % of sodium reabsorption from tubules to blood through the basolateral membrane

occurs in a thick ascending loop other 75 % occurs in the apical membrane through the proximal convoluted tubule, distal convoluted tubule, and collecting duct from urine to tubules (23). Nakamura et al. suggested that the modulation of intestinal mineralocorticoid receptors, which influence sodium absorption in the colon and correlate with changes in epithelial sodium channel expression, suggests a new avenue for studying the molecular mechanisms underlying hypertension and cardiovascular disease (22).

Thus, the study further elucidates the molecular mechanisms underlying DAP's actions by examining its impact on sodium transporters. The downregulation of MR, ENaC, NCC, NHE, and $Na^+K^+$ ATPase in DAP-treated rats compared to DOCA-salt rats provides valuable insights into how DAP influences sodium reabsorption and excretion. ENaC in endothelial cells influences vascular tone by increasing intracellular $Na^+$, stabilizing f-actin, and inhibiting endothelial nitric oxide synthase (eNOS), leading to endothelial stiffening, and reduced nitric oxide production (282). The NO produced by eNOS within the kidney enhances natriuresis and diuresis by dilating the renal vasculature and suppressing transport throughout the nephron (283). Therefore, we evaluated the influence of ENaC inhibition with dual activation of Mas and pGCA receptors on eNOS activation in the kidney. In addition to this, the result suggested that DAP is also upregulating the eNOS protein expression. These findings collectively suggest that DAP's ability to inhibit sodium channels plays a pivotal role in promoting sodium excretion and maintaining sodium balance, further highlighting its potential as a therapeutic agent for conditions associated with mineralocorticoid excess.

## *6.6 Conclusion*

In summary, the findings elucidated in this study underscore the diverse mechanisms through which DAP mitigates the adverse impacts of DOCA-salt administration in a rat. These results collectively emphasize the potential of dual activation of Mas and pGCA receptors as a promising therapeutic option for addressing hypertension, organ hypertrophy, renal injury, fibrosis, and disruptions in sodium balance. DAP's unique mode of action, involving the dual activation of Mas and pGCA receptors and inhibition of ENaC expression, holds promise for the treatment of salt-sensitive hypertension. Further research and clinical studies are necessary to fully explore the clinical efficacy of DAP in human subjects.

# *Chapter 7*

# *Co-Activation of Mas And pGCA Receptors Suppresses Endothelin-1-Induced Endothelial Dysfunction via Nitric Oxide/cGMP System*

## *7.1 Graphical abstract*

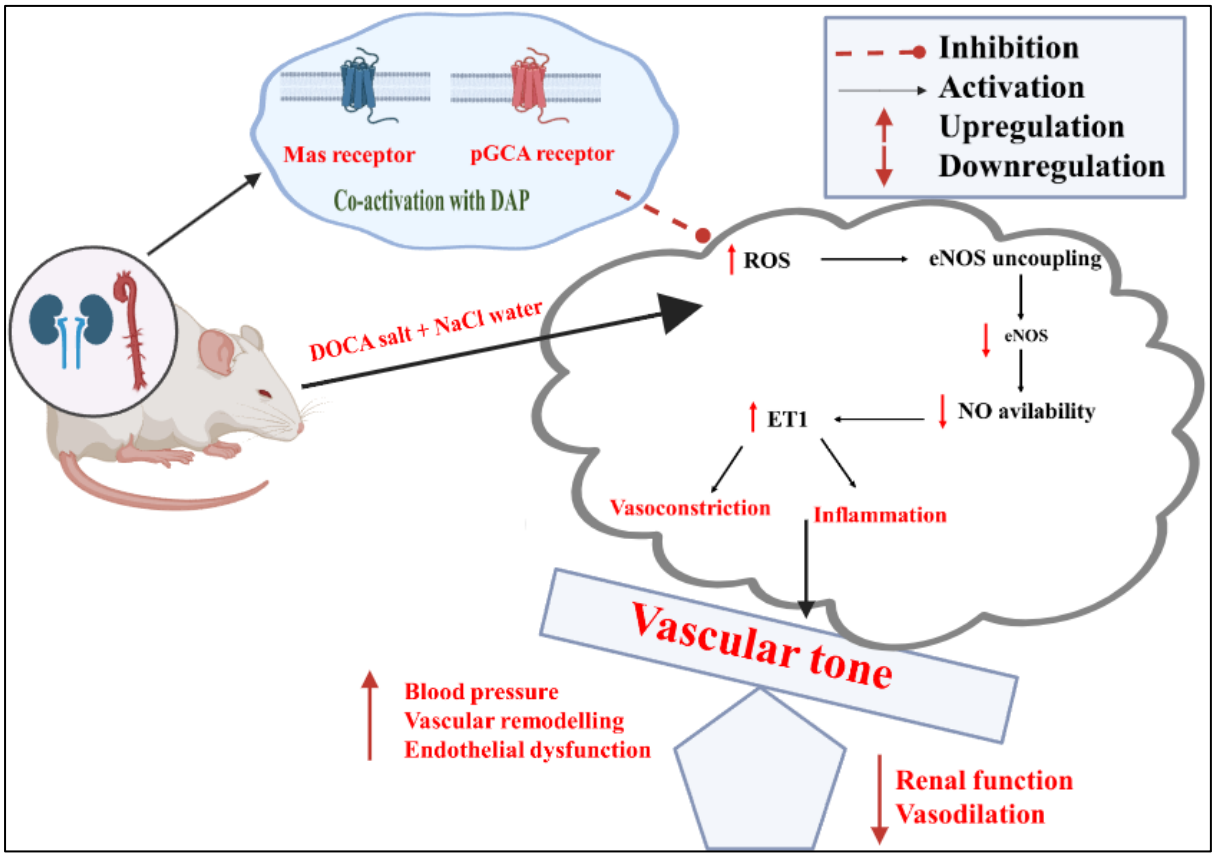



*Figure 7. 1: Graphical abstract for chapter 7*

## *7.2 Introduction*

Endothelial dysfunction, a key indicator of cardiovascular disease, manifests primarily through RAAS abnormalities, shear stress, inflammation, oxidative stress, and a decrease in nitric oxide production. Endothelial dysfunction is associated with vascular inflammation and platelet aggregation inhibition, in addition to impaired vasodilation (284). Furthermore, it is an independent contributor to the exacerbation of cardiovascular complications (88). While current therapeutic approaches, such as RAAS inhibitors and other antihypertensive drugs, have been extensively studied, their effectiveness is primarily limited to lowering blood pressure. These treatments have shown limited efficacy in addressing endothelial dysfunction in hypertensive patients (63). As a result, the need for a multivalent therapeutic strategy to manage endothelial dysfunction becomes more evident, playing a critical role in the overall management of CVD and its complications.

The recently identified Mas receptor axis and pGCA receptor have been recognized independently for their cardiac and renal protective properties via RAAS pathway modulation

(65). Furthermore, these receptors, Mas and pGCA, are linked to the NO/cGMP (285) and ET1 pathways (270). Endothelial-derived NO is essential for protective vasodilation, with the NO/cGMP signalling cascade preserving endothelial and vascular function (286). Nitric oxide not only regulates vasodilation but also contributes to blood vessel remodeling. ET1, on the other hand, is a potent vasoconstrictor that plays an important role in regulating arterial health and blood pressure. Elevated ET1 levels have been linked to vascular damage, remodeling, inflammation, and kidney disease (287). Increased ET-1 expression has been linked to increased nicotinamide adenine dinucleotide phosphate hydrogen oxidase activity and uncoupling of endothelial nitric oxide synthase. The delicate balance of nitric oxide and ET1 is critical for vascular function, and any imbalance between these two factors can contribute to endothelial dysfunction (288).

According to previous published studies, the Mas and pGCA receptors have counter-regulatory effects against the vasoconstrictor AngII, protecting the kidney and heart. Recent research has shown that Ang1-7 mimetics and angiotensin receptor blockers can improve endothelial dysfunction by increasing Ang1-7 levels (65). Similarly, in heart failure, a combination of BNP receptor agonists and neprilysin inhibitors has been shown to improve vasodilation and diuresis by increasing BNP levels (245). Given the individual cardioprotective pathways, therapeutic strategies that address multiple pathways at the same time have the potential to effectively improve endothelial dysfunction complications.

In this study, we investigated a multivalent strategy that activated the NO/cGMP pathway while simultaneously inhibiting the ET1 pathway. The goal was to see if this approach improved therapeutic efficacy and reduced remodeling associated with endothelial dysfunction. This is the first study to investigate two critical issues: the effect of co-activating Mas and pGCA receptors on endothelial dysfunction and vascular damage, and the role of dual activation in maintaining the delicate balance between the NO and ET1 signalling pathways. The findings provide valuable insights for future therapeutic interventions by elucidating a detailed mechanism of dual pathway activation, effectively balancing ET1 and nitric oxide.

## *7.3 Materials and methods*

In this study, we used Wistar rats, weighing between 180-220 g, were ethically obtained with the approval of the IAEC under protocol number BITS-Hyd-IAEC-2022-07. After a minimum of one week of acclimatization, the rats underwent experimental induction of hypertension using DOCA salt and 1 % NaCl drinking water. After induction of hypertension, the rats were

treated with Ang1-7, BNP, DAP, and Losartan. Weekly assessments of body weight, water intake, and blood pressure were conducted. After two weeks of peptide treatment, blood and urine were collected for biochemical parameter analysis. The study also involved various assays such as isometric tension studies on rat aorta, measurement of plasma cGMP and cAMP levels, qPCR, western blotting and histopathological examinations. The detailed protocols for each procedure can be found in Chapter 4, Method Section. Data analysis was performed using one-way ANOVA followed by post hoc Bonferroni's test, and a p-value <0.05 was considered statistically significant. Researchers are encouraged to refer to Chapter 3 for comprehensive protocol details.

## *7.4 Results*

### 7.4.1 DAP administration improves biochemical parameters

As shown in Table 7.1, rats after a 4-week DOCA-salt treatment had an increase in serum LDL levels (control: 44.61 ± 3.28, DOCA-salt: 67.55 ± 3.78, N = 4, $p<0.05$), which was mitigated by DAP treatment (DAP: 49.18 ± 3.74, N = 4, $p<0.05$). Serum HDL levels decreased in DOCA-salt rats and increased slightly after DAP treatment, but the differences were statistically insignificant. Following DOCA-salt treatment, serum total cholesterol, triglycerides, CkMB, albumin, and creatinine concentrations all increased significantly. The elevated TC/HDL and TG/HDL ratios indicated a lipid profile abnormality and the progression of arterial diseases. Similarly, Surprisingly, DAP treatment significantly reduced the the TC/HDL and TG/HDL ratios.

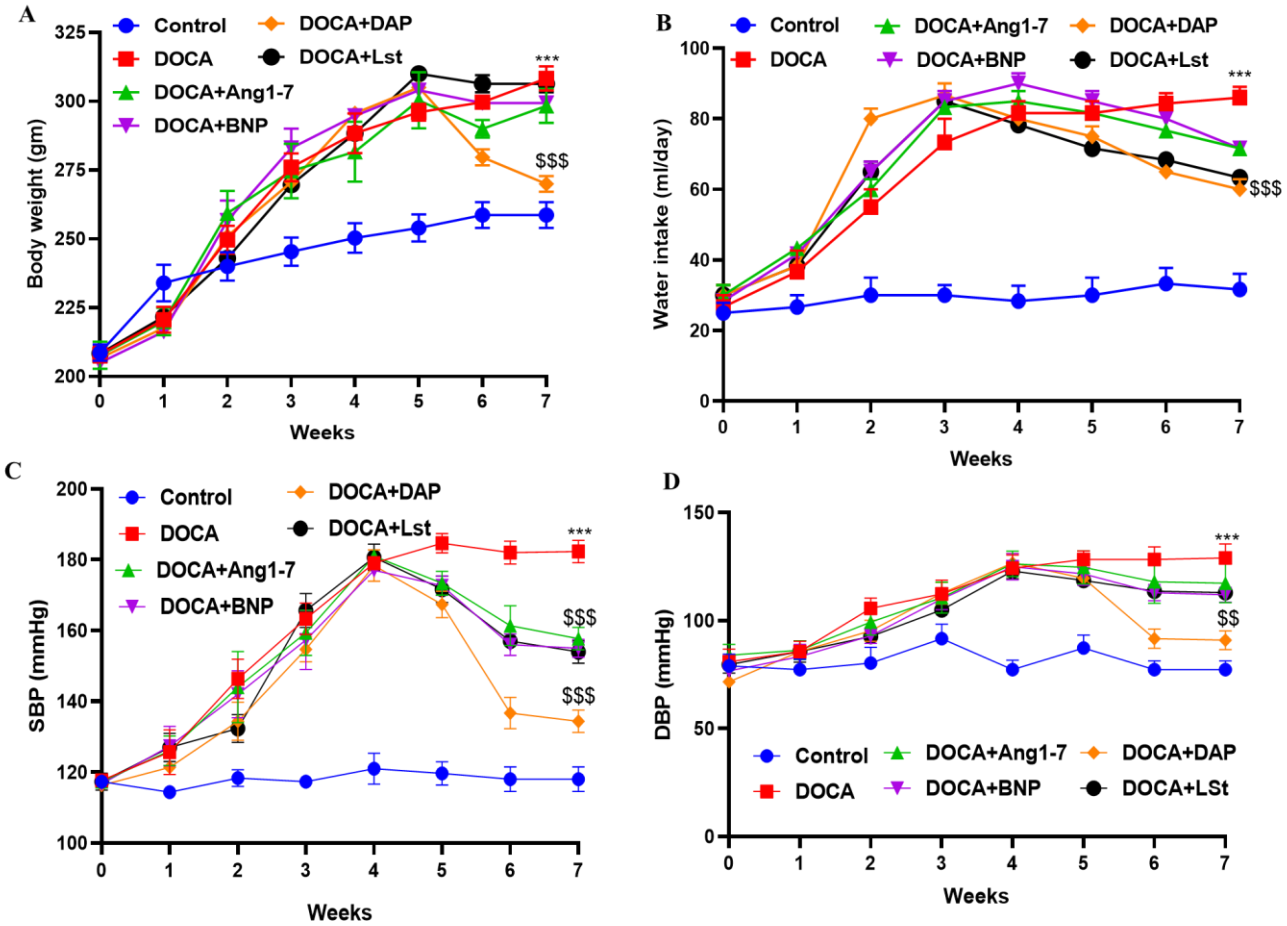
*Table 7. 1: Serum and urine biochemical parameters*

| Samples | Parameter | Symbol and units | Control (N=4) | DOCA (N=4) | DOCA+DAP (N=4) |
|---|---|---|---|---|---|
| Serum | Low-density lipoprotein | LDL (mg/dL) | 45.61 ± 3.28 | 67.55 ± 3.78$^{*}$ | 49.18 ± 3.74$^{\$}$ |
| | High-density lipoprotein | HDL (mg/dL) | 28.58 ± 0.19 | 24.00 ± 1.75 | 27.44 ± 0.43 |
| | Total cholesterol | TC (mg/dL) | 87.48 ± 2.29 | 121.89 ± 6.65$^{**}$ | 91.54 ± 2.81$^{\$}$ |
| | Triglycerides | TG (mg/dL) | 76.33 ± 6.13 | 126.57 ± 5.44$^{***}$ | 91.78 ± 4.85$^{\$\$}$ |
| | Total cholesterol/High-density lipoprotein | TC/HDL | 3.06 ± 0.731 | 5.07 ± 8.523$^{*}$ | 3.33 ± 6.039$^{\$}$ |
| | Triglycerides/High-density lipoprotein | TG/HDL | 2.67 ± 0.747 | 5.27 ± 3.469$^{**}$ | 3.34 ± 4.577$^{\$}$ |
| | Creatine kinase-myocardial band | CKMB (IU/L) | 4.93 ± 0.07 | 12.75 ± 0.52$^{***}$ | 8.59 ± 0.32$^{\$}$ |

Data were expressed as mean ± SEM (where N = 4). Data were analysed using ordinary one-way ANOVA followed by Bonferroni multiple comparison test, where $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. respective control, $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ vs. DOCA.

### 7.4.2 DAP administration reduces blood pressure in DOCA-Salt rats

Treatments, including Ang1-7, BNP, and losartan, demonstrated efficacy in lowering blood pressure over 2 weeks in the context of DOCA-salt-induced hypertension. However, DAP had a significant impact, resulting in a significant decrease in blood pressure. DAP treatment caused significant reductions in body weight and saltwater intake in hypertensive Wistar rats after only 2 weeks of intervention, as shown in Figures 7.2A and 7.2B. Concurrently, DAP administration resulted in significant reductions in systolic and diastolic blood pressure when compared to DOCA-salt-treated groups (Figures 7.2C and 7.2D). Surprisingly, DAP showed a promising ability to lower mean blood pressure in the DOCA-salt group, as shown in Figure 7.2E. Furthermore, as shown in Figure 7.2F, DAP treatment effectively reduced the elevated heart rate levels observed in DOCA-salt rats.



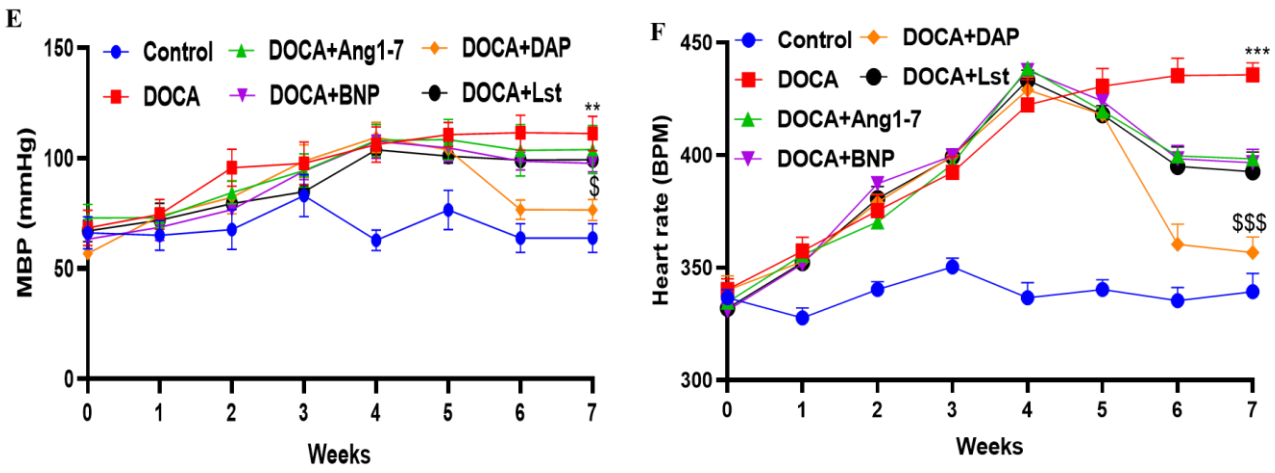



*Figure 7. 2: DAP Administration Reduces Blood Pressure In DOCA-Salt Rats*

After 28 days of DOCA treatment randomly few rats received Ang1-7, BNP, DAP and Lst. After 2 weeks of treatment, we observed (A) Body weight (B) Water intake (C) SBP (D) DBP (E) MBP (F) Heart rate. Data were expressed as mean ± SEM (where N = 4). Data were analysed using ordinary one-way ANOVA followed by Bonferroni multiple comparison test, where **p < 0.01, ***p < 0.001 vs. respective control, $^{\$}$p < 0.05, $^{\$\$}$P < 0.01, $^{\$\$\$}$p < 0.001 vs. DOCA.

### 7.4.3 DAP treatment increases Mas and pGCA receptor gene expression

We looked at the mRNA expression of Mas and pGCA receptors in rat aorta and kidney tissues after a 4-week DOCA-salt treatment. Notably, mRNA levels of Mas and pGCA receptors were significantly reduced in DOCA-salt-treated rats' kidney and aorta samples, as shown in Figures 7.3A and 7.3B. When compared to the DOCA-salt group, DAP demonstrated remarkable efficacy in reversing this downregulation in both kidney and aorta, resulting in a significant upregulation of Mas and pGCA receptor mRNA, as shown in Figure 7.3C-7.3F.

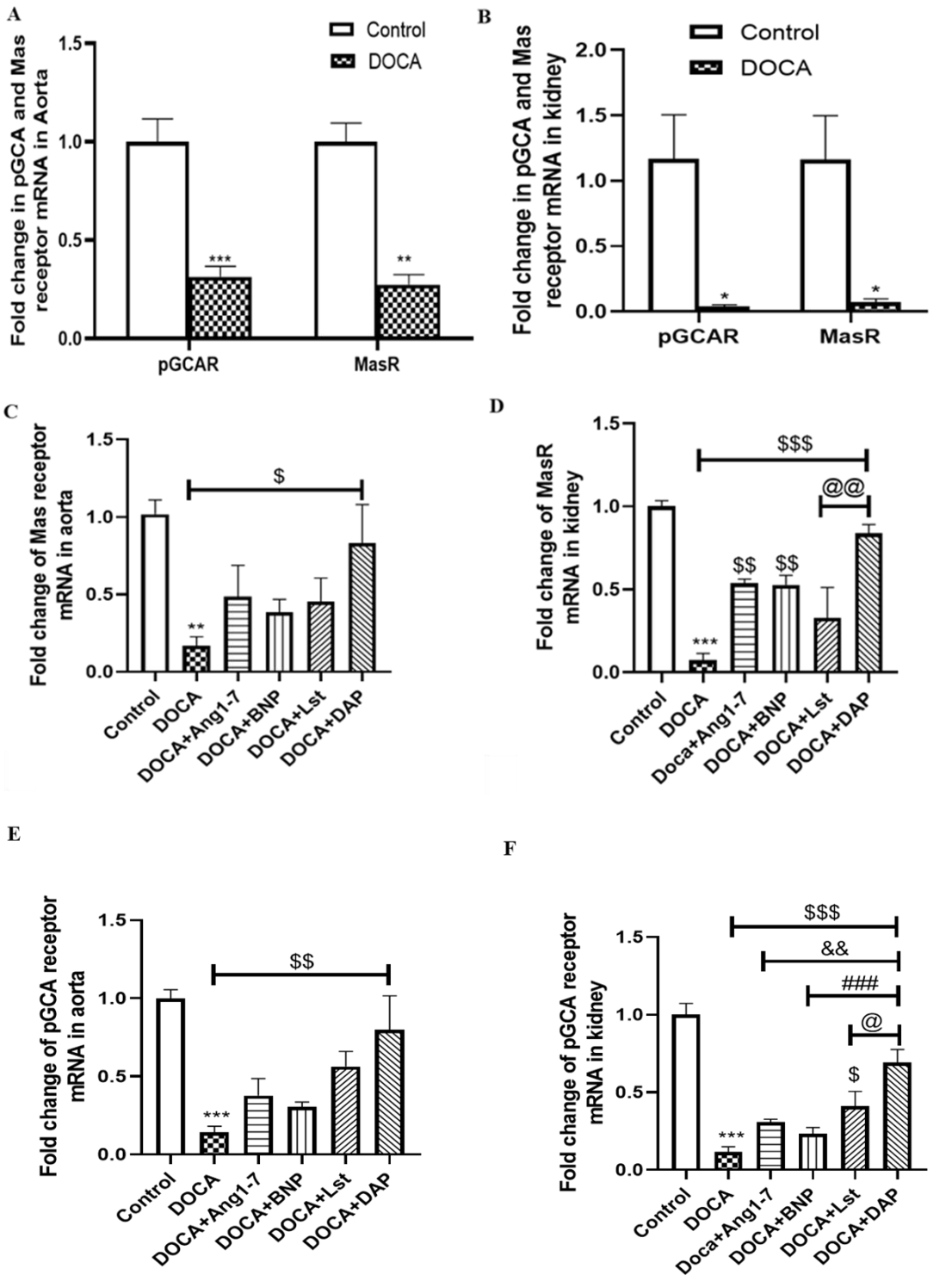



Figure 7. 3: DAP Treatment Increases Mas And pGCA Receptor Gene Expression

After 4 weeks of DOCA-salt treatment, gene expression analysis of (A) pGCA and Mas receptor in aorta cDNA (B) pGCA and Mas receptor in kidney cDNA. After 2 weeks of Ang1-7, BNP, DAP and Lst treatment we measured (C) The gene expression of the Mas receptor in the aorta (D) The gene expression of the Mas receptor in the kidney (E) The gene expression of the pGCA receptor in the aorta (F) The gene expression of the pGCA receptor in the kidney

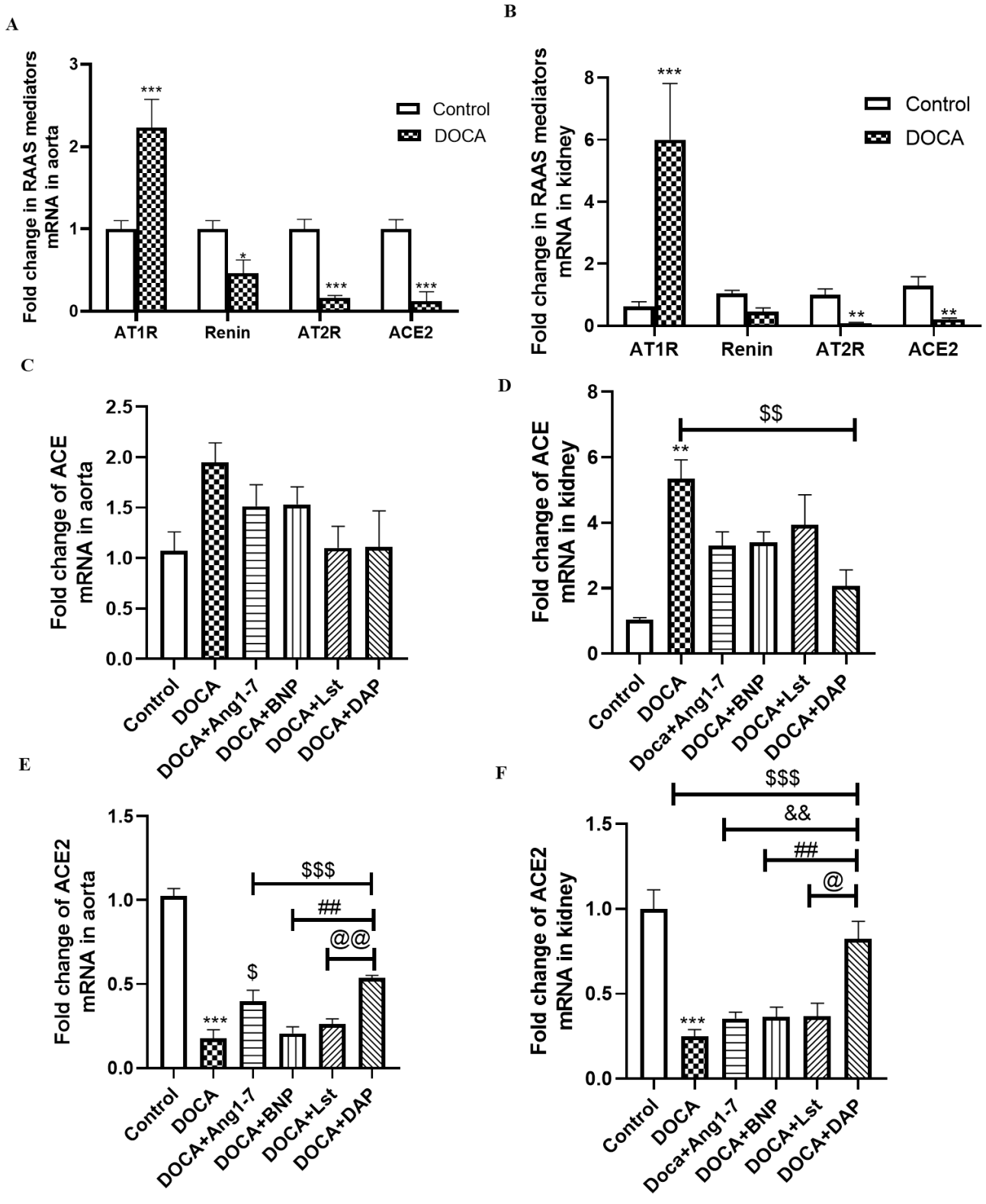
by qPCR. Data were expressed as mean ± SEM, where N = 4. Data were analysed using ordinary one-way ANOVA followed by Bonferroni multiple comparison test, where *p < 0.05, **p < 0.01, ***p < 0.001 vs. respective control, $^{\$}$p < 0.05, $^{\$\$}$p < 0.01, $^{\$\$\$}$p < 0.001 vs. DOCA, $^{\&\&}$p < 0.01 vs. DOCA+Ang1-7, $^{\#\#\#}$p < 0.001 vs DOCA+BNP, $^{@}$p < 0.05, $^{@@}$p < 0.01 vs. DOCA+Lst.

### 7.4.4 DAP treatment modulates RAAS pathway gene expression

Following DOCA treatment, we found a significant increase in the expression of the AT1R gene in both the aorta and kidney. In contrast, AT2R, renin, and ACE2 gene expression was significantly reduced in both organs (Figure 7.4A and 7.4B), confirming the successful induction of hypertension via the RAAS pathway. Figure 7.4C shows that ACE mRNA expression increased in the aorta of DOCA-salt rats, with a non-significant decrease after DAP treatment. However, as shown in Figure 7.4D, there was a significant decrease in ACE expression in the kidneys of DAP-treated rats. DOCA-salt rats' ACE2 expression was significantly reduced in both kidney and aorta tissues. DAP treatment, however, effectively reversed this effect, increasing ACE2 expression (Figures 7.4E and 7.4F).

Our findings are consistent with previous research linking the ACE/ACE2 ratio to key parameters in human hypertension such as SBP, proteinuria, blood glucose, and serum creatinine levels. In DOCA-salt-induced hypertensive rats, the aortic and kidney ACE/ACE2 gene expression ratios were significantly higher than in control rats. DAP treatment, on the other hand, significantly reduced this ratio, as shown in Figures 7.4G and 7.4H.

The DOCA-salt model, which mimics human hypertension with high aldosterone and low renin, revealed a decrease in renin protein expression in the kidney after DOCA-salt administration. Importantly, DAP treatment significantly reversed this trend as shown in Figures 7.4I and 7.4J. These findings show that a 2-week DAP treatment can effectively modify RAAS mediators in both kidney and aorta tissues.


A
Fold change in RAAS mediators mRNA in aorta
Control
DOCA
AT1R
Renin
AT2R
ACE2
B
Fold change in RAAS mediators mRNA in kidney
C
Fold change of ACE mRNA in aorta
Control
DOCA
DOCA+Ang1-7
DOCA+BNP
DOCA+Lst
DOCA+DAP
D
Fold change of ACE mRNA in kidney
Doca+Ang1-7
E
Fold change of ACE2 mRNA in aorta
F
Fold change of ACE2 mRNA in kidney

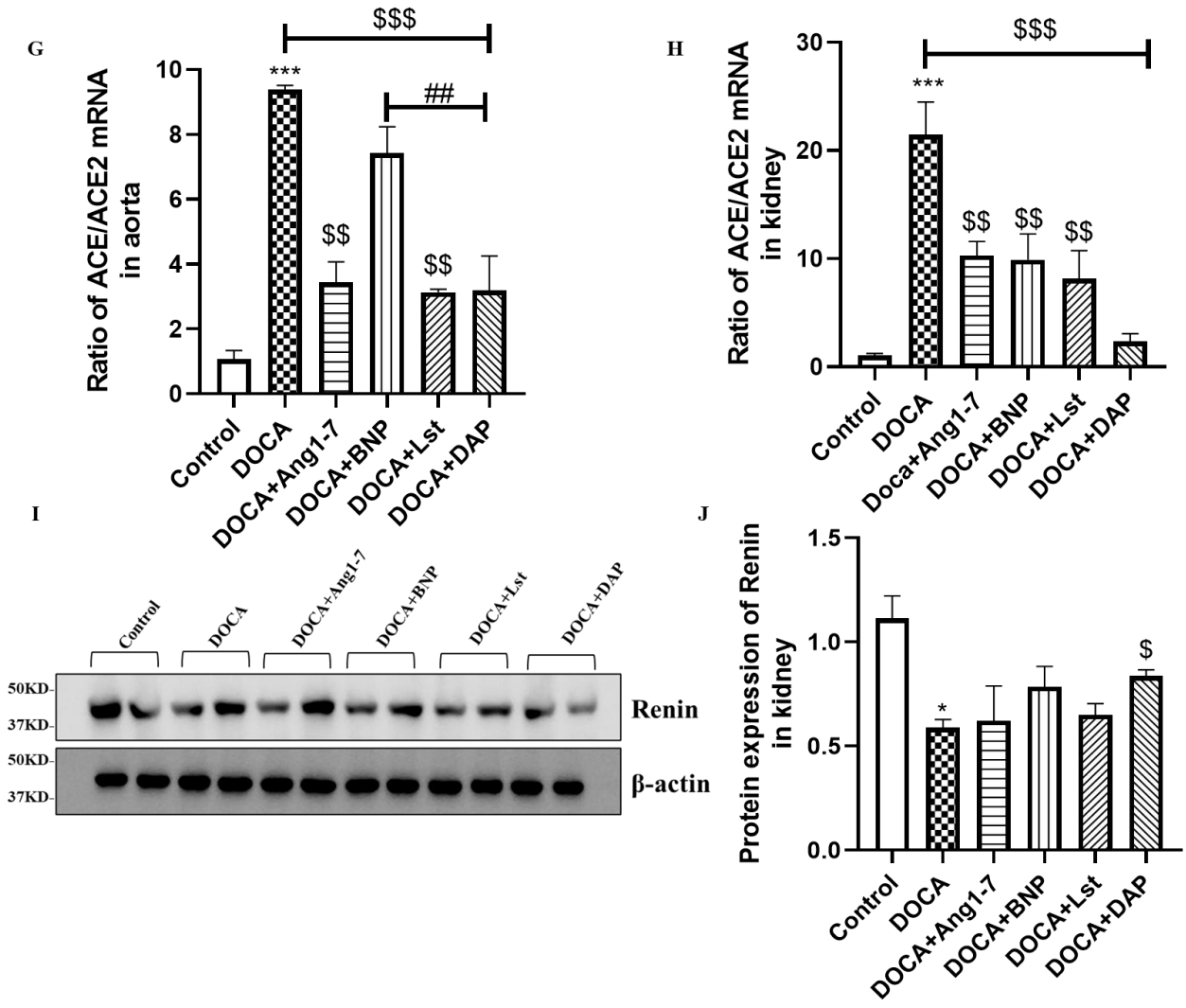


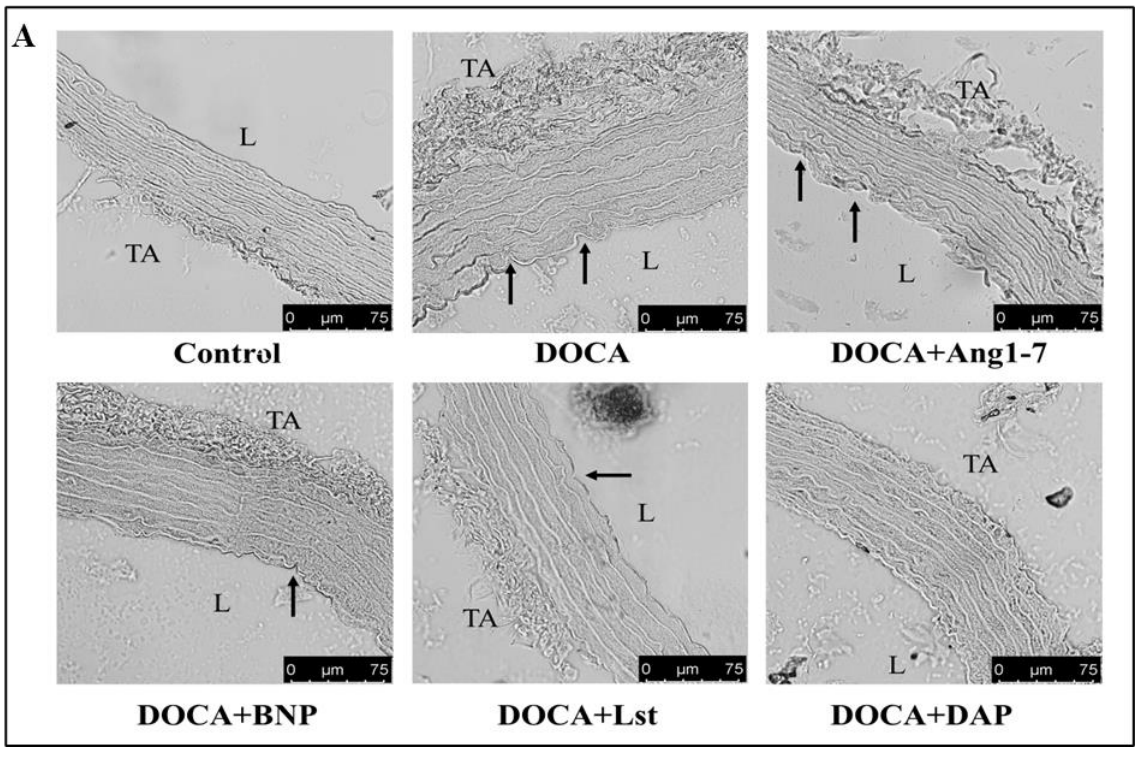

Figure 7. 4: DAP Treatment Modulates RAAS Pathway Gene Expression

Gene expression analysis of AT1, renin, AT2 and ACE2 in (A) aorta (B) Kidney after 4 weeks of DOCA-salt treatment by qPCR. After confirming the successful development of hypertension, we treated rats with peptide and losartan. (C) Gene expression analysis of ACE in aorta (D) Gene expression analysis of ACE in Kidney by qPCR. (E) Gene expression analysis of ACE2 in aorta (F) Gene expression analysis of ACE2 in the kidney by qPCR. (G) ACE/ACE2 ratio in the aorta (H) ACE/ACE2 ratio in the kidney. (I) Renin levels were measured by western blot analysis using rat kidney protein samples. (J) Quantification data on protein expression of renin from images in I. Data were expressed as mean ± SEM, where N = 4. Data were analysed using ordinary one-way ANOVA followed by Bonferroni multiple comparison test, where, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. respective control, $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ vs. DOCA, $^{\&\&}p < 0.01$ vs. DOCA+Ang1-7, $^{\#\#}p < 0.01$ vs DOCA+BNP, $^{@}p < 0.05$, $^{@@}p < 0.01$ vs. DOCA+Lst.

### 7.4.5 DAP treatment alleviates vascular remodeling and protects endothelial integrity

Endothelial dysfunction is critical in the progression of CVDs. We investigated vascular damage and its implications to better understand the underlying mechanisms. Vascular remodeling, a key factor in the development and exacerbation of endothelial dysfunction, was thoroughly studied. DOCA-salt-treated rats had internal aortic lining distortions, which were significantly reduced in both control and DAP-treated rats (Figure 7.5A). This remodeling was primarily caused by increased aortic thickness, which resulted in a smaller lumen diameter. When compared to control rats, DOCA-salt treatment significantly increased aortic thickness. DAP treatment, on the other hand, emerged as a powerful intervention, effectively reducing aortic thickness when compared to other peptides and losartan (Figure 7.5B and 7.5D). As a result, the lumen diameter of DOCA-salt rats decreased and was successfully restored in the DAP-treated group (Figure 7.5C and 7.5E).

We calculated the W/L to quantify these changes. Surprisingly, the W/L ratio increased significantly in DOCA-salt-treated rats, highlighting the extent of vascular damage. When compared to individual peptides and losartan, DAP treatment reduced the W/L ratio significantly (Figure 7.5F). These findings highlight the importance of DOCA-salt-induced vascular damage in endothelial dysfunction. Notably, our synthesized DAP demonstrated promising potential in preserving vascular structural and functional integrity, implying its involvement in critical vascular health mechanistic pathways.



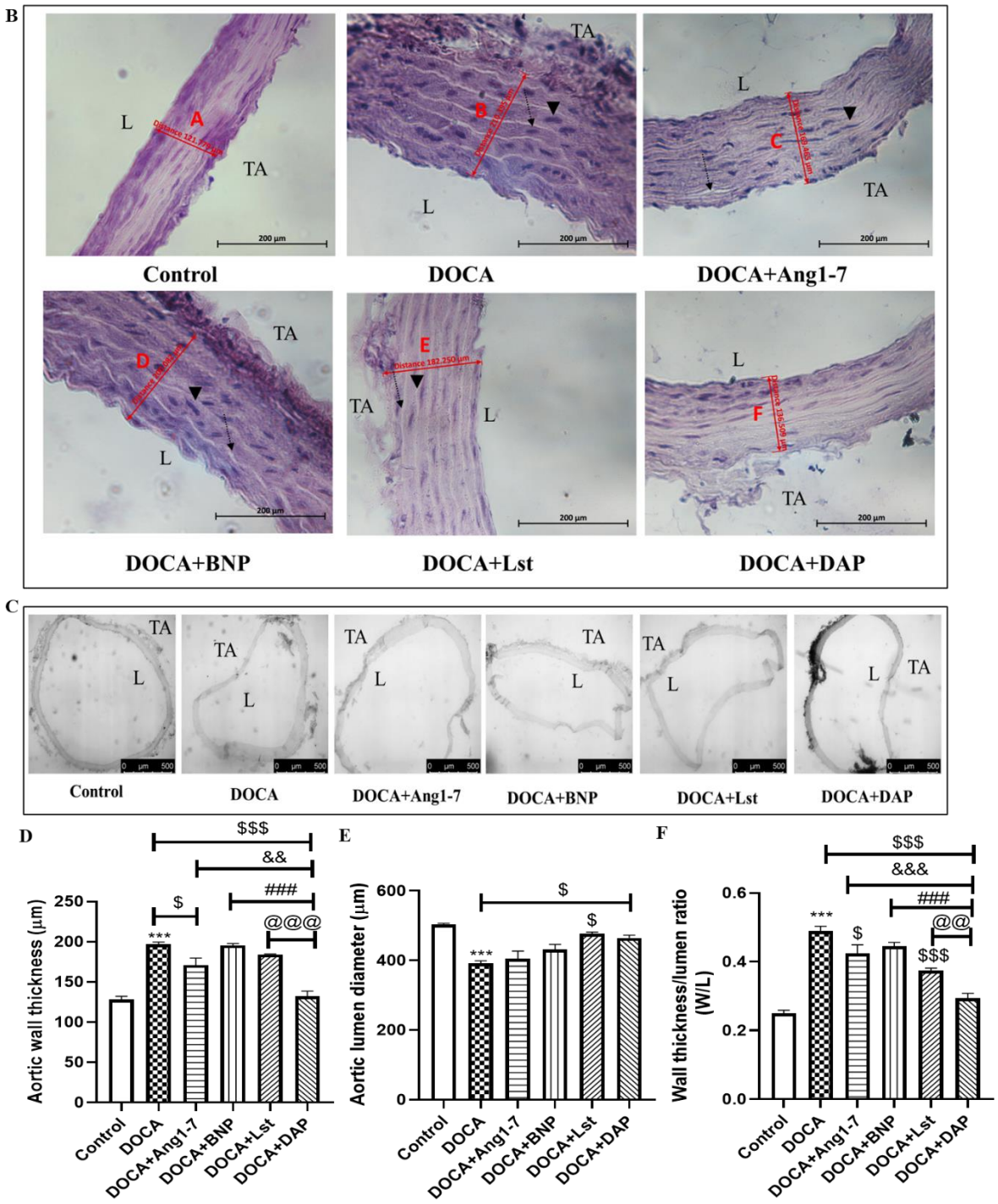


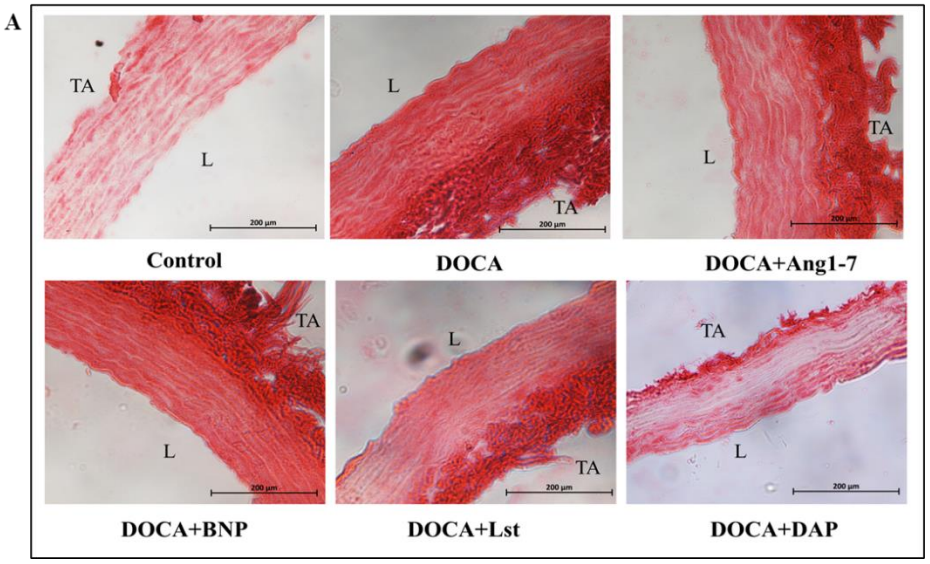

*Figure 7. 5: DAP Treatment Alleviates Vascular Remodeling and Protects Endothelial Integrity*

Changes in media thickness and lumen diameter define vascular remodeling. (A) Representative brightfield cross sections of rat aorta. The black arrow shows the distortion of the inner lining of the aortic wall. Scale bars, 75 µm. (B) Representative cross-sections of rat aorta with haematoxylin-eosin staining. The red arrow shows the area considered for measuring the thickness of the aorta. Here, A, B, C, D, E, and F represent thickness. (Units in µm) A:

121.77, B: 210.36, C:160.47, D: 200.03, E: 182.25, F: 136.50. The dashed arrow shows increased perinuclear space. The arrowhead indicates flattened nuclei. Scale bars, 200 µm. (C) Representative cross-sections of whole rat aorta from each group. Vessel narrowing and media thickness increased in hypertension rats. Scale bars, 500 µm. (D) Quantification data on aortic wall thickness (E) Quantification data on aortic lumen diameter (F) Representative data on aortic wall thickness/lumen diameter ratio (W/L ratio). Data were expressed as mean ± SEM (where N = 4). Data were analysed using ordinary one-way ANOVA followed by Bonferroni multiple comparison test, where ***$p < 0.001$ vs. respective control, $^{\$}p < 0.05$, $^{\$\$\$}p < 0.001$ vs. DOCA, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ vs. DOCA+Ang1-7, $^{\#\#\#}p < 0.001$ vs. DOCA+BNP, $^{@@@}p < 0.001$ vs. DOCA+Lst. Here L indicates the lumen side of the aorta and TA indicates tunica adventitia.

### 7.4.6 DAP treatment reduces fibrosis in DOCA-salt rats

Blood vessel fibrosis and calcification contribute to stiffness and dysfunction. Using Sirius red staining, we discovered a significant increase in collagen deposition (a fibrosis marker) in DOCA-salt-induced rats. DAP treatment, on the other hand, resulted in a significant twofold reduction in collagen deposition (Figure 7.6A and 7.6B). Serum cAMP, a fibrosis regulator, decreased in DOCA-salt rats but increased significantly after DAP treatment (Figure 7.6C). The expression of α-SMA, a fibrotic protein, was higher in DOCA-salt groups but decreased with DAP treatment (Figures 7.6D and 7.6E). Aortic calcification was also increased in DOCA-salt rats, but DAP showed promise in reducing it (Figures 7.7A and 7.7B). These findings indicate that DAP activation of dual receptors has potent anti-fibrotic and anti-calcification effects, outperforming the outcomes of single receptor activation.



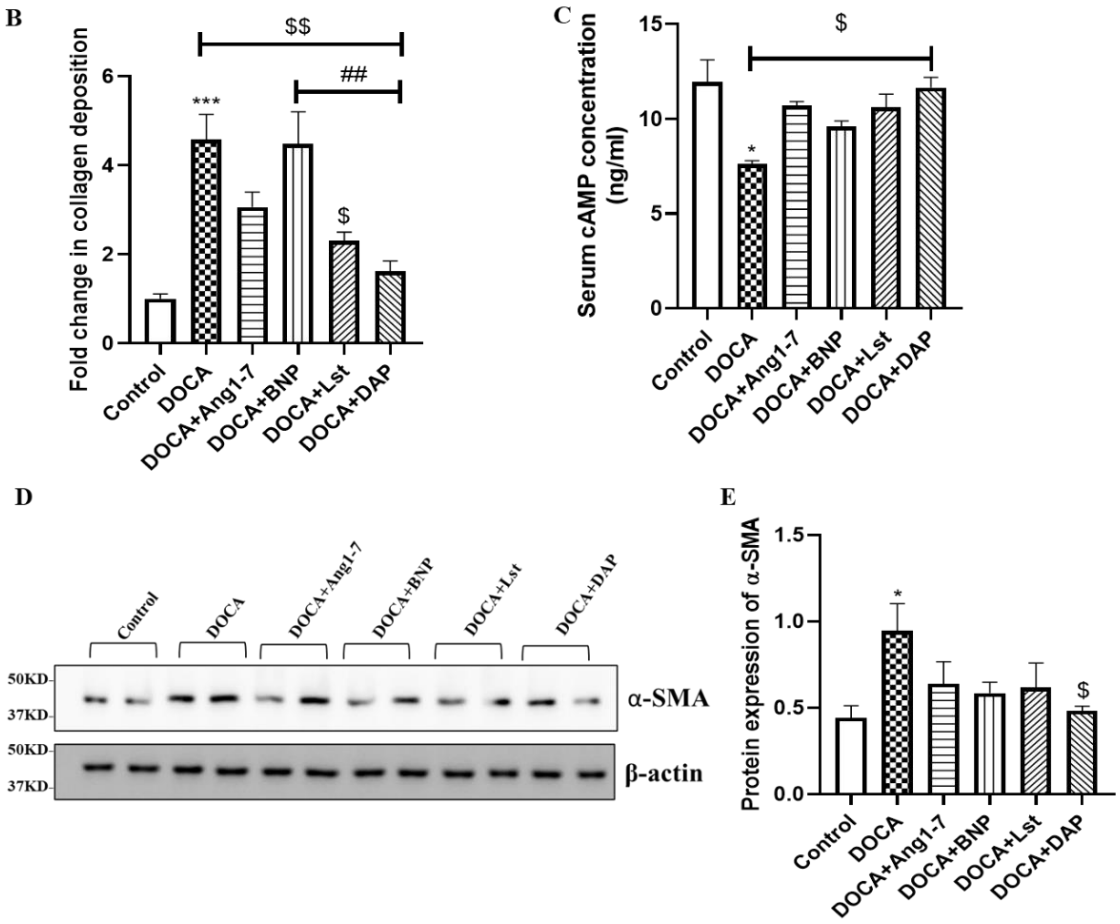



*Figure 7. 6: DAP Treatment Reduces Fibrosis In DOCA-Salt Rats*

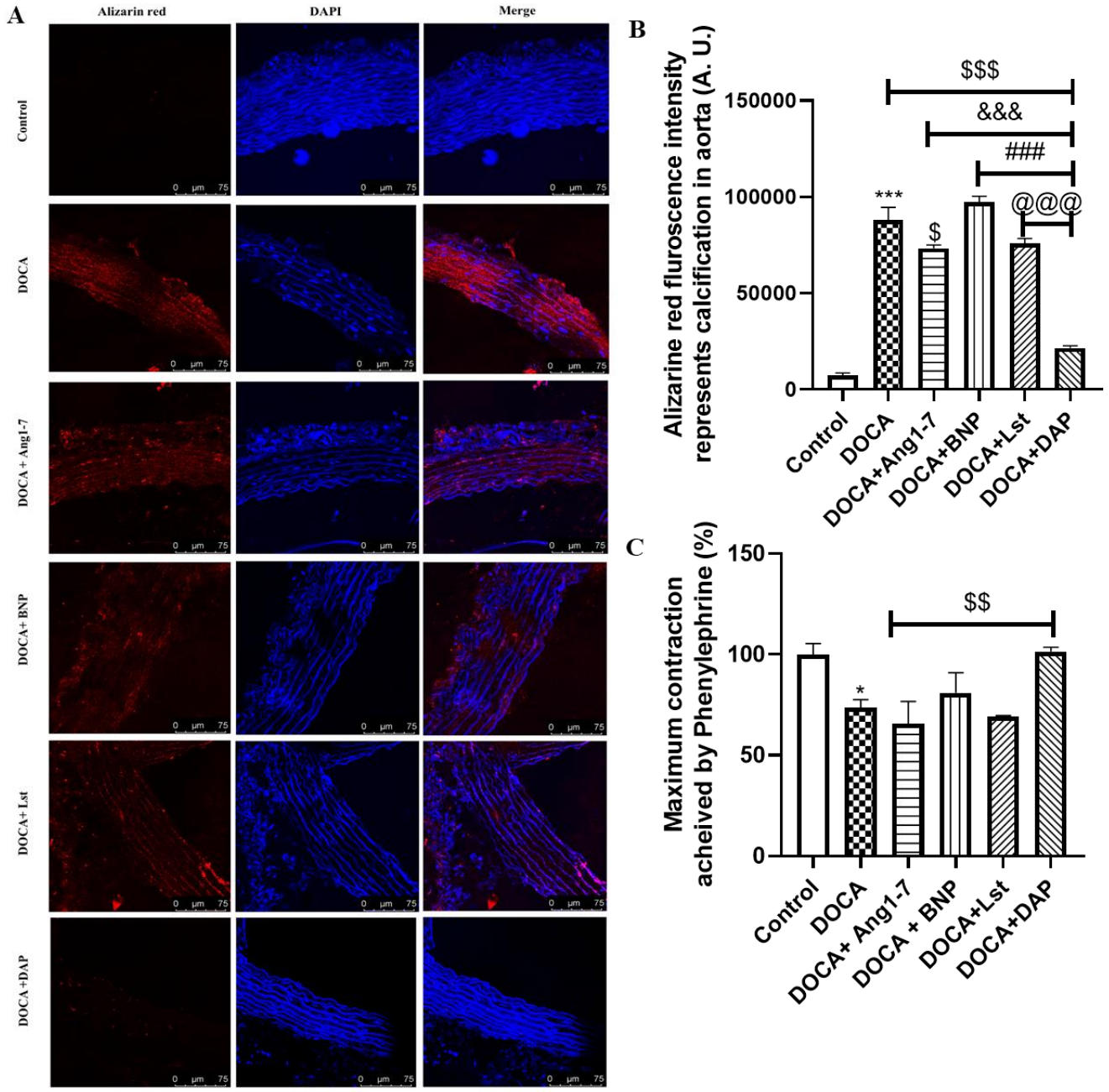
(A) Representative cross sections of rat aorta by picrosirius red staining for collagen deposition. Scale bars, 200 μm. (B) Quantified data on fold change in aortic collagen deposition from Sirius red staining. (C) Serum cAMP was measured with an assay kit. (D) α-SMA levels measured by western blot analysis using rat aorta protein samples. (E) Quantification data on protein expression of α-SMA from images in D. Data were expressed as mean ± SEM (where N = 4). Data were analysed using ordinary one-way ANOVA followed by Bonferroni multiple comparison test, where $^{*}p < 0.05$, ***$p < 0.001$ vs. respective control, $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ vs. DOCA, $^{\#\#}p < 0.01$ vs. DOCA+BNP. Here L indicates the lumen side of the aorta and TA indicates tunica adventitia.

### 7.4.7 DAP alleviates endothelial dysfunction in DOCA-salt rats

Vascular tone dysfunction is associated with increased wall thickness, decreased lumen diameter, vascular fibrosis, and calcification (289). Figure 7.7A and 7.7B suggests that DAP is promising in reducing the vascular calcification in DOCA-salt induced rat. Aortic rings isolated from DOCA-salt rats showed a reduced contractile response to PhE, which was restored in

DAP-treated rats (Figure 7.7C). Ach-induced endothelial relaxation was significantly reduced in aortic rings from DOCA-salt-induced rats compared to control rats, potentially contributing to weakened contraction. DAP treatment, on the other hand, increased Ach-induced vasorelaxation by 40 % compared to DOCA-salt rats (Figure 7.7D).

Furthermore, cGMP production in the serum of DOCA-salt rats was lower than in the control group (7.83 ± 0.05 vs 17.52 ± 0.46 pmol/ml, respectively; N = 4; $p<0.001$). DAP, on the other hand, significantly increased cGMP production (16.00 ± 1.08 pmol/ml, N = 4, $p<0.001$) (Figure 7.7E). These findings prompted a more in-depth investigation of the effect of dual receptor activation on the eNOS-dependent vasorelaxation mechanism.



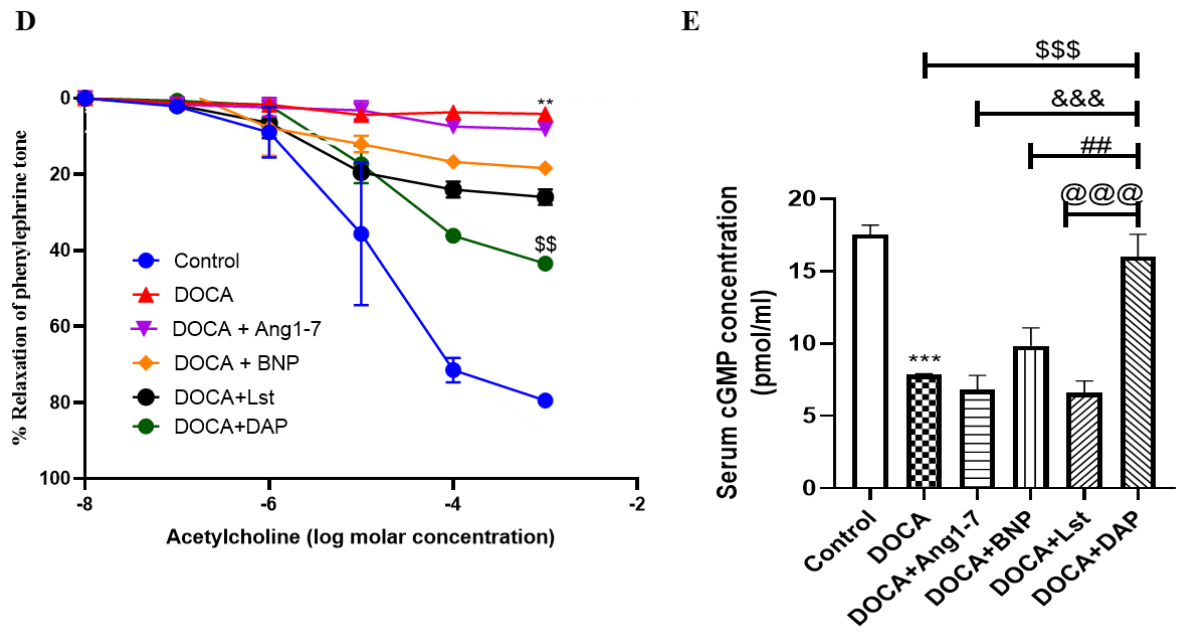



*Figure 7. 7: DAP Alleviates Endothelial Dysfunction In DOCA-Salt Rats*

(A) DAP reduces calcification, where the rat aorta is incubated with alizarin red staining. Scale bars, 75 µm (B) Quantified data on aortic calcification from alizarin red staining**.** DOCA-salt treatment alters the vascular tone of the aorta. Concentration-related responses were obtained to acetylcholine in phenylephrine-precontracted rings in control, disease and treatment groups. (C) Maximum contraction achieved by phenylephrine (Units in percentage). (D) Quantified data on relaxation responses achieved by acetylcholine in the aorta by isometric artery tension studies. (E) Serum cGMP was measured with an assay kit. Data were expressed as mean ± SEM, where N = 4. Data were analysed using ordinary one-way ANOVA followed by Bonferroni multiple comparison test, where $^{*}p < 0.05$, $^{**}p < 0.01$, ***$p < 0.001$ vs. respective control, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ vs. DOCA, $^{\&\&\&}p < 0.001$ vs. DOCA+Ang1-7, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. DOCA+BNP, $^{@@@}p < 0.001$ vs. DOCA+Lst. Here L indicates the lumen side of the aorta and TA indicates tunica adventitia.

### 7.4.8 Dual activation of the DAP administration initiates the eNOS-dependent pathway

DAP administration initiates the eNOS-dependent pathway, which is important in the development of various cardiovascular diseases. Our study found that DOCA-salt rats had downregulated eNOS protein expression than the control group, whereas DAP treatment resulted in a significant upregulation of eNOS protein expression (see Figure 7.8A and 7.8B). We investigated the eNOS downstream signalling pathway to better understand how dual activation causes eNOS-dependent vasorelaxation. Our findings show a decrease in PKG

protein expression in the DOCA-salt group, followed by an increase after DAP treatment as illustrated in Figures 7.8A and 7.8C. Notably, neither DOCA nor DAP had a significant effect on VEGF protein expression (see Figures 7.8A and 7.8D).

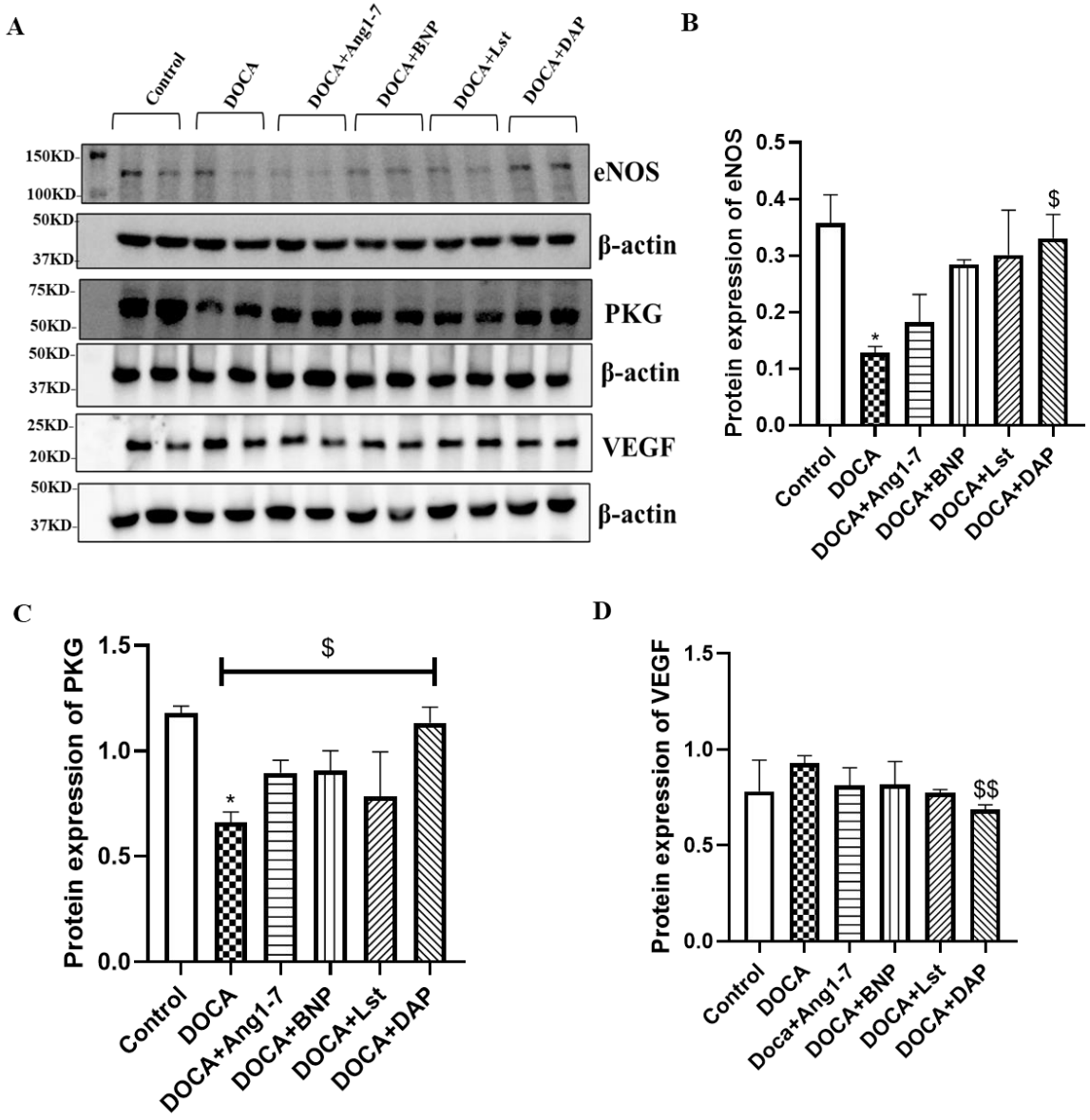


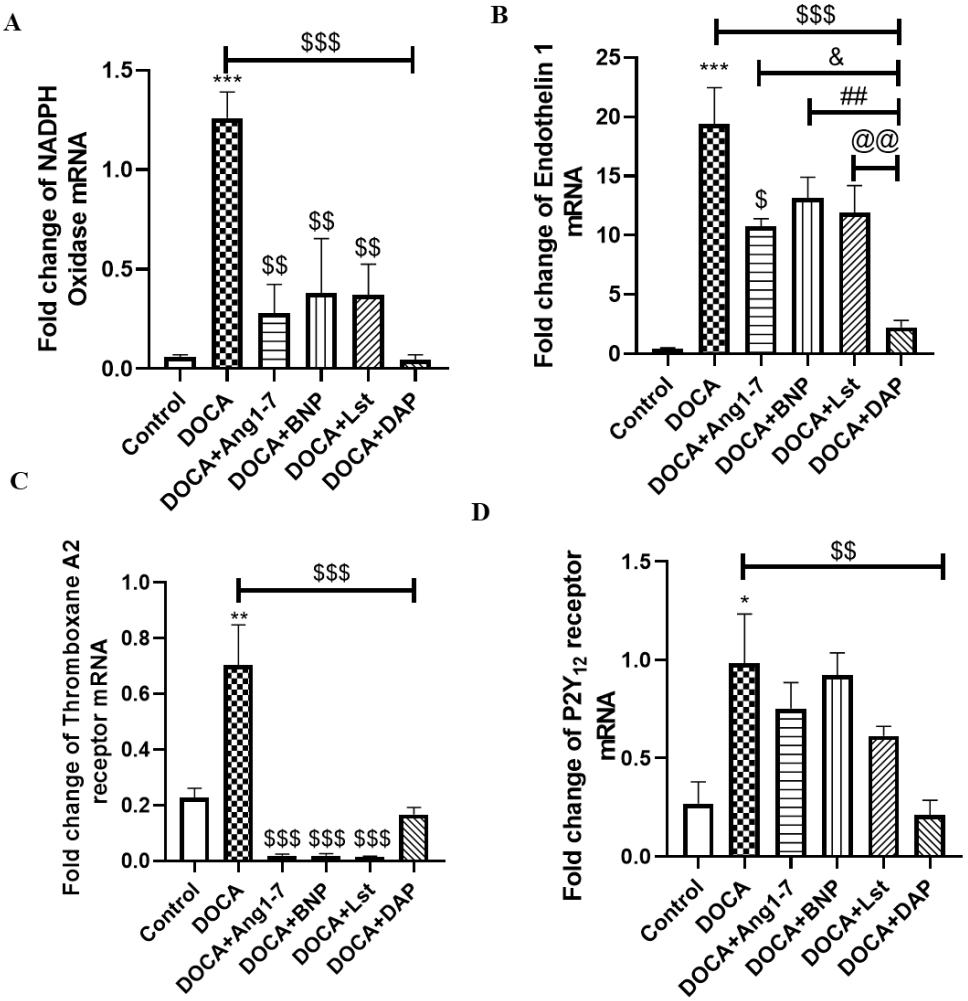

*Figure 7. 8: Dual Activation of The DAP Administration Initiates The eNOS-Dependent Pathway*

(A) Aortic levels of eNOS, PKG and VEGF were measured by western blot analysis. (B) Quantification data on protein expression of eNOS from images in A. (C) Quantification data on protein expression of PKG from images in A. (D) Quantification data on protein expression of VEGF from images in A. Data were expressed as mean ± SEM (where N = 4). Data were

analysed using ordinary one-way ANOVA followed by Bonferroni multiple comparison test, where $^{*}p < 0.05$ vs. respective control, $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ vs. DOCA.

### 7.4.9 DAP exerted inhibitory effects on ET1-dependent pathways in DOCA-salt treated rats

Using qPCR, we investigated the effect of NO activation on ET1 pathways. The gene expression of NADPH oxidase, ET1, Thromboxane A2 receptor, and P2Y12 receptor upregulated significantly in groups treated with DOCA-salt compared to the control group. However, DAP significantly reduced the gene expression of these receptors, outperforming the effects of Ang1-7, BNP, and losartan (refer to Figure 7.9A-7.9D). Surprisingly, neither DOCA-salt nor DAP treatment had any effect on Prostaglandin I2 synthase (see Figure 7.9E). Furthermore, in DOCA-salt rats, DAP effectively reduced the protein expression of the inflammation marker JNK (see Figure 7.9F-7.9H). These findings suggest that DAP takes advantage of the interrelationship between NO and ET1, thereby balancing downstream pathways and enhancing its overall effectiveness.



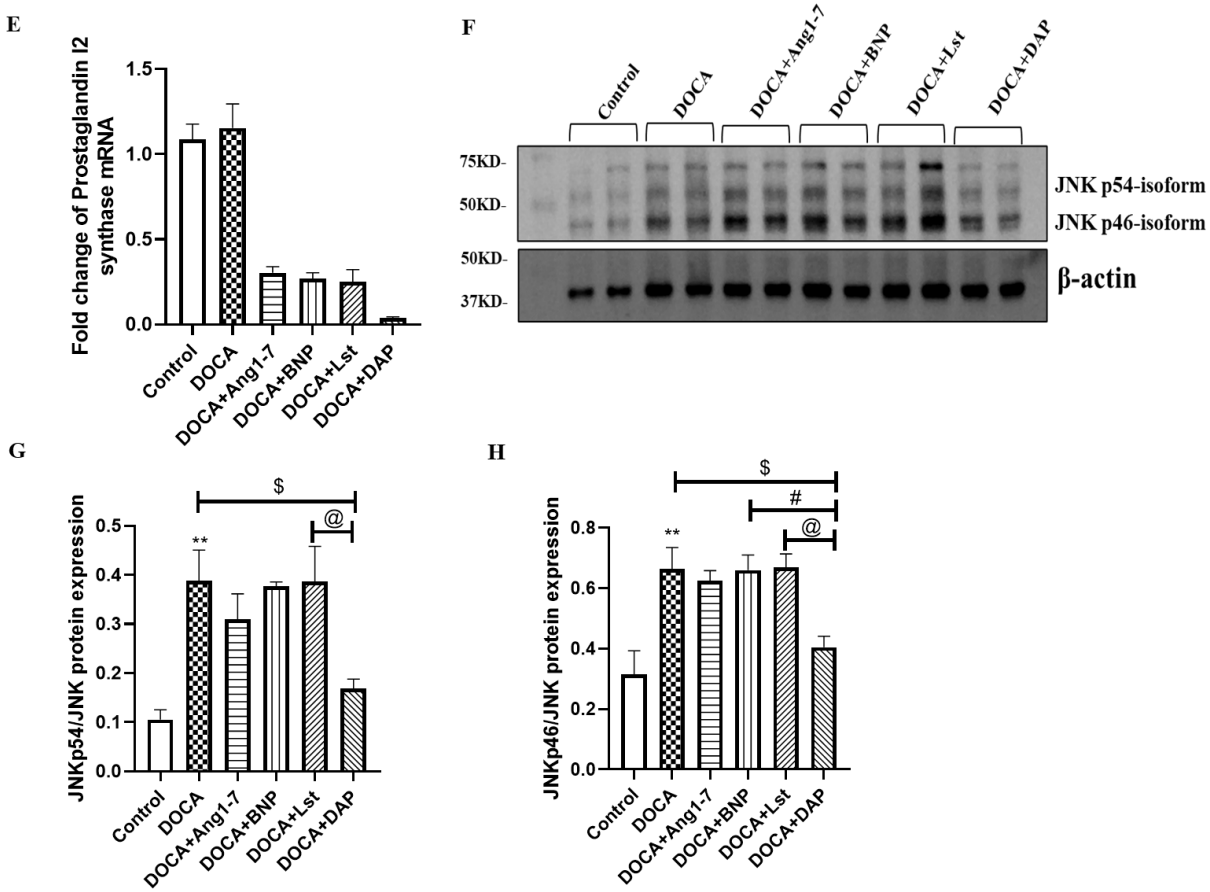



*Figure 7. 9: DAP Exerted Inhibitory Effects on ET1-Dependent Pathways In DOCA-Salt Rats*
(A) qPCR analysis of NADPH oxidase mRNA (B) qPCR analysis of Endothelin I mRNA (C) qPCR analysis of Thromboxane A2 receptor mRNA (D) qPCR analysis of $P2Y_{12}$ receptor mRNA (E) qPCR analysis of Prostaglandin I2 synthase mRNA expression (F) Aortic protein levels of JNK measured by western blot analysis (G) Quantification data on protein expression of JNK54 isomer from images in F. (H) Quantification data on protein expression of JNK46 isomer from images in F. Data were expressed as mean ± SEM (where N = 4). Data were analysed using ordinary one-way ANOVA followed by Bonferroni multiple comparison test, where $^{*}p < 0.05$, $^{**}p < 0.01$, ***$p < 0.001$ vs. respective control, $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ vs. DOCA, $^{\&}p < 0.05$ vs. DOCA+Ang1-7, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. DOCA+BNP, $^{@}p < 0.05$, $^{@@}p < 0.01$ vs. DOCA+Lst.

### 7.4.10 DAP elevates nitric oxide levels in primary rat endothelial cells treated with aldosterone

In this study, primary endothelial cells were treated with 1nM aldosterone to mimic the *in-vivo* DOCA-salt hypertension conditions. DAF-FM analysis showed that cells treated with aldosterone produced less NO than the control group. Notably, DAP showed significant efficacy in stimulating NO production under these DOCA-salt conditions (see Figure 7.10A-7.10D). This discovery provides critical insights into our research, implying that increased NO production contributes to vasorelaxation.

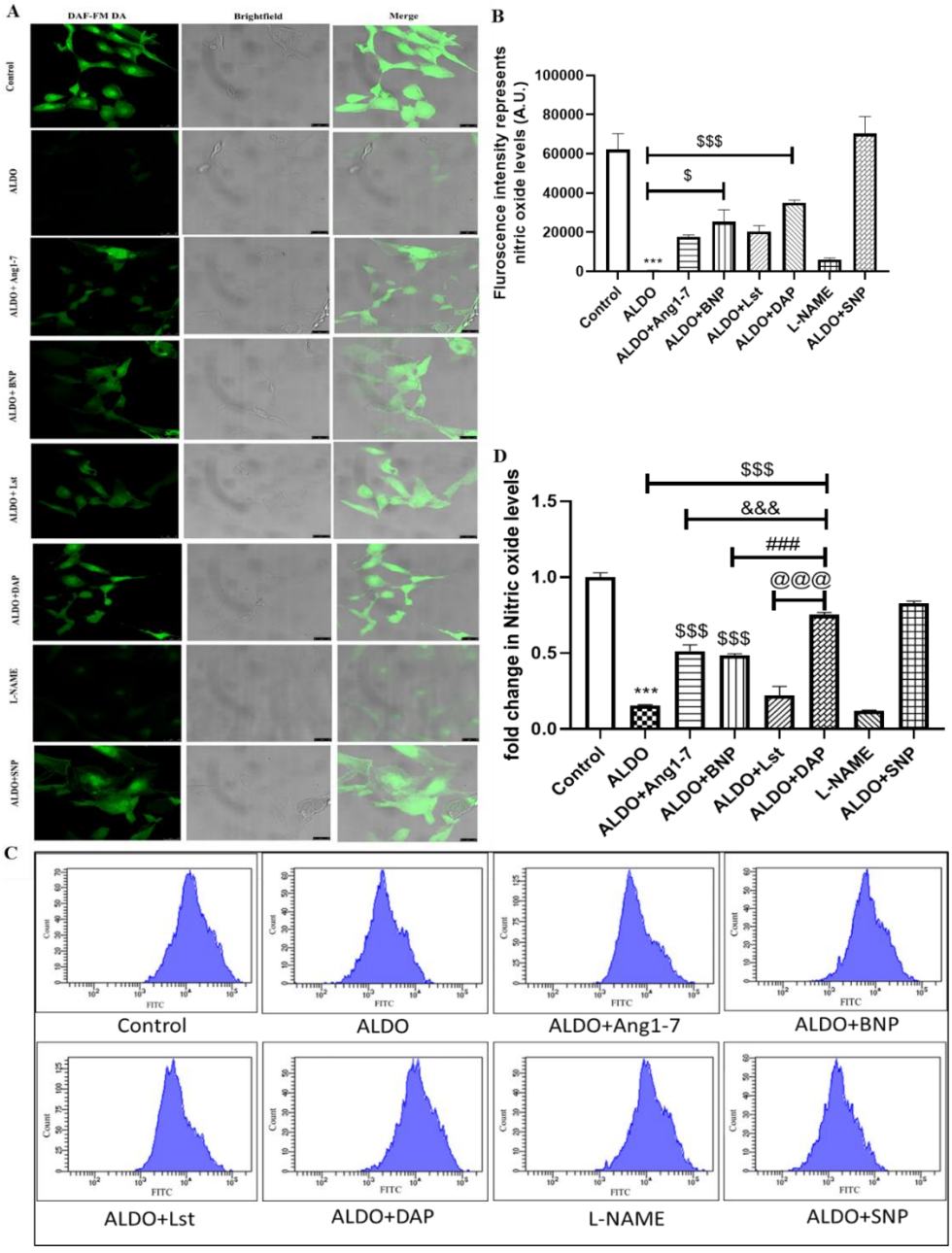
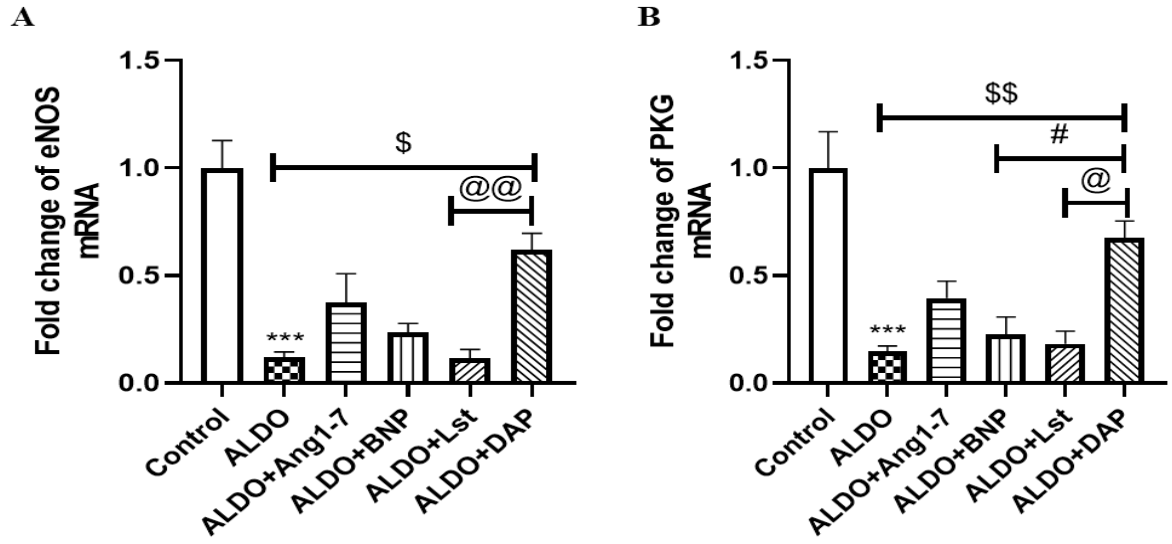



*Figure 7. 10: DAP induces Nitric oxide production in aldosterone-treated primary rat endothelial cells*

(A) Fluorescence intensity of NO levels in cells labelled with DAF-FM DA dye by confocal microscopy. Scale bars, 25 µm. (B) Quantification data on NO levels fluorescent intensity with DAF-FM DA dye from images in A. (C) DAP induces NO production in rat primary endothelial cells labelled with DAF-FM DA dye by flow cytometry. (D) Quantification data on NO production by flow cytometry. Data were analysed using ordinary one-way ANOVA followed by Bonferroni multiple comparison test, where ***$p < 0.001$ vs. respective control, $^{\$}p < 0.05$, $^{\$\$\$}p < 0.001$ vs. DOCA, $^{\&\&\&}p < 0.001$ vs. DOCA+Ang1-7, $^{\#\#\#}p < 0.001$ vs. DOCA+BNP, $^{@@@}p < 0.01$ vs. DOCA+Lst.

### 7.4.11 DAP regulates the balance of the NO/cGMP And ET1 pathways in primary rat endothelial cells

Our research found that aldosterone treatment reduced eNOS and PKG gene expression compared to the control, but DAP treatment increased these expressions significantly (see Figure 7.11A and 7.11B). We also measured the concentrations of cGMP in each group's cell lysates. While cGMP concentrations decreased in aldosterone-treated cells, DAP replicated the *in-vivo* results by effectively increasing cGMP concentrations as shown in Figure 7.11C. We used qPCR to determine the effect of DAP on ET1 gene expression. Treatment with aldosterone upregulated ET1 gene expression significantly. Interestingly, BNP, losartan, and DAP effectively suppressed ET1 gene expression in the aldosterone group, demonstrating their efficacy as shown in Figure 7.11D.



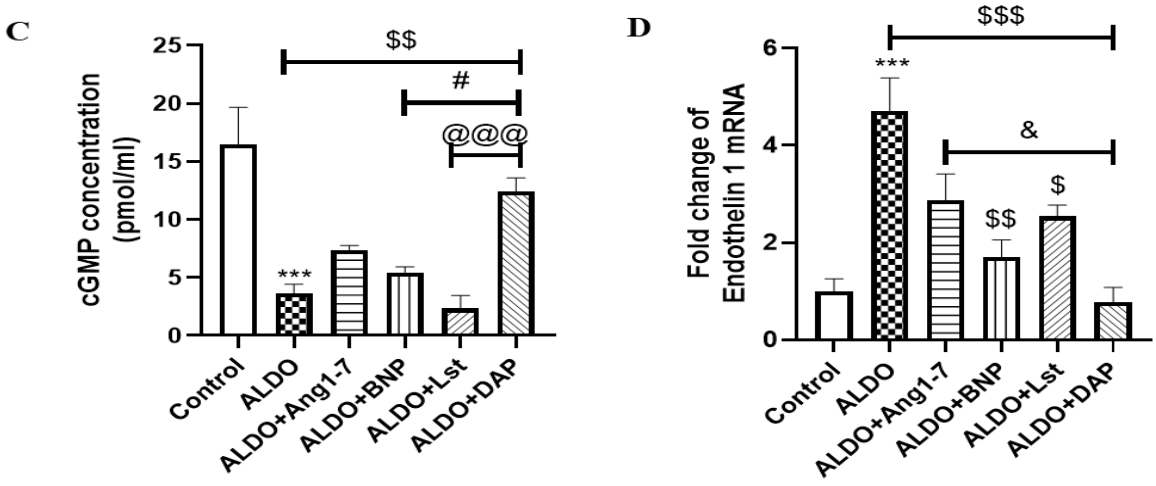



*Figure 7. 11: DAP Regulates the Balance of The NO/cGMP And ET1 Pathways In Primary Rat Endothelial Cells*

(A) qPCR analysis of eNOS mRNA in rat aortic primary endothelial cells. (B) qPCR analysis of PKG mRNA in rat aortic primary endothelial cells. (C) Levels of cGMP in cell lysates of rat aortic primary endothelial cells were measured with an assay kit (D) qPCR analysis of Endothelin I mRNA in rat aortic primary endothelial cells. Data were expressed as mean ± SEM (where N = 4). Data were analysed using ordinary one-way ANOVA followed by Bonferroni multiple comparison test, where ***$p < 0.001$ vs. respective control, $^{\$}p < 0.05$, $^{\$\$}p < 0.05$, $^{\$\$\$}p < 0.001$ vs. DOCA, $^{\&}p < 0.05$ vs. DOCA+Ang1-7, $^{\#}p < 0.05$ vs. DOCA+BNP, $^{@}p < 0.05$, $^{@@}p < 0.01$, $^{@@@}p < 0.01$ vs. DOCA+Lst.

## *7.5 Discussion*

Endothelial dysfunction is a significant risk factor for CVD, contributing to impaired vascular tone, endothelial damage, inflammation, and fibrosis. These complications are intricately linked to conditions like hypertension, atherosclerosis, and various cardiovascular complications. Our findings show that simultaneous activation of Mas and pGCA receptors acts as a strong protective mechanism against endothelial dysfunction, maintaining a balance between the NO/cGMP and ET1 pathways.

The potent mineralocorticoid activity, deoxycorticosterone acetate is recognized for its significant impact on raising blood pressure, inducing vascular injuries, hypertrophy, and inflammation (290). DOCA-salt-induced vascular dysfunction is important in the development of endothelial dysfunction, hypertension, renal injuries, and cardiovascular diseases (291). In this study, we used an *in-vivo* model of DOCA-salt-induced hypertension in Wistar rats. In this

study, we found that DOCA-salt significantly increased body weight, water intake, blood pressure, and heart rate. Earlier investigations proposed that abnormal lipid metabolism is evident in DOCA-salt hypertensive rats, leading to elevated levels of TC and TG, as well as HDL oxidation, ultimately contributing to endothelial dysfunction (292). Furthermore, the ratios of TC/HDL and TG/HDL serve as indicators of the risk of heart disease. In accordance with these findings, our data align with and support this information, suggesting elevated lipid biomarkers and various biochemical parameters in both serum and urine of DOCA-salt hypertensive rats. Furthermore, DOCA-salt reduced the mRNA levels of protective receptors like MasR, pGCAR, AT2R, ACE2, and renin while increasing the mRNA levels of the hypertension marker AT1 receptor in the kidney and aorta. The dysregulation of the RAAS pathway caused by DOCA-salt is a critical mechanism that contributes to hypertension, renal damage, and endothelial dysfunction.

In our study, administration of Ang1-7, BNP, and losartan resulted in significant reductions in water intake, body weight, blood pressure, and heart rate, presenting valuable benefits for hypertension management. It also resulted in reduced levels of lipid biomarkers and other biochemical parameters in both serum and urine. While these compounds have shown promising results in animal models, it is important to note that both Ang1-7 and BNP have a short duration of action and are ineffective against the complications associated with hypertension (65). Losartan, while effective, has several undesirable side effects. Researchers have investigated strategies to prolong the action of peptides and increase their availability within the body to improve their efficacy. One promising approach involves simultaneously activating two distinct protective receptors, which has the potential to have a more profound impact than activating a single receptor (293). This strategy has been strengthened by the approval of LCZ696, Sacubitril/valsartan, for the treatment of heart failure (157). LCZ696, a dual angiotensin receptor-neprilysin inhibitor, is effective in treating hypertension in large-scale clinical trials (168). Following this paradigm, we synthesized a DAP by fusing Ang1-7 and BNP, with the goal of simultaneously activating both the Mas receptor and the pGCA receptor.

According to our findings, DAP administration significantly reduced water intake, body weight, blood pressure, and heart rate. Similarly, DAP outperformed individual peptides, Ang1-7 and BNP, in terms of protective effects and therapeutic benefits. Notably, DAP treatment resulted in increased Mas, pGCA, and ACE2 expression, as well as increased kidney renin expression. Parent peptides of DAP, Ang1-7 upregulates MASR by positive feedback

mechanism (294). Conversely, ligand-binding seems to allosterically regulate increased specific activity of the GC catalytic domains of pGCA receptor (210). It is worth noting, however, that DAP treatment reduced ACE gene expression in the kidney. The delicate balance between ACE and ACE2 is critical for maintaining normal bodily function in both healthy and diseased conditions (295). Certain disease conditions cause disruptions in this balance, and our findings confirm that DOCA-salt disrupts the equilibrium between ACE and ACE2. Furthermore, our findings show that DAP treatment restores this balance.

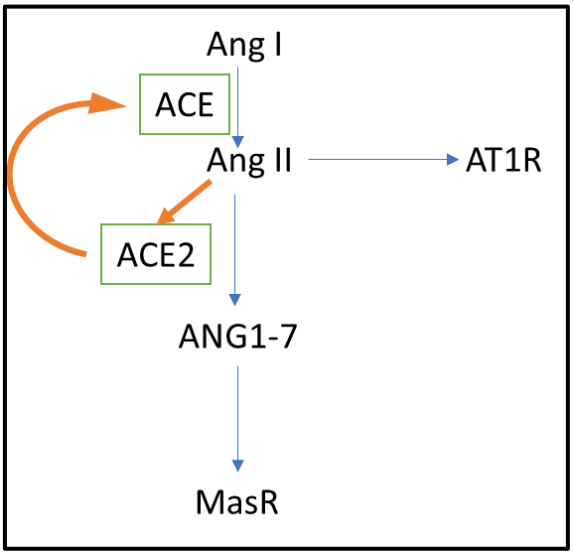



*Figure 7. 12: ACE and ACE2 balance in healthy individuals*

ACE enzyme cleaves Angiotensin I into Angiotensin II, and ACE2 converts Angiotensin II into Ang1-7. In healthy individuals, ACE activity is counterbalanced by ACE2. In disease conditions, Angiotensin II negatively regulates ACE2 expression (Indicated by red arrow). On the other hand, increased ACE2 levels block ACE expression (Indicated by red arrow).
Figure is reproduced from reference (295)

Endothelial dysfunction, characterized by blood vessel damage, artery wall distortion, increased thickness, decreased diameter, fibrous tissue accumulation, and calcium deposition, disrupts normal vascular tone (85). Significant aortic wall distortions, increased wall thickness, decreased diameters, and increased fibrosis, collagen, and calcium deposits were observed in rats with DOCA-salt-induced hypertension, impacting aortic contraction and relaxation. This condition causes an increase in the wall thickness to lumen diameter ratio, which increases vascular resistance and worsens hypertension (224). Notably, DAP treatment had a protective effect in these rats, improving vessel function. DAP specifically improved phenylephrine and acetylcholine responses, both of which were impaired in DOCA salt-induced rats.

In the aortic environment, the vascular tone is maintained by the release of various vasodilators such as nitric oxide and vasoconstrictors. Nitric oxide is generated through two pathways: the eNOS-dependent pathway and the eNOS-independent pathway involving substances like nitrate and nitrite. The primary source of blood NO arises from L-arginine via the eNOS pathway. These pathways may trigger long-term transcriptional regulation by activating transcription factors or other mechanisms. NO can easily diffuse into smooth muscle cells, activating soluble guanylate cyclase (sGC) and protein kinases, thereby inducing vasodilation (296). Additionally, the transcriptional impact of VEGF contributes to the steady-state expression of eNOS mRNA and protein. Dysfunction in the eNOS pathways has been demonstrated to result in vascular dysfunction and hypertension (117).

Furthermore, in DOCA-salt rats, DAP treatment reversed the decline in eNOS and PKG expression, as well as serum cGMP concentrations. eNOS and cGMP are important in blood pressure regulation, with eNOS acting as a powerful vasodilator that releases the secondary messenger cGMP. In hypertension, cGMP production is compromised due to eNOS uncoupling and reduced NO availability. Our findings show that DAP works by activating eNOS and cGMP, two important molecules for blood vessel health. Targeting the eNOS/cGMP pathway has emerged as a stand-alone treatment in recent years, lowering blood pressure and improving overall cardiovascular health through NO release (297). We also observed a decrease in oxidative stress, as evidenced by the downregulation of NADPH oxidase mRNA expression. These findings suggest that DAP improves endothelial function by activating the eNOS/cGMP/PKG signalling pathway. Notably, VEGF expression remained unchanged, implying a minor, if any, role in the DAP-mediated mechanism of action.

The balance of nitric oxide and endothelin-1 is critical for maintaining vascular function, but it is disrupted under chronic stress conditions (298). ET-1 mRNA expression was found to be significantly higher in DOCA-salt-induced rats in our study. Elevated ET-1 levels in patients have been linked to cardiovascular disease and hypertension. An imbalance between NO and ET-1, characterized by decreased NO and increased ET-1, contributes to hypertension (299). Blood vessel narrowing is caused by endothelial dysfunction, which is accompanied by platelet aggregation and thrombosis. The $P2Y_{12}$ receptor, which responds to ADP, is involved in this process (300). In DOCA-salt-induced hypertensive rats, we found increased Thromboxane A2 receptor and $P2Y_{12}$ receptor mRNA expression, which contributed to vessel constriction and impaired endothelial function. In DOCA-salt rats, co-treatment with DAP effectively reduced ET1, Thromboxane A2 receptor, and $P2Y_{12}$ receptor mRNA expression.

DAP did not affect Prostaglandin I2 synthase mRNA expression, which is important for blood vessel function. Furthermore, blocking the $P2Y_{12}$ receptor in human endothelial cells reduces inflammation and endothelial dysfunction caused by lipopolysaccharide (301). In addition, DAP treatment protected against DOCA-salt-induced inflammation by downregulating JNK protein expression, which is associated with arterial wall inflammation.

Subsequently, we investigated the effects of downregulated eNOS expression on NO production and investigated the underlying molecular mechanisms. We isolated and examined primary endothelial cells from Wistar rats to better understand these processes. Our findings confirm that aldosterone reduces NO production, which is consistent with our *in-vivo* findings. Previous research suggested that Ang1-7 and BNP both stimulate NO/cGMP pathways independently. Surprisingly, our research shows that DAP strongly induces NO production, with a significantly greater impact than the individual peptides Ang1-7 and BNP. Increased NO levels act as a negative regulator of the endothelin-1 signalling pathway. These findings are consistent with previous research showing that increased NO inhibits ET1-induced hypertrophy in cultured cells (302). In primary endothelial cells, aldosterone significantly increased ET1 mRNA expression while decreasing eNOS mRNA expression, as well as PKG and cGMP levels. Surprisingly, DAP treatment effectively reversed all of these effects.

## *7.6 Conclusion*

In conclusion, our findings show that simultaneously activating two receptors protects blood vessels from damage in DOCA-salt-induced hypertensive rats, effectively preventing endothelial dysfunction. The key mechanisms underlying this vascular protection are the inhibition of ET1 and the activation of the Nitric Oxide/cGMP pathways, both of which are required for optimal blood vessel function. These findings strongly suggest that the dual activation strategy holds great promise as a novel approach to lowering the risk of cardiovascular disease.

# *Chapter 8*

# *Conclusion and Future Scope of Work*

## *8.1 Conclusion*

Hypertension and associated cardiovascular diseases are highly prevalent and major health challenge around the world. Person with elevated blood pressure doubles the risk of vital organ damage and associated cardiovascular events compared to normal blood pressure. Hence, early detection, awareness, and proper care in initial stages of hypertension is essential. Alarming prevalence and inadequacy of traditional therapies has demanded the investigation of novel approaches for hypertension management. The primary goal of this thesis is to investigate the multivalent strategy co-activating two receptors simultaneously and evaluate the molecular mechanism of protective effects associated with dual receptor activation.

Initially, dual activation achieved by DAP was screened against $H_2O_2$ induced oxidative stress in renal epithelial cells. The study findings revealed DAP is promising against oxidative stress induced hypertrophy, fibrosis and inflammatory responses. It has also inhibited the AT1R, a cardiovascular disease marker in $H_2O_2$ treated NRK52E cells. Additionally, DAP is more promising than its parent peptides Ang1-7 and BNP. However, Inhibitor study with specific blocking of these peptides suggests that DAP is acting through Mas and pGCA receptor activation. These *in-vitro* results provided insights for further *in-vivo* studies.

In conjunction with the above study, dual activation achieved by second peptide NP was evaluated in the primary aortic cells i.e. VSMC and EC. The key findings of our study show that downregulation of Mas and pGCA receptors plays a significant role in $H_2O_2$-induced vascular phenotypic change, vascular damage, and endothelial injury. This could be mediated by an imbalance in the NO/sGC/cGMP/PKG and eNOS pathways. Furthermore, NP inhibits $H_2O_2$-mediated dysfunction by activating both Mas and pGCA receptors. As a result, the experimental evidence from our studies suggests that Mas and pGCA receptors play an important role in the development of vascular pathology, and that targeting Mas and pGCA receptors in vascular pathology could be a beneficial therapeutic approach for mitigating its negative consequences.

Conversely, the findings of *in-vivo* study highlight multifaceted actions of dual activation with DAP in mitigating the negative effects of DOCA-salt administration in a rat animal model. This study also features natriuresis and inhibition of ENaC as a distinct feature of dual activation of Mas and pGCA receptors. These findings support potential of dual activation as a promising therapeutic candidate for the treatment of hypertension, organ hypertrophy, renal injury, fibrosis, and impaired sodium handling. Also, data suggested that DAP is superior in protective actions that the standard drug losartan and its parent peptides.

More research and clinical trials are needed to determine the full clinical potential of DAP in humans.

Further, *in-vivo* study findings indicate that simultaneous activation of Mas and pGCA receptors can effectively protect blood vessels from damage in DOCA-salt-induced hypertensive rats, thereby preventing endothelial dysfunction. The key mechanisms underlying this vascular protection include ET1 inhibition and the activation of the Nitric Oxide/cGMP pathways, which are required for optimal blood vessel function. These findings suggest that the dual activation strategy has significant promise to reduce the risk of cardiovascular disease.

Therefore, our overall study concludes dual activation of Mas and pGCA receptors shows promising protective effects, also the overall effect of dual activation is superior to its parent peptides.

## *8.2 Future scope of research*

- The current study provides a strong foundation for the development of a novel peptide-based strategies for hypertension treatment. Future research should build upon these findings to advance toward clinical applications and contribute to the evolving landscape of cardiovascular medicine.
- Exploration of the efficacy of the dual activation in related cardiovascular conditions beyond hypertension could be explored. Conditions such as heart failure, vascular disorders or neurological disorders may benefit from the cardioprotective properties demonstrated by dual activation.
- Conducting long-term safety and tolerability studies is crucial to ascertain the sustained therapeutic benefits of dual activation without causing adverse effects. This includes investigating potential effects on organs beyond the cardiovascular and renal systems and monitoring for any unforeseen complications.
- Further research could focus on optimizing the formulation and delivery methods of the dual-acting peptide. This may involve exploring different dosages, delivery routes, and potential combination therapies to enhance the efficacy and minimize any potential side effects.
- Given the multifaceted nature of hypertension, combining DAP with existing antihypertensive medications or exploring synergistic approaches with other emerging therapies could be a strategic avenue for further investigation. This may enhance the overall efficacy of treatment while minimizing potential side effects.

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# *Appendix I*

## *I.I Amino acid sequence of Ang1-7, BNP and DAP*

*Table I. I: Amino acid sequence and molecular weights of Ang1-7, BNP and DAP*

| | |
|---|---|
| **Angiotensin1-7 (Ang1-7): 7**-amino-acid heptapeptide | 899.02 Da |
| **DRVYIHP** | |
| **Brain natriuretic peptide (BNP32):** 32 amino acids in length | 3464.08 Da |
| SPKMVQGSGCFGRKMDRISSSSGLGCKVLRRH | |
| **Dual-acting peptide (DAP):** 30 amino acids in length | 3473.07 Da |
| **DRVYIHP**CFGRKMDRISSSSGLGCKVLRRH | |

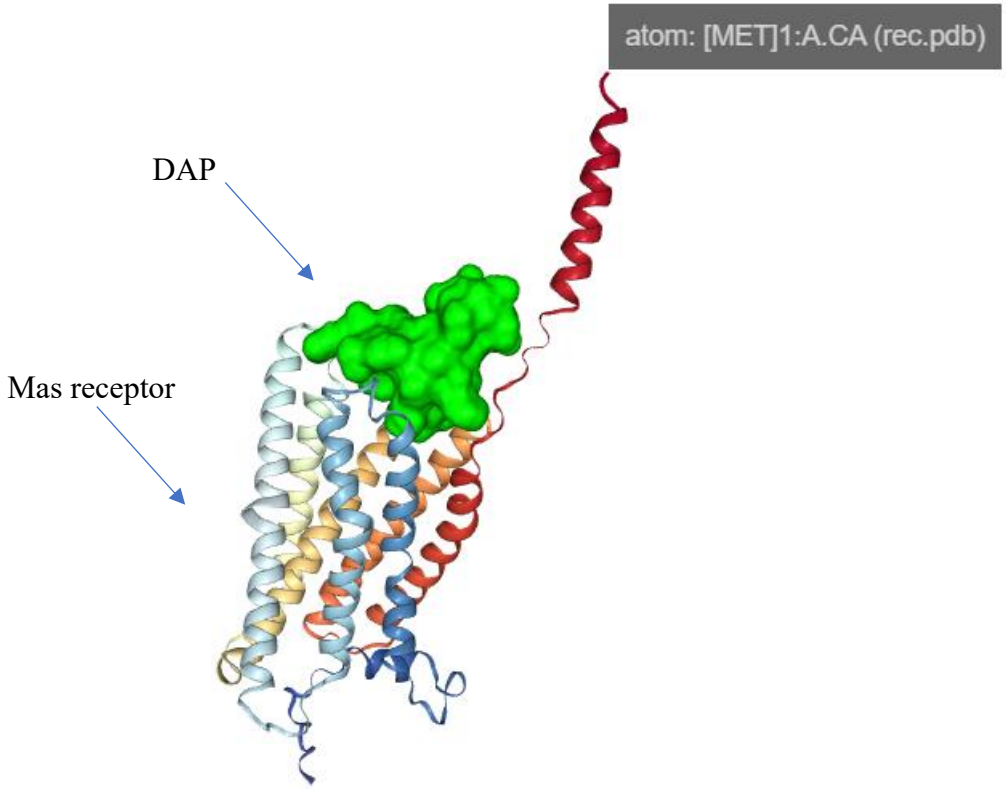
Amino acid sequences of peptides (Ang1–7, BNP and DAP). Here, capital letter = L-amino acid residues, amino acid residues are either underlined or italicized to depict that the design of DAP peptide sequence is inspired from Ang1–7 and BNP peptide. Lines connecting two cysteine residues indicate disulphide linkage.

*Table I. II: List of amino acids and their abbreviations.*

**This table included for reference for Table I.I.**

| Amino acid | 3-letter abbreviation | 1-letter abbreviation |
|---|---|---|
| Arginine | Arg | R |
| Asparagine | Asn | N |
| Aspartic acid | Asp | D |
| Cysteine | Cys | C |
| Glutamine | Gln | Q |
| Glycine | Gly | G |
| Histidine | His | H |
| Isoleucine | Ile | I |
| Leucine | Leu | L |
| Lysine | Lys | K |
| Methionine | Met | M |
| Phenylalanine | Phe | F |
| Proline | Pro | P |
| Serine | Ser | S |
| Tyrosine | Tyr | Y |
| Valine | Val | V |

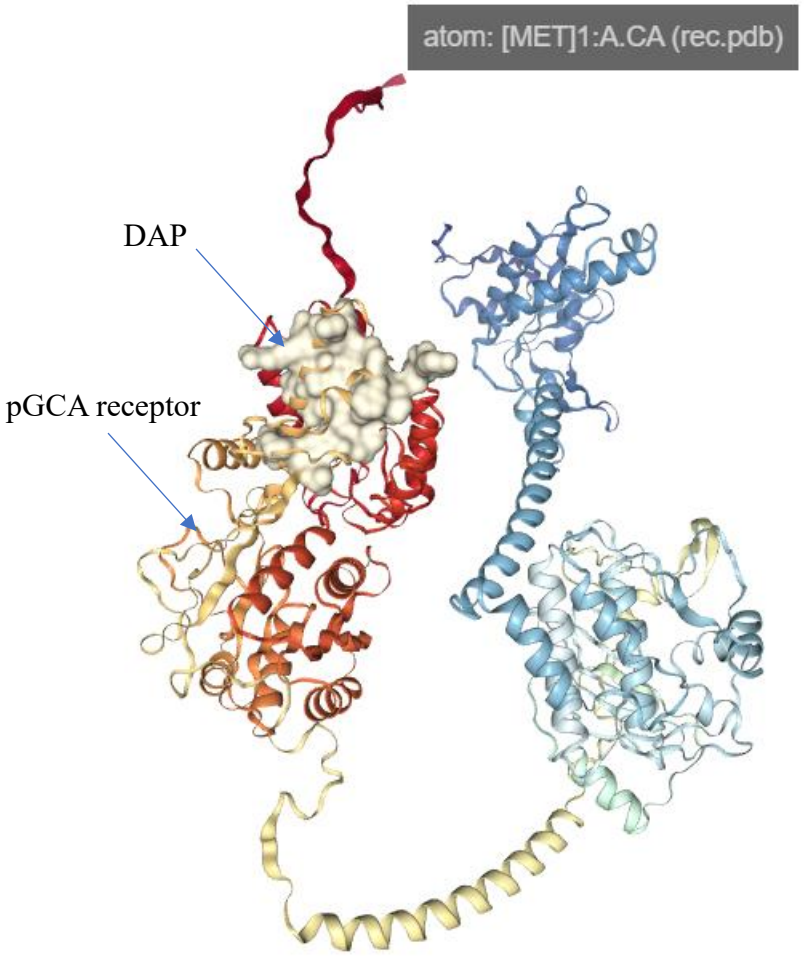
## *I.II Molecular docking of DAP with Mas and pGCA receptor*




| | |
|---|---|
| **Docking Score** | -265.85 |
| **Confidence Score** | 0.9103 |
| **Ligand rmsd (Å)** | 38.30 |

*Figure I. I: Molecular Docking of DAP With Mas Receptor*

DAP (Ligand) structure homology model visualization generated by Swiss model. Mas receptor model is taken from AlphaFold Protein Structure Database. Ligand-receptor docked complex using the HDOCK server; Mas (receptor) in rainbow colour and the DAP (ligand) in Green. Evaluation metrics suggest that when the confidence score is above 0.7, the two molecules would be very likely to bind.




| | |
|---|---|
| **Docking Score** | -272.58 |
| **Confidence Score** | 0.9207 |
| **Ligand rmsd (Å)** | 35.68 |

*Figure I. II: Molecular Docking of DAP With pGCA Receptor*

DAP (Ligand) structure homology model visualization generated by Swiss model. pGCA receptor model is taken from AlphaFold Protein Structure Database. Ligand-receptor docked complex using the HDOCK server; pGCA (receptor) in rainbow colour and the DAP (ligand) in White. Evaluation metrics suggest that when the confidence score is above 0.7, the two molecules would be very likely to bind.

## *I.III Gating Strategies for Quantification of Oxidative Stress in cells*

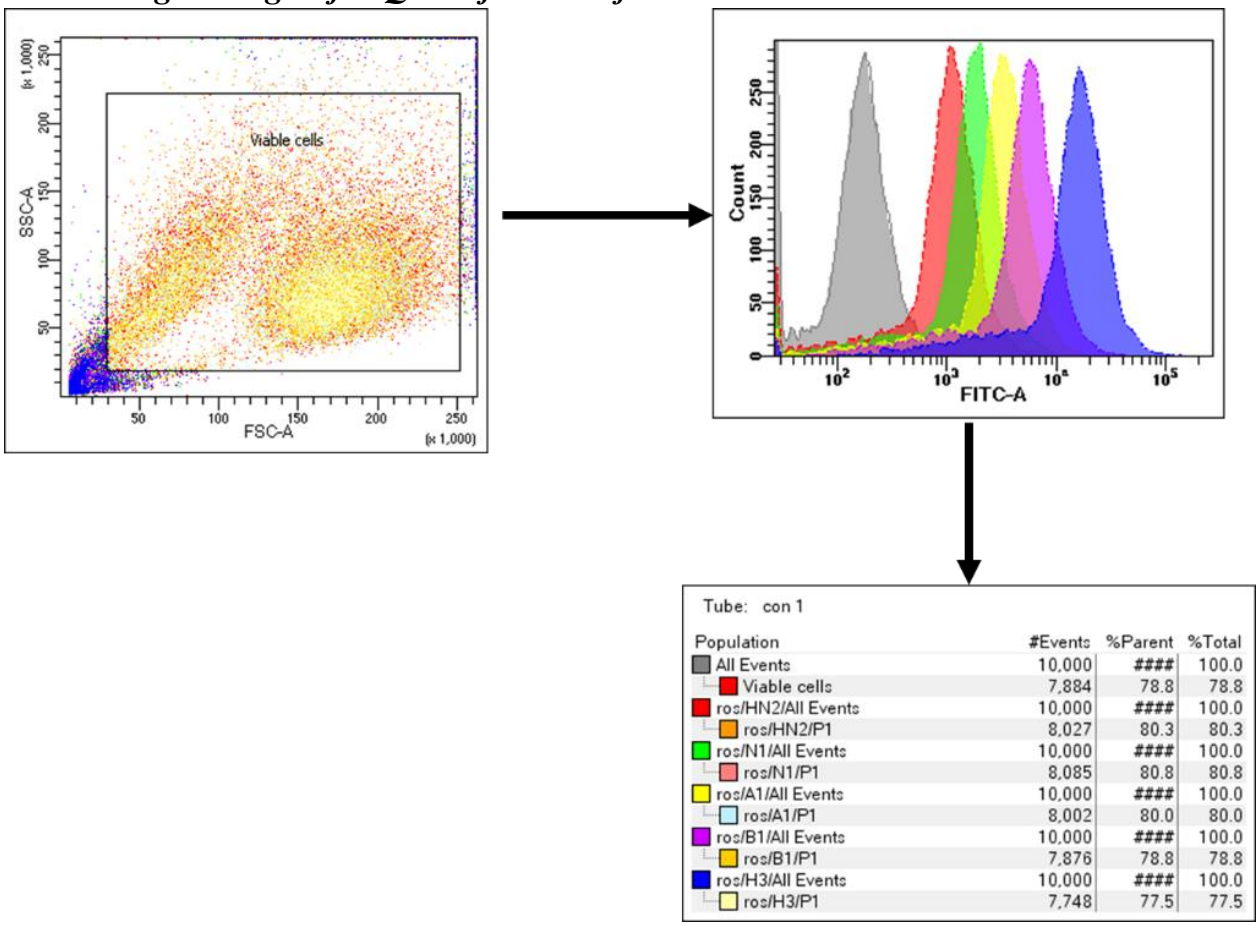



*Figure I. III: Gating strategy for oxidative stress*

VSMC and NRK52E cells were treated with $H_2O_2$, with or without peptide treatment. The cells were then stained with CM-H2DCFDA) (5 µmol/L) for 30 min at 37 °C. 10,000 cells were analysed to measure the ROS present in the cells. We have carefully selected only the viable, lived cells for our analysis. Representative histograms depicting the ROS-induction in the $H_2O_2$ treated cells.

## *I.IV Gating strategies for quantification of nitric oxide levels in endothelial cells*

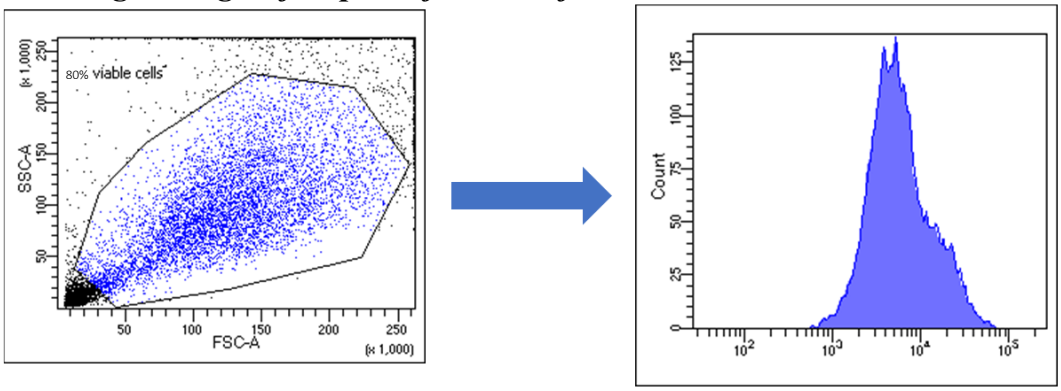



*Figure I. IV: Gating strategies for Quantification of Nitric Oxide levels in endothelial cells*

10,000 cells were analysed to measure the nitric oxide present in the cells. We have carefully selected only the viable, lived cells for our analysis. The gating strategies employed to identify and include only the living cell population.

### *I.V Gating strategies for quantification of apoptosis in NRK52E cells*

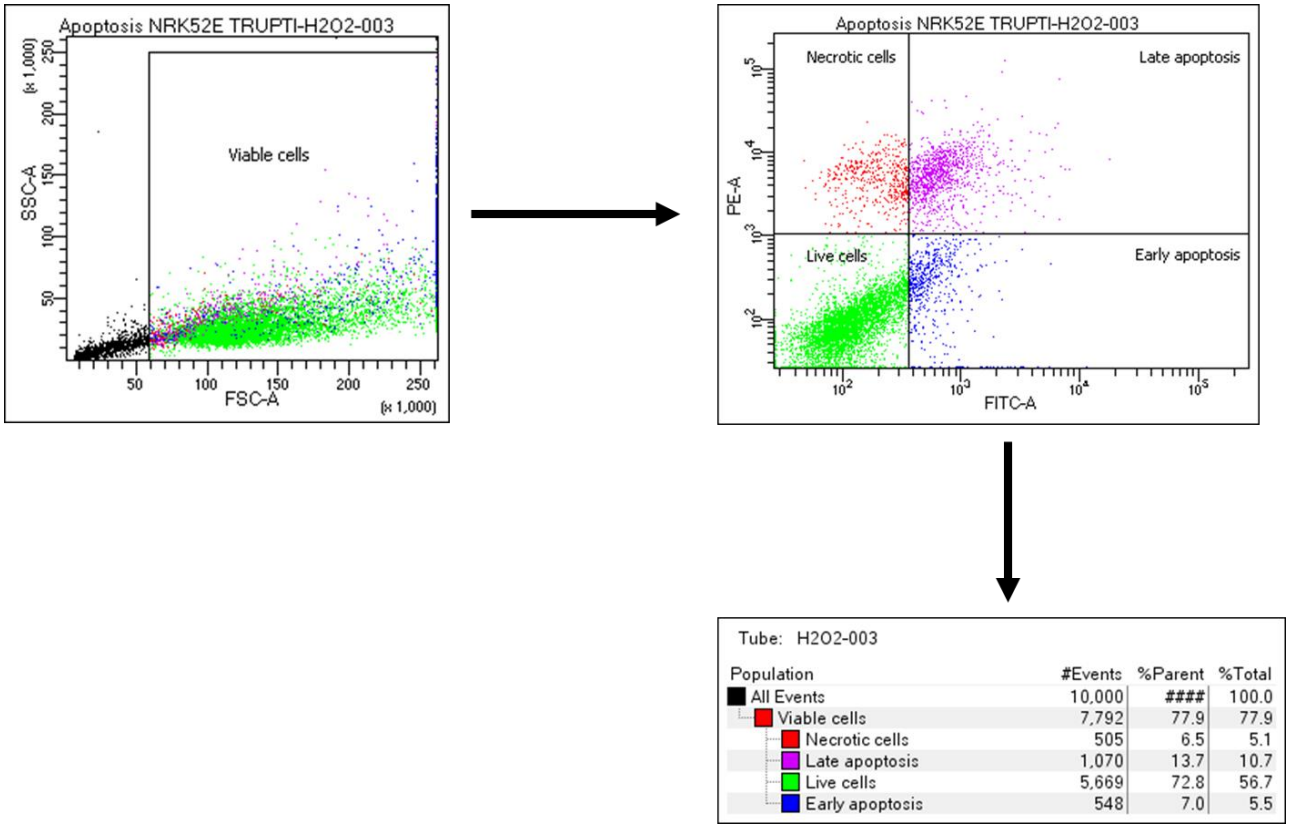



*Figure I. V: Gating Strategies for Quantification of Apoptosis in NRK52E cells*

NRK52E cells were treated with $H_2O_2$ to induce apoptosis, with or without peptide treatment. The cells were then stained with FITC-annexin V and PI. 10,000 cells were analysed to measure the ROS present in the cells. Apoptotic cells positive for annexin V can be seen in the bottom right quadrant and dead cells positive for both annexin and PI in the top right quadrant. Healthy cells are negative for both stains. The levels of apoptotic cells and dead cells vary depending on which FSC and SSC gate is used.

# *Annexure*

***Publications from Ph.D. thesis***

1. **Ghatage, T.,** Goyal, S.G., Dhar, A. *et al.* Novel therapeutics for the treatment of hypertension and its associated complications: peptide- and nonpeptide-based strategies. *Hypertens Res* 44, 740–755 (2021).
2. **Ghatage T,** Singh S, Mandal K, Jadhav KB, Dhar A. Activation of Mas and pGCA receptor pathways protects renal epithelial cell damage against oxidative-stress-induced injury. Peptides. 2023 Apr; 162:170959. doi: 10.1016/j.peptides.2023.170959. Epub 2023 Jan 21. PMID: 36693526.
3. **Ghatage T,** Singh S, Mandal K, Dhar A. MasR and pGCA receptor activation protects primary vascular smooth muscle cells and endothelial cells against oxidative stress via inhibition of intracellular calcium. J Cell Biochem. 2023 Jul;124(7):943-960. doi: 10.1002/jcb.30422. Epub 2023 May 21. PMID: 37210727.
4. **Ghatage T,** Singh S, Mandal K, Dhar A. Co-activation of Mas and pGCA Receptors Suppresses Endothelin-1-Induced Endothelial Dysfunction via Nitric Oxide/cGMP System. Biochim Biophys Acta Mol Basis Dis. 2024 Mar 8:167110.

***Publications under revision***

1. **Ghatage T,** Singh S, Mandal K, Dhar A. Dual activation of Mas and pGCA receptors promotes natriuresis in DOCA-salt-treated rats.
2. Deepika Dasari, Ganesh Panditrao Lahane, **Trupti Ghatage,** Jegadheeswari Venkadakrishnan, Indu Dhar, Dharmarajan Sriram, Arti Dhar. Selective inhibition of SGLT-1 by KGA-2727 attenuates Diabetic cardiomyopathy in-vitro in cultured cardiomyocytes and *in-vivo* in wistar rats.
3. Ganesh Lahane, **Trupti Ghatage**, Jegadheeswari V, Twisha Dube, Dishank Arondekar, Rakesh Kumar, Arti Dhar, Audesh Bhat. Role of Nanoparticle Formulation for the Combination Delivery of Multiple Antigens. (Book chapter)

***Other publications***

1. Li L, Mangali S, Kour N, Dasari D, **Ghatage T**, Sharma V, Dhar A, Bhat A. Syzygium cumini (jamun) fruit-extracted phytochemicals exert anti-proliferative effect on ovarian

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2. Bhat A, **Ghatage T**, Bhan S, Lahane GP, Dhar A, Kumar R, Pandita RK, Bhat KM, Ramos KS, Pandita TK. Role of Transposable Elements in Genome Stability: Implications for Health and Disease. Int J Mol Sci. 2022 Jul 15;23(14):7802. doi: 10.3390/ijms23147802. PMID: 35887150; PMCID: PMC9319628.
3. Dasari D, Bhat A, Mangali S, **Ghatage T**, Lahane GP, Sriram D, Dhar A. Canagliflozin and Dapagliflozin Attenuate Glucolipotoxicity-Induced Oxidative Stress and Apoptosis in Cardiomyocytes via Inhibition of Sodium-Glucose Cotransporter-1. ACS Pharmacol Transl Sci. 2022 Mar 9;5(4):216-224. doi: 10.1021/acsptsci.1c00207. PMID: 35434529; PMCID: PMC9003386.

***Book chapters***

1. **Trupti Ghatage**, Srashti Goyal, Deepika Dasari, Jayant Jain, Arti Dhar, Audesh Bhat. Nanomaterial for Kidney Disease Management, Functional Nanomaterials for Regenerative Tissue Medicines, Taylor and francis group, 2021, 1st edition.

***Papers presented at national/international conferences***

1. **Ghatage T**, Singh S, Mandal K, Jadhav KB, Dhar A. "Dual-acting Peptide Protects Against Oxidative Stress-induced Injury in Renal Epithelial Cells Via Mas And pGCA Receptor Co-activation" presented at 9th Indian Peptide Symposium and INDIA PEPTIDE SHOW, Feb 2023, Bits Pilani Goa campus.
2. **Ghatage T**, Singh S, Mandal K, Jadhav KB, Dhar A. "Novel peptide protects primary aortic cells against oxidative stress via inhibition of intracellular calcium" presented at International Conference on INNOVATE-2024, March 2024, Bits Pilani, Pilani Campus.
3. Anusha Vemana, **Trupti Ghatage**, Arti Dhar. "Therapeutic Potential of Angiotensin1-7 and BNP to Mitigate AngII-Induced TLR4-Mediated Hypertensive Responses in Renal Cells" presented at International Conference on INNOVATE-2024, March 2024, Bits Pilani, Pilani Campus.

# Trupti Santosh Ghatage

## *Biography*

Trupti Santosh Ghatage accomplished her undergraduate studies in pharmacy at Bharati Vidyapeeth College of Pharmacy in Kolhapur in 2017. Following this, she excelled in the GPAT examination and was awarded an AICTE fellowship for her postgraduate studies. Pursuing her interest in pharmacology, she obtained her master's degree from the University Department of Pharmaceutical Sciences at Rashtrasant Tukadoji Maharaj Nagpur University in 2019. Her master's research focused on investigating the "Role of Transcription Factor SREBP1 in the Neuroprotective Effect of Cerebrolysine in Delayed Hours of Cerebral Ischemia," showcasing her proficiency in molecular and cellular research techniques.

In 2020, Trupti embarked on her doctoral journey at the Department of Pharmacy, BITS-Pilani, Hyderabad campus, under the guidance of Dr. Arti Dhar. Her commitment and hard work were acknowledged when she was honoured with the ICMR-SRF fellowship in December 2022, providing crucial support for her ongoing doctoral research. Trupti's dedication to advancing pharmaceutical knowledge is evident in her impressive track record, including the publication of over five papers in international journals and contributions to two book chapters. She has actively shared her research findings by presenting at various conferences, and one of her notable research contributions has been featured in Stemcell Science News. Trupti Santosh Ghatage stands as a testament to her unwavering commitment to advancing the field of Pharmacology and making significant contributions to pharmaceutical knowledge.

# Prof. Arti Dhar

## *Biography*

Dr. Arti Dhar presently serves as a Professor in the Department of Pharmacy at Birla Institute of Technology and Science (BITS), Pilani, Hyderabad campus. Her academic journey culminated in the attainment of a Ph.D. from the University of Saskatchewan, Canada, in the year 2010. Throughout her doctoral pursuit, Dr. Dhar received prestigious scholarships from the Heart and Stroke Foundation of Canada (HSFC) and the Arthur Smith Memorial Scholarship from the University of Saskatchewan. Notably, she garnered multiple travel awards from the Canadian Physiological Society and the Canadian Hypertension Society.

Dr. Dhar's remarkable achievements continued with her postdoctoral training at Lakehead University, Ontario, Canada, and the University of Saskatchewan, Canada, spanning from 2010 to 2013. Her doctoral thesis earned a nomination for the Governor General's Gold Medal, and her groundbreaking research was featured on CBC Channel Canada and the campus news channel in March 2011.

Since joining BITS Pilani, Hyderabad Campus in 2014, Dr. Dhar has been a recipient of research funding from various prestigious sources, including DST under the Young Scientist scheme, CSIR extramural research funding, ICMR, and an early career award, in addition to grants from BITS under the competitive grant program. Her primary research focus revolves around identifying novel therapeutic targets for cardiovascular and metabolic disorders.

Under her mentorship, four students are currently pursuing their Ph.D., a testament to her commitment to nurturing the next generation of researchers. Dr. Arti Dhar has amassed a significant number of National and International publications, showcasing her impactful contributions to the field of pharmacy and cardiovascular research.