Role of Double Stranded RNA Dependent Protein Kinase (PKR) in Hypertension: Mechanism and Prevention

THESIS

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by

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CERTIFICATE

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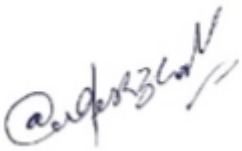

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ABSTRACT

Diabetes and cardiovascular diseases are the major health concerns worldwide. Approximately, more than fifty percent of individuals with diabetes suffer from accompanying cardiovascular complications. Hypertension is quite often related with diabetes. According to previous report around sixty percent of diabetic individuals are hypertensive and near by twenty percent of individuals with hypertension are diabetic. Hypertension is one of the leading causes of cardiovascular mortality and morbidity and is associated with target/end-organ-damage. It severely affects renal system, induces cardiac and vascular dysfunction. Hypertension induced end-organ-damage is often associated with inflammation, excessive oxidative damage and cellular apoptosis. Reportedly, considerable vascular dysfunction, cardio-renal damage, increase in the markers of inflammation, elevated oxidative load and cellular apoptosis has been well illustrated in the spontaneously hypertensive rats. Alterations in these three severly affected organs supports the role of oxidative damage and inflammation in the pathogenesis of hypertension. Several lines of evidence indicate that inflammation in the renal epithelial cells of rat kidney and vasculature are the most important changes occurring in the development and progression of HTN.

Double stranded RNA dependent protein kinase R (PKR), is a serine/threonine kinase. PKR is known to be a key inducer of inflammation, insulin resistance and is known to cause impairment in glucose homeostasis in obese patients. Current reports suggest that PKR can also act in response to metabolic stress in in-vivo mouse model as well as in humans. Several forms of cellular stress inducing agents such as oxidative stress, elevated metabolic burden, mechanical stress, inflammatory mediators and excess nutrients are known to activate PKR. Despite of its primary role in regulating translation initiation, PKR acts as an upstream marker for activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and c-Jun N-terminal kinases (JNK) activation. Activation of NF-κB and JNK are well recognised for initiating inflammatory responses and promoting programmed cell death. Although the clear underlying mechanism is still

not fully elucidated. Henceforth, in the present study we were determined to examine the effect of high fructose (HF) on PKR upreguation in cultures of renal epithelial cells (NRK-52E) originated from rat kidney and primary vascular smooth muscle cells (VSMCs) isolated by enzymatic digestion method using rat thoracic aorta. Further, we aim to explore whether inhibition of PKR could prevent any harmful effects of HF in cultures of these cells. Confocal microscopy and western blotting was done to determine PKR expression. Reactive oxygen species (ROS) production and cellular apotosis were measured by flow cytometry. In response to HF, significant increase in PKR expression, generation of ROS and cellular apoptosis were noted in cultures of renal epithelial cells and VSMCs, attenuation was observed with selective inhibition of PKR by imoxin treatment. Additionally, treatment with imoxin also attenuated remodelling of vasculatutre and phenotypic switching in VSMCs. After successful establishment of role of PKR in renal epithelial cells and primary VSMCs under hypertensive disease condition, next we were determined to look for exact changes of PKR expression in in-vivo disease model. Till date, no experimental evidences have been gained so far to determine the role of PKR in in-vivo model of hypertension and related cardiovascular complications. In the present study L-NAME (40mg/kg, p.o by oral gavage) was used to induce hypertension. Male Wistar rats were treated with (o.5 mg/kg, i.p) of imoxin along with the administration of L-NAME for four weeks to investigate its influence on hypertension. Changes in physiological parameters such as changes in systolic blood pressure (SBP) and diastolic blood pressure (DBP) along with alterations in heart rate (HR) and mean arterial pressure (MAP) were assessed by recording non-invasive blood pressure (NIBP) measurement. Biochemical estimations were done for serum creatinine, blood urea nitrogen, nitrite/nitrate, high density lipoprotein, triglycerides, total cholesterol and intracellular calcium level using assay kits. Compared to control significant changes were observed in physiological and serum parameters in L-NAME administered rats. Improvement was observed with imoxin treatment. Endothelium damage, aortic and cardiac remodelling are the characteristic hallmark of progressive hypertension. Results of isometric tension studies on L-NAME or with imoxin treated rats showed significant improvement in

endothelium dependent relaxation in rat aortic rings pre-contracted with phenyleprine in response of acetylcholine in L-NAME treated rats. In agreement with this we also report significant decrease in endothelial nitric oxide synthase (eNOS) expression in rat kidney and aorta isolated from L-NAME rats, reversal was achieved with imoxin treatment. We also report changes in luminal thickening and myofibril derangement in aorta and heart sections of L-NAME rats, whereas improvement was noted with imoxin treatment. Renin angiotensin system (RAS) is known to get activated under hypertensive disease state. In this study we also checked whether PKR could induce renin and angiotensin (Ang-II) expression or not. Ang-II is a major component of RAS a potent vasoconstrictor, inducer of inflammation and fibrosis. In-vivo Ang-II is known to activate renal NF-κB pathway via up-regulation of several pro-inflammatory genes, initiating a local inflammatory response. Several pieces of evidence indicate complete in-vivo antagonism of Ang-II by treatment with angiotensin receptor blockers (ARB's). But how Ang-II initiates inflammatory cascade in a PKR dependent manner in hypertension associated end-organ-damage is still not known. PKR is a serine-threonine kinase which regulates inflammatory signalling cascade through upregualtion of NF-κB and MAPKs. Members of MAPKs such as extracellular regulated kinase1/2 ($ERK_{1/2}$), and JNK are the crucial contributors involved in regulating cell proliferation and apoptosis. NF-κB activation and pathogenic role of JNK has been well described in hypertension related cardiovascular and renal damage. We investigated that PKR activation in L-NAME administered rats caused significant up regulation in Ang-II together with the significant increase in its precursor renin, attenuation in the expression of Ang-II and renin were observed in rats treated with imoxin. Ang-II and ROS act in a corroborated manner to upregulate TGF-β1. Role of TGF-β1 has been well established in fibrogenesis by augmenting apoptotic pathway, collagen deposition, and matrix protein synthesis. We also report increased expression of TGF-β1 and inflammatory mediators in L-NAME treated rat kidney, aorta and heart, attenuation was observed with imoxin treatment. This is for the first time we report that the changes in the kidney, heart and vasculature could be mediated in part by upregulation of the PKR pathway. PKR activation acts as a chief key mediator

associated in the pathogenesis of renal and vascular inflammation. PKR activation is further connected with the induction of cardiac and aortic remodelling in L-NAME model of experimental hypertension. In conclusion PKR acts as a principal contributor in the pathogenesis of L-NAME induced end-organ-damage and PKR inhibition by imoxin has a therapeutic potential for combating hypertension and its associated cardiovascular complications.

DECLARATION

I hereby declare that, the present work embodied in this thesis entitled, **"Role of Double Stranded RNA Dependent Protein Kinase (PKR) in Hypertension: Mechanism and Prevention"** was carried out by me during the course of investigation (August 2016-January 2021) under the direct supervision and guidance of Prof. Arti. Dhar, Department of Pharmacy, BITS-Pilani, Hyderabad campus, Hyderabad, India and co-supervision of Dr. Audesh Bhat, Assistant Professor, Center for Molecular Biology, Central University of Jammu, Jammu, India.

This work has not been done / submitted in part or full to any other university or institute for award of any degree or diploma and is not concurrently submitted in candidature for any other degree.

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Jaspreet Kaur Kalra

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LIST OF ABBREVIATIONS

Hypertension: HTN

Cardiovascular: CV

World Health Organization: WHO

Renin-Angiotensin-Aldosterone System: RAAS

Sympathetic Nervous System: SNS

Angiotensin II: Ang-II

Reactive Oxygen Species: ROS

Tumor Growth Factor: TGF-β1

Double-stranded RNA dependent protein kinase: PKR

Nuclear Factor kappa-Light-Chain-Enhancer of Activated B cells: NF-Kb

c-Jun N-terminal Kinases: JNK

Type 2 Diabetes: T2D

Renal Tubular Epithelial Cells/Proximal Tubular Cells: NRK-52E

Vascular Smooth Muscle Cells: VSMCs

High Fructose: HF

Nitric Oxide: NO

Methyl Ester of N(Gamma)-Nitro-L-Arginine: L-NAME

Blood Pressure: BP

Heart Rate: HR

Diabetes Mellitus: DM

American College of Cardiology: ACC

American Heart Association: AHA

Autonomic Nervous System: ANS

Cardiac Output: CO

Peripheral Vascular Resistance: PVR

Parasympathetic Nervous System: PNS

Calcium Channel Blockers: CCBs

Renin Angiotensin System: RAS

Angiotensin-Converting Enzyme: ACE

Anti-diuretic Hormone: ADH

Endothelial-Derived Relaxing Factors: EDRF

Spontaneously Hypertensive Rat: SHR

Deoxy-corticosterone Acetate: DOCA

sGC: Soluble Gunalyl Cyclase

cGMP: Cyclic Guanosine Monophosphate

Angiotensin Receptor Blockers: ARBs

Nitric Oxide Synthase: NOS

Angiotensin Receptor 1: AT1

High Glucose: HG

Insulin Receptor Substrates: IRS

Inhibitor of Kappa B Kinase: IKK

Double-Stranded RNA: dsRNA

Eukaryotic Translation Initiation Factor 2-α: eiF2α

Mitogen Activated Protein Kinases: MAPKs

Selective PKR Inhibitor: imoxin/C16

New Onset Diabetes: NOD

Insulin Growth Factor-1: IGF-1

Congestive Heart Failure: CHF

High Fructose: HF

Institutional Animal Ethics Committee: IAEC

Hanks Balanced Salt Solution: HBSS

Paraformaldehyde: PFA

Polyvinylidene Difluoride: PVDF

Enhanced Chemiluminescence Reagent: ECL

Bovine Serum Albumin: BSA

Dichlorofluorescein Diacetate: DCFDA

Dimethyl Sulfoxide: DMSO

Propidium Iodide: PI

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

Indian National Science Academy: INSA

Body Weight: B.wt

Systolic Blood Pressure: SBP

Diastolic Blood Pressure: DBP

Mean Arterial BP: MAP

Phenylephrine: PhE

Acetylcholine: Ach

Blood Urea Nitrogen: BUN

Triglycerides: TGs

High density lipoprotein: HDL

Total Cholesterol: TC

Protease Inhibitor: PI

Phenyl Methyl Sulfonyl Fluoride: PMSF

Optimal Cutting Temperature: OCT

Terminal deoxynucleotidyl transferase dUTP nick end labeling: TUNEL

Deoxynucleotidyl Transferase: TdT

Diaminobenzidine: DAB

Alizarin Red Staining: ARS

Extracellular Matrix: ECM

Fetal bovine serum: FBS

Dubelco's Modified Eagle Medium: DMEM.

Dedicated To

My Beloved Grandparents,

Sardarni Kesara Kaur Kalra

&

Sardar Hazoor Singh Kalra,

for always blessing me from the heaven…

Chapter-1

INTRODUCTION

Hypertension (HTN) is a common, chronic disease which frequently entails debilitating cardiovascular (CV) damage and renal complications. HTN is referred to sustained elevation in systemic blood pressure (BP), which exceeds the usual range of 120/80 mmHg to 140/90 mmHg. HTN is a silent killer and a significant paramount for CV and renal diseases [1-3]. Several shreds of evidence signify that the BP values of 115/75 mmHg or above are associated with increased CV and renal risk [4-8]. Unlike other disease conditions, people with HTN remain asymptomatic. However, few cases of HTN causes symptoms like dizziness, headache, palpitations of the heart, chest pain, shortness of breath and nose bleeds. These symptoms cannot be under look, but at the same time these symptoms can't be relied on to signifying HTN. World Health Organization (WHO) reports HTN as an alarming situation that indicates a significant requirement of changes in lifestyle.

HTN is a prime global concern owing to its increased prevalence and sequential incidences of morbidity and mortality associated with CV disease, end-stage renal disease, and socio-economic burden on society [9]. According to the reports made available by WHO, approximately 1 billion adults were suffering from HTN in the year 2000, and this number has been projected to escalate to 1.56 billion by the end of the year 2025 [10]. Estimates suggest that nearly 7.5 million people die due to HTN every year, accounting for 12.8% of the mortality rate [11]. Though HTN is a significant contributor for CV and renal diseases still the exact molecular mechanism underneath its poor prognosis and pathogenic development is still uncertain. Incidence of raised BP in a smaller portion of patients (approximately 2% to 5%) is associated with underpinning renal or adrenal disease abnormalities also known as “Secondary HTN.” However, in the rest 90% to 95% hypertensive patients, none of the particular sole cause is clearly defined, and their disease state is often termed as "Essential HTN." Numerous physiological mechanisms participate in the maintenance and regulation of normal BP, any discrepancies occurring in these mechanisms may contribute in the progression and sustenance of essential HTN [12]. Among several reported factors excessive salt intake, insulin resistance, sympathetic nervous system (SNS), renin-angiotensin-aldosterone system (RAAS) over-activity has been studied extensively [13, 14]. However, recent trends are

currently shifting towards exploring the role of intrauterine nutrition, low birth weight, neuro-vascular abnormalities, renal inflammation, endothelial dysfunction, vascular remodelling, and inflammation in the pathogenesis of HTN. Vascular and cardiac remodelling along with the hypertensive nephropathy are the most common complications associated with chronic HTN [15, 16]. Since decades neurohumoral systems are held accountable for HTN induced cardiovascular fibrosis, remodelling, and nephropathy [17]. However, the molecular mechanism for unraveling the activation of neurohumoral systems, initiation of local inflammatory and fibrotic responses is still not precisely known. Reports from the previous studies suggest that angiotensin II (Ang-II), a foremost peptide of RAS and reactive oxygen species (ROS) act in a corroborated manner to induce tumor growth factor-1 (TGF-β1). It is the postulate that Ang-II promotes TGF-β1 release in a time and concentration-dependent manner [18-20]. Once activated, these pathways share standard networks that directly promote cardiac, vascular remodelling, and renal injury through the onset of inflammatory and fibrotic responses [21]

Double stranded RNA dependent protein kinase R (PKR), is a serine/threonine kinase [22]. PKR is known to be a key inducer of inflammation, insulin resistance causing impairment in glucose homeostasis in obese patients. Current reports suggest that PKR can also act in response to metabolic stress in in-vivo mouse model as well as in humans. Several forms of cellular stress inducing agents such as oxidative stress, elevated metabolic burden, mechanical stress, inflammatory mediators and excess nutrients are known to activate PKR [23-26]. Despite of its primary role in regulating translation initiation, PKR acts as an upstream marker for activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and c-Jun N-terminal kinases (JNK) activation [26,27]. Activation of NF-κB and JNK are well recognised for initiating inflammatory responses and promoting programmed cell death [28,29]. Although the clear underlying mechanism is still not fully elucidated. Extensive research supports the role of PKR in obesity and type 2 diabetes (T2D) as illustrated by several in-vitro, in-vivo, and gene knock out models [25, 20]. However, till date no experimenatal evidence is available which demonstrates the role of PKR in the deregulation of BP homeostasis, endothelial

damage, HTN associated CV alterations, nephropathy, and tubular calcification. As reported, HTN is associated with diabetes. According to previous report around sixty percent of diabetic individuals are hypertensive, and near by twenty percent of individuals with hypertension are diabetic. [1]. Therefore, based on the above-reported pieces of evidence two approaches were followed in the discussed work to investigate the role of PKR in HTN. The first approach follows in-vitro strategies using renal tubular epithelial cells (NRK-52E) and primary vascular smooth muscle cells (VSMCs) to confirm the role of PKR in renal inflammation, vascular inflammation, remodelling and oxidative stress under high fructose ((HF), nutrient excess conditions). The second approach follows in-vivo studies using nitric oxide (NO) synthase inhibitor (L-NAME; Methyl Ester of N(Gamma)-Nitro-L-Arginine) treated male Wistar rats. L-NAME model of experimental HTN mimics the actual clinical condition of essential HTN [4]. In experimental animals, L-NAME produces the impairment in vascular tone, endothelium-dependent relaxation, increase in BP, inflammation accompanied by cardiorenal and vascular fibrosis togethor with the intimal thickening and changes of the aortic vessel wall structure [31-33]. L-NAME administration implicates with the activated RAS, production of superoxide's and ROS, which further reduces the availability of NO and augments the production of peroxynitrite [34, 35].

Therefore in the discussed study, we sought to examine: (i) The effects of HF on PKR activation in NRK-52E cells and primary VSMCs, (ii) Pathogenic association of PKR signalling in maintenance and regulation of BP homeostasis in L-NAME administered rats; (ii) The effects of PKR activation on endothelium-dependent relaxation and underlying mechanism; (iii) Path-morphologic alterations related with PKR activation that comprise cardiorenal alterations, vascular fibrosis, glomerulosclerosis and changes in lumen diameter.

CHAPTER-2

LITERATURE REVIEW

2.1. Hypertension

HTN is defined as sustained elevation in systemic BP, which exceeds the usual range of 120/80 mmHg to 140/90 mmHg. HTN is a silent killer and a significant paramount for CV and renal diseases [36-38]. Unlike other disease conditions people with HTN remain asymptomatic. However, few cases of HTN causes symptoms like dizziness, headache, palpitations of the heart, chest pain, shortness of breath and nose bleeds. These symptoms cannot be under look, but at the same time these symptoms can't be relied on to signify HTN [39]. According to WHO, HTN is an alarming situation that indicates a significant requirement of changes in lifestyle. Incidence of raised BP in a smaller portion of patients (approximately 2% to 5%) is associated with underpinning renal or adrenal disease abnormalities also known as "Secondary HTN" [41]. However, in the rest 90% to 95% hypertensive patients, none of the particular sole cause is clearly defined, and their disease state is often termed as "Essential HTN" [42].

2.2. Essential/Primary hypertension

Essential/primary HTN (also called idiopathic HTN) is the standard type of HTN that has no known cause. It affects 90%-95% of the total hypertensive population. It often runs in families presenting a group of genetically based diseases or ailments accompanied by biochemical alterations inherited [42-45]. The incidence of essential HTN is highly prevalent among people with advancing age. Younger individuals with comparatively high BP are also vulnerable to the development of critical HTN [46-48]. Numerous pathophysiologic factors are the crucial contributors associated in the genesis of essential HTN: over activated SNS, increased production of aldosterone and vasoconstrictor agents, excessive dietary intake of sodium; augmented renin release and elevated Ang-II, NO deficit, modifications in other natriuretic peptides; that modulates vascular tone and renal sodium handling; selective lesions/fibrotic scarring in the renal microvasculature; HR modulating changes in adrenergic receptors, contraction/relaxation cycle of heart, vascular reactivity; and significant alterations occurring in cellular ion transport are some of the prominent contributors involved in the pathogenesis of HTN. Along with these factors, hyperglycemia, insulin resistance syndrome and the amplified activity of

growth factors are also found to be closely associated with the genesis of HTN [49]. Modulations in vascular tone, endothelial damage, higher ROS generation, and remarkable remodelling in vasculature are major pathogenic features that represent hypertensive disease condition. Numerous factors are implicated in the progression and development of HTN or BP regulation. Previous reports suggest that among all known factors, mechanisms about renal alterations and vascular remodelling are the prime culprits involved in causing end-organ damage [50].

2.3. Secondary Hypertension

Prevalence of Secondary HTN is restricted only to 5% to 10% of total hypertensive cases. It is most commonly associated with the underlying reversible cause [51-53]. The vulnerability of this form of HTN is around 30% in people with a younger age group. Approximately people with an age group of 18 to 40 years of age are majorly affected by this form of HTN. Due to high-cost of testing, low yield, and higher incidences of false-positive results, secondary HTN goes unwarranted in most of the clinical cases. However, patients with early signs are recommended to undergo the test [54-55]. Although the precise reasons for the secondary HTN are not known, some crucial factors may play an essential counterpart in its progression and development; the list includes a sedentary lifestyle, excessive salt intake, smoking, diabetes, obesity, stress, and aging [56]. HTN is also a predisposing risk factor for end-stage renal disease/chronic renal disease, heart failure, ischemic heart disease, and stroke [57, 58].

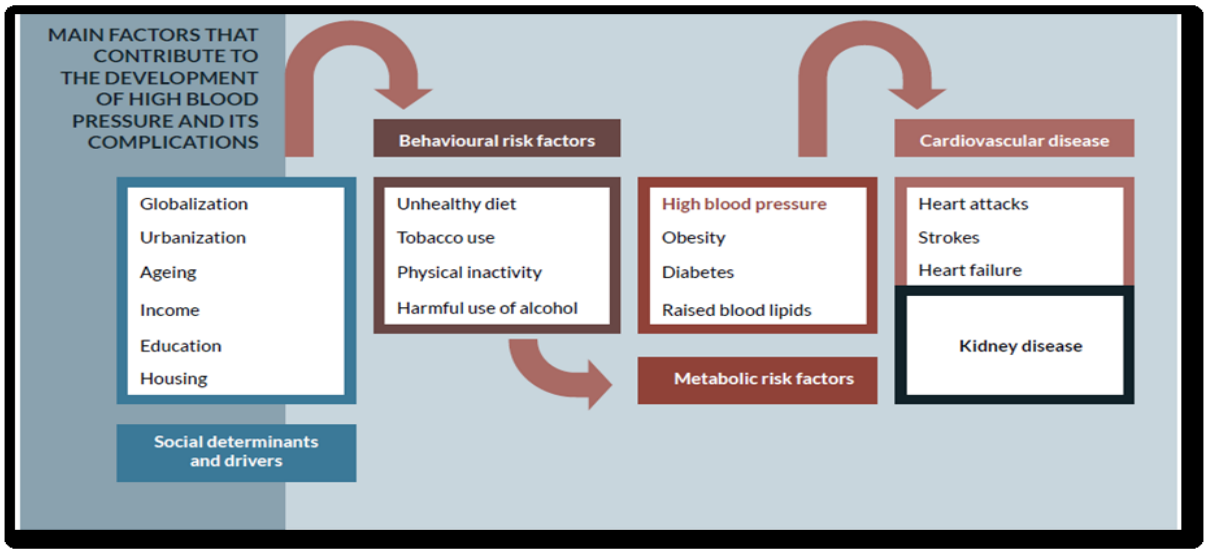


Fig.1. Main contributors responsible for elevated blood pressure [63].

2.4. Classification of Hypertension

American College of Cardiology (ACC) and American Heart Association (AHA) has revised the old guidelines: comprehensive set 2003 for categorizing the stages of HTN, including its detection, prevention, management, and treatment. The newer instructions describe HTN with patients having either elevated or stage I HTN and have eliminated the pre-HTN from the categorical representation. Interestingly, these guidelines also emphasize on proper BP monitoring and measuring techniques using optimized and validated devices. These guidelines also value on the necessary training of the health care providers to underpin the causes lying underneath "white-coat HTN" [59].

Category	**SBP (mm Hg)**	**DBP (mm Hg)**
Normal	Less than 120	Less than 80
Elevated	120-129	Less than 80
Stage 1	130-139	80-89
Stage 2	140	90
Hypertensive crisis	>180	>120

Table.1. Classifical representation of hypertension according the new guidelines revised by ACC and AHA. Hypertensive crisis, >180 systolic and >120 diastolic, with patients requiring alterations in medication in the absence of any other indications, or patients requiring urgent hospitalization in case of organ damage [60].

2.5. Prevalence

HTN is a major global concern owing to its increased prevalence and sequential incidences of morbidity and mortality associated with CV disease, end-stage-renal disease and socio-economic burden on society [61]. According to the reports made available by WHO, approximately 1 billion adults were suffering from HTN in the year 2000, and this number has been projected to escalate to 1.56 billion by the end of

the year 2025 [10, 62]. It has been estimated that nearly 7.5 million people die due to HTN every year, accounting for 12.8% of the mortality rate [63].

According to the Fourth National Family Health Survey, nearly 11.3% Indian population is living with HTN, comprising 13.8% Indian males of 15–49 years of age and 8.8% Indian female's aged between 15–54 years. Currently, one more survey conducted on age group of or equal to 18 years or older further reveals higher incidence of HTN in men (27.4%, men 112 million) compared to women (20.0%, women 95 million). one of the leading studies which identifies worldwide burden of diseases, report that in 2016, nearly 1.63 million people died in India with associated cause of HTN, whereas in 1990, deaths reported due to HTN were 0.78 million [64]. Henceforth, there exists a significant urge for proper screening and control of HTN and its associated CV and renal risks.

2.6. Regulation of BP

The control of BP is a multifactorial physiologic function. It depends on a series of actions of cardio-renal, vascular, and neuroendocrine systems. Therefore, it is vital to understand the role and contribution of each factor involved in the vicious process of BP regulation for a thorough investigation of the pathological process associated with HTN. Moreover, gained pathological insights may prove helpful in exploring the newer horizons for subclinical abnormalities and improved therapeutic targeting. BP control is usually governed by local, global neural and renal, endocrine mechanisms. At the local level, vasoconstriction and dilatation mechanisms are linked with acute BP control. However, chronic control is connected with the change in number in BP values and structural alterations in blood vessels. Global regulation of BP associates with modulations in cardiac output, which is mediated by the autonomic nervous system (ANS). Cardiac output (CO) depends upon the volume of the blood. Higher blood volume directly correlates with increased rate of blood return to the heart, ultimately leading to high CO [65]. Peripheral vascular resistance (PVR) is an essential contributor to BP regulation. Vascular resistance totally depends upon vessel diameter, vessel length, and thickness of blood. More the lumen diameter, lesser will be the exerted resistance, whereas large vessel size is related to increased resistance. In turn, the higher the vessel radius more will be the

BP. The smooth and pulsatile flow of blood is also influenced through the accumulation of fat on arterial walls and increased viscosity of blood [66]. Reports suggest that increased blood viscosity is related to elevated BP levels. SNS is crucial for global neural control of arterial HTN, whereas cardiac functions are primarily regulated through the parasympathetic nervous system (PNS). The renal-endocrine systems are the most prominent regulator of BP [67, 68]. The majority of the chronic hypertensive cases are related to the impairment in renal, endocrine systems, alterations in this system disrupts body fluid homeostasis mechanisms vital for control of HTN [67].

2.6.1. Sympathetic Nervous System Overactivity

Sustenance of a regular BP is attributed to the equilibrium balance between the CO and PVR. The majority of the patients with essential HTN exhibit standard CO but an elevated PVR [69]. PVR does not depend on the alterations in large arteries or the capillaries; instead, it is highly dependent on the alterations in small arterioles, which are internally lined with VSMCs. The contraction of VSMCs is well correlated with an increase in calcium concentration, which also associates with vasodilatory effects achieved by the use of calcium channel blockers (CCBs). Long-standing strain in smooth muscle is considered to cause narrowing or thickening of lumen diameter, and intimal thickening in the arteriolar vessel walls. Noted structural changes in vasculature are probably associated with the rise in Ang-II, causing an irreversible rise in PVR. According to previous report it is assumed that in initial stages of HTN, a rise in BP is related to the raised CO but not with the increase in PVR, which is associated with the over-activity of the SNS. The following rise in PVR could be attributed to the compensatory mechanism to stop the elevated BP to reach to the capillary bed, where it can significantly influence cell homeostasis. Increased sympathetic overactivity primarily occurs in initial stages of primary HTN, including other forms of HTN such as those which are associated with obesity, early T2DM, pre-diabetes, kidney disease, and failure of heart to pump and perform vital functions. Increased sympathetic activity is attributed to the activation of central and peripheral mechanisms. In addition to this, physical and emotional stress also activates sympathoadrenal activity and causes a rise in BP [70-72].

2.6.2. Hormonal Mechanisms: Renin Angiotensin System

The RAS is the most important of the endocrine systems, acts as a key contributor involved in the control of BP. Regulation of RAS is governed by numerous hormones that influence a rise in blood volume and PVR. The significant component of RAS is renin. Renin is released by the juxtaglomerular cells present in the kidney against diminished salt intake and glomerular under perfusion. The overactivity of RAS is also coupled with the increased production and secretion of renin. Renin converts angiotensinogen to Ang-I, an inactive physiological substance, capable of getting converted easily to Ang-II in lungs by the angiotensin-converting enzyme (ACE) enzyme. Ang-II, a well known potent vasoconstrictor of the arterioles, thus causes an increase in BP. Ang-II also causes an increase in the myocardial force of contraction by increasing Ca^{2+} influx. Ang-II upregulation is linked with the enhanced production and secretion of antidiuretic hormone (ADH). ADH acts as an essential contributor to BP regulation. It increases water reabsorption in the kidney tubules, ultimately causing increased fluid volume, CO, increased vascular resistance, with the overall increase in arterial pressure [69]. Further, it also augments the aldosterone release from the adrenal gland. Increased aldosterone is directly related to the increased BP, which may be coupled with the upregulation of Na+/K+ pumps of the distal convoluted tubule and collecting duct within the nephron, causing enhanced sodium and water retention. However, an increase in BP cannot be solely ascribed to the circulating RAS in essential HTN. For instance, elderly and black people have been reported to exhibit decreased renin and Ang-II, and therapeutic options that specifically block the activity of RAS do not claim to be an effective treatment strategy for them. Several reports demonstrate the existence of non-circulating local RAS, which is an essential regulator for BP control. Kidney, arterial tree, and the heart are known to possess local renin systems. These local renin systems may share substantial roles in regulating regional blood flow [73-75].

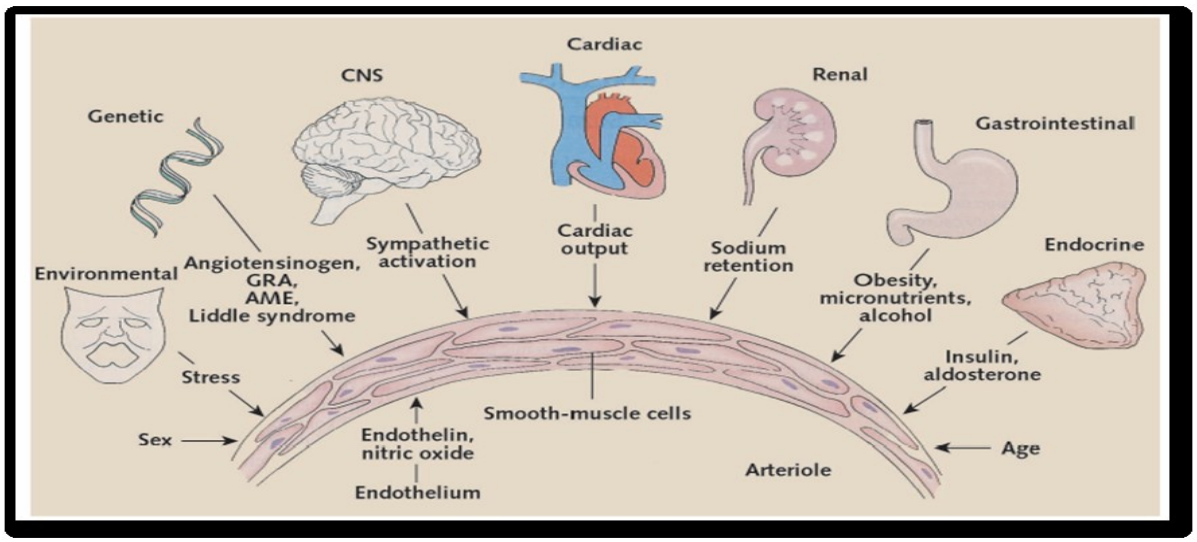


Fig.2. Underlying pathophysiologic mechanisms involved in development of hypertension. AME: apparent mineralocorticoid excess; CNS: central nervous system; GRA: glucocorticoid-remediable aldosteronism [439].

2.6.3. Vascular Mechanisms

Vascular endothelial cells are the key participants involved in CV regulation by producing local vasoactive agents. NO, a signifcant vasodilator and highly vasoconstrictor peptide endothelin are locally produced vasoactive agents. Endothelial dysfunction has been indicated in clinical cases of essential HTN. Endothelial dysfunction is recognized by decreased release of vasodilator NO and augmented release of pro-thrombotic, pro-inflammatory, growth factors, and endothelial-derived vasoconstricting agents such as endothelin, thromboxane, and TGF-β1. Endothelium of a healthy individual regularly releases NO against the flow of blood. NO mediates endothelium-dependent vasodilatation and directly lowers the vascular resistance. However, endothelial dysfunction not merely affects endothelium-dependent vasorelaxation but also induces the chronic release of inflammatory mediators in endothelium [76]. Hence, from those as mentioned above, it's clear that restoration of endothelial function could be a significant paramount for therapeutic strategy in attempting to diminish various imperative complications of the HTN. Currently, available antihypertensive medications are capable of restoring impaired production of NO but fail to revive the damaged endothelium-dependent vascular reactivity to highly specific endothelial agonists.

This implicates that dysfunctioning of endothelium is a principal event which often becomes permanent as HTN develops and persists [69].

2.6.4. Insulin sensitivity

Several epidemiological studies demonstrate that there's a crosslinking of plausible factors, such as hyperlipidemia, insulin resistance, obesity, glucose intolerance, DM, and HTN. Altogethor these factors are commonly identifed as metabolic syndrome X. Previous report suggests metabolic syndrome as one of the chief participant involved in elevated BP and vascular pathology. In some cases, hypertensive patients that are non-obese may represent resistance to insulin. Although there exists a series of objections to the present hypothesis, it should explain why the jeopardy of CV events is synergistic instead of being just additive [69].

2.7. Animal models of HTN

Animal models have proved to be useful in elucidating the cause and progression of HTN. HTN is diverse because of the variability occurring in methods **to** induce it in animals [77]. Variables like food and fluid intakes, structural alterations, environment,pharmacological intervention and importantly, genetics are manipulated to induce experimental HTN in animals. Many models help to elucidate the various causes of HTN in humans, like the spontaneously hypertensive rat (SHR) [78], transgenic rat (Ren2)27 (TGR(Ren2)27, Goldblatt [79], and deoxycorticosterone acetate (DOCA) HTN rats [80].

2.7.1. Spontaneously Hypertensive Rat (SHR)

A hypertensive genetic model, the SHR, is the most frequently used model of HTN [81]. Inbred from Wistar and Wistar-Kyoto non-hypertensive controls, SHR develop HTN at 4-6 weeks old without intervention [82], within the early stages of HTN, SHR maintains total peripheral resistance, but have increased flow rate**.** However, as HTN is established, hypertrophic vessels increase total peripheral resistance, the flow rate returns to normal, and remodelling within the heart occurs [83, 84]. Like in essential human HTN, the precise cause is unknown, but SHR shows changes in SNS activity, alterations in NO availability, and endothelial dysfunction [85, 86]. Levels of sGC and cGMP were found to be significantly lower in young SHR compared with age-matched Wistar-Kyoto [87]. Increases in arterial wall

renin are observed in SHR. While increases in Ang-II haven't been noted, sensitivity to Ang-II is seen in SHR [88].

2.7.2. TGR(Ren2)27HypertensiveRat

Like SHR, the (Ren2)27 TGR(Ren2)27 is an experimental model of HTN that also shows sensitivity to Ang-II. Created by Mullins et al., the TGR (Ren2)27 rats were developed through the introduction of the mouse Ren2 gene into rats [89]. It had been previously reported that injection of purified mouse renin elevated pressure level**,** and thus the Ren2 gene had shown high expression during a transgenic mouse model [90]. Homozygous TGR(Ren2)27 rats develop severe HTN at an early age and reach maximum levels at nine weeks old**.** Alike the SHR, the mechanisms behind the increase in pressure level within the TGR(Ren2)27 rats haven't been elucidated. Interestingly, the report suggests that circulating renin levels remained unchanged or decreased as compared to heterozygous normotensive littermates [91]. Also, the ductless gland of the TGR(Ren2)27 is hypertrophic accompanied by significant increases in local renin and aldosterone [92]. Ang-II levels are found to be elevated, but more importantly, the VSMCs were shown to own increased sensitivity to Ang-II, similar to the changes observed in SHR [93]. In young, but not aged, TGR(Ren2)27 rats plasma steroid levels and secretion are enhanced and will be involved within the development of HTN [94]. Aged TGR(Ren2)27 rats show diminished NO release suggesting endothelial dysfunction. The sum of those changes is remodelling, hypertrophy, and end-organ damage. Studies involving TGR(Ren2)27 rats give the further study of HTN and its pathophysiology, but might not be representative of human HTN because of its early onset and severe nature [95]. As noted above, genetics plays a very important role in the development and sustenance of HTN. However, structural changes, food, and fluid intake are integral factors in some form of HTN, as observed within the Goldblatt and DOCA animal models.

2.7.3. The Goldblatt Hypertensive rats

The first animal model of HTN was developed by Harry Goldblatt in 1934 when he clipped the arterial blood vessel of a dog and produced a hypertensive state [96]. As an experimental model of renal HTN**,** it can include one in all the following: two-kidney one clip (2K1C) where both kidneys remain intact, and one arterial blood

vessel is constricted with a clamp, one-kidney one-clip (1K1C) where one kidney is removed, and therefore the arterial blood vessel of the remaining kidney is clamped, or two-kidney two-clip (2K2C) where both kidneys are intact but either the aorta or both renal arteries are clamped. The clamps reduce renal perfusion pressure, which successively stimulates renin and Ang-II synthesis [97]. This causes endothelial dysfunction, increased peripheral resistance, and subsequent increases in pressure level**.** Unlike the 2K1C, the 1K1C model is taken into account to be sodium-fluid volume-dependent due to the absence of the healthy kidney and its absent compensatory elevated sodium and water excretion, resulting in fluid retention [98]. Additionally, the 2K2C exhibit severe renal ischemia, accounting for increased RAS and SNS activity [99]. In rats, the Goldblatt hypertensive model induces a chronic hypertensive state with increased renin and Ang-II, similar to the conditions that are observed in humans with unilateral arterial blood vessel stenosis [100]. In contrast to the high renin levels noted within the Goldblatt hypertensive models, the administration of DOCA salt induces the low renin form of HTN [101].

2.7.4. Deoxycorticosterone acetate-salt (DOCA-salt) Hypertensive Model

The DOCA-salt model uses synthetic mineralocorticoid steroids and NaCl to mimic aldosterone overload induced volume overload, and subsequent HTN via retention of sodium and water [102, 103]. It is characterized by endothelial dysfunction, elevated RAS activity, and oxidative stress [104]. DOCA-salt rats exhibit low renin and don't respond well to RAS inhibitors, like angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs) [105]. However, DOCA-salt HTN does respond well to diuretics and aldosterone inhibitors [118]. DOCA salt rats develop severe hypertrophy and end-organ damage [106]. This low renin, volume overload model of HTN is productive to review because it mimics the result of chronic human high BP**.** The DOCA-salt hypertensive model and several other animal models allow further elucidation of the various unknown mechanisms driving human HTN. Notably, a key element in this model is modulation in Ang-II or the alterations in sensitivity to Ang-II. L-NAME induced HTN is another well-known method used for inducing experimental HTN.

2.7.5. N-ω-nitro-L-arginine methyl ester (L-NAME)-induced HTN

Experimental models of HTN are significant within the exploration of HTN. One such established model is L-NAME induced experimental HTN in rats [107]. Routes of effective administration include intravenous, intraperitoneal, and oral. L-NAME produces a hypertensive state reflective of the dysfunction seen in high BP via several mechanisms, including inhibition of NO, SNS activity increasing total peripheral resistance, oxidative stress, and arterial remodelling [108]. Classically, L-NAME **is** an inhibitor of nitric oxide synthase (NOS), resulting in decreased NO [109]. Acute and chronic inhibition of NO produces endothelial dysfunction, which has been clearly demonstrated by the L-NAME model [110]. Moreover, the inhibition of NO by L-NAME in young SHR produces similar results to it of naturally aged SHR, supporting the role of NO inhibition within the study of high BP [111]. In addition to the reduction of NOS activity by L-NAME, the assembly of $O2^{-}$ by L-NAME may further decrease NO through its peroxynitrite (ONOO- -) mediated uncoupling of NOS [112]. L-NAME may directly alter baroreceptor sensitivity in SNS, resulting in progression and development of HTN [113]. It has been hypothesized that NO is an inhibitory modulator of SNS outflow [114]. Large volumes of evidence show that stimulation of SNS activity in L-NAME-induced HTN is often modulated by reduced NO availability [115]. SNS activity may be a contributor to acute and chronic L-NAME-induced HTN [116 117]. Additionally**,** to the attenuation of NO, evidence has also shown that L-NAME stimulates the RAS [118, 119]. As in high BP**,** RAS is stimulated at many levels by L-NAME. Studies of chronic L-NAME administration indicate increased mRNA expression of renin. Moreover, elevated levels and activity of renin are also reported within the plasma of L-NAME treated rats [120, 121]. Alterations in activity of ACE, an enzyme responsible for the conversion of Ang I to Ang-II, have also been observed in L-NAME administered rats [122]. Evidently, prolonged L-NAME treatment in rats is related to increase ACE activity in plasma and tissue [123, 124]. Both antagonists of the angiotensin receptor 1 (AT1) and inhibitors of ACE prevented the event of L-NAME induced HTN. Henceforth indicates the importance of Ang-II [125]. The report further suggests L-NAME administration causes increased release of Ang-II

and its mediated effects [126]. In fact, recent reports show that plasma Ang-II is elevated within three weeks of treatment with L-NAME [127]. Ang-II induction is accompanied by enhanced generation and production of ROS. Subsequently, oxidative stress is a significant contributor involved in the pathogenesis of L-NAME induced HTN [128, 129]. Vascular remodelling, fibrosis, inflammation, hypertrophy, decreased NO, increased Ang-II, and increased ROS are some of the significant events which have been critically noted in the L-NAME induced HTN [130]. Alike essential HTN, L-NAME induces an inter-connected pathophysiological response that needs an anti-hypertensive treatment capable of modifying multiple causal and responsive factors.

2.8. Double-stranded RNA-dependent Protein Kinase R: PKR

PKR is a known inducer of inflammation and apoptosis. PKR mediates inflammatory NF-κB and JNK activation [26, 27], promotes fibrosis and cell death [28, 29]. Extensive research implicates the role of PKR in obesity and T2D in several in-vitro, in-vivo, and gene knock out models [25, 20]. Till present, the role of PKR in HTN and associated end-organ damage has not been studied.

2.8.1. PKR: Structure and Function

Earlier, PKR was known by different names such as double-stranded RNA-dependent protein kinase; double-stranded RNA activated inhibitor (DAI); double-stranded RNA-dependent kinase (dsl) and double-stranded RNA activated p68 protein kinase (p68), including several other names. Now, its name has been standardized and termed as RNA regulated protein kinase i.e., PKR, and to date, PKR is known by this name [131]. Human PKR is encoded by a 551 amino acid protein. It has two distinct domains, N-terminal regulated dsRNA-binding domain (dsRBD) and C-terminal kinase domain, known for its catalytic activity. The dsRBD contains two dsRNA-binding motifs (dsRBM), each motif is made up of about residues of 65 amino acids detached by a 20 amino acid linker [132].

Besides dsRNA, endoplasmic reticulum (ER) stress, mechanical stress, high fat diet, pathogens, environmental stress, heme limitation, cytokines (TNF-α, IL-1β), and irradiation also possess the ability to activate PKR [133]. PKR activation causes

autophosphorylation of many downstream substrates, causing excessive cell damage and DNA fragmentation [133]. In addition, researchers have found that the PACT is a plausible cellular activator of PKR which can cause the PKR phosphorylation [134], while others have demonstrated that PKR-associated protein X (RAX), the murine counterpart for PACT [135], is accountable for the activation of PKR at the cellular level when stimulated by any extracellular stress stimuli [136-138]. Table.2. shows the list of some PKR activators that are associated with the substrate phosphorylation and physiological process linked with substrate phosphorylation. It has been elucidated that PACT consists of three independent domains; first, two of them are similar to dsRBM, and the last one is (PACTd3), which happens to have 66 residues, earlier PACTd3 seems to be an unidentified domain. Several studies carried on mutational analyses have confirmed that even a weak binding of PACTd3 to PKR can cause its activation. However, reports also suggest that the first two domains are responsible for mediating strong linking between PACT and PKR [139].

PACT has been recognized as a unique activator of PKR. Interaction of PACT with PKR is illustrated by means of a two-hybrid screening for yeast. All the interferon (IFN) independent tissues express PACT. Although PACT contains the dsRNA-binding domain, it can cause PKR activation even in the absence of dsRNA. In mammalian cells, PACT mediated PKR activation phosphorylates eIF2α and inhibits protein synthesis [140]. RAX encoded by the prkra gene is a murine ortholog for PACT, which possesses the potential for activating PKR [141]. Several studies have reported that PACT and RAX have similar amino acid sequences; out of 313 recognized amino acid residues, they differ only in six residues, which are un-identical with four similar substitutions. These two (PACT/RAX) activators of PKR gets activated under the conditions of cellular stress; they are also implicated in regulating several signalling pathways and protein synthesis. Arsenite, thapsigargin, and H2O2 are strong chemical inducers of stress. These inducers cause rapid phosphorylation of RAX followed by subsequent interaction of RAX with PKR leading to PKR activation, and ultimately they cause inhibition of protein synthesis [138, 142, 143]. It has been reported that the serine 18 phosphorylation is responsible

for inducing conformational changes in RAX/PACT, which is required for the association of dsRBM with PKR and successive PKR activation [141].

PKR activator	**Substrate**	**Physiological process linked with substrate phosphorylation**	**Reference**
Interferon (type I predominantly α/β and type II γ)	Phosphorylation Fas-associated death domain (FADD)	Receptor-interacting protein (RIP)1/RIP3 kinase-mediated necrosis	[144]
Protein activator of PKR (PACT)/ protein kinase R (PKR)-associated protein X RAX	Ser 18 phosphorylation of RAX	Inhibition of protein translation and cell cycle arrest	[145]
short dsRNA (ss-dsRNA)	Phosphorylation of eukaryotic initiation factor-2 α-subunit (eIF2α).	Inhibiting translation or inducing RNA interference (RNAi)	[146, 147]
Indomethacin	Phosphorylation of eukaryotic initiation factor-2 α-subunit (eIF2α).	Inhibition of viral protein synthesis and blockade of viral replication	[148]
3-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-5,7-dihydroxy-4H-chromen-4-one (DHBDC)	Phosphorylation of eIF2α, IκBα, tumor suppressor p53, nuclear protein 90	Translation inhibition	[149]
1H-Benzimidazole-1-ethanol, 2,3-dihydro-2-	Phosphorylation of eukaryotic	Inhibiting translation or inducing RNA	[150]

imino-alpha-(phenoxymethyl)-3-(phenylmethyl)-monohydrochloride (BEPP monohydrochloride)	initiation factor-2 α-subunit (eIF2α).	interference (RNAi)	
Heparin	PKR dimerization	Phosphorylation of the eIF2α, K296R substrate, disruption of PKR monomer–dimer equilibrium, translation inhibition	[151]
Viral ribonucleoprotein (vRNP); influenza virus	Phosphorylation of eukaryotic initiation factor-2 α-subunit (eIF2α).	Inhibition of translation	[152]
Infections Eg: Chlamydia trachomatis	Alterations in TLR4 and MyD88 signalling	Increase in IRE1α RNAse activity	[153]
Inflammation	Phosphorylation of inhibitor of kappa B kinase (IKK), and serine phosphorylation insulin receptor substrates	Hinderance in insulin signalling, Insulin resistance	[1]

Table.2. PKR activators, their phosphorylated substrates, and physiological process linked with specific substrate phosphorylation.

Upon activation, PKR phosphorylates several downstream substrates such as protein phosphatase 2A (PP2A) and eukaryotic translation initiation factor eIF-2α that are involved in modulating protein synthesis [133, 154]. Notably, PKR is implicated in regulating various physiological and pathological functions such as cell growth, apoptosis, stress, transcription, and many types of malignancies [155]. PKR has been ascribed as an anticipated regulator that is involved in maintaining the innate immune response for safeguarding against the offensive viral infections that are common in higher eukaryotes [156]. Further, reports suggest that there exists extensive networking between PKR regulated inflammatory signalling and metabolic diseases [157]. It has been reported that PKR is also activated by infections and pathogen-induced stress, which stimulates two intracellular serine kinases JNK and IκB kinase (IKK), that are involved in initiating an inflammatory cascade [44]. JNK and IKKb work collaboratively in eliciting an inflammatory feedback mechanism and inducing insulin resistance. PKR induced inflammatory signalling pathways are well correlated with the insulin resistance observed in obesity cases.

Although JNK and IKK are involved in regulating and maintaining innate immune response against viral infection, nevertheless, remodelling in any of these kinases can alter the overall pathway for cell signalling and translational regulation [158-159]. Hence, the above fact demonstrates that PKR induced inflammation is detrimental for cell growth and sustenance of tissue population [158]. Therefore, there exists a significant urge for disclosing the role of PKR in metabolic diseases, including hypertensive pathology, as they may direct the discovery and development of newer treatment strategies that are aimed at targeting PKR.

2.8.2. PKR and oxidative stress

Environmental changes, together with the sedentary lifestyle, genetic, and epigenetic behavior, reveals that oxidative stress plays a central role in multiple pathological conditions. Oxidative stress is a widely accepted term used to describe an imbalance in ROS generation and its degradation, allied with the impairment in cellular antioxidant defense mechanism [160]. It has been verified that systemic oxidative stress shares undeviating interdependence with the components of metabolic diseases such as dyslipidemia, insulin resistance, and glucose intolerance [141-143].

Biomolecules and cells are highly susceptible to damage by the ROS generated during oxidative stress conditions. Lipid peroxidation, cell damage, DNA fragmentation, and apoptosis are some of the destructive events that are directly associated with the excess ROS [142]. A higher incidence of oxidative damage has been noted in patients having a profile of Met S. Obese individual represents elevated body Mass Index (BMI) and body fat percentage, posing higher incidence for oxidative stress-induced cellular damage [161]. Under extreme conditions of oxidative stress, the rapid decline was observed in the anti-inflammatory adipokine and adiponectin levels within the adipose tissue [162, 163]. Oxidative stress has a negative influence on insulin resistance, glucose intolerance, and hyperglycemia, including the number of diabetic and CV complications [164]. For instance, the results of an in-vitro study have demonstrated that the cells treated with high glucose (HG) manifest massive oxidative stress. In HG treated cells, oxidative stress is accompanied by marked cell death, neuronal damage, and disruption of endothelial cells while the untreated cultures have shown the more significant number of well-developed neurons and more enacted endothelial cells [160]. In addition, oxidative stress is further accompanied by a series of adverse outcomes such as impairment in gene transcription, translocation of GLUT4 [165], increased levels of Di-acyl-Glycerol (DAG), activation of PKC and its several isoforms [166, 167], PKC isoforms display a wide variety of cellular signals, ranging from elevated ROS level to the increased expression of inflammatory markers such as NF-κB [168]. Oxidative stress induces several stress-activated signalling pathways/kinases, representing integrated signalling between inflammation and apoptosis. Further, it has been reported that activation of stress-activated signalling pathways such as NF-κB, inducible nitric oxide synthase (iNOS), Intercellular Adhesion Molecule 1 (ICAM-I), Mitogen-activated protein kinase (p38 MAPK) and JNK is linked with the several cells destructing events that favor early apoptosis in most of the prevailing disease conditions [169].

Remarkably, oxidative stress and activated PKR are the most prominent risk factors involved in the progression and development of numerous diseases, ranging from neurodegenerative disorders such as Alzheimer's disease to a broad array of

interconnected metabolic diseases including several forms of malignancies [170, 171]. Studies of Bahl et al., and Udumala et al., have illustrated the role of PKR in CV diseases [133]. PKR is activated by multiple stimuli, which may be either internal or external such as endoplasmic reticulum stress, nutrient excess, and many other factors [24]. High nutrient intake has been correlated well with oxidative stress-induced obesity, insulin resistance, hyperglycemia, T2D, abnormalities in adipocytes, chronic inflammatory responses, and decreased level of endogenous antioxidant enzymes [172-174]. Induction of PKR by oxidative stress or vice versa is associated with the activation of two intracellular kinases such as (i) JNK and (ii) IκB kinase (IKK) that are engaged in metabolic homeostasis and insulin resistance [23]. Like oxidative stress, PKR is very competent in producing alterations in insulin signalling pathways such as phosphatidylinositol-3 kinase (PI3-K)/Akt pathway or ERK kinases [175]. Overproduction of ROS activates insulin signalling, induces metabolic changes, and causes activation of downstream signals. Eventually, PKR increases the threshold of glucose uptake in adipocytes and facilitate GLUT4 translocation and biosynthesis of specific lipid molecules in adipocytes [176]. Therefore, from the above-discussed facts, it can be well understood that PKR and oxidative stress play an important role in the pathogenesis of CV diseases, representing PKR as a novel target involved in the development and progression of CV diseases.

2.8.3. PKR and inflammation

Inflammation is a preliminary tissue response to a wide range of physical, chemical, or biological injury/insult. Chronic low-grade inflammation is a characteristic feature of distinct forms of metabolic diseases and uncountable CV complications [177]. As described earlier, met-inflammation is a collective term that is commonly used to represent the inflammation of metabolic tissues in vast metabolic diseases [178, 179]. Adipose tissue, skeletal muscles, liver, gut, and brain are the most studied metabolic tissues which are known to express the most severe form of met-inflammation [180]. The acute inflammatory response is concerned with the accumulation of plasma proteins and white blood cells at the site of injury. Generally, acute inflammatory response is generated by the mast cells and

macrophages which releases pro-inflammatory cytokines, chemokines (TNF-α, IL-6, and IL-1b), vasoactive amines (histamine and serotonin), eicosanoids (prostaglandins, prostacyclins, thromboxanes, leukotrienes, and epoxyeicosatrienoic acids), and products of proteolytic cascades [181]. Interestingly, it has been found that activation of JNK and NF-kB pathways in peripheral tissues creates hindrance in insulin signalling; thus, causes insulin resistance [182]. Moreover, sub-chronic met-inflammation is considered as the most vital variable that positively affects insulin resistance and CV complications [183-185].

Several studies have elucidated that C-reactive protein (CRP) levels are elevated in individuals with cardiovascular diseases. Furthermore, the majority of prospective studies have revealed that CRP and high sensitivity CRP (hsCRP) are also linked to the development and progression of diabetes. Nonetheless, they both are considered as a critical clinical criterion for cardiovascular diseases [186, 187]. Growing evidence indicates that inflammatory responses are responsible for causing insulin resistance and inducing alterations in nutrient metabolism. In the presence of an inflammatory condition, insulin receptor substrates (IRS) are phosphorylated on serine residues causing dampening of the insulin signalling. However, under normal conditions, IRS's are phosphorylated on tyrosine residues. It has been demonstrated that among all known IRS modifying enzymes, JNK, an inhibitor of kappa B kinase (IKK) and protein kinase C (PKC) are well recognized for inducing deleterious effects on insulin action due to phosphorylation of IRS-1 at serine residues [188]. The immune response pathway regulates nutrient metabolism and is fundamentally dependent on inflammatory signals; after the initiation of inflammation, immune responses are known to alter the fate of nutrient metabolism [189]. Linkage of inflammatory responses and nutrient metabolism with PKR is further elaborated by Nakamura and his lab mates. In response to threats by a viral infection, PKR confers cell defense through restricting the synthesis of general proteins, imparting innate immune response [23].

PKR activation is governed by varying stress responses; nevertheless, it is of significant interest to state that PKR is also activated by auto-phosphorylation due to

the presence dsRNA. Moreover, the earlier name of PKR i.e., double-stranded RNA dependent protein kinase, has also been derived from the same fact [189].

Eukaryotic translation initiation factor 2-α (eiF2-α), is a chief PKR substrate involved in monitoring the protein synthesis [190]. PKR is a Ser/Ther protein kinase implicated in mediating the signalling pathways through interacting with the different proteins such as protein phosphatase-2 (PP2A), Mitogen-activated protein kinases (MAPKs) and NF-ƙB proteins [168]. IKK is a predominant kinase, having two catalytic subunits: IKKα and IKKβ. These subunits can form either homo or heterodimers such as NF-κB inducing kinase, MAP kinase one which regulates the phosphorylation of IKKα and IKKβ [191]. Phosphorylation of IκB involves a multimeric complex of about 700 to 900-kD, ascribed as IκB kinase complex [192,193]. Reportedly, IKKβ is the foremost effector responsible for IκB phosphorylation in response to cytokines [194,195].

Phosphorylation of IκB is associated with the activation of NF-ƙB along with its subsequent translocation into the nucleus. PKR mediated overexpression of NF-ƙB causes transcription of many apoptosis-regulating genes such as first apoptotic signal ligand (FasL), p53, and cellular inhibitor of apoptosis (cIAPs) that are also found to be up-regulated [190]. Similar to PKR, the NF-ƙB transcription pathway is also activated by pro-inflammatory cytokines, chemokines, pathogens, viral products, dsRNA, and oxidative stress [196, 197]. Several studies have demonstrated that PKR is implicated in transcriptional stimulation due to the activation of NF-ƙB [191]. Several cellular genes are known to take part in the progression of the cell cycle; at the same time, they are also implicated in promoting cell death. Ideally, c-Myc is involved in cell proliferation in the presence of growth factors, appears to be associated with apoptosis under stress conditions [192]. C-Myc-mediated apoptosis has also been reported in the pancreatic cells of transgenic mice [193]. Induction of apoptosis by c-Myc is related with the stimulation of wide range of pro-apoptotic target genes, for example, Bax and FasL [192], perhaps C-Myc is also involved in the down-regulation of different survival proteins including Bcl-2 [194], However, an association of c-fos, an immediate-early response gene is well acknowledged in multiple stress stimuli that lead to apoptosis [195]. C-Myc-mediated apoptosis has

also been reported in the pancreatic cells of transgenic mice [193]. Most importantly, it has been reported that competitive inhibition of PKR by 2-aminopurine (selective PKR inhibitor) significantly inhibits the induction of c-myc and c-fos genes preventing programmed cell death [196]. Nakamura et al., has shown that mouse embryo fibroblasts (MEFs) derived from PKR knockout mice (PKR0/0) exhibits a significant reduction in IFN-β expression. However, MEFs derived from PKR+/+ mice, in which PKR was activated by interacting with dsRNA, showed a substantial increase in the expression of IFN-β [23].

2.8.4. PKR and apoptosis

The term apoptosis is derived from the Greek words "απο" and "πτωσιζ," which means falling off. Apoptosis was first described by Curie and her students in 1972, to elucidate the concept of programmed cell death and fragmentation of genomic DNA [197, 198]. During apoptosis, multiple morphological changes occur in apoptotic cells, distinguishing them from a normal/healthy cell. Cytoplasmic condensation, nuclear karyopyknosis, changes in cell shape, membrane blebbing, cytoskeletal disruption, and formation of apoptotic bodies are some of the characteristic features of an apoptotic cell. The newly formed apoptotic bodies are quickly phagocytized by the macrophages without initiating an immune response [199, 200]. Apoptosis is an essential event involved in the development and aging process, contributing a fundamental role in regulating homeostasis and the sustenance of tissue cell population. Apoptosis is often known to precede the defense mechanism in response to immune reactions or noxious stimuli [201-203]. Besides its functional role in regulation and maintenance of homeostasis, it is relevant to state that apoptosis can be triggered by a broad range of physiological and pathological stimuli causing cell death, although, it need not be essential that all the cells will respond equally and die on interacting with the similar stimulus. It has been quite explicit that apoptosis is a highly orchestrated cell death process, which plays an essential role in multiple pathological conditions that are linked with the alterations in the central nervous system and CV system [204, 205].

Over the past two decades, it has been assumed that molecular mechanisms leading to apoptosis entail intimate interaction between various apoptotic and pro-

apoptotic factors. Advancements in this field have led to the elucidation of specific mechanisms that are accountable for cell death. Remarkably, apoptotic mechanisms are solely dependent upon intrinsic (caspase-dependent/mitochondria-mediated) and extrinsic (death receptor-associated) pathways [206]. Moreover, some studies have reported that these two pathways are regulated by an effector caspase (caspase 3) for executing the morphological and biochemical modifications [207-209]. Binding of the death ligands such as FasL or TNF-α to the membrane-bound death receptors causes activation of the death receptor pathway. Activated death receptors often lead to the accumulation of the Fas-associated death domain (FADD). Several reports constellate that FADD has been a major contributor to the pathogenesis of CV disorders [204]. The role of PKR in inducing apoptosis signal cascade and programmed cell death in metabolic diseases is explored and defined by many researchers [204, 210, 211]. Fig.3. depicts the primary pathway involved in PKR mediated apoptosis.

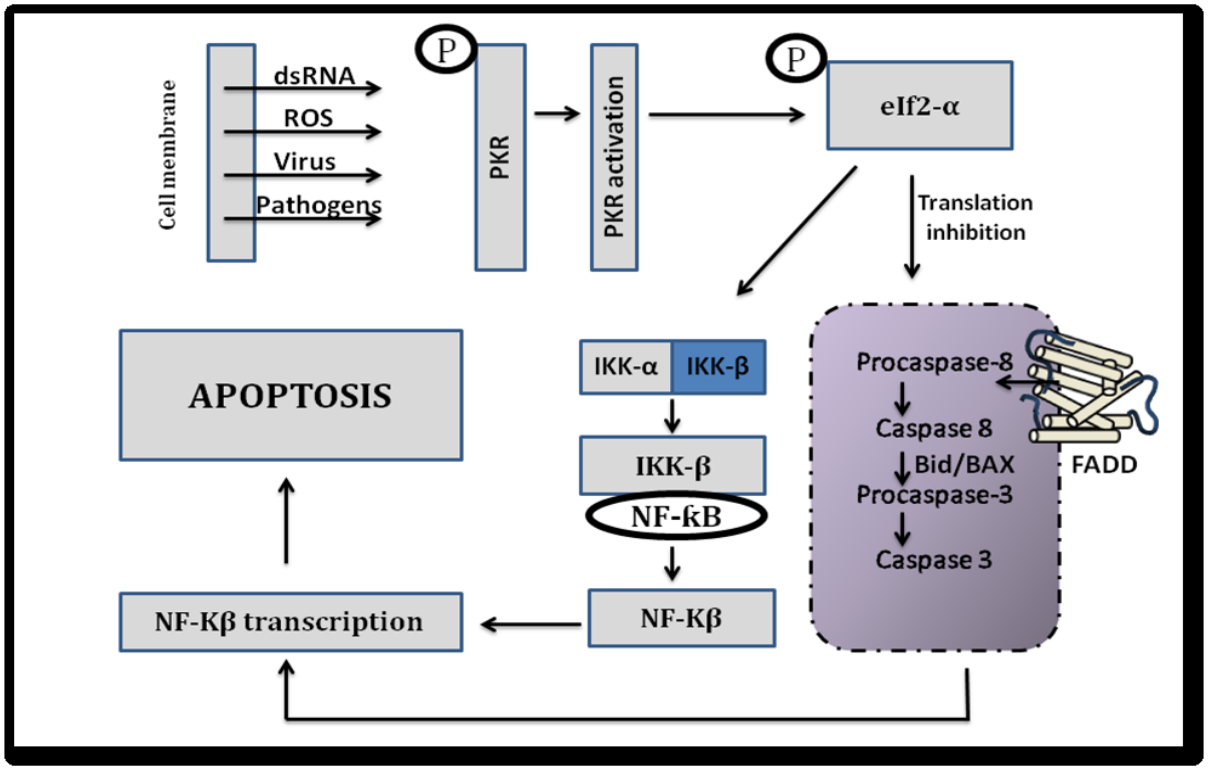


Fig.3. Primary pathway involved in PKR mediated apoptosis

It has been elucidated that the apoptosis-inducing property of PKR is attributed to its ability to activate NF-κB, having a critical role in controlling gene transcription [210, 211]. Reportedly, PKR causes up-regulation in Fas mRNA transcription. An

increase in Fas mRNA transcription leads to apoptotic cell death, which in turn confers the role of PKR in mediating apoptosis [212, 213]. Accordingly, it can be well understood that FADD is presumed in PKR-mediated apoptosis. PKR regulated apoptosis is dependent on the well-known death receptors, such as Fas, TNFR1, and DR3, that require FADD for their activation [214, 215]. In contrast, the study of Gil and Esteban states that none of these death receptors are involved in PKR induced apoptosis, and moreover, it is independent of Fas and TNFR1 death receptors.

PKR bears functional importance in the phosphorylation of three major MAPKs, such as (i) JNK, (ii) ERK, and (iii) p38, under ribotoxic stress conditions [21]. Although, the molecular mechanisms behind the interaction of PKR with MAPKs and hypothesis regarding its role, whether apoptotic or anti-apoptotic, remains unclear. Phosphorylation of MAPKs is attributed to the activation of NF-ƙB signalling through PKR interaction [216]. Results from the various disease models have concluded PKR as a significant regulator linked with the increased expression of the broad range of inflammatory markers while, PKR blockade can revert the increased expression of inflammatory markers with the substantial decrease in cytokine production in a variety of disease models [217, 218].

2.8.5. PKR Inhibitors

At present, approximately 80 serine/threonine inhibitors were identified using docking and statistical modeling. These 80 Identified inhibitors are classified into (indol-3 yl)pyrimidin-2-amines, pyrazolo[3,4-d]pyrimidines pyrimido[5,4-b]indoles, 6-heterocyclylamino indazoles, triazolo[3,4-f]pyridazines, and (3-methylene-5-oxo-pyrazolidin-4-ylidene) hydrazinosulfonamides. In addition to this, other classes include highly potent and specific PKR inhibitors such as indirubin three oximes, CAMK inhibitor, and analogs of JNK inhibitor. The identified compounds were screened for their inhibitory concentration (IC50) values using luciferase assay. Among all, 46 compounds with IC50 >100 µM, 20 compounds with 20<IC50 <100 µM, six compounds with <0.25 µM < IC50<20 µM and seven compounds with IC50 <0.1 µM were found. These identified compounds were known to be potent PKR inhibitors. However, for specific selectivity and efficacy, selective PKR inhibitor

imoxin (C16) has been used in the present study. 2-Aminopurine is another well known specific inhibitor available for PKR [219].

2.9. Gaps in existing research

2.9.1. What is already known?

For treating HTN, ACE inhibitors and ARBs are the first choices of drugs when used in combination with thiazide diuretics, CCBs, and beta-blockers, thus constituting a multidrug therapy whose regimen lasts for several months to years. However, several studies have reported that the use of diuretics, CCBs, and beta-blockers are linked with the progression and development of New-Onset Diabetes (NOD), dyslipidemia, impairment in glucose homeostasis and insulin resistance. Moreover, combining the statins for achieving the lipid-lowering effects is coupled with the deteriorating effects on glucose metabolism [220-223]. Thus, there exists a need for newer therapy, which can effectively combat all of these issues related to the use of current drug therapy.

2.9.2. What does this study add?

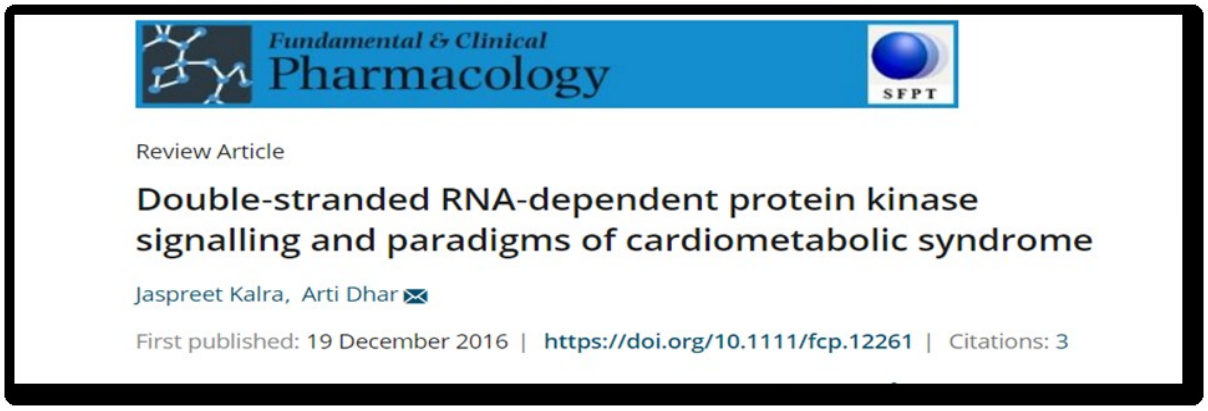
PKR has ubiquitously expressed serine/threonine-protein kinase, which is activated under several stress conditions, including metabolic abnormalities and oxidative stress [224]. It has been verified that systemic oxidative stress shares undeviating interdependence with the components of metabolic diseases such as dyslipidemia, insulin resistance and glucose intolerance. Moreover, similar kind of alterations has also been reported for HTN along with the activation of RAS, increased SNS activity, endothelial dysfunction [225]. Interestingly, researchers in our lab have successfully established the role of PKR in these metabolic abnormalities, and further its selective inhibition by small-molecule novel inhibitors is capable of reversing all these metabolic abnormalities and attenuating all the changes in serum lipid profile and glucose homeostasis in an animal model of diabetes. Moreover, we have also found that it is also capable of restoring structural and morphological changes in the heart, indicating its potential benefits [226, 227]. Therefore, an attempt has been made in the present study to establish the role of PKR in HTN. It has also been hypothesized that inhibition of PKR by its selective inhibitor imoxin (C16) could be one of the

potential therapeutic strategies to treat HTN with no risk of dyslipidemia, insulin resistance, and glucose intolerance.

2.9.3 Clinical significance?

The ultimate goal of any hypertensive therapy is to reduce overall mortality and reduce the global burden. HTN treatment follows multidrug therapy whose regimen lasts for several months to years. According to previous reports, older adults are more susceptible to HTN, and multidrug regimen may affect medication compliance, posing a severe threat to combating this disease [228-230]. If we succeed in our hypothesis, we propose that PKR targeted interventions in the near future may offer the synergistic benefit with multidrug therapy. Thus, targeting PKR may represent a new synergistic strategy to develop a novel approach for effective treatment strategies. With alarm increasing in the number of HTN and associated CV mortality, there is a need to explore a new approach to overcome this global burden. PKR is activated by diet, nutrient excess, pathogens, endoplasmic reticulum stress, inflammatory mediators, viruses, and interferon. But how does inflammatory pathways/stress factors contribute to hypertension? What type of mechanistic approach would be a connecting link in its pathology? Currently, these questions are poorly understood, presenting an urgency to elucidate the necessary mechanism underlying this pathological state.

Outcome: Review article out of literature survey



CHAPTER-3

RATIONALE, HYPOTHESIS, AND OBJECTIVES

3.1. Rationale

HTN is a progressive CV syndrome arising from complex and interrelated etiology. Early markers of the syndrome are often present before BP elevation sustains; therefore, HTN cannot be classified solely by discrete BP elevation [231]. Cardiometabolic disruptions such as insulin resistance, obesity, hyperglycemia, and dyslipidemia are known to increase the risk for high BP, including several CV and metabolic diseases such as T2D, and atherosclerosis [232]. The epidemic of HTN, diabetes, and obesity broadly attributes to an excess of caloric intake and a lack of adequate physical activity. Though numerous mechanisms have been put forward still the precise molecular mechanisms lying underneath are far from clear. Chronic inflammation is a feature of congestive heart failure (CHF) and can contribute to the development of numerous diseases, including HTN, atherosclerosis, insulin resistance, T2D, and cancer. Metabolic and immune signalling pathways are intimately linked, and understanding how nutrient excess promotes cellular inflammation is of considerable importance in understanding the pathogenesis of many chronic diseases [233]. Recently, PKR was identified as a core component of a "metabolic inflammasome" linking stress signalling to metabolic disorders [233]. PKR is a serine/threonine-protein kinase that is activated by dsRNA, cytokines, stress signals, interferons, and viral infection and it also plays a vital role in the nutrient/pathogen sensing interface. PKR acts as a critical modulator of chronic metabolic inflammation, insulin sensitivity, and glucose homeostasis [234]. Moreover, it is an essential regulator of cellular inflammation, proliferation, and apoptosis and may be involved in several CV diseases associated with chronic inflammation [233, 234]. Role of PKR in T2D and obesity has been well studied in numerous in-vitro, in-vivo, and gene knock out models [234, 133] however, till date, minimal research has been done to elucidate the role of PKR in HTN and associated target organ damage in kidney, aorta, and heart. Our laboratory has reported that H9C2 cardiomyocytes exhibit increased expression of PKR after exogenous exposure to HG, followed by increased production of ROS and gene markers of inflammation. The harmful effect of HG on cardiomyocytes was prevented by co-administration of indirubin 3-oxime, a PKR inhibitor in these cells, thus proving that HG-induced

pathological changes are linked to PKR activation [234]. PKR activation causes ROS production by reducing the expression of Bcl-2 and by increasing the Bax expression. The overexpression of Bax is responsible for apoptosis. PKR is reported to mediate apoptosis by multiple mechanisms, including interaction with Fas-associated death domain protein upregulation of the proapoptotic factor Bax, downregulation of Bcl-2, and by activation of the different caspases (caspase-8/caspase-3). Activation of PKR prevents the pro-proliferative effects of insulin growth factor-1 (IGF-1) by activating JNK and disrupting the IRS1/PI3K/Akt signalling pathway. Wang et al. reported that CHF patients contain more PKR expression in heart than in a healthy heart. There was a significant increase in myocardial expression and translocation of PKR in human patients and mice suffering from CHF [235].

Epidemiological data clearly demonstrates that hyperinsulinemia, insulin resistance, obesity, and HTN are interrelated processes [236, 237]. Further, Udumala et al. have reported that PKR gets activated in response to stressful environments such as nutrient excess and participate in mediating inflammation, insulin sensitivity, and maintenance of glucose homeostasis [234]. The aforementioned reports confirm the PKR expression in the heart, hence indicating its prospects as a potential target for HTN associated target organ damage. Like other cardiac abnormalities arising due to excess nutrients/stress or in response to inflammatory insult, HTN is also associated with kidney and heart hypertrophy, inflammation, changes in vasculature. These changes are connected through vicious pathways that promote the failure of compensatory mechanisms causing apoptosis [233]. Till present, the role of PKR in HTN and the underlying mechanism is not known. Therefore, in this study, we sought to investigate whether PKR plays a role in high fructose (HF)-induced hypertensive changes in kidney and aorta and to explore the mechanisms involved and its prevention by using novel inhibitors of PKR in-vitro. After mimicking the HTN conditions in in-vitro models, next, we aimed to for the confirmation of the role of PKR in HTN in in-vivo conditions. Experimental HTN was induced by L-NAME administration; the choice for this model is based upon the fact that it mimics the exact requirements of essential HTN, which are relatable to

humans. Therefore, PKR targeting will present a newer perspective for treating and combating this disease condition.

3.2. Hypothesis

It has been hypothesized that oxidative stress, inflammation, and HTN are interrelated with each other. In all the animal models of HTN, oxidative stress and chronic inflammation are allied with elevated arterial pressure. Amelioration of oxidative stress and chronic inflammation can remarkably produce a reduction in BP and may exert beneficial effects on the improvement of target organ damage and path-morphological alterations coupled with HTN.

(i) We predict that PKR activation has confounding influence in triggering the pathological changes associated with HTN. Exogenous exposure to HF, in-vitro/L-NAME, in-vivo will possibly lead to PKR activation. Activated PKR will mediate the inflammatory responses and may produce the pathogenic alterations which are responsible for the progression and development of HTN.

(ii) We assume that selective inhibition of PKR with imoxin (C16), an inflammation directed intervention will inhibit the activity of PKR and its harmful effect on kidney, aorta, and heart.

3.3. Objectives and experimental approach

The main goals of the proposed research are as follows:

- **To correlate the pathogenic process of HTN with changes in PKR expression in aorta/kidney and the related signal pathways:** As aforementioned, kidney and aorta are the primarily affected organs under the hypertensive condition. To correlate our hypothesis, firstly, we followed an in-vitro approach using cultures of renal epithelial/proximal tubular cells and primary VSMCs. We demonstrate significant upregulation of PKR, local inflammatory responses apoptosis along with substantial renal and vascular remodelling in HF treated cultures. In contrast, significant attenuation was observed on co-incubating the cells with imoxin/C16.
- **To investigate the mechanisms underlying the role of PKR in the development of HTN:** Based on the results obtained from cellular and molecular insights using an in-vitro approach. Next, we followed the in-vivo

approach using L-NAME model of experimental hypertension to investigate hypertension associated target/end-organ-damage in kidney, heart and aorta.

- **To investigate the potential of small molecule PKR inhibitors and examine their effectiveness in the prevention of development of HTN:** Effectiveness of imoxin in combating L-NAME induced pathological changes in kidney, aorta, and heart was determined by using non-invasive BP measurement, isometric tension studies, biochemical estimations, protein expression studies by western blot and immunohistochemistry. Pathomorphological alterations were assessed by using H&E staining in an in-vivo approach. We demonstrate significant up-regulation of PKR in kidney, aorta, and heart along with the significant increase in SBP, DBP, MAP, HR, and B.wt. activated RAS components along with a significant rise in pathomorphological disruptions and biochemical parameters in L-NAME administered rats, attenuation was observed with imoxin treatment along with concomitant administration of L-NAME.

CHAPTER-4

MATERIAL AND METHODS

4.1. Chemicals

PKR specific inhibitor C16 (imoxin, cat no: I9785), Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME hydrochloride; Cat no: N5751), Angiotensin-II Human (cat no: A9525), 4',6-diamidino-2-phenylindole (DAPI, cat no: D9542,), Bicinchonic acid assay kit (BCA, cat no: BCA-1), RIPA buffer (cat no: R0278), Protease inhibitor (cat no: P8340), Phenyl methyl sulfonyl fluoride (PMSF, cat no: P7626), luminol (cat no: A8511), p-Coumaric acid (cat no: C9008), 4',6-diamidino-2-phenylindole (DAPI, cat no: D9542,), 2,7 dichlorofluorescein diacetate (DCFDA, product no: D6883), Griess reagent (cat no: G4410), alizarin red S (cat no: A5533), rhodamine 6G (cat no: R4127), Eosin (cat no: E4009), and hematoxylin (cat no: 51275), poly-L-lysine solution (0.1 % (w/v) in H2O); cat no: P8920), thiazolyl Blue Tetrazolium Bromide (MTT, cat no: M 2128), 5-Bromo-2'-deoxyuridine (BrdU, cat no: B5002) were purchased from Sigma Aldrich Chemicals (St. Louis, MO, USA). Immun-Blot PVDF Membrane was purchased from Bio-Rad (Hercules, CA, USA). Confocal dishes (cat no: 100350) were procured from (SPL life sciences, USA). Collagenase Type II (cat no: TC212), elastase, (cat no: TC311), soybean trypsin inhibitor (cat no: TC251), fructose (cat no: DD017), Hanks balanced salt solution (HBSS, cat no: TL1010), Trypsin-EDTA Solution 1X (cat no: TCL007), Penicillin/Streptomycin, (cat no: A001), fetal bovine serum (FBS, Cat no: RM10432), Dulbecco's Modified Eagle's medium (DMEM, cat no: AL007A) and 40-micron cell strainers (cat no: TCP024), Trypsin-EDTA Solution 1X (cat no: TCL007), were procured from Himedia Laboratories (Mumbai, India). Dpx mountant media (Ref:Lamb/Dpx).

4.2. Antibodies and kits

Primary antibodies for protein kinase R (PKR; sc-708, lot no:G0215), anti-α-smooth-muscle actin (sc-32251, lot no: J0417), caspase-3 (sc-7148, lot no: K2410), JNK (sc-137018, lot no:K1809) p-JNK (G-7) (sc-6254, lot no:J1810), NF-Ķb (sc-8008, lot no:C2118), MMP-9 (sc-13520, lot no: C1318), ERK1/2 (sc-154302, lot no:H1018), p-ERK1/2 (sc-81492, lot no:A0918), BrdU (II-B5) (sc- 32323, lot no:D0418), Ang-II (H-12) (sc-374511, lot no:A0819), Renin (B-12) (sc-133145, lot no:E0319), eNOS (A-9) (sc-376751, lot no:I1517), TGF-β (sc-130348, lot no:D0219), p-CREB (sc-NLRP3 (sc-134306, lot no:JI617), TNF-α (52B83) (sc-52746, lot no:B1518), GAPDH (sc-32233, lot no: J0518)

and β-actin (sc-47778, lot no:H3016), goat anti-mouse IgG-HRP (sc-2005, lot no-G0215) were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA), AGEs; ab-23722 lot no:GR3215361-1(Abcam), anti-h-iNOS: Mab-9502; clone: 2D2-B2, lot no: CRF0817091, R&D, Minneapolis, MN 55413, USA), Rabbit polyclonal antibody for p-PKR Thr 451 (cat no: 44-668G, lot no: sc242370), all secondary antibodies (Texas red goat anti rabbit cat no: 2767, lot no:8639 and Alexa Flour 488 goat-anti mouse (cat no: A11001, lot no:948490) were procured from Invitrogen, Thermo fisher scientific (CA, USA). TUNEL assay kit (cat no:ab206386) and Masson's trichrome kit (cat no:ab150686) were purchased from Abcam (Milton, Cambridge, UK). Annexin V apoptosis kit was purchased from (INVITROGEN), Thermo Fischer Scientific (USA). Commercial kits for assessment of serum creatinine, blood urea nitrogen, (BUN), Auto span Liquid Gold LDG (Lactate Dehydrogenase, cat no: 74LS100-25, lot no: 4000017643), Total cholesterol (TC), Triglycerides (TG'S), High-density lipoproteins (HDL), Low-density lipoproteins (LDL), were purchased from Arkray Health care Pvt. Ltd, Surat, Gujrat, India.

4.3. Cell culture: in-vitro approach

Hypertensive disease state primarily affects kidney, aorta, and heart, including other organs which are less severely affected. Kidney and aorta are vital organs responsible for the maintenance of BP homeostasis. So based on the reported evidence, we have chosen rat kidney cultures of NRK-52E cells. Secondly, we opted for vascular smooth muscle cells (VSMCs) isolated from rat aorta for studying the role of PKR associated changes in-vitro in hypertensive conditions induced by HF.

Cell line: NRK-52E was obtained from National Centre for Cell Sciences (NCCS), Pune, India and were cultured on T25 flasks and on coverslips using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin antibiotic solution. Cultures were maintained in a humidified atmosphere of 95% air and 5% CO2 at 37℃. $5x10^6$ cells per ml cells were seeded in T25 flasks, 80% confluent cells up to 5-6 passages were used for further experiments. Before the start of treatment with imoxin/C16 (5μM) or in combination with HF (5mM), cells were maintained in FBS free DMEM for 12 h. Dose of C16 was selected on the basis of previously reported literature [238].

4.4. Isolation of vascular smooth muscle cells from vascular smooth muscle cells

For the isolation of primary VSMCs, we have used male Wistar rats approximately weighing 180-220 g, not older than eight weeks of age. For the experimental research ethical clearance for protocol no BITS-HYD/IAEC/2017/05 was granted by an Institutional Animal Ethics Committee (IAEC). Enzymatic digestion method was adopted for isolation of primary VSMCs from rat thoracic aorta. For every use enzyme solution was freshly prepared. Composed of enzyme solution is collagenase 10 mg and 10 mg soybean trypsin inhibitor, dissolved in 10 ml of Hanks Balanced Salt Solution (HBSS). To this solution~0.354 ml of elastase and 100µl of penicillin/streptomycin (1%) were added to make a final concentration of 0.744 units/ml. Prior to the start of isolation procedure all animals were anesthetized by using isoflurane and sacrificed immediately by cervical dislocation. Aseptic conditions were maintained throughout. Abdominal cavity was exposed by giving incision on the abdomen. Thorax was cut open and aorta was harvested. Harvested aortas were rapidly kept in ice cold HBSS. Extraneous and fatty tissue was gently scraped off using a scalpel blade and cleaned aortas were moved to the new petri dish with fresh HBSS. Thereafter, using 1ml syringe luminal walls of aortas were washed twice with incomplete DMEM. Following the wash with incomplete DMEM, inner side of aorta was exposed by making a longitudinal cut. Layer of endothelial cells was removed gently by rubbing the scalpel blade on the exposed side with the soft hand. Aortas were once again shifted to the fresh batch of HBSS and quickly chopped into small pieces of 3-4mm and transferred immediately to a T25 flask containing prewarmed enzyme solution at 37^{o}C. T25 flask was then positioned vertically in a humidified incubator maintained at 37^{o}C with a constant supply of 5% CO2. The process of enzymatic digestion can carried out for not more than four h. The flask was placed with the cap loosened for vigorous enzymatic digestion. To increase cell dissociation process gentle pipetting should be done after every one h. After four h of digestion process enzyme solution was collected in a 15 ml falcon tube was centrifuged. Resultant pellet was washed by centrifugation in HBSS, thereafter final pellet was again resuspended in 20% FBS supplemented DMEM. Depending on the visible dissociated cells, cell suspension was seeded in in 2-6 wells

of 48 well plates. Once the confluency is achieved, cells were passaged and cultured in six-well plates. In the present study maximum up to 6-8 passages of primary VSMCs were used for subsequent experiments. Cells were maintained in 2% FBS supplemented DMEM during the 24h treatment schedule. Treatment with HF (5mM) and PKR selective inhibitor C16) (5μM, based on reported literature) alone and in combination respectively was maintained for 24 h in cultured cells [238].

Two different inducing agents have been used for in-vitro as well as in-Consumption of HF is associated with the onset of CV complications including HTN. Therefore, HF was selected as a choice of inducing agent to study PKR up-regulation in NRK-52E and VSMCs. Moreover, HF induced hypertension has been well studied in animal model as well but in our in-vivo study, we have used L-NAME model for inducing HTN as we solely wanted to explore the endothelium dependent changes. L-NAME exerts its effect by inhibitionof eNOS. Generation of eNOS is related with the vaso-relaxing effects observed in hypertensive disease state. However, endothelium dependent changes are difficult to be studied in case of HF induced HTN as occurring endothelial damage is due to the other underlying causes.

4.5. Alpha smooth mucle actin staining and protein expression: characteristic hallmark of VSMCs

We performed immunoflorescence staining and Western blot to investigate the protein expression for alpha smooth mucle actin. For immunocytochemistry, cultures of VSMCs with a cell confluence of around 70-80% grown in a confocal dish were used. Media was discarded and cells were followed by washing with HBSS. Cell fixation was done with the help of 4% paraformaldehyde (PFA) for 15 min at normal room temperature. Cells were washed thrice with HBSS, and were subjected to permeabilization for 5 min by adding a mild detergent such as 0.1% Triton X-100 for dissolving the cell membrane. Blocking was done for 1h using 3% blocking solution prepared with the help of bovine serum albumin (BSA). Primary mouse monoclonal specific antibody (1:50 dilution) was added and kept for overnight incubation at 4^{o}C. The next morning, gentle washings three times for each sample

with HBSS were given and Alexa flour 488 conjugated secondary antibody (1:2000 dilution) was added and allowed to incubate for 2h at room temprature. Following three more washes, cells were stained with nucleus specific dye DAPI (1µg/ml) at room temperature for 10 min. Cells were washed twice and visualized under confocal laser-scanning microscopy (Leica DMi8, Leica Microsystems, Germany). Settings were kept constant throughout for three independent experimensts.

For protein expression studies, protein sample (40 µg) from control cells was loaded on the 8% SDS page. Wet transfer method using polyvinylidene difluoride (PVDF) membrane (Bio-Rad) was used for transferring the proteins. 5% non-fat milk in PBS-T was used for blocking. Mouse monoclonal anti-α-smooth-muscle actin specific primary antibody (1:1000 dilutions) was added onto the membranes and kept for overnight incubation at 4ºC. The following morning, the membrane was subjected to three washings with PBS-T for 15-20 min time for each wash. Thereafter, membrane was incubated with goat antimouse secondary antibody (1:2000 dilutio) for two h. After incubation with secondary antibody, three more washes with PBS-T with 15 min time cycle for each wash was given. Using Enhanced Chemiluminescence Reagent immunoreactivity to the specifc protein was detected [239].

4.6. Confocal Laser Scanning Microscopy

Immunocytochemistry was done according to the aforementioned method for identification of VSMCs. PBS was used for NRK-52E cultures, while HBSS was explicitly used for primary cultures of VSMCs.

4.7. Measurement of Reactive Oxygen Species (ROS)

DCFDA assay was performed with the help of flow cytometry for the measurement ROS production. For the assay, cells were seeded in six-well tissue culture plates with an approximate cell population of (4×10^5 cells/well). 70-80% confluent cells were then treated with HF (5mM) or with imoxin alone and in combination with HF in 2% heat inactivated FBS supplemented DMEM (in case of VSMCs) and 0.5% FBS supplemented DMEM for NRK-52E cells and kept aside for 24h in a humidified incubator maintained at 37ºC with 5% Co2 supply. Untreated cells were referred as

control. 24h post treatment, DMEM was discarded followed by three washes either with HBSS/PBS. Cells were maintained in PBS/HBSS and 2',7'-dichlorofluorescein diacetate (CM-H2DCFDA, 10 µmol/L), was added on the cells and placed in an humidified incubator at 37°C for 25 min. Membrane permeable dye 2',7'-dichlorofluorescein diacetate when loaded on the cells gets converted into 2',7'-dichlorofluorescein with the help of enzyme cytosolic esterases. Later on, this is then oxidized by reactive nitrogen species/ROS to form florescent dichlorofluorescein (DCF). Excitation wavelength of 488 nm was used to measure the fluorescent intensity of DCF using an Amnis FlowSight, flow cytometer (Amnis FlowSight, Millipore, USA). The data was analysed with the help of IDEAS software (version 6.0). Mean of three independent experiments (n=3) was used for the graphical representation plotted as a histogram.

4.8. Rhodamine 6g staining

Mitochondrial membrane potential was determined with the help of this stain. cell population of(4×10^5 cells/well) were seeded on the six-well tissue culture plates allowed to grow till 70-80% of confluence. Growth medium was discarded and cells were washed twice with HBSS. Cells were loaded with rhodamine 6G (100nm) and kept for incubation period of 30 min at 37°C. Images were observed and collected with the help of Leica DMi8, Leica Microsystems, Germany.

4.9. Estimation of peroxynitrites in cell supernatant

The amount of released nitrite/nitrate in cell supernatant is one of the markers for the production of nitric oxide. Nitrite/nitrate levels in cell supernatant were determined by a colorimetric assay using Griess reagent. An equivalent volume (100µl) of cell supernatant and Griess reagent were mixed gently in a 96 well plate, and the resulting mixture kept aside for 10 min at room temperature. The absorbance was recorded at λmax 540nm using Spectramax Plus 384, microplate reader (Molecular Devices, California, USA).

4.10. Cell proliferation essay

Cell proliferation was determined by the MTT assay. Briefly, 1×10^4 cells/well, were placed in sterile 96-well culture plates and incubated to 70-80 percent confluence. The cells were serum-starved for the next 24h after that, followed by incubation with HF (5mM) alone and in combination with imoxin, (5µM), for 24 h. Untreated cells

served as control. The next morning, the medium was discarded and 200 µl of MTT solution (5 mg/mL in PBS) was added in each well and kept aside for an incubation period of 4 h. After incubation, the MTT solution was discarded and 200 µL of dimethyl sulfoxide (DMSO) was added in each well. Absorbance was recorded by a microplate reader at λmax of 570 nm and 620 nm using UV−visible spectroscopy (Spectramax Plus 384, Molecular Devices, California, USA).

4.11. 5-Bromo-deoxyuridine (BrdU) assay

$3x10^4$ cells/well were seeded on confocal dishes, and the following morning, cells were treated with HF alone or in combination with imoxin for 24 h. On the next day, cells were incubated with BrdU (10µg/ml) for 24 h at 37°C. After the incubation period, cells were subjected to fixation and permeabilization, followed by treatment with 2N HCl at 37°C for 30 min. Blocking was done using 3% BSA, and the samples were kept for overnight incubation with anti-BrdU antibody (1:200 titer) at 4°C and an Alexa flour 488-anti-mouse antibody (1:2000 titer) for one h at room temperature. Images were captured using confocal imaging (Leica DMi8, Leica Microsystems, Germany).

4.12. Scratch test for Migratory behaviour

The cells (4×10^5 cells/well) were allowed to grow until confluence in six-well tissue culture plates. Cells were serum-starved for 24 h with DMEM supplemented with 2% FBS. Then, a scratch was made with 200µl tip, and bright-field images were captured at 0 h after those cells were treated with different groups either alone with HF or in combination with C16 for 24 h and again images were captured using Leica microscope.

4.13. Assessment of cellular hypertrophy by hematoxylin and Eosin (H & E) staining

Briefly, cells were grown in sterile six-well tissue culture plates until they became confluent. Cultured cells were incubated with HF alone, and in combination with imoxin (C16) for 24 h, untreated cells served as control. The following morning, the growth medium was discarded; cells were washed thrice with HBSS and fixed with 4% paraformaldehyde for 15 min at room temperature. The cells were then treated with two changes of 95% ethanol for 3 min before being stained with 0.1% Mayers Haematoxylin for 1 min and rinsed in running distilled water for 5 min. The cells

were then stained with Eosin for 30 sec and washed with distilled water. After washing, the cells were dehydrated in increasing concentration (50%, 70%, and 95%) of ethanol, 30 sec for each cycle. Stained cells were visualized by bright field microscopy using an inverted microscope (Carl Zeiss microscopy, Germany).

4.14. lactate dehydrogenase activity

Lactate dehydrogenase (LDH) is considered as one of the crucial markers for cytotoxicity. LDH activity was measured in cell supernatants of different treatment groups. LDH activity was noted as a mean change in absorbance at λmax 340nm using commercially available AUTOSPAN, Liquid Gold LDH, Optimised DGKC, Kinetic Assay. Absorbance change was pointed out with the help of a microplate reader. Spectramax Plus 384 (Molecular Devices, California, USA).

4.15. DAPI staining and agarose gel electrophoresis for assessment of chromatin condensation

VSMCs ($3x10^4$ cells/well) were grown in 35 mm confocal dishes and incubated with different groups for 24 h in serum-free DMEM. Untreated cells served as control. After that, the growth medium was discarded, and the cells were washed with HBSS thrice. They were then fixed with four %PFA for 15 min and permeabilized with 0.1% Triton X 100 for 5 min at room temperature. The cells were further incubated with DAPI (1µg/ml) for 10 min in the dark at room temperature, washed with HBSS, and visualized under the confocal microscope (Leica DMi8, Leica Microsystems, Germany). For agarose gel electrophoresis, 1µg DNA samples were loaded on 1% agarose gel and ran at 70 V for 25 min. The gel was visualized using a molecular imager, gel doc XR+ imaging system, BioRad.

4.16. Annexin V/fluorescein isothiocyanate (FITC) /propidium iodide (PI)staining

Annexin V and PI double staining were carried out to differentiate the normal cells from apoptotic cells. Cultured NRK-52E cells were incubated alone or in combination with different treatment groups for 24 h; cells were trypsinized and harvested under cool conditions. An obtained pellet for each sample was resuspended in 500µl AnnexinV conjugate/binding buffer mixture, which was

prepared by diluting Annexin V–FITC conjugate (INVITROGEN), 1 in 100 in Annexin V binding buffer. Mixtures were placed in the dark at room temperature or 20 min. Later on100µl of 50µg/ml propidium iodide (PI) solution was added to each sample mixture, and the final sample mixtures were immediately transferred onto the ice. Apoptotic cell percentage was detected by using the Amnis imaging flow cytometer, and results were quantified by using IDEAS software.

4.17. Vascular calcification :Alizarin red staining

4×10^5 cells/well were briefly seeded in sterile six-well culture tissue plate and maintained until they became 70-80% confluent. Thereafter, cells were incubated with HF alone, and in combination with imoxin (C16) for 24 h, untreated cells served as control. After incubation, the cells were washed thrice with HBSS and subjected to fixation with 4% paraformaldehyde (PFA) at room temperature for 15 min, followed by three washings with HBSS. Excess HBSS was drained off, and 1 ml of 1 % alizarin red solution (ARS) in 10% ammonium hydroxide was added into each well and incubated for 40 min at room temperature with gentle shaking. ARS was aspirated, and the cells were washed with distilled water (5 times). Stained cells were visualized by bright field microscopy using an inverted microscope (Carl Zeiss microscopy, Germany).

4.10 Western blotting for PKR and its downstream markers

For Western blotting, 40 µg of a protein sample of untreated cells was loaded on an 8%-10% SDS page and subjected to transfer on to a PVDF membrane (Bio-Rad). Following a transfer, the membrane was blocked with 5% skimmed milk in PBS-T (for phosphorylated proteins 3% BSA in PBS-Twas used forblocking) for one h at room temperature and incubated overnight with primary antibodies (1:1000 titer) at 4^oC. The next morning, the membrane was washed thrice with PBST, 15 min for each period, and incubated with HRP conjugated secondary antibody for two h. After incubation, the membrane was washed thrice again, and immunoreactivity to the desired protein was detected with the help of an ECL Reagent.

4.18. In-vivo approach

4.18.1. Experimental animals

Thirty-two, eight weeks old male Wistar rats weighing 180-230 g were used in the present study. Animals were procured from the registered Committee for the purpose of contrl and Supervision of Experiments on Animals (CPCSEA) breeders. Animals were housed in a group of three in polypropylene cages. They were maintained following the recommended laboratory conditions; temperature (22±1 °C), relative humidity (55-60%) with 12 h light/dark cycle having excess to food and water ad libitum. Before the start of the experiment, animals were acclimatized to laboratory conditions for one week. Recordings for non-invasive BP (NIBP) were taken between 9:00 and 17:00 h. The experimental protocol for the present study was approved by an IAEC, bearing a protocol no BITS-HYD/IAEC/2017/06. All the experimental procedures are carried out following the guidelines issued by the Indian National Science Academy (INSA) for the use of experimental animals.

4.18.2. Induction of Hypertension and Experimental protocol

Experimental HTN in Wistar rats was induced by the administration of L-NAME (40 mg/kg b.w, p.o) for four weeks [240]. A selective PKR inhibitor (Imoxin/C16) (0.5mg/kg, i.p) was administered for four weeks. Animals were randomly allocated into the following four groups with n=8 rats in each group: Group I: untreated, served as control (CON), Group II: L-NAME (40mg/kg, orally), Group III: L-NAME+C16 (the same dose of L-NAME was administered with concomitant administration of C16 (0.5mg/kg, i.p), Group IV: C16 only (0.5mg/kg, i.p).

4.18.3. Body weight , non invasive Blood pressure (BP) and heart rate (HR)measurement

B.wt, systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) and HR were measured in conscious rats on day 0, day 7th, day 14th, day 21st, and day 28th for each rat in an individual group. Before the actual BP measurement, rats were habituated to the rat restrainer for four days. Rats were positioned in a restrainer; tail occlusion cuff was placed on a tail, and rats were permitted to acclimatize for 10 min. After that, readings for SBP, DBP, MAP, and HR were noted for ten consecutive measurements using the MRBP system (IITC Life

Science, Woodland Hills, CA). The mean of six measurements for each animal having approximately the same/nearby values was taken for the graphical presentation.

4.19.4 Blood sampling and Biochemical measurements

After BP measurement on the following days: day 0 (for determination of basal values), and day 28th (for determination of endpoint values), blood samples were withdrawn and collected from the rat orbital sinus, allowed to clot for 30 min. After that, samples were subjected to centrifugation at 3000 rpm for 15 min; serum supernatant was collected and stored in multiple aliquots at –80^{o}c for biochemical estimations. On the 28th day, animals in each group were sacrificed. Kidney, heart, and aorta from animals of each group were harvested and stored at -80^{o}c for further experiments.

4.19.5. Ex-vivo isometric tension on rat aortic rings

Isometric tension studies will be carried out on rat aortic rings according to the previously described method [241]. Briefly, the thoracic aorta from a rat will be cut into rings 3-4 mm in length and mounted in 10 mL organ baths containing Krebs solution (in mM: NaCl 118, KCl 4.7, MgSO4 1.2, KH2PO4 1.2, NaHCO3 22, glucose 5, CaCl2 2.5), maintained at 37° C and bubbled with 95%O2/5%CO2). Aortic rings will be equilibrated for up to 60 min at a resting tension of 2 g. Tissues will be contracted with a sub-maximal dose of Phenylephrine (PhE) (0.3 μM). At the plateau phase of contraction, the cumulative concentration-response curves (CRC) of acetylcholine (Ach) will be built. Endothelium was kept functionally undamaged in most experiments unless otherwise specified, which was confirmed by the Ach (1 μM)-induced vascular tissue relaxation. Four to six rings will be mounted at the same time from a single rat. Isometric tension will be recorded with isometric force transducers using the 'Chart' software and Power Lab equipment (AD Instruments Pvt. Ltd., India).

4.19.6. Biochemical estimations

Serum creatinine, blood urea nitrogen (BUN), triglycerides (TGs), high-density lipoprotein (HDL), total cholesterol (TC), nitrite/nitrate (peroxynitrites), intracellular

calcium were measured by commercially available kits as per the manufacturer's instructions.

4.19.7. Determination of Kidney and heart weight :a preliminary indicator for assessing hypertrophy

Following sacrifice, both the kidneys and heart were isolated and harvested. Kidneys were de-capsulated, washed twice with saline. After that, kidney and heart were dried on gauze and weighed to determine the kidney/heart to body weight ratio as a measure of kidney/heart hypertrophy.

4.19.8. Western blot of PKR and its downstream markers

Kidney samples were homogenized in Radioimmunoprecipitation assay buffer (RIPA) cocktail buffer, composed of RIPA buffer (1ml)+protease inhibitor (PI, 10 µl) + phenyl methyl sulfonyl fluoride (PMSF, 10 µl), sample tubes were kept in ice and incubated for 1h with constant agitation. After that, tissue homogenates were centrifuged for 15 min at 10,000 rpm, and the supernatant was collected obtained protein samples were quantified by using the BCA method. On 8-12% of the SDS page, 40 µg of protein samples were loaded allowed to separate based on molecular weight. Protein samples were transferred on to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Membranes were blocked with 5% non-fat milk (3% BSA was used for blocking of phosphorylated proteins) for 1h at room temperature. Membranes were incubated overnight with the primary antibody (1:1000 titer) at 4^{o}C. The next morning, the membrane was washed thrice with PBS-T, 15 min for each period, and incubated with horseradish peroxidase (HRP) conjugated secondary antibody (1:2000 titer) for two h. After incubation, the membrane was washed thrice again, and immunoreactivity to desired protein was detected with the help of an Enhanced Chemiluminescence Reagent (composed of ECL reagent: 10ml (0.1 M Tris, pH 8.6; 55µl luminol (250 mM); 25µl p-Coumaric acid (90 mM); 3µl H_2O_2 30% (w/v). Markers for inflammation and vasoconstriction: Ang-II, Renin, NF-ķB, JNK, p-JNK, TNF-α, and NLRP3. Markers for fibrosis, phenotypic transition, and apoptosis: TGF-β, α-smooth muscle actin (α-SMA) ERK1/2, and caspase 3. For western blot, 1:1000 titer was used for all primary antibodies, and 1:2000 titer was used for secondary antibody dilution.

4.19.9. Immunohistochemistry for PKR and its downstream markers

Kidney/heart and aorta tissues embedded in optimal cutting temperature (OCT) medium were used for immunohistochemistry. 4µm sections were cut onto the glass slides and stored at 4^{o}c for overnight (samples can also be stored at 4^{o}c for one week). Before the start of the procedural step, slides were incubated at room temperature for 30 min, fixed in ice-cold acetone for 5 min at -20^{o}c air-dried the slides for 30 min at room temperature. After that, slides were rinsed twice with PBS, 2 min for each wash. Blocking was done at room temperature for 1 hr using 3% BSA. Tissue sections were incubated overnight with primary antibody (1:200 titer) washed thrice with PBS. Fluorescent labeled secondary antibody (1:2000 titer) was added, and sections were incubated at room temperature for two h. After that, slides were washed thrice, counter-stained with DAPI for 10 min at room temperature, and washed with PBS-T. Coverslip was mounted. Images were captured at 20X magnification using confocal laser-scanning microscopy. For AGEs, images were captured at 10X magnification using confocal laser-scanning microscopy (Leica DMi8, Leica Microsystems, Germany).

4.19.10. Histological examination

After fixation, 4-5 µm sections of paraffin-embedded kidney/heart tissues were cut down onto the glass slides and dried overnight in a hot air oven at 60^{o}c. Slides were washed in 3 changes of xylene for 10 min each followed by two changes in absolute alcohol. After that, the slides were washed twice with distilled water. Next, tissue sections were incubated with Hematoxylin stain for 3-5 min washed thrice with distilled water and after that washed with acid alcohol (70% ethanol in 1%HCl) for 2-3 min. Again slides were rinsed with distilled water and stained with Eosin for 30 s followed by subsequent washes with distilled water. Lastly, slides were rinsed with two changes of absolute alcohol and mounted with the help of Dpx mountant media. Images were captured at 40X; sections were visualized by bright field microscopy using an upright microscope (Carl Zeiss microscopy, Germany).

4.19.11. Sirius red and Masson's trichrome staining for assessment of fibrosis and collagen deposition

4-5µm kidney/heart sections were cut on to the slides and are dried in a hot air oven at 55^{o}c for overnight. The next morning, slides were deparaffinized with xylene, hydrated by two consecutive washes with distilled water. For Sirius red staining, 200 µl of picro-Sirius stain was applied onto each section, kept for incubation at room temperature for one h. After that, slides were rinsed in two changes of 0.5% acetic acid solution and washed twice with distilled water. Finally, slides were washed in two changes of absolute alcohol and mounted with the help of Dpx mounting media. Masson's trichrome staining was carried out in accordance with the protocol suggested by the manufacturer. Blue coloration represents the collagen deposition in the tissue section. Images were captured at 40X and visualized by bright field microscopy using an upright microscope (Carl Zeiss microscopy, Germany).

4.19.12. In situ detection of DNA strand breaks: TUNEL assay for tubular cell apoptosis

To quantitate nuclei with fragmented DNA, the deoxynucleotidyl transferase (TUNEL) assay was performed according to the manufacturer's instruction. Briefly, paraffin-embedded sections (4µm) were cut onto a glass slide. Sections were rehydrated with dips of xylene followed by changes in decreasing concentration of ethanol. After that, sections were permeabilized with proteinase K, and quenching was done for endogenous peroxidases. Samples were equilibrated, labeled with deoxynucleotidyl transferase (TdT) enzyme for 1.5 h at room temperature. The reaction was ended by adding stop buffer, and slides were washed with tris buffer saline (TBS) followed by blocking. After that, slides were developed by adding working diaminobenzidine (DAB) solution. Finally, the slides were counter-stained with methyl green and incubated at room temperature for 1-3 min. Slides were dipped in two changes of 100% ethanol, and coverslips were mounted. Images were captured at 40X magnification using the Zeiss microscope (Carl Zeiss microscopy, Germany).

4.19.13. Alizarin Red Staining (ARS): For assessment of nephrocalcification

As abovementioned, paraffin-embedded kidney sections (4µm) cut onto a glass slide. Sections rehydrated with dips of xylene followed by changes in decreasing concentration of ethanol. After that, slides were stained with 1% ARS for 4-5 min excess dye was tapped off and washed with distilled water. Slides were dehydrated with 5-6 dips of acetone and clear in xylene and mounted with the Dpx mountant media. Images were captured at 10X magnification, and sections were visualized by bright field microscopy using an upright microscope (Carl Zeiss microscopy, Germany).

CHAPTER-5

RESULTS

5.1. Statistical analysis

Data obtained from independent experiments are expressed as mean±SD/SEM. Statistical analysis was performed using Two-way ANOVA or either by one-way ANOVA followed by Bonferroni's post hoc test. P value of less than 0.05 was considered to be statistically significant.

5.2. IMOXIN ATTENUATES HIGH FRUCTOSE INDUCED OXIDATIVE STRESS AND APOPTOSIS IN RENAL EPITHELIAL CELL VIA DOWN REGULATION OF PKR PATHWAY

Diabetes and CV disorders are health problems of epidemic proportions worldwide. More than half of those with diabetes also develop CV complications. HTN is frequently linked with diabetes. Evidently, almost 60% of diabetic patients are hypertensive, and approximately 20% of individuals with HTN are diabetic [242]. Several lines of evidence indicate that oxidative stress and inflammation are interrelated in the pathogenesis of HTN [243]. Interestingly, significant increase in the renal interstitial inflammation and level of oxidative stress markers has been observed in the spontaneously hypertensive rats (SHRs), which clearly indicates the possible role of oxidative insult and inflammation in the development and progression of HTN [244]. Recently, reports suggest that inflammation in the renal proximal tubules of rat kidney is one of the major causes involved in the development of HTN [245, 246]. Studies of Wu and colleagues have shown that activation of inflammatory signalling molecules JNK and IKK are the major contributors in the development of HTN in Sprague dawley rats [247]. Thus, inhibition of these inflammatory pathways confers the protection against several destructive events which play an important role in the development of HTN [248-251].

PKR is a ubiquitously expressed serine/threonine protein kinase [252], which can be activated by excessive nutrient intake and numerous forms of stress signals such as mechanical stress, metabolic stress, oxidative insult and cytokines [253-257]. It has been reported that activation of PKR via oxidative stress is related with the induction of two intracellular inflammatory kinases, JNK and IKK which are involved in the modulation of metabolic homeostasis and insulin resistance [258].

Remarkably, PKR activation also exerts the negative influence on insulin signalling pathways abnormalities in adipocytes and chronic metabolic inflammation [259]. Moreover, PKR activation is also linked with the excessive intake of certain nutrients such as palmitic acid and HG/HF playing a crucial role in the development of insulin resistance, glucose intolerance and metabolic inflammation [260]. HF treated rats is a well-established model of HTN. Prevailing literature supports the fact that HTN is coupled with oxidative stress and inflammation, together with insulin resistance, glucose intolerance, and metabolic inflammation which altogether contributes in the tubulointerstitium inflammation of rat kidney [261]. Notably, all of these adverse effects such as glucose intolerance, insulin resistance, and metabolic inflammation are also linked with the PKR activation. Moreover these effects were significantly inhibited either by gene silencing or PKR knockdown [254, 260, 262], presenting a newer therapeutic option for treating diabetes and its associated CV complications including HTN.

Although several treatment strategies are available which are aimed to act via inhibiting the increased oxidative burden and inflammatory markers, but associated side effects and poor efficacy have limited their potential for use. Hence, there exists a significant urge to develop safer therapeutic agents whose long term use is related with maximal efficacy and minimal side effects. Till date numerous studies are being carried out to elucidate the underlying mechanism in the development of HTN and its effect on kidney, but the molecular and cellular linkage of PKR in HTN is not yet reported. PKR is known to be activated by excess nutrients such as high fructose (HF), which is a known model for HTN. The role of PKR in cultures of renal tubular epithelial cells NRK 52E is still not investigated. Aim of the present study is to investigate the effect of HF on PKR pathway and underlying molecular mechanism in renal tubular kidney cells.

Cell culture

Normal rat kidney epithelial cell line (NRK-52E) was obtained from National Centre for Cell Sciences, Pune, India and were cultured on T25 flasks and on cover slips using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with containing 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin antibiotic solution.

Cultures were maintained in a humidified atmosphere of 95% air and 5% CO2 at 37℃. 5x10^6 cells per ml were seeded in T25 flasks, upto 80% confluent cells upto 5-6 passages were used. Before the start of treatment alone selective PKR inhibitor imoxin (5µM) or in combination with HF (5mM), cells were maintained in FBS free DMEM for 24 h.

RESULTS

Effect of imoxin on PKR expression in HF treated NRK 52E cells

To investigate effect of HF on PKR we performed immunofluorescence staining and western blotting quantified by Image J software. Incubation of cultured NRK 52E cells with HF for 24 h significantly increased PKR protein expression as demonstrated by immunocytochemistry and western blotting (Fig.5.2.1.A. and 5.2.1.B.). The effect of HF was significantly attenuated by imoxin co-incubated with HF group.

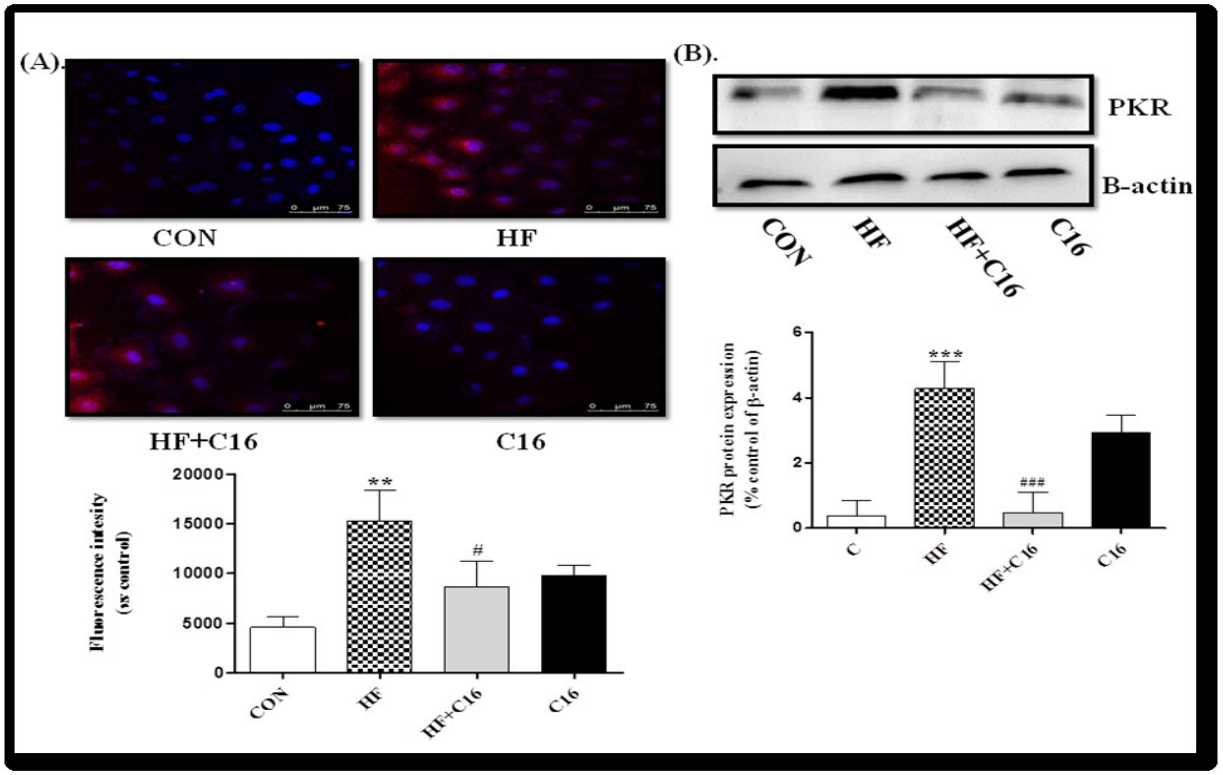


Fig.5.2.1. PKR expression is increased in HF treated renal NRK 52E epithelial cells, attenuation by imoxin: Cultured renal NRK 52E epithelial cells were incubated with normal culture medium (CON) or medium containing fructose (HF; 5 mM) for 24 h. Imoxin (5 µM) was incubated alone or with HF for 24 h. PKR expression was measured by immunofluorescence staining (A) and western blot (B). n=4 for each treatment. **P<0.01 vs. respective control (CON), #P<0.05 vs. respective HF treated group.

Effect of imoxin on caspase-3 expression in HF treated NRK 52E cells

To investigate the effect of HF on caspase-3 we performed immunofluorescence staining and western blotting, quantified by Image J software. Incubation of cultured NRK 52E cells with HF for 24 h significantly increased caspase-3 protein expression as demonstrated by immunocytochemistry and western blotting (Fig.5.2.2.A. and 5.2.2.B.). The effect of HF was significantly attenuated by imoxin co-incubated with HF group

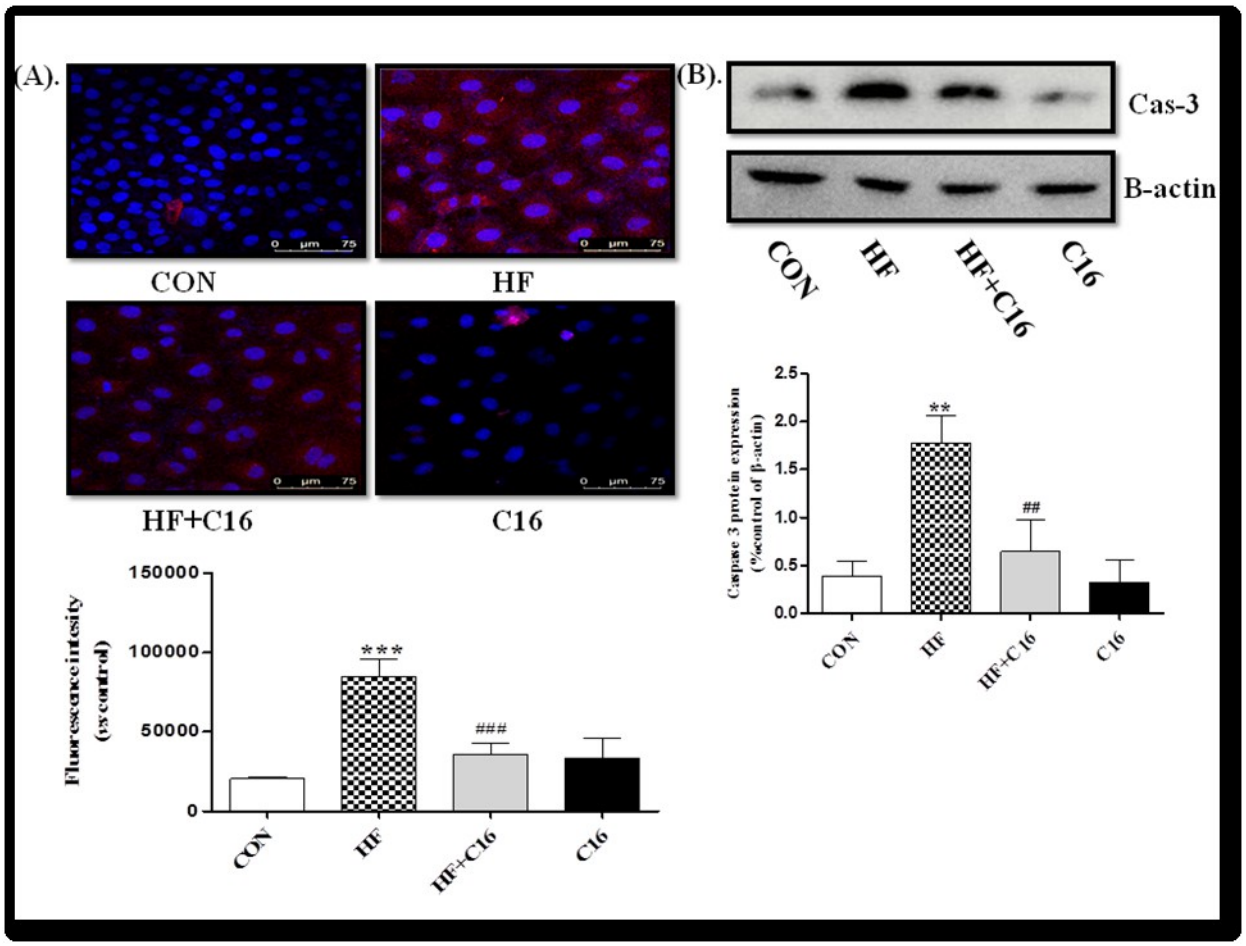


Fig.5.2.2. Caspase-3 expression is increased in HF treated renal NRK 52E epithelial cells, attenuation by imoxin: Cultured renal NRK 52E epithelial cells were incubated with normal culture medium (CON) or medium containing fructose (HF; 5 mM) for 24 h. Imoxin (5 μM) was incubated alone or with HF for 24 h. Caspase-3 expression was measured by immunofluorescence staining (Fig.4.1.2.A.), and western blotting (Fig.4.1.2.B.). n=4 for each treatment. **P<0.01 vs. respective control (CON), #P<0.05 vs. respective HF treated group.

Effect of imoxin on intracellular production of reactive oxygen species (ROS) and JNK expression in HF treated NRK 52E cells

Incubation of cultured NRK 52E with HF (5 mM) for 24 h significantly increased ROS production and JNK expression whereas the ROS production and JNK expression was significantly attenuated by imoxin (5 μM) co-incubated with HF.

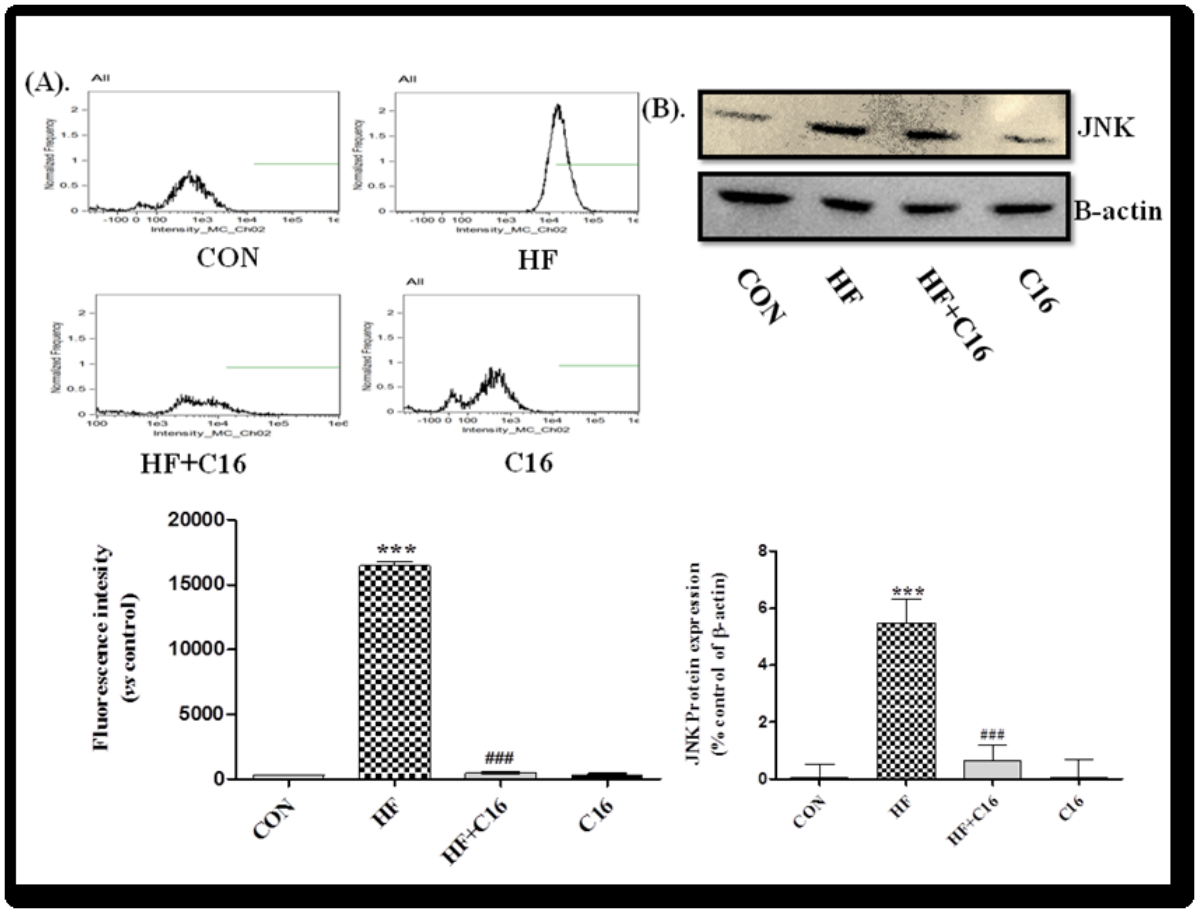


Fig.5.2.3. Increased reactive oxygen species (ROS) production and JNK expression in cultured renal NRK 52E epithelial cells, attenuation by imoxin: Cultured renal NRK 52E epithelial cells were incubated with normal culture medium (CON) or medium containing fructose (5 mM) for 24 h. Imoxin (5 μM) was incubated alone or with HF for 24 h. Fig.5.2.3.A. ROS production was measured by flow cytometry n=6 for each treatment. Fig.5.2.3.B. JNK expression was determined by western blotting, n=3. ***P<0.001 vs. respective control (CON), ###P<0.001 vs. respective HF treated group.

Effect of imoxin on annexin V FITC/PI binding assay in HF treated NRK 52E cells

Annexin V- FITC/PI assay was carried out to determine the apoptotic cell death in NRK 52E cells. In this method, each cell was determined individually by using flow cytometer. Apoptotic cell density was significantly increased in HF treated cells and

most of the cells were in early apoptotic phase (live cells: 18.12%, early apoptotic: 65.57%, late apoptotic: 15.61%) compared to the control group in which maximum cells were live and intact (live cells: 81.99%, early apoptotic: 3.1%, late apoptotic: 0%). Further on treatment with C16 showed significant improvement in the apoptosis rate (live cells: 26.47%, early apoptotic: 52.1%, late apoptotic: 12.16%) in C16 treated group co-incubated with HF.

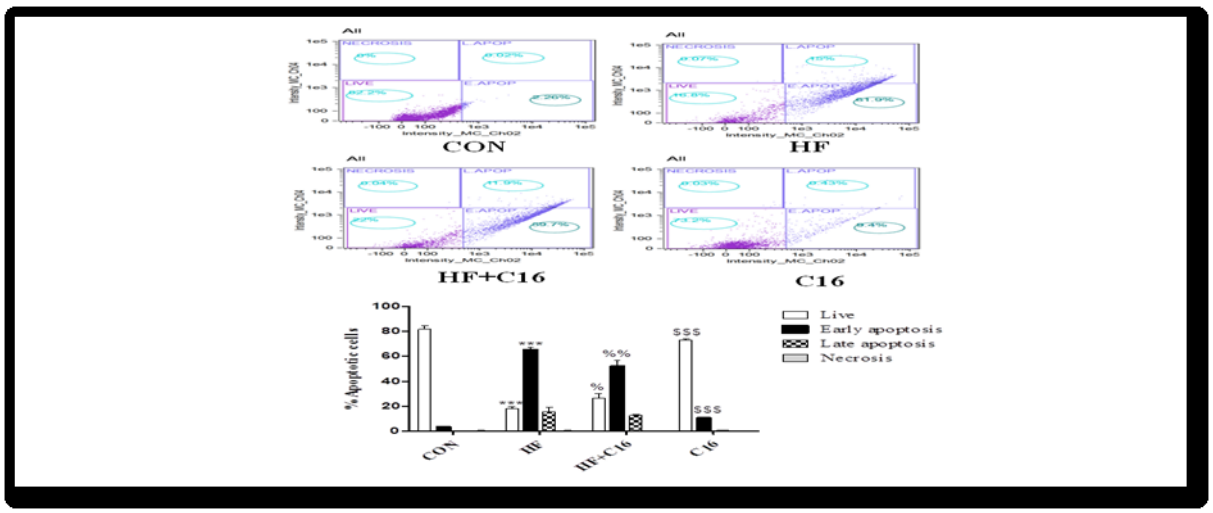


Fig.5.2.4. Exogenous HF induces apoptosis in cultured renal NRK 52E epithelial cells, attenuation by imoxin: Cultured renal NRK52-E epithelial cells were incubated with normal culture medium (CON) or medium containing fructose (5 mM) for 24 h. Imoxin (5 µM) was incubated alone or with HF for 24 h. Apoptosis was measured by FACS analysis using Annexin-IV assay kit. n=6 for each treatment. **P<0.01, ***P<0.001vs respective control (CON),%P<0.05,%%P<0.01vs respective HF group. (Graphical data has been plotted against the mean values obtained from three independent experiments while the Fluorescence-activated cell sorting (FACS) image for apoptosis is represented by data obtained from single experiment).

DISCUSSION

Diabetic and CV disorders are major health problems worldwide. Profound knowledge and understanding of the underlying molecular and cellular associations of these disorders may contribute to the discovery and development of novel drug candidates for the prevention and treatment of these diseases. In the present study, we have investigated the beneficial role of selective PKR inhibitor imoxin (C16) against HF induced increased PKR expression, oxidative damage, and apoptosis in cultured renal epithelial cells (NRK 52E). In in-vivo and in-vitro studies, activation of PKR has been linked with the initiation of several pathological events, such as

insulin resistance, glucose intolerance, and increased expression of inflammatory and oxidative stress markers in diabetes and CV complications [11]. PKR knockdown has been reported to attenuate and inhibit the above mentioned harmful effects [13]. In the present study, we report for the first time increased PKR activation in cultured renal epithelial cells after exposure to HF and attenuation of PKR activation with selective PKR inhibitor, imoxin (C16) [Fig.5.2.1.]. We also report that pharmacological inhibition of PKR by imoxin (C16) attenuates HF induced ROS production [Fig.5.2.3]. In the FACS assay, we found that inhibition of PKR has significantly reduced the increased generation of ROS in HF treated cultured renal epithelial cells. Interestingly, a significant reduction in inflammatory and apoptotic markers has also been observed in HF treated cultured renal epithelial cells co-incubated with imoxin (C16) [Fig.5.2.2., and Fig.5.2.3.]. HF is often linked with oxidative stress [263, 264], and in the present study, we have demonstrated that HF increases ROS generation in renal epithelial cells via activation of PKR pathway.

Previous studies have reported HF fed SD rats to exhibit increased vascular inflammation,which is attributed to increased expression of NF-Ƙb. The NF-Ƙb expression is correlated with increased oxidative stress and ROS production [265]. Moreover, PKR is reported to be involved in the regulation and maintenance of several cellular pathways, including NF-Ƙb. Further, it has been elucidated that PKR activates NF-Ƙb and PKR- - knockout selectively inhibits activation of NF-Ƙb [266] Thus, it can be postulated that inhibition of PKR will possibly contribute to the inhibition of numerous destructive pathways associated with increased ROS generation. In our study, we also found that imoxin (C16) inhibits the HF induced ROS production, indicating the functional role of PKR in diabetes-related vascular complications.

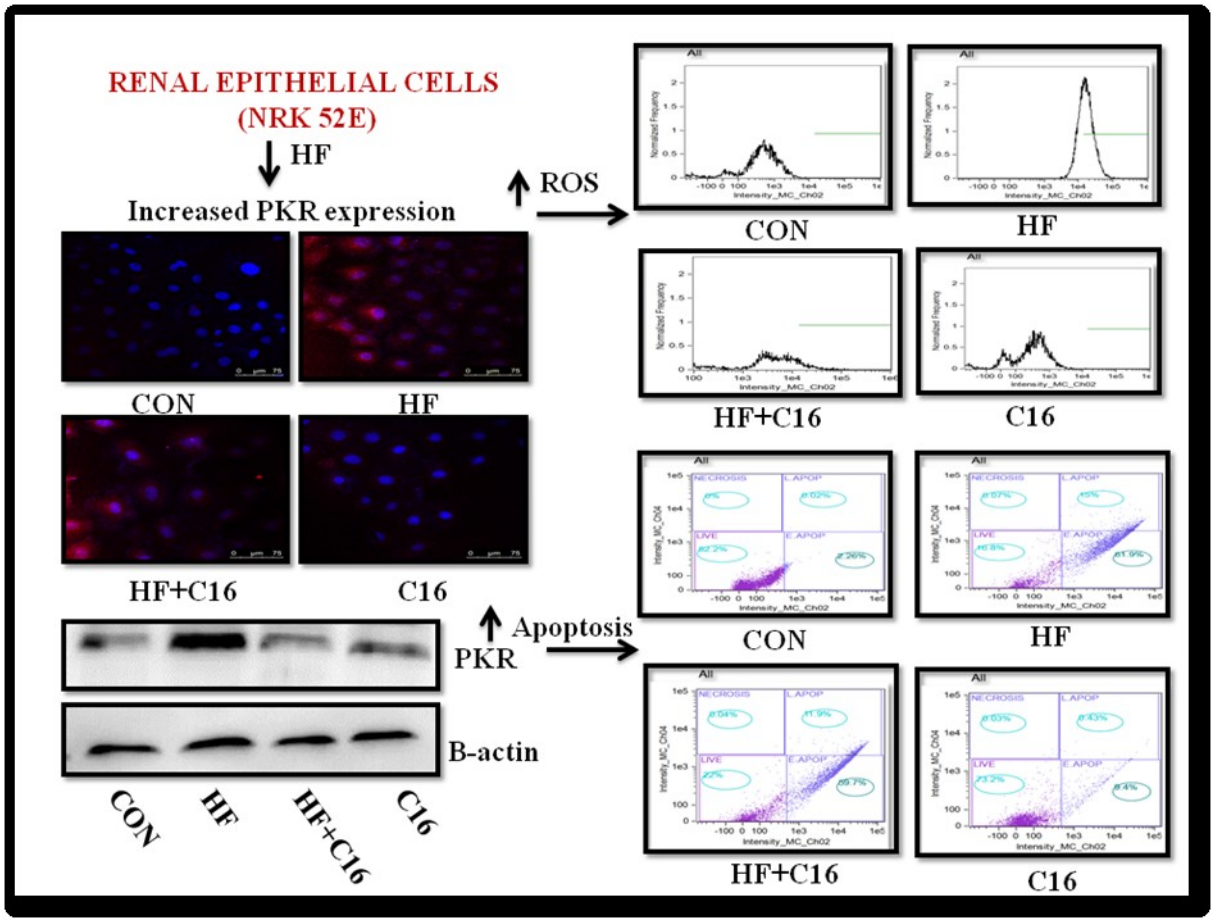
We also observed an increase in early, as well as late apoptosis in HF treated renal epithelial cells which were attenuated by PKR inhibitor imoxin [Fig.5.2.4.]. Several putative mechanisms have been linked in the PKR mediated cell death. Recently, it has been reported that activation of PKR causes an increase in the transcription of Fas mRNA. Increased transcription of Fas mRNA is associated with the interaction of FADD [267, 268]. Additionally, activation of pro-apoptotic factor

Bax and caspase-8/caspase-3 pathway are also known to play a vital role in the PKR mediated apoptosis [269, 270]. Activation of PKR is associated with the activation of two inflammatory cellular kinases JNK and IKK, notably these kinases are involved in the regulation of metabolic homeostasis and insulin resistance [13, 271]. JNK activation is also frequently associated with the generation of ROS and has been considered as one of the critical hallmarks of oxidative insult and inflammation, which altogether contribute to apoptosis and cell death [272]. Interestingly we investigated that expression of caspase-3 and JNK has been upregulated in HF treated cultured renal epithelial cells; however, significant reduction has been observed in the expression of caspase-3 and JNK on co-incubating the HF treated cultured renal epithelial cells with imoxin (C16).

Apoptotic cell death is a common condition that plays a chief role in the worsening of many disease conditions including diabetes-related vascular complications and HTN [273]. Recently, PKR mediated activation of NF-ƙb is considered as the primary culprit involved in the apoptosis-inducing property of PKR [274, 275]. FACS analysis was carried out to determine the apoptotic cell death in cultured renal epithelial cells. We found that in HF treated renal epithelial cells the majority of the cells were in early apoptotic and late apoptotic phase; however, a significant reduction was observed in cell density of early and late apoptotic phase on co-incubating the cells with imoxin (C16). Hence, it can be concluded that PKR mediated oxidative damage and apoptotic cell death in renal epithelial cells and its selective inhibition by imoxin (C16) may represent a novel approach for treating diabetes-related vascular complications and HTN.

The present study demonstrates the functional role of PKR in inducing oxidative damage and apoptotic cell death in renal epithelial cells (NRK 52E). Selective inhibition of PKR attenuates all the destructive effects representing a novel therapeutic strategy for the development of newer and safer drug candidates to combat this disease condition.

SUMMARY



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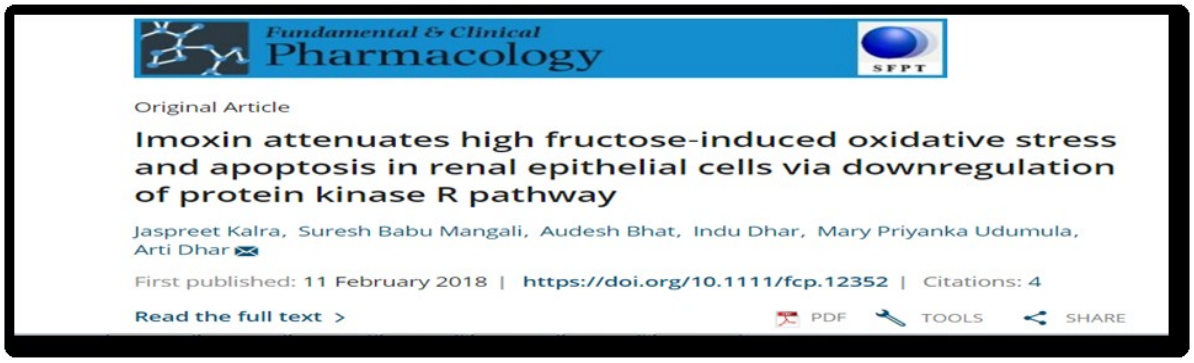

Fundamental & Clinical Pharmacology

Original Article

Imoxin attenuates high fructose-induced oxidative stress and apoptosis in renal epithelial cells via downregulation of protein kinase R pathway

Jaspreet Kalra, Suresh Babu Mangali, Audesh Bhat, Indu Dhar, Mary Priyanka Udumula, Arti Dhar

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5.3. SELECTIVE INHIBITION OF PKR IMPROVES VASCULAR REMODELLING AND INFLAMMATION IN HIGH FRUCTOSE TREATED VASCULAR SMOOTH MUSCLE CELLS

PKR is known to be activated or induced by multiple forms of cell stress including oxidative stress, metabolic stress, mechanical stress, inflammatory signals and excess nutrients. In addition to regulating translation initiation, PKR mediates NF-κB and JNK activation [26,27], promotes chromatin condensation and apoptosis [276,277]. Role of PKR in type 2 diabetes and obesity has been well studied in numerous in-vitro, in-vivo, and gene knock out models [25, 20] however, till date minimal research has been done to elucidate the role of PKR in VSMCs and its role in vascular pathology.

VSMCs the major contractile cells of the vascular system are critical for regulating BP and blood flow throughout the body. Chronic inflammation in vessel structure is a pathological process that acts as a crucial contributor to the development of HTN [278]. Modulation in these signalling pathways causes phenotypic transition in VSMCs which allows them to dedifferentiate, proliferate and migrate from the medial layer to intimal layer where they survive and form the neo-intima which may hinder the blood flow [279]. Vascular inflammation in medial VSMCs is accompanied by the release of pro-inflammatory mediators; synthesis and secretion of various extracellular matrix proteins and mitosis factors-that may further promote calcification and progression of vascular pathology, eventually leading to undesirable clinical outcomes [280].

NF-κB, extracellular signal-regulated kinase 1 and 2 ($ERK_{1/2}$) and matrix metalloproteinase (MMP-9) have been proposed to regulate the inflammation and

proliferation of VSMCs [281, 282]. Recent studies have demonstrated that treatment of cultured VSMCs in-vitro with diabetogenic agents such as HG and advanced glycation end products (AGEs) can increase the activities of MAPKs and NF-ķB, which are associated with vascular inflammation and exhibit increased rates of proliferation and migration relative to those cultured in normal glucose [283, 284]. Events of chromatin remodelling and enhanced inflammatory gene expression in HG-induced monocytes were also reported [285, 286]. Recently, our lab has also established the involvement of PKR in NF-ķB and JNK mediated inflammation in rat cardiomyocytes and renal epithelial cells indicating it as a major contributor in CV pathologies [287, 288].

The present study aims to elucidate the mechanism by which PKR signalling causes impairment in vessel structure. We adopted primary VSMCs isolated from rat thoracic aorta to investigate: (i) The effects of HF on PKR activation and mito-oxidative damage; (ii) The effects of PKR activation on proliferation of primary VSMCs and its underlying mechanism; (iii) The actions of PKR activation on apoptosis or necrosis (if any); or on chromatin condensation and its underlying molecular pathway.

Cell culture

Experimental animals

Eight week old, male Wistar rats weighing 180-220 g were used for the isolation of primary VSMCs. The animals used in the experimental research were approved by an Institutional Animal Ethics Committee (IAEC) bearing a protocol no BITS-HYD/IAEC/2017/05.

Isolation of primary rat aortic vascular smooth muscle cells (VSMCs)

VSMCs from rat thoracic aorta were isolated by enzymatic digestion. The enzyme solution was prepared by dissolving 10 mg of collagenase and 10 mg soybean trypsin inhibitor in 10 ml of HBSS. ~0.354 ml elastase and 100µl of penicillin/streptomycin (1%) were added to make a final concentration of 0.744 units/ml. The enzyme solution was freshly prepared for every use. Eight week old male Wistar rats were used for the isolation, with the aortas from two rats isolated each time. The rats were anaesthetized by thiopental sodium (100mg/kg, i.p) and

sacrificed immediately. Aortas were harvested under aseptic conditions by giving incision on ventral portion of thorax and abdomen. The isolated aortas were immediately submerged in HBSS and all the fatty tissue scraped off before shifting them to a fresh batch of HBSS. The aortas were then washed twice with incomplete DMEM internally with the help of a 1ml syringe. A longitudinal cut was made in each aorta to expose its inner side, and the endothelial cells were removed by gentle rubbing with the help of a scalpel. The aortas were cut into small pieces and were quickly transferred to a T25 flask containing enzyme solution. It was then placed vertically in an incubator maintained at 37^{o}c and 5% CO2 with the cap loosened for enzymatic digestion. Enzymatic digestion was carried out for 4 h with gentle pipetting every 1 h to enhance dissociation. The enzyme solution was centrifuged and the resulting pellet was washed by centrifugation in Hanks solution then, the pellet was suspended in DMEM supplemented with 20% heat inactivated FBS. Cells were seeded in 4 wells of 48 well plates. Confluent cells were split among 6 well plates; VSMCs up to 6-8 passages were used for further experiments. The cultured cells were incubated in 2% FBS supplemented DMEM with HF (5mM) or with selective PKR inhibitor imoxin (C16) (5µM, based on reported literature) alone and in combination respectively for 24 h [289]. Mannitol was used as a negative control for comparing the effects of HF [290].

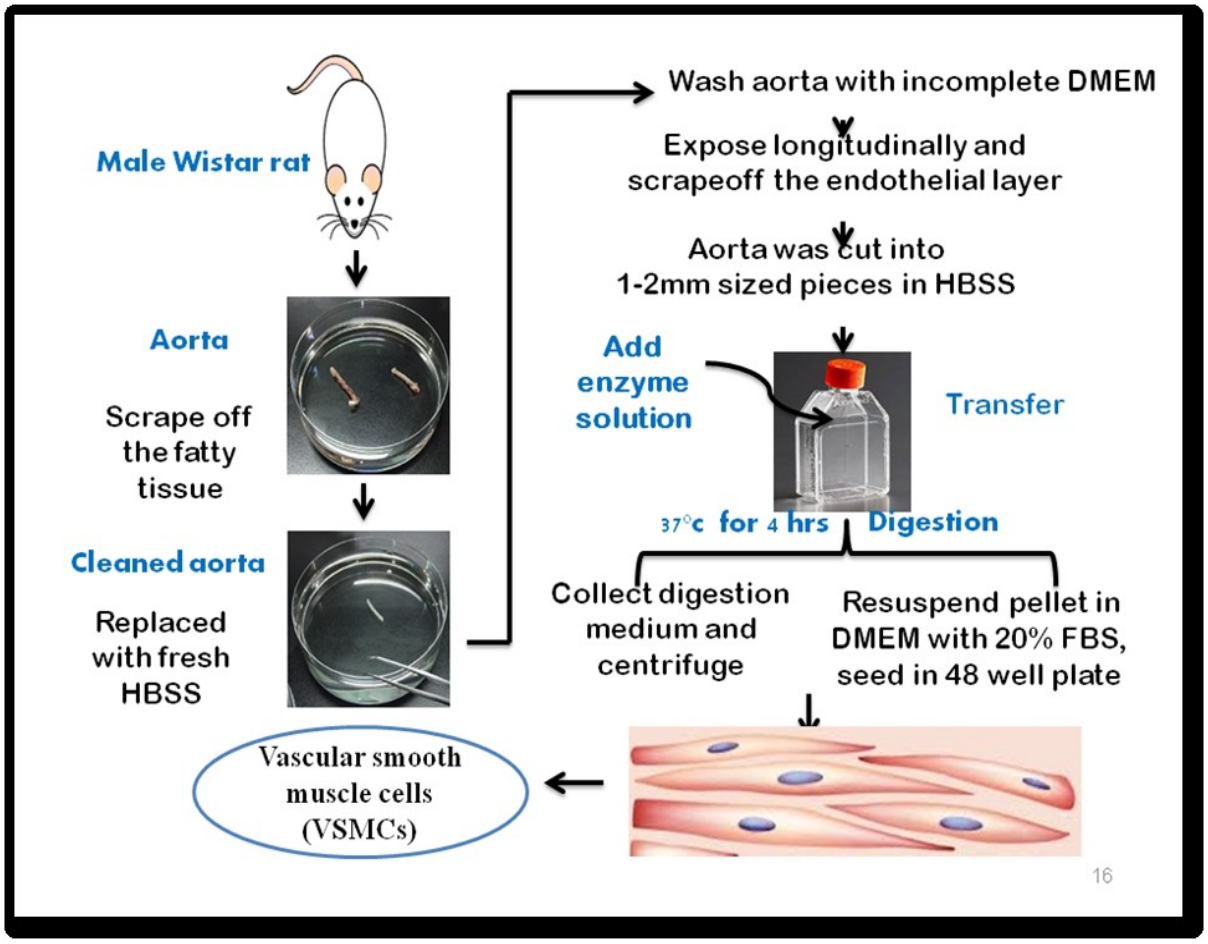


Fig.5.3.1. Schematic representation of the method of isolation of rat aortic vascular smooth muscle cells (VSMCs) using enzymatic digestion method

RESULTS

Characterization and identification of contractile phenotype of rat aortic primary VSMCs and HF triggered PKR expression

Rat aortic primary VSMCs were isolated according to the aforementioned method [291]. Our first aim was to characterize the isolated VSMCs. VSMCs contractile phenotype is characterised by the presence of α-smooth muscle actin (α-SMA), calponin, smoothelin, smooth muscle myosin heavy chains SM-1 and SM-2. They are essential for normal contraction and relaxation of vasculature. This phenomenon enables the blood vessels to adjust the luminal diameter and to develop and sustain normal BP [292, 293]. Results of the western blotting (Fig.5.3.2.A.) in an un-treated protein sample of isolated VSMCs confirmed the expression α-SMA an indicative of contractile VSMCs. The results of immunofluorescence staining (Fig.5.3.2.B.) for α-

SMA further revealed that the isolated VSMCs are spindle shaped and elongated. Next, we examined whether HF treatment to the VSMCs can cause up-regulation in the expression of phospho-PKR (p-PKR) and PKR. We found significant increase in p-PKR (Fig. 5.3.2.D.) and PKR expression (Fig. 5.3.2.C. and D.) in HF treated VSMCs and un-treated VSMCs serving as control, the effect was attenuated in HF treated VSMCs co-incubated with imoxin. No significant change was observed in the mannitol (used as a negative control) treated group compared to the control.

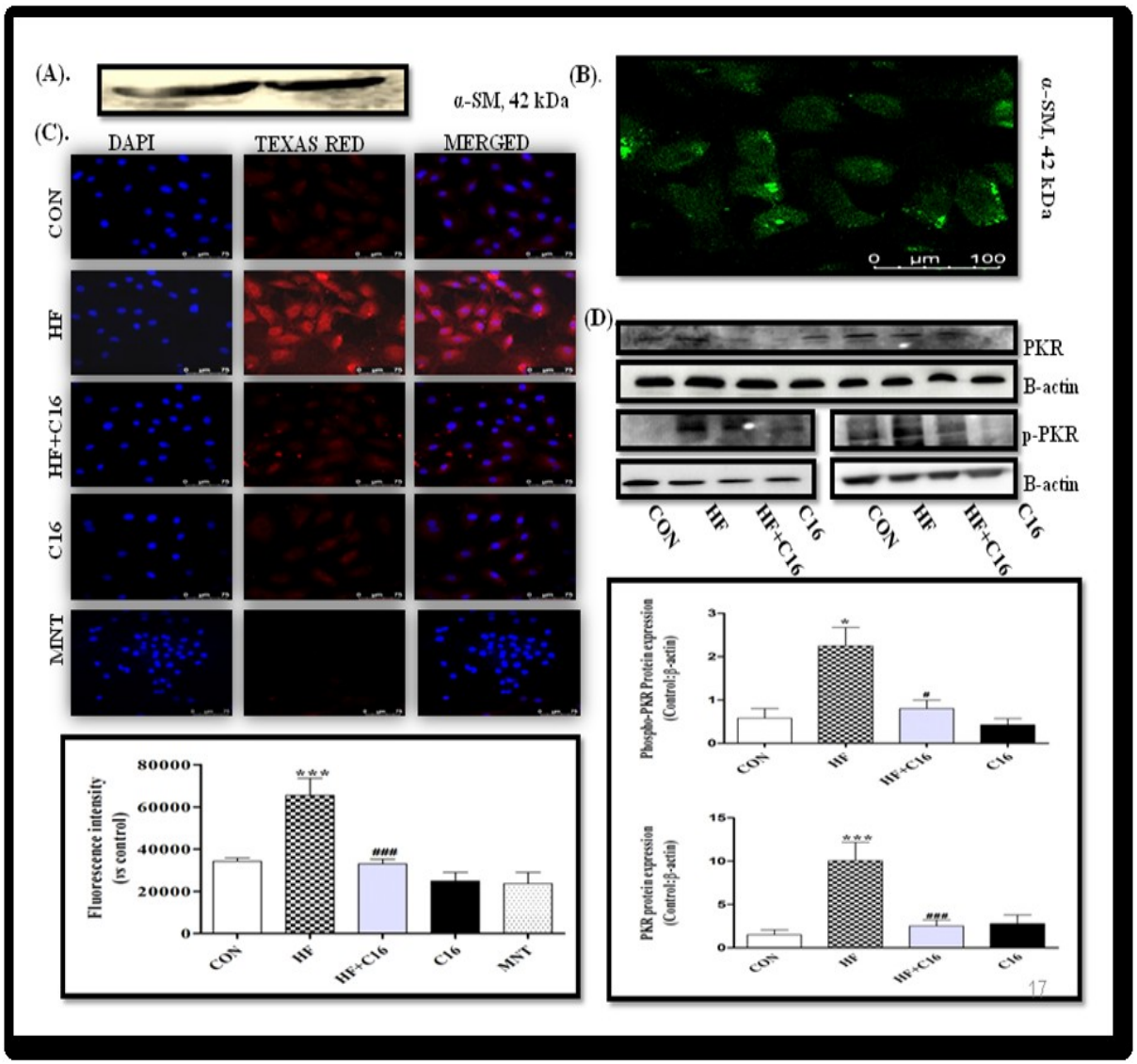


Fig.5.3.2. Identification and characterization of VSMCs and HF triggered increase in PKR expression, attenuation by C16: Fig. **5.3.2**.A. Western blotting was done for alpha-smooth muscle actin for identification of VSMCs. Fig.5.3.2.B. Immunofluorescence staining was performed for alpha-smooth muscle actin: a characteristic marker for contractile phenotype of VSMCs. Fig.1.C. Protein kinase R (PKR) expression is increased in HF-treated VSMCs, attenuation by imoxin (C16): Cultured VSMCs were incubated with normal culture medium (C) or medium

containing high fructose (HF 5 mM) for 24 h. Imoxin (5μM) was incubated alone or with HF for 24 h. PKR expression was measured by immunofluorescence staining Fig.5.3.2.C. and western blotting Fig.5.3.2.D. n = 3 for each treatment. Data expressed as ±SD: ***P < 0.001 vs. C, ###P < 0.001 vs. HF+C16 treated group.

Imoxin attenuates HF triggered ROS production, facilitates iNOS expression in VSMCs

PKR is a known inducer of oxidative stress under excessive nutrient intake. The VSMCs were stimulated with HF to elucidate the role of PKR in mediating oxidative damage. We report significant increase in ROS production in HF treated VSMCs compared to control, imoxin co-incubation along with HF treatment has significantly attenuated the increase in ROS production (Fig.5.3.3.A.). Under oxidative stress conditions, the iNOS can be expressed and may contribute in inflammatory responses. In our study, we found that VSMCs showed increased expression of iNOS and nitrate/nitrite levels in response to HF as evidenced by results of Griess assay and immunofluorescence staining. This increase was significantly reduced in imoxin co-incubated cells along with HF. We relate this reduction in iNOS expression and nitrite/nitrate level to the inhibition of PKR (Fig.5.3.3.A and B.). No significant change was observed in the mannitol (used as a negative control) treated group compared to the control.

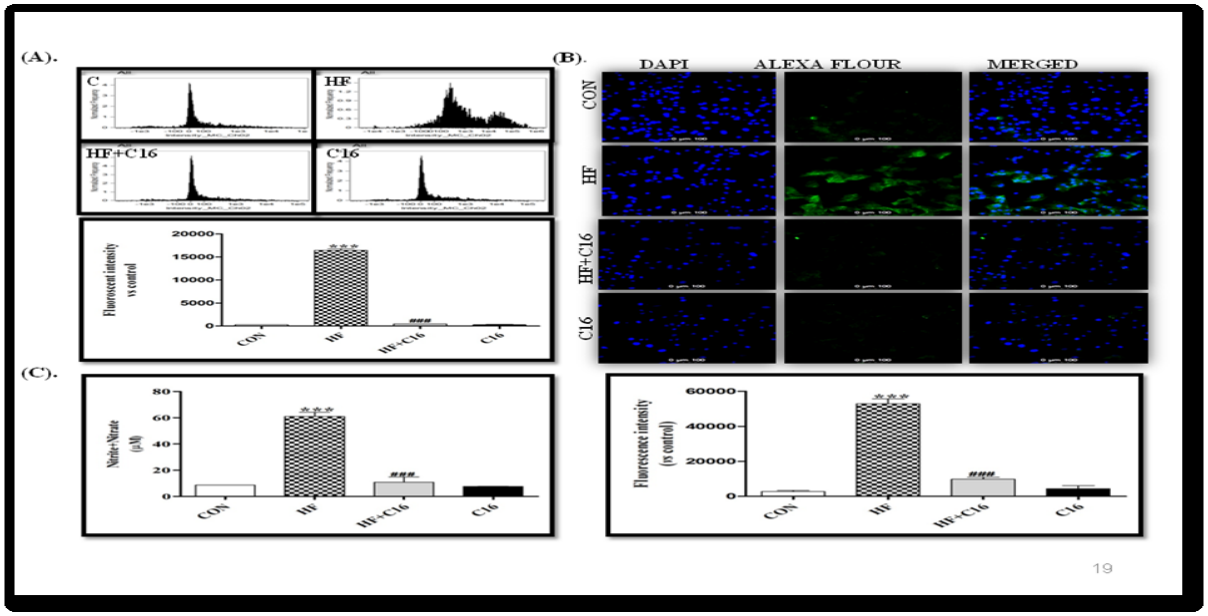


Fig.5.3.3. Increased ROS production, iNOS expression and nitrite/nitrate levels in HF-treated VSMCs and restoration by imoxin (C16): Cultured VSMCs were incubated with normal culture medium (C) or medium containing high fructose (HF 5 mM) for 24 h. Imoxin (5µM) was incubated alone or with HF for 24 h. Fig.5.3.3.A. DCFDA assay was performed for flow cytometric determination of production of reactive oxygen species (ROS). ROS production was increased in cultured HF-treated VSMCs, attenuation by imoxin. Data expressed as ±SD: ***P < 0.001 vs. C, ###P < 0.001 vs. HF+C16 treated group. Fig.5.3.3.B. immunofluorescence staining was performed for iNOS expression. Significant differences in iNOS expression were observed between HF incubated VSMCs and un-treated cells, attenuation by imoxin. Data expressed as ±SD: ***P < 0.001 vs. C, ###P < 0.001 vs. HF+C16 treated group. Fig.5.3.3.A. Nitrite/nitrate levels were determined by Griess assay in cell supernatant of cultured VSMCs incubated with normal culture medium (C) or medium containing high fructose (HF 5 mM) for 24 h. Imoxin (5µM) was incubated alone or with HF for 24 h. n = 3 for each treatment. Data expressed as ±SD: ***P < 0.001 vs. C, ###P < 0.001 vs. HF+C16 treated group.

Imoxin attenuates HF triggered inflammatory responses in VSMCs

PKR is well recognised for initiating inflammatory responses through the phosphorylation and activation of JNK and NF-ķB activation. Present findings show that HF treatment induces inflammatory response in VSMCs as demonstrated by increased fluorescence intensity and protein expression of NF-ķB (Fig.5.3.4.A. and B.). We also observed substantial increase in expression of phospho-JNK (p-JNK) (Fig.5.3.4.C.) and JNK in HF treated VSMCs, followed by its attenuation in imoxin co-incubated cells along with HF treatment (Fig.5.3.4.C. and D.) No significant change was observed in the mannitol (used as a negative control) treated group compared to the control.

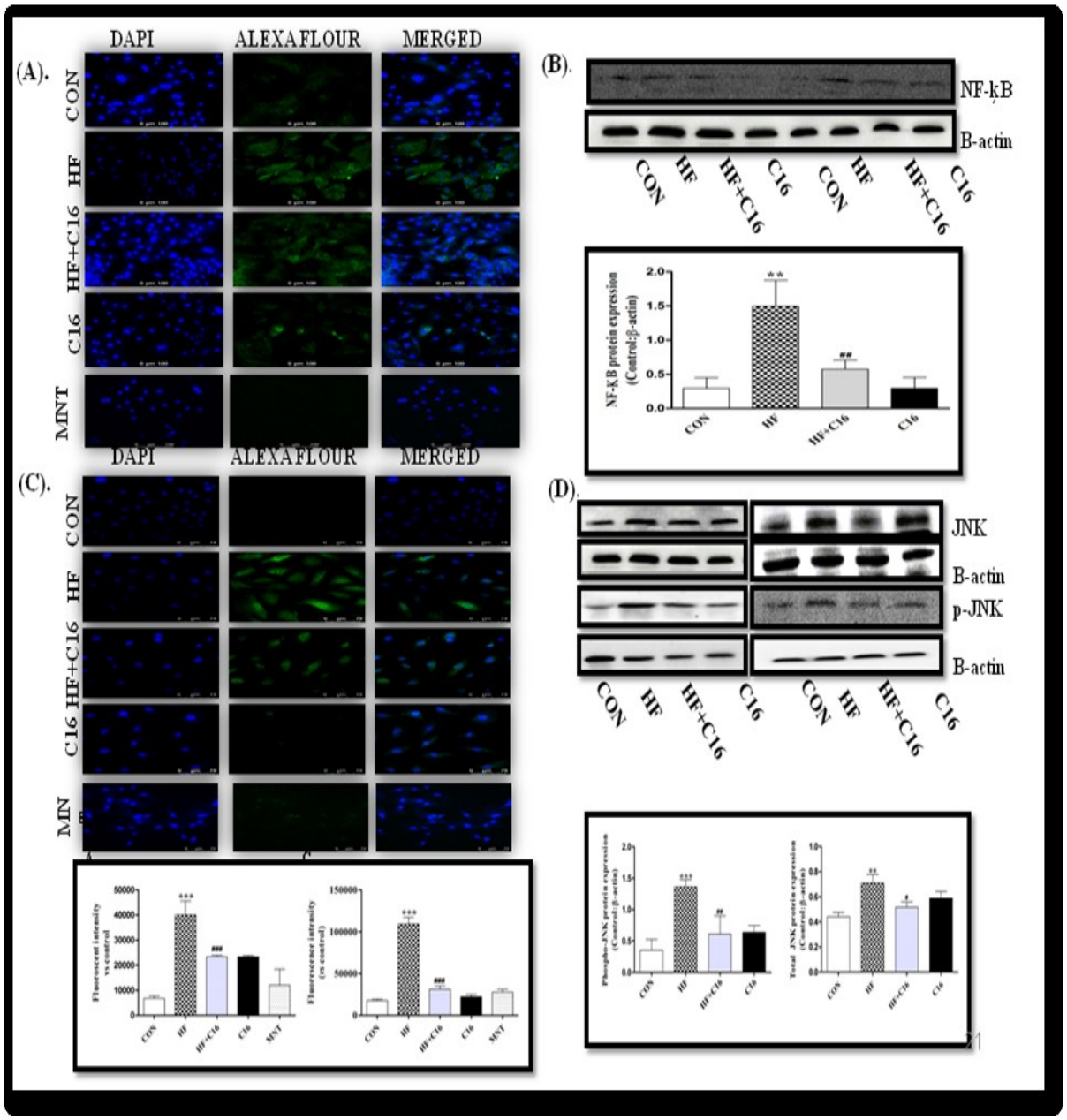


Fig.5.3.4.Nuclear factor kappa B (NF-ķB) and c-Jun N-terminal kinases (JNKs) expression is increased in HF-treated VSMCs, attenuation by imoxin (C16): Cultured VSMCs were incubated with normal culture medium (C) or medium containing high fructose (HF 5 mM) for 24 h. Imoxin (5μM) was incubated alone or with HF for 24 h. Fig.5.3.4.A. Increase in NF-ķB expression was demonstrated by increase in relative fluorescent intensity in HF treated VSMCs compared to un-treated cells, significant attenuation by imoxin, shown by immunofluorescence staining, data expressed as ±SD: ***P < 0.001 vs. C, ###P < 0.001 vs. HF+C16 treated group, and western blotting Fig.5.3.4.B. data expressed as ±SD: **P < 0.01 vs. C, ##P < 0.01 vs. HF+C16 treated group. Fig.5.3.5.C. Increase in JNK expression was demonstrated by increase in relative fluorescent intensity in HF treated VSMCs compared to un-treated cells, significant attenuation by imoxin is measured by immunofluorescence staining. Data expressed as ±SD: ***P < 0.0001 vs. C, ###P < 0.0001 vs. HF+C16 treated group. Fig.5.3.6.D. (western blotting) Data expressed as ±SD: **P < 0.001 vs. C, #P < 0.05 vs. HF+C16 treated group. n = 3 for each treatment.

Imoxin attenuates HF induced cellular hypertrophy, proliferative and migratory behaviour in VSMCs

Vascular remodelling in case of hypertensive disease pathology is linked with the degradation of ECM components along with hypertrophy, enhanced proliferative and migratory behaviour. Our findings obtained from H&E staining clearly indicate cellular hypertrophy demonstrated by significant increase in hypertrophic area in HF treated VSMCs; attenuation in hypertrophic area was noted in HF treated VSMCs co-treated with imoxin Fig.5.3.4.A. After observing the enhanced cellular hypertrophy, we aimed at investigating whether observed hypertrophy is due to vascular remodelling. MMP-9 up-regulation is well established in causing vascular remodelling and degradation of ECM components [294]. Intriguingly, we found enhanced expression of MMP-9 protein (Fig.5.3.4.B.), higher proliferation rates (Fig.5.3.4.C.), increased cell division and DNA synthesis (Fig.5.3.4. D. and E.), in HF treated VSMCs compared to control. However, a significant decrease was observed in HF treated VSMCs co-treated with imoxin. Results of the scratch test has further revealed the increased migratory behavior noticed by reduced scratch width in HF treated cells compared to control, where as the decrease was observed in HF treated cells co-incubated with C16 (Fig.5.3.4. D).

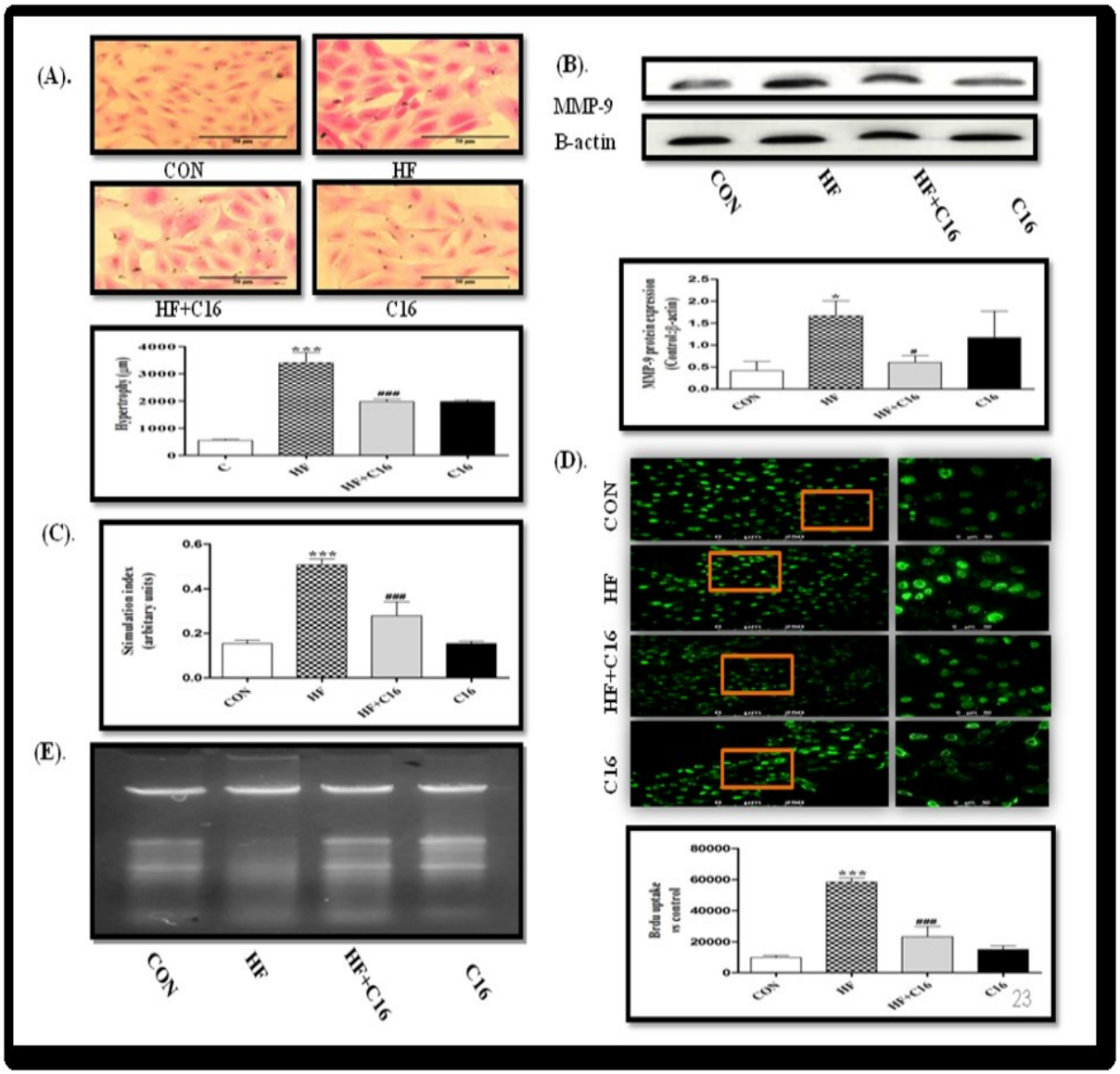


Fig.5.3.5. Assessment of cellular hypertrophy, proliferative and migratory behaviour: Vascular remodelling is accompanied by hypertrophy and degradation of ECM components [279]. We checked whether HF induced hypertrophy in VSMCs could be reversed by imoxin or not. After observing the significant attenuation in hypertrophy (Fig.5.3.4.A.), we wanted to check the involvement of MMP-9, MMP-9 is the major matrix metalloproteinase's involved in migration as well as vascular remodelling process in-vitro as well as in-vivo. Therefore, we primarily aimed to look for the expression of MMP-9 in VSMCs in the discussed study. MMP-9 (Fig.4.B.), is known to be a key contributor in case of vascular remodelling which facilitates the degradation of matrix components, So next, we checked for the expression of MMP-9 (Fig.5.3.4.B.). After establishing the significant contribution of MMP-9 in HF induced vascular remodelling and hypertrophy we aimed to look for whether this HF induced hypertrophy in VSMCs is causing any change in their proliferative rates, as hypertrophy could be related with increased proliferation of VSMCs (Fig.5.3.4.C.). In turn, increased proliferative rates are attributed to increased DNA synthesis. Nuclear incorporation of BrdU is among one of the most common parameter used for assessment of DNA synthesis [279-281]. So, here we sought to

assess the preliminary examination of DNA on agarose gel electrophoresis; we hypothesized that if cells were in non-proliferative state we may observe a DNA smear or if they were exhibiting proliferative nature then DNA must be intact and we may note a sharp band for HF treated group (Fig.5.3.4.E). Based on this assumption we did this experiment and found that the results are in tune with the later assumption, as no DNA breakage/smear was detected/observed in HF treated group compared to other experimental groups. Hence, we conclude that it could possibly be due to the rapid proliferation occurring in cells. For providing insights at the molecular level BrdU labelling assay was done for the same (Fig.5.3.4.D.) Fig.5.3.4.A. Hematoxylin and eosin staining was performed for assessment of cellular hypertrophy. Fig.4.A. Cellular hypertrophy/growth has been observed in cultured HF-treated VSMCs, attenuation by imoxin (C16): Cultured VSMCs were incubated with normal culture medium (C) or medium containing high fructose (HF 5 mM) for 24 h. Imoxin (5μM) was incubated alone or with HF for 24 h. Fig.5.3.4.B. Increase in MMP-9 protein expression: PKR down regulator and contributor for increased proliferation rates. Upregulation in MMP-9 expression has been observed in cultured HF-treated VSMC's, attenuation by imoxin (C16): Cultured VSMCs were incubated with normal culture medium (C) or medium containing high fructose (HF 5 mM) for 24 h. Imoxin (5μM) was incubated alone or with HF for 24 h. Data expressed as ±SD: *P < 0.05 vs. C, #P < 0.05 vs. HF+C16 treated group. Fig.5.3.4.C. MTT assay was carried out to determine the proliferative behavior. Increased proliferation rate/stimulation index was noted in cultured HF-treated VSMCs, attenuation by imoxin (C16). Data expressed as ±SD: ***P < 0.001 vs. C,##P < 0.001 vs. HF+C16 treated group. (Fig.5.3.4.D. and Fig.5.3.4.E.) BrdU labelling and agarose gel electrophoresis was performed for to determine the cell division and enhanced DNA synthesis. Increased cell division and enhanced DNA synthesis were observed in cultured HF-treated VSMCs, attenuation by imoxin. n = 3 for each treatment. Data expressed as ±SD: ***P < 0.001 vs. C, ##P < 0.001 vs. HF+C16 treated group.

Imoxin attenuates HF triggered drop in mitochondrial membrane potential, apoptotic and cytotoxic effects in VSMCs

Apoptosis is a programmed event of cell death. Increased activity of enzyme LDH, chromatin condensation, drop in mitochondrial membrane potential and activation of effector caspases are significant paramount's involved in cell death. LDH enzyme activity was significantly increased in cell supernatant of HF treated VSMCs, significant attenuation was observed in HF treated VSMCs co-incubated along with the imoxin Fig.5.3.6.A. The results of DAPI staining have also revealed nuclear fragmentation in the HF treated group compared to control, attenuation was noted in nuclear fragmentation/chromatin condensation in HF treated VSMCs co-incubated along with the imoxin Fig.5.3.6.B. Significant drop in mitochondrial

membrane potential was noted in HF treated group compared to control, the drop was attenuated in cells co-treated with imoxin Fig.5.3.6.C. We also found significant increase in protein expression and fluorescent intensity of caspase-3, a death effector caspase in HF treated VSMCs and its inhibition was observed in imoxin treated group (Fig.5.3.6.A. and Fig.5.3.6.B.). No significant change was observed in the mannitol (used as a negative control) treated group compared to the control.

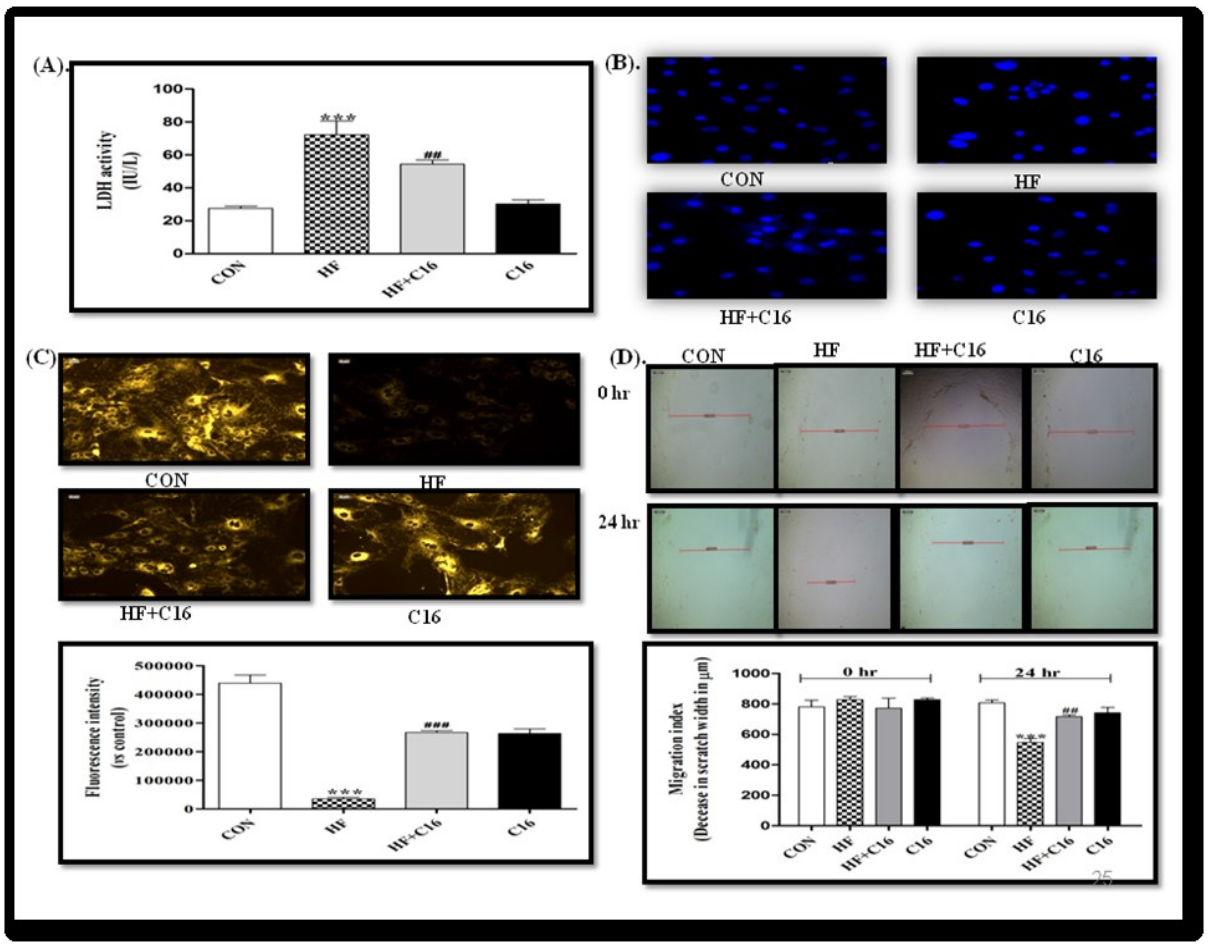


Fig.5.3.6. HF triggered drop in mitochondrial membrane potential and cytotoxic effects in VSMCs, attenuation by VSMCs: Cultured VSMCs were incubated with normal culture medium (C) or medium containing high fructose (HF 5 mM) for 24 h. Imoxin (5µM) was incubated alone or with HF for 24 h. Fig.5.3.6.A. Increased enzyme activity of lactate dehydrogenase: marker for cytotoxicity was reported in HF treated VSMCs, attenuation by imoxin. Data expressed as ±SD: ***P < 0.001 vs. C, ##P < 0.01 vs. HF+C16 treated group. Fig.5.3.6.B. Nuclear staining with DAPI was performed to investigate chromatin condensation. Higher incidence of chromatin condensation was observed in HF treated VSMCs, attenuation by imoxin. Fig.5.3.6.C. Results of rhodamine 6G staining demonstrates significant drop in mitochondrial membrane potential in HF treated VSMCs, attenuation by imoxin. n = 3 for each treatment. Data expressed as ±SD: ***P < 0.001 vs. C, ###P < 0.001 vs. HF+C16 treated group. Fig.5.3.6.D. Results of scratch test demonstrates significant increase in migration index demonstrated by decrease in scratch width in HF treated VSMCs,

attenuation by imoxin. n = 3 for each treatment. Data expressed as ±SD: ***P < 0.001 vs. C, ##P < 0.01 vs. HF+C16 treated group.

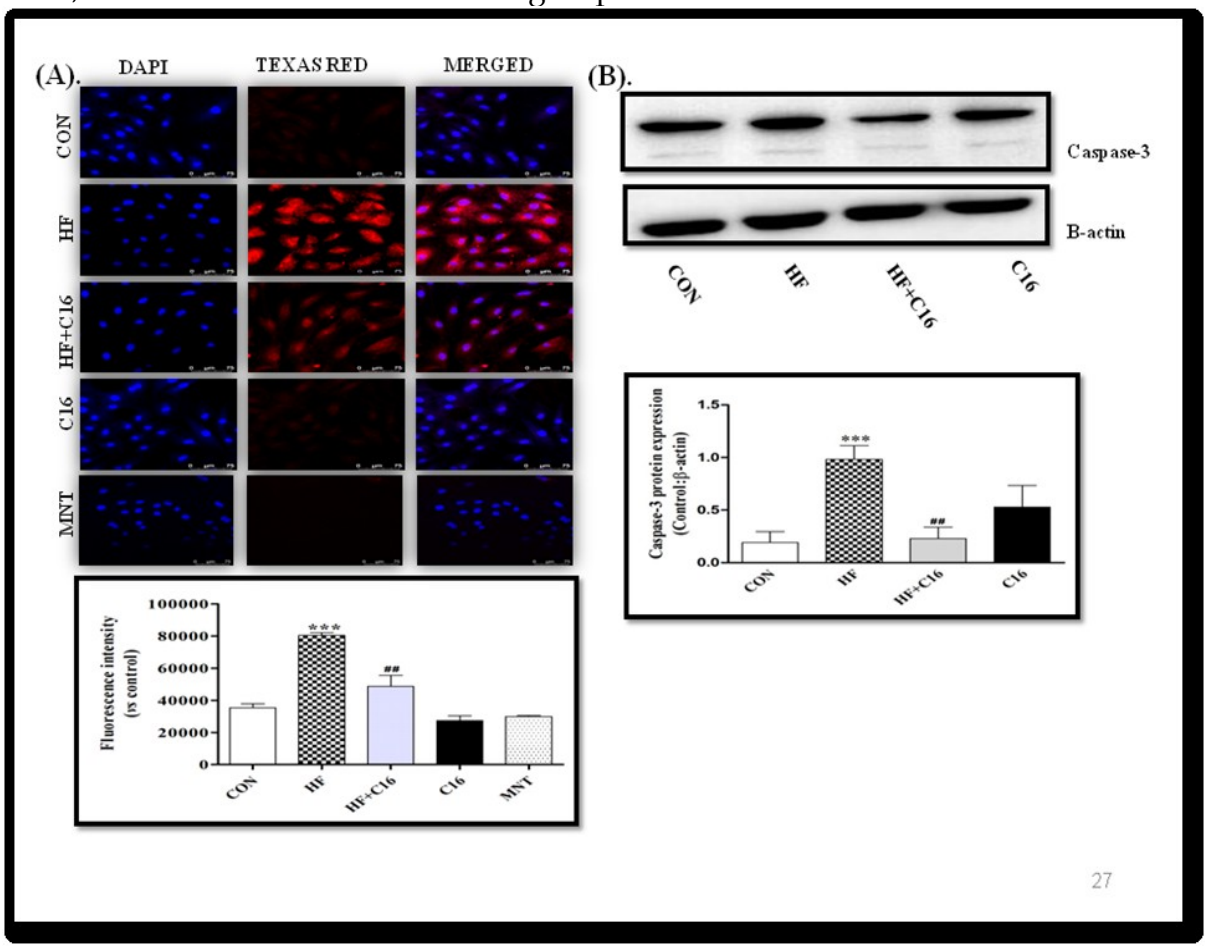


Fig.5.3.7. HF triggered caspase-3 expression and apoptosis in VSMCs, attenuation by imoxin: Cultured VSMCs were incubated with normal culture medium (C) or medium containing high fructose (HF 5 mM) for 24 h. Imoxin (5μM) was incubated alone or with HF for 24 h. Fig.5.3.7.A. Result of immunofluorescence staining: Increase in relative fluorescent intensity of caspase-3 expression in HF treated VSMCs, attenuation by imoxin. Data expressed as ±SD: ***P < 0.001 vs. C, ##P < 0.01 vs. HF+C16 treated group. Fig.5.3.7.B. Increased protein expression of caspase-3 in HF treated VSMCs, attenuation by imoxin. Data expressed as ±SD: ***P < 0.001 vs. C, ##P < 0.01 vs. HF+C16 treated group. n = 3 for each treatment.

Imoxin attenuates HF triggered vascular calcification, AGEs accumulation and phenotypic modulation by inhibition of p-ERK$_{1/2}$ and ERK$_{1/2}$ in VSMCs

Vascular calcification, deposition of AGEs, decreased expression of contractile markers and higher proliferation rate are the prominent features of phenotypic switch in VSMCs observed in hypertensive disease pathology. In the present study, HF caused significant vascular calcification and accumulation of AGEs (Fig.5.3.8.A. Fig.5.3.8.B.) in HF treated VSMCs. The treatment with imoxin in HF treated cells

resulted in significant reduction of calcification and deposition of AGEs. We found that the protein expression of phospho-ERK (p-$ERK_{1/2}$) and $ERK_{1/2}$ was also significantly raised in VSMCs incubated with HF compared to control, attenuation in p-$ERK_{1/2}$ and $ERK_{1/2}$ expression was observed on co-incubating the HF treated cells with imoxin Fig.5.3.8.C.

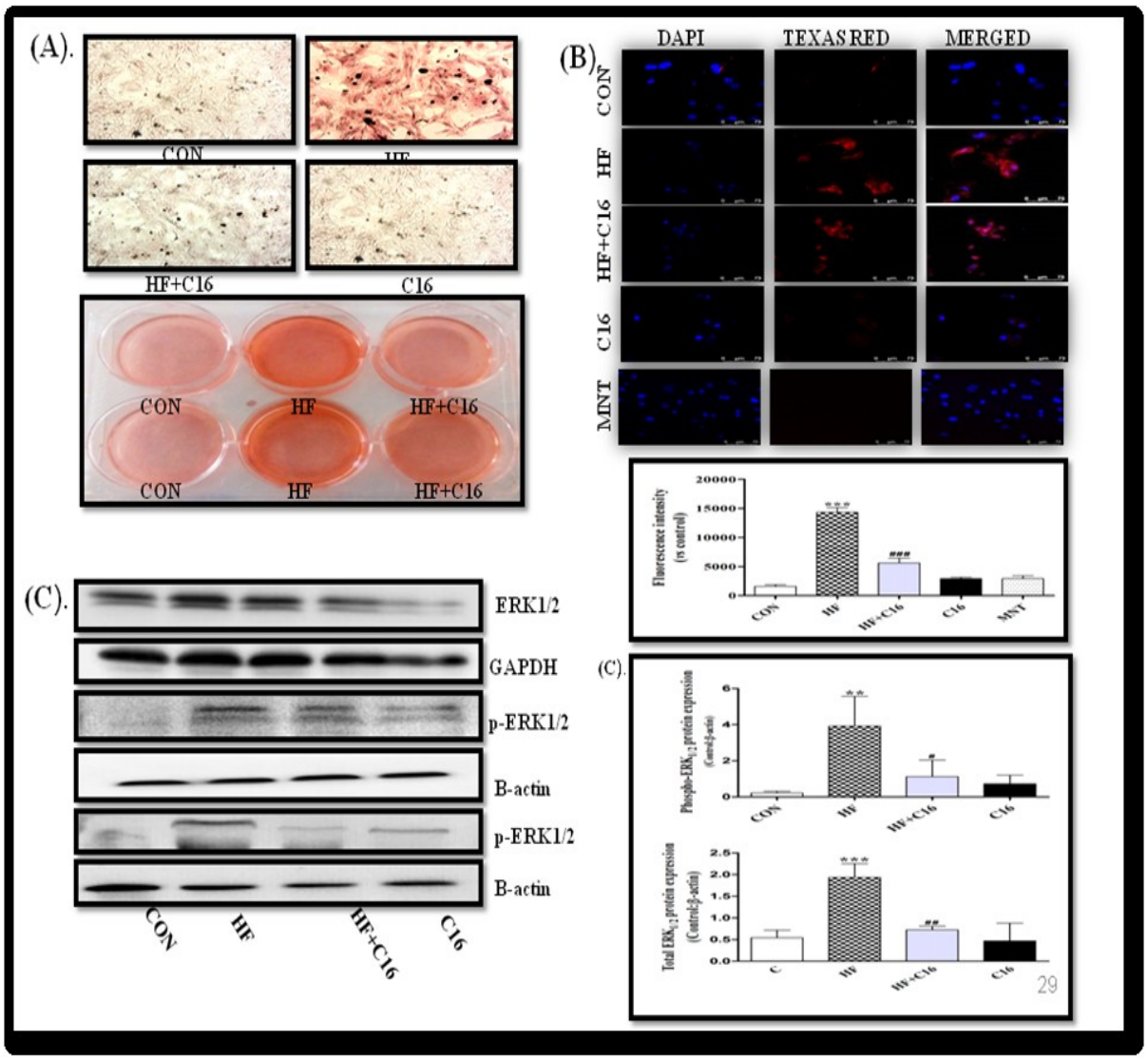


Fig.5.3.8.HF triggered vascular calcification, AGEs accumulation and up-regulation of ERK1/2 in VSMCs, attenuation by imoxin. Cultured VSMCs were incubated with normal culture medium (C) or medium containing high fructose (HF 5 mM) for 24 h. Imoxin (5µM) was incubated alone or with HF for 24 h. Fig.5.3.8.A. Result of alizarin red staining: Increase in calcium deposition/vascular calcification in HF treated VSMCs, attenuation by imoxin. Fig.5.3.8.B. Result of immunofluorescence staining: Increase in relative fluorescent intensity of AGEs expression in HF treated VSMCs, attenuation by imoxin. Data expressed as ±SD: ***P < 0.001 vs. C, ###P < 0.001 vs. HF+C16 treated group. Fig.5.3.8.C. Western blot was performed for ERK1/2: significant up-regulation has been observed in expression of

ERK1/2 in HF treated VSMCs, attenuation by imoxin. Data expressed as ±SD: ***P < 0.001 vs. C, ##P < 0.01 vs. HF+C16 treated group. n = 3 for each treatment.

DISCUSSION

In the present study we demonstrate for the first time that HF causes VSMCs inflammation and remodelling by activating the NF-ķB and $ERK_{1/2}$/ MMP-9 pathway in a PKR dependent manner. Increased expression of p-PKR (Fig.5.3.2.D) and PKR in HF-treated VSMCs was abolished by selective inhibition of PKR by imoxin (Fig.5.3.2. C and D.). Treatment with HF increased NF-ķB (Fig.5.3.4.A and B.) p-JNK and JNK expression (Fig.5.3.4.C and D)-a critical regulator of vascular inflammation and apoptosis, increase was attenuated when VSMCs were co-treated with imoxin, a selective PKR inhibitor. PKR up-regulates several transcriptional genes implicated in inflammation through activation of inhibitor of κB (IκB) kinase (IKK) and MAPKs. Members of MAPKs family play a vital role in cell proliferation and apoptosis. Interestingly, cellular proliferation and apoptosis are the common contributors to vascular remodelling [27, 295, 296]. Activation of NF-κB due to translocation by excessive dietary factors could induce the ROS generation [297]. Furthermore, elevated ROS is attributable to numerous hallmarks indicated in vascular damage and remodelling including higher proliferative rates, cellular hypertrophy, increased migratory behaviour and apoptosis [298-300]. In the present study, HF-treated VSMCs exhibited increased expression of iNOS, nitrite/nitrate levels along with significant increase in ROS production, significant decrease was noted in all of the above-stated markers for oxidative stress in VSMCs co-incubated with imoxin (Fig.5.3.3. A., B., and C.). The findings of our study robustly claim that NF-ķB activation through PKR dependent signalling is associated with vascular inflammation.

Increase in cellular hypertrophy was observed in HF-treated VSMCs, indicated by an increase in a hypertrophic area (Fig.5.3.5. A.). This increase was attenuated in HF-treated VSMCs co-incubated with imoxin. Several studies have elucidated that vascular insulin resistance is critically related to the pro-atherogenic changes occurring in vasculature [301]. Findings of Hitomi et al., reveal that

aldosterone promotes insulin resistance in rat VSMCs through down regulation of insulin receptor substrate-1(IRS-1) [302]. Evidently, VSMCs also expresses abundant insulin-like growth factor-1 receptor (IGF1R) compared to insulin receptors [303]. Increased IGFIR is also reported in aortas isolated from diabetic animals [304]. Numerous studies have reported that IR and IGF1R subunits form hybrid receptors, having greater affinity to IGF1R than insulin [305]. According to previous reports, cellular hypertrophy in VSMCs could be related with insulin resistance and over expression of IGF1 in response to HF [306-308].

Interestingly, Lightell et al. in their studies have reported that insulin receptor gene silencing in VSMCs is related with the reduced Akt phosphorylation and increased expression of phospho-ERK, ultimately leading to increased rates of cellular proliferation [309]. Our study further identifies that proliferation; migration and apoptosis are tightly regulated processes in VSMCs. We explored PKR as a major activator for MAPKs, PKR activates two members of MAPKs family, including ERK1/2; which might be responsible for cellular proliferation and migratory behavior, whereas JNKs has been reported to induce cellular apoptosis in vascular injury [310-313]. Notably, the findings of our study indicate the activation and phosphorylation of $ERK_{1/2}$ pathway in VSMCs treated with HF, phospho-$ERK_{1/2}$ (p-$ERK_{1/2}$) and $ERK_{1/2}$ expression was significantly reduced in imoxin co-incubation in HF treated VSMCs (Fig.5.3.8.C.). Previous reports suggest that activation of $ERK_{1/2}$ also contributes to the excessive accumulation of AGEs, which could apparently lead to increased ROS production and activation of inflammatory responses [314]. In our study expression of AGEs has been significantly increased in HF treated VSMCs; attenuation was achieved by imoxin co-incubation indicating it as an important target involved in the pathogenesis of vascular remodelling.

In the present study, cellular proliferation is determined by MTT assay (Fig.5.3.4.C., results are presented as stimulation index) and migration index is done by scratch test (Fig.5.3.6.D.). Higher proliferation rates and increased migration index were observed in HF treated VSMCs, and significant reduction was observed in HF-treated cells co-incubated with imoxin. Previous reports suggest increased

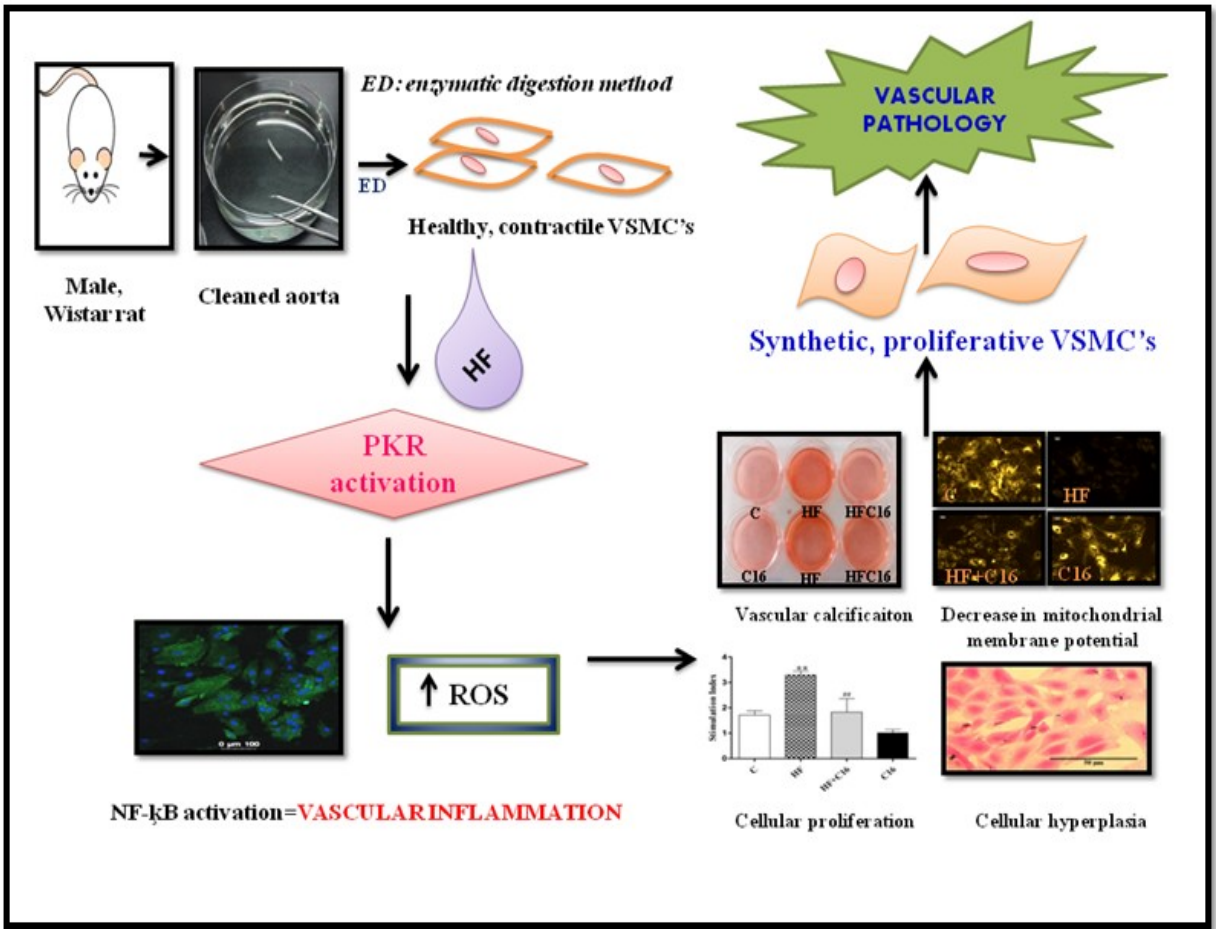
proliferation rate and migratory behaviour are attributing to the increased DNA synthesis [315]. Nuclear incorporation of BrdU is among one of the most common parameter used for assessment of DNA synthesis [316]. Here, increase in nuclear incorporation of BrdU (DNA synthesis) in HF treated VSMCs and its attenuation in VSMCs co-incubated along with C16 in presence of HF was evidenced with the help of BrdU labelling assay (Fig.4.D.). For providing the better insights for the same we have also performed the agarose gel electrophoresis (Fig.4.E.). results of which clearly indicates no breaks in DNA band obtained for HF treated samples, which could be a indicator for higher proliferation rates and increased migratory behavior in VSMCs. Next, we examined the role of PKR in HF-induced VSMC apoptosis. Apoptosis is a significant paramount that accounts for the normal physiology as well as it may act as a key contributor in the development of numerous disease pathologies [317]. The molecular mechanisms associated with cell death in vascular pathologies are still far from clear. However, activation of death receptor pathways and mechanical stress induced pathways are of considerable importance for inducing apoptosis as they both lead to activation of executioner caspase, caspase-3 [318, 319]. Results of the present study reveal that the supernatants of HF-treated VSMCs exhibit increased activity of LDH compared to un-treated cells, which served as control, and attenuation by imoxin. Increased activity of LDH is related to elevated cytotoxicity and cell death (Fig.5.3.6.A.). In normal physiological conditions cytoplasmic enzyme LDH is present in almost every single cell, however under excessive stress conditions, increased levels of LDH has been reported which could be possibly due to the release of LDH enzyme into extracellular space. Chromatin condensation and drop in mitochondrial membrane potential two major hallmarks for apoptotsis [320]. The results of DAPI staining demonstrates nuclear fragmentation in the HF treated group, attenuation in nuclear fragmentation/ chromatin condensation was observed in HF treated VSMCs co-incubated along with the imoxin (Fig.5.3.6.B.). Results of rhodamine 6G staining further illustrates drop in mitochondrial membrane potential, indicated by decreased fluorescent intensity in HF treated cells. However, this drop was attenuated in cells treated with HF in combination with imoxin (Fig.5.3.6.C.). Increased caspase 3 expression in HF-

incubated cells, attenuation by imoxin, was also reported in the present study (Fig.5.3.7.A. and B.).

Healthy ECM is crucial for structural integrity of blood vessels, and metalloproteinase's (MMPs) are among one of the ECM components that regulate the normal functioning and contractile phenotype of VSMCs [321]. In vascular pathology, excessive production of MMPs is proposed to be involved in degradation of elastin and other components of ECM, promoting widespread VSMC proliferation and migration in the vascular remodelling process involved in the development of hypertension [293]. In the present study, MMP-9 expression was significantly increased in HF treated VSMCs, significant decrease was noted in the expression of MMP-9 in VSMCs co-incubated along with the C16 (.5.3.5.B.). Several studies have reported up-regulation of MMP-9 is associated with the increased incidence of vascular calcification [322, 323]. Here for the first time, we report the involvement of PKR pathway in vascular calcification in HF incubated VSMCs and its attenuation by selective PKR inhibition by imoxin co-incubation in HF-treated VSMCs (Fig.5.3.8.A.). A possible explanation for calcification observed in our findings could be the decreased elasticity evidenced by up-regulation in MMP-9 activity and the degradation of matrix components which may contribute to the poor elasticity and higher vascular stiffness- a characteristic hallmark of phenotypic modulation noted in hypertension [324].

In conclusion, the salient findings of our study demonstrate that PKR is a major contributor involved in HF-induced vascular inflammation and remodelling. This inflammatory and proliferative behaviour could be mediated in part by activation of NF-ķB and $ERK_{1/2}$/MMP-9 pathway in a PKR dependent manner. Additionally, we also propose that these PKR mediated perturbations are blocked by selective inhibition of PKR by imoxin. Therefore, experimental evidence of our studies indicates the significant role of PKR in the development of vascular pathology and targeting PKR in vascular pathology could be a beneficial approach in combating the destructive events associated with it.

SUMMARY



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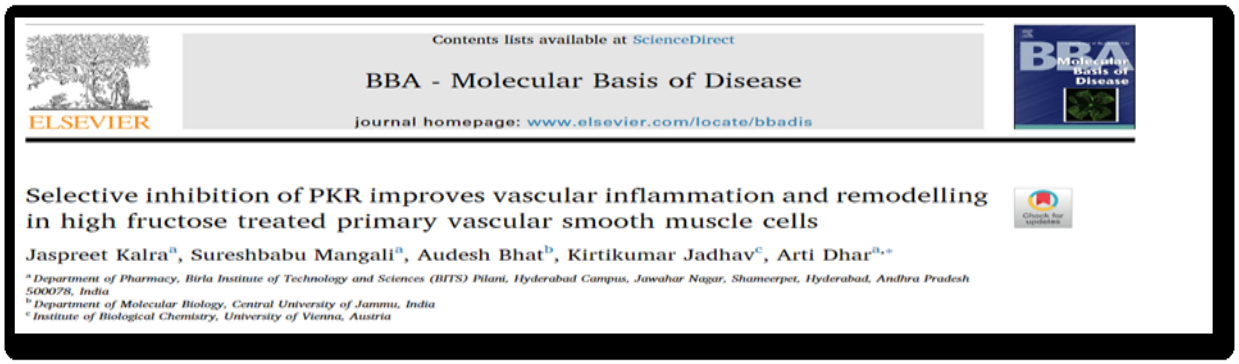

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Selective inhibition of PKR improves vascular inflammation and remodelling in high fructose treated primary vascular smooth muscle cells

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5.4. UPREGULATION OF PKR PATHWAY CONTRIBUTES TO RENAL INFLAMMATION AND TUBULAR APOPTOSIS IN L-NAME INDUCED EXPERIMENTAL HYPERTENSION

PKR mediates inflammatory signalling through NF-κB and JNK activation [26,27], promotes fibrosis and cell death. Extensive research implicates the role of PKR in obesity and T2D in several in-vitro, in-vivo, and gene knock out model [25,20]. Till present, the role of PKR in hypertensive nephropathy and tubular calcification has not studied. Impairment of renal function as a consequence of hemodynamic and cellular factors during hypertensive damage causes the replacement of functional nephrons by fibrotic lesions [325]. The RAS is considered as the critical factor known for BP regulation, volume homeostasis, and an important risk factor for end-organ damage occurring due to HTN through direct effects on cardiac, vascular, and renal tissues [326]. Increased RAS activity is a pathogenic hallmark of HTN and kidney damage [327]. These multifaceted pathologies emerge to stimulate a harmful cycle of swelling, inflammation and fibrosis. Interstitial inflammation, fibrotic scarring, glomerular damage and tubular cell apoptosis are some of the final outcome of this inflammatory cascade [328]. Emerging evidence shows that Ang-II promotes inflammation and is a key mediator of HTN associated kidney damage. It also acts as an essential contributor to trigger ROS production, stimulate cell growth factors, and evoke the release of inflammatory mediators [329-331]. Ang-II in-vivo causes increase in expression of TNF-α, alongwith the increase in numerous other pro-inflammatory mediators, such as NF-κB, JNK, MAPK's pathways which are well correlated with the increased interstitial inflammatory cells in hypertensive kidney [332, 333]. Intriguingly, crosstalk between the role of vasoconstrictor, Ang-II and fibrosis promoter TGF-β1 has been well recognized in the animal models of renal

fibrosis [334]. Ang-II/NF-κB and TGF-β1 pathways are interconnected in the progression of inflammation, fibrosis and disease worsening owing to their capability to facillitate the recruitment and accumulation of inflammatory cells into the glomerulus as well as into the tubulointerstitium. These mediators promote the massive synthesis of ECM and its components that are essentially required to minimize the effects of proteases that are critical for ECM degradation [335]. Previously, we have successfully demonstrated the association of PKR in activating inflammatory responses, cellular apoptosis and phenotypic transition in cultures of NRK-52E and VSMCs [224, 336]. In continuation of our previous study we are the first to establish the role of PKR in L-NAME induced hypertensive nephropathy. Therefore we aimed to examine: (i) Pathogenic association of PKR pathway in hypertensive nephropathy using in-vivo model of HTN; (ii) Pathomorphologic changes associated with PKR activation that comprise renal inflammation, fibrosis and its fundamental mechanism; (iii) The effects of PKR up-regulation on nephrocalcification, tubular cell death, and underlying mechanism

Experimental Animals

Thirty-two, eight weeks old male Wistar rats with a weight range of 180-230 g were employed in the present experiment. Animals were procured from registered CPCSEA breeders. They were maintained following the recommended laboratory conditions having free excess to food and water ad libitum. Before the start of the experiment, animals were allowed to habituate to laboratory environmental conditions for one week. Recordings for non-invasive blood pressure (NIBP) were taken between regular working hrs i.e between 9:00 and 17:00 h. For usage of the experimental animal's ethical clearance with a protocol no BITS-HYD/IAEC/2017/06 was issued by Institutional Animal Ethics Committee (IAEC), BITS Pilani, Hyderabad Campus, India.

Induction of hypertension and experimental protocol

To induce experimental HTN, rats were administred with a (40 mg/kg b.w, p.o) daily dose of L-NAME for four weeks [337]. Imoxin/C16 (0.5mg/kg, i.p) was administered concomitantly for four weeks. Animals were indiscriminately allocated into the respective experimental groups with n=8 rats in each group: Group I:

control (CON), Group II: L-NAME (40mg/kg, p.o), Group III: L-NAME+C16 (same dose of L-NAME was administered with concomitant administration of C16 (0.5mg/kg, i.p), Group IV: C16 only (0.5mg/kg, i.p).

RESULTS

Development of experimental HTN in L-NAME administered rats and subsequent nephropathy, attenuation by imoxin

The overall increase in mean aortic blood pressure (MAP) and elevated serum creatinine and BUN levels are the characteristic hallmark of hypertensive nephropathy. The previous report suggests that the severity of kidney damage associates to the HTN related magnitude of arterial pressure exposure of renal microvasculature. Under conditions where an increase in aortic pressure is longstanding and exceeds the maximal threshold of compensatory mechanisms, it transmits to the glomerular capillaries. Increased pressure in glomerular capillaries causes glomerular injuries with fibrinoid necrosis [338]. In the present study, rats were administered orally with L-NAME to induce HTN resulting from inhibition of NOS. Significantly increased MAP, change in MAP (Fig.5.4.1.A and B.), serum creatinine and BUN values (Table.3.) along with the reduced expression of eNOS (Fig.5.4.1.C.), were noted in the L-NAME rats, against the comparison with control rats, attenuation was observed with imoxin treatment. Alterations in these parameters suggested chronic renal impairment due to the development of HTN.

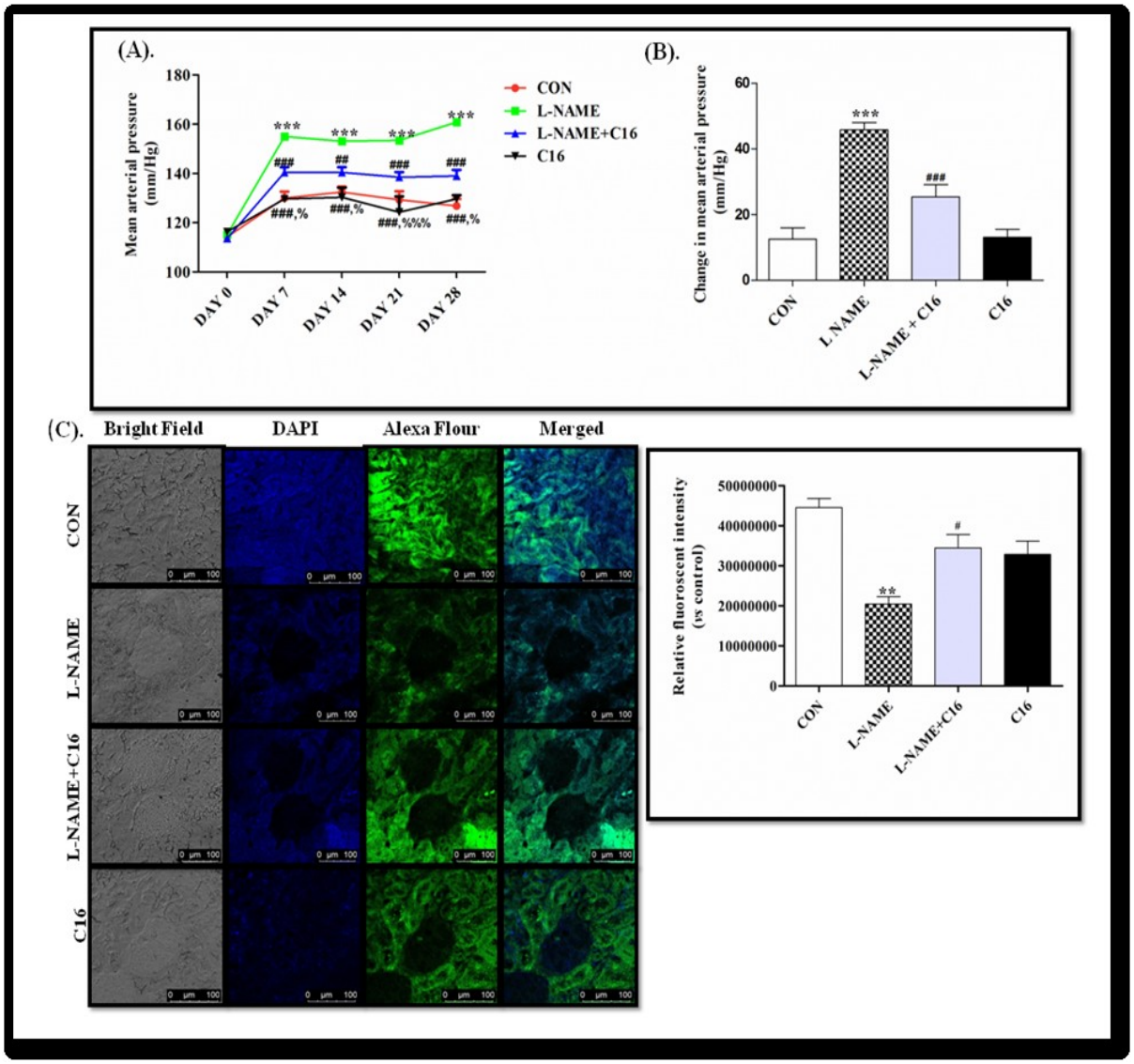


Fig.5.4.1. Induction of experimental hypertension, associated kidney damage and attenuation by imoxin (C16): Fig.5.4.1.A. Mean arterial blood pressure (MAP) was recorded for each rat in an individual group using non-invasive blood pressure measurement technique, n=6. Fig.5.4.1.B. Represents change in MAP following the induction of hypertension from day 0-day 28, n=6. Two-way ANOVA followed by Bonferroni post hoc analysis was used for statistical analysis. Data expressed as mean ±SEM: *P<0.05, **P<0.01, ***P < 0.001 vs. CON, #P < 0.05, ##P<0.01, ###P < 0.001 vs. L-NAME+C16 treated group. Fig.5.4.1.C. Immunohistochemical analysis was done for determination of the expression of eNOS in L-NAME treated renal tissues, attenuation by imoxin, n=3, scale bar represents 100µm. n=3, one-way ANOVA followed by Bonferroni post hoc analysis was used for statistical analysis. Data expressed as mean ±SEM: *P<0.05, **P<0.01, ***P < 0.001 vs. CON, #P < 0.05, ##P<0.01, ###P < 0.001 vs. L-NAME+C16 treated group.

EXP GP	Serum Cr (mg/dl)		Serum BUN (mg/dl)	
	DAY 0	DAY 28	DAY 0	DAY 28
CON	0.67±0.0723	0.79±0.0374	0.46±0.0867	0.42±0.0629
L-NAME	0.69±. 0.0948	1.41±0.7467***	0.48±0.0725	1.57±0.4664***
L-NAME+C16	0.72±. 0.0875	0.84±0.1004#	0.41±0.0722	1.17±0.4525#
C16	0.69±. 0.074	0.72±0.1123	0.42±0.1080	0.59±0.0711

Table.3.Preliminary assessment of hypertension associated nephropathy: Serum creatinine and BUN values were determined in L-NAME treated rats, attenuation by imoxin, n=6. One-way ANOVA followed by Bonferroni post hoc analysis was used for statistical analysis. Data expressed as mean ±SEM: *P<0.05, **P<0.01, ***P < 0.001 vs. CON, #P < 0.05, ##P<0.01, ###P < 0.001 vs. L-NAME+C16 treated group.

Imoxin attenuates L-NAME triggered PKR expression in renal tissues

PKR is known to get activated under stress conditions. Once activated, it causes oxidative stress and initiates an inflammatory signalling cascade. Results of western blot analysis (Fig.5.4.2.A.), and immunohistochemistry studies (Fig.5.4.2.B.), indicate significant up-regulation in renal PKR expression in L-NAME treated group, we observed attenuation with imoxin administration.

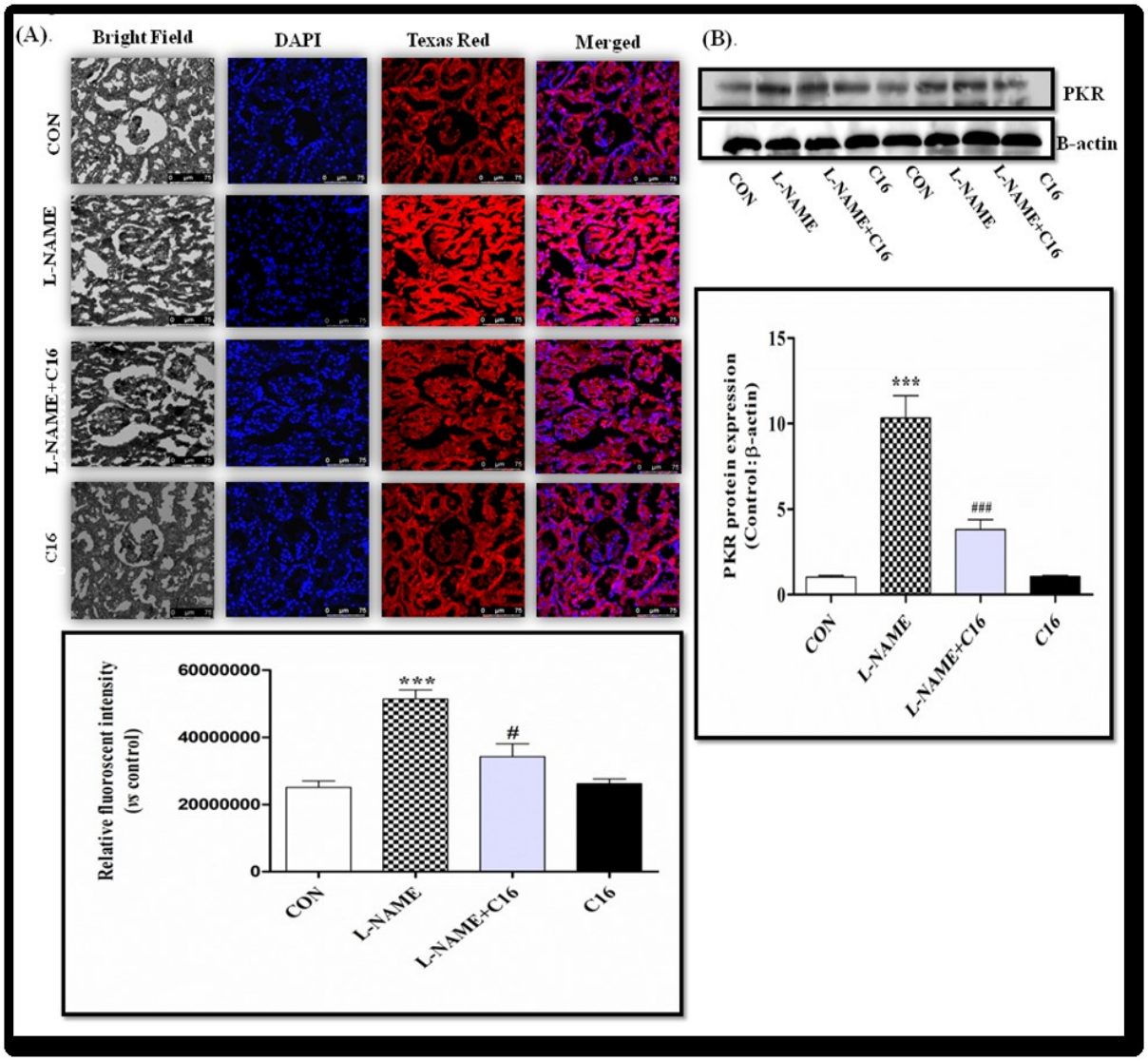


Fig.5.4.2. **Increased PKR expression in L-NAME-treated rats and restoration by imoxin (C16):** Fig.5.4.2.A. Immunohistochemistry was performed for determination of expression of PKR. Upregualtion of PKR in LNAME-administered rats, attenuation by imoxin, n=3. Scale bar represents 75µm. Fig.5.4.2.B. Increase in PKR expression was observed between L-NAME-administered rats and CON rats, attenuation by imoxin, n=4. One-way ANOVA followed by Bonferroni post hoc analysis was used for statistical analysis. Data expressed as mean ±SEM: *P<0.05, **P<0.01, ***P < 0.001 vs. CON, #P <0.05, ##P<0.01, ###P <0.001 vs. L-NAME+C16 treated group.

Imoxin attenuates L-NAME triggered RAS activation

We aimed to look whether PKR activation can cause Ang-II up-regulation or not. Results obtained from western blot and immunohistochemistry reveals significant increase in renal Ang-II (Fig.5.4.3.A. and B.), and its precursor renin ((Fig.5.4.3. C.

and D.), in L-NAME treated rats whereas significant down regulation was reported with the imoxin treatment.

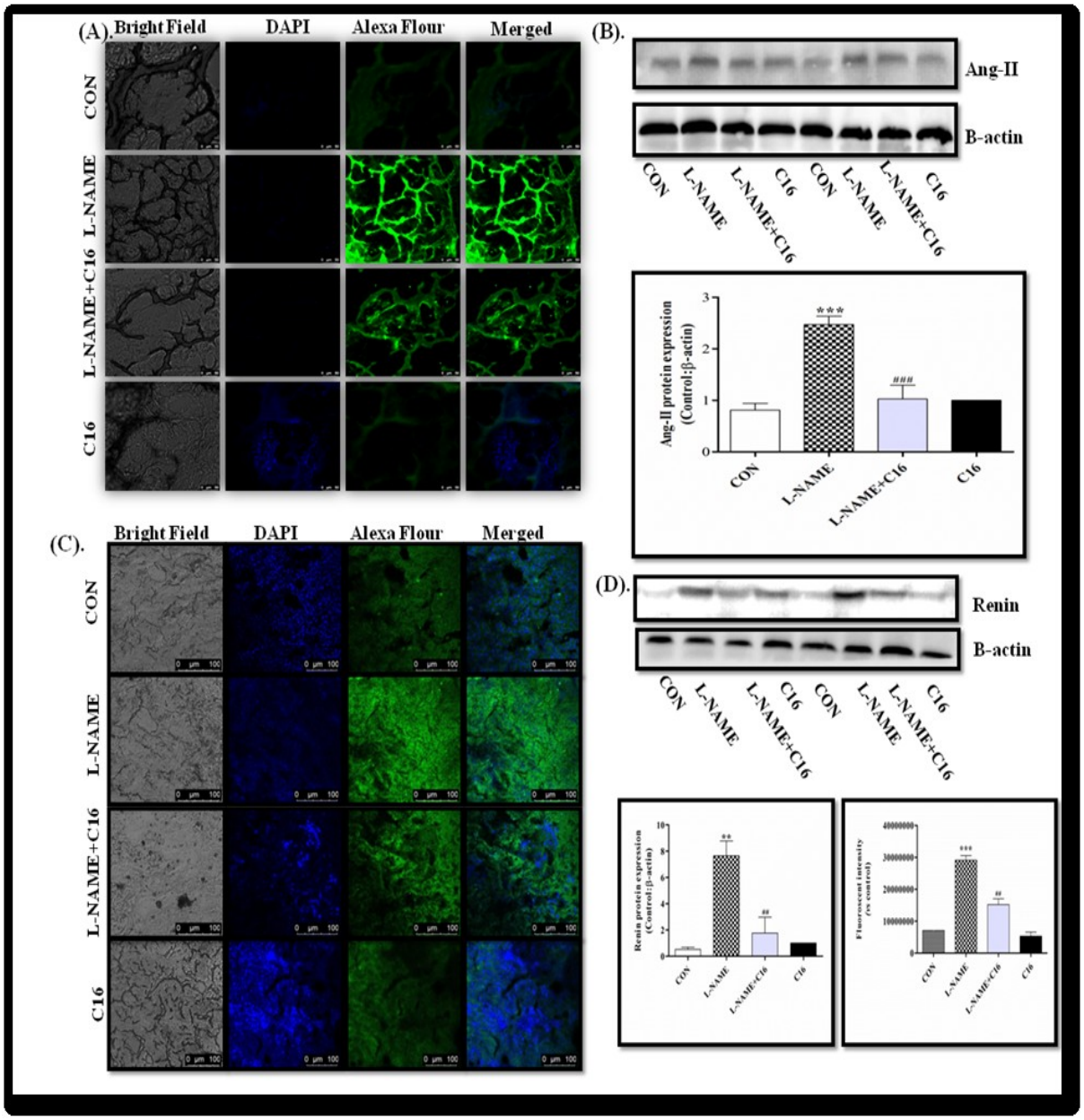


Fig.5.4.3. Increase in angiotensin-II (Ang-II) and renin in LNAME-administered rats, reversal by imoxin (C16): Fig.5.4.3.A. Immunohistochemistry reveals increase in Ang-II expression in L-NAME treated rats compared to CON rats, significant attenuation by imoxin, n=3, scale bar represents 50µm. Fig.5.4.3.B. Demonstration of increased Ang-II protein expression by western blotting in L-NAME group compared to CON group, significant attenuation by imoxin n=4. Fig.5.4.3.C. Immunohistochemistry demonstrates increase in renin expression in L-NAME group compared to CON, significant inhibiton was achieved with imoxin treatment, n=3, scale bar represents 100µm. Fig.5.4.3.D. Demonstration of increased renin protein expression by western blotting in L-NAME group compared to CON rats, significant attenuation by imoxin n=3. One-way ANOVA followed by Bonferroni post hoc analysis was used for statistical analysis. Data expressed as mean ±SEM: *P<0.05,

**P<0.01, ***P < 0.001 vs. CON, #P < 0.05, ##P<0.01, ###P < 0.001 vs. L-NAME+C16 treated group.

Imoxin attenuates L-NAME induced tubulointerstitial fibrosis, phenotypic transition by inhibition of TGF-β, and expression of AGEs in rat kidney

Fibrogenic TGF-β is a core contributor in enhancing ECM synthesis. It is an essential paramount that contributes to glomerulosclerosis and interstitial fibrosis. In addition to this AGEs are also known to promote fibrosis via enhancement of ECM deposition [339]. We observed enhanced fibrosis in L-NAME kidney (Fig.5.4.4.A.), along with the substantial increase in TGF-β (Fig.5.4. 4.B.), and AGEs (Fig.5.4.5.E.), in L-NAME treated rats. However, we observed considerable improvement in fibrosis with imoxin treatment. We relate this improvement with the inhibition of TGF-β and AGEs in a PKR dependent manner. After that, we hypothesized that ECM accumulation also promotes fibrosis deposition, causing the phenotypic transition of interstitial fibroblast to myofibroblast and collagen loading. α-SMa is one of the crucial indicators for the same. We report a significant increase in α-SMa expression (Fig.5.4.4.C.), and collagen deposition (blue coloration deposits, (Fig.5.4.4.D.), in kidney samples of L-NAME treated rats, attenuation was observed with imoxin treatment.

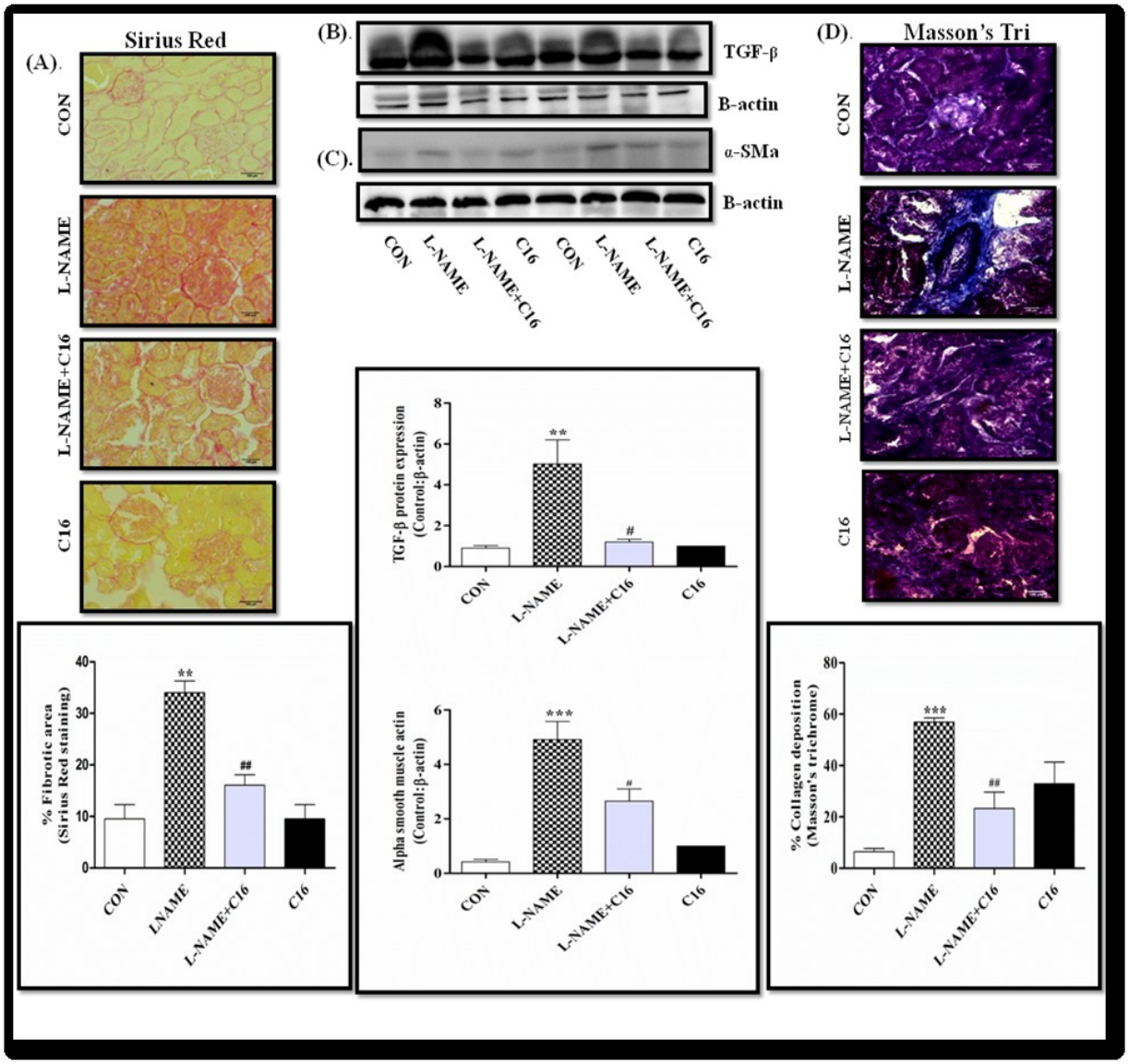


Fig.5.4.4. Imoxin attenuates L-NAME induced tubulointerstitial fibrosis, phenotypic transition by inhibition of TGF-β in rat kidney: Fig.5.4.4.A. Sirius red staining was performed for assessment of tubulointerstitial fibrosis. L-NAME group signifies enhanced fibrosis in L-NAME group vs CON, attenuation with imoxin, n=3, scale bar represents 100µm. Fig.5.4.4.B, Upregulation in TGF-β is reported in L-NAME group inhibition by imoxin (C16), n=3. Fig.5.4.4.C. Increased expression of α-SMa is a characteristic feature of fibroblastic transition into myofibroblasts. Increase in α-SMa was observed in L-NAME group, inhibiton by imoxin (C16), n=3. Fig.5.4.4.D. Masson's trichrome staining was performed to determine collagen deposition in L-NAME treated rat kidney. Increased cell collagen accumulation is evident by remarkable blue coloration in L-NAME-treated rats, attenuation by imoxin (C16), n=3 for each treatment, scale bar represents 100µm. One-way ANOVA followed by Bonferroni post hoc analysis was used for statistical analysis. Data expressed as mean ±SEM: *P<0.05, **P<0.01, ***P < 0.001 vs. CON, #P < 0.05, ##P<0.01, ###P < 0.001 vs. L-NAME+C16 treated group.

Imoxin attenuates L-NAME induced inflammatory markers

PKR mediates inflammation through the activation of NF-ķB and JNK. Significant up-regulation was reported in the expression of p-JNK (Fig.5.4.5.A.), JNK (Fig.5.4.5.B.), in L-NAME group, attenuation was observed with imoxin administration. Intriguingly, NF-ķB (Fig.5.4.5.C.) and TNF-α (Fig.5.4.5.D.) were also found to be up-regulated in L-NAME treated rats followed by its attenuation with imoxin co-treatment.

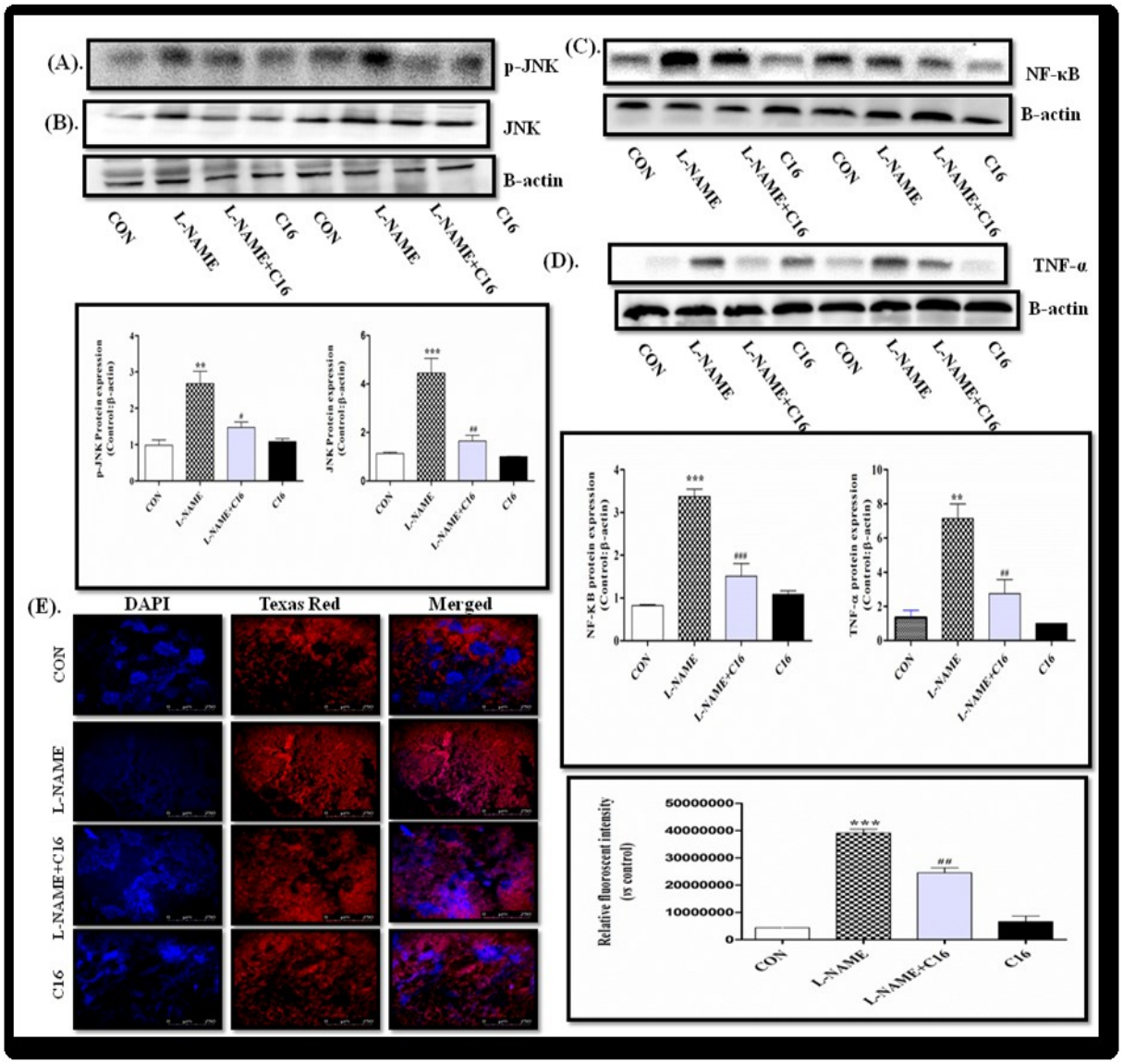


Fig.5.4.5. L-NAME up regulates inflammatory responses and expression of AGEs in rat kidney, attenuation by imoxin: PKR is a known mediator of inflammation. Fig.5.4.5.A. Increased expression of p-JNK: marker for renal inflammation was reported in L-NAME-treated rats, attenuation by imoxin, n=3. Fig.5.4.5.B. Western blot for total JNK was also performed for further confirmation. Higher expression of JNK was noted in L-NAME rats, attenuation by imoxin, n=3. Fig.5.4.5.C. Western

blot for NF-ķB expression demonstrates significant up regulation in L-NAME-treated rats, inhibition by imoxin, n=3 for each treatment. Fig.5.4.5.D. Western blot results for TNF-α expression demonstrates significant increase in its protein expression in L-NAME-treated rats, inhibition by imoxin. n=3 for each treatment. Fig.5.4.5.E. Increase in AGEs expression was revealed by immunohistochemistry in L-NAME treated rats compared to CON rats, inhibition was achieved by imoxin. n=3, scale bar represents 250µm. One-way ANOVA followed by Bonferroni post hoc analysis was used for statistical analysis. Data expressed as mean ±SEM: *P<0.05, **P<0.01, ***P < 0.001 vs. CON, #P < 0.05, ##P<0.01, ###P < 0.001 vs. L-NAME+C16 treated group.

Imoxin attenuates L-NAME induced pathomorphological changes

Activated RAS and inflammatory mediators are also known to be associated for inducing pathomorphological changes. Glomerular HTN causes an increase in glomerular capillary stretching, damage to renal endothelial cells and highly increased protein filtration ultimately leading to the glomerular collapse, focal segmental necrosis and glomerulosclerosis. Therefore, next, we sought to investigate structural and morphological alterations in kidneys of L-NAME treated rats. We demonstrate significant glomerulosclerosis, tubular dilation, cast formation, and with evident collapsed glomeruli and significant dilation in renal corpuscle in kidney sections of L-NAME administered rats (Fig.5.4.6.A.) against the respective control, inhibition was noted with imoxin co-treatment along with the L-NAME treatment. Earlier reports suggest that glomeruli collapse could be attributed to decreased renal perfusion, whereas increased glomerular capillary pressure leads to either glomerular sclerosis or necrosis [340]. Extensive hypertrophy as demonstrated in kidneys of L-NAME rat's against control is also a preliminary marker for enhanced fibrosis and increased BP, inhibition was noted with imoxin treatment(Fig.5.4.6.B.)

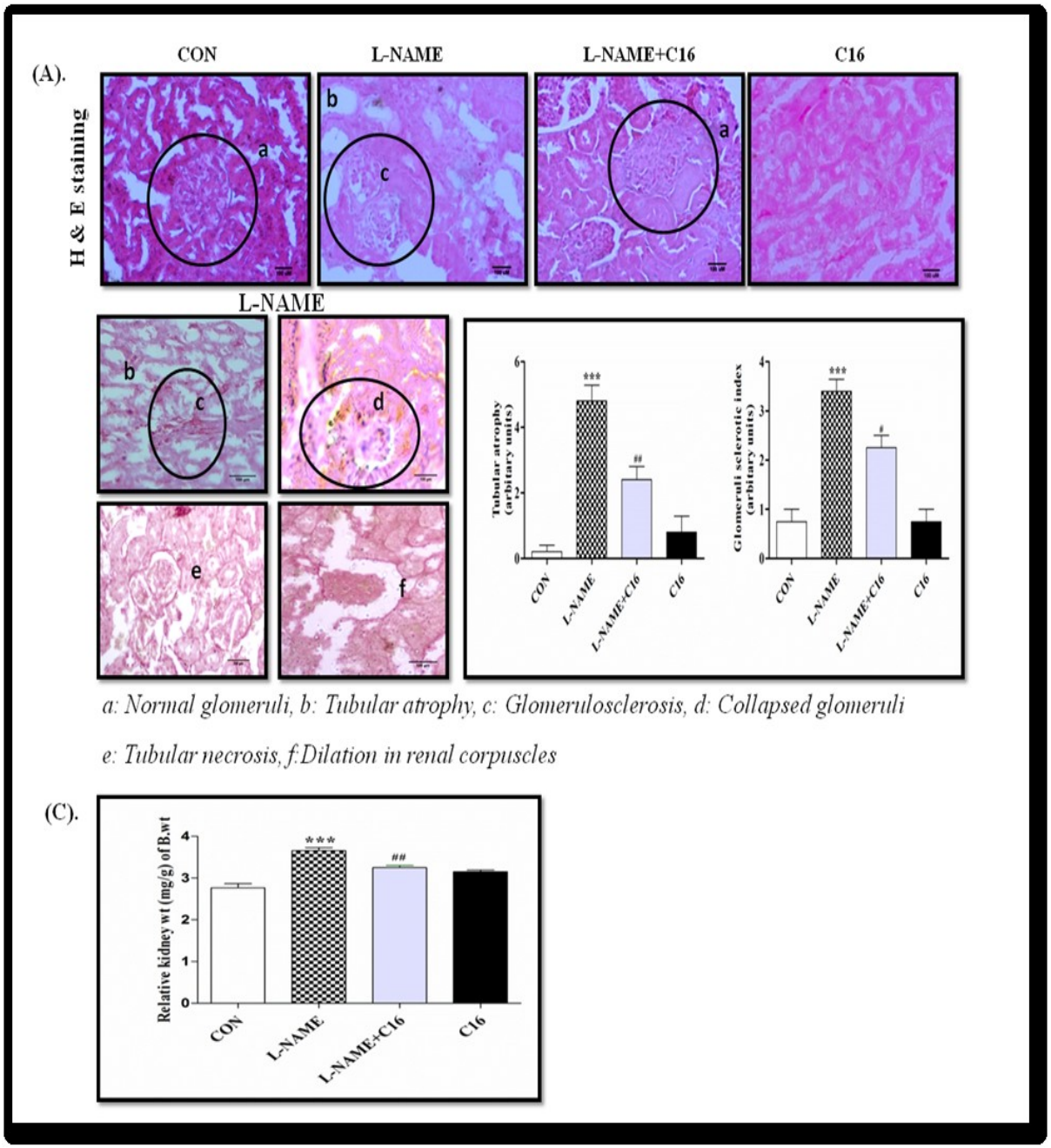


Fig.5.4.6. Imoxin attenuates L-NAME induced tubular atrophy, glomerulosclerosis and kidney hypertrophy: Fig.5.4.6.A. Result of Hemtoxylin and Eosin staining: Significant tubular atrophy was observed in sections of L-NAME-treated rat kidney, attenuation by imoxin, n=5, scale bar represents 100µm. Fig.5.4.6.B. Increased incidence of glomerulosclerosis was observed in sections of L-NAME-treated rat kidney, attenuation by imoxin n=5, scale bar represents 100µm. Fig.5.4.6.C. After sacrifice rat kidneys were isolated, decapsulated and dried on gauge and weighed. Relative kidney weight was calculated as a measure of index for kidney hypertrophy, n=5. One-way ANOVA followed by Bonferroni post hoc analysis was

used for statistical analysis. Data expressed as mean ±SEM: *P<0.05, **P<0.01, ***P < 0.001 vs. CON, #P < 0.05, ##P<0.01, ###P < 0.001 vs. L-NAME+C16 treated group.

Imoxin attenuates L-NAME induced nephrocalcification, tubular cell apoptosis by inhibition of ERK1/2and caspase-3

Ang-II directly exerts its effect on raising intracellular calcium release along with the activation of MAPKs followed by oxidative stress and apoptosis. Based on this assumption we stained L-NAME treated rat kidney sections with alizarin red stain (ARS) and surprisingly we found higher number of calcification centers compared to control, attenuation was observed with selective inhibition of PKR with imoxin (Fig.5.4.7.A). For exploring its underlying mechanism we checked for $ERK_{1/2}$ expression, we report significant increase in $ERK_{1/2}$ in L-NAME rat kidney compared to control, inhibition was achieved with imoxin treatment along with concomitant administration of L-NAME (Fig.5.4.7.B). Finally, based on the above gathered experimental evidences we were interested in unraveling apoptosis, if any is occurring due to the activation and up regulation of above noted markers. Results of our study illustrates that L-NAME treated rat exhibits significant tubular cell apoptosis and apoptotic glomeruli compared to control (Fig.5.4.7.C.), observed apoptosis was further supported by the elevated protein expression of caspase-3, where as attenuation was observed with imoxin treatment along with concomitant administration of L-NAME (Fig.5.4.7.D.),

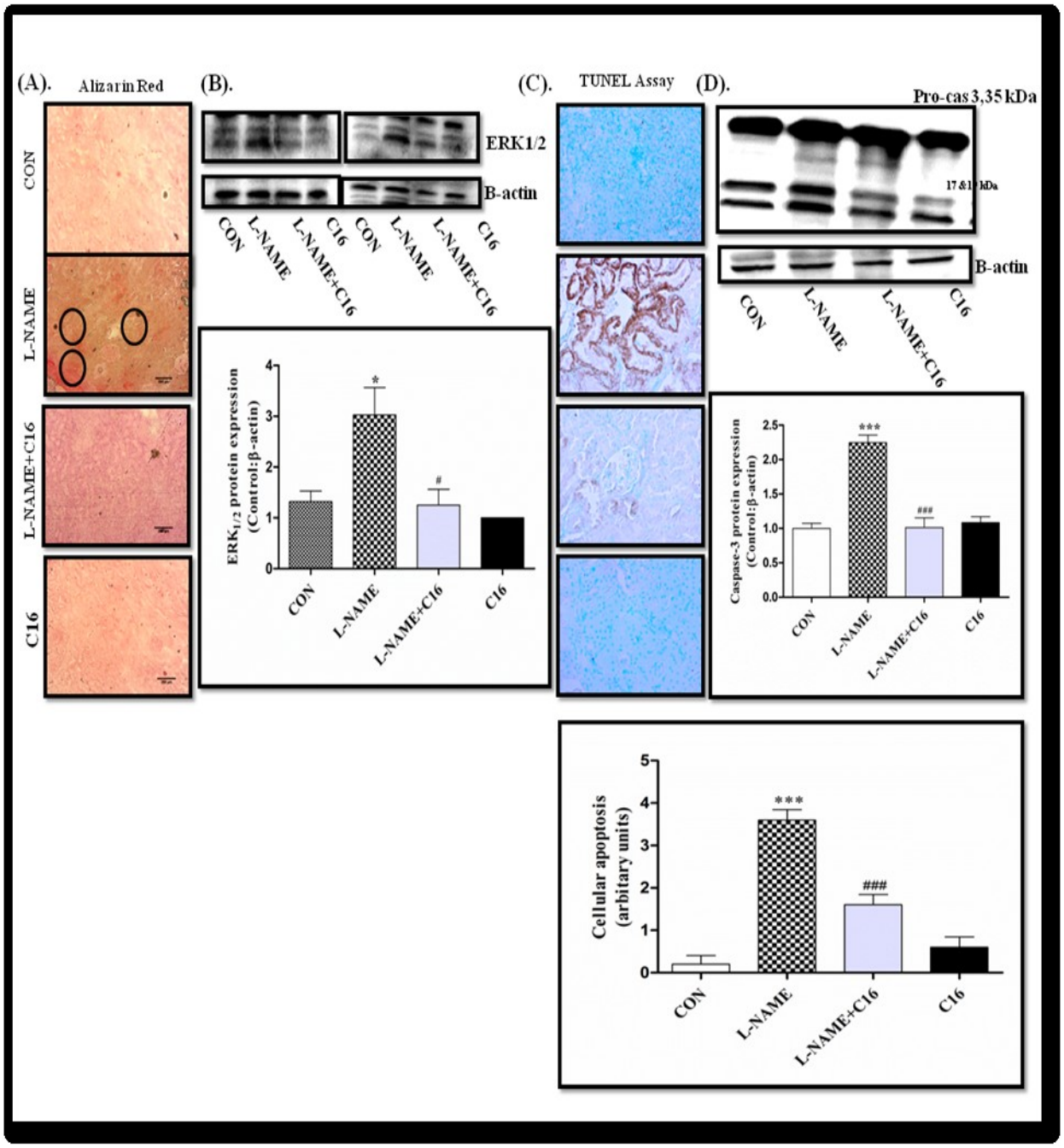


Fig.5.4.7. L-NAME triggered nephro-calcification, increased $ERK_{1/2}$ and cellular apoptosis, inhibition by imoxin. Fig.5.4.7.A. Staining was performed with Alizarin Red. Nephro-calcification in L-NAME rat kidney, inhibition by imoxin, n=3. Scale bar represents 250µm Fig.5.4.7.B. Western blot indicates up-regulation of $ERK_{1/2}$ in L-NAME-treated rat kidney, attenuation by imoxin, n=3. Fig.5.4.7.C. TUNEL assay was performed to quantitate nuclei with fragmented DNA: significant cellular apoptosis has been observed in TUNEL assay in L-NAME-treated rat kidney, attenuation by imoxin, n=5. Fig.5.4.7.D. Western blot was done to determine the caspase-3 expression in L-NAME treated rats, attenuation by imoxin, n=3. One-way ANOVA followed by Bonferroni post hoc analysis was used for statistical analysis. Data expressed as mean ±SEM: *P<0.05, **P<0.01, ***P < 0.001 vs. CON, #P < 0.05, ##P<0.01, ###P < 0.001 vs. L-NAME+C16 treated group.

DISCUSSION

We report that treatment with imoxin (Fig.5.4.2.A. and B), attenuated hypertensive nephropathy associated tubulointerstitial fibrosis and cell apoptosis in L-NAME administered rats by partial attenuation of Ang-II/TGF-β in PKR dependent manner. In consistent with previous studies, we observed chronic NOS inhibition by L-NAME in wistar rats induces HTN in time dependent manner (Fig.5.4.1.). Experimental HTN was confirmed by significant increase in MAP, (Fig.1.A. and B.), in L-NAME treated rats, MAP increase induced by L-NAME was attenuated by selective PKR inhibitor imoxin. Elevated serum creatinine, and BUN values (Table.3.) demonstrated HTN induced nephropathy, attenuation was observed with imoxin administration. Further confirmation of nephropathy was observed by substantial decrease in renal endothelial eNOS expression in L-NAME rats, restoration in eNOS expression was observed with imoxin co-treatment in L-NAME treated rats (Fig.5.4.1.C.).

Chronic activation of RAS is implicated in the deregulation of BP homeostasis and HTN induced kidney damage. Ang-II, one of the byproducts of activated RAS system is a potent vasoconstrictor and inducer of inflammation and fibrosis. In-vivo Ang-II evokes the renal NF-κB expression via up-regulation of several pro-inflammatory genes, initiating a local inflammatory response [330]. Several pieces of evidence indicate complete in-vivo antagonism of Ang-II by treatment with ARB's reduces the expression of NF-κB in renal cells, glomerulus, and tubular cells and has significantly improved BP, glomerular and tubular cell damage [339-341]. Since PKR is a known inducer of inflammation, whether PKR influences the expression/activity of Ang-II which in turn partly contributes to deleterious effects of PKR in L-NAME induced hypertensive nephropathy is still not known. We observed Ang-II up-regulation in kidney harvested from L-NAME treated rats and attenuation by imoxin (Fig.5.4.3.) which confirms PKR to be upstream of Ang-II in induction of hypertensive nephropathy.

PKR evokes inflammatory responses through NF-κB and MAPKs. Members of MAPKs such as $ERK_{1/2}$ and JNK are crucial regulators responsible for cellular proliferation and apoptosis [342]. NF-κB activation and pathogenic role of JNK has

been well described in renal diseases [343]. We reported that PKR activation in L-NAME treated rats caused significant up regulation in Ang-II together with the significant increase in its precursor renin (Fig.5.4.3.A.&B.) (Fig.5.4.3.C.&D.), improvement was observed with imoxin treatment. We also report a substantial increase in p-JNK, JNK, NF-κB and TNF-α (Fig.5.4.5.A., and B.), in L-NAME treated rats and attenuation with imoxin. An increase in NF-κB and TNF-α (Fig.5.4.5.C., and D.), in renal tissue is associated with the initiation of local inflammatory responses [6]. Hence, the above results indicate that PKR activates the Ang-II, induces increase in MAP and stimulates local inflammatory pathways and selective inhibiton by PKR diminishes the activity of Ang-II (Fig. Fig.5.4.3.A. and C.)

In the glomerular endothelium, overactivated RAS and TGF-β is the core contributor in the pathogenesis of glomerulonephritis and hypertensive renal damage [345-347]. Next, we aimed to look for PKR associated tubulointerstitial fibrosis and epithelial-to-mesenchymal transition in HTN induced kidney damage. Ang-II promotes the deposition of ECM by the augmented release of endogenous profibrotic growth factors, such as TGF-β [343]. In the present study L-NAME treated rat kidney's showed remarkable kidney hypertrophy **(**Fig.5.4.6.C.), up-regulation of TGF-β and (Fig.5.4.6.B.), increased collagen deposition (Fig.5.4.6.D.), significant inhibition was observed in rats treated with imoxin. Hence, there exists a considerable crosstalk between Ang-II and TGF-β and all of these effects were diminished by selective PKR inhibitor.

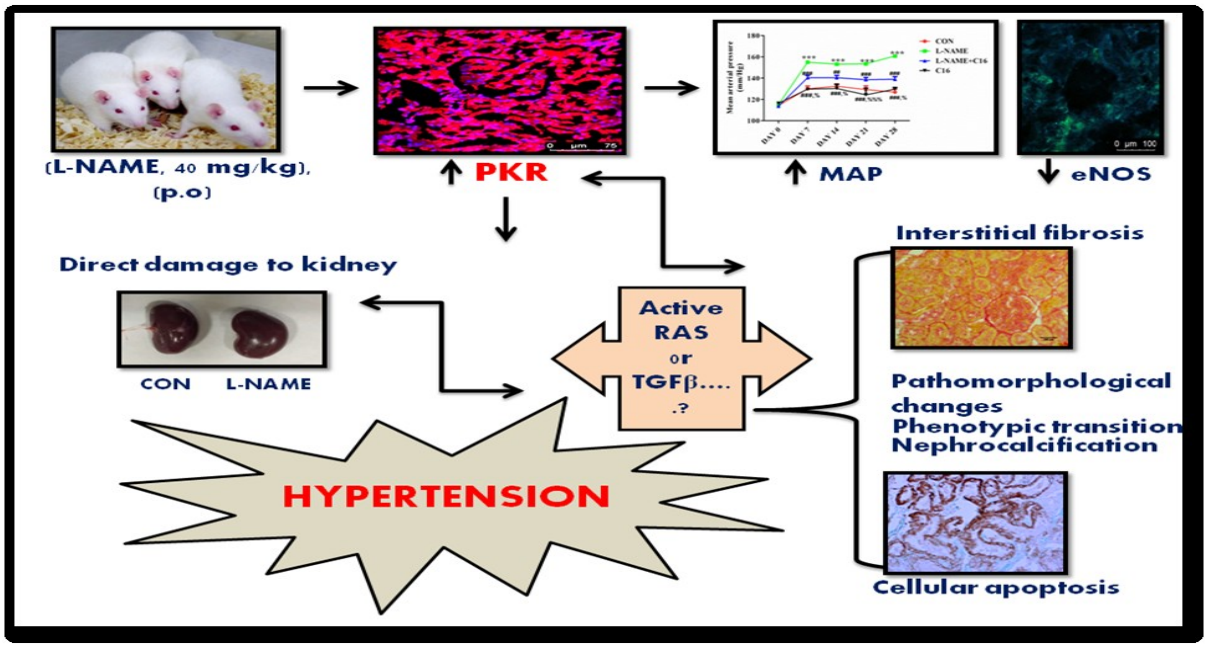
Tubulointerstitial fibrosis and cellular apoptosis are the prominent features of kidney damage. Fibrotic changes in renal tubules are followed by the phenotypic transition of healthy fibroblasts into myofibroblasts, which further promotes ECM deposition and mesenchymal transition. α-SMA is one of the prominent marker which signifies enhanced tubulointerstitial fibrosis [348, 349]. In our study, L-NAME, challenged rats exhibit remarkable tubulointerstitial fibrosis demonstrated by activated Ang-II/TGF-β pathway. Sirius red staining visualizes the significant fibrosis (Fig.5.4.4.A.) increased red coloration deposits in renal tubules and glomeruli), whereas (Fig.5.4.4.C.), shows significant increase in α-SMA expression in L-NAME treated rats; significant reduction in renal fibrosis and α-SMA expression

was observed with imoxin treatment. Patients with glomerular sclerosis and proliferative glomerulonephritis present an increased number of apoptotic glomeruli. The cells of apoptotic glomeruli possess active caspase-3 with rich DNA strand breaks. Similar results were also seen for expression of caspase-3 (Fig.5.4.7.C.), with the presence of TUNEL positive tubular and glomeruli cells, (Fig.5.4.7.D.), attenuation was observed with imoxin treatment. Tubular cells are considered as the vital contributors involved in interstitial fibrosis and scarring [350, 351]. It has been well reported that L-NAME causes severe structural and morphological alterations in rat kidney. Histological examination reveal devastating pathomorphological changes such as increased glomerulosclerosis, and tubular dilation in L-NAME treated rats, significant reversal was observed with imoxin treatment (Fig.5.4.6.A). Ang-II is a crucial contributor implicated in activation of MAPKs. MAPKs such as $ERK_{1/2}$ and JNK including AGEs have been known to regulate proliferation, differentiation, inflammation, and fibrosis. We hypothesize that the observed pathomorphological alterations in the kidney of L-NAME administered rat could be attributed to the Ang-II mediated activation of MAPKs in a PKR dependent manner. Surprisingly, we are the first one to unravel the nephroclacification in L-NAME treated rats, attenuation or zero calcification centers were observed with imoxin treatment (Fig.5.4.7.A). Increased calcium deposits in kidneys of L-NAME administered rats could possibly be due to elevated intracellular calcium levels augmented by overactivated Ang-II and its mediator in a PKR dependent pathway. We suggest that further studies should be carried out for exploring the exact mechanism.

According to Arany et al., NRK-52E cells challenged with cisplatin undergo cellular apoptosis through EGFR/Src/ERK-dependent mechanisms, with no role of JNK in tubular cell death [352]. Contrary to this report, authors of the same group have also reported the protective role of $ERK_{1/2}$ in response to H_2O_2 mediated cellular death. Further, they also proposed that over activated JNK activity is held accountable for cell death and toxicity [353-355]. However, in contrast to both the reports, we found increased expression of p-JNK, JNK as well as for $ERK_{1/2}$ in rat kidney of L-NAME treated rat; attenuation was seen in the expression of p-JNK, JNK

(Fig.5.4.5.A. and B.), $ERK_{1/2}$ (Fig.5.4.7.B.), and AGEs (Fig.5.4.5.E.) with imoxin treatment. Therefore, in light of the above experimental evidences we propose that L-NAME treated rat develop substantial HTN and associated nephropathy through a marked up regulation of Ang-II/TGF-β in a PKR dependent manner. Successful reversal of HTN, and pathomorphological changes in kidney was achieved through selective inhibition of PKR with imoxin in L-NAME administered rats. Hence, this study presents PKR as a potential therapeutic target for HTN and associated renal complications.

SUMMARY



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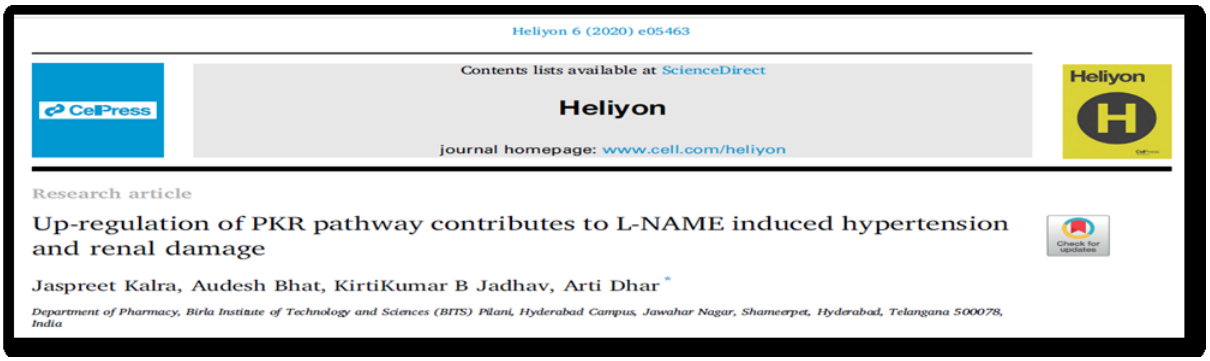


5.5. In-Vivo Inhibition of PKR Improves Blood Pressure Homeostasis, And Cadiovascular Remodelling In L-NAME Treated Wistar Rats And Ex-Vivo Modulates Endothelium Dependent Relaxation In Rat Aortic Rings

HTN is a common disease worldwide and a significant risk factor for pathological CVDs [356]. Vascular and cardiac remodelling accompanied by fibrosis are the most common complications associated with chronic HTN [2, 3]. Since decades neurohumoral systems are held accountable for HTN induced CV fibrosis, and remodelling. However, the molecular mechanism for unravelling the role of neurohumoral mechanisms, including the RAS and TGF-β1 is still not precisely known [356-360]. Reports from the previous studies suggest that Ang-II and ROS act in a corroborated manner to induce TGF-β1. Further, it is a postulate that Ang-II promotes TGF-β1 release in a time and concentration-dependent manner [357, 361-363]. Once activated, these pathways share standard network and directly promotes cardiac and vascular remodelling through the initiation of inflammatory and fibrotic responses [356-360].

Inflammation associated with PKR activation is coupled with NF-κB, TGF-β, and JNK increase [364, 365], which commonly contribute towards fibrosis and celluar death [366, 367]. Previously we established the role of PKR in mediating damage to cardiomyocyte and renal epithelial cells along with deteriorating effects on VSMCs [368-370]. Till date there exist a gap in research to clearly describe the

association of PKR in the HTN related deregulation of BP homeostasis, endothelial damage, and target organ damage to heart and vasculature.

L-NAME mimics the actual clinical condition of essential HTN in experimental rats [371]. L-NAME produces impaired vascular tone and endothelium-dependent relaxation, produces increase in BP, inflammation accompanied by left ventricular hypertrophy, myocardial fibrosis with intimal thickening and changes of the aortic vessel wall structure [372-375]. During cardiac remodelling process myocardial hypertrophy occurs as a compensatory response against pressure over load to sustain norrmal cardiac function. L-NAME-treated rats found to exhibit myocardial fibrosis [376]. The molecular mechanisms underneath myocardial fibrosis and vice versa activation of downstream inflammatory and [377] and oxidative load carriers are still not explicit [378, 379]. Under L-NAME induced HTN, a pro-inflammatory cytokine, TNF-α gets activated in response to oxidative load. These downstream inflammatory signals act in orchestrated manner to initiate the pro fibrotic mediator TGF-β [380]. Role of TGF-β1 has been well established in fibrogenesis by augmenting apoptotic pathway, collagen deposition, and matrix protein synthesis [381-383]. For alterations in vessel wall structure it has been recognized as an adaptive response to an increase in wall tension [384]. Moreover, it also finds it correlation with degradation of ECM components i.e. elastin fragments, increased expression of MMP-9 in vasculature. Recently we have also reported that MMP-9 acts as a core contributor in vascular remodelling process in-vitro in hypertensive disease condition [369]. L-NAME administration further implicates with the activated RAS, production of superoxides and ROS, which further reduces the availability of nitric oxide and enhances the formation of peroxynitrite [385, 386]. We were determined to explore the PKR pathway in L-NAME induced modulations in BP homeostasis, cardiovascular fibrosis, and aortic wall remodelling.

Hence in the discussed study we aimed to examine and explore: (i) Pathogenic association of PKR pathway in BP homeostasis in L-NAME administered rats; (ii) PKR activation and its affect on endothelium-dependent relaxation and underlying mechanism; (iii) Structural and morphological alterations related with

PKR activation that comprise cardiac and vascular fibrosis and changes in lumen diameter.

RESULTS

Changes in Physiological Parameters: BP, HR, B.wt, and Lipid Profile in L-NAME administered rats, improvement by imoxin

L-NAME inhibits the NOS activity and biosynthesis of NO, causes HTN. L-NAME treatment to rats causes damage to the vascular endothelium, and also produces undesirable CV abnormalities. At the start of the experimental studies baseline SBP and DBP values were almost similar for all groups. Rise in SBP was noted on day 14 in L-NAME treated rats compared to CON, however significant rise in DBP was reported with the disease progression i.e. on day 21 in L-NAME rats compared to CON. This increase in SBP and DBP was sustained till day 28 in L-NAME administerd rats compared to the CON, attenuation in SBP and DBP were observed with imoxin (C16) treatment (Fig.5.5.1.A. and B.). Similarly, no changes in baseline b.wt and HR were observed in any of the experimental groups; however from day 14 onwards significant decrease in B.wt and HR were reported in L-NAME adminstered rats. This decrease was sustained till the end of the experiment in L-NAME rats compared to CON, significant improvement was seen with imoxin administration (Fig.5.5.1.C. and D.). In addition, increased triglyceride levels (Fig.5.5.1.E.), were noted in the L-NAME group, compared to CON, inhibition was observed with imoxin treatment. No changes were reported in HDL and total cholesterol levels among all the groups.

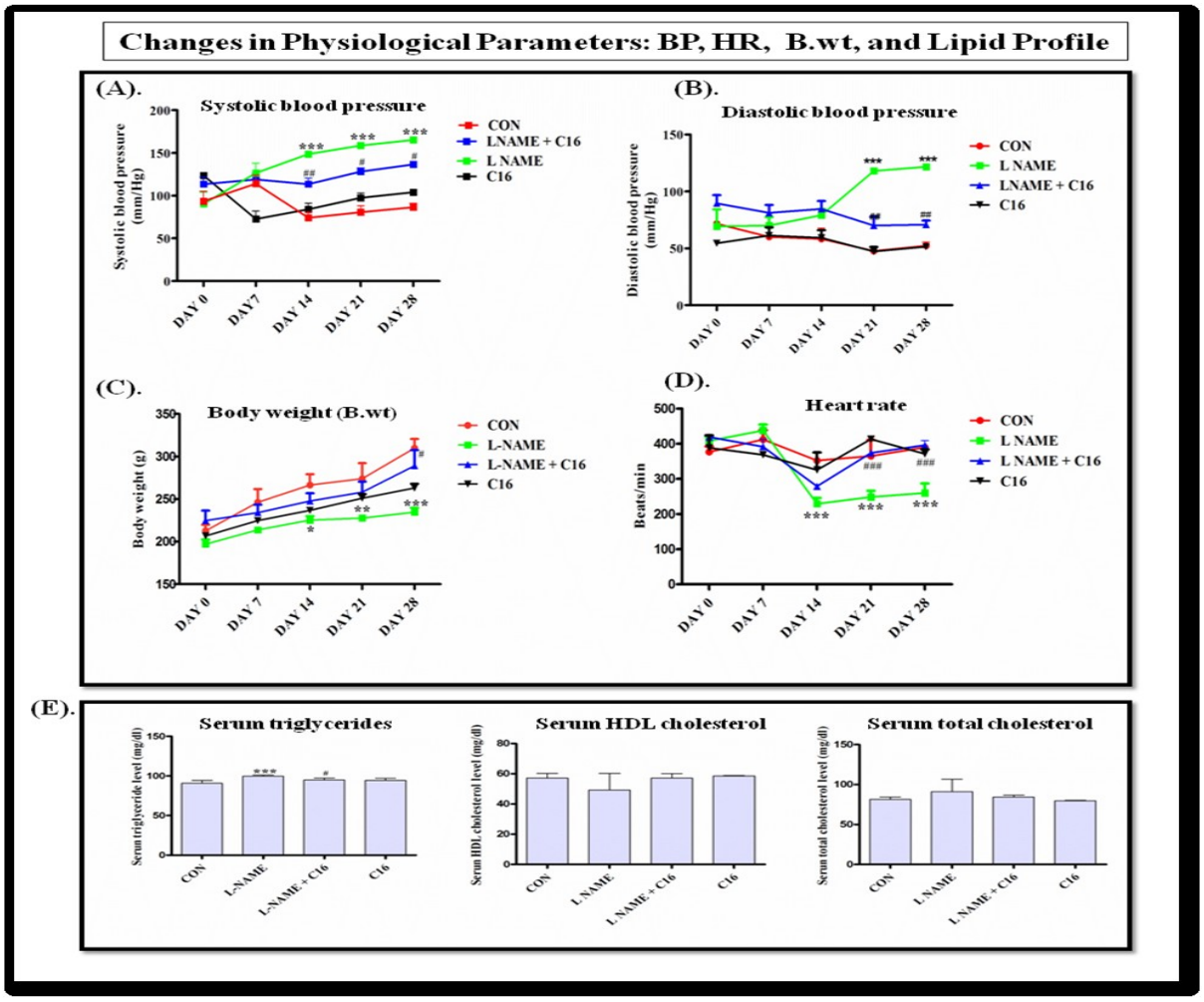


Fig.5.5.1. Changes in Physiological Parameters: BP, HR, B.wt, and Lipid Profile in L-NAME administered rat, improvement by imoxin Fig.5.5.1. (A, B,D) SBP, DBP, and HR was measured for each rat in an individual group using non-invasive tail cuff blood pressure measurement technique, following the induction of hypertension from day 0-day 28, n=6. Fig.5.5.1.C. Body weight (B.wt) for each rat in an individual group was measured on day 0,7, 14, 21 and 28. Two-way ANOVA followed by Bonferroni post hoc analysis was used for statistical analysis. Data expressed as mean ±SEM: *P<0.05, **P<0.01, ***P < 0.001 vs. CON, #P < 0.05, ##P<0.01, ###P < 0.001 vs. L-NAME+C16 treated group. Fig.5.5.1.E. Serum HDL, TGs and total cholesterol values were determined in L-NAME treated rats, attenuation by imoxin, n=6. One-way ANOVA followed by Bonferroni post hoc analysis was used for statistical analysis. Data expressed as mean ±SEM: *P<0.05, **P<0.01, ***P < 0.001 vs. CON, #P < 0.05, ##P<0.01, ###P < 0.001 vs. L-NAME+C16 treated group.

Imoxin attenuates L-NAME triggered PKR expression in heart and aorta

Once activated, PKR causes oxidative stress and initiates an inflammatory signalling cascade. Till date no study describes the association of PKR in HTN induced CV complications. We report connection of PKR in L-NAME induced cardiac and aortic remodelling. Results of immunohistochemistry (Fig.5.5.2.A. and C.), and western

blot (Fig.5.5.2.B.), indicate a significant increase in cardiac and aortic PKR expression in L-NAME treated group, attenuation was observed with imoxin administration.

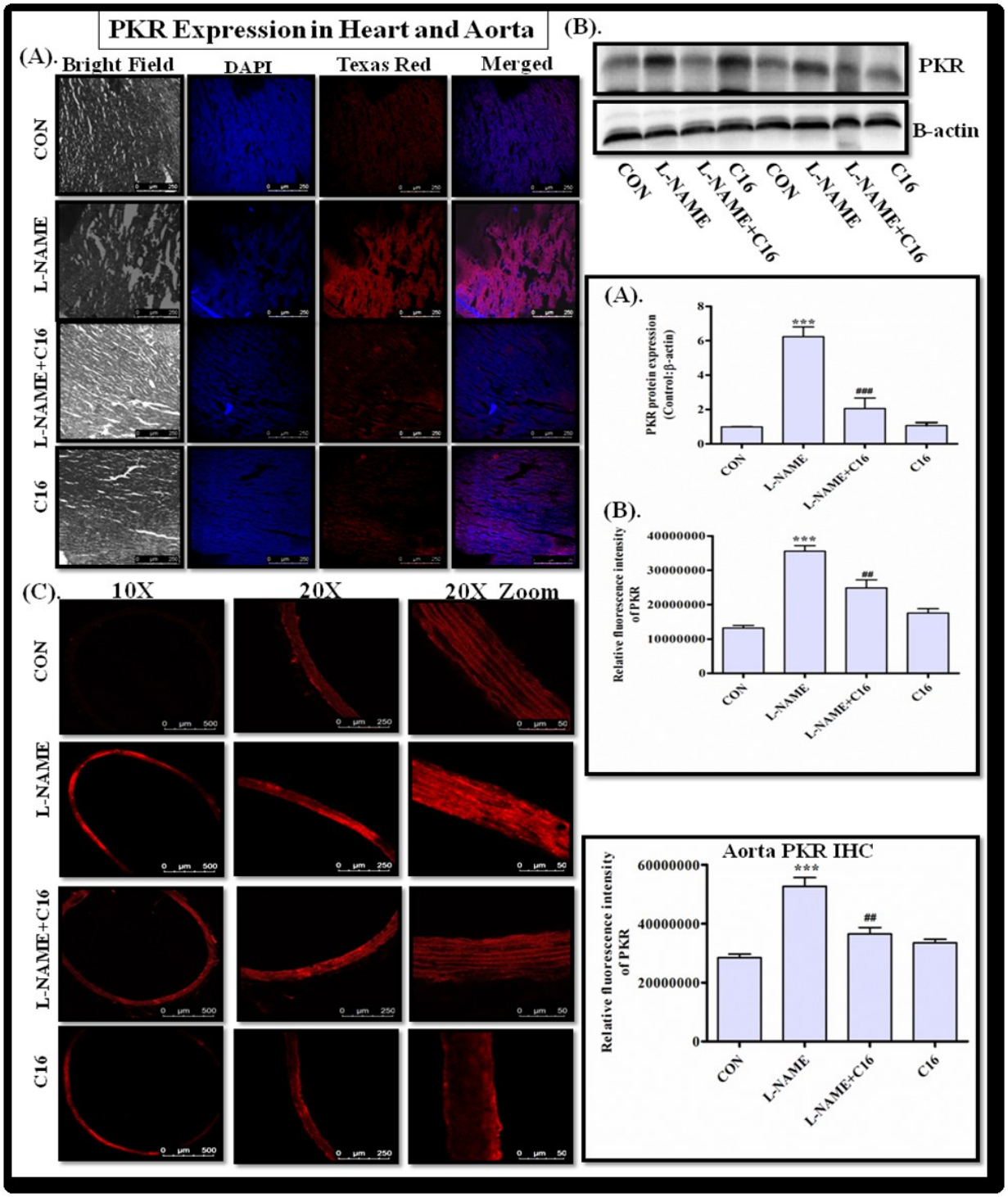
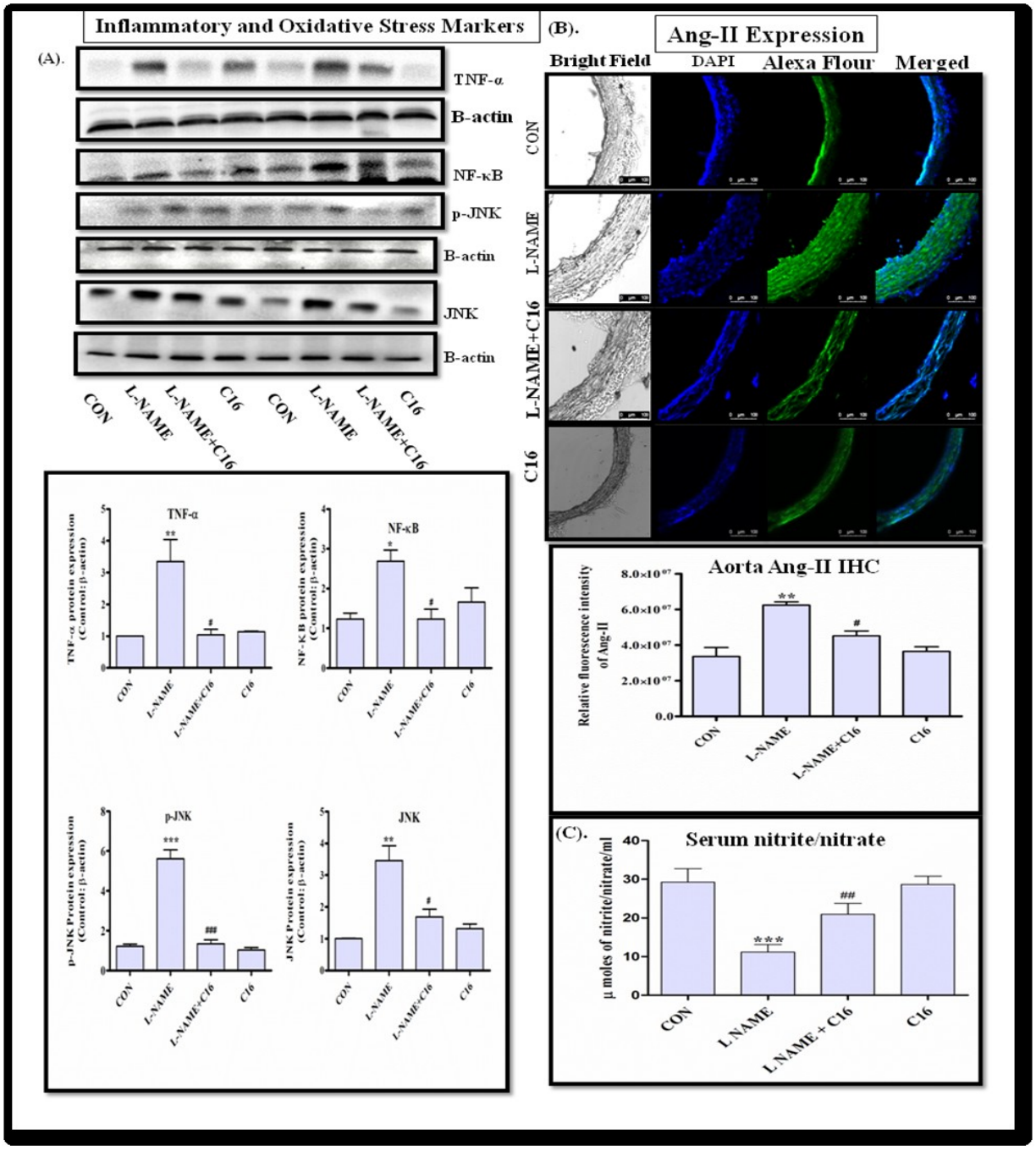


Fig.5.5.2. Imoxin attenuates L-NAME triggered PKR expression in heart and aorta
Fig. 5.5.**2**.A. Immunohistochemistry reveals increased expression of PKR in LNAME rats, inhibition by imoxin, n=3. Scale bar represents 250µm. Fig. 5.5.2.B. Western blot indicates significant differences in PKR expression in L-NAME-treated rats and compared to CON, inhibition by imoxin, n=3. Fig.5.5.2.C. Immunohistochemistry was performed for determination of expression of PKR. PKR expression was increased in LNAME-treated rats, inhibition by imoxin, n=3, scale bar represents

50µm. One-way ANOVA followed by Bonferroni post hoc analysis was used for statistical analysis. Data expressed as mean ±SEM: *P<0.05, **P<0.01, ***P < 0.001 vs. CON, #P <0.05, ##P<0.01, ###P <0.001 vs. L-NAME+C16 treated group.

Imoxin attenuates L-NAME triggered inflammatory mediators and up regulation of Ang-II expression

We were determined to investigate changes in the expression of cytokines (e.g., NF-ķB, and TNF-a), inflammatory and pro-fibrotic factors such as p-JNK, and JNK. Results obtained from western blot reveal increased NF-ķB, TNF-a, p-JNK and JNK expression in L-NAME treated rats whereas significant attenuation was reported with the imoxin treatment (Fig.5.5.3.A.). Ang-II is the principal vasoconstrictor peptide responsible for the maintenance and regulation of BP and electrolyte homeostasis. We report significant increase in Ang-II in rat aorta harvested from L-NAME administered rats compared to CON, attenuation was observed with imoxin treatment (Fig.5.5.3.B.). Increased peroxynitrites are one of characteristic hallmark of oxidative load, elevated serum nitirites/nitrates were reported in L-NAME adminstered rats compared to CON, whereas reduction was observed with imoxin treatment (Fig.5.5.3.C.).



Imoxin attenuates L-NAME triggered inflammatory mediators and up regulation of Ang-II expression Fig.5.5.3.A. Demonstration of increased NF-Ķb, JNK and TNF-α protein expression by western blotting in L-NAME adminstered rats compared to CON rats, significant attenuation by imoxin n=3. Fig.5.5.3.B. Increase in Ang-II expression in L-NAME administered rats compared to CON rats, inhibition by imoxin as demonstrated by immunohistochemsitry, n=3, scale bar represents 100μm. Fig.5.5.3.C.Serum nitrites/nitrate was determined in L-NAME treated rats, attenuation by imoxin, n=6. One-way ANOVA followed by Bonferroni post hoc analysis was used for statistical analysis. Data expressed as mean ±SEM: *P<0.05, **P<0.01, ***P < 0.001 vs. CON, #P < 0.05, ##P<0.01, ###P < 0.001 vs. L-NAME+C16 treated group.

L-NAME induced fibrosis , cardiac and arterial remodelling, increase in heart weight, improvement by imoxin.

In response to inflammatory mediators, activated TGF-β induces characteristic fibrotic lesions and collagen deposition. Next, we aimed to look for plausible changes occurring due to activated TGF-β. Results obtained from Sirius red staining and Masson's trichrome staining reveals excessive fibrosis (enhanced red coloration deposits) (Fig.5.5.4.A.), and collagen deposition (marked blue coloration deposits) (Fig.5.5.4.B.), in heart sections of L-NAME adminstered rats compared to CON, attenuation was reported with the imoxin treatment. In addition to this observed changes in the present study are also accompanied by an increase in relative heart weight compared with the respective CON, attenuation in these effects was inhibited by imoxin (Fig.5.5.4.C.). HTN has been reported to induce changes in intracellular signalling pathways causing activation of numerous genes held accountable for enhanced protein synthesis, cardiomyocyte growth, ultimately causing alterations in vessel wall structure, left ventricular (LV) remodelling and dysfunctioning of myocardium. LV remodelling corresponds to the ventricular changes occurring in response to volume overload or pressure load [13]. Therefore, next, we sought to investigate structural and morphological alterations in heart and aorta of L-NAME treated rats. We demonstrate significant myofibril derangement and myocardial necrosis in heart sections of L-NAME administered rats (Fig.5.5.4.D.), compared to the CON, attenuation was observed with imoxin. In addition to this we also report significant derangement of elastin fragments (Fig.5.5.4.E.)

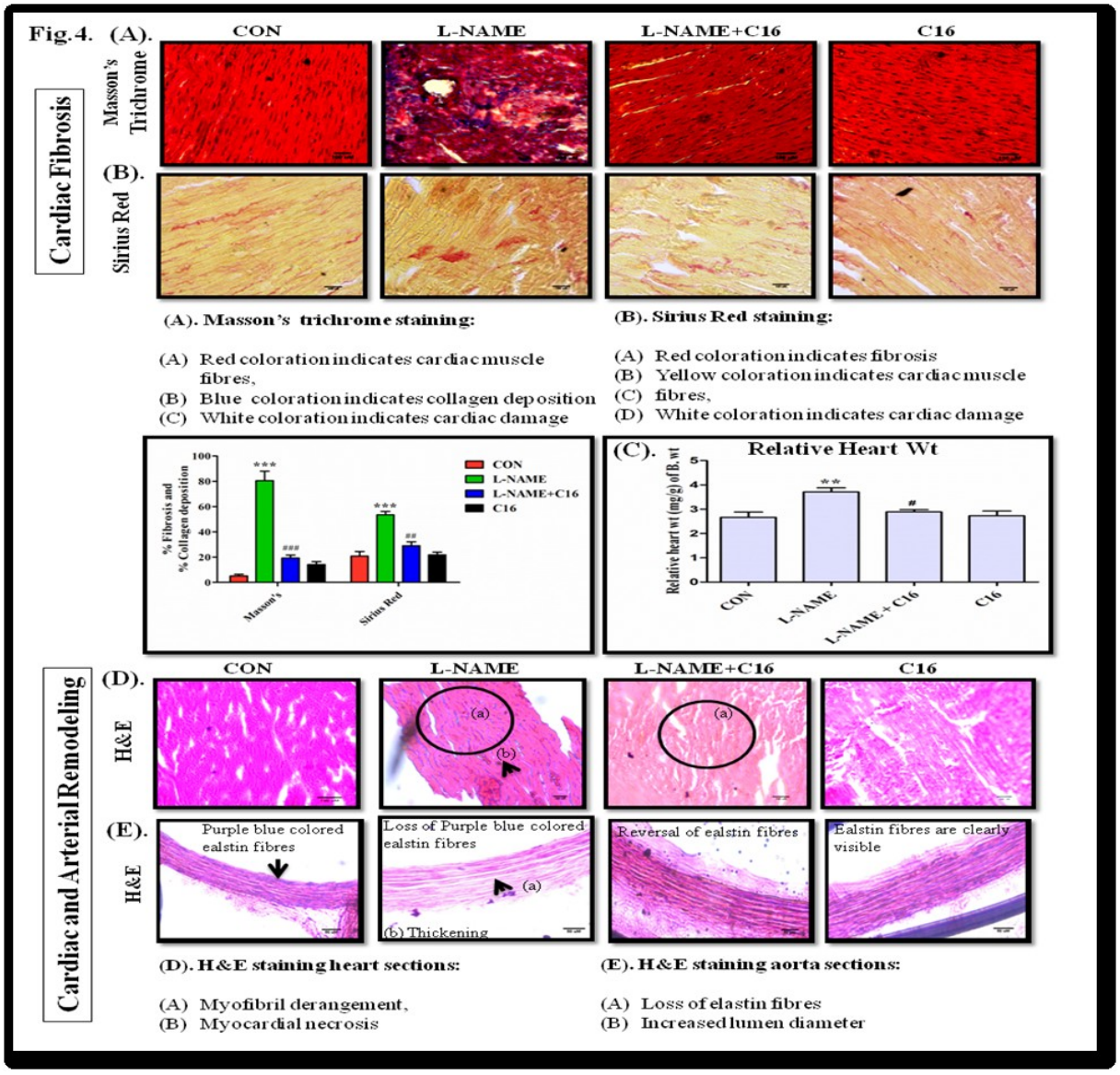


Fig.5.5.4. L-NAME induced fibrosis, cardiac and arterial remodelling, increase in heart weight, improvement by imoxin Fig.5.5.4.A. Masson's trichrome staining was performed to determine collagen deposition in L-NAME treated rat heart. Increased collagen accumulation as evident by remarkable blue coloration was reported in L-NAME-adminstered rats, inhibition by imoxin (C16), n=3 independent experiments, scale bar represents 100µm. Fig.5.5.4.B. Sirius red staining was carried out for determination of cardiac fibrosis. Enhanced fibrosis was reported in L-NAME-adminstered rats vs CON, attenuation with imoxin, n=3, scale bar represents 100µm. Fig.5.5.4.C. Changes in heart weight: preliminary marker of hypertrophy. Increase was observed in L-NAME-adminstered rats, attenuation by imoxin (C16), n=6. Fig.5.5.4.D. Result of Hematoxylin and Eosin staining: Significant myofibril derangement was reported in sections of L-NAME-adminstered rat heart, attenuation by imoxin, n=5, scale bar represents 100µm. Fig.5.5.6.B. Increased incidence of loss of elastin fibres was observed in sections of L-NAME-treated rat aorta, attenuation by imoxin n=5, scale bar represents 50µm. One-way ANOVA followed by Bonferroni post hoc analysis was used for statistical analysis. Data

expressed as mean ±SEM: *P<0.05, **P<0.01, ***P < 0.001 vs. CON, #P < 0.05, ##P<0.01, ###P < 0.001 vs. L-NAME+C16 treated group.

Imoxin attenuates L-NAME triggered TGF-β upregulation in heart and aorta

In addition to raised BP, L-NAME administration causes CV remodelling including myocardial fibrosis, left ventricular hypertrophy, and narrowing of the vasculature walls [16]. Myocardial fibrosis is a complex process which is associated with activation of downstream inflammatory mediators which activates profibrotic TGF-β. Fibrogenic TGF-β actively participates in enhancing ECM synthesis. Results of immunohistochemistry and western blot demonstrate significant increase in TGF-β expression in heart (Fig5.5.5.A. and B.), isolated from L-NAME administered rats, attenuation was reported with imoxin treatment. HTN related structural changes in vessel wall are associated with increased expression of profibrotic markers. TGF-β upregulation was noted in rat aortic tissue sections isolated from L-NAME administered rats compared to CON, significant attenuation was observed with imoxin treatment (Fig.5.5.5.C.).

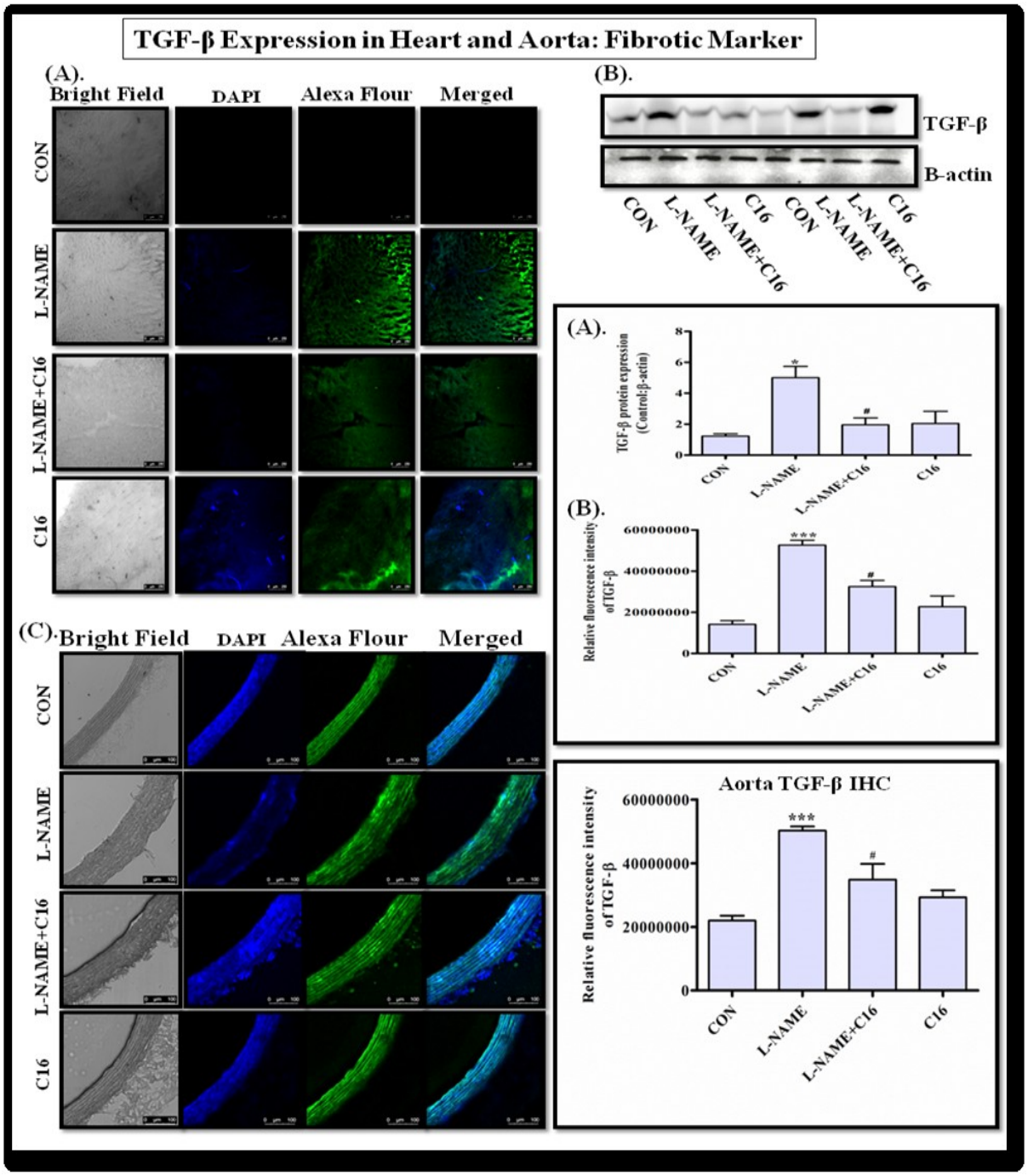


Fig.5.5.5. Imoxin attenuates L-NAME triggered TGF-β expression in heart and aorta Fig.5.55.A. Immunohistochemistry was performed for determination of expression of TGF-β. TGF-β expression was increased in LNAME-treated rats, attenuation by imoxin, n=3. scale bar represents 250µm. Fig.5.5.5.B. Western blot shows significant up-regulation in TGF-β expression in L-NAME-administered rats compared to CON rats, inhibition by imoxin, n=3. Fig.5.5.5.C. Immunohistochemistry was carried out on rat aorta forn TGF-β. TGF-β was upregulated in LNAME-adminstered rats, attion by imoxin, n=3. Scale bar represents 100µm. One-way ANOVA followed by Bonferroni post hoc analysis was used for statistical analysis. Data expressed as mean ±SEM: *P<0.05, **P<0.01, ***P < 0.001 vs. CON, #P <0.05, ##P<0.01, ###P <0.001 vs. L-NAME+C16 treated group.

Imoxin improves endothelium dependent relaxation ex-vivo and eNOS expression and changes in luminal diameter in L-NAME administered rats

Modulations in endothelial function, increased vascular contraction, and arterial remodelling are some of the prominent features of HTN. In the CON group, acetylcholine (Ach) significantly relaxed rat aortic rings pre-contracted with phenylephrine (PhE) (Fig.5.5.6.D). As hypothesised, the four-week treatment of L-NAME considerably reduced endothelium-dependent relaxations, in response to Ach, whereas endothelium dependent relaxation was significantly improved in imoxin treated alone group or when co-administered with L-NAME rats compared to CON, attenuation was observed with imoxin treatment (Fig.5.5.6.C.). eNOS expression was measured in isolated rat aortic tissue sections, we found substantial reduction in eNOS expression in L-NAME adminstered rats compared to CON, however restoration in eNOS expression was observed with imoxin treatment (Fig.5.5.6.B.). Moreover we also observed increased luminal diameter (Fig.5.5.6.C.) in L-NAME administered rats compared to CON, reversal was achieved with imoxin administration.

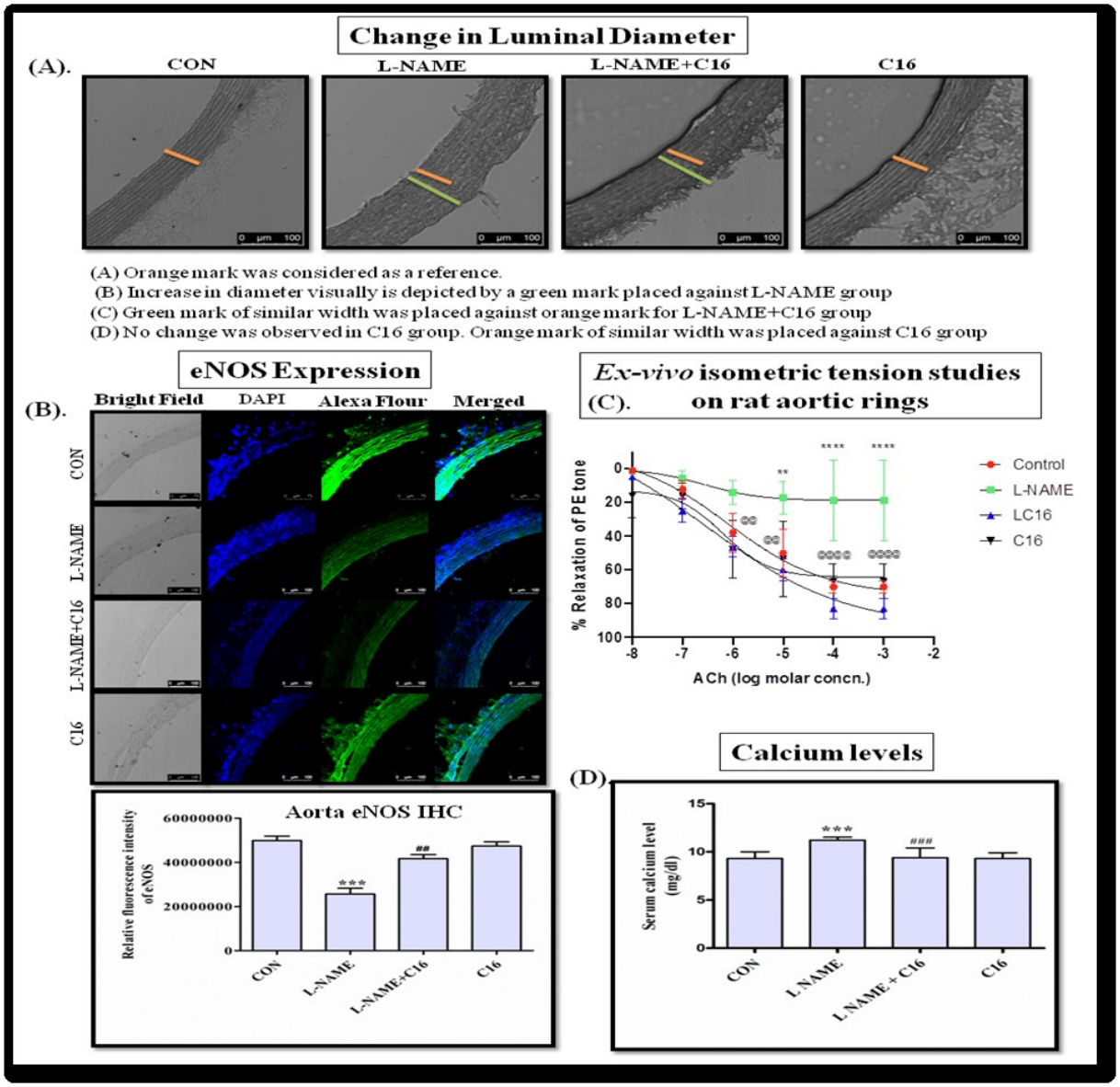


Fig.5.5.6. Imoxin improves endothelium dependent relaxation ex-vivo and eNOS expression and changes in luminal diameter in L-NAME administered rats: Endothelial damage and lumen narrowing is a characteristic feature of progressive HTN Fig.5.5.6.A. Bright field microscopy was performed for rat aorta. Visual increase in lumen diameter was reported in L-NAME-adminstered rats, improvement by imoxin, n=5. Scale bar represents 100µm. Fig.5.5.6.B. Immunohistochemistry was performed for determination of expression of eNOS. eNOS expression was decreased in LNAME-treated rats, restoration by imoxin, n=3, scale bar represents 100µm. Fig.5.5.6.C. Ex-vivo studies on rat aortic rings demonstrates significant decrease in endothelium dependent relaxation in L-NAME-treated rats, attenuation by imoxin,. n = 3 for each treatment. Fig.5.5.6.D. Western blot results for TNF-α expression demonstrates significant increase in its protein expression in L-NAME-treated rats, inhibition by imoxin. n=3 independent experiments were performed. One-way ANOVA followed by Bonferroni post hoc analysis was used for statistical analysis. Data expressed as mean ±SEM: *P<0.05, **P<0.01, ***P < 0.001 vs. CON, #P < 0.05, ##P<0.01, ###P < 0.001 vs. L-NAME+C16 treated group.

Imoxin attenuates L-NAME induced changes in expression of advanced glycation end products(AGEs), expression of CREB and intracellular calcium levels.

According to some reports inhibition of nitric oxide synthase associates with increased vascular remodelling, peroxynitrites and elevated intracellular calcium levels. Based on the previous studies we were determined to examine the effect of imoxin on deposition of AGEs in aorta. We found enhanced deposition of AGEs in aorta of L-NAME administered rats compared to CON, where as significant decrease in AGEs accumulation was noted with imoxin treatment (Fig.5.5.7.A. and B.). Similarly, our findings also demonstrate significantly higher levels of intracellular calcium in L-NAME administered rats compared to CON, attenuation was observed with imoxin treatment (Fig.5.5.6.D.).

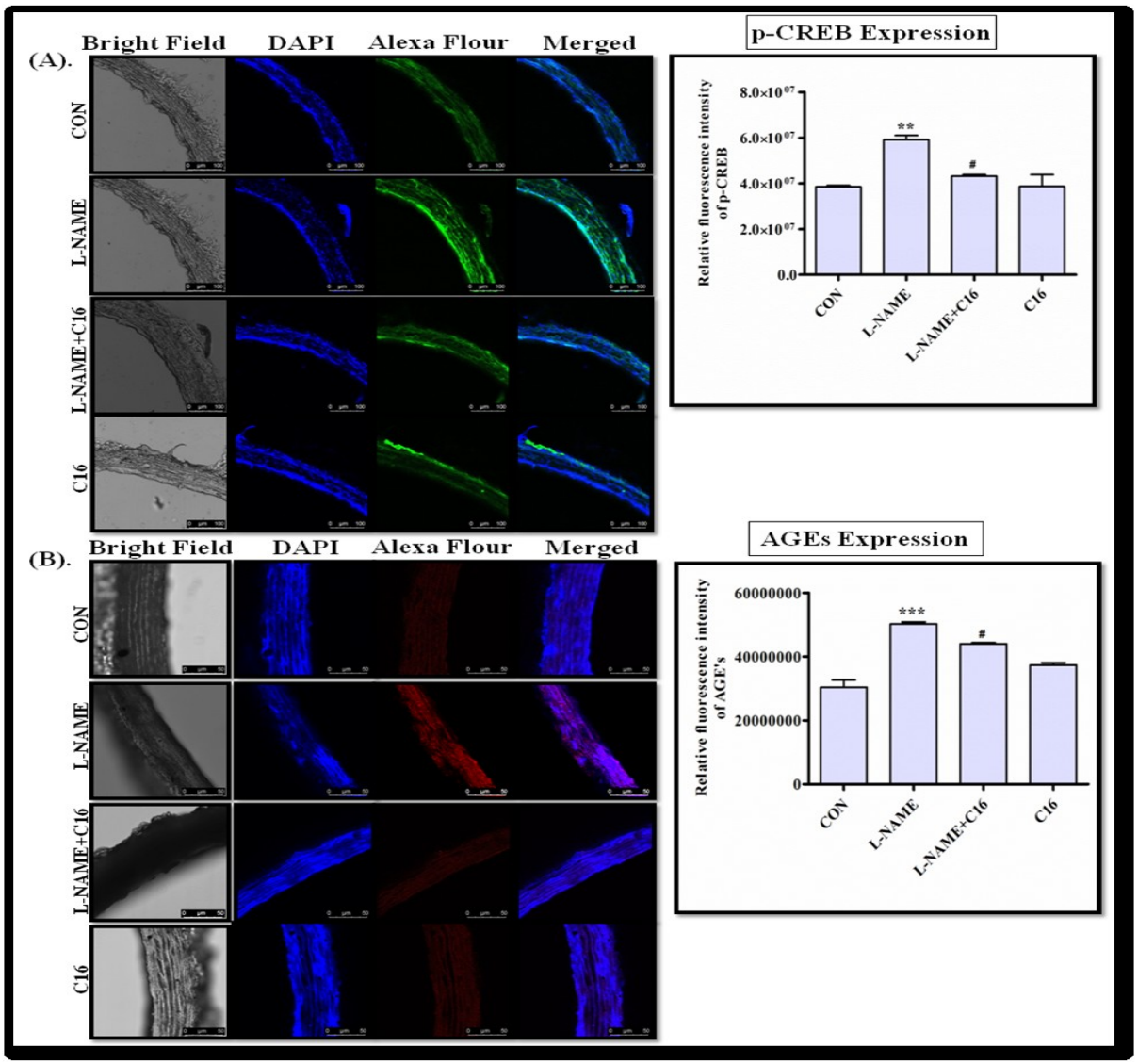


Fig.5.5.7. L-NAME triggered up-regulation of p-CREB, AGEs, and apoptosis, attenuation by imoxin. Fig.5.5.7.A. Immunohistochemistry was performed for

determination of expression of p-CREB. P-CREB expression was increased in LNAME-treated rats, restoration by imoxin, n=3, scale bar represents 100µm. Fig.5.5.7.B. Immunohistochemistry was performed for determination of expression of AGEs. AGEs expression was increased in LNAME-treated rats, restoration by imoxin, n=3, scale bar represents 50µm. Fig.7. C. and D. Result of western blot: Increase in protein expression of caspase 3 and Inos in L-NAME-treated rat heart, attenuation by imoxin, n=3. One-way ANOVA followed by Bonferroni post hoc analysis was used for statistical analysis. Data expressed as mean ±SEM: *P<0.05, **P<0.01, ***P < 0.001 vs. CON, #P < 0.05, ##P<0.01, ###P < 0.001 vs. L-NAME+C16

DISCUSSION

We demonstrate that PKR inhibition (Fig.5.5. 3.A., B., and C.) by imoxin helped in improving inflammation, cardiac fibrosis, arterial remodelling, and BP homeostasis in L-NAME adminstered Wistar rats. Compared to any other experimental models, L-NAME animal model of HTN is the easiest and convenient model to explore inflammatory pathways with end-organ harm [387]. Using the NIBP measurement technique, we observed that NOS inhibition by L-NAME administration in rats caused significant HTN. The significant rise in SBP and DBP confirmed induction of experimental HTN, (Fig.5.5.1.A. and B.), reduced expression of eNOS in L-NAME treated rat aorta and reduced endothelial-dependent relaxation (Fig.5.5.6.B.), ex-vivo in rat aortic rings (Fig.5.5.6.C.), harvested and mounted from L-NAME adminstered rats compared to CON. Additionally, treatment with imoxin showed improvement in BP reduction, pathological, and morphological changes in the myocardium and arterial vessel wall structure. According to previously published reports, L-NAME induces CV remodelling, cellular hypertrophy, extracellular collagen deposition, and fibrosis, including the release of inflammatory cytokine, oxidative load markers, and decreased NO availability in response to pressure overload [388, 389]. Tension builds up on the arterial wall is then distributed equally throughout the major arteries, and induces the spread of inflammation and fibrosis to surround in whole of the heart [390]. Damage to vasculature is well recognized by arterial remodelling increased expression of vasoconstrictor agents such as Ang-II and inflammatory markers [391-393]. These changes ultimately lead to development of cardiac remodelling and disease worsening [394, 385].

Various researchers have further claimed that exogenous administration of Ang-II or over activation of intracellular Ang-II cause's activation of TGF-β1 in hearts and arteries in-vivo [396, 397]. However, how Ang-II initiates inflammatory and fibrotic cascade in a PKR dependent manner in L-NAME induced deregulation of BP homeostasis, cardiac fibrosis, and arterial remodelling is still not known.

The pathogenic role of NF-κB activation and JNK has been well described in HTN associated CV damage [398]. We investigated that PKR activation in L-NAME administered rats has led to the considerable up-regulation of Ang-II in rat aorta compared to CON, attenuation was achieved with imoxin treatment (Fig.5.5.3.B). Moreover, a significant increase in serum nitrite/nitrate (Fig.5.5.3.C.), was also reported in L-NAME adminstered animals; imoxin treatment reversed the changes. We propose that PKR mediated Ang-II increase could be related to oxidative damage. Analogs of l-arginine facilitate eNOS uncoupling, causing a massive increase in superoxide generation and production [399], these superoxides quickly react with NO to generate peroxynitrite and induce ROS [400]. Consequently, it leads to a decrease in NO bioavailability [401] [46]. ROS production in endothelial cells is linked with the mediation of inflammatory responses through the regulation of NF-κB in-vitro and in-vivo. NF-κB-responsive elements are confirmed in the promoter region of the AT1 receptor gene [402, 403], signifies the role of ROS and iNOS involved in the regulation of Ang-II expression. Further, NF-κB is known to provoke local inflammatory responses [404]. We also report a substantial increase in p-JNK, JNK, NF-κB, and TNF-α (5.5.2.A.), in L-NAME administered rats, attenuation was reported with imoxin. An increase in NF-κB and TNF-α could be associated with the initiation of local inflammatory responses [405]. Our studies further reveal that PKR activation mediates an increase in SBP, DBP, and stimulates local inflammatory pathways via acting as an upstream regulator.

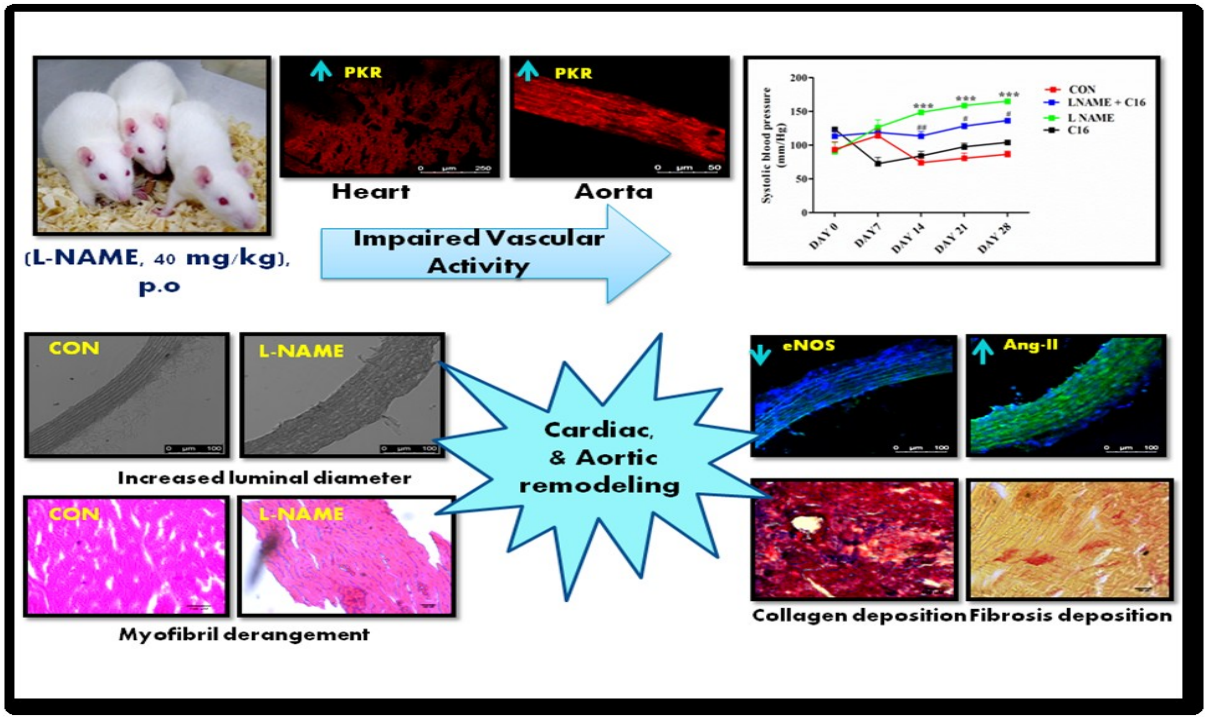
In continuation with the above findings we further sought to determine whether the L-NAME–induced overactivity of PKR could lead to cardiac and vascular fibrosis, we examined the effect of PKR inhibitor on alterations in TGF-β expression. TGF-β is a critical marker of fibrogenesis in response to inflammatory stress. Up-regulated TGF-β is an important pathological hallmark for cardiac and

vascular remodelling processes [406, 407]. In the present study, L-NAME treatment caused significant up-regulation in TGF-β protein expression in heart tissue (Fig.5.5.5.A. and B.), as well as in aorta (Fig.5.5.5.C.), of L-NAME administered rats compared to CON, reduction was noted with imoxin treatment. We propose that the mechanism underlying enhanced TGF-β production is related to the overall increase in the activity of PKR mediated local inflammatory responses. Our hypothesis for Ang-II mediated production of TGF-β is also supported by numerous other studies that claim the same [408-410]. In the discussed study we observed the major effect of the L-NAME, on myocardial fibrosis which was confirmed by an abundance of red coloration as shown by Sirius staining (Fig.5.5.4.B.), and increase in collagen load in the interstitium as demonstrated by Masson's trichrome staining, (Fig.5.5.4.A.), attenuation was observed with imoxin treatment. L-NAME treated rats exhibited derangement in cardiac muscle fibers with huge gaps in-between. Excessive interstitial gaps are connected with the disturbances in collagen metabolism, causing reduced degradation, augmented accumulation of collagen in ECM, abnormalities in structure and dysfunctioning of the heart [411-413]. The aforementioned changes ultimately lead to cardiac remodelling and progression of long term myocardial fibrosis. Observed changes in the present study are also accompanied by an increase in relative heart weight compared with the respective CON, attenuation in these effects was inhibited by imoxin. Attenuation in the fibrotic process solely associates with the inhibition of TGF-β in a PKR dependent manner.

Histopathological examination was done for investigating the structural changes in rat heart, and aorta of L-NAME treated rats. An increase in lumen diameter (Fig.5.5.6.A.), degradation, and disorientation of elastin fragments (Fig.5.5.6.E.) was prominent in L-NAME rat aorta, whereas the abundance of myocardial necrosis and derangement in myocardial fibers (Fig.5.5.4.D.), were extensively noticed in L-NAME rat heart when compared to appropriate CON, significant reversal was achieved with imoxin treatment. In addition to this, we also report increased expression of AGEs in rat aorta and heart in L-NAME adminstered rats (Fig.5.5.7. A and B.). We further report reduction in body weight, heart rate in L-NAME adminstered rats, where as reversal was achieved with imoxin treatment.

Next, we were determined for investigating the changes if any occurring in lipid profile. We observed elevated serum triglycerides level in L-NAME adminstered rats compared to CON, attenuation was noted with imoxin treatment. However, we did not report any of the significant changes in serum HDL and total cholesterol levels in between any of the group.

SUMMARY



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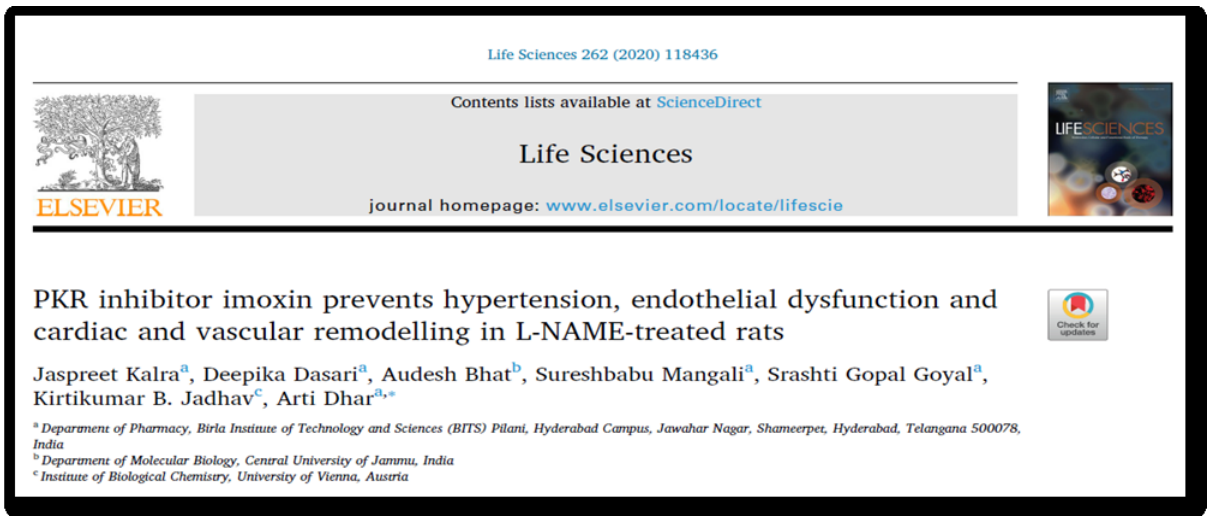

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PKR inhibitor imoxin prevents hypertension, endothelial dysfunction and cardiac and vascular remodelling in L-NAME-treated rats

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CONCLUSION

CV disorders are the major health concern across the globe. Majority of the patients (approximately more than 50% of patients) suffering from CV diseases are often presented with HTN and its associated cardio-renal complications. Arterial HTN represents a most important CV epidemic condition in developed and as well as in developing world, more often arterial HTN remains unidentified until late in its development course. HTN is a complex and multifactorial disease condition arising from interrelated etiology. Due to its complex pathogenesis it is difficult to identify the specific markers of HTN but according to the available reports some unspecific markers are represented before the visible alterations in BP elevation. Hence, HTN can not be correlated alone with the sustained rise in BP [1]. Cardiometabolic disruptions such as insulin resistance, obesity, hyperglycaemia, and dyslipidemia increase the susceptibility for likelyhood of increased BP including numerous CV and metabolic diseases such as T2DM and atherosclerosis [414]. As an outcome of HTN, peculiar organs experience damage which may result in cardiac disease, vascular dementia, cerebrovascular disease, renal dysfunction, vascular remodelling and dysfunction. Therefore, HTN is more often referred as end organ disease/target organ damage disease [415]. On the contrary, end stage renal disease is the most widespread form of secondary HTN and increasing evidence indicate that it is an independent factor responsible for CV morbidity and mortality [416-419]. HTN associated end-organ-damage involve complex pathogenesis interconnected with tightly regulated process including endothelial dysfunction, vascular inflammation and remodelling, excessive collagen deposition, activation of matrix metalloproteinases (MMPs) [420-422]. Development and progression of end-organ-damage is further exaggerated by increased arterial stiffness [423, 424], concomitant changes in heart rate [425], sympathetic overactivity [426], and modulations in components of RAS. Though high BP is the major contributor to HTN associated end-organ-damage, lots of evidence indicated that there are several other mediators that are also essentially involved. Hence, these observations have raised significant concern to warrant the multifactorial strategy, which aim at achieving target BP levels along with the overall reduction in development and progression of end-organ-damage and substantially minimizing the CV risk [427, 428].

In addition to its role in mediating inflammatory responses we report PKR as an important contributor to cellular proliferation and apoptosis. It could emerge as an essential target for exploring its association in numerous CV that are also linked with chronic inflammation [3, 4]. Excess of caloric intake and a lack of adequate physical activity are considered as vital parameters for the onset of HTN and related metabolic disorders. The exact mechanism is still not understood. Using HF in our study we have successfully demonstrated that metabolic and immune signalling pathways are intimately linked with each other. Kidney and vasculature primarily are the most severly affected organs under hypertensive disease condition. Hence, cellular insights were unraveled to explore on mechanisms related to nutrient excess/high caloric intake induced reno-vascular inflammation and remodelling using the cultures of renal epithelial cells and VSMCs. Evidently we report that in response to HF-induced changes in renal cells and VSMCs, PKR upregulation alongwith ROS, cell death apoptosis including other destructive events were observed whereas attenuation was achieved by imoxin treatment. Recently PKR was recognized as a major factor of "metabolic inflammasome" connecting stress signalling to metabolic diseases which can be activated by excess of caloric intake including stress signals [3]. Chronic inflammation, insulin sensitivity and glucose homeostasis can be affected by the PKR upregulation [4]. Previous reports confirm the expression of PKR in the heart and its role in CV diseases. PKR pathway was not explored and understood in renal epithelial cells, VSMCs, kidney and as well as in aorta. As aforementioned, HTN severely affects kidney and aorta as its major target. Several lines of evidence indicate that increased vascular and aortic remodelling or aortic stiffness and alterations in BP are leading correlates for potential CV diseases and renal complications [429]. Moreover, aortic stiffening is often considered an end result of sustained HTN [430, 431]. Arterial HTN has been described as principal origin of end-stage kidney disease and a potential contributor involved in development of glomerular and tubulo-interstitial fibrosis and apoptosis [432]. The role of kidneys in maintenance of long-term BP regulation has been well recognized [433]. We have successfully established the PKR pathways and its role in inducing HTN associated renovascular complications in HF treated NRK 52E cells and VSMCs

obtained from rat aorta. In brief, we report PKR upregulation as a significant paramount for causing inflammation, oxidative stress, aopotosis, vascular inflammation; phenotypic switching and remodelling in HF treated NRK 52E cells and primary VSMCs. Thus, therapeutically targeting PKR using selective PKR inhibitors could emerge as an effective and beneficial approach for treating the CV complications.

After the preliminary investigation we conducted in-vivo studies on male Wistar rats. L-NAME-induced experimental HTN was chosen as an animal model of choice, owing to fact that it mimics the exact condition of human HTN. Longstanding BP is also responsible for microvascular lesions in highly perfused organs such as kidney, heart and brain. Numerous studies suggest increased BP and aortic stiffness is also associated with the higher incidence of hemodynamic load on the heart which is held accountable for LV remodelling, hypertrophy, and impaired diastolic function [434-436]. We summarise that PKR activation is linked with deregulation of BP homeostasis, CV fibrosis, aortic remodelling/stiffening and hypertensive nephropathy in L-NAME-induced experimental animals, attenuation was achieved with imoxin treatment.

Vascular and cardiac remodelling accompanied by fibrosis are the most common complications associated with chronic HTN [2, 3]. Since decade's neurohumoral systems, more specifically RAS are held accountable for HTN induced renal injury, CV fibrosis, and aortic remodelling. Impairment of renal function as a consequence of hemodynamic and cellular changes during hypertensive damage causes the replacement of functional nephrons by fibrotic lesions. Reports from the previous studies suggest that Ang-II and ROS act in a corroborated manner to induce TGF-β1. Further, it is a postulate that Ang-II promotes TGF-β1 release in a time and concentration-dependent manner [2, 6-8]. Once activated, these pathways share standard network and directly promotes cardiac and vascular remodelling through the initiation of inflammatory and fibrotic responses [1-5]. These multifaceted pathologies emerge to stimulate a harmful cycle of swelling, inflammation and fibrosis. Interstitial inflammation, fibrotic scarring, glomerular damage and tubular cell apoptosis are some of the final outcome of this

inflammatory cascade where as it causes myofibril derangement in heart and narrowing of lumen in aorta. Ang-II in-vivo causes increase in the production of TNF-α and also up-regulates other pro-inflammatory mediators, including NF-κB, JNK and, MAPK's pathways which are associated with the presence of glomerular and interstitial inflammatory cells in the affected organs

Our study successfully reports the beneficial effect of imoxin in improving BP homeostasis, endothelium dependent relaxation, inflammation, fibrosis, arterial remodelling, and pathomorphological changes in kidney, heart and aorta in L-NAME induced hypertensive Wistar rats. L-NAME administration causes impairment in endothelium dependent relaxation, and induces the expression of PKR and ROS generation. Induction of PKR relates to the up regulation of Ang-II, NF-kB, and TGF-β in L-NAME treated rats. Disrupted endothelium, elevated ROS and local inflammatory responses are the underlying mechanisms which are held accountable for the PKR mediated end-organ-damage in HTN.

Our studies indicate PKR as a major participant in L-NAME caused hypertensive nephropathy. PKR activation further relates to the evidences of cardiac and aortic remodelling in L-NAME adminstered rats. The observed changes in three major organs i.e. kidney, heart and aorta are possibly caused by activation of the PKR pathway. PKR mediated modulations are selectively inhibited by imoxin. We conclude that selective inhibition of PKR induced alterations warrant clinical investigation for the HTN associated end-organ-damage.

CHAPTER-7

FUTURE SCOPE OF WORK

- The present study provides the role of PKR in development of hypertension. Selective inhibition of PKR is achieved by using imoxin. Effects of imoxin were studied in high fructose treated renal epithelial cells and primary vascular smooth muscle cells in-vitro and in-vivo in L-NAME model of hypertension.
- In the present study, selective PKR inhibitor was used. Activity of numerous other novel PKR inhibitors can also be evaluated on high fructose treated renal epithelial cells and primary vascular smooth muscle cells for the comparative studies.
- In addition to this role of PKR can also be explored for other cardiovascular disease conditions such as myocardial infacrction, stroke chronic heart failure.
- Pharmacokinetics studies of imxoin in both healthy and disease rat/mouse models could also be evaluated.

CHAPTER-7

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CHAPTER-8

8.1. PUBLICATIONS FROM PHD THESIS

1. **Kalra J**, Mangali S, Bhat A, Jadhav K, Dhar A. Upregulation of PKR Pathway Contributes To Renal Inflammation and Tubular Apoptosis in L-NAME Induced Experimental Hypertension. Heliyon. 2020 Nov 18;6(11):e05463.
2. **Kalra J**, Mangali S, Bhat A, Jadhav K, Dhar A. PKR inhibitor imoxin prevents hypertension, endothelial dysfunction and cardiac and vascular remodelling in L-NAME-treated rats Life Sci. 2020 Dec 1;262:118436.
3. **Kalra J**, Mangali S, Bhat A, Jadhav K, Dhar A. Selective inhibition of PKR improves vascular inflammation and remodelling in high fructose treated primary vascular smooth muscle cells. Biochim Biophys Acta Mol Basis Dis. 2020 Mar 1;1866(3):165606.
4. **Kalra J**, Babu MS, Bhat A, Dhar I, Udumula MP, Dhar A. Imoxin attenuates high fructose induced oxidative stress and apoptosis in renal epithelial cell via downregulation of PKR Pathway. Fundam Clin Pharmacol. 2018 Jun;32(3):297-305. **(IF:2.3)**
5. **Kalra J,** Dhar A. Double-stranded RNA-dependent protein kinase signalling and paradigms of cardiometabolic syndrome. Fundam Clin Pharmacol. 2018

8.2. OTHER PUBLICATIONS

1. **Kalra J,** Mangali SB, Dasari D, Bhat A, Goyal S, Dhar I, Sriram D, Dhar A. SGLT1 Inhibition Boon or Bane for Diabetes-Associated Cardiomyopathy. Fundam Clin Pharmacol. 2019 Nov 7. **(IF:2.3).**
2. **Jaspreet Kalra,** Vandana Krishna, BollaReddy Sri Varsha Reddy, Arti Dhar, Venkata Vamsi Krishna Venuganti, Audesh Bhat. chapter 10, Nanoparticles in Medical Imaging, in book entitled as Nanoparticles in Analytical and Medical Devices. Publisher Elsevier
3. **Kalra J,** Reddy, BRSVR, Krishna V, Dhar A. Emerging Perspectives for the Development of Coronary Artery Stents as a Replacement Tool for Coronary Artery Bypass Grafting.(Communicated)
4. Mangali S, Bhat A, Jadhav K, **Kalra J**, Sriram D, Vamsi Krishna Venuganti V, Dhar A.. Upregulation of PKR Pathway Mediates Glucolipotoxicity Induced Diabetic Cardiomyopathy In Vivo in Wistar Rats and In Vitro in Cultured Cardiomyocytes. Biochem Pharmacol. 2020 Apr 3:113948.
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8.3.PAPERS PRESENTED AT NATIONAL/INTERNATIONAL CONFERENCES

1. **Kalra J,** Udumula MP, Babu MS, Bhat A, Dhar I, , Dhar A . "Imoxin Attenuates High Fructose Induced Oxidative Stress And Apoptosis In Renal Epithelial Cell Via Downregulation Of Pkr Pathway". Presented at International Conference on Challenges in Drug Discovery and Development, from March 2-4, 2017, BITS-Pilani campus.
2. **Kalra J**, Mangali S, Bhat A, Jadhav K, Dhar A. Selective inhibition of PKR improves vascular inflammation and remodelling in high fructose treated primary vascular smooth muscle cells. Presented at Global Health summit: ICONICA 2020 at Panjab University Chandiagrh, from Feb 12-14 , 2020
3. **Kalra J**, D. Dasari, Bhat A, Dhar A. Selective Inhibition of PKR Ameliorates Hypertensive Nephropathy and Aortic Remodelling in L-Name Treated Wistar Rats. Presented at ISN World Congress of Nephrology, at Abu Dhabi, UAE from Mar 26-29 ,2020

BIOGRAPHY OF JASPREET KAUR KALRA

Jaspreet Kaur Kalra completed her Bachelor of Pharmacy from SBS Post Graduate Institute of Biomedical Sciences and Research, Dehradun, India of in year 2012 and M. Pharm (Pharmacology), from ISF college of Pharmacy, Moga, Punjab, India, in year 2014. She gained one year of research experience during her job in Pharmacology and Toxicology Division, IVRI, Izatnagar, Bareilly. In 2016, she joined for her doctoral research at Department of Pharmacy, Birla Institute of Technology and sciences (BITS-Pilani), Hyderabad campus, under the supervision of Dr Arti Dhar. She was awarded with CSIR-SRF fellowship in May 2018 for pursing her doctoral research work. She has more than 15 international publications and 1 book chapter to her credit. She had presented papers at various National and International Conferences. She has won the title of best outstanding oral presenter for her work entitled as Selective inhibition of PKR improves vascular inflammation and remodelling in high fructose treated primary vascular smooth muscle cells, presented at Global Health summit: ICONICA 2020 at Panjab University Chandiagrh, from Feb 12-14, 2020. She also received DST-International Travel grant for presenting her research work at ISN-WCN 2020, Abu Dhabhi, UAE.

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BIOGRAPHY OF PROF. ARTI DHAR

Dr Arti Dhar is currently working as an Associate Professor in the Department of Pharmacy at Birla Institute of Technology and Science (BITS), Pilani, Hyderabad campus. Dr. Dhar received her Ph.D from University of Saskatchewan, Canada in the year 2010. During her PhD she received scholarships from Heart and Stroke Foundation of Canada (HSFC) and Arthur Smith Memorial Scholarship from University of Saskatchewan, Canada. Dr. Dhar also won numerous travel awards from Canadian Physiological Society and Canadian Hypertension Society. Her PhD thesis was nominated for Governor General's Gold medal and her thesis work was presented on CBC channel Canada and on campus news channel in March 2011. Dr. Dhar did her postdoctoral trainings from Lakehead University, Ontario, Canada and University of Saskatchewan, Canada from the year 2010 to 2013. After joining BITS Pilani, Hyderabad Campus in 2014, she has received research funding from DST under Young scientist scheme, CSIR extramural research funding, ICMR, and early career award and from BITS under additional competitive grant. Her prime research interests are centered on novel therapeutic targets for cardiovascular and metabolic disorders. Currently 5 students are pursuing PhD under her guidance. She has several National and International publications to her credit.

BIOGRAPHY OF DR. AUDESH BHAT

Dr. Audesh Bhat is currently working as an Assistant Professor in the Centre for Molecular Biology, Central University of Jammu, India. He has more than 12 years of research/teaching experience and has around 40 publications to his credit. Dr. Bhat qualified CSIR-UGC NET Exam twice in 2002, 1st time for lectureship and 2nd time JRF among top 20%. After completing his Ph.D from University of Jammu in collaboration with Jawaharlal Nehru University (JNU), New Delhi, Dr. Bhat joined Washington University, Saint Louis, Missouri as a postdoctoral fellow where he worked on telomere biology. Later he moved to Canada and joined University of Saskatchewan (UfS) as a postdoctoral fellow and then became Research Officer, where he continued work on the characterization of factors responsible for maintaining stability of human genome. He was awarded a 2 years postdoctoral fellowship by Saskatchewan Health Research Foundation (SHRF) to carry out research on Translesion Synthesis Polymerases. In 2014, he returned back to India and briefly worked as a Senior Scientific Writer at Novartis Healthcare Pvt. Ltd, Hyderabad before joining Central University of Jammu. His research work encompasses diverse areas of human biology such as human genetic diversity, genetics of Type II diabetes, cancer biology, cell biology, immunology etc. He currently has two ICMR sponsored research projects worth 150 lakhs. He has completed 2 research projects, one sponsored by UGC, India and 2nd by SHRF, Canada. Dr. Bhat is recipient of Venus International Faculty award and Bharat Vikas award. Besides research, Dr. Bhat is actively involved in teaching and other scientific activities. He has developed several interdisciplinary courses on Molecular Biology that are being offered to the students of other streams. He organises scientific activates on the eve of National Science Day and guest lectures every year for the students. His other passions include voluntary work. He participated in "Leukemia and Lymphoma Society, Canada fund raising initiatives; volunteered for "Let's Talk Science" and "Saskatoon regional science fair" initiative by UfS; organised diabetes detection camp etc.