Role of Epigenetics in Modulating Inflammatory Response Mediated by NF-κB through AT_1 or AT_2 receptors in the Development of Renal Failure under Type 2 Diabetic Condition

THESIS

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CERTIFICATE

This is to certify that the thesis entitled **"Role of Epigenetics in Modulating Inflammatory Response Mediated by NF-κB through AT_1 or AT_2 Receptors in the Development of Renal Failure under Type 2 Diabetic Condition"** and submitted by **Ms. Anuradha** ID No**. 2013PHXF0423P** for award of Ph.D. of the institute embodies original work done by her under my supervision.

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Table of content

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ANURADHA

Abstract

Background: Type 2 diabetes induced nephropathy, one of the major causes of end stage renal failure globally has been known to be associated with severe hemodynamic imbalance which in turn activates renin angiotensin system (RAS). Angiotensin II (Ang II) is the most potent component responsible for widespread biological actions by acting majorly through Ang II type 1 receptors (AT_1) and Ang II type 2 receptors (AT_2). Though a vast scale of studies have been performed regarding the role of AT_1 receptor inhibition in treatment of diabetic nephropathy and its role in controlling nuclear factor κB (NF-κB) mediated inflammatory cascade, the view regarding the role of AT_2 receptors in regulation of NF-κB signaling remained mystified. Existing studies showed that NF-κB signaling was regulated by different Ang II receptor sub-types in different tissues and conditions, thereby urging us to determine the exact roles of Ang II receptor sub-types in regulation of this pathway in diabetic nephropathy. Also the effect of AT_1 and AT_2 receptors' activity on expression of Angiotensin Converting Enzyme (ACE) and Angiotensin Converting Enzyme 2 (ACE2) remained unknown, despite their significant roles as components of deteriorative and protective axes of RAS. Another important gap in the existing literature was that though angiotensin receptor blockers and ACE inhibitors are the mainstay of currently available pharmacological interventions, their effects on epigenetic modifications have not been studied in-depth. As suggested by the recent studies, genetic penchant alone may not be sufficient to explain the pathogenesis of diabetic nephropathy and thus, epigenetic mechanisms must be studied thoroughly to understand the complete phenomenon. Posttranslational histone modifications, involving covalent modifications of histones play a substantial role in regulation of gene transcription and are extremely important to unearth the exact pathogenic basis of diabetic nephropathy as well as its treatments. Hence, in the current study we aimed to check the effect of pharmacological interventions acting through AT_1 and AT_2 receptors on posttranslational histone modifications.

Methodology: A non-genetic animal model for type 2 diabetes induced nephropathy was developed in adult, male Wistar rats (160-180 gm body weight) using chronic high fat diet (HFD) feeding and low dose Streptozotocin [(STZ) 35mg/kg, *intra peritoneal*] treatment. The diabetic rats were administered with various treatments

including i) study 1: AT_1 receptor antagonist (Telmisartan, 10mg/kg, *per oral*), AT_2 receptor antagonist (PD123319 ditrifluoroacetate, 10mg/kg, *sub-cutaneous*) or the combination of both the receptor antagonists in study 1 and ii) study 2: AT_1 receptor antagonist (Telmisartan, 10mg/kg, *per oral*), AT_2 receptor agonist [Compound 21 (C21) at three different doses- 0.075mg/kg, 0.15mg/kg or 0.3mg/kg *per oral*] and or the combination of Telmisartan (10mg/kg, *per oral*) and C21 (0.3mg/kg, *per oral*). The effect of these agents on progression of diabetic nephropathy was evaluated by checking various biochemical, hemodynamic and morphrometric parameters. The microscopic alterations in the renal architecture were assessed by the hematoxylin and eosin or Picro Sirius red staining to estimate the glomerular damage score and collagen deposition area in kidney sections. Also, the effects of these treatments on expression of mRNA and proteins involved in progression of pathological cascades like inflammation, renal fibrosis and apoptosis were studied by quantitative RT-PCR, immunoblotting and immunohistochemistry. Histones were extracted from kidney and their covalent modifications were studied by Western blotting studies. The expression of enzymes involved in orchestrating posttranslational histone modifications like histone H3 lysine 4 (H3K4) methyltransferase SET7/9, histone acetyl transferase, P300/CBP-associated factor (PCAF), histone H2A and H2B specific ubiquitinases and deubiquitinases were also studied. The enrichment of histone H2AK119 monoubiqutination (Ub) at promoter regions of inflammatory and fibrotic genes, monocyte chemoattractant protein 1 (*Mcp1*) and transforming growth factor 1 (*Tgfb1*) was studied using chromatin immunoprecipitation assay.

Results: The current study showed that inhibition AT_2 receptors deteriorated the biochemical, morphological, microscopic and hemodynamic parameters in diabetic nephropathy rats while AT_1 receptors antagonist, Telmisartan improved them. The conjugation of C21 with Telmisartan further improved the effects while the the combination of Telmisartan and PD123319 did not show any alterations showing that counterregulatory effect of the receptors on each other. It was also found that ACE and ACE2 are differentially regulated by AT_1 and AT_2 receptors and C21 and Telmisartan both improve *Ace/Ace2* ratio in diabetic kidney, thereby indicating that promotion of protective axis of RAS may be one of the mechanisms for renoprotective effects shown by these agents and their combination. NF-κB signaling

pathway was also found to be differentially regulated by the Ang II receptor subtypes. AT_1 receptors exacerbate while AT_2 receptors pacify the inflammatory signaling by NF-κB pathway and thereby control the production of proinflamatory chemokines (MCP1), cytokines (interleukin 6, tumor necrosis factor alpha) and adhesion molecules (vascular chemoadhesion molecule 1). Renal fibrosis indicated by increased extra cellular matrix deposition- collagen and fibrotic markers like transforming growth factor (TGF-β1) and fibronectin and renal apoptosis shown by the expression of markers including cleaved caspase 3, cleaved PARP1, caspase 3 and caspase 7 were significantly reduced by Telmisartan and C21 and even more efficiently by their combination, thus proving the anti-fibrotic potential of the combination regimen. The aberrations in posttranslational histone modifications- histone H3 lysine (K) acetylation and PCAF expression, histone H3 methylation and SET7/9 expression, H2AK119Ub and H2BK120Ub and their respective ubiquitinases and deubiquitinases were also reverted by the treatment with C21 and its combination with Telmisartan, while these were found to be exacerbated by PD123319 treatment. Histone H2AK119Ub was found show an increased occupancy at the promoter regions of inflammatory gene, *Mcp1* and fibrotic gene, *Tgfb1* in kidney samples from both insulin resistant and type 2 diabetic rats. The treatment with AT_1 and AT_2 receptor antagonists reduced and exaggerated the elevated H2AK119Ub in type 2 diabetic rats' kidney, respectively.

Conclusion: Based on the abovementioned results, it could be concluded that ACE, ACE2 and NF-κB signaling are differentially regulated by Ang II receptor subtypes. Simultaneous stimulation of AT_2 receptors and inhibition of AT_1 receptors reduces ACE/ACE2 ratio and NF-κB mediated inflammation in diabetic kidney. Also, the combination regimen provides more efficient anti-fibrotic and anti-apoptotic action than the individual molecules. Moreover, C21 either alone or in combination with Telmisartan reverts the posttranslational histone modifications promoted by diabetic nephropathy. Therefore, Telmisartan and C21 combination appears to be a potential therapeutic combination to treat diabetic nephropathy which combats inflammation, fibrosis, apoptosis as well as epigenetic alterations and further research needs to be done to propel the molecule, C21 or its combination with Telmisartan to further stages of drug development.

List of tables

List of figures

List of abbreviations

Activator protein-1 (AP1)

Advanced glycation end products (AGEs)

Alkaline phosphatase (ALP)

Angiotensin A (Ang A)

Angiotensin 1-7 (Ang 1-7)

Angiotensin converting enzyme 2 (ACE2)

Angiotensin II (Ang II)

Angiotensin II type 1 receptor (AT_1)

Angiotensin II type 2 receptor (AT_2)

Apolipoprotein-E knockout (ApoE-/-)

AT_1 receptor antagonist/blocker (ARB)

B-cell lymphoma 2 (Bcl2)

Blood urea nitrogen (BUN)

cAMP-response-element-binding protein (CREB)

CC-chemokine ligand (CCL)

Cyclic guanosine monophosphate (cGMP)

Endothelin-1 (ET1)

Enhanced chemiluminescence (ECL)

Epidermal growth factor (EGF)

Extracellular matrix (ECM)

Glomerular filtration rate (GFR)

Glucose transporter 1 (GLUT-1)

High fat diet (HFD)

Inhibitor of kappa B **(**IκB)

intercellular adhesion molecule 1 (ICAM1)

Interleukin 6 (IL6)

IκB kinase (IKK)

Kelch ECH associating protein1 (Keap1)

Mas-related genes (MrgD)

Mitogen activated protein kinase (MAPK)

Monocyte chemoattractant protein (MCP1)

NADPH oxidase (NOX)

Nitric oxide (NO)

Nitric oxide synthase (NOS)

Nitrix oxide (NO)

Normal Pellet Diet (NPD)

Nuclear factor of activated T-cells (NFAT)

Nuclear Factor-κB (NF-κB)

Phospholipase C (PLC)

Plasma albumin (PAL)

Plasma creatinine (PCr)

Plasma glucose (PGL)

Plasma insulin (PI)

Plasma total cholesterol (PTC)

Plasma triglyceride (PTG)

Plasma triglyceride (PTG)

Poly ADP ribose polymerase (PARP)

Protein kinase C (PKC)

Regulated on activation, normal T cell expressed and secreted (RANTES)

Renin angiotensin system (RAS)

Streptozotocin (STZ)

Superoxide dismutase (SOD)

Systolic blood pressure (SBP)

Thiobarbituric acid reactive substrate (TBARS)

Transforming growth factor-β (TGF-β1)

Tumor necrosis factor alpha (TNF-α)

Ubiquitin specific protease (USP)

Urine albumin-to-creatinine ratio (UACR)

Vascular cell adhesion molecule-1 (VCAM1)

Vascular endothelial growth factor (VEGF)

Zucker diabetic fatty (ZDF)

1. Introduction

Type 2 diabetes, a leading cuase of morbidity and mortality, throughout the world due to changes in lifestyle, lack of exercise and faulty food habits is characterised by chronic hyperglycemia and hyperinsulinemia. Diabetic nephropathy, a secondary complication inflicted by type 2 diabetes is one of the leading causes of end stage renal failures globally (Ghaderian *et al.*, 2015). Diabetic nephropathy mainly involves inflammatory changes including alteration in levels of cytokines, chemokines and leukocyte populations and increased oxidative stress (Anders *et al.*, 2011). Inflammation is closely associated with tissue repair, regeneration of parenchymal cells, and filling of tissue defects with fibrous tissue which leads to scar formation and results in progressive fibrosis along with loss of tissue structure and function (Meng *et al.*, 2014). Not only fibrosis, hyperglycemia and an increased level of inflammatory mediators also increases apoptosis in the kidney cells. In both type 1 and type 2 diabetes, hyperglycemia, free radical stress and disturbed hemodynamics promote apoptosis of kidney podocytes, epithelium, glomerular and proximal tubular cells through induction of B-cell lymphoma 2 (Bcl2)/ Caspase/ poly ADP ribose polymerase (PARP) pathway (Kumar *et al.*, 2004; Susztak *et al.*, 2006; Verzola *et al.*, 2007; Habib, 2013). The progression of diabetic nephropathy thus involves an abrupt increase in renal inflammation, fibrosis and apoptosis which deteriorate the renal functioning and lead to end stage renal dysfunction. Hemodynamic alterations such as hyperfiltration and hyperperfusion caused by hyperglycemia are considered major kidney injury factors, but such alterations are only one aspect of a complex series of pathophysiological alterations related to the presence of glucose metabolism defects (Kumar *et al.*, 2014; Martín-Timón *et al.*, 2014).

Hemodynamic balance is regulated by various systems in body that control blood pressure, blood volume, urine formation or water retention in the body. One of the most important systems playing a pertinent role in maintaining the hemodynamic balance is renin angiotensin system (RAS) which has pressor as well as depressor components to keep the blood pressure under check. It consists of two balancing arms acting as pressor axis [Angiotensin II (Ang II)/ Angiotensin Converting Enzyme (ACE)/ Ang II type 1 receptor (AT_1)] and depressor axis [Angiotensin Converting Enzyme 2 (ACE2)/Angiotensin (1-7)/Mas receptor] which are also termed as

deteriorative and protective axes, respectively (Ocaranza *et al.*, 2012; Montezano *et al.*, 2014). Ang II, the most potent molecule from RAS possesses hypertrophic, pro-fibrotic, pro-inflammatory and proliferative roles which in turn make it an important player in pathogenesis of nephropathy in type 2 diabetes (Benigni *et al.*, 2010; Kim *et al.*, 2017). It also has a prominent role in activation of Nuclear Factor-κB (NF-κB) signaling pathway in diabetic nephropathy (Lee *et al.*, 2004b). The NF-κB family of transcription factors has a crucial role in rapid responses to stress and pathogens as well as in development and differentiation of immune cells (Sen *et al.*, 2010; Model, 2015).

A number of *in vivo* and *in vitro* studies showed that Ang II regulates the production of pro inflammatory cytokines like interleukin-1β (IL1β) (Alique *et al.*, 2015) and chemokines such as monocyte chemoattractant protein-1 (MCP1), transforming growth factor-beta (TGF-β), intercellular adhesion molecule 1 (ICAM1), vascular cell adhesion molecule-1 (VCAM1), regulated on activation, normal T cell expressed and secreted (RANTES) and tumor necrosis factor alpha (TNF-α) (Ruiz-Ortega *et al.*, 2002). NF-κB activity in macrophages, glomerular and tubular parenchymal cells is linked with severity parameters of diabetic nephropathy such as proteinuria, oxidative stress or inflammation (Mezzano *et al.*, 2004).

The effects of Ang II are mediated majorly by two receptors, referred to as the AT_1 and Ang II type 2 (AT_2) receptors (Dasgupta *et al.*, 2011; Ocaranza *et al.*, 2012; Montezano *et al.*, 2014). According to various studies, Ang II mediated NF-κB activation is carried out through different receptor subtypes in different cells. In vascular smooth muscles (Ruiz Ortega *et al.*, 2001) and mesangial cells (Ruiz-Ortega *et al.*, 2001) both AT_1 and AT_2 ; in tubuloepithelial cells AT_1, while in endothelial cells AT_2 (Wolf *et al.*, 2002b) were reported to be associated with the NF-κB pathway activation. It has been reported that the production of cytokines involved in the process of inflammation is regulated differentially, ie, in glomerular epithelial cells, RANTES expression is regulated by AT_2 (Wolf *et al.*, 1997a) while expression of pro inflammatory genes like IL6, VCAM1, MCP1 is controlled by AT_1 receptors (Ruiz-Ortega *et al.*, 2003). The study performed by Esteban et al., confirmed the role of differential regulation of NF-κB by Ang II under diseased condition. In the kidney of wild type mice (C57BL/6) with unilateral ureteral obstruction, treatment with AT_1

receptor antagonist (Losartan) or AT_2 receptor antagonist (PD123319) partially decreased NF-κB activation, whereas only the AT_2 receptor blockade diminished monocyte infiltration, which implicates that both the receptor subtypes are involved in activating NF-κB signaling pathway while AT_2 receptors orchestrate the monocyte mediated inflammation (Esteban *et al.*, 2004). ***However, role of Ang II receptor subtype in activation of NF-κB signaling in the kidney of type 2 diabetes animals still remains elusive. Therefore, there is a need to identify the receptor subtype orchestrating the NF-κB signaling pathway in type 2 diabetic kidney. This will give us a clear insight into the regulatory mechanism of the signaling pathway.***

The increased load of inflammatory mediators and free radicals in the cell starts a feedback or compensatory mechanism through which the stress could be ameliorated and this is where the protective axis of RAS comes into the picture (Ocaranza *et al.*, 2012). ACE2, an endogenous enzyme sharing 40% homology with ACE is expressed abundantly in adult kidney. It degrades Ang II to Ang (1–7) and Ang I to Ang (1–9), that is subsequently converted to Ang (1–7) by ACE. ACE2 has been found to show beneficial effects against hypertension, cardiac dysfunction, fibrosis, inflammation, atherosclerosis and diabetic complications due to its counter regulating effect on the ACE/Ang-II/AT_1 receptor axis (Liu *et al.*, 2011; Nadarajah *et al.*, 2012). ACE2 contributes to the cell protective action by reducing oxidative stress, hypertrophy (Jin *et al.*, 2012), fibrosis and inflammation through inhibition of various pathways like TGF-β1, macrophage migration inhibitory factor (Simoes e Silva *et al.*, 2013), and mitogen-activated protein kinases/ NF-κB pathway (Meng *et al.*, 2013). The overexpression of ACE2 has been reported to reduce the oxidative stress by activating the anti-oxidant enzymes like superoxide dismutase and inhibiting NAD(P)H oxidase (NOX) in a dose dependent manner thus proving useful in controlling the hemodynamic imbalance (Bindom *et al.*, 2009). The Ang II receptor subtypes involved in regulation of ACE2 expression varies from tissue to tissue. A study performed by Ali et al., 2013 showed that in obese Zucker rats, a long term AT_2 receptor agonist, CGP42112A treatment led to attenuation of AT_1 receptor function and increment in the of activity of ACE2/Ang-(1-7)/Mas axis (Ali *et al.*, 2013). Also, in the paraventricular nucleus, the centre of cardio-regulation in brain shows an overexpression of ACE2 along with reduced AT_1 receptor and ACE expression and

increased AT_2 receptor and Mas expression under Ang II induced hypertensive condition (Sriramula *et al.*, 2011). The administration of AT_1 receptor antagonist, Telmisartan was found to increase the expression of ACE2 in kidney arterioles (tunica media) which in turn increases the Ang II degradation and culminates into the antihypertensive effect (Soler *et al.*, 2009). It was seen that both AT_1 receptors and AT_2 receptors were involved in the regulation of ACE2 expression in brain regions controlling blood pressure (Obr *et al.*, 2006). The alteration in ACE2 expression has been known to participate in the pathogenesis of diabetes and diabetic nephropathy (Nadarajah *et al.*, 2012). ***However, the regulation of ACE and ACE2 expression by Ang II receptor subtypes in type 2 diabetic kidney still remains an enigma.***

Diabetic nephropathy, a chronic inflammatory condition involves an increase in macrophage infiltration which culminates into elevated production of cytokines and chemokines (Soetikno *et al.*, 2011; Elmarakby *et al.*, 2012). CC-Chemokine Ligand 2 (CCL2), also known as monocyte chemoattractant protein-1 (MCP1) recruits and activates monocytes/macrophages and triggers a series of reactions upregulating inflammation and fibrosis in diabetic kidney. It is known that macrophage infiltration is regulated by the RAS through NF-κB signaling pathway and its activation has been reported to be controlled by different Ang II receptor subtypes in different tissues under different conditions (Ruiz-Ortega *et al.*, 2003; Esteban *et al.*, 2004). However, which of the Ang II receptor subtypes is involved in its orchestration under type 2 diabetic nephropathy, remains mysterious. Renal fibrosis triggered by elevated MCP1 involves transforming growth factor-β1 (TGF-β1), which prompts the onset of reactions mediated by fibrotic proteins leading to an accelerated production of extracellular matrix (Zi *et al.*, 2012). Despite being the biomarkers for diabetic nephropathy, the epigenetic mechanisms regulating the expression of MCP1 and TGF-β1 in diabetic nephropathy are incompletely understood.

A myriad of signals and inputs modulate the covalent modifications of the histones to facilitate or hinder the binding of promoters to the binding site by remodelling the chromatin structure. These posttranslational histone modifications occur generally at the N terminal amino acid functional groups in the tail region of the histones (Grunstein, 1997; Cheung *et al.*, 2000; Cremer *et al.*, 2001). The well-known posttranslational histone modifications are acetylation, methylation, phosphorylation,

ubiquitination, sumoylation, ADP-ribosylation, deamination and proline isomerization (Sterner *et al.*, 2000; Kouzarides, 2007). The part played by histone H3 acetylation, methylation and phosphorylation in alteration of gene expression in diabetic kidney has been studied to a certain extent. However, histone H2A and H2B lysine monoubiquitination have not garnered much attention despite their prominent role in numerous biological pathways.

The process of addition of ubiquitin moiety called ubiquitination decides the fate of various proteins in the cells, by either directing them towards proteasomal degradation or participation in several cell signaling pathways (Shaid *et al.*, 2013). The first protein which was found to be ubiquitinated was H2A and it was found that about 5–15% of total H2A was in ubiquitinated state in a normal mammalian cell. Though the functions of H2A monoubiquitination remain unclear, abundant instances indicate its role in gene repression and deoxyribonucleic acid damage repair and cell cycle progression (Vissers *et al.*, 2008). Recent studies showed that hyperglycaemic state promoted histone H2AK119Ub and reduced the H2BK120Ub in glomerular mesangial cells, which in turn elevated the TGF-β1 mediated pathways leading to fibrotic gene expression. These alterations occurring in hyperglycaemic state were significantly controlled by exposure to a 26s proteasome inhibitor, MG132 which could partially be attributed to its ability to limit NF-*κ*B mediated inflammatory signaling through inhibiting the IκBα sumoylation and ubiquitination (Gao *et al.*, 2013; Huang *et al.*, 2013).

Though the Ang II type 1 receptor blockers (ARB) and ACE inhibitors are the most commonly prescribed agents for the treatment of diabetic nephropathy, the studies investigating their underlying epigenetic mechanisms which may be the cues to understand the reasons for their efficacy remain limited. The effects of angiotensin receptors' activity on the posttranslational histone modifications which are crucial for deciding the gene transcription have not been studied in-depth. Recently, it was reported that Losartan attenuated key parameters of diabetic nephropathy and increased histone H3 lysine 9/14 acetylation (H3K9/14Ac) at receptor for advanced glycation endproducts (RAGE), plasminogen activator inhibitor-1 (PAI1), and MCP1 promoters in mesangial cells cultured under diabetic conditions it could not ameliorate other modifications like H3K36 trimethylation (Me3) at the MCP1 gene or

H3K4Me1/2/3 at RAGE, PAI1 and MCP1 (Reddy *et al.*, 2014a). Such reports emphasize the need of studying the posttranslational modifications inflicted by the ARBs. ***Despite the important role of Ang II receptor subtypes in pathogenesis as well as treatment of diabetic nephropathy, the epigenetic effect of agents acting through AT_1 and AT_2 receptors have also not been studied in depth till date.***

Based on the abovementioned information, the current study was conceived so as to understand the role of Ang II receptor subtypes in regulation of ACE and ACE2 expression and also in activation of NF-κB signaling pathway in type 2 diabetes induced renal failure. The current study also aimed to unearth the effects of Ang II receptor subtypes and the interventions acting through these receptors on posttranslational histone modifications, which play a major role in orchestration of type 2 diabetes induced nephropathy.

2. Review of literature

2.1. Diabetic nephropathy: A global epidemic

2.1.1. Epidemiology of diabetic nephropathy

According to the World Health Organization projection, about 7.7 percent of the total world population, i.e., 439 million people will be suffering with diabetes by 2030. The drastic changes in lifestyle, increase in aged population and rapid urbanization have led to a three-fold increment in the number of diabetic patients over the past three decades, globally (Inzucchi *et al.*, 2012; Forouhi *et al.*, 2014). Among the 10 countries with the largest numbers of people predicted to have diabetes mellitus by 2030, five are in Asia, thereby justifying its title as the 'diabetes epicentre of the world' (China, India, Pakistan, Indonesia and Bangladesh) (Chen *et al.*, 2012; Zimmet *et al.*, 2016). Type 2 diabetes accounting for 90–95% of those with diabetes, previously referred to as non–insulin-dependent diabetes or adult onset diabetes, encompasses individuals who have insulin resistance or relative insulin deficiency (American Diabetes, 2011; 2014). Common factors influencing type 2 diabetes include genetic make-up, age, high fat/ high calorie intake, sedentary lifestyle and obesity (American Diabetes, 2011; Fukuda *et al.*, 2012; Mori *et al.*, 2014). Type 2 diabetic patients have been reported to be at a high risk of developing microvascular complications like coronary artery diseases, peripheral artery disease or stroke or macrovascular complications including diabetic nephropathy, neuropathy, and retinopathy (Alaboud *et al.*, 2016). Approximately 20–50% of patients with type 1 or type 2 diabetes develop nephropathy (Matavelli *et al.*, 2011; Zhuo, 2014; Thomas *et al.*, 2016).

2.1.2. Pathological changes and clinical manifestations in diabetic nephropathy

Diabetic nephropathy, characterized by a progressive decline in renal function represents one of most important causes for end stage renal disease throughout the world (Fineberg *et al.*, 2013; Thomas *et al.*, 2016). Diabetic nephropathy is clinically defined by macroalbuminuria or microalbuminuria and abnormal renal function as represented by an abnormal serum creatinine, calculated creatinine clearance, or a rapid decline in glomerular filtration rate (GFR) (Ruiz-Ortega *et al.*, 2002; Reutens, 2013). Microalbuminuria, defined as urinary albumin excretion rate of 20–200 μg/min (30–300mg per day) or an albumin to creatinine ratio of 2.5–25.0 mg/mmol in males

and 3.5–35.0 mg/mmol in females varies markedly depending upon the physical work out, health, heart diseases, blood pressure, pregnancy, infections and menstruation. Due to the high probabaility of variations in urinary albumin excretion rate, at least two specimens (preferably first morning midstream void), collected over a 3–6 months' period should be abnormal before considering a patient to have microalbuminuria or macroalbuminuria (MacIsaac *et al.*, 2014). The mesangial fractional volume is correlated with albumin excretion rate and GFR in both type 1 and type 2 diabetes (Tervaert *et al.*, 2010). Another newly identified parameter for identification of renal failure includes, cystatin C level, a potential predictive marker for diagnosing patients with unchanged or regressed microalbuminuria (Fineberg *et al.*, 2013).

The initial evidences of morphological alterations in diabetic nephropathy include lesions of glomerular basement membrane, mesangial and arteriolar expansion. Diabetic nephropathy is characterized by mesangial expansion which may be nodular, so-called Kimmelstiel-Wilson nodules, hyaline in both afferent and efferent arterioles, and markedly thickened glomerular basement membrane by electron microscopy (Wolf, 2004; Tervaert *et al.*, 2010; Ritz *et al.*, 2011; Gonzalez Suarez *et al.*, 2013; Reidy *et al.*, 2014). Podocyte loss may be a crucial contributor to this progressive sclerosis (Pourghasem *et al.*, 2015). Tubular basement membranes thicken in parallel to glomerular basement membrane. Early interstitial inflammation with predominantly mononuclear cells is followed by later increased interstitial fibrosis and tubular atrophy (Najafian *et al.*, 2011; Pourghasem *et al.*, 2015; Qi *et al.*, 2017). The extraglomerular lesions, including tubulointerstitial lesions, tubular atrophy, interstitial inflammation, and tubulointerstitial fibrosis, are closely related to renal function loss in the progression towards end stage renal dysfunction in patients with preexisting renal insufficiency. In diabetic microangiopathy, hyalinosis occurring in efferent arteriole is a typical lesion used to differentiate from hypertensive nephropathy (Fioretto *et al.*, 2007; Jain, 2012). According to Renal Pathology Society, diabetic nephropathy is divided into four hierarchical glomerular lesions. Although the evaluation of interstitial and vascular changes has been separated, in this classification, the damage inflicted by glomerular lesions is the lowest in group one but increases throughout the groups. Glomerular alterations as most important lesions

were classified as follows: class I: glomerular basement membrane thickening; class IIa: mild mesangial expansion; class IIb: severe mesangial expansion; class III: nodular sclerosis and class IV: global glomerulosclerosis in >50% of glomeruli (Tervaert *et al.*, 2010; Pourghasem *et al.*, 2015; Qi *et al.*, 2017). Chronic renal failure induced pathological derangements like impaired osmotic balance, renin-angiotensin-aldosterone and sympathetic activity, anemia, bone and mineral metabolism, uremia, toxin accumulation along with progressive hyperglycemia and oxidative stress is also one of the important risk factors for development of cardiovascular diseases which increase the morbidity and mortality linked with diabetes even further (Tomey *et al.*, 2014).

2.2. Pathogenesis of diabetic nephropathy: Focusing the molecular mechanisms

The development of nephropathy in type 2 diabetes is a multifactorial, complex process orchestrated by chronic hyperglycemia and reduced insulin sensitivity or insulin resistance. Chronic hyperglycemia and persistent hyperinsulinemia activate various biological cascades triggering pathogenic mechanisms by tipping off the redox and metabolic balance to initiate fibrosis, inflammation, apoptosis and endothelial dysfunctions which form the pillaring pathways culminating into renal dysfunction in diabetic condition (Cao *et al.*, 2011; Vinod, 2012; MacIsaac *et al.*, 2014; Gnudi *et al.*, 2016; Furukawa *et al.*, 2017).

2.2.1. Metabolic and redox imbalance

It has been found that diabetic pattients with end stage renal dysfunction show significantly higher advanced glycation end products (AGEs) concentration than the patients without renal failure due to increased production rate and reduced clearance rates (Kumar Pasupulati *et al.*, 2016). The accumulation of AGEs in the extracellular matrix and glomerular basement membranes promote the development of lesions or renal injury. The interaction of AGEs with RAGE and activation of protein kinase C (PKC) increases the activity of phospholipase C (PLC) with an increase in intracellular Ca^{2+} and diacylglycerol. PKC is also known to initiate endothelial dysfunction by decreasing nitrix oxide (NO) bioavailability, elevating endothelin-1 (ET1), and vascular endothelial growth factor (VEGF) (Teng *et al.*, 2014). AGEs play an important role in increasing the intracellular oxidative stress which in conjugation

with increased glucose metabolism and auto-oxidation further upregulate the extracellular matrix deposition and activates various transcription factors, like NF-κB, activator protein-1 (AP1), and specificity protein 1 leading to transcription of genes encoding cytokines, growth factors, and extracellular matrix proteins (ECM) (Vlassara *et al.*, 2013; Nowotny *et al.*, 2015).

Accumulating research suggests that oxidative stress is a significant contributor to the pathogenesis of diabetic nephropathy (Giacco *et al.*, 2010; Kashihara *et al.*, 2010; Gnudi *et al.*, 2016). Under normal physiological conditions, the antioxidant systems neutralize the free radicals generated due to the high metabolic activity of the kidney which in diseased diseased states gets disturbed and promotes renal damage concomitant with worsening glucose metabolism and vascular dysfunction (Gnudi *et al.*, 2016). NAD(P)H oxidase (NOX), AGE, defective polyol pathway, uncoupled nitric oxide synthase (NOS) and mitochondrial respiratory chain *via* oxidative phosphorylation increase the accumulation of free radicals including superoxide anion (O_2^-), hydroxyl racial ($HO^·$), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl), NO and lipid radicals (Vasavada *et al.*, 2005) which oxidize various tissue biomolecules, such as, DNA, protein, carbohydrates and lipids to to produce DNA double or single strand breaks which further exacerbate the oxidative stress (Kashihara *et al.*, 2010; Mima, 2013; Sil, 2015; Gnudi *et al.*, 2016; Miranda-Díaz *et al.*, 2016). Excess amounts of ROS modulate activation of PKC, mitogen-activated protein kinases (MAPK), and various cytokines and transcription factors which eventually cause increased expression of ECM genes with progression to fibrosis and end stage renal disease (Giacco *et al.*, 2010; Kashihara *et al.*, 2010; Elmarakby *et al.*, 2012).

2.2.2. Inflammation

Chronic inflammation plays an important role in the development of diabetes and its secondary complications (Mima, 2013; Sun *et al.*, 2013; Wada *et al.*, 2013; Duran-Salgado *et al.*, 2014; Donate-Correa *et al.*, 2015). The inflammation in kidney is highlighted by infiltration of inflammatory cells in glomerular and tubulointerstitial spaces. These infiltrates degranulate and release proinflammatory and firotic cytokines. Numerous cytokines released into the renal microenvironment further propagate renal failure. For example, interleukin 1 (IL-1), IL6, IL-18, IL-13 increases

vascular permeability, mesangial cells proliferation and matrix deposition, podocytes proliferation, increased expression of adhesion molecules on endothelial cells and vascular smooth muscle cells (VSMC) (Donate-Correa *et al.*, 2015; Elsherbiny *et al.*, 2016). The inflammatory markers like tumor necrosis factor-α (TNF-α) and MCP1 serve as important biomarkers for nephropathy progression in diabetes and have also been in implied as vasodilator and vasoconstriction mediators contributing to alterations in glomerular capillary permeability barrier (Barutta *et al.*, 2015). Taken together, these cytokines cause albuminuria and disturbances of sodium homeostasis (Donate-Correa *et al.*, 2015). Hyperglycemic milieu activates transcription factors such as upstream stimulatory factor 1 and 2, AP1 (activator protein 1), NF-κB, CREB (cAMP-response-element-binding protein), NFAT (nuclear factor of activated T-cells) and SP1 (stimulating protein 1) which cause inflammation and ECM turnover (Sanchez *et al.*, 2009). Among the transcription factors, NF-κB is the most important in the pathogenesis of diabetic nephropathy activated by a wide variety of nauxious stimuli (Oeckinghaus *et al.*, 2011; Shi *et al.*, 2014).

2.2.1. NF-κB mediated inflammation in diabetic nephropathy

NF-κB, a pleiotropic transcription factor, activated by cytokines, bacterial and viral products, UV irradiation and oxidative stress plays a pivotal role in regulating multiple biological functions, such as inflammation, immunity, cell proliferation, and apoptosis (Oeckinghaus *et al.*, 2011; Shi *et al.*, 2014). The NF-κB family of transcription factors has a crucial role in rapid responses to stress and pathogens as well as in development and differentiation of immune cells (Sen *et al.*, 2010; Model, 2015). Nuclear NF-κB p65 protein and mRNA expressions in isolated peripheral blood mononuclear cells were well correlated with urinary MCP1, Regulated upon Activation, Normal T cell Expressed, and Secreted (RANTES) and the severity of nephropathy under type 2 diabetic condition (Figure 1).

The transcriptional co-acivators initiate covalent modifications of NF-κB subunits and promotes their nuclear translocation so as to start the process of gene transcription (Sen *et al.*, 2010; Smale, 2011). NF-κB super family consists of at least 5 genes encoding the members RelA (p65), RelB, RelC, p50, and p52. The inactive form of NF-κB is localized in the cytoplasm and consists of the DNA-binding p50 and p65 subunits and an inhibitory subunit called inhibitor of kappa B **(**IκB), which is bound

to p65. The phosphorylation at Ser32 and Ser36 by the IκB kinase (IKK) complex releases IκB-α from the complex and unmasks the nuclear localisation sequence to promote nuclear translocation of p50/p65 complex and initiate the transcription (Rompe *et al.*, 2010).

NF-κB-dependent pathways play an important role in macrophage infiltration and kidney injury associated with several inflammatory diseases, including atherosclerosis, insulin resistance, metabolic syndrome, and diabetes and its secondary complications (Baker *et al.*, 2011; Tornatore *et al.*, 2012). In diabetic kidney disease, proteinuria, hyperglycemia and oxidised low density lipids activate NF-κB in tubular, endothelial and mesangial cells. (Wada *et al.*, 2013). It plays an important role in production of pro inflammatory cytokines, such as TNF-α, MCP1, interleukins, interferon β, granulocyte–monocyte colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor G-CSF; cell-surface molecules such as VCAM1, intracellular cell adhesion molecule-1 (ICAM1), E-selectin, IL2 receptor a-chain and major histocompatibility complex (HSC) class I and II; acute-phase proteins such as serum amyloid A and enzymes such as the inducible forms of NOS and cyclooxygenase and the anti-oxidant defence enzyme Mn-superoxide dismutase (SOD) (Gómez-Garre *et al.*, 2001; Tornatore *et al.*, 2012; Huang *et al.*, 2013; Bhatt *et al.*, 2014)

NF-κB mediated inflammation in diabetic nephropathy involves an increase in macrophage infiltration which further elevates production of cytokines and chemokines including CC-chemokine ligand (CCL) 2, CCL3, CCL4, CCL5 and CXC-chemokine ligand (CXCL) 2 (Soetikno *et al.*, 2011; Elmarakby *et al.*, 2012). MCP1 recruits and activates monocytes/macrophages and triggers a series of reactions upregulating inflammation and fibrosis in diabetic kidney. Renal fibrosis triggered by elevated MCP1 involves TGF-β1, which prompts the onset of reactions mediated by SMAD proteins leading to an accelerated production of extracellular matrix (ECM) (Zi *et al.*, 2012). ***Despite being biomarkers for diabetic nephropathy, the exact underlying mechanisms regulating the expression of* MCP1 *and* TGF-β1 *in type 2 diabetes induced nephropathy are incompletely understood.***

2.2.3. Fibrosis

Hyperglycemia induces an abnormal activation of PKC, MAPK and JNK which stimulate the fibrotic and inflammatory signaling cascades (Kanwar *et al.*, 2011; Kanasaki *et al.*, 2013). Hyperglycemia induced TGF-β1 in mesangial cells stimulate mRNA and protein glucose transporter 1 (GLUT-1) to increase glucose-induced metabolic abnormalities (Inoki *et al.*, 1999). The connective tissue growth factor (CTGF) promotes glomerulosclerosis to induce transient actin cytoskeleton disassembly in mesangial cells, high production of fibronectin, collagen types I and IV, and mesangial cell hypertrophy (Schena *et al.*, 2005). Chronic hyperglycemia activates the resident and non resident renal cells to produce humoral mediators, cytokines and growth factors responsible for triggering increase in ECM deposition and functional alterations like shear stress leading to increased glomerular permeability (Cao *et al.*, 2011; Vinod, 2012; MacIsaac *et al.*, 2014). Chronic hyperglycemia causes redox imbalance and raises free oxidative and nitrosative radicals which initiate biological pathways involved in structural and functional changes including glomerular hyperfiltration, glomerular and tubular epithelial hypertrophy, and microalbuminuria, followed by the development of glomerular basement membrane thickening, accumulation of mesangial matrix, overt proteinuria, glomerulosclerosis and end stage renal disease (Schena *et al.*, 2005; Vinod, 2012).

2.2.4. Hemodynamic imbalance

Type 2 diabetes is also closely linked with an elevated risk of hemodynamic imbalance, which is also an independent risk factor for development of nephropathy in type 2 diabetic subjects (Martín-Timón *et al.*, 2014). The endothelial dysfunction involving reduced nitric oxide bioavailability, poorly compensated for by increased production of prostacyclin and/or endothelium-dependent hyperpolarizations, and increased production or action of endothelium-derived vasoconstrictors, abnormal angiogenesis, and impaired endothelial repair (Shi *et al.*, 2017) (Figure 2).

Endothelial dysfunction has also been shown to lead to an uncoupling of the VEGF-NO axis in glomeruli and tubules resulting in enhanced inflammation, proliferative, macrophage infiltration effects (Dellamea *et al.*, 2014). Endothelin-1 mediated inhibition of vasodilators like NO and prostacyclins which leads to reduction in blood flux, GFR, inhibition of salt and water reabsorption, glomerular cellular proliferation

and accumulation of ECM (Dellamea *et al.*, 2014; Kohan *et al.*, 2014; Gagliardini *et al.*, 2015). The alterations in the endothelial functioning trigger various feedback mechanisms which act to bring back the blood pressure to normalcy, one of these important pathways is RAS. Overactivation of RAS may lead to metabolic alterations that not only impact blood pressure but also insulin resistance through increasing vasoconstriction, increasing renal sodium reabsorption, and stimulating aldosterone hormone secretion (Hsueh *et al.*, 2011). A number of reports showed that hyperglycemia mediated oxidative stress stimulates angiotensin generation from kidney cells and this step is a crucial perpetrator for development of diabetic nephropathy in type 2 diabetics (Ribeiro-Oliveira *et al.*, 2008; Kamiyama *et al.*, 2013). Ang II orchestrates pathophysiological processes implicated in the development of diabetic nephropathy (haemodynamic changes, hypertrophy, ECM accumulation, growth factor/cytokine induction, ROS formation, podocyte damage, proteinuria, interstitial inflammation) due to its ability to activate NF-κB pathway, TGF-β mediated fibrosis, podocyte damage and hypertrophy (Ribeiro-Oliveira *et al.*, 2008; Benigni *et al.*, 2010; Ritz *et al.*, 2011; Kamiyama *et al.*, 2013; Bernardi *et al.*, 2016) (Figure 1).

2.3. Role of renin angiotensin system in diabetic nephropathy

RAS is the major control system for blood pressure and fluid balance (Sparks *et al.*, 2014). The classical view of RAS has evolved to organ- and tissue- based systems which perform paracrine/autocrine functions. Local RAS exists in different organs including the kidney, heart, pancreas and bone marrow. In the kidney, all of the RAS components are present in resident kidney cells (Lai *et al.*, 2011). RAS comprises of two balancing arms- deteriorative (ACE/AngII/AT_1 receptor) and the other protective axis (ACE2/Ang-(1-7)/Mas). The deteriorative axis, also called the pressor arm consists of angiotensinogen, renin, aldosterone, angiotensin I (Ang I), ACE, Ang II (Ang II), Ang II type 1 receptors (AT_1 receptor) and the depressor arm comprises of Ang II type 2 receptors (AT_2 receptor), angiotensin converting enzyme related carboxypeptidase (ACE2), Ang(1-7) and Mas receptors (Gironacci *et al.*, 2014).

2.3.1. Classical renin angiotensin system

Classically, it is comprised by angiotensinogen, an α-2-globulin produced mainly by the liver, which is cleaved in the amino-terminal portion by renin, an aspartyl protease

produced in the kidney and secreted into the bloodstream forming angiotensin I (Ang I), a decapeptide with no known biological function. The monocarboxypeptidase ACE removes the dipeptide His-Leu from the C-terminal portion of Ang I, converting it into Ang II (Ang II), the major peptide of this classical axis. Other enzymes, like cathepsins and chymases are also known to produce functional Ang II intracellularly (Sparks *et al.*, 2014) (Figure 3). The octapeptide Ang II acts as a vasopressor agent exerting its action by direct effects on arteriolar smooth muscle and also stimulates production of aldosterone by zona glomerulosa of adrenal cortex which helps in sodium reabsorption in the kidney. In the kidneys, efferent arterioles are constricted more than afferent, forcing blood to build up in the glomerulus and increasing glomerular pressure. Ang II is also a potent growth modulator and pro-inflammatory peptide involved in pathogenesis of atherosclerosis, vascular and myocardial remodelling, renal failure and congestive heart failure (Muthalif *et al.*, 2000; Dikalov *et al.*, 2013; Montezano *et al.*, 2014).

The effects of Ang II are mediated by two receptors, referred to as the AT_1 and AT_2 receptors (Ocaranza *et al.*, 2012; Montezano *et al.*, 2014). The Ang receptors AT_1 and AT_2 fully meet classification criteria, with IUPHAR Receptor Code of 2.1.Ang.01.000.00.00 and 2.1 Ang.02.000.00.00. They are seven transmembrane domains rhodopsin subclass G protein-coupled receptor (GPCR). The human genome contains single genes AGTR1 and AGTR2, which encode AT_1 and AT_2 receptors, respectively (Humphrey *et al.*, 1998; Karnik *et al.*, 2015; Singh *et al.*, 2016).

2.3.1.1. AT_1 receptors

Most of the known effects of Ang II are related to AT_1 receptor activation. AT_1 receptors belong to seven-membrane-domain superfamily of G-protein coupled receptors and are expressed in VSMCs, heart, lung, brain, liver, kidney and adrenal glands (Guo *et al.*, 2001). AT_1 receptors are coupled to a variety of intracellular signaling molecules, including the phospholipases A2, C and D, adenylate cyclase, voltage dependent Ca^{2+} channels and to a variety of kinases involved in phosphorylation cascades (Nickenig *et al.*, 2002) (Figure 2).

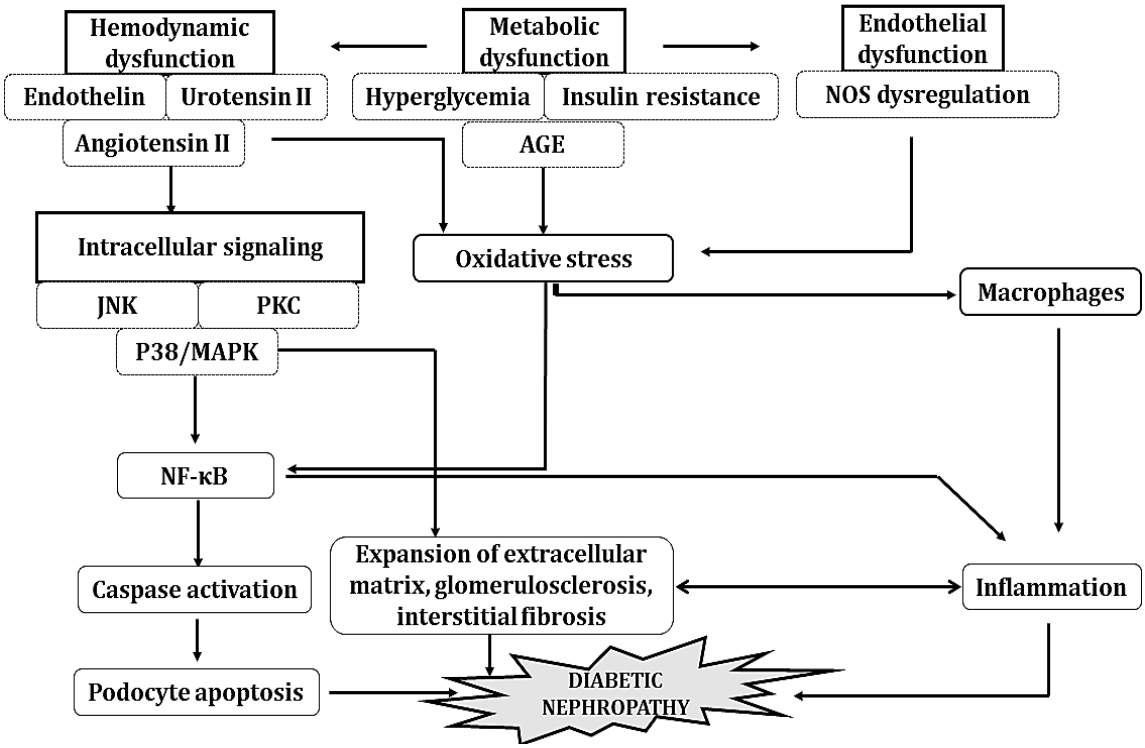


Figure 1. Schematic representation of pathogenesis of diabetic nephropathy.

The pathogenesis of diabetic nephropathy is likely to be as a result of metabolic and hemodynamic and endothelial abnormalities interacting with each other and with various reactive oxygen species-dependent pathways. Both gene regulation and activation of transcription factors are influenced by these factors leading to functional changes like proteinuria, increased glomerular permeability and structural changes like glomerular lesions and scarring leading to the classical hallmarks of diabetic nephropathy.

AT_1 receptor activity is mediated by Gq/11-proteins activating the Ca^{2+} signal and the PKC-mediated system (Singh *et al.*, 2016). The effects of Gq-protein-mediated activation of the AT_1 receptor varies in different tissues, including vasoconstriction, aldosterone release, renal sodium reabsorption, adrenergic facilitation, vascular smooth muscle hypertrophy and cardiac myocyte hyperplasia. PKCs are serine/threonine kinases whose substrates include proteins that are important in cellular proliferation. The activation of phospholipases through both G-protein-dependent and G-protein-independent mechanisms causes phosphatidylinositol 4,5-bisphosphate (PIP2) to be hydrolyzed to two important molecules, IP3 and diacylglycerol which in turn activates PKC. Ang II activates numerous tyrosine phosphorylated proteins, which trigger the response to numerous growth factors and

proinflammatory cytokines (Schieffer *et al.*, 1996; Guo *et al.*, 2001; Karnik *et al.*, 2015) (Figure 2).

Depending on the cell and organ type, stimulation of these signal-transduction pathways leads to cellular contraction, hypertrophy, proliferation, and/or apoptosis. It has become apparent that one of the most important effects of AT_1 receptor activation, particularly in the cardiovascular system, is the production and release of ROS (Nadarajah *et al.*, 2012) which is implicated in many pathophysiological conditions in the cardiovascular system including cigarette-smoking induced damage, hypercholesterolemia, diabetes, hypertension, and heart failure (Liu *et al.*, 2011; Wang *et al.*, 2014; Furukawa *et al.*, 2017). Recent studies suggest that AT_1 receptor mediated ROS production is linked to activation of the PLC mediated superoxide-radical-producing NAD(P)H oxidase, subunit NOX4 activation in kidney cells (Fazeli *et al.*, 2012). Under diseased conditions, the increased Ang II mediated activation of AT_1 receptor promotes hypertrophy of mesangial cells and tubular epithelial cells by increasing the production of the pro-sclerotic cytokine TGF-β, MCP1 leading to promotion of ECM formation and glomerulosclerosis. Oxidative stress mediated NF-κB activation and up-regulation of the pro-inflammatory genes are also closely related with proteinuria and interstitial cell infiltration, adding further insult to the kidney (Kawai *et al.*, 2017).

2.3.1.2. AT_1 receptor antagonists in diabetic nephropathy

An appropriate technique to prevent microalbuminuria, a significant marker for development of diabetic nephropathy is to achieve metabolic control and reduce hypertension in diabetic patients. The most commonly recommended drugs for preventing diabetic nephropathy include ACE inhibitors, ARBs and thiazide diuretics (Sharma *et al.*, 2011). A pool of evidence suggests that ARBs, not only reduce the elevated blood pressure but also show renoprotective effects to safely and efficiently delay the progression of diabetic nephropathy, thus becoming the first line treatment in people with or at risk of developing renal diseases (Lewis *et al.*, 2001; Miura *et al.*, 2010). The results from various clinical trials showed that ARBs are generally well tolerated except a few cases of hyperkalemia, which led to discontinuation of the study while in case of ACE inhibitors, 20% of the general population were found to be intolerant (Einhorn *et al.*, 2009). Owing to their potent antihypertensive and

renoprotective benefits similar to those of ACE inhibitors, ARBs surely are useful alternative treatment options in this large group of ACE inhibitor-intolerant patients (Sharma *et al.*, 2011).

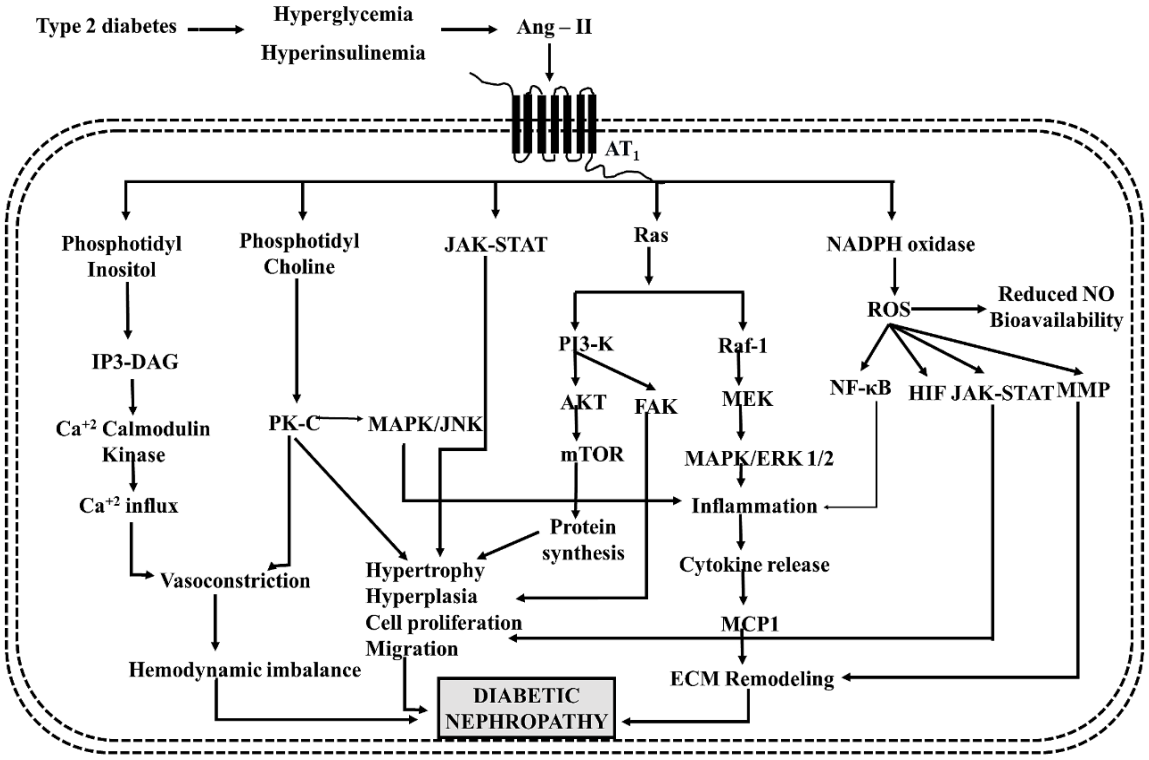


Figure 2. The role of AT_1 receptors in pathogenesis of diabetic nephropathy.

Ang II type 1 receptors (AT_1 receptor) are heptahelical G protein coupled receptors which activate phosphatidyl inositol, phosphotidyl choline, JAK/STAT, Ras mediated AKT or MAPK pathway and NADPH oxidase. The activation of AT_1 receptors leads to vasoconstriction, hypertrophy, hyperplasia, proliferation, inflammation and fibrosis which play an important role in pathogenesis of diabetic nephropathy.

Telmisartan is a non-competitive, insurmountable, non-peptide ARB with the strongest receptor binding affinity among all the ARBs. It is orally active, possesses good oral absorption and tolerability. It is a lipophilic compound widely distributed to tissues and is highly bound to plasma proteins, up to 99.5% (Deppe *et al.*, 2010; Verdecchia *et al.*, 2011) (Table 3). Telmisartan 1-O-acylglucuronide is its principal metabolite in humans which is mainly excreted through biliary faecal routes. Another property which makes Telmisartan, a lucrative therapeutic option in diabetic nephropathy is its ability to elicit hydrophobic interaction with the ligand binding domain of peroxisome proliferator-activated receptor gamma (PPAR-γ) (Lakshmanan

et al., 2011). Telmisartan has a longer half-life, better efficacy and greater antihypertensive effect as compared to Valsartan or Losartan which make it a drug of choice for the alleviation of diabetic nephropathy (Wienen *et al.*, 2000; Goebel *et al.*, 2006; Cao *et al.*, 2012).

A number of clinical trials have been conducted to determine the efficacy of Telmisartan in treatment of diabetic nephropathy. In Diabetics Exposed to Telmisartan And enalaprIL (DETAIL®) study it was found that Telmisartan improved the decline in GFR over 5 years in patients with type 2 diabetes and early nephropathy which was determined using the iohexol based direct measurement of GFR (Barnett, 2004). A prospective 1-year trial to compare Micardis® versus losArtan in hypertensive type 2 DiabEtic patients with Overt nephropathy (AMADEO™) suggests that Telmisartan produced a 29.8% reduction in urine albumin-to-creatinine ratio, whereas that with Losartan was only 21.4% (Bakris *et al.*, 2008). The INcipieNt to Overt: Ang II receptor blocker, telmisartan, Investigation On type 2 diabetic Nephropathy (INNOVATION®) study, conducted on Japanese patients with microalbuminuria, demonstrated that 2 year treatment with Telmisartan (40 or 80mg) arrested the transition to overt nephropathy in a dose dependent manner and this improvement was statistically significant as compared to the placebo control group (Makino *et al.*, 2007). ONTARGET®, which evaluated 25,620 high risk patients demonstrated that Telmisartan controlled albuminuria more efficiently than ACE inhibitor, Ramipril and the effect was similar to their combination (Liebson *et al.*, 2009). The Telmisartan versus Ramipril in renal ENdothelium DYsfunction (TRENDY®) study showed that 9 week treatment with Telmisartan improved the resting renal plasma flow and endothelial function of renal vasculature in better fashion as compared to that of the ACE inhibitor, Ramipril (Ritz *et al.*, 2010). Clinical studies also showed that combination of Telmisartan with ACE inhibitor (Ramipril) increased the occurrence of adverse events without leading to any other improvements in therapeutic potential (Mann *et al.*, 2008). ***These reports from clinical trials suggest that a combination of Telmisartan with other pharmacological interventions may be one of the intelligent ways to tackle and treat diabetic nephropathy.***

2.3.2. Non-classical renin angiotensin system

Recently, newer molecules constituting the protective axis have emerged. The hydrolysis of Ang II by ACE2 or other peptidases, including prolylendopeptidase (PEP) and prolyl-carboxipeptidase (PCP) generates **Ang (1-7)**, a biologically active 7-amino acid peptide (Asp-Arg-Val-Tyr-Ile-His-Pro) which elicits protective effects like vasodilatory substances' release (NO, prostaglandin E2, bradykinin) (Dasgupta *et al.*, 2011; Sparks *et al.*, 2014). ACE2 can also convert Ang I into Ang-(1-9) which can be hydrolyzed by ACE or neutral endopeptidase to produce Ang-(1-7). In addition, Ang-(1-7) can be formed directly from Ang I by action of endopeptidases. The biological activities of Ang-(1-7) include NO-dependent vasodilation, anti-arrhythmogenesis, anti-thrombogenesis, antifibrogenesis, and improvement in glucose and lipid metabolism. Ang-(1-7) is currently classified as an endogenous hormone with anti-proliferative properties, and it is being tested in clinical trials phases I and II. The actions of Ang-(1-7) are strictly related to Mas activation, another seven transmembrane domain G-protein-coupled receptor (Carey, 2013; Etelvino *et al.*, 2014; Villela *et al.*, 2014).

Another important component of the ACE2/Ang-(1-7)/Mas axis is **Angiotensin A** (Ang A). It is an octapeptide sharing a similar peptide structure with Ang II, except having alanine 1 in the place of aspartate1 and thereby showing similar for AT_1 receptor and a slightly higher affinity for AT_2 receptors. Its major effects include vasoconstriction in isolated perfused rat kidney and renal vasoconstriction in anesthetized normotensive and hypertensive rats (Carey, 2013; Etelvino *et al.*, 2014; Hrenak *et al.*, 2016).

Recently, a newly discovered endogenous peptide component of RAS named **alamandine [Ala1-Ang-(1-7)]** was discovered and found to be present in both human plasma as well as rat heart. It could be a product of either ACE2 mediated hydrolysis of Ang II or decarboxylation of N-terminal aspartate residue of Ang (1-7) (Hrenak *et al.*, 2016). The expression of alamandine was found to be significantly higher in nephropathy patients (Carey, 2013). Despite acting on different receptors, majority of biological actions of alamandine and Ang (1-7) are similar like endothelial-dependent vasodilation in rat and mice aortic rings, modulation of baroreflex sensibility after intra-cerebro ventricular infusion, selective increase in phenylephrine-evoked

bradycardia, long-lasting antihypertension in spontaneously hypertensive rats and a decrease of collagens I, III, and fibronectin deposition in isoproterenol-treated rats (Leao *et al.*, 2016).

Alamandine binds selectively to the **MrgD** (Mas-related genes), a part of G protein coupled receptor family and not with the mas receptors (Figure 4) (Etelvino *et al.*, 2014; Villela *et al.*, 2014; Tetzner *et al.*, 2016). MrgD is widely expressed most abundantly in sensory neurons in the dorsal root spinal ganglia, and in lower levels in different parts of the body including brain, parts of male and female reproductive system, alimentary canal, urinary bladder, blood vessels, uterus, eyeball, trachea, thymus, heart, lung, diaphragm, skeletal muscleand adipose tissue (https://www.proteinatlas.org/ENSG00000172938-MRGPRD/tissue). The details of various receptors involved in RAS are represented in figure 3.

2.3.2.1. ACE2: An important protective tool from RAS pathway

A chemically related enzyme, ACE-related carboxypeptidase, also known as ACE2, has 42% homology with ACE at the metalloprotease catalytic domain but differs from ACE in having only one enzymatic site and also is not inhibited by the ACE specific inhibitors. It degrades Ang II to Ang 1–7 and Ang I to Ang 1–9, that is subsequently converted to Ang 1–7 by ACE (Liu *et al.*, 2011; Nadarajah *et al.*, 2012). In humans, ACE2 transcripts have been found to be ubiquitously found in various organs including heart, kidney, and testis, lungs, liver, central nervous system and placenta (Wolf *et al.*, 2002b). The renal tissue is known to exhibit abundant expression of both ACE as well as ACE2 which co-localize in the brush border of mouse proximal tubules. In glomeruli, ACE2 is mainly present in glomerular epithelial cells and sparsely in mesangial cells (Mizuiri *et al.*, 2008; Mizuiri *et al.*, 2015).

Though ACE2 remained an unsung hero for quite a long time despite its early discovery, its importance as a component of protective arm of RAS has now been acknowledged. ACE2 has been shown to elicit beneficial roles in various diseases which could be attributed to its ability to convert Ang II into Ang (1-7) which in turn reduces the vasoconstriction caused by Ang II (Rentzsch *et al.*, 2008). ACE2 contributes to the cell protective action by reducing oxidative stress, hypertrophy (Jin *et al.*, 2012), fibrosis and inflammation through inhibition of various pathways like

TGF-β1, macrophage migration inhibitory factor (Simoes e Silva *et al.*, 2013), mitogen-activated protein kinases/ NF-κB pathway (Meng *et al.*, 2013). ACE2 induces natriuresis, reduced oxidative stress, vasodilation, antiproliferative activity, and diuresis by upregulating the concentrations of nitric oxide and prostaglandins (Mizuiri *et al.*, 2015). For instance, in spontaneously hypertensive stroke prone rats vascular ACE2 overexpression reduced hypertension, probably by locally degrading Ang II and improving endothelial function (Rentzsch *et al.*, 2008). The adenovirus mediated overexpression of ACE2 in a rabbit model of atherosclerosis reduced monocyte infiltration and fibrosis indicated by increased expression of matrix metalloproteases 3 and 9, collagen, leading to greater plaque and higher plaque stability scores. Administration of A-779, a selective Ang (1-7) antagonist significantly attenuated the beneficial effects achieved by this ACE2 overexpression (Dong *et al.*, 2008). In a monocrotaline induced pulmonary hypertension rat model, activation of pulmonary ACE2 enzyme by XNT (1-[(2-dimethylamino) ethylamino]-4-(hydroxymethyl)-7-[(4-methylphenyl) sulfonyl oxy]-9H-xanthene-9-one) prevented pulmonary hypertension and vascular remodelling (Ferreira *et al.*, 2009).

The increased load of inflammatory mediators and free radicals in the cell starts a feedback or compensatory mechanism through which the stress could be ameliorated and this is where the protective axis of RAS, AT_2 receptor/ ACE2/Mas axis comes into picture (Ocaranza *et al.*, 2012). The overexpression of ACE2 has been reported to reduce the oxidative stress by activating the SOD and inhibiting NOX in a dose dependent manner thus proving useful in controlling the hemodynamic parameters (Bindom *et al.*, 2009). The Ang II receptor subtypes involved in regulation of ACE2 expression varies from tissue to tissue. A study performed by Ali et al., 2013 showed that in obese Zucker rats, a long term AT_2 receptor agonist [CGP42112A, 10nM] treatment led to attenuation of AT_1 receptor function and increment in the of activity of ACE2/Ang-(1-7)/Mas axis (Ali *et al.*, 2013). Also, in the paraventricular nucleus, the centre of cardio-regulation in brain shows an overexpression of ACE2 along with reduced AT_1 receptor and ACE expression and increased AT_2 receptor and Mas expression under Ang II induced hypertensive condition (Sriramula *et al.*, 2011). The administration of AT_1 receptor antagonist, Telmisartan was found to increase the expression of ACE2 in kidney arterioles (tunica media) which in turn increases the

Ang II degradation and culminates into the antihypertensive effect (Soler *et al.*, 2009). It was seen that both AT_1 receptor and AT_2 receptor are involved in the regulation of ACE2 expression in brain regions controlling blood pressure (Obr *et al.*, 2006).

The alteration in ACE2 expression has been known to participate in the pathogenesis of diabetes and diabetic nephropathy (Nadarajah *et al.*, 2012). In diabetic nephropathy, the expression of ACE and ACE2 get disturbed elevated ACE/ACE2 ratio has served as a severity index for renal damage. The level of ACE2 expression has been reported to have increased in kidney cortex and tubular region of *db/db* mice and treated diabetic mice (Ye *et al.*, 2004a; Leehey *et al.*, 2008), while it was found to have lowered in glomeruli of *db/db* mice (Ye *et al.*, 2006a; Leehey *et al.*, 2008).

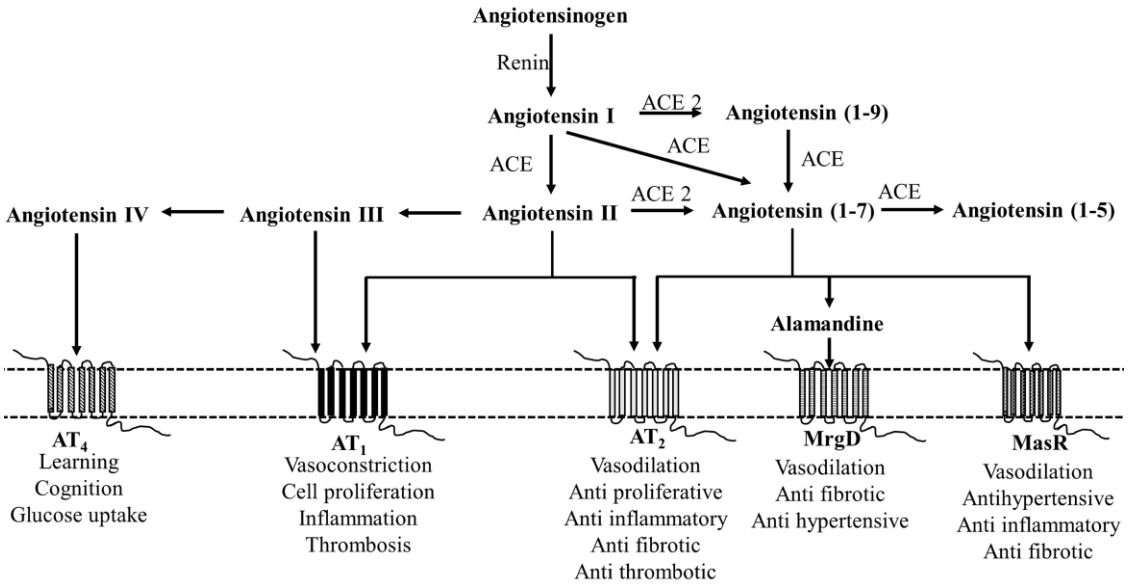


Figure 3. Schematic representation of renin angiotensin system and its receptors.

Renin angiotensin system comprises of angiotensinogen, renin, aldosterone, angiotensin I (Ang I), angiotensin converting enzyme (ACE), angiotensin II (Ang II), Ang II type 1 receptors (AT_1 receptor) and while the later comprises of Ang II type 2 receptors (AT_2 receptor), angiotensin converting enzyme related carboxypeptidase (ACE2) and Mas receptors. Renin, produced by juxtaglomerular cells in the kidney lyses angiotensinogen to form Ang I which is acted upon by ACE to get converted into Ang II which acts on AT_1 and AT_2 receptors to elicit pathogenic and protective actions, respectively.

Another *in vivo* study demonstrated that consumption of high salt diet increased ACE expression in rat kidney more drastically than that of ACE2 expression, leading to oxidative stress and subsequent renal damage (Bernardi *et al.*, 2011). Chang et al

showed that AT_2 receptor deficiency accelerates the development of diabetic nephropathy, which appears to be mediated, at least in part, via heightened oxidative stress and ACE/ACE2 ratio in renal proximal tubules (Chang *et al.*, 2011). The ACE/ACE2 ratio is an equally important cardiac health parameter as indicated by a study conducted in patients with dilated or ischemic cardiomyopathy. It was found that ratios of ACE/ACE2 mRNA and ACE/ACE2 protein were lower in the myocardium of patients with mild heart failure than those in normal myocardium but higher in patients with moderate to severe heart failure (Wang *et al.*, 2015a). Not only preclinical studies, the clinical studies also showed a prominent role of ACE and ACE2 balance in diabetic nephropathy progression. The kidney samples obtained from diabetic nephropathy patients showed a drastic reduction in mRNA as well as protein expression of ACE2 and increase in ACE (Mizuiri *et al.*, 2008; Reich *et al.*, 2008). These reports show that the balance between expression of ACE and ACE2 is an imperative factor involved in maintenance of renal functioning.

ACE inhibitors and ARBs, the mainstay of pharmacological therapies for diabetic nephropathy have not yet been studied thoroughly to establish their effects on alteration of ACE2 expression. Chen et al showed that AT_1 receptor blockade improved cardiac remodelling and increased ACE2 and Ang (1–7) in infarcted hearts of normotensive Lewis rats, whereas vascular ACE2 was limited to the endothelium in small intracoronary vessels (Chen *et al.*, 2012) Another study showed that increased ACE2 and Ang (1–7) in association with altered dimensions of the thoracic aorta but not carotid arteries in response to Olmesartan treatment provides evidence that this pathway is regulated by AT_1 receptors and may be important in mediating the pressure-independent vascular remodeling effects of angiotensin peptides (Igase *et al.*, 2005). The exact role of Ang II receptor subtypes in regulation of ACE2 expression and activity, especially in type 2 diabetic kidney has not yet been understood completely. ***Hence in the current study, an attempt was made to understand the relationship between Ang II receptor subtypes (AT_1/AT_2) and ACE2 expression which can be an important link to understand the complete effect of manipulating these receptors in pathogenesis of type 2 diabetes induced nephropathy.***

2.3.2.2. AT_2 receptor: signaling pathways

The signaling pathways through which AT_2 receptor acts involves phospholipase A2, phosphotyrosine phosphatase which in turn inhibits mitogen-activated protein kinase phosphatase-1 which inhibits ERK1/2 leading to reduction in fibrosis and proliferation (Recarti et al., 2015). Elbaz et al showed that AT_2 receptor mediated inhibition of tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) and signal-regulatory protein (SIRPα1) and prevents subsequent association of both IRS-1 and SIRPα1 with Src homology 2 containing tyrosine phosphatase (SHP-2) (Elbaz *et al.*, 2000). Reports also suggest that AT_2 receptors negatively cross-talk with receptor tyrosine kinases such as fibroblast growth factor, endothelial growth factor, and insulin receptors by targeting autophosphorylation of the receptor. AT_2-interacting protein (ATIP1) cooperates with AT_2 to trans-inactivate receptor tyrosine kinases. This inhibition of insulin receptor was postulated to be responsible for growth inhibitory action of AT2 receptor (Nouet *et al.*, 2004). Also, AT_2 receptor is involved in controlling renal nitric oxide, guanosine cyclic 3′5′-monophosphate (cGMP), and kinins levels which control vasodilation and redox balance (Recarti *et al.*, 2015). Recent research has shown that AT_2 receptor ushers a significant antioxidant, anti-inflammatory (Sabuhi *et al.*, 2011), anti-fibrotic, anti-hypertrophic (Ma *et al.*, 1998; Nabeshima *et al.*, 2006) and anti-apoptotic properties (de Gasparo *et al.*, 1999; Steckelings *et al.*, 2005; McCarthy *et al.*, 2012; Namsolleck *et al.*, 2014; Recarti *et al.*, 2015). AT_2 receptor has been seen to be upregulated by insulin and insulin like growth factor-1 and downregulated by Ang II, epidermal growth factor (EGF), and platelet derived growth factor in diabetes. AT_2 receptors are also upregulated in various clinical conditions such as Na^+ depletion and renal ischemia reperfusion (Ayele *et al.*, 2010). AT_2 receptors regulate vasodilation, nitric oxide synthesis and cGMP production (Ayele *et al.*, 2010) which are equally important in curbing renal diseases and thus researchers pointed towards the addition of AT_2 receptor agonists to the treatment regimen for diabetic nephropathy (Figure 4).

2.3.2.3. Molecules acting through AT_2 receptors

Unlike AT_1 receptors, AT_2 receptors remained undervalued despite its discovery in late 1980s due to the lack of sufficient agents which could be used as specific agonists or antagonists to the receptor (Steckelings *et al.*, 2005; Recarti *et al.*, 2015). However,

the design and synthesis of selective AT_2 receptor antagonists PD123319, EMA401, and agonists like CGP42112A, M024/C21 led to an improvement in understanding of the role of this receptor and also further promoted design and synthesis of molecules possessing beneficial effects (Kellici *et al.*, 2015).

2.3.2.3.1. AT_2 receptor antagonist: PD123319

PD123319 ditrifluoroacetate is a potent, selective, non-peptide angiotensin AT_2 receptor antagonist with an IC_{50} values of 34 and 210 nM in rat adrenal tissue and brain respectively (Blankley *et al.*, 1991). PD123319 or its radioiodinated, fluorescent, and biotinylated analogs have been important tools for defining Ang II receptor subtypes in tissues from various species, including human but their use has been limited to experimental studies only (Hodges *et al.*, 1993; Wexler *et al.*, 1996). Other chemical details for PD123319 are mentioned in Table 3. Waseda et al had shown that in mouse model of bleomycin-induced pulmonary fibrosis, PD123319 treatment reduced lung fibrosis score, hydroxyproline level, macrophage, lymphocyte, and neutrophil counts in bronchoalveolar lavage fluid along with TNF-α and MCP1 levels, on day 7. On the 14^{th} day, the cell infiltration and macrophage inflammatory protein levels were normalized but the TGF-β levels could not be pacified (Waseda *et al.*, 2008). Another study showed that at low concentrations, PD123319 attenuates cardiopulmonary injury by reducing alveolar septal thickness, pulmonary influx of inflammatory cells, including macrophages and neutrophils, medial wall thickness of small arterioles, and extravascular collagen III deposition and pulmonary arterial hypertension- induced right ventricular hypertrophy but does not affect alveolar and vascular development in newborn rats with experimental bronchopulmonary dysplasia (Wagenaar *et al.*, 2014).

2.3.2.3.2. AT_2 receptor agonists

CGP42112A, a peptide AT_2 receptor agonist widely used for AT_2 receptor related studies could not translate into a clinically successful molecule owing to its poor *in vivo* stability and its partial antagonism (Steckelings *et al.*, 2012). The other AT_2 receptor agonists currently available are enlisted in table 1 (Rosenström *et al.*, 2005; Ohinata *et al.*, 2009; Jones *et al.*, 2011; Danyel *et al.*, 2013; Wagenaar *et al.*, 2013; Guimond *et al.*, 2014). In a study conducted by Bosnyak et al, the order of affinity of

ligands at AT_2 receptor in HEK (human embryonic kidney)-293 cells stably transfected with either AT_1 receptor or AT_2 receptor was found to be: CGP42112> AngII≥AngIII>C21≥PD123319≫AngIV>Ang-(1–7). It also showed that AT_2 receptoors showed lesser affinity towards AngIV and Ang-(1–7) than Ang II, but AngIV, Ang-(1-7) and Ang III showed a high degree of selectivity towards AT_2 receptors (Bosnyak *et al.*, 2011).

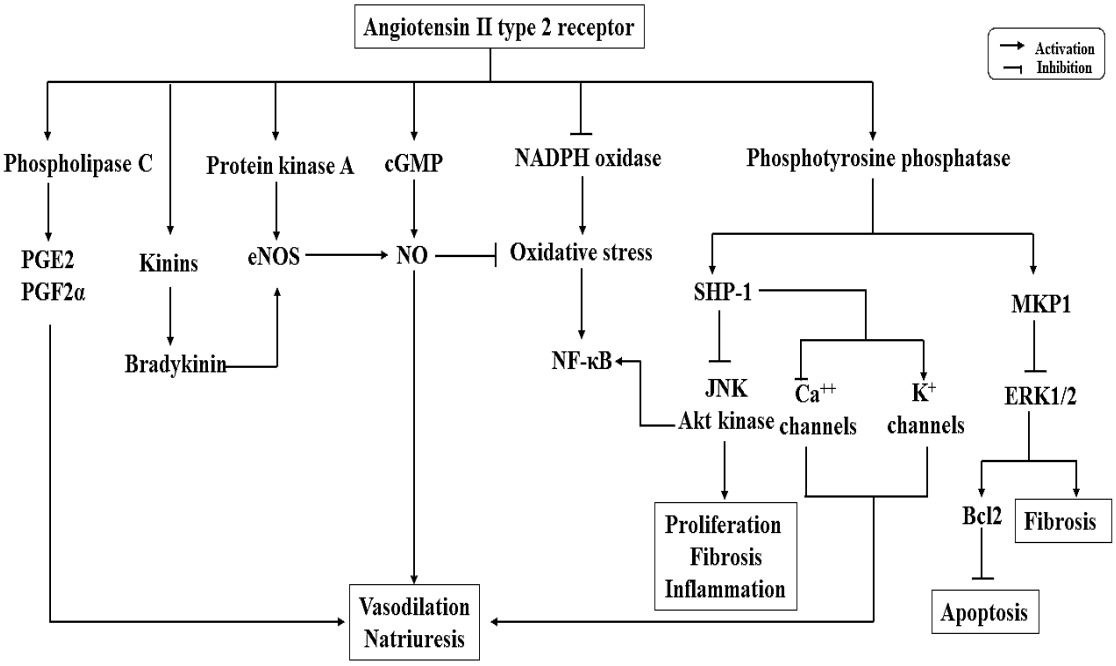


Figure 4. AT_2 receptors: signaling pathways

The signaling pathways through which AT_2 receptor acts involves activation of phospholipase A2, and phosphotyrosine phosphatase which in turn inhibit vasoconstriction and mitogen-activated protein kinase phosphatase-1. This leads to a reduction in ERK1/2 expression and thus controls apoptosis as well as fibrosis. AT_2 receptor is involved in controlling renal nitric oxide, guanosine cyclic 3′5′-monophosphate, and kinins levels which in turn are responsible for vasodilation and antioxidant potential. The antioxidant action is also mediated through another pathway, NADPH oxidase mediated pathway which plays a major role in activation of NF-κB signaling involved in regulation of inflammation.

2.3.2.3.3. Compound 21: A novel AT_2 receptor agonist

In 2004, the AT_2 receptor related research gained momentum with the advent of the novel non peptide AT_2 receptor agonist named C21 which was orally and systemically active (Steckelings *et al.*, 2012). The chemical details for C21 are mentioned table 3. It showed an oral bioavailability of about 30% and has a plasma half-life of almost 4-

6 hours. C21, modelled on the C-terminal pentapeptide structure of Ang II lacks AT_1 receptor affinity and was demonstrated in human embryonic kidney cells to have 4000-fold selectivity to AT_2 receptor (Wan *et al.*, 2004; Bosnyak *et al.*, 2011). Owing to its vast biological activities including anti-fibrotic, anti-inflammatory, anti-apoptotic, anti-oxidant and anti-hypertensive properties, C21 showed a profound beneficial effect in heart failure, myocardial infarction, chronic inflammatory diseases, and neurological diseases such as ischemic stroke (McCarthy *et al.*, 2012; Rehman *et al.*, 2012; Chow *et al.*, 2016).

C21 has also recently been approved by European Medicines Agency (EMA) and Food and Drug Administration (FDA) for the treatment of pulmonary fibrosis, a rare disease which entitled the molecule to be granted an orphan drug status (Bruce *et al.*, 2015; Rathinasabapathy *et al.*, 2015). According to Vicore Pharma Annual report 2016, in clinical trials, conducted by Vicore Pharma in collaboration with the Åbo, a Finland based clinical contract research organization, increasing doses of C21 (0.3mg to 100mg) were given to healthy human volunteers, first in single doses and then in repeated doses. The data from the clinical trials showed that C21 was well tolerated with no serious side-effects, shows an acceptable degradation profile and consistent blood levels

(http://vicorepharma.com/wp-ontent/uploads/2017/04/Årsredovisning2016_eng.pdf).

2.3.2.4. C21 in kidney diseases and diabetic nephropathy

Accumulating evidences suggest that AT_2 receptor stimulation with C21 not only reduced the increment in inflammatory cytokines, inflammatory cell infiltration, ECM deposition, vimentin expression in kidney but also improved the brain damage and survival rate in high salt diet fed spontaneously hypertensive stroke prone rats (Gelosa *et al.*, 2009). In acute disease models like, 2-kidney-1-clip hypertensive rat model for ischemic kidney failure, C21 treatment lowered the levels of inflammatory mediators and cell infiltrates, and enhanced production of NO and cGMP in the ischemic kidneys, without altering the blood pressure and these effects were partially antagonised by PD123319 (Matavelli *et al.*, 2011). Another study showed that, C21 treatment in spontaneously hypertensive rats led to renal vasodilatation and increased natriuresis more prominently in females as compared to males due to higher expression of AT_2 receptor in the female rats' kidney as compared to their male

counterparts (Silva-Antonialli *et al.*, 2004; Hilliard *et al.*, 2012). Recently, Castoldi et al demonstrated that C21 treatment in cyclosporine induced nephropathy significantly reduced the inflammatory cell infiltration and fibrosis of glomeruli and renal tubules which is the most potent predictor of renal disease progression (Castoldi *et al.*, 2016). Recent studies have shown that endothelial dysfunction is a major culprit in pathogenesis of renal diseases. A study conducted by Rehman et al showed that C21, either alone or in combination with Losartan abrogates the development of hypertension and vascular damage in stroke-prone spontaneously hypertensive rats (Rehman *et al.*, 2012). C21 alone or in combination with losartan was shown to improve endothelial function, vascular composition through amelioration of oxidative stress, fibrotic markers, and inflammatory cell infiltration (Rehman *et al.*, 2012). Another study showed that C21 significantly controlled TNF-α induced and HFD-induced endothelial inflammation in human umbilical vein endothelial cells, as well as intact mouse aortae which thus showed an anti-atherosclerotic potential of the compound (Sampson *et al.*, 2016). The aforementioned reports show that C21 is a potential alleviator of different pathological pathways involved in impairment of renal function in kidney diseases. These pathological pathways have also been found to be disturbed in renal failure occurring in both type 1 and type 2 diabetic patients. C21 has been shown to reduce diabetic nephropathy in various preclinical studies which have tried to delineate the underlying mechanisms through which C21 ameliorates diabetic nephropathy (Table 4).

In both type 1 and type 2 diabetes, hyperglycemia, free radical stress and disturbed hemodynamics promote apoptosis of kidney podocytes, epithelium, glomerular and proximal tubular cells through induction of caspase mediated apoptosis (Kumar *et al.*, 2004; Susztak *et al.*, 2006; Verzola *et al.*, 2007; Habib, 2013). C21 treatment is known to attenuate myocardial infarction in Wistar rats due to its anti-apoptotic and anti-inflammatory actions (Kaschina *et al.*, 2008). Another study conducted by Matavelli and colleagues showed that streptozotocin (STZ) treated Sprague Dawley rats showed renal function was severely disrupted, i.e. urine albumin to creatinine ratio was elevated. C21 treatment for 4 weeks was found to improve the urine albumin to creatinine ratio which showed the apparent improvement in renal function. The inflammatory mediators in renal interstitial fluid including TNF-α, IL6, NO,

cGMP, and 8-isoprostane, and in kidney like TNF-α and IL6. However, the increment in blood pressure could not be improved with C21 treatment. The expressions of AT_2 receptor mRNA and protein were increased in diabetic rats as compared with NC, but this increment was not reduced by C21 treatment. The study demonstrated that C21 improved renal function by hindering the renal inflammatory cascade and improving NO and cGMP production (Matavelli *et al.*, 2015). Homozygous ApoE mice are devoid of ApoE protein thus leading to a normally developed mice with a severely increased serum plasma cholesterol and spontaneous atherosclerotic plaques. Apolipoprotein-E knockout (ApoE-/-) mice treated with STZ serve as appropriate murine models for diabetes and associated atherosclerotic lesions (Shen *et al.*, 2007; Zaragoza *et al.*, 2011).

Table 1. List of AT_2 receptor agonists and their current stages of development.

	AT_2 receptor agonists	**Stage of drug development**	**References**
1.	Compound 21 (C21) [Vicore Pharma, Sweden]	Completed phase 1 clinical trials and granted orphan drug status for treatment of idiopathic pulmonary fibrosis	(Danyel, et al., 2013; Bruce et al., 2015)
2.	Lanthipeptide 2 (LP2-3) [MorphoSys AG, Germany]	Completed phase 1 clinical trials	(Wagenaar et al., 2013)
3.	β-amino acid substituted angiotensin peptides	*In vitro* and *in vivo* studies	(Jones et al., 2011)
4.	Benzodiazepine based gamma-turn mimetics incorporated into Ang II	*In vitro* studies	(Rosenström et al., 2005)
5.	Novokinin (RPLKPW)	*In vivo* studies	(Ohinata *et al.*, 2009)
6.	Sarile and sarlasin	*In vitro* studies	(Guimond *et al.*, 2014)

Chow et al, showed that STZ treated ApoE-/- mice showed a drastic increase in the renal dysfunction evidenced as increase in albuminuria and mesangial hypertrophy. With C21 treatment over a 20-week period albuminuria, mesangial area and glomerulosclerosis were significantly controlled as compared to that of the diabetic mice. C21 attenuated the diabetes induced upregulation of various inflammatory genes including MCP1, cluster of differentiation 11 (CD11) which are known to further enhance the rate of renal damage (Chow *et al.*, 2014). Sustained inflammation further enhances the albumin permeability of glomerular basement membrane and increase ECM deposition leading to proteinuria, glomerulosclerosis and finally tubulointerstitial fibrosis (Wada *et al.*, 2013). The expression of fibrotic genes like TGF-β1, connective tissue growth factor, collagen type I, collagen type IV and fibronectin were also found to have been controlled by C21. These improvements in inflammation, fibrosis and overall renal functioning were not based on C21 mediated AT_2 receptor upregulation because the AT_2 receptor expression was not found to have been altered by C21 treatment, thus showing that the mechanism of action remains at the downstream level (Chow *et al.*, 2014).

Another study conducted by McKelvey et al showed that AT_1 receptor antagonist, Candesartan further improved the renoprotection ushered by C21 in STZ treated ApoE-/- mice by reducing albuminuria and glomerulosclerosis. However, there were no significant changes in body weight, blood glucose, acetylated haemoglobin or systolic blood pressure upon C21 treatment (Mckelvey *et al.*, 2014). Koulis et al showed that C21 treatment significantly attenuated diabetes mellitus–induced elevated levels of cystatin C, albuminuria, mesangial expansion, and glomerulosclerosis in six-week-old STZ treated ApoE−/− mice. Moreover, C21 markedly inhibited the expression oxidative stress, inflammation, and fibrosis related markers. These findings validate the potential antioxidant, anti-inflammatory cum anti-fibrotic roles of C21 in diabetes induced renal failure (Koulis *et al.*, 2015). Zucker diabetic fatty (ZDF) rats show a significant glomerular, tubulointerstitial and perivascular fibrosis accompanied by an upsurge of inflammatory mediators, including cytokines like TNF-α and macrophage infiltration. C21 treatment not only improved the fibrosis but also decreased albuminuria in ZDF rats only up to an age of 11 weeks but this effect vanished in 20 week old ZDF rats, as nephropathy further progressed. The treatment

with Losartan decreased albuminuria (upto 15 weeks) macrophage infiltration, TNF-α expression, and renal glomerular and perivascular fibrosis, restored glomerular nephrin expression, but did not reduce tubulointerstitial fibrosis, a severity marker in renal diseases. The combination of Losartan and C21 showed the best reduction in albuminuria in ZDF rats at the end of the 20 week protocol suggesting this combination as a novel pharmacological tool to slow the progression of nephropathy in type 2 diabetes (Castoldi *et al.*, 2014). The studies related to C21 and its role in diabetic nephropathy are compiled in table 2.

2. 4. Ang II mediated NF-κB pathway activation in type 2 diabetes induced nephropathy: AT_1 receptor or AT_2 receptor?

Ang II is a renal growth factor that enhances cell growth and ECM synthesis (Habib, 2013) and contributes to the recruitment of infiltrating inflammatory cells into the kidney as it leads to adhesion of circulating cells to endothelial and mesangial cells (Chaves *et al.*, 2009). This process of upregulation of adhesion molecules, cytokines and chemokines by Ang II involves upregulation of various proinflammatory genes, such as VCAM1, ICAM1, IL6 and MCP1, through the activation of several intracellular signaling systems, including the NF-κB, MAPK cascade, Rho proteins and redox maintenance pathways (Gai *et al.*, 2015; Park *et al.*, 2015). Though reports suggest a potent role of Ang II in regulation of cell proliferation, apoptosis, fibrosis, and the inflammatory response via NF-κB dependent pathways (Rehman *et al.*, 2012), the exact underlying mechanisms and the Ang II receptor subtypes orchestrating the activation of NF-κB pathway by Ang II have not yet been understood completely (Figure 5). The current literature shows a divided view on the role of the Ang II receptor subtypes in activation of NF-κB signaling pathway through various *in vivo* and *in vitro* studies (Mezzano *et al.*, 2001; Luft, 2002; Ruiz-Ortega *et al.*, 2006).

Table 2. Role of C21 in diabetic nephropathy.

#	**Animal model used/ Dose C21 administration**	**Key findings**	**References**
1.	ZDF rats C21 (0.3mg/kg/day, *ip*) and/ or Losartan (10mg/kg/day, in drinking water) for 15 weeks	↓hemodynamic imbalance, ↓glomerular, tubulointerstitial and perivascular fibrosis, ↓inflammatory mediators and macrophage infiltration, no change in blood glucose levels even with C21 and losartan combination	(Castoldi *et al.*, 2014)
2.	STZ treated ApoE-/- mice C21 (1 mg/kg/day) and/or Candesartan (4.3 mg/L) for 20 weeks	↓albuminuria and glomerulosclerosis, no significant changes in body weight, blood glucose, HbA1c or systolic blood pressure	(Mckelvey *et al.*, 2014)
3.	STZ treated ApoE-/- mice C21 (1 mg/kg/day) for 20 weeks	↓albuminuria, mesangial area and glomerulosclerosis, ↓ inflammatory mediators (MCP1, CD11), ↓ fibrosis markers (TGF-β, collagen and fibronectin)	(Chow et al., 2014)
4.	STZ treated ApoE-/- mice C21 (1 mg/kg/d) treatment via daily gavaging for 20-weeks	↓cystatin C, albuminuria, mesangial expansion, and glomerulosclerosis, ↓oxidative stress, inflammation, and fibrosis, No effect on metabolic parameters and blood pressure	(Koulis *et al.*, 2015)
5.	STZ (65mg/kg, *ip*) treated male Sprague Dawley rats C21 (0.3 mg/kg/day, *sc*) for 4 weeks	↓renal inflammatory mediators in renal interstitial fluid (TNF-α, IL6) and 8-isoprostane) and in whole kidney (TNF-α, IL6), ↑nitric oxide and cGMP levels, ↑urine albumin to creatinine ratio, no alteration in body weight, blood glucose, total kidney mass index, urine output, and SBP	(Matavelli et al., 2015)

In 1998, Ruiz Orega and colleagues showed for the first time that in a model of immune complex nephritis, Ang II mediates recruitment of inflammatory cell infiltrates through NF-κB activation and MCP1 expression by renal cells (Ruiz-Ortega *et al.*, 1998). Another study by Wolf et al showed that Ang II mediated increase in inflammatory cytokines like RANTES in glomerular endothelial cells, both *in vivo* and vitro occurs through activation of AT_2 receptors (Wolf *et al.*, 1997b). An *in vivo* study performed by Ruiz Orega and colleagues employing subcutaneous mini-osmotic pumps for systemic infusion of Ang II into normal rats showed that NF-κB recruitment in infiltrating cells was increased significantly and the treatment with ARB, Losartan diminished NF-κB activity in glomerular and tubular cells, activator protein 1 (AP-1) expression in renal cells, improved tubular damage and normalized the arterial blood pressure. On the other hand, PD123319 diminished mononuclear cell infiltration and NF-κB activity in glomerular and inflammatory cells, without altering AP-1 and hemodynamic imbalance. This study suggested the involvement of AT_1 receptors in NF-κB mediated tubular injury while AT_2 receptors regulate monocyte infiltration (Ruiz-Ortega *et al.*, 2001).

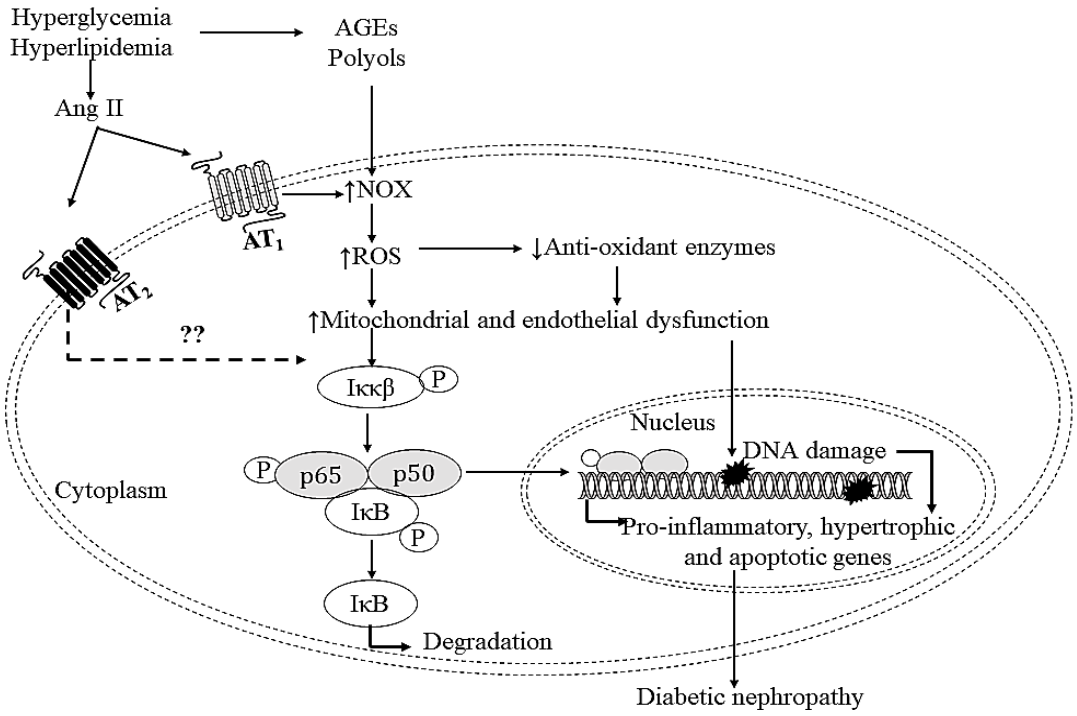


Figure 5. Ang II receptor subtypes and NF-κB activation in diabetic nephropathy.

Though the role of AT_1 receptors in activation of NF-κB through NAPD oxidase is well established, the exact role of AT_2 receptors in the activation of NF-κB has not yet been

understood completely, especially in type 2 diabetic kidney due to various reports indicating the involovement of these receptors in varied manner, depending upon the tissue type and conditions.

In an *in vitro* study performed by Wolf et al, where COS7 cells were made to express AT_1 and AT_2 receptors it was found that both AT_1 as well as AT_2 receptors played a significant role in activation of NF-κB (Wolf *et al.,* 2002b). The PC12 cells, which express only the AT_2 receptors showed an increased expression of NF-κB upon exposure to Ang II and the treatment of AT_2 receptor blocker, PD123319 led to an inhibition of NF-κB signaling (Wolf *et al.,* 2002b). In rats, glomerular endothelial cells which express both AT_1 and AT_2 receptors prominently, the activation was found to be guided by both AT_1 as well as AT_2 receptors (Wolf *et al.,* 2002b). This study showed the importance of controlling AT_2 receptor activation in addition to AT_1 receptor antagonism in order to limit Ang II-mediated proinflammatory renal and cardiovascular effects (Tea *et al.,* 2000). In VSMCs, the NF-κB signaling pathway is activated by AT_2 receptors. The antioxidants and ceramide inhibtors blocked the pathway through action on both AT_1 as well as AT_2 receptors whereas tyrosine kinase showed the action through AT_1 receptors only (Kaschina *et al.,* 2008). In another study using AT_{1a} receptor knockout mice, the Ang II mediated activation of NF-κB signaling was found to be intact, thus proving the role of AT_2 receptor in its activation (Lorenzo *et al.,* 2002; Esteban *et al.,* 2003). However, the role of the AT_{1b} receptor could also be speculated in this observation (Luft, 2002; Chang *et al.,* 2011). In tubuloepithelial cells, this was mainly by AT_1 receptor, while in endothelial cells it was via AT_2 receptor (Ayele *et al.,* 2010). In uninephroctomized Wistar Kyoto rats treated with high dose of bovine serum albumin induced renal failure evinced by proteinuria and renal lesions. The treatment with both Losartan and PD123319 reduced the tubular epithelial inflammation mediated by NF-κB, thus showing a potent role of both the AT_1 and AT_2 receptors in controlling the inflammatory cascades (Gómez-Garre *et al.,* 2001). The study performed by Esteban et al., confirmed the role of differential regulation of NF-κB by Ang II under diseased condition. In the kidney of wild type mice (C57BL/6) with unilateral ureteral obstruction, treatment with AT_1 receptor (Losartan) or AT_2 receptor antagonist (PD123319) partially decreased NF-κB activation, whereas only the AT_2 receptor

blockade diminished monocyte infiltration, which implicates that both the receptor subtypes are involved in activating NF-κB signaling pathway while AT_2 receptor orchestrates the monocyte mediated inflammation (Esteban *et al.*, 2004). A study by Wu et al showed that treatment with AT_2 receptor agonist CGP42112A in the fetal VSMCs, which express AT_2 receptors endogenously reduced the expression of macrophage infiltration marker, MCP1 to indicate the role of AT_2 receptor in TNF-α induced inflammation (Wu *et al.*, 2004). It was found that treatment with either AT_1 receptor antagonist Valsartan, the Ang II type 2 receptor antagonist PD123319, or with the NF-κB inhibitor pyrrolidine dithiocarbamate, blocked renal monocyte infiltration, NF-κB activation and upregulation of NF-κB-related pro inflammatory genes (Lee *et al.*, 2004a; Patel *et al.*, 2009). ***Despite the vast ongoing research, the exact role of Ang II receptor subtypes in regulating NF-κB signaling in type 2 diabetic kidney is not yet clearly understood. Hence, the current study aimed at delineating the mechanisms underlying activation of NF-κB mediated inflammatory cascade in type 2 diabetic kidney.***

2.5. Posttranslational histone modifications in diabetic nephropathy

The abovementioned literature survey clearly indicates a vast role of NF-κB in pathogenesis of nephropathy under type 2 diabetc condition. NF-κB other than directing the synthesis of proinflammatory cytokines also dictates the histone modifications and regulates the post translational modifications involved in gene expression during pathogenic response and thus acts as the key transcription factor in inflammatory diseases . An upregulation in this *p65* expression is linked with persistently increased histone H3 lysine 4 methylation (H3K4Me) at its promoter but not with H3 lysine 4 di-methylation (H3K4Me2) or H3 lysine 4 tri-methylation (H3K4Me3) (Paneni *et al.*, 2015). The increase in NF-κB p65 gene expression is associated with persisting epigenetic marks that are maintained when the cell is removed from its hyperglycemic environment, providing evidence that epigenetic modifications contribute to altered gene expression and could form the basis for physiologic hyperglycemic memory (Villeneuve *et al.*, 2010). The process of transcription of proinflamatory genes involves a combined activity of lineage defined transcription factors and inducible factors so as to evade the heterochromatin structure. Stimulus-dependent expression of IL1β gene requires a well sequenced

cooperative binding of three transcription factors: NF-κB, interferon regulatory factor 3 or interferon regulatory factor 7 (IRF3/IRF7), and activating transcription factor **2** (ATF-2/c-JUN) (Bhatt *et al.*, 2014). These transcription factors then promote the binding of histone acetyltransferase (HAT) complex, p300/ cAMP response element-binding protein binding protein (CBP) which then unwinds the DNA and triggers the respective gene transcription. Also, NF-κB has been shown to recruit GCN5 acetyltransferase complexes, which primarily modify histone H4K5/K8/K12 lysines (Bhatt *et al.*, 2014). It has also been shown that chromatin histone H3K4 methyltransferase, SET7/9, is a coactivator of NF-κB mediates the expression of proinflammatory factors under hyperglycemic conditions and plays a vital role in pathogenesis of type 2 diabetes induced vascular dysfunctions (Li *et al.*, 2008; Paneni *et al.*, 2015). These reports together suggest that NF-κB not only mediates the expression of numerous inflammatory cytokines but also is linked closely with the epigenetic modifications which are yet to be understood completely.

Gene transcription and activation are dynamic processes involving the conversion of compact heterochromatin into transcription factor-accessible euchromatin and thus regulating expression of genes (Zentner *et al.*, 2013). Epigenetics, the study of heritable changes in gene function without a change in the nucleotide sequence, includes DNA methylation, posttranslational histone modifications and microRNAs as the major mechanisms (Grunstein, 1997; Cheung *et al.*, 2000; Cremer *et al.*, 2001; Jung, 2016). The alterations in epigenetic states caused by environmental factors, diets, and physical activities are known to trigger gene deregulation and pathological outcomes which play an important role in progression of various diseases. In eukaryotes, the regulation of genetic information is packaged as chromatic DNA coiled into a complex, higher-order structure made up of a series of subunits named nucleosome. Each nucleosome has the folding of 147-bp linear DNA around the octamers comprising two copies of core histone proteins H2A, H2B, H3, and H4 (Sterner *et al.*, 2000; Kouzarides, 2007; Bannister *et al.*, 2011; Iwasaki *et al.*, 2013; Venkatesh *et al.*, 2015). Histone tails can undergo more than 60 different types of modification; dynamic chromatin structure is modulated by posttranslational translational modifications on histones including acetylation and methylation of lysine (K) and arginine (R), phosphorylation of serine (S) and threonine (T), ubiquitination

and sumoylation of lysines, ribosylation, deamination and proline isomerization (Vissers *et al.*, 2008; Bannister *et al.*, 2011; Sadakierska-Chudy *et al.*, 2015). These signals and inputs modulate the covalent modifications of histones to facilitate or hinder the binding of transcriptional factors to the promoters binding site by remodelling the chromatin structure. These posttranslational histone modifications occur generally at the N terminal amino acid functional groups in the tail region of the histones (Iwasaki *et al.*, 2013; Zentner *et al.*, 2013). Alterations in epigenetic chromatin marks such as histone methylation, acetylation and ubiquitination have been identified in kidney cells in response to the high glucose environment (Brennan *et al.*, 2013).

2.5.1. Histone methylation

The pioneer studies reporting methylation of lysine residues were reported in flagellin protein of Salmonella typhimurium (Ambler *et al.*, 1959) while histone methylation was first depicted in 1964 (Murray, 1964). Histone methylation taking place on both arginine and lysine residues may be mono-, di-, or trimethylated is associated with either gene expression or repression. It plays a significant role in modulating gene activity which in contrast to acetylation, is more constant and long-standing. In general, H3K36Me2/3, H3K4Me1/2/3, and H3K79Me2 are relevant to the activation of gene transcription. However, H3K9Me2/3, H3K27Me3, and H4K20Me3 are considered as repressive chromatin markers (Sun *et al.*, 2014; Sun *et al.*, 2017).

It has been reported that hyperglycemia induces histone modifications and changes in gene transcription (Shi *et al.*, 2014; Sun *et al.*, 2014; Yuan *et al.*, 2016; Sun *et al.*, 2017). Decreased histone H3 methylation, along with increased expression of pro-inflammatory genes, has been also reported in VSMCs from diabetic animals even after normalization of glycemia (Kiernan *et al.*, 2003). SET7, an H3K4 methyltransferase, was implicated in NF-κB-mediated inflammatory gene expression in TNF-α treated cell line, Tamm-Horsfall Protein 1 (THP-1) monocytes, and in macrophages from diabetic mice (Li *et al.*, 2008). A study has shown that TGF-β1 may control the expression of ECM genes in mesangial cells, exposed to high glucose concentrations, via increasing or decreasing histone methylations (H3K4Me or H3K9Me) at promoters of genes coding for the ECM components (Brasacchio *et al.*, 2009). These results prove that metabolic memory exists in the vascular dysfunction

arising from hyperglycemic exposure due to H3K4Me. Experimental data and studies in diabetic patients have shown that histone H3 methylation and acetylation may be key epigenetic events associated with pathogenesis of diabetes (Villeneuve *et al.*, 2010). The induction of key pro fibrotic genes by hyperglycemia and its downstream effectors TGF-β1 was found to be associated with increase in activation marks (H3K4Me, H3K4Me2, and H3K4Me3) and decrease in repressive marks (H3K9Me and H3K9Me2) at these gene promoters. Changes in repressive histone post translational modifications were associated with collagen III gene expression in aging induced nephropathy (Krupa *et al.*, 2010). In type 1 diabetes model, OVE26 mice, the renal gene expression of cyclooxygenase 2, S100A4/FSP1, and vimentin was upregulated and this found to be associated with increased H3K4Me2 levels (Komers *et al.*, 2013). Recently, it was found that post translational histone modifications are closely linked with metabolic memory in type 1 diabetic subjects. It revealed high correlations between the same active marks (H3K9Ac and H3K4Me3) in monocytes and lymphocytes, modest correlations between the two active marks in same cell types, and poor correlations between repressive mark (H3K9Me2) and either of the active marks. Among the 38 case hyperacetylated promoters, the expression of over fifteen genes related to the NF-κB inflammatory pathway including TNF-α, lipoteichoic acid (LTA), signal transducer and activator of transcription 1 (STAT1) and caspase recruitment domain-containing protein 8 (CARD8) were all induced by hyperglycemia (Miao *et al.*, 2014). A study by Tu et al showed that histone modifications are intricately linked with change in gene expression involved in progression of diabetes. Their results showed that progression of type 2 diabetes is associated with increased H3K9Me2 and H3K4Me levels and decreased H3 acetylation and this could be correlated with decreased *Glut2* gene expression (Tu *et al.*, 2015). Also, transient hyperglycemia has been observed to cause altered histone H3 methylation in vascular endothelial cells which was associated with persistent expression of pro-atherogenic genes (Gai *et al.*, 2015). In rat mesangial cells cultured in diabetic conditions or pretreated with TGF-β1, expressions of a methyltransferase SET7/9 increased, along with the enrichment of SET7/9 at fibrotic gene *Col1a1/Col4a1* promoter regions. Also, SET7/9 gene silencing was found to suppress *Col1a1/Col4a1* gene expression (Yuan *et al.*, 2016).

A study showed that reduced H3K79Me at renal epithelial sodium channel promoter increased gene expression in response to aldosterone signaling (Zhang *et al.*, 2006). Histone H4K20Me, comparatively less studied histone modification regulates DNA replication and DNA damage repair while the trimethylation acts as a repressive mark. In an induced diabetic retinopathy model, increased occupancy of H4K29Me3 at mitochondrial *Sod* gene was found to be correlated with poor glycemic control. In an induced rat model, poor glucose control can lead to retinal key antioxidant (Zhong *et al.*, 2011). Both mouse cortical collecting duct M1 cells models and diabetic nephropathy patients can develop polyuria due to the upregulation of aquaporin 5, an aquaporin 2 binding partner and decreased H3K79Me2 (Wu *et al.*, 2013). These reports clearly suggest the active involvement of histone methylation in inflammation and fibrosis, which are the pillars for development of secondary complications of diabetes. Responsive epigenetic marks identified include H3KMe active and repressive marks (Sun *et al.*, 2010a) and H2A/H2B ubiquitination (Gao *et al.*, 2013).

2.5.2. Histone ubiquitination

The process of ubiquitination involves the addition of ubiquitin, a highly conserved and abundantly expressed 76-amino-acid protein, onto a protein (Swatek *et al.*, 2016). It has also been called “kiss of death” because it often commits the labelled protein to proteasomal degradation to decide the fate of various proteins in the cells, by either directing them towards proteasomal degradation or participation in several cell signaling pathways (Shaid *et al.*, 2013). The addition of ubiquitin moiety to a protein substrates involves a set of three enzymes, E1, E2 and E3 which activate, conjugate and ligate the ubiquitin moiety to the target protein in a tightly regulated and sequential manner (Pickart *et al.*, 2004; Komander, 2009). E3 ligases are the most important and widely studied enzymes in the ubiquitination pathway. The C-terminus of ubiquitin can be conjugated to one of the seven internal lysine residues (K6, K11, K27, K29, K33, K48, K63) on another ubiquitin, forming polyubiquitin chain of different linkages (Komander *et al.*, 2012). Ubiquitination is revocable, and the ubiquitinated proteins can be proteolytically deubiquitinated by specific lineages of deubiquitinating enzymes called deubiquitinases (Pickart *et al.*, 2004; Komander, 2009).

Histone ubiquitination is a prominent epigenetic mark that may influence changes in gene expression and involves a variety of chromatin-based events, such as gene silencing and repair of DNA damage (Kouzarides, 2007). The majority of histone ubiquitination occurs on chromatin by the addition of a single ubiquitin molecule via isopeptide linkage to a specific lysine residue on the C-terminal tail of histones H2A and H2B. To a lesser extent, histones H1, H3, and H4 can be ubiquitinated *in vivo* and ubiquitination of different histones has distinct functions (Higashi *et al.*, 2010). The first protein which was found to be ubiquitinated was H2A and it was found that about 5–15% of total H2A was in ubiquitinated state in a normal mammalian cell. Though the functions of H2A ubiquitination remain unclear, abundant instances indicate its role in gene repression and DNA damage repair and cell cycle progression (Vissers *et al.*, 2008; Zhou *et al.*, 2008). H2AK119Ub is mediated by the B lymphoma Mo-MLV insertion region 1 homolog (Bmi-1)/Ring1A protein found in the human polycomb complex and is associated with transcriptional repression. In contrast, H2BK120Ub is mediated by human ring finger protein 20 (RNF20)/RNF40 and E2 ubiquitin-conjugating enzyme E1 (Ube2e1) and is required for active transcription (Cao *et al.*, 2005; Zhu *et al.*, 2005). Bmi-1, an E3 ligase, play an important role in H2A ubiquitination and homeobox gene silencing through H3K27Me by increasing methyltransferase enhancer of zeste homolog 2 (EZH2) and H2AK119 ubiquitination (Cao *et al.*, 2005).

Moreover, H2B ubiquitination is associated with the activated and transcribed regions of highly expressed genes (Minsky *et al.*, 2008). Overexpression of RNF20, an E3 ligase specific for H2B, subsequently increased the levels of H3K4 and H3K79 methylation, and stimulation of HOX gene expression. In contrast, inhibition of RNF20/40 complex reduced H2B monoubiquitination, lowers H3K4 and H3K79 methylation, and repressed HOX gene expression (Zhu *et al.*, 2005). In yeast, histone H2BK123 monoubiquitination, mediated by the E2-conjugating enzyme radiation sensitivity protein 6 (Rad6) and the E3 ligase Bre1 in yeast is a prerequisite for histone H3K4 and H3K79 methylation (Wood *et al.*, 2003) and deubiquitination of H2BK123 is required for histone H3K36Me (Henry *et al.*, 2003).

A study showed that hyperglycemia may cause cell damage, induce the ubiquitination of histone H2A, and reduce the ubiquitination of histone H2B in mesangial cells to

accelerate and dampen the progression of diabetic nephropathy, respectively (Li *et al.*, 2008). Recent studies showed that hyperglycaemic state promoted monoubiquitination of histone H2AK119 and reduced the monoubiquitination of histone H2BK120 in glomerular mesangial cells, which in turn elevated the TGF-β mediated pathways leading to fibrotic gene expression. These alterations occurring in hyperglycaemic state were significantly controlled by exposure to a 26s proteasome inhibitor, MG132 which could partially be attributed to its ability to limit NF-*κ*B mediated inflammatory signaling through inhibiting the I*κ*B*α* sumoylation and ubiquitination (Gao *et al.*, 2013; Huang *et al.*, 2013).Reecently, the role of histone ubiquitination in regulation of methylation marks was reported by Goru et al, who showed that both H2AK119Ub and H2BK120Ub were increased in whole kidney, while both were reduced in the glomerular fraction. Also, decreased occupancy of H2AK119Ub and H2BK120Ub were observed on the promoters of *Set7/9* and *Suv39h1* in diabetic kidney. In addition, methylation marks regulated by H2AK119Ub (H3K27Me2 and H3K36Me2) and H2BK120Ub (H3K4Me2 and H3K79Me2) were also found to be altered on the promoters of *Set7/9* and *Suv39h1* showing the role of H2AK119Ub and H2BK120Ub in regulating histone H3K4Me2 and H3K9Me2 through modulating the expression of SET7/9 and SUV39H1 in the development of diabetic renal fibrosis (Goru *et al.*, 2016). ***However, the effects of histone monoubiquitination on pathogenesis of diabetic nephropathy are incompletely understood and need to be studied in depth so as to understand the posttranslational histone modifications and their cross-talks more clearly.***

Histone ubiquitination has also been found to be closely linked with histone acetylation (Zhu *et al.*, 2007). Histone H2A deubiquitinase (2A-DUB, or KIAA1915/MYSM1) interacts with PCAF to regulate transcription by coordinating histone acetylation and deubiquitination, and destabilizing the association of linker histone H1 with nucleosomes so as to trigger androgen receptor-dependent gene transcription, which in association with reduced H2A ubiquitination serves as an important prostate cancer related mark (Zhu *et al.*, 2007).

2.5.3. Histone acetylation

The levels of histone acetylation are tightly controlled by histone acetyl transferases (HATs) and histone deacetylases (HDACs) (Bayarsaihan, 2011). HAT transfers acetyl

group from acetyl-CoA to ε-amino group of lysine residues on N-terminal region histones in tail regions. This relaxes the chromatin structure and allows transcription to proceed (Loidl, 1994) while deacetylation catalyzed by HDAC restores positive charge on histone coupling negatively charged DNA to it (Ruthenburg *et al.*, 2007). In general histone and other proteins' acetylation promotes the gene transcription and deacetylation leads to repression or gene silencing (Wolffe, 1996; Xu *et al.*, 2007). The pathogenesis of diabetic nephropathy is linked with alterations in histone acetylation levels.

Hyperglycemia induced inflammatory gene expression was associated with increased promoter H3/H4KAc, and enhanced recruitment of acetyl transferase, CBP/P300 as well as the pro inflammatory transcription factor NF-κB in monocytes (Miao *et al.*, 2004). The diabetic patients showed a significant increment in H3K9/14Ac and H4K5/8/12Ac TNF-α and cycloxygenease 2 inflammatory genes promoters in human blood monocytes (Miao *et al.*, 2004). HDAC2 activity was increased in kidneys of diabetic animals and inhibition of HDAC activity blocked diabetes-induced fibrotic gene expression (Noh *et al.*, 2009). In a rat model of type 2 diabetes-induced diabetic nephropathy, hyperglycemia in the kidneys was associated with reduced levels of active chromatin marks H3KAc and Ser10 phosphorylation at the fibrillin 1 and collagen type III alpha1 gene promoters. Changes in global histone modifications were seen in the kidneys of uninephrectomized diabetic *db/db* mice (Sayyed *et al.*, 2010). PCAF, a transcriptional adaptor protein and HAT functions as catalytic subunit of transcriptional co-activator complex to acetylate histone H3 and H4 (Shi *et al.*, 2014). It facilitates transcriptional activation of NF-κB by reducing its binding to IκBα enhancer and enhancing its nuclear localization (Kiernan *et al.*, 2003). Additional *in vitro* and *in vivo* studies have suggested a possible role for HDAC in TGF-β1 mediated ECM production and kidney fibrosis (Badal *et al.*, 2014). The role of elevated acetylated histone in vascular injury and remodeling has also been reported in type 2 diabetic patients (Bugger *et al.*, 2014). In a recent study, diabetic C57BL/6J male mice showed significantly and progressively increased expression of H3K9/14Ac, as compared with age-matched controls, at both the 3 month and 6 month time points; however, the C66 or JNK inhibitor for 3 months showed significant attenuation of the diabetes-related increase in the expression of

H3K9/14Ac levels, which persisted through the 3 months after the end of treatment. The diabetes induced elevation in p300/CBP occupancy on the promoters of the *Ctgf, Tgfb1, Pai1* was significantly controlled by curcumin analogue 66 (Wang *et al.*, 2015b).

The importance of studies related to various histone modifications and their mechanisms is further highlighted by a recent study conducted by Shao et al whose studies on panoramic expression profile of 164 enzymes in 19 human and 17 murine tissues showed that the differential expression of histone modification enzymes in various tissues including cardiovascular, immune and other tissues. They also found that heart and T cells are the tissues in which histone acetylation/deacetylation, and histone methylation/demethylation are in the highest varieties whereas the Treg cells are having more downregulation than upregulation of histone modification enzymes under metabolic diseases. These results have demonstrated that the few upregulated enzymes may be potential novel therapeutic targets in metabolic diseases and Treg activity (Shao *et al.*, 2016).

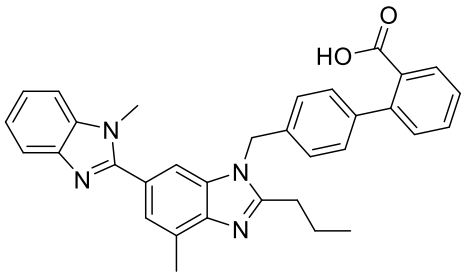
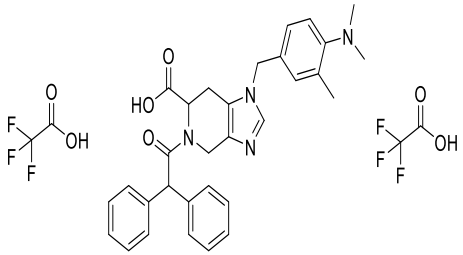
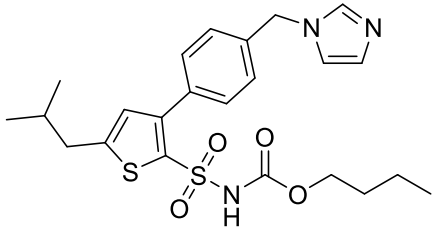
2.6. Effects of Ang II receptors' activity on posttranslational histone modifications in diabetic nephropathy

The abovementioned reports demonstrate the importance of studying RAS and posttranslational histone modifications in diabetic nephropathy. Despite the fact that ARBs and ACE inhibitors are the mainstay of the pharmacological interventions currently used to delay the progression of diabetic nephropathy, the epigenetic effects of these agents have not yet been studied in-depth. In a recent study, ARB, Candesartan or Irbesartan reduced methylation of the nephrin promoter in murine glomeruli of an adriamycin nephropathy model with recovery of KLF4 (Kruppel-like factor 4) expression and a decrease in albuminuria. In podocyte-specific KLF4 knockout mice, the effect of ARB on albuminuria and the nephrin promoter methylation was attenuated. In cultured human podocytes, Ang II reduced KLF4 expression and caused methylation of the nephrin promoter with decreased nephrin expression. In patients, nephrin promoter methylation was increased in proteinuric kidney diseases with decreased KLF4 and nephrin expression. KLF4 expression in ARB treated patients was higher in patients with than without ARB treatment (Hayashi *et al.*, 2015). Zhong et al suggested that since the conjugation of ACE

inhibitor (benzapril) and HDAC inhibitor (Vorinostat) reduced the renal damage due to abnormal gene expression observed in human immunodeficiency virus-associated nephropathy, this could be a potential combination for the treatment of various other chronic kidney diseases (Zhong *et al.*, 2013). There are only few reports regarding the influence of ARBs on posttranslational histone modifications. Reddy et al showed that the renal damge in *db/db* mice could be attributed to elvated RNA polymerase II recruitment and permissive histone marks as well as decreased repressive histone marks at the promoter regions of RAGE and PAI genes. Though treatment with ARB, Losartan attenuated key parameters of diabetic nephropathy and increased H3K9/14Ac at RAGE, PAI1, and MCP1 promoters in mesangial cells cultured under diabetic conditions it could not ameliorate other modifications like H3K36Me3 at the MCP1 gene or H3K4Me1/2/3 at RAGE, PAI1 and MCP1 (Reddy *et al.*, 2014a). Xu et al showed that in rats with myocardial hypertrophy, the expression of HDAC2 was increased in contrast to the levels of HDAC5 and HDAC9 and these epigenetic changes were reversed by treatment with Valsartan (Xu *et al.*, 2015). Thus, combination therapies targeting epigenetic regulators and AT_1 receptors could be evaluated for more effective treatment of diabetic nephropathy. ***These studies clearly show that the studies clarifying the effects of agents acting on RAS, especially the ACE inhibitors and ARBs on the post translational histone modifications are limited and hence urge us to to dig deeper into the matter and unearth the relationship between Ang II receptor subtypes and histone modifications.***

Taken together, the abovementioned literature survey shows that despite the significant role of Ang II receptor subtypes in diabetic nephropathy and their roles in regulation of NF-κB mediated inflammation, the exact roles of the receptor subtypes still needs to be demystified. Therefore, in the current study an attempt was made to establish the relationship between the key effectors of type 2 diabetes induced nephropathy including Ang II receptors, NF-κB mediated signaling pathways and the posttranslational histone modifications.

Table 3. Chemical details of Telmisartan, PD123319 ditrifluoroacetate, and Compound 21

Parameter	**Molecule**		
	Telmisartan	**PD123319 ditrifluoroacetate**	**Compound 21**
IUPAC name	2-[4-[[4-methyl-6-(1-methylbenzimidazol-2-yl)-2-propylbenzimidazol-1-yl]methyl]phenyl]benzoic acid	1-[[4-(Dimethylamino)-3-methylphenyl]methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridine-6-carboxylic acid ditrifluoroacetate	Butyl N-[3-[4-(imidazol-1-ylmethyl)phenyl]-5-(2-methylpropyl)thiophen-2-yl]sulfonylcarbamate
Chemical structure			
Molecular weight	514.629 g/mol	736.67 g/mol	475.16 g/mol
Molecular formula	$C_{33}H_{30}N_4O_2$	$C_{31}H_{32}N_4O_3.2CF_3CO_2H$	$C_{23}H_{29}N_3O_4S_2$

3. Background and objectives

3.1. Background

As it is clear from the above mentioned literature survey, Ang II receptors and NF-κB have a pronounced role in progression of chronic inflammation and associated renal failure in type 2 diabetes. Though vast scale studies have been performed regarding the role of AT_1 receptor antagonists in treatment of diabetic nephropathy and their role in controlling NF-κB mediated inflammatory cascade, the views regarding the role of AT_2 receptors in regulation of NF-κB signaling remain contradictory. Existing studies showed that NF-κB signaling was regulated by different Ang II receptor sub-types in different tissues and conditions, thereby urging us to determine the exact roles of Ang II receptor sub-types in regulation of this pathway in diabetic nephropathy. Also the effect of AT_1 and AT_2 receptors' activity on expression of ACE and ACE2 remained unknown, despite their significant roles as components of deteriorative and protective axes of RAS, respectively.

Another important gap in the existing literature was that though ARBs and angiotensin ACE inhibitors are the mainstay of currently available pharmacological interventions, their effects on epigenetic posttranslational modifications have not yet been studied in-depth. As suggested by recent studies, genetic penchant alone may not be sufficient to explain the pathogenesis of diabetic nephropathy and thus, epigenetic mechanisms must be studied thoroughly to understand the complete phenomenon. Posttranslational histone modifications like histone H3 acetylation, H3 methylation and H2A and H2B monoubiquitnation play a substantial role in regulation of gene transcription and are extremely important to unearth the exact pathogenic basis of diabetic nephropathy. The imact of therapeutic interventions acting through Ang II receptor subtypes on histone covalent modifications must also be evaluated so as to check the epigenetic basis of their activities. The scarce information available regarding the relationship between the Ang II receptor activity and the posttranslational modifications inflicted by these agents urges us to conceive the current study aimed to connect this missing link in existing literature (Figure 6).

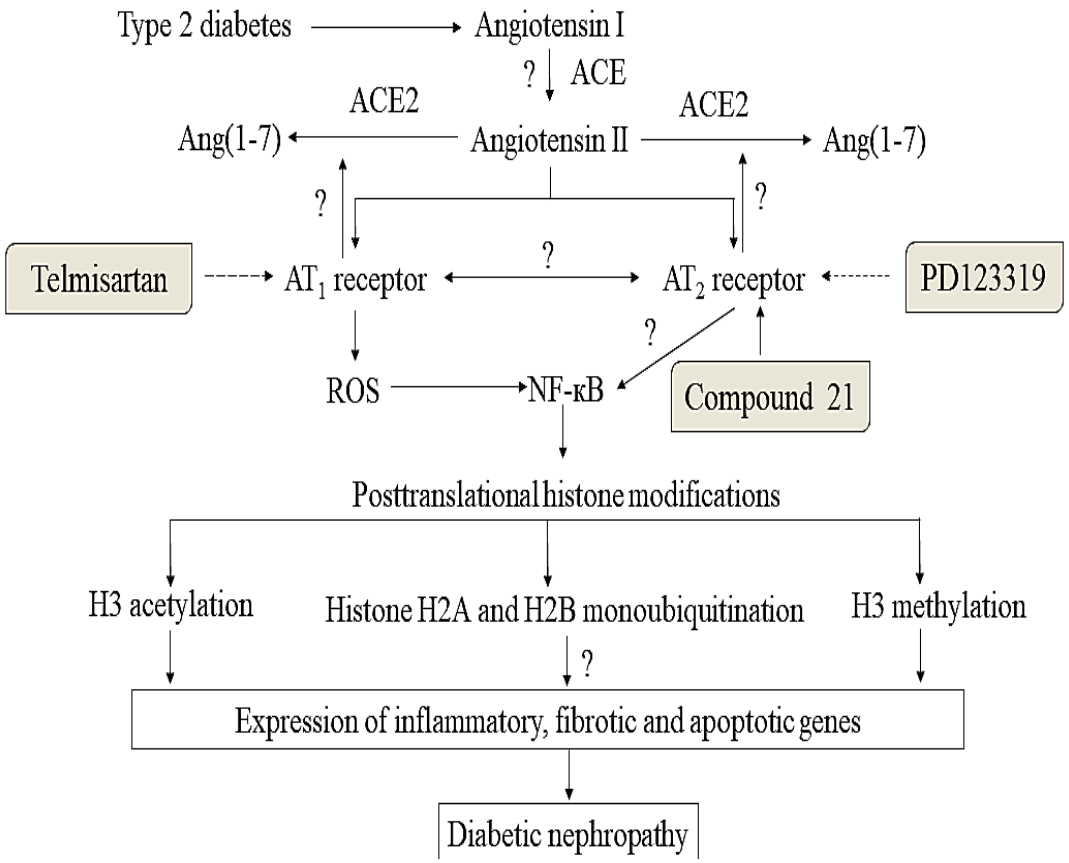


Figure 6. Gaps in existing research.

3.2. Objectives

- To understand the differential effects of Ang II receptor subtypes on the expression of ACE and ACE2 in type 2 diabetic nephropathy.
- To delineate the role of Ang II receptor subtypes in regulation of NF-κB mediated inflammatory signaling in type 2 diabetic nephropathy.
- To understand the role of Ang II receptor subtypes in modulating the posttranslational histone modifications and their role in regulating inflammation in type 2 diabetic nephropathy.

4. Materials and Methods

4.1. Materials

4.1.1. Chemicals

PD123319 ditrifluoroacetate was procured from Tocris Biosciences, (Bristol, UK). Streptozotocin (STZ) was procured from Sigma-Aldrich (St. Louis, MO, USA). Compound 21 (C21) has been obtained as a gift sample, kindly provided by Anders Ljunggren, Vicore Pharma., Sweden. Antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Cell Signaling Technology (Danvers, MA, USA). For biochemical estimation, spectrophotometric kits purchased from Accurex (Accurex Biomedical Pvt. Ltd., Mumbai, India) and ultra-sensitive rat insulin kit obtained from Crystal Chem (Downer's Grove, IL, USA). Enhanced chemiluminescence reagent was purchased from Thermo Fisher Scientific (Waltham, MA, USA).

4.1.2. Instruments

Major instruments used to conduct present experiments methods are enlisted bellow

Table 4. List of instruments used in the study.

Name of instruments	**Make**	**Country**
Non-invasive blood pressure (NIBP) system	AD Instruments	Australia
Microtome	Leika	Germany
Microscope	Olympus	USA
Vertical gel electrophoresis unit	Bio-Rad	USA
Semi-dry transfer apparatus	Bio-Rad	USA
Amersham Hypercassette	GE Healthcare	USA
Thermocycler	BR Biochem	India
Light cycler-96	Roche	Germany
DynaMag-2	Thermo Fisher Scientific	USA

4.2. Methods

4.2.1. Animal studies

Adult male *Wistar* rats (160-180g) were procured from the Central Animal Facility of the institute, Birla Institute of Technology and Science Pilani (BITS Pilani). The animals were maintained under standard environmental conditions and were provided with feed and water *ad libitum*. All the animals were fed on normal pellet diet (NPD) one week prior to the experimentation. A prior permission was sought from the Institutional Animal Ethics Committee, BITS Pilani, for conducting the study (IAEC/RES/17/04/ Rev-2/19/36 and IAEC/RES/17/04/ Rev-2/19/36).

4.2.2. Development of animal model for type 2 diabetic nephropathy

The animal model for type 2 diabetes induced nephropathy was developed using HFD and administration of low dose of STZ (Gaikwad *et al.*, 2010). Briefly, the rats were divided into two groups- NPD and HFD fed rats (58% fat, 25% protein and 17% carbohydrate, as a percentage of total kcal) *ad libitum* respectively for an initial period of 2 weeks. After 14 days of dietary manipulation, the HFD fed rats were treated with low dose of STZ (35mg/kg, *intra peritoneal*) dissolved in ice cold citrate buffer (0.1 M, pH 4.4) to induce type 2 diabetes. The induction of type 2 diabetes was confirmed by analyzing the plasma glucose levels after 48 hours of induction. After induction of diabetes, the rats were allowed to be fed on HFD diet for 24 weeks (Figure 7).

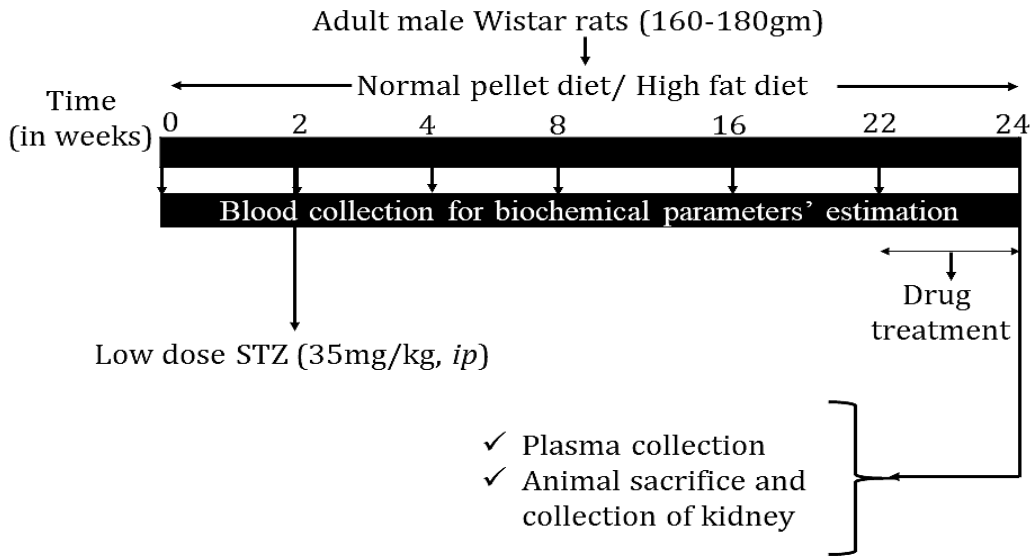


Figure 7. Study plan for induction of type 2 diabetes induced renal failure in Wistar rats.

4.2.3. Drug treatment

The HFD+STZ treated rats were administered with Telmisartan (10mg/kg, *per oral*) (Khan *et al.*, 2011; Al-Rejaie *et al.*, 2015; Heeba *et al.*, 2015), PD123319 ditrifluoroacetate (10mg/kg, *subcutaneously*) (Jones *et al.*, 2012; Umschweif *et al.*, 2014) or both or vehicle (0.5% w/v sodium carboxy methyl cellulose) for 14 days prior to the study termination. In the group receiving both Telmisartan and PD123319, both the agents were administered one after the other, without any significant time lag. The dose of Telmisartan was selected based upon the literature that suggests that Telmisartan (10 mg/kg/day, *per oral*) reduces the pace of progression of glomerulosclerosis, and significantly improved interstitial cell infiltration, interstitial fibrosis, dilation and atrophy of renal tubules, normalized plasma lipids (total cholesterol and triglyceride), thus showing its renoprotective effect (Ohmura *et al.*, 2012).

In study 2, the HFD+STZ rats were treated with Telmisartan (10mg/kg, *per oral*), Compound 21, their combination or vehicle. According to the reports provided by Vicore Pharma, C21 shows non- selectivity above the dose 0.3mg/kg thus prompting us to choose 0.3mg/kg as the highest dose for our study. Since, the studies related to the orally effective dose of C21 in type 2 diabetic nephropathy were not available, three doses of C21 were selected- 0.075, 0.15 and 0.3 mg/kg. In the group receiving both Telmisartan (10mg/kg, *per oral*) and C21 (0.3mg/kg, *per oral*), both the agents were administered one after the other, without any significant time lag. Animal body weights and kidney weights were noted and analyzed at the end of study. The animals were divided into different groups, so that the number of animals maintained in each experimental group was 6. The experimental groups for study 1 and study 2 are mentioned in Figure 8 and 9, respectively.

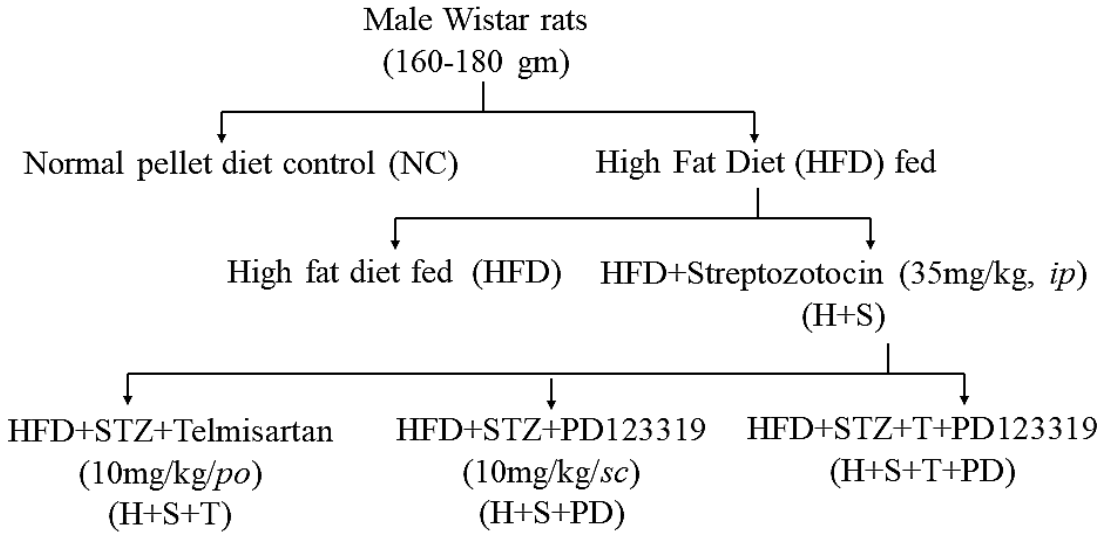


Figure 8. Experimental groups for study 1.

(A) Normal control (NC) (B) High fat diet fed (HFD) (C) High fat diet+Streptozotocin (35mg/kg, *ip*) (H+S) (D) High fat diet+STZ+Telmisartan treated (10mg/kg/*po*) (H+S+T) (E) High fat diet+STZ+PD123319 ditrifluoroacetate treated (10mg/kg/*s.c.*) (H+S+PD) (F) High fat diet+STZ+Telmisartan+PD (H+S+T+PD).

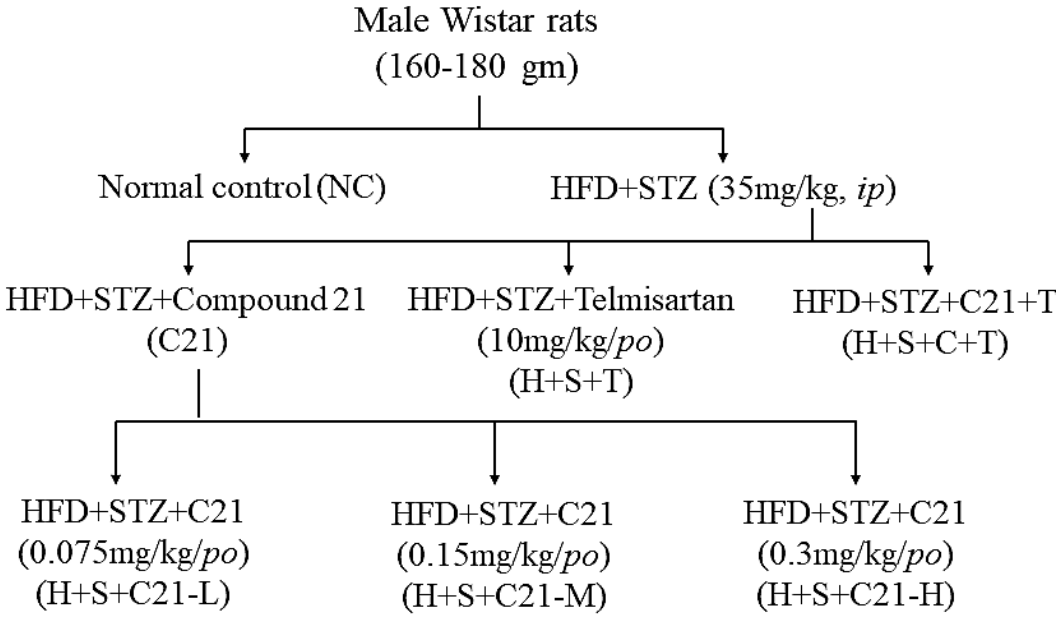


Figure 9. Experimental groups for study 2.

(A) Normal control (NC) (B) High fat diet+STZ treated (35mg/kg,*ip*) (H+S) (C) High fat diet+STZ+Telmisartan treated (10mg/kg/*po*) (H+S+T) (D) High fat diet+STZ+C21 treated (0.075mg/kg/*po*) (H+S+C21-L) (E) High fat diet+STZ+C21 treated (0.15mg/kg/*po*) (H+S+C21-M) (F) High fat diet+STZ+C21 treated (0.3mg/kg/*po*) (H+S+C21-H), also mentioned as HSC at few points in the text (G) High fat diet+STZ treated+C21 treated (0.3mg/kg/*po*)+Telmisartan (10mg/kg/*po*) (H+S+C+T).

4.2.4. Assessment of type 2 diabetes and renal function

Venous blood (0.5mL) was collected from animals fasted overnight and plasma was separated from the blood by centrifuging the blood samples at 2000 x g for 15 min, at 4°C. The blood samples were collected and plasma was analyzed for glucose (PGL), triglycerides (PTG), total cholesterol (PTC), blood urea nitrogen (BUN), creatinine (PCr), albumin (PAL) and alkaline phosphatase (ALP) (using commercially available kits, Accurex, Mumbai, India) (Khan *et al.*, 2016; Nanaware *et al.*, 2017). Insulin determination was made by ELISA kit using rat insulin as the standard (Rat ELISA kit, Crystal Chem, USA) (Sims-Robinson *et al.*, 2016; Griffin *et al.*, 2017).

4.2.5. Assessment of systolic blood pressure

Systolic blood pressure (SBP) was recorded at end of treatment period in the pre-acclimatized animals, using a tail cuff blood pressure recorder (AD Instruments, Bella Vista, NSW, Australia) (Yiallourou *et al.*, 2012; Bhaswant *et al.*, 2015).

4.2.6. Protein Determination

Protein content in the kidney samples was measured by the Lowry's method using bovine serum albumin [(BSA) 1 mg/ml] as a standard (Lowry *et al.*, 1951). Briefly, kidney samples were homogenized and the diluted supernatant was made up to 1 ml using distilled water and Lowry's reagent was added to it. The thoroughly mixed contents were allowed to stand for 15-30 minutes at room temperature followed by addition of Folin-Ciocalteu reagent. The solution thus formed was vortexed vigorously and incubated at room temperature for 30 minutes. The standard curve was plotted using BSA. The protein content in the kidney samples was determined spectrophotometrically at 660 nm.

4.2.7. Estimation of oxidative stress level in kidney

The estimation of oxidative stress was carried out by estimating the lipid peroxide and reduced glutathione levels in the kidney. Briefly, after sacrificing the animals, the kidneys were excised, decapsulated and rinsed with normal saline and weighed. The lipid peroxide level in animal tissues was measured according to method described by Ohkawa et al., 1979. After weighing, kidney tissue was minced properly and the homogenate was prepared in 1 mL of cold phosphate buffered

saline (pH 7.4) and centrifuged at 700×*g*. Supernatant was collected and used for estimation of thiobarbituric acid reacting substances (TBARS) by using spectrophotometric method at 532nm (Ohkawa *et al.*, 1979; Palma *et al.*, 2014).

The reduced glutathione (GSH) content was estimated according to Ellman's method (Boyne *et al.*, 1972). For reduced glutathione, kidney tissues were homogenized in 10 ml ice-cold homogenizing buffer combined with sulphosalicylic acid and the homogenate was centrifuged. Ellman's reagent was added to the supernatant to produce yellow colour of 5-thio-2-nitrobenzoate-SH. The absorbance was taken at 412 nm.

4.2.8. Estimation of nitrosative stress in kidney

The accumulation of nitrite in the supernatant, an indicator of the production of nitrite (NO), was determined by a colorimetric assay using Griess reagent (0.1% N-(1-naphthyl) ethylenediamine dihydrochloride, 1% sulfanilamide, and 2.5% phosphoric acid) (Green *et al.*, 1982). Briefly, equal volumes of supernatant and Griess reagent were mixed and the mixture was incubated for 10 minutes at room temperature in dark and the absorbance was determined at 540nm spectrophotometrically. The concentration of nitrite in the supernatant was determined from a sodium nitrite standard curve and expressed as micromoles per milligrams protein present in kidney samples.

4.2.9. Histopathological evaluation

Histopathology was performed as per the protocol described previously (Gaikwad *et al.*, 2010). Briefly, the kidney tissue was fixed in 10% (v/v) formalin in phosphate buffered saline and embedded in paraffin. 5µm sections were deparaffinized with xylene (2 times, three minutes each) and rehydrated using gradient percentages of ethanol (100%, 95%, 70%, 50%; 3 minutes each) and hematoxylin and eosin staining was performed (Liu *et al.*, 2016). Histopathological images were captured by using Olympus microscope (Model no. BX41, NY, USA). Glomerular damage was assessed using a semi quantitative score by a blinded observer as follows: 0 = no lesion, 1 = <25% damage, 2 = 25-49 % damage, 3 = 50-74% damage, 4 = 75-100% damage, respectively (Ninichuk *et al.*, 2008).

For study of deposition of ECM, the sections were washed under running tap water and stained with 0.5% Picrosirius Red (PSR) stain using saturated picric acid solution in distilled water for 1 hr. After staining with PSR, slides were treated with acidified water (5ml glacial acetic acid in 1000ml distilled water; 2 times, 5 minutes each), then the sections were washed under running tap water and dehydrated using ethanol (100%; 3 changes, 3 minutes each), placed in xylene and proceeded for mounting using di-N-Butyl Phthalate in Xylene (DPX) media (Tsuda *et al.*, 2013; Kang *et al.*, 2015). At least 25 kidney sections from each group (4-5 sections from each animal) were observed for deposition of ECM (collagen), and percentage fibrotic (collagen positive) area was analyzed using ImageJ software and the data analysis was carried out by using GraphPad 5 Prism software.

4.2.10. Immunohistochemistry

Immunohistochemistry was performed (Karpe *et al.*, 2014; Khan *et al.*, 2015; Kumar *et al.*, 2016). Briefly, kidney sections (5 µm) were taken from paraffin blocks and deparaffinized with xylene, followed by antigen retrieval by heating in citrate buffer (10 mmol/L for 45 minutes). After, antigen retrieval, the sections were cooled down to room temperature and treated with H_2O_2 (3%) for 15 minutes (to block endogenous peroxides), washed with tris buffered saline (1X TBS) and blocked by using BSA (5%) solution. After blocking, the sections were incubated with the primary antibodies mentioned in table 5 (12 hrs at 4°C) and rinsed thrice with TBS. Further, these sections were incubated (1 hr at room temperature) with respective secondary antibodies, followed by detection with diaminobenzidine (DAB) as a chromogen. The sections were counterstained with hematoxylin, dehydrated with alcohol and xylene, and mounted in DPX (Sigma Aldrich). All the images were analyzed using ImageJ software for calculating DAB positive area. The list of antibodies used has been mentioned in table 5.

Table 5. List of primary antibodies used in immunohistochemistry.

Cell signaling technology, USA	**Santa Cruz Biotechnology, USA**
TGF-β1 NF-κB p65 Phosphorylated NF-κB p65 (Ser536) Phosphorylated IκB-α (Ser32) Ki67 (*Dilution used-1:200)*	AT_1 receptor AT_2 receptor ACE ACE2 Keap1 SET7/9 MCP1 Fibronectin *(Dilution used-1:200)*

4.2.11. Histone isolation and Western blotting

Histone isolation and western blotting were performed (Gaikwad *et al.*, 2010; Kumar *et al.*, 2016). Briefly, tissues was dissected manually, homogenized in Buffer-A [12% w/v sucrose, 10mM EDTA, 5mM NaCl, 10mM Tris, 1% PMSF (0.1M), 0.1% NaBr (1M), and pH-7.2]. After filtration this homogenate was layered on Buffer-B [15%w/v sucrose, 10mM EDTA, 5mM NaCl, 10mM Tris, 1% PMSF (0.1M), 0.1% NaB (1M), and pH-7.4] and centrifuged at 4000 rpm. Subsequent layering was done by adding 1% Triton-X solution, after centrifugation nuclear palette was re-suspended in modified LSB. Further treatment with concentrated HCl, this solution was sonicated and centrifuged at max speed, then supernatants was collected and 25% trichloroacetic acid was added for precipitation of protein. After final centrifugation at max speed palette which contain histone protein was dissolved in water. Isolated histones were subjected to 14% SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Amersham, GE healthcare Bio-Science, Pittsburgh, PA, USA). Immunoblot analysis was performed by using specific rabbit monoclonal antibodies mentioned in the table 6. Anti-rabbit IgG, HRP-linked antibody was used as secondary in 1:20,000 (v/v) dilution (Cell Signaling Technology, Danvers, MA, USA). Proteins were detected by the ECL system and ECL Hyperfilm. Immunoblots were quantified by densitometric analysis using ImageJ software and the exposures were in linear dynamic range, each modification was normalized by respective total H3 and H2A blot, then data

analysis was performed by using Prism software (version 5.0; GraphPad, San Diego, CA, USA) and results were expressed as fold over NC.

Table 6. List of primary antibodies used in Western blotting.

Cell signaling technology, USA		**Santa Cruz Biotechnology, USA**
H2AK119Ub	Cleaved PARP	β –actin
H2BK120Ub	Cleaved caspase 3	MCP1
H2A total	Caspase 3	
H3 total	PCAF	
H3K4Me2		
H3K36Me2		
H3K79Me2		
H3K9Ac		
H3K14Ac		
H3K18Ac		
H3K28Ac		
H3K56Ac		

4.2.12. RNA isolation and Real Time-Polymerase Chain Reaction (RT-PCR)

RNA was isolated from kidneys by using commercially available kit (Ambion™ PureLink™ RNA Mini Kit, Life Technologies, USA). 5 µg of RNA was taken and incubated with 1 µl (2U) of recombinant DNase1 for 30 min at 37°C [Ambion™ Recombinant DNase I (RNase-free), Life Technologies, USA] to remove the single or/and double stranded DNA, chromatin and RNA-DNA hybrids present in the sample. Further, DNase1 was inactivated by heating the samples at 75°C along with 5mM of EDTA. cDNA was synthesized by using cDNA kit (GeneSure™ First Strand cDNA Synthesis Kit, Puregene, Genetix brand, USA). The samples were incubated at 25°C for 5 min, 42° C for 60 min followed by inactivation at 70° C for 5 min. Quantitative real-time polymerase chain reaction of the samples was performed as per the protocol described previously on Light Cycler® 96 Real-Time PCR System using the Fast Start Essential DNA Green Master and results were analyzed by Light Cycler® Software (Roche, Mannheim, Germany). Primers were designed (Table 7) and obtained from Eurofins, Bengaluru, India. After

amplification, a melting curve analysis was performed to verify the specificity of the reaction. Levels of mRNA were normalized to their respective *18s* contents. Experiments were carried out in triplicate (n=3) for each sample and results were expressed as fold change over respective control (Gaikwad *et al.*, 2010; Kumar *et al.*, 2016).

4.2.13. Chromatin-Immunoprecipitation (ChIP) assay

ChIP assay was performed as per the protocol provided by the supplier (Gaikwad *et al.*, 2010). MAGnify™ Chromatin Immunoprecipitation System (Thermo Fisher Scientic, CA, USA) according to manufacturer's guidelines. Briefly, kidneys were chopped into small pieces, re-suspended in phosphate buffered saline (PBS) and cross linked with 1% (v/v) formaldehyde for 10 minutes. Cross linking reaction was stopped by adding 0.125M glycine, washed thrice with PBS containing protease inhibitors and lysed in SDS lysis buffer. Chromatin was sonicated for 10 s and the lysate was allowed to cool for 60s over ice. This procedure was repeated for six cycles to obtain chromatin size of 0.5-1 kb. Lysates were incubated for 2 h with H2AK119Ub antibody. Before the addition of antibody, input samples were removed from the lysate and stored at -20^{o}C until extraction. Following incubation with antibody, protein-DNA complexes were eluted, and the cross-links were reversed using cross linking buffer. The DNA obtained was purified using magnetic beads. ChIP enriched DNA samples and input DNA samples were analyzed by quantitative PCR with SYBR reagent in Light cycler 96 Real-time PCR machine (Roche, Mannheim, Germany) using promoter specific forward and reverse primers for *Mcp1* and *Tgfb1* (Table 8) (Eurofins, Bengaluru, India). Anti-IgG antibody was used as negative control for ChIP experiment. Results were expressed as fold change over control rats.

4.2.14. Statistical analysis

Experimental values were expressed as means ± S.E.M. Statistical comparison among different groups was performed using one way analysis of variance to detect the difference in observations between all groups. If F value was significant then multiple comparisons were done by Tukey test using Prism software (version 5.0; GraphPad, San Diego, CA) for Windows. Data was considered to be statistically significant if $p<0.05$. Immunohistochemical scores were analyzed using Kruskal

Wallis ANOVA on ranks, followed by the Tukey test. Data was considered statistically significant if $p < 0.05$.

Table 7. List of primer sequences used for qRT-PCR.

Gene name	**Primer sequences for qRT-PCR**	**Accession ID.**
Ace	Forward 5'-CGCAGCTCTTCGCTGAC-3' Reverse 5'-TCTCCTCCGTGATGTTGGTG-3'	NM_012544.1
Ace2	Forward 5'-ATGAAGCGGGAGATCGTTGG-3' Reverse 5'-TGGAACAGAGATGCAGGGTC-3'	NM_001012006.1
At_1	Forward 5′-CTCTGCCACATTCCCTGAGTT-3′ Reverse 5′-CTTGGGGCAGTCATCTTGGA-3′	NM_030985.4
At_2	Forward 5′-AACCGGCAGATAAGCATTTG-3′ Reverse 5′- CAGCCACAGCCAGATTGAAG-3′	NM_012494.3
Casp7	Forward:5'-ACCGCTCCACCATCATCTCA-3' Reverse:5'- CGGACATCCATACCTGTCGCT-3'	NM_022260.3
Casp8	Forward:5'- CGAACGATCAAGCACAGAGAG-3' Reverse:5'- AGATCAGACAGTACCCCCGA3'	NM_022277.1
Col1a1	Forward 5'-TGGCAACCTCAAGAAGTCCC-3' Reverse 5'- ACAAGCGTGCTGTAGGTGAA-3'	NM_053304.1
Il6	Forward:5'-GGATACCACCCACAACAGAC-3' Reverse:5'-GAAACGGAACTCCAGAAGAC-3'	NM_012589.2
Mcp1	Forward 5'- GTCTCAGCCAGATGCAGTTA-3' Reverse 5'- CCTTATTGGGGTCAGCACAG-3'	NM_031530.1
Nfkb p65	Forward:5'-CATCACACGGAGGGCTTC-3' Reverse:5'-GAACGATAACCTTTGCAGGC-3'	NM_199267.2
Parp1	Forward:5'-TTGGTGGAGTACGAGATTGACC-3' Reverse:5'- TGGGGGATGAGGGTGTAGAA-3'	NM_013063.2
Pcaf (Kat2b)	Forward 5'- TTCCCCCTCCAGCGTGTTAG-3' Reverse 5'- GGATTAGTGTTCTCGCTCCCAG-3'	XM_003750617.4
Rnf168	Forward 5'-CCACACGCTCTGTAACCCAT-3' Reverse 5'- CTGGCTGGTACTCATCAACGAT	NM_001127597.2
Rnf2	Forward 5'-ACAGCGCACAGACCAGATACA-3'	NM_00102566

	Reverse 5'-AGACCCCACCACACCACTTG-3'	7.1
Sod1	Forward 5'-CACTCTAAGAAACATGGCG-3' Reverse 5'-CTGAGAGTGAGATCACACG-3'	NM_017050.1
Tgfb1	Forward 5'- CTGCTGACCCCCACTGATAC-3' Reverse 5'- AGCCCTGTATTCCGTCTCCT-3'	NM_021578.2
Tnfa	Forward:5'-GATCGGTCCCAACAAGGAGG-3' Reverse:5'-CTTGGTGGTTTGCTACGACG-3'	NM_012675.3
Usp16	Forward 5'-GCCGTCTCACCGGATTGTA-3' Reverse 5'-CCCCTTTGTTCGTTTCTTTCCC-3'	NM_001100501.1
Usp21	Forward 5'-TGGAGCGAGAAGACAGCAAG-3' Reverse 5'-CGGTCACATACTGGGGCATT-3'	NM_001127638.1
Usp22	Forward 5'-AACTGCACCATAGGTCTGCG-3' Reverse 5'-GTACGGAATGTGTGGGGAGC-3'	NM_001191644.1
Usp7	Forward 5'-ATGGAGGACGACACCAGT-3' Reverse 5'-CACAAAACACGGAGGGCTA-3'	NM_001024790.1
Vcam1	Forward:5'-ACACCTCCCCCAAGAATAC-3' Reverse:5'-CCAGATTCACTCCTTCACAC-3'	NM_012889.1

Table 8. List of primer sequences used for ChIP-qRT-PCR.

Gene name	**Primer sequence for ChIP-qRT-PCR**	**Accession No.**
Mcp1	Forward 5'- ACCAAGGACTCAGTGGACTA-3' Reverse 5'- AGAGGAAGTGGCCAAGAAAC-3'	NM_031530.1
Tgfb1	Forward 5'- TTCGCGCTCTCCGAAGTT-3' Reverse 5'- CGGGCGTCAGCACTAGAA-3'	NM_021578.2

5. Results

5.1. To understand the differential effects of Ang II receptor subtypes on the expression of ACE and ACE2 in type 2 diabetic nephropathy.

5.1.1. Effect of Ang II receptor(s)' blockade on activity on metabolic parameters, renal function parameters and systolic blood pressure in type 2 diabetes induced nephropathy

The development of type 2 diabetes was confirmed by performing plasma biochemical estimations. After 24 weeks of study, HFD fed rats showed a significant rise in the plasma glucose (PGL), total cholesterol (PTC), triglycerides (PTG) and insulin (PI) levels whereas the HFD+STZ treated rats showed severe hyperglycemia, elevated cholesterol and triglyceride levels along with a significant reduction in plasma insulin levels as compared to NC. These observations showed the development of hyperglycemia and insulin resistance in HFD fed rats and type 2 diabetes in HFD+STZ treated rats. The reduced plasma insulin levels in HFD+STZ treated animals were significantly improved by the Telmisartan treatment, whereas PD123319 treatment did not make any difference. However, Telmisartan when administered with PD123319 in HFD+STZ treated animals, elevated the plasma insulin level as compared to that of the PD123319 treated animals. The elevation in plasma glucose, triglyceride and total cholesterol levels were markedly controlled by the treatment with Telmisartan whereas the treatment with PD123319 did not alter the values of these parameters, as compared to the HFD+STZ treated rats. The co-administration of AT_1 and AT_2 receptors' antagonists does not show any significant difference in plasma glucose and triglyceride levels as compared to HFD+STZ treated rats but is significantly higher than the Telmisartan treated animals (Table 9).

Blood urea nitrogen (BUN) and Plasma Creatinine (PCr), the markers of renal dysfunction were increased significantly and plasma albumin (PAL) levels were decreased in HFD+STZ treated animals while only BUN was increased in case of HFD fed rats as compared to NC (Table 9). This indicates that low dose STZ treatment followed by chronic HFD feeding leads to development of renal failure in HFD+STZ treated animals, whereas chronic high fat diet feeding alone does not cause renal dysfunction. The treatment with AT_1 receptor antagonist, Telmisartan

normalizes the levels of BUN and PCr as compared to that of the HFD+STZ treated rats. It was further noted that AT_2 receptor antagonist, PD123319 did not alter the PCr levels but elevated the BUN levels significantly as compared to the HFD+STZ treated rats, thus showing its role in worsening the renal failure induced by type 2 diabetes. The combination of Telmisartan with PD123319 could not alter the BUN levels as compared to the HFD+STZ treated rats. However, the PCr levels were significantly reduced in H+S+T+PD group as compared to the HFD+STZ treated rats (Table 9). The reduction in the PAL level was improved by Telmisartan treatment but it was not altered by PD123319 treatment. The simultaneous administration of both the antagonists does not show any significant difference from the HFD+STZ treated rats, but these values differed significantly from that of the Telmisartan treated animals (Table 9). These results clearly suggest that the treatment with Telmisartan controls the BUN, PCr as well as PAL levels which denote a significant amelioration of renal failure and its renoprotective role in type 2 diabetes. The treatment with PD123319 was found to exacerbate only the BUN levels, whereas the co-administration of Telmisartan and PD123319 significantly could not change the levels of PAL as compared to the HFD+STZ treated rats.

The micro and macrovascular complications of diabetes lead to severe hemodynamic changes. HFD fed and HFD+STZ treated rats have shows ignificantly elevated systolic blood pressure (SBP) when compared with normal pellet fed rats (Table 9). The Ang II mediated vasoconstriction that leads to rise in the blood pressure is known to be enacted through Ang II/AT_1 receptor axis (Bradford *et al.*, 2010). This fact is supported by our results which show a significant decline in systolic blood pressure upon treatment with AT_1 receptor antagonist as compared to the HFD+STZ treated rats. The blockade of AT_2 receptor resulted into an elevation in SBP but the combined administration of the antagonists does not affect the blood pressure significantly, when compared with the disease control rats. The dramatically increased SBP upon PD123319 treatment could be attributed to the increase in the expression of AT_1 receptor which occurs as a consequence of AT_2 receptor blockade.

Table 9. Effect of Ang II receptor(s) blockade on metabolic, renal function and hemodynamic parameters.

Groups	Metabolic parameters				Renal failure markers			Hemodynamic parameter
	PGL (mmol/L)	**PTG (mg/dL)**	**PTC (mmol/L)**	**PI (pmol/mL)**	**BUN (mmol/L)**	**PAL (gm/L)**	**PCr (mg/dL)**	**SBP (mm Hg)**
NC	6.1 ± 0.8	55 ± 3.0	1.2 ± 0.1	3.3 ± 0.2	3.3 ± 0.7	30 ± 1.7	1.5 ± 0.2	90 ± 5.2
HFD	8.0 ± 0.3	78 ± 3.6*	4.6 ± 0.2*	6.7 ± 0.3*	5.7 ± 0.4	31 ± 1.1	1.7 ± 0.3	110 ± 2.9*
H+S	30 ± 1.3$^{*\$}$	165 ± 7.5$^{*\$}$	16 ± 1.2$^{*\$}$	0.8 ± 0.1$^{*\$}$	11 ± 0.8$^{*\$}$	18 ± 1.6$^{*\$}$	4.9 ± 0.2$^{*\$}$	137 ± 4.9$^{*\$}$
H+S+T	17 ± 1.2$^{\#\$}$	87 ± 4.2$^{\#\$}$	6.8 ± 0.7$^{\#}$	2.2 ± 0.2$^{\$\#}$	6.3 ± 0.7$^{\#}$	31 ± 2.1$^{\#}$	2.8 ± 0.1$^{\#}$	86 ± 2.0$^{\$\#}$
H+S+PD	41 ± 1.8$^{\#@\$}$	178 ± 2.8$^{@\$}$	18 ± 0.1$^{\$@}$	0.7 ± 0.1$^{\$@}$	14 ± 0.6$^{\$\#@}$	18 ± 1.3$^{\$@}$	5.9 ± 0.1$^{\#\$@}$	157 ± 3.9$^{\$\#@}$
H+S+T+PD	31 ± 1.4$^{\$@\,\alpha}$	154 ± 4.8$^{@\,\$\,\alpha}$	9.8 ± 0.9$^{\$\#\alpha}$	1.3± 0.3$^{\$@\alpha}$	12 ±0.4$^{@\$}$	23 ± 0.5$^{\$@}$	3.1± 0.3$^{\#,\alpha}$	104 ± 1.5$^{\#@\alpha}$

Note: Data represented in mean ± SEM (n=6). *p<0.05 Vs Normal Control (NC), #p<0.05 Vs High fat diet+STZ (35mg/kg, *ip*) (H+S), $p<0.05 Vs High fat diet control (HFD), @p<0.05 Vs High fat diet+STZ+Telmisartan (H+S+T), α p<0.05 Vs High fat diet+STZ+PD123319 (H+S+PD); High fat diet+STZ+Telmisartan +PD123319 (H+S+T+PD). Abbreviations: Plasma glucose (PGL); plasma triglyceride (PTG); plasma total cholesterol (PTC); plasma insulin (PI); blood urea nitrogen (BUN); plasma albumin (PAL); plasma creatinine (PCr) and systolic blood pressure (SBP)

5.1.2. Effect of Ang II receptor(s)' blockade on activity on morphometric and microscopic parameters in type 2 diabetes induced nephropathy

Diabetic nephropathy has been characterized by a drastic fall in body weight along with a remarkable increase in the kidney weight. To study the morphological changes associated with the development of diabetic nephropathy, the alterations in body weight (BW), kidney weight (KW) and relative kidney weight [(KW/BW)*1000] were studied. The HFD fed rats showed a significantly higher body weight and kidney weight as compared to the NC. This result is in line with the previous reports which showed that long term exposure to HFD food promotes obesity and renal hypertrophy. In HFD+STZ treated rats, an increased kidney weight along with reduced body weight was observed. The relative kidney was found to have dipped significantly in the HFD+STZ treated rats as compared to HFD fed rats as well as those of the NC (Table 10). In comparison with HFD+STZ treated rats, the average body weight was improved significantly by Telmisartan, but it was further reduced by PD123319. In HFD+STZ treated rats, AT_1 receptor antagonist prevented body weight loss, whereas AT_2 receptor antagonist further augmented the weight loss. There was no difference between the body weights of the rats from HFD+STZ and the combination treated groups (Table 10). The significant increase in kidney weight of HFD+STZ treated rats was considerably controlled by Telmisartan treatment, but there was no difference among the kidney weights of PD123319 treated, combination treated rats and type 2 diabetic rats. The relative kidney weight showed a remarkable increase in the HFD+STZ treated rats which was ameliorated by Telmisartan treatment and augmented by PD123319. However, the administration of AT_1 receptor and AT_2 receptor blockers simultaneously, does not change the morphometric parameters as compared to the HFD+STZ treated rats (Table 10). This shows that Telmisartan not only improves the plasma biochemical parameters indicative of lipid and carbohydrate metabolism and renal functioning but also improves the body weight loss linked with progression of type 2 diabetes condition.

Glomerular hypertrophy, glomerular basement membrane thickening, mesangial expansion, renal lesions and deposition of ECM (collagen) are the common features of diabetic nephropathy (Alsaad *et al.*, 2007). For the evaluation of the glomerular damage, hematoxylin and eosin (H&E) staining was performed. The marker for

cellular proliferation, Ki67 was checked to study the glomerular hypertrophy. The HFD fed and HFD+STZ treated rats showed striking features of glomerular damage as compared to the NC: (1) a significantly high glomerular damage as shown by microscopic changes like increase in urinary space, tubular damage, glomerular hypertrophy and vacuolations as compared to NC, as shown by the H&E staining (Figure 10A and C) (2) The Ki67 expression was found to be significantly upregulated, as shown by the increased percentage of Ki67 positive area (Figure 10B and D). These histopathological changes further confirm the development of renal failure in HFD+STZ treated animals. The glomerular damage and cellular proliferation were attenuated by the administration of Telmisartan and further heightened by PD123319 whereas the concurrent usage of both the antagonists significantly reduced the glomerular damage as compared to PD123319 treated rats. These results confirmed the protective role of AT_2 receptor in diabetic nephropathy and pro-fibrotic, pro-hypertrophic face of AT_1 receptor and further show the counter-regulatory effect of AT_1 receptor and AT_2 receptor in diabetic kidney.

Table 10. Effect of Ang II receptor(s) blockade on renal morphometric parameters.

Groups	**Morphometric parameters**		
	Body weight (gm)	**Kidney weight (gm)**	**(KW /BW)* 1000**
NC	254 ± 8.07	1.36 ± 0.03	5.35 ± 0.01
HFD	335 ± 1.84*	1.78 ± 0.09	5.34 ± 0.05*
H+S	174 ± 3.34$^{*\$}$	2.33 ± 0.11$^{*\$}$	13.3 ± 0.02$^{*\$}$
H+S+T	232 ± 2.37$^{\#\$}$	1.43 ± 0.03$^{\#}$	6.12 ± 0.01$^{\#}$
H+S+PD	156 ± 2.21$^{\#\$@}$	2.66 ± 0.03$^{\$@}$	17.0 ± 0.02$^{\$@}$
H+S+T+PD	190 ± 2.27$^{\#\$\alpha}$	2.25 ± 0.10$^{\$@\alpha}$	11.8 ± 0.04$^{\$@\alpha}$

Note: Data represented in means ± SEM (n=6). *p<0.05 Vs Normal Control (NC), #p<0.05 Vs High fat diet+STZ (H+S), $p<0.05 Vs High fat diet control (HFD)**,** @p<0.05 Vs High fat diet+STZ+Telmisartan (H+S+T), α p<0.05 Vs High fat diet+STZ+PD123319 (H+S+PD); High fat diet+STZ+Telmisartan +PD123319 (H+S+T+PD).

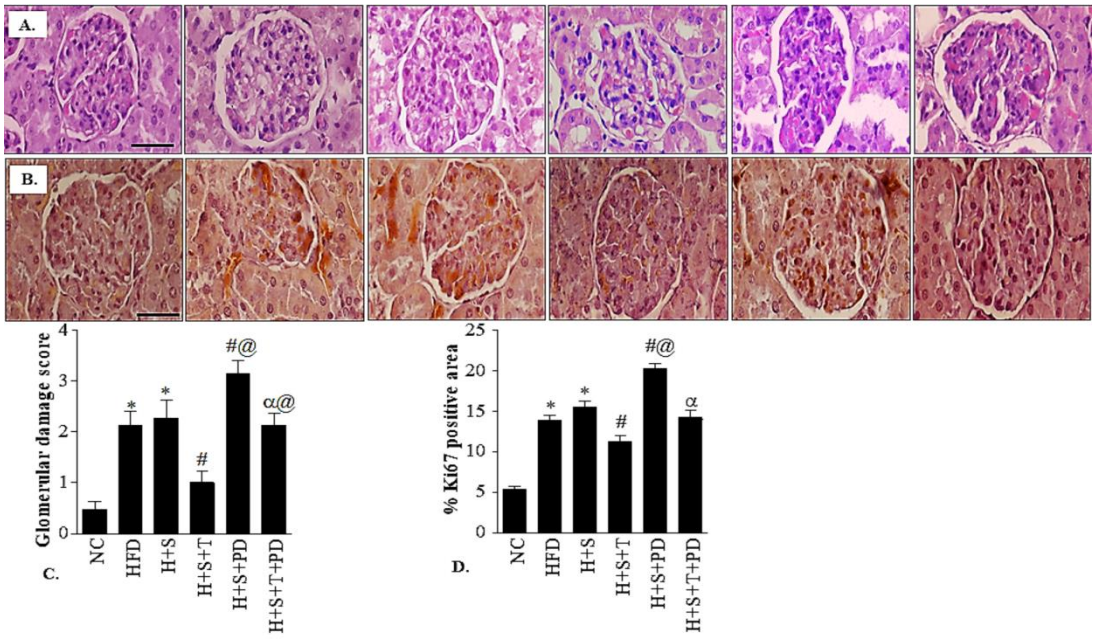


Figure 10. Effect of Ang II receptor(s) blockade on glomerular damage and glomerular proliferation.

The representative images for (A) Hematoxylin/eosin staining and (C) glomerular damage score (B) The light microscopic images for immunohistochemistry of Ki67 and (D) quantification of %Ki67 positive area. The scale bar represents 50µm (original magnification,×100). **Note:** Data represented in mean ± SEM (n=6). *p<0.05 Vs Normal Control (NC), #p<0.05 Vs High fat diet+STZ (35mg/kg, *ip*) (H+S), $p<0.05 Vs High fat diet control (HFD)**,** @p<0.05 Vs High fat diet+STZ+Telmisartan (H+S+T), α p<0.05 Vs High fat diet+STZ+PD123319 (H+S+PD); High fat diet+STZ+Telmisartan +PD123319 (H+S+T+PD).

5.1.3. Combination of AT_1 receptor antagonist and AT_2 receptor agonist improves metabolic, hemodynamic and morphometric parameters in type 2 diabetes induced nephropathy

5.1.3.1. Dose standardization for AT_2 receptor agonist, Compound 21

The AT_2 receptor agonist, C21 has been known to show good oral absorption and bioavailability. However, the oral dose at which C21 might show protection in diabetes induced nephropathy has not yet been discussed. The AT_2 receptor agonist C21 (0.075, 0.15, 0.3 mg/kg, *po*, n=6) or vehicle (normal saline, n=6) was administered for 14 days prior to study termination. It was found that C21 significantly improved plasma glucose, total cholesterol, and insulin more efficiently at 0.3mg/kg dose as compared to that of the lower and medium doses. However, the plasma triglyceride levels, significantly elevated in type 2 diabetic rats

were not improved by C21. The renal function parameters, BUN and PAL did not show any reduction in the lower and the medium dose C21 treatment groups, but the exposure to highest dose reduced the BUN levels significantly. PCr was found to be improved markedly at both 0.15 and 0.3mg/kg dose. Alkaline phosphatase (ALP), an important marker for renal dysfunction was increased drastically in type 2 diabetic rats and was found to be improved remarkably at all the three doses of C21 (Table 11). The systolic blood pressure, elevated profoundly in type 2 diabetic rats also could not be improved by C21, which shows the inability of C21 to improve the hemodynamic parameters, *in vivo*. The loss of body weight in type 2 diabetic rats was improved markedly at 0.15 and 0.3mg/kg doses, but the kidney weights were not controlled by the treatment. However, the relative kidney weights were found to be improved significantly by C21 in a dose dependent manner. Thus, 0.3mg/kg dose was selected for further combination with Telmisartan, to check whether hyperglycemia, renal failure and hemodynamic alterations occurring in type 2 diabetic rats could be controlled in a better manner with the combination than the individual treatments.

5.1.3.2. C21 and Telmisartan combination reduces metabolic and renal dysfunction in type 2 diabetic rats

The plasma biochemical parameters are important markers of carbohydrate and lipid metabolism and hence act as important indicators of type 2 diabetes development. The HFD fed low dose treated rats were found to show a significant increase in PGL, TG, PTC and a drastically low PI concentration as compared to the control rats. Treatment with Telmisartan could improve PGL, TG, PTC and PI levels as compared to the type 2 diabetic rats. The treatment of type 2 diabetic rats with C21 (0.3mg/kg) could improve TG, PTC and PI levels but not the PGL levels. However, the combination of Telmisartan and C21 reduced the elevations in TG, PTC levels and simultaneously elevated the PI levels more efficiently than the individual treatments (Table 11). The combination regimen also efficient in improving the PGL levels in type 2 diabetic rats, which was not improve by the solo treatment with C21, thus indicating a potential role of the combination in controlling hyperglycemia, a prominent feature of type 2 diabetes (Table 11).

The renal function parameters, including PAL, BUN, and PCr were found remarkably disturbed in HFD fed low dose treated rats. Telmisartan treatment in type 2 diabetic rats improved the renal function parameters. C21 treatment was also found to alleviate renal dysfunction to a certain degree but their combination showed a more significant improvement in renal dysfunction, thus showing a potential additive effect of the molecules. ALP, BUN and PCr, significantly elevated in type 2 diabetic rats were more efficiently reduced by C21 as compared to Telmisartan. However, the combination regimen further improved the renal dysfunction as compared to the individual treatments (Table 11).

5.1.3.3. C21 and Telmisartan combination improves renal morphometric and hemodynamic parameters in type 2 diabetic rats

In type 2 diabetic rats, the body weight dipped down whereas the kidney weight showed a significant elevation, as compared to that of NC. Though the relative kidney weight (kidney weight-body weight ratio) increment indicating renal hypertrophy in type 2 diabetic rats was ameliorated significantly by both the molecules, the efficiency of Telmisartan was more than that of C21. The combination regimen restored the body weight loss as well as the kidney weight to improve the relative kidney weight ratio in a more efficient manner than either of the treatments (Table 12).

Concurrent with the previous reports, the SBP was found to have elevated drastically in type 2 diabetic rats. Telmisartan treatment could reduce the elevation in SBP. The treatment of type 2 diabetic rats with C21 could not improve the blood pressure disturbances but its co-treatment with Telmisartan was found to improve the effect of Telmisartan quite phenomenally, thus indicating a potential role of this combination in alleviating hypertension linked with type 2 diabetes (Table 12).

Type 2 diabetic rats were found to show a prominent glomerular as well as tubular damage, evinced by thickening of basement membrane and narrowing of Bowman's capsule and percentage collagen positive area in glomerular and tubular region. The histochemical analysis showed that treatment with either C21 or Telmisartan ameliorated glomerular damage to some extent but the most prominent improvement was rendered by the combination which reduced both the glomerular and tubular alterations significantly (Figure 11).

Table 11. Effect of Telmisartan and C21 on plasma parameters for metabolism and renal failure.

Groups	Metabolic parameters				Renal parameters			
	PGL (mmol/L)	**TG (mg/dL)**	**PTC (mmol/L)**	**PI (pmol/L)**	**BUN (mmol/L)**	**PAL (gm/L)**	**PCr (mg/dL)**	**ALP (IU/L)**
NC	7.9 ± 0.7	63 ± 6.7	1.2 ± 0.3	304 ± 4.39	4.5 ± 0.4	39 ± 1.1	0.8 ± 0.2	40.0 ± 3.22
H+S	37 ± 2.7*	175 ± 10 *	18 ± 0.9*	107 ± 11.4 *	8.5 ± 0.7*	22 ± 1.4*	3.5 ± 0.4*	287 ± 7.45*
H+S+C21-L	30 ± 1.5	149 ± 3.2	13 ± 1.6	125 ± 11.2	8.1 ± 0.6	25 ± 0.9	2.2 ± 0.2$^{\#}$	151 ± 5.83$^{\#@}$
H+S+C21-M	27 ± 2.1	149 ± 5.6	9.2 ± 0.7$^{\#}$	169 ± 18.3$^{\#}$	6.7 ± 0.5	30 ± 1.1	1.5 ± 0.2$^{\#\$}$	158 ± 8.71$^{\#@}$
H+S+C21-H	21 ± 2.0$^{\#}$	138 ± 11	9.3 ± 0.6$^{\#}$	176 ± 14.1$^{\#}$	5.2 ± 0.3$^{\#@}$	31 ± 1.4$^{\#}$	1.5 ± 0.1$^{\#@}$	121 ± 6.25$^{\#@}$
H+S+T	17 ± 1.2$^{\#}$	105 ± 4.2$^{\#}$	6.8 ± 0.7$^{\#}$	158 ± 8.5$^{\#}$	6.4 ± 0.6$^{\#}$	27 ± 1.8$^{\#}$	2.8 ± 0.1$^{\#}$	249 ± 2.91$^{\#}$
H+S+C+T	9.2 ± 0.8$^{\#@\$}$	98 ±3.7$^{\#\$}$	7.7 ± 3.7$^{\#}$	292± 8.81$^{\#@\$}$	4.6 ± 0.4$^{\#@\$}$	35 ± 1.2$^{\#@\$}$	0.6 ± 0.1$^{\#@\$}$	78.0 ± 3.18$^{\#@\$}$

Note: Data represented in means ± SEM (n=6). *p<0.05 Vs normal control (NC), #p<0.05 Vs HFD+STZ (35mg/kg, *ip*), @p<0.05 Vs High fat diet+STZ treated+Telmisartan treated (10mg/kg/*po*) (H+S+T), $p<0.05 Vs High fat diet+STZ treated+C21 treated (0.3mg/kg/*po*) (H+S+C21-H), High fat diet/ STZ /low dose C21 (0.075mg/kg/*po*) (H+S+C21-L), High fat diet/STZ treated/C21 treated (0.15mg/kg/*po*) (H+S+C21-M), High fat diet/ STZ treated/C21 (0.3mg/kg/*po*)/Telmisartan (10mg/kg/*po*) (H+S+C+T). Abbreviations: Plasma glucose (PGL); plasma triglyceride (PTG); plasma total cholesterol (PTC); plasma insulin (PI); blood urea nitrogen (BUN); plasma albumin (PAL); plasma creatinine (PCr) and alkaline phosphatase (ALP).

Table 12. Effect of Telmisartan and Compound 21 on morphometric parameters and systolic blood pressure.

Groups	Morphometric parameters			Hemodynamic parameter
	Body weight (gm)	**Kidney weight (gm)**	**(KW /BW)*1000**	**SBP (mm Hg)**
NC	215 ± 6.58	1.2 ± 0.1	5.5 ± 0.2	90.2 ± 5.19
H+S	137 ± 3.19*	2.3 ± 0.1*	17 ± 1.3*	131 ± 4.89*
H+S+C21-L	183 ± 7.41*	1.9 ± 0.2	11 ± 0.5$^{\#}$	115 ± 3.02
H+S+C21-M	226 ± 2.61*	2.3 ± 0.2	11 ± 0.8$^{\#}$	119 ± 5.09
H+S+C21-H	220 ± 3.34#	2.3 ± 0.1	10 ± 0.6#	120 ± 7.23
H+S+T	223 ± 5.85#	1.4 ± 0.2#	7.3 ± 0.2#	88.1 ± 2.00$^{\#}$
H+S+C+T	232 ± 6.59#	1.4 ± 0.1#$	6.2 ± 0.2#$	95.1 ± 5.54$^{\#\$}$

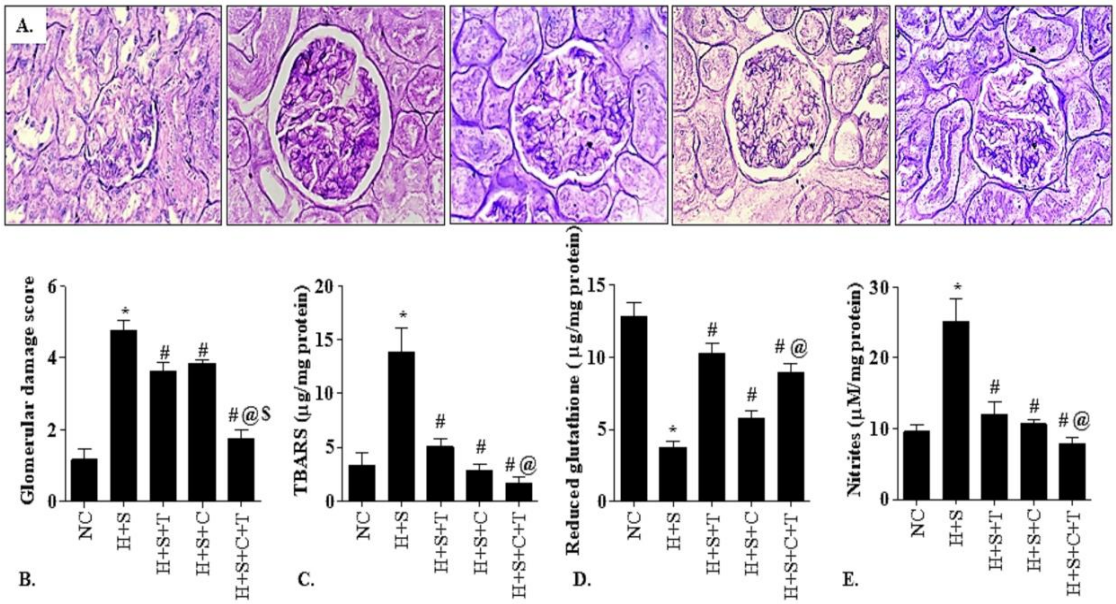


Figure 11. Effect of Compound 21 and Telmisartan combination on glomerular damage score and oxidative stress.

Light microscopic pictures of hematoxylin and eosin staining to estimate glomerular damage score and quantification. Oxidative stress markers (A) Thiobarbituric acid reactive substrate (TBARS) (B) reduced glutathione (GSH) (C) Nitrites in kidney homogenate. Note for table 12 and figure 11 : Data represented in means ± SEM (n=6). *p<0.05 Vs normal control (NC), #p<0.05 Vs HFD+STZ (H+S) (35mg/kg, *ip*), @p<0.05 Vs High fat diet+STZ

treated+Telmisartan treated (H+S+T), $p<0.05 Vs High fat diet+STZ treated+C21 treated (H+S+C), High fat diet+STZ treated+C21+Telmisartan (H+S+C+T).

5.1.4. Effect of Ang II receptors' activity on oxidative stress in type 2 diabetic nephropathy

5.1.4.1. AT_2 receptor antagonist aggravates renal oxidative stress in type 2 diabetic nephropathy

Oxidative stress markers like TBARS and GSH were measured in the kidneys of all the rats. TBARS was found to have increased remarkably in HFD fed rats and HFD+STZ treated rats (Figure 12C) whereas the reduced glutathione was decreased as compared to the normal rats (Figure 12D). This heightened load of free radicals was attenuated by treatment with Telmisartan and PD123319 treatment did not change the free radical load. The simultaneous exposure to both the antagonists led to a significant reduction in oxidative stress as compared to PD123319 treated group and HFD+STZ treated group.

To further confirm the increase in the oxidative stress, Keap1 expression was studied using immunohistochemistry technique. The transcription factor NF-E2–related factor 2 (Nrf2), and its negative regulator, Keap1 maintain cellular defence mechanisms by regulating the expression of anti-oxidant genes. It was seen that the expression of Keap1 was increased significantly in both HFD fed rats and HFD+STZ treated rats as compared to that of the NC. The treatment with Telmisartan attenuated the expression whereas PD123319 treatment further elevated it (Figure 12A-B). However, the combination of the antagonists does not change Keap1 levels as compared to the HFD+STZ treated rats. This result shows that the increase in oxidative stress and reduction in antioxidant enzyme level is associated with the increased expression of Keap1, which may be an adaptive change to overcome the cellular stress which is in accordance with the previous reports that demonstrated similar changes in spontaneously hypertensive rat kidney (Erejuwa *et al.*, 2012).

Further, to check the effect on the expression of antioxidant enzymes, we checked the mRNA level of antioxidant enzyme, superoxide dismutase 1 (*Sod1*). In the current study we found that *Sod1* mRNA level was significantly elevated in HFD and HFD+STZ treated rats. This elevation could be significantly reduced by the

Telmisartan treatment and was further raised by PD123319. However, the combination did not show any significant alteration as compared to the HFD+STZ treated animals (Figure 12E). This result confirms that hyperglycemia induced oxidative stress in type 2 diabetes induced nephropathy is associated with the activation of Ang II/AT_1 receptor axis of RAS.

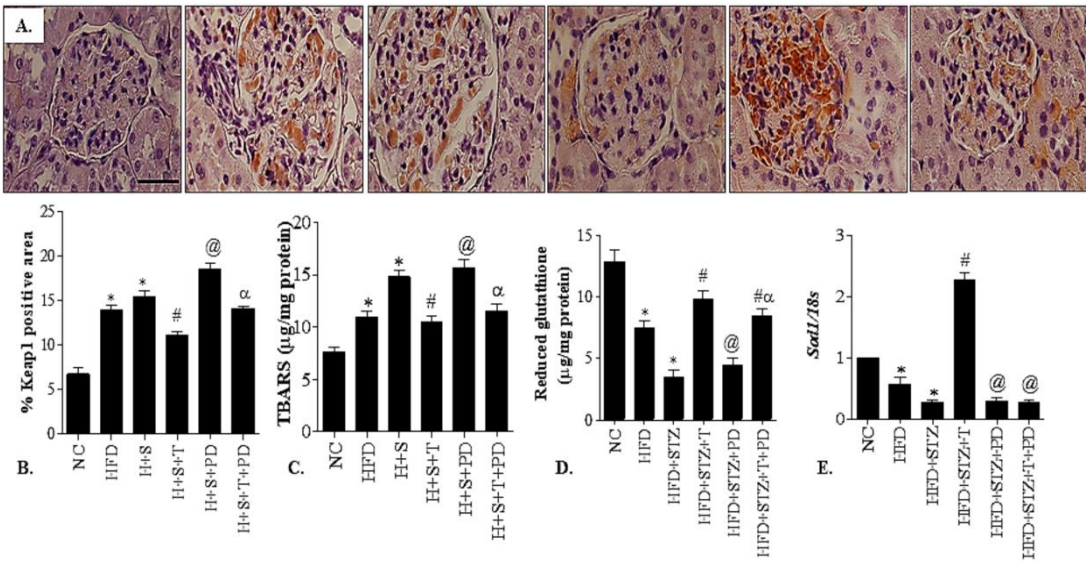


Figure 12. Effect of Ang II receptor(s) blockade on renal oxidative stress markers.

(A) Representative images of immunohistochemistry performed to analyse Keap1 protein expression in rat kidney sections. The scale bar represents 50µm (original magnification, ×100) and (B) % Keap1 positive area in the kidney sections. Thiobarbituric acid reactive substrate (TBARS) (D) reduced glutathione (GSH) (E) Fold change in mRNA expression of *Sod1*. Note: Data represented in mean ± SEM (n=6). *p<0.05 Vs Normal Control (NC), #p<0.05 Vs High fat diet+STZ (35mg/kg, *ip*) (H+S), $p<0.05 Vs High fat diet control (HFD), @p<0.05 Vs High fat diet+STZ+Telmisartan (H+S+T), α p<0.05 Vs High fat diet+STZ+PD123319 (H+S+PD); High fat diet+STZ+Telmisartan +PD123319 (H+S+T+PD).

5.1.4.2. Effect of Telmisartan and C21 combination on renal oxidative stress markers

Oxidative stress marker, TBARS and GSH were measured in the kidneys of all the rats. TBARS was found to have increased remarkably in type 2 diabetes treated rats' kidney whereas the reduced glutathione was decreased as compared to the NC. The redox imbalance in type 2 diabetic kidney was pacified by Telmisartan, which show has been known to show potent antioxidant property. The reduction in TBARS was

more prominent in C21 treatment, while improvement in reduced glutathione levels was better in Telmisartan treated groups. The combination treated group showed the most promising reduction in TBARS level while in the case of reduced glutathione, the effects of combination and Telmisartan treatment are almost comparable. Free nitrite load is also an important indicator of free radical stress. The elevated free nitrite levels, elevated in type 2 diabetic rats' kidney were found to be controlled significantly by Telmisartan as well as C21 and the effect of Telmisartan was found to be improved, thus showing a potentiation of the antioxidant activity (Figure 11C-E).

5.1.5. Effect of Telmisartan, PD123319 and C21 on the expression of Ang II receptor subtypes

AT_1 receptors, which have been known to show the detrimental effects of Ang II, were drastically upregulated in HFD fed and HFD+STZ treated control rats as compared to NC. This elevation could be significantly reduced by AT_1 receptor antagonist, Telmisartan and further augmented by AT_2 receptor antagonist, PD123319 (Figure 13A). Our results demonstrated that AT_2 receptor is significantly upregulated in both HFD fed and HFD+STZ treated rats (Figure 13B). The expression of AT_2 receptor was further enhanced by the administration of the AT_1 receptor antagonist as compared with the HFD+STZ treated rats, which was in accordance with the previously reported data, which suggest that the blockade of AT_1 receptor leads to upregulation of AT_2 receptor (Bregonzio *et al.*, 2008). The expression of AT_2 receptor was reduced significantly by the exposure to PD123319. On the contrary, the simultaneous exposure to both the antagonists, increased the expression of AT_2 receptor in type 2 diabetic kidney as compared to PD123319 treated rats. At mRNA level, the elevation in At_1 as well as At_2 expression was increased drastically and it was reduced significantly by Telmisartan treatment. PD123319 increased the At_1 expression markedly while no significant alteration was seen in At_2 expression (Figure 13).

The elevated mRNA expression of *At1* receptors was found to show a reducing trend by the treatment with all the three treatments, i.e., Telmisartan, C21 and their combination, however no significant difference was observed. The expression of *At2* receptors was found to be increased in type 2 diabetic rats drastically as

compared to NC. The treatment with Telmisartan increased the expression of *At2* receptors whereas C21 did not show any significant influence over the mRNA expression. The combination of Telmisartan and C21 significantly improved the expression of *At2* receptors as compared to the diabetic rats, which may be the reason for an improved protective effect of the combination in treatment of type 2 diabetes induced renal failure (Figure 13G-H).

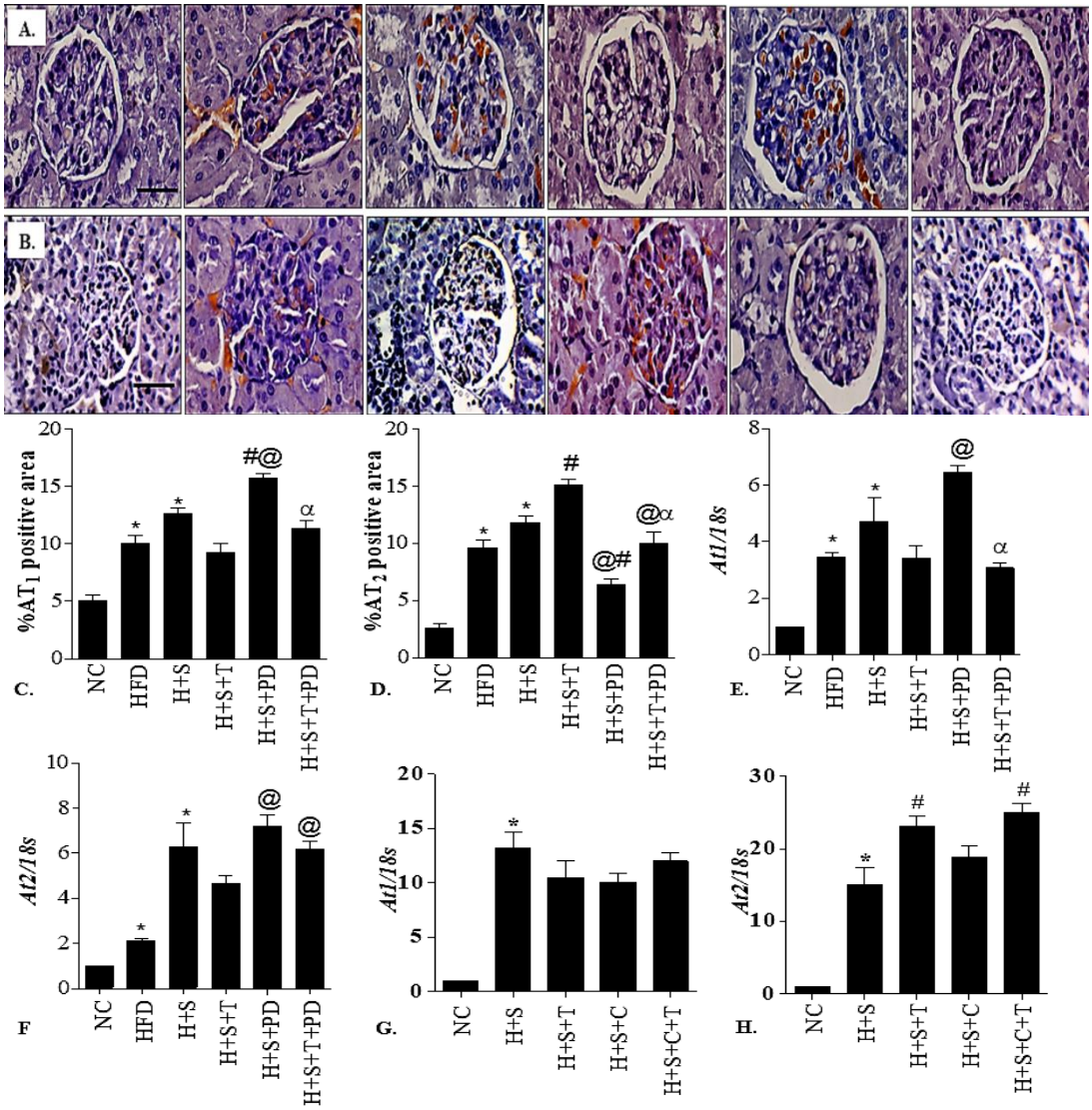


Figure 13. Effect of the Telmisartan, PD123319 and C21 or their combinations on expression of Ang II receptor subtypes.

The light microscopic pictures illustrating the immunostaining for (A) AT_1 receptor and (B) AT_2 receptor and quantification of (C) % AT_1 receptor positive area (D) % AT_2 receptor positive area in the kidney sections. The bar graphs (E-F) show *At1* and *At2* in study 1 and (G-H) represent the *At1* and *At2* mRNA expression in study 2. The scale bar represents 50µm (original magnification, ×100).

5.1.6. Effect of Ang II receptors' activity on ACE and ACE2 expression

5.1.6.1. Effect of Telmisartan and PD123319 on ACE and ACE2 expression in type 2 diabetic nephropathy

In order to check the effect of Ang II receptor blockade on expression of ACE and ACE2, immunohistochemistry staining was carried out. The elevated expression of ACE2 in HFD fed and HFD+STZ treated rats was normalized by Telmisartan treatment and further augmented by PD123319 administration. The simultaneous administration of both antagonists shows a reduction in the ACE2 expression when compared to that of the PD123319 treatment but does not differ remarkably from the type 2 diabetic kidney. This shows that the ACE2 expression is enhanced by diabetic nephropathy and the blockade of AT_2 receptor further signals the increase in its expression (Figure 14). This elevation of expression of ACE2 may be the feedback mechanism of the cells to combat the aggravated pathological conditions. These results indicate that ACE2 expression gets augmented by treatment with AT_2 receptor antagonist and reduced by AT_1 receptor treatment, thus ACE2 expression could be inversely related with AT_2 receptor activity and positively correlated with the AT_1 receptor.

The mRNA expression of *Ace* was increased drastically in HFD fed as well as the HFD+STZ treated rats' kidney and this elevation was reduced significantly by Telmisartan but remained unaltered by PD123319 or Telmisartan and PD123319 combination treatment. The expression of *Ace2* was reduced in HFD and HFD+STZ treated rats' kidney. Telmisartan increased the *Ace2* levels while PD123319 and its combination with Telmisartan did not cause any major alteration in *Ace2* mRNA expression (Figure 14). The mRNA and protein expressions were found to be different from each other, thus showing a potential role of epigenetic mechanisms regulating the gene expression.

5.1.6.2. Effect of C21 and Telmisartan on *Ace and Ace2* expression in type 2 diabetic nephropathy

Though the elevated mRNA expression of *Ace* in type 2 diabetic kidney was controlled markedly by the treatment with Telmisartan and C21 alone, their combination was found to be more efficient in reducing the increment, thus showing its ability to limit the exacerbation of deteriorative axis. The protective axis

component, *Ace2* was reduced significantly in type 2 diabetic rat kidney and was improved by the treatment with Telmisartan, C21 as well as their combination (Figure 14G-H).

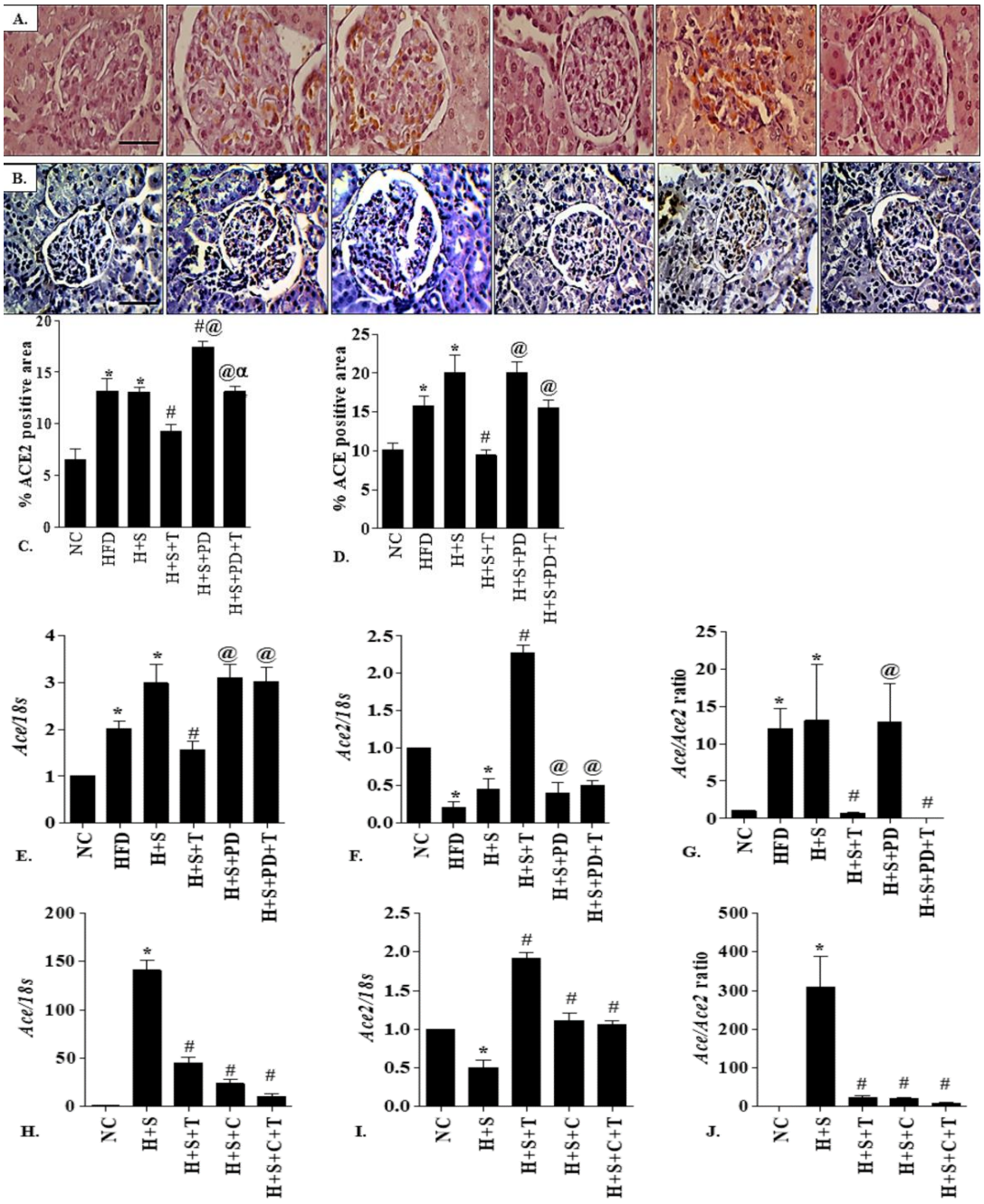


Figure 14. Effect of Ang II receptors' activity on expression of renal ACE and ACE2 in type 2 diabetic rats.

The light microscopic pictures illustrating the immunostaining for (A) ACE2 and (B) ACE and quantification of (C) % ACE2 (D) ACE positive area in the kidney sections. Bar charts represent the (E-G) mRNA expression of *Ace* and *Ace2* and their ratio for study1 and (H-J) show *Ace, Ace2 and Ace/Ace2* ratio for study 2, respectively.

The Ace/Ace2 ratio, a marker of severity of renal failure was found to be raised enormously in type 2 diabetic kidney and it was normalized most efficiently in all the three treatment groups. Though there was no significant difference between the three treatment groups' effect on Ace/Ace2 ratio, slight reducing trend was observed in the combination treated animals (Figure 14).

5.2. To delineate the role of Ang II receptor subtypes in regulation of NF-κB mediated inflammatory signaling in type 2 diabetic nephropathy.

5.2.1. Effect of Ang II receptor subtype(s)' blockade on NF-κB mediated inflammatory signaling and TGF-β1 mediated fibrosis in type 2 diabetic nephropathy

NF-κB p65, phosphorylated NF-κB p65 and phosphorylated IκB-α subunits were significantly elevated in the kidneys of HFD fed as well as HFD+STZ treated animals. These results show that the expression of the active form was significantly enhanced under type 2 diabetes related nephropathy which may be due to the increased oxidative stress. AT_1 receptor antagonist treatment significantly reduced NFκB p65, phosphorylated NFκB p65 and phosphorylated IκB-α level in diabetic kidney. PD123319 severely augmented the expression of the NF-κB p65, phosphorylated NF-κB p65 and IκB-α subunits. The co-administration of the two antagonists does not change the expression level as compared to the HFD+STZ treated rats but was significantly higher than Telmisartan treated group. Thus, it can be concluded that the activation of NF-κB under type 2 diabetic nephropathy was found to be orchestrated by AT_1 receptor. However, since the blockade of AT_2 receptor showed a significant increase in the expression of phosphorylated forms, it may be concluded that AT_2 receptor also plays a significant role in controlling this signaling pathway.

MCP1, a marker of macrophage infiltration has been found to be an indicator of degree of severity of renal failure in type 2 diabetes. In our study, we observed a significant increase in the expression of MCP1 at both mRNA as well as protein levels in HFD fed and HFD+STZ treated rats which could be ameliorated by Telmisartan treatment to a considerable amount. The blockade of AT_2 receptor further elevated the expression in type 2 diabetic rats indicating a probable role of AT_2 receptor in regulation of macrophage infiltration whereas the combination of

both the antagonists did not lead to any major difference (Figure 15D, 15H and 15K).

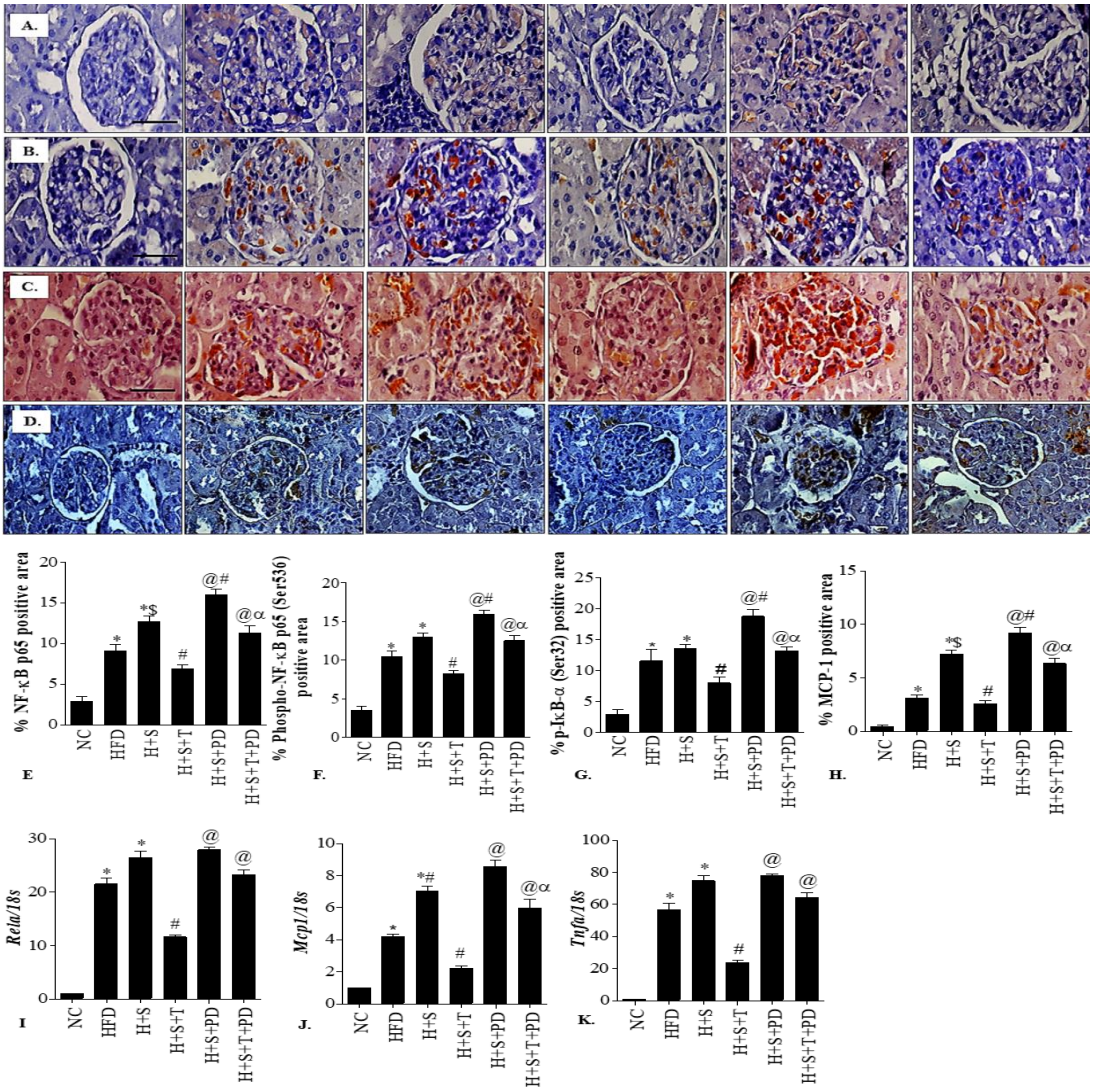


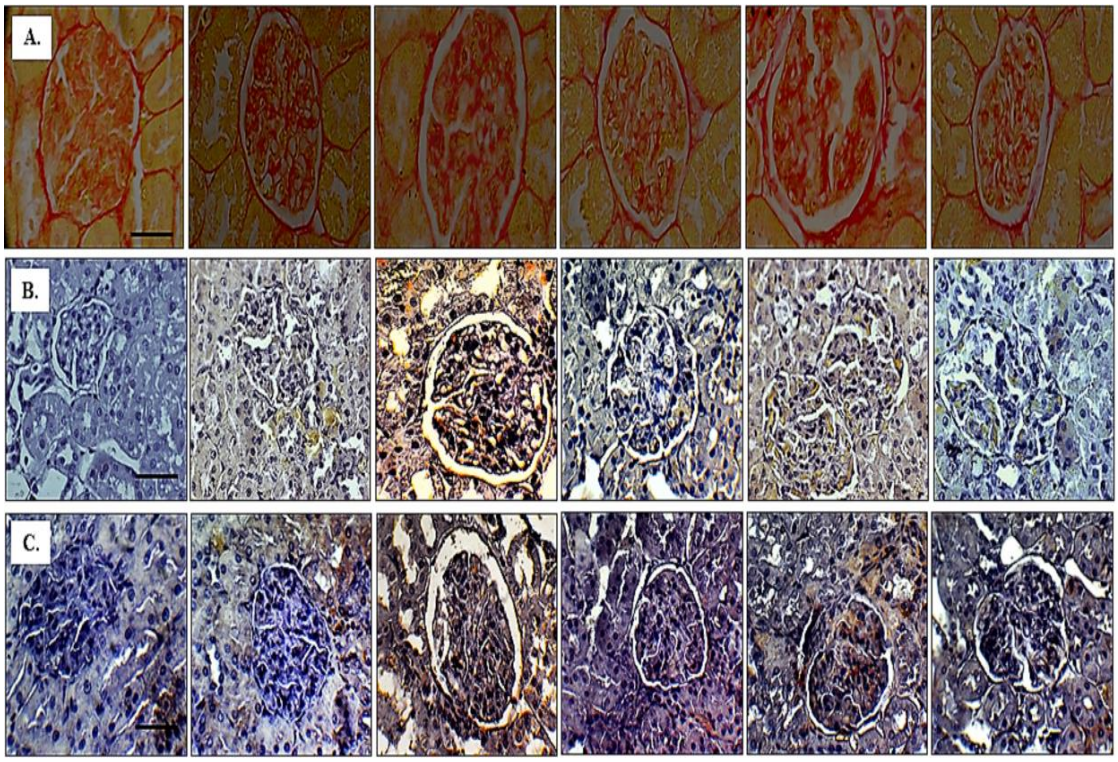
Figure 15. Effect of Telmisartan and PD123319 on expression of NF-κB signaling pathway in type 2 diabetic kidney.

Representative images for immunostaining of (A) NF-κB p65 (B) phospho NF-κB p65 (Ser536) (C) phospho-IκB-α (Ser32) (D) MCP1 and their quantifications (E-H). The bar charts show fold change in mRNA levels of (I) *Rela* (J) *Mcp1* (K) *Tnfa*. Note: Data represented in mean ± SEM (n=6). *p<0.05 Vs Normal Control (NC), #p<0.05 Vs High fat diet+STZ (35mg/kg, ip) (H+S), $p<0.05 Vs High fat diet control (HFD), @p<0.05 Vs High fat diet+STZ+Telmisartan (H+S+T), α p<0.05 Vs High fat diet+STZ+PD123319 (H+S+PD); High fat diet+STZ+Telmisartan +PD123319 (H+S+T+PD).

The mRNA expression of pro inflammatory cytokine like *Rela* (Nfkbp65 subunit) and *Tnfa* was found to be enhanced manifolds in HFD and treated rats and was

significantly reduced by Telmisartan treatment. The treatment with PD123319 and its combination with Telmisartan did not alter the cytokine levels as compared to the type 2 diabetic controls (Figure 15).

In order to determine renal fibrosis linked with type 2 diabetes, protein expression of TGF-β1 and fibronectin and mRNA expression of *Tgfb1* and *Col1a1* were checked and it was seen that the expression of these fibrotic markers was increased markedly in the HFD fed and HFD+STZ treated rats as compared to NC. The treatment with AT_1 receptor blocker could significantly revert the expression levels. The PD123319 treatment elevated these expression levels but it was not statistically significant from that of the HFD+STZ treated rats. The treatment with both the antagonists showed marked reduction in the fibrotic markers as compared to the HFD+STZ treated rats. These results demonstrate that Ang II mediated fibrotic cascade in type 2 diabetic nephropathy may be activated through AT_1 receptor and not through the AT_2 receptor (Figure 16).



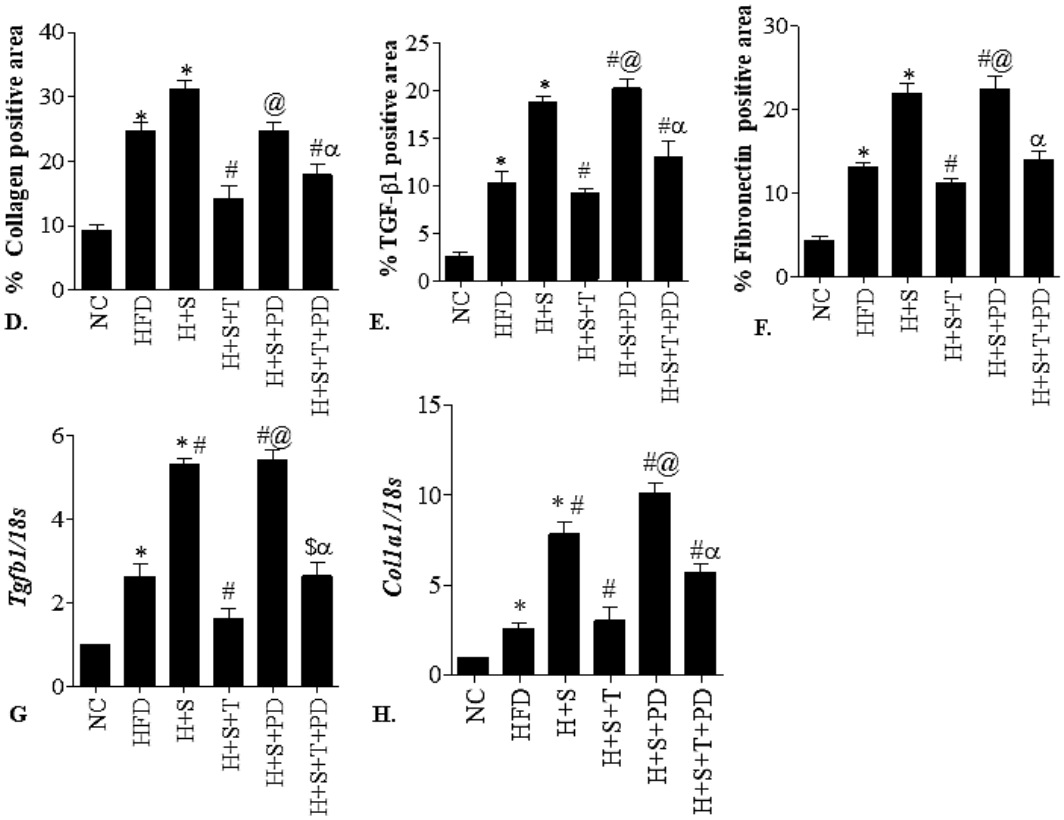


Figure 16. Effect of Telmisartan and PD123319 on expression of renal fibrosis.

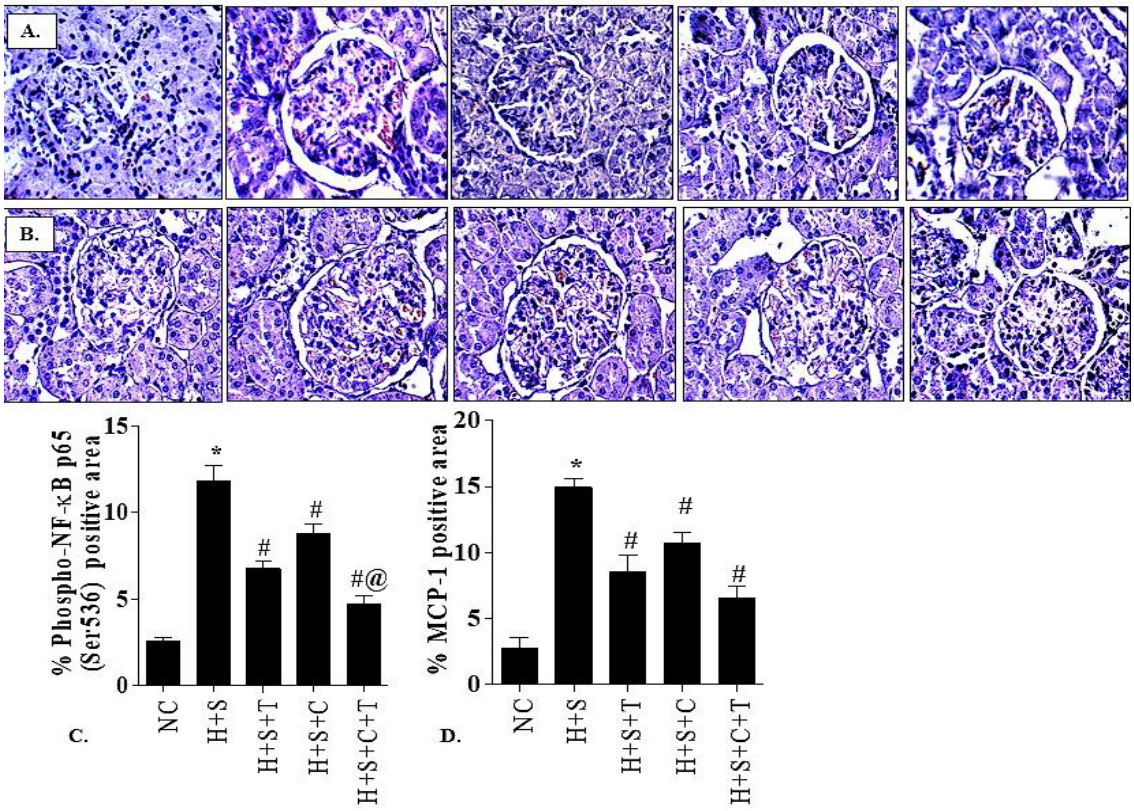
The light microscopic pictures illustrating the (A) Picro-Sirius red staining to quantify collagen deposition and immunostaining for (B) TGF-β1 and (C) fibronectin and their respective quantifications (D-F). The scale bar represents 50μm (original magnification, ×100). The bar chart represents the fold change in mRNA expression of (G) Tgfb1 (H) Col1a1. **Note:** Data represented in mean ± SEM (n=6). *p<0.05 Vs Normal Control (NC), #p<0.05 Vs High fat diet+STZ (35mg/kg, ip) (H+S), $p<0.05 Vs High fat diet control (HFD)**,** @p<0.05 Vs High fat diet+STZ+Telmisartan (H+S+T), α p<0.05 Vs High fat diet+STZ+PD123319 (H+S+PD); High fat diet+STZ+Telmisartan +PD123319 (H+S+T+PD).

5.2.2. C21 and Telmisartan combination ameliorates NF-κB mediated renal inflammation, fibrosis and apoptosis in type 2 diabetes

We found that type 2 diabetic kidney showed drastic activation of the NF-κB signaling pathway, as demonstrated by the multi-fold increase in expression of phosphorylated NF-κB p65 (Ser536) protein, downstream molecule, MCP1 (Figure 17A-D) and mRNA expression of *Rela* (Figure 17E). NF-κB signaling was controlled most significantly by the combination treatment, followed by Telmisartan and C21. This further confirms that concomitant treatment with AT_1 receptor

blocker and AT_2 receptor agonist pacifies the NF-κB mediated inflammatory cascade in type 2 diabetic kidney.

Inflammation is marked by an elevation of the assisting cytokines and chemokines which help in accelerating and propagating the cellular damage. In this study, mRNA expression of cytokines including interleukin 6 (*Il6*), tumour necrosis factor alpha (*Tnfa*); adhesion molecule, vascular cell adhesion molecule 1 (*Vcam1*) and chemokine, monocyte chemoattractant protein1 (*Mcp1*) was significantly upregulated in type 2 diabetic rats' kidneys. The treatment with Telmisartan alleviates the abrupt surge in cytokine and chemokine load in type 2 diabetic kidney. Though, monotherapy with C21 treatment reduced only the inflammatory cytokines *Il6* and *Tnfa*, the combination improved the cytokines, adhesion molecules as well as the chemokines more efficiently than the individual treatments. This shows that Telmisartan and C21 combination controls the NF-κB mediated signaling pathway at mRNA level and reduces the production of pro inflammatory molecules in type 2 diabetic nephropathy (Figure 17E-I).



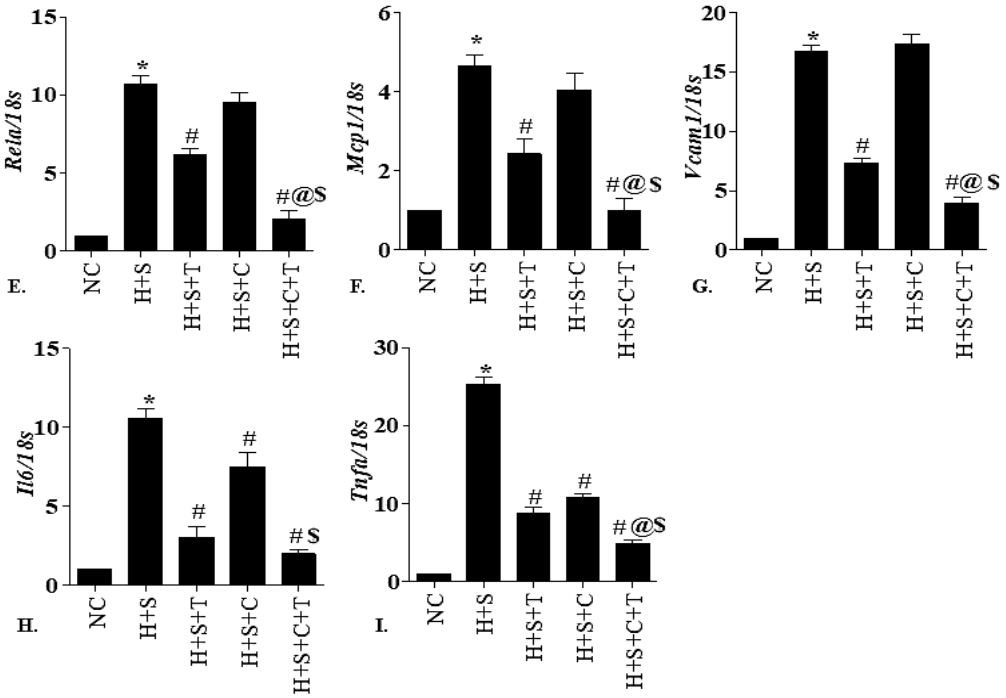


Figure 17. C21 and Telmisartan combination ameliorates NF-κB mediated renal inflammation in type 2 diabetes.

Representative images for immunostaining (A) phosphorylated NF-κBp65 (Ser536) and (B) MCP1 and their respective quantifications (C-D). The bar charts represent the fold change in mRNA expression of (E-I) *Rela*, *Mcp1*, *Vcam1*, *Il6* and *Tnfa.* Note: *p<0.05 Vs normal control (NC), #p<0.05 Vs HFD+STZ (35mg/kg, ip), @p<0.05 Vs High fat diet+STZ treated+Telmisartan treated (H+S+T), $p<0.05 Vs High fat diet+STZ treated+C21 treated (H+S+C), High fat diet+STZ treated+C21+Telmisartan (H+S+C+T).

In study 2, type 2 diabetic rats showed prominent proliferation and fibrosis markers, TGF-β1 and collagen deposition. Telmisartan treatment reduced the expression of TGF-β1 as well as collagen in glomerular and tubular regions which is in concordance with the previous reports. C21 also reduced renal fibrotic markers' expression and it also further improved the anti-fibrotic activity of Telmisartan (Figure 18). At mRNA level, the expressions of *Tgfb1* and *Col1a1* were found to have exacerbated in type 2 diabetic rats. This increase in *Tgfb1* mRNA expression levels was controlled significantly by Telmisartan and C21 and their effects were further improved when given in conjugation. The *Col1a1* level was improved significantly by Telmisartan but not by C21 alone. The combination of Telmisartan with C21 showed a significant improvement in *Col1a1* levels in type 2 diabetic rats as compared to the individual treatments (Figure 18). These results indicate that AT_1

receptor antagonist and AT_2 receptor agonist combination is a potent anti-fibrotic regimen in type 2 diabetes.

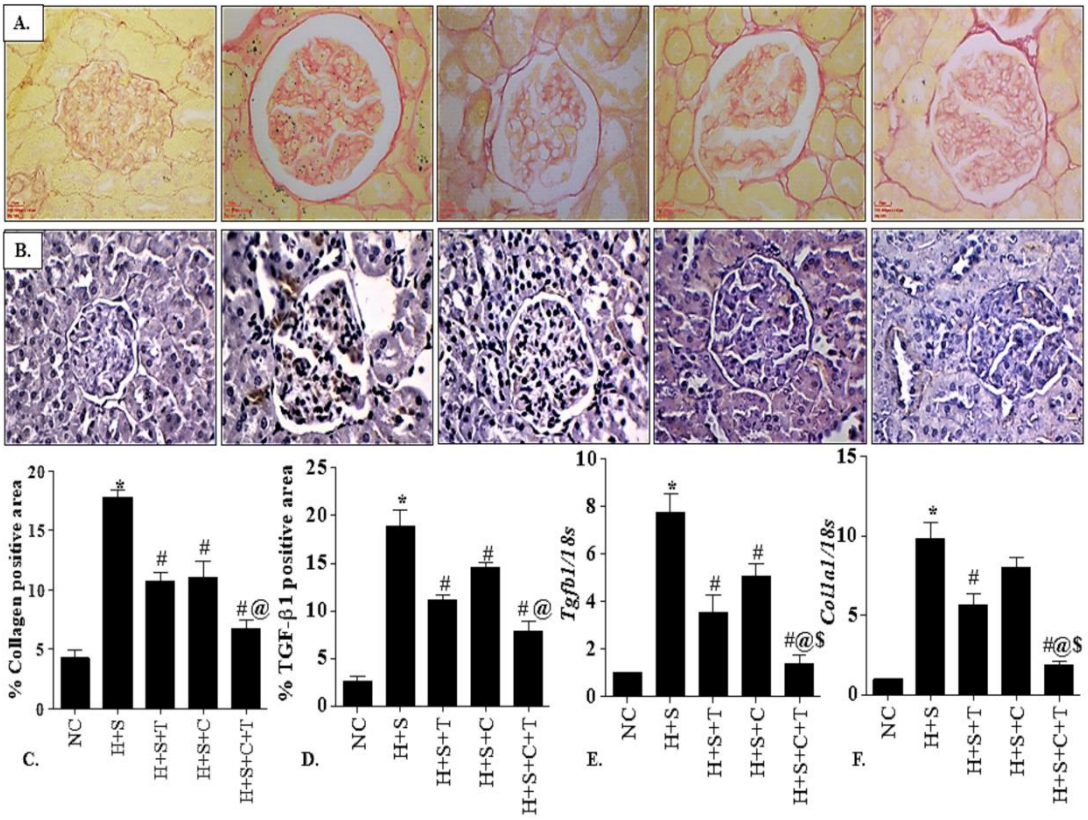


Figure 18. Effect of Telmisartan and C21 on renal fibrosis markers in type 2 diabetes
(A) Representative images of Picro Sirius Red staining and (C) % collagen positive area estimation (B) TGF-β1 immunohistochemistry (D) % TGF-β1 positive area. Fold change in (E) *Tgfb1* and (F) *Col1a1* mRNA expression. Note: Data represented in means ± SEM (n=6). *p<0.05 Vs normal control (NC), #p<0.05 Vs HFD+STZ, @p<0.05 Vs High fat diet+STZ treated+Telmisartan treated (H+S+T), $p<0.05 Vs High fat diet+STZ treated+C21 treated (H+S+C), High fat diet+STZ treated+C21+Telmisartan (H+S+C+T).

The expression of cleaved caspase 7 was found to have increased significantly in the immunohistochemistry. The treatment with C21 and Telmisartan reduced the elevated cleaved caspase 7 expression and their combination further ameliorated glomerular apoptotic marker's expression (Figure 19A-B).

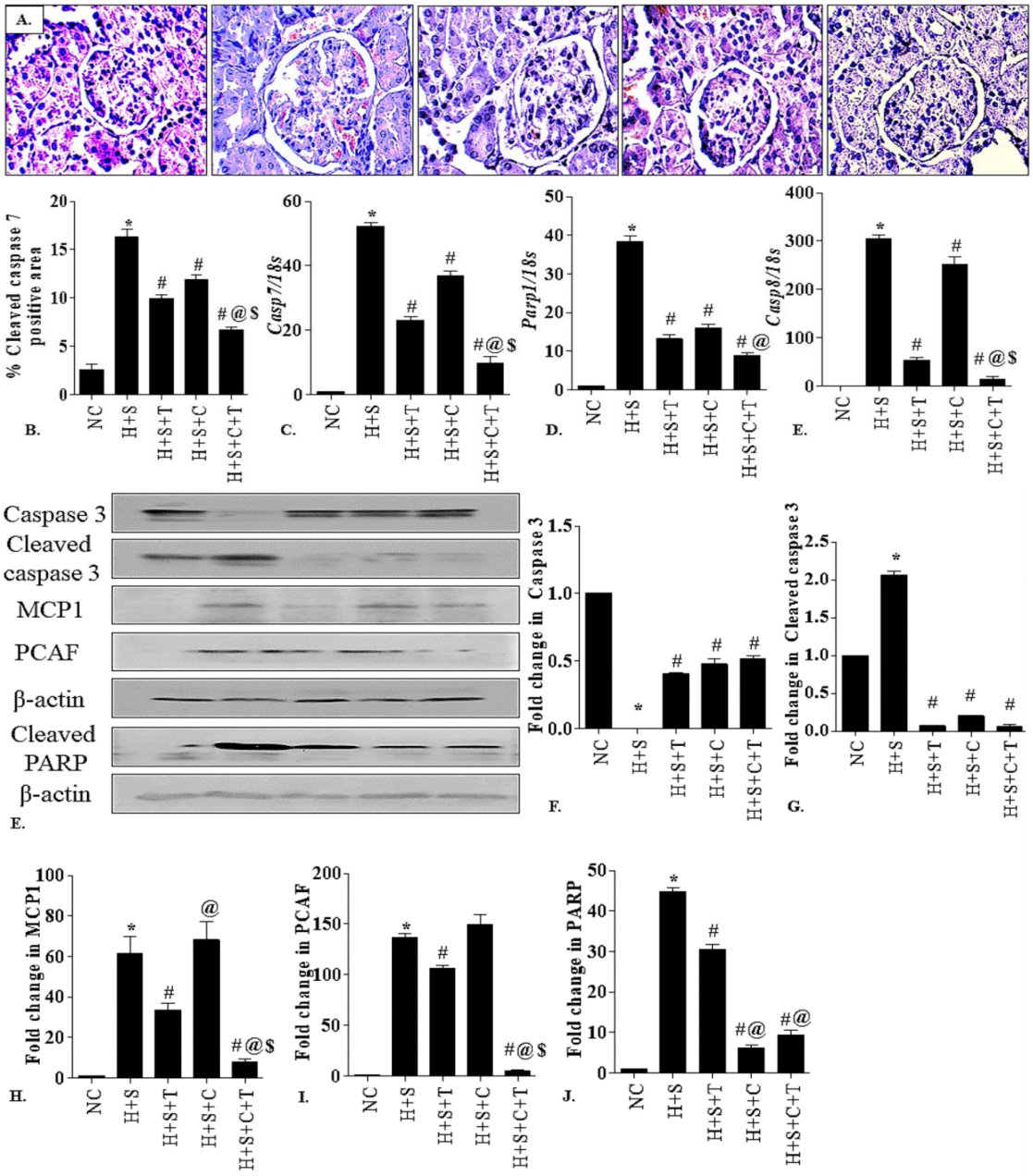


Figure 19. C21 and Telmisartan combination reduces renal apoptosis in type 2 diabetes.

(A) Representative images for immunohistochemistry of cleaved caspase 7 and (B) bar chart representing quantification of % cleaved caspase 7 positive area. (C-E) represent fold change in mRNA expression of *Casp7, Parp1* and *Casp8* respectively. (F) Representative Western blots of Caspase 3, Cleaved caspase 3, PARP; chemokine, MCP1 and PCAF, which are normalized against respective β-actin blots and (G-H) represent their respective densitometric analysis. Note: *p<0.05 Vs normal control (NC), #p<0.05 Vs HFD+STZ, @p<0.05 Vs High fat diet+STZ treated+Telmisartan treated (H+S+T), $p<0.05 Vs High fat

diet+STZ treated+C21 treated (H+S+C), High fat diet+STZ treated+C21+Telmisartan (H+S+C+T).

In this study, type 2 diabetic kidney was found to show profound increase in protein expression of cleaved PARP, and cleaved caspase 3 whereas the expression of caspase 3 was found to have attenuated. The expression of these apoptotic markers was improved markedly by both C21 and Telmisartan treatments. C21 was found to further promote the anti-apoptotic potential of Telmisartan, as evidenced by the potent reduction in apoptotic markers in the combination treated group. This shows that the expression of apoptotic proteins in type 2 diabetic kidney is significantly controlled by the AT_1 receptor blocker and AT_2 receptor agonist (Figure 19). In order to further check the effect of treatment at mRNA levels, mRNA expression of apoptotic genes, *Casp7, Casp8 and Parp1* was checked (Figure 19C-E). Here, mRNA expression of *Casp7, Casp8 and Parp1*, elevated drastically in type 2 diabetic rats was improved by both Telmisartan and C21. The combination regimen was found to ameliorate apoptotic markers' gene expression more significantly as compared to that of the individual treatments, thus showing its potent anti-apoptotic potential.

5.3. To understand the role of Ang II receptor subtypes in modulating the posttranslational histone modifications and their roles in regulating inflammation in type 2 diabetic nephropathy.

5.3.1. Effect of Ang II receptors' activity on histone H3 methylation

In this study, we found that SET7/9 is upregulated in HFD fed as well as HFD+STZ treated rats as compared to the NC. The treatment with Telmisartan reduces the upsurge in SET7/9 expression, PD123319 further accelerates the expression, whereas the combination of the two does not show any significant difference in the expression from the HFD+STZ treated rats (Figure 20). Thus, it may be concluded that both AT_1 and AT_2 receptors are involved in regulation of macrophage infiltration under type 2 diabetic nephropathy and this regulation may be associated with the epigenetic alterations. The increase in the methyltransferase is known to be linked with histone H3 methylation. The histone H3 dimethylation at lysine 4, 36 and 79, considered as transcription enhancer markers were increased in HFD fed and HFD+STZ treated rats. The treatment with Telmisartan reversed the

modifications, PD123319 further increased the methylation whereas the combination did not show any significant difference from that of the PD123319 treated group. This shows that histone H3 methylation is significantly altered by modulating AT_1 and AT_2 receptor activity and PD123319 increases global histone methylation (Figure 20).

The treatment with Telmisartan reduced global histone H3K4 dimethylation quite significantly as compared to the diabetic control as well as that of the C21 treatment. The combination of Telmisartan and C21 further ameliorated the increment in elevated histone H3K4Me2 and thus showing that the combination regimen is the most efficient treatment regimen, owing to its ability to revert the H3K4 hypermethylation (Figure 21B).

5.3.2. Effect of Ang II receptors activity on histone H3 acetylation in type 2 diabetic nephropathy

5.3.2.1. Telmisartan reverts the increased histone H3 acetylation in type 2 diabetic rats' kidney samples

Our study showed that the type 2 diabetic kidney shows an increase in the histone H3 acetylation at lysine 9, 14 and 27. This augmentation in histone H3 acetylation could be significantly reversed by the treatment with AT_1 receptor blocker, Telmisartan and further augmented by AT_2 receptor blocker, PD123319. However, their co-administration did not lead to any significant alterations in the acetylation of H3 as compared to the HFD+STZ treated group. The H318Ac was not found to show any significant elevation in HFD fed or the HFD+STZ treated rats, but Telmisartan could reduce the elevation in H3K18Ac as compared to HFD+STZ treated rats (Figure 21). Our results confirm that histone H3K9/K14Ac get accelerated under diabetic conditions. Thus, the therapeutic activity shown by Telmisartan, may be partially attributed to its ability to reduce the elevated histone H3 acetylation in diabetic nephropathy.

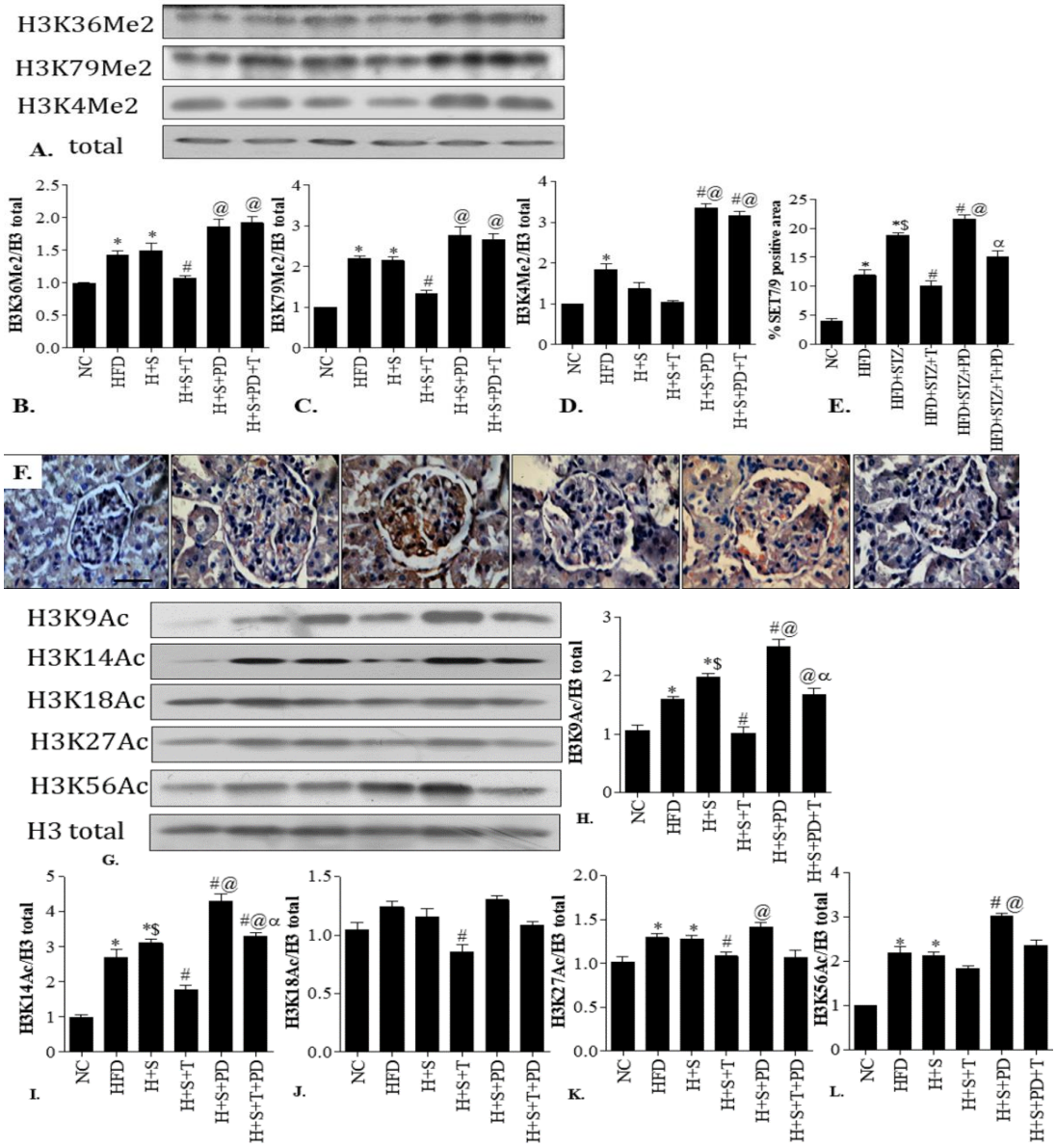


Figure 20. Effect of Ang II receptor blockade on expression of histone H3 lysine dimethylation and acetylation.

(A) Representative Western blots of histone H3 dimethylation at lysine 36, 79 and 4 (B-D) show their respective quantitative analysis. (F) Representative images of SET7/9 immunohistochemistry and (E) shows % SET7/9 positive area. (G) Representative Western blots of H3 acetylation at lysine 9, 14, 18, 27 and 56 (H-I) densitometric analysis of respective blots. Note: Each data point is represented as mean ± SEM, n=3 blots/protein. *p<0.05 Vs Normal Control (NC), #p<0.05 Vs High fat diet+STZ (H+S), $p<0.05 Vs High fat diet control (HFD), @p<0.05 Vs High fat diet+STZ+Telmisartan (H+S+T), α p<0.05 Vs High fat diet+STZ+PD123319 (H+S+PD); High fat diet+STZ+Telmisartan +PD123319 (H+S+T+PD).

5.3.2.2. C21 and Telmisartan combination reverts histone H3 acetylation at lysine 14 and 27 and histone acetyltransferase expression

Histone H3 acetylation at both lysine 14 and 27 increases in type 2 diabetic rats' kidneys. H3K14Ac was reduced more significantly by Telmisartan as compared to C21 and the combination improved the effect of Telmisartan whereas H3K27Ac was reduced equally efficiently by both Telmisartan and C21 (Figure 27A-C). The HAT, PCAF also called lysine acetyl transferase 2B (KAT2B) was significantly upregulated in type 2 diabetic rats' kidneys and it was reduced significantly by Telmisartan and its combination with C21 further lowered the protein expression (Figure 22E). The mRNA expression of *Pcaf* was also found to be reduced by the Telmisartan and C21 combination more efficiently than the individual treatments (Figure 21).

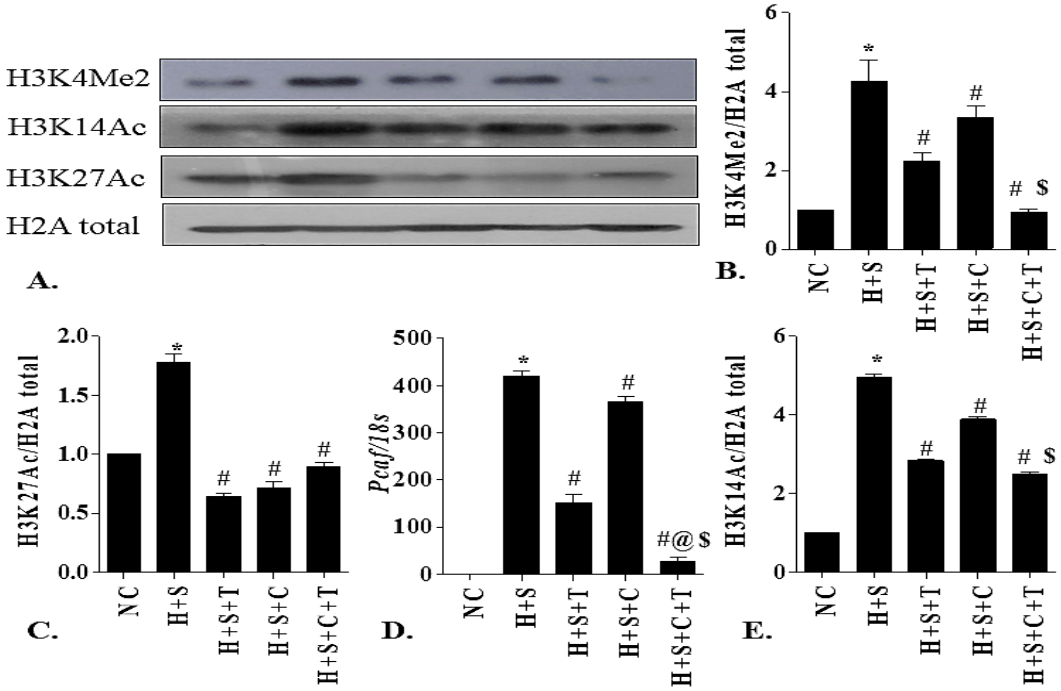


Figure 21. C21 and Telmisartan combination reverts histone H3 lysine 4 dimethylation and lysine 14/27 acetylation. Panel A: Representative Western blots for H3K14 and H3K27 acetylation normalized against H2A total. The bar charts represent the quantification of (B) H3K27 (C) H3K14 acetylation and (C) Fold change in mRNA expression of *Pcaf*. Note: Each data point is represented as mean ± SEM, n=3 blots/protein. Note: Data represented in means ± SEM (n=6). *p<0.05 Vs normal control (NC), #p<0.05 Vs HFD+STZ, @p<0.05 Vs High fat diet+STZ treated+Telmisartan treated (H+S+T), $p<0.05 Vs High fat diet+STZ treated+C21 treated (H+S+C), High fat diet+STZ treated+C21+Telmisartan (H+S+C+T).

5.3.3. Effect of Ang II receptors activity on histone monoubiquitination

5.3.3.1. AT_1 receptor antagonist reverts H2A lysine 119 and H2BK120 monoubiquitination in type 2 diabetic nephropathy

We found that H2AK119Ub gets increased in the HFD+STZ treated and HFD fed rats. The treatment with Telmisartan leads to a reduction in ubiquitination, whereas PD123319 further enhanced the H2AK119Ub. The combination of the two antagonists led to a significant reduction in histone H2AK119Ub as compared to that of the PD123319 treated animals, but was slightly more than that of the Telmisartan treated animals (Figure 22).

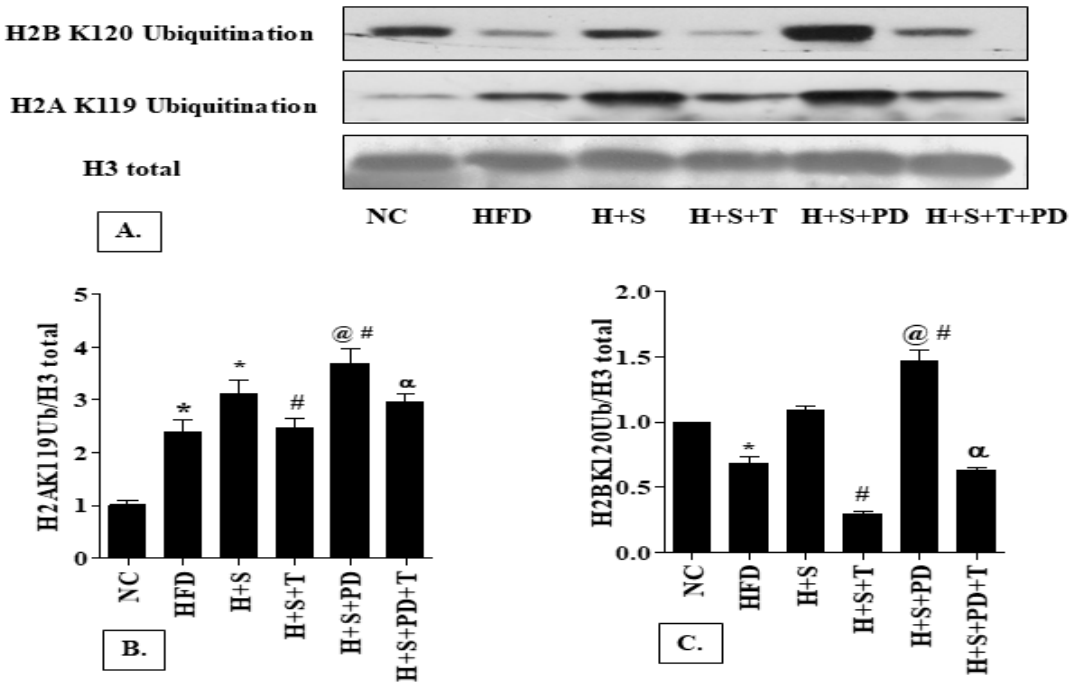


Figure 22. Effect of Telmisartan and PD123319 on expression of histone H2AK119 and H2BK120 monoubiquitination.

Western blots for (A) H2AK119Ub and H2BK120 monoubiquitination (B&C) quantitative analysis using ImageJ software. Note: Each data point is represented as mean ± SEM, n=3 blots/protein. *p<0.05 Vs Normal Control (NC), #p<0.05 Vs High fat diet+STZ (35mg/kg, ip) (H+S), $p<0.05 Vs High fat diet control (HFD), @p<0.05 Vs High fat diet+STZ+Telmisartan (H+S+T), α p<0.05 Vs High fat diet+STZ+PD123319 (H+S+PD); High fat diet+STZ+Telmisartan +PD123319 (H+S+T+PD).

Histone ubiquitination is a tightly regulated, reversible process guided basically by two enzymes- E3 ubiquitin ligases, responsible for addition of ubiquitin to protein substrate and deubiquitinases which help in removal or detachment of ubiquitin

moiety from the substrate. We also found changes in the expression of epigenetic enzymes that catalyse some of the diabetic nephropathy altered histone modifications in the HFD+STZ treated rats. Notably mRNA expression of the H2A specific E3 ubiquitin ligases like *Rnf2* and *Rnf168* were elevated significantly in the HFD fed and HFD+STZ treated rats. This rise in the mRNA expression is reversed significantly by the treatment with Telmisartan and further elevated by the PD123319 exposure. The treatment with both AT_1 receptor and AT_2 receptor antagonists showed an mRNA expression level similar to that of the HFD+STZ treated rats. The H2A specific deubiquitinases, *Usp21* and *Usp16* were found to have reduced significantly in the HFD fed and HFD+STZ treated rats, thus supporting our observation that H2AK119Ub gets enhanced in pre-diabetic and type 2 diabetic kidney and this alteration could be reversed to a considerable extent by Telmisartan treatment and was adversely affected by the PD123319 exposure (Figure 23). The mRNA expressions of H2B specific ubiquitinases and deubiquitinases did not show any signficant alterations as compared to that of the diabetic rats, in which H2BK120Ub was found to have raised significantly when compared with the NC (Figure 23).

5.3.3.2. Targeting AT_1 receptor ameliorates macrophage infiltration and renal fibrosis in diabetic nephropathy through modulation of H2AK119Ub

We next examined whether blocking Ang II receptors could alter H2AK119Ub recruitment at the promoter regions of *Mcp1* and *Tgfb1* using chromatin immunoprecipitation-QPCR assays. The results indicated that H2AK119Ub levels at *Mcp1* and *Tgfb1* promoters in the diabetic nephropathygroup were markedly lower as compared with the NC. The occupancy of H2AK119Ub was found to increase significantly by the treatment with Telmisartan whereas PD123319 treatment led to a further drop in the occupancy. These results suggest that Ang II mediated induction of *Mcp1* and *Tgfb1* expression in the kidneys of type 2 diabetic rats may be attributed to reduced occupancy of H2AK119Ub at their respective promoter regions, which in turn indicates a reduction in inhibition of expression (Figure 23 G-H).

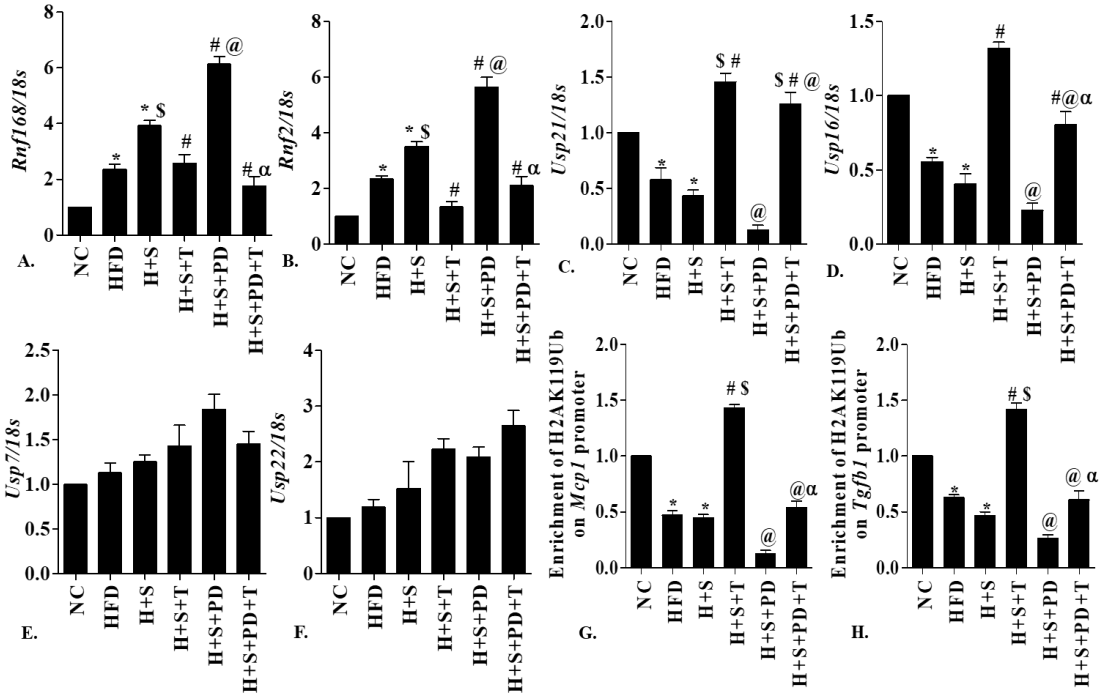


Figure 23. Effect of Telmisartan and PD123319 on mRNA expression of histone ubiquitinases and deubiquitinases.

Increased mRNA expression of H2A specific E3 ubiquitin ligases- (A) *Rnf168* and (B) Rnf2 and decreased mRNA expression of H2A specific deubiquitinases (C) *Usp21* and (D) *Usp16* in type 2 diabetic kidney. H2B specific deubiquitinases (E) *Usp7* and (F) *Usp22* did not show any significant changes in their mRNA expression. ChIP assay based determination of fold change in occupancy of H2AK119Ub at (G) *Mcp1* and (H) *Tgfb1* promoter. Note: Each data point is represented as mean ± SEM, n=6. *p<0.05 Vs Normal Control (NC), #p<0.05 Vs High fat diet+STZ (H+S), $p<0.05 Vs High fat diet control (HFD), @p<0.05 Vs High fat diet+STZ+Telmisartan (H+S+T), α p<0.05 Vs High fat diet+STZ+PD123319 (H+S+PD); High fat diet+STZ+Telmisartan +PD123319 (H+S+T+PD).

5.3.3.3. C21 and Telmisartan combination reverts histone H2A and H2B monoubiquitination

Histone H2AK119Ub and H2BK120Ub are repressive and permissive histone modifications, respectively. In type 2 diabetic kidney, both these modifications are found to have increased markedly as compared to the NC (Figure 24).

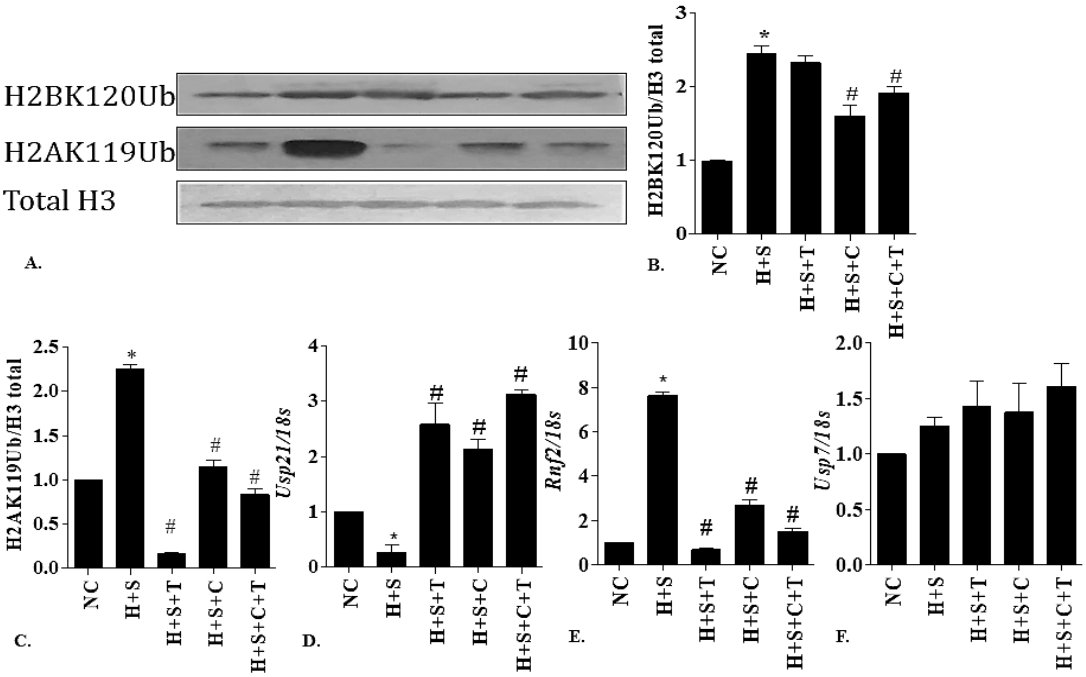


Figure 24. C21 and Telmisartan combination reverts histone H2AK119 and H2BK120 monoubiquitination.

Panel A: Reprentative Western blots for H2AK119 and H2BK120 monoubiquitination normalized against H3 total. The bar charts (B-C) represent their respective quantification and (D) *Usp7* specific for H2BK120 monoubiquitination (E-F) *Usp21* and *Rnf2* specific ubiquitinase and deubiquitinase towards H2AK119Ub. Note: Each data point is represented as mean ± SEM, n=3 blots/protein. *p<0.05 Vs normal control (NC), #p<0.05 Vs HFD+STZ, @p<0.05 Vs High fat diet+STZ treated+Telmisartan treated (H+S+T), $p<0.05 Vs High fat diet+STZ treated+C21 treated (H+S+C), High fat diet+STZ treated+C21+Telmisartan (H+S+C+T).

H2AK119Ub was reduced significantly by C21 alone but not by Telmisartan whereas the combination normalized the level of monoubiquitination. H2BK120 monoubiquitination was reduced more significantly in the Telmisartan treated group as compared to C21. Though the combination could not improve the Telmisartan effect on H2A and H2B monoubiquitination, it reduced the monoubiquitination significantly as compared to the type 2 diabetic kidneys. The histone H2B specific deubiquitinase, *Usp7* did not show any significant alterations in any of the groups whereas the H2A specific deubiquitinase *Usp21* reduced significantly in type 2 diabetic kidney and was improved significantly by C21, Telmisartan as well as the combination and there was no significant difference between the treatment groups. The E3 ligase specific for H2AK119Ub, *Rnf2* increased manifolds as compared to

that of the NC. Telmisartan treatment normalized the *Rnf2* expression level, C21 also reduced the levels quite significantly and the combination treatment showed that the impact of C21 on H2AK119Ub is markedly improved by Telmisartan (Figure 24).

6. Discussion

Diabetic nephropathy is one of the major reasons for end stage renal dysfunction and comorbidity throughout the world (Thomas *et al.*, 2016; Papadopoulou-Marketou *et al.*, 2017). The underlying mechanisms triggering its pathogenesis include hyperglycemia or hyperinsulinemia induced pathogenic mechanisms including redox imbalance, mitochondrial and endoplasmic reticulum stress and activation of several secondary molecules which further pump up the cascades like inflammation, fibrosis and apoptosis in various renal cells (Grudden *et al.*, 2005; Cao *et al.*, 2011; Badal *et al.*, 2014; Reidy *et al.*, 2014; Ramalingam *et al.*, 2017). Diabetic nephropathy and hypertension are closely linked and have been known to show a bidirectional relationship with each other, thus making hemodynamic aberration an important feature of advanced diabetic nephropathy. The altered hemodynamics due to glomerular hyper filtration and poor osmoregulation activates the RAS, which initially acts to maintain homeostasis but with progression of disease, gradually starts to worsen the renal functioning (Kamiyama *et al.*, 2013; Kobori *et al.*, 2013; Mercier *et al.*, 2014).

The currently available therapies like ARBs (Telmisartan) or ACE inhibitors (Ramipril) or even their combinations for diabetic nephropathy delay or slacken off the progression of renal dysfunction but have not yet been able to curb its root cause (Fernandez Juarez *et al.*, 2013; Makani *et al.*, 2013; Ruggenenti, 2017). Itmay be due to i) lack of understanding of Ang II receptors' role in expression of ACE and ACE2 (the two most important enzymes of RAS) and NF-κB mediated inflammatory cascade or ii) incomplete understanding of epigenetic mechanisms (posttranslational histone modifications) in diabetic nephropathy and the effects of therapeutic interventions acting through modulation of Ang II receptors' activity.

The current study showed that a combination of novel AT_2 receptor agonist, Compound 21 and AT_1 receptor antagonist, Telmisartan ameliorates diabetic nephropathy by improving renal inflammation, fibrosis and apoptosis through normalization of ACE and ACE2 levels and reversal of epigenetic modifications (posttranslational histone modifications).

6.1. Simultaneous AT_1 receptor blockade and AT_2 receptor activation ameliorates biochemical aberrations, oxidative stress and hemodynamic imbalance in type 2 diabetic nephropathy

The main risk factors for diabetic nephropathy are hypertension, hyperlipidemia, and hyperglycemia (Rachmani *et al.*, 1999; MacIsaac *et al.*, 2014). In the present study, the HFD+STZ treated rats demonstrated typical characteristics of type 2 diabetes, such as hyperglycemia, elevated plasma triglyceride and total cholesterol levels. The progression of renal damage was confirmed by the increased blood urea nitrogen and serum creatinine, and reduced plasma albumin which are taken as indices of altered GFR in diabetic nephropathy (Ferguson *et al.*, 2012; Sandilands *et al.*, 2013; Lopez-Giacoman *et al.*, 2015). The renal tissue damage and collagen deposition were examined by histopathological studies by staining with hematoxylin and eosin which showed an increased glomerular damage in diabetic kidney. The administration of AT_1 receptor and AT_2 receptor antagonists has shown opposite effects by ameliorating and deteriorating the pathological features of type 2 diabetic nephropathy, respectively. The co-administration of both the antagonists minorly attenuates but does not improve it significantly as compared to that of the diabetic controls, which confirms the counter-regulatory biological effects elicited by AT_1 and AT_2 receptors.

The individual treatment with Telmisartan and C21 reduced PGL, PTC and PI in diabetic rats, but their combination showed further improvement in carbohydrate and lipid metabolism indicating that it could be a better therapeutic intervention than C21 and Losartan which could not revert the elevated blood glucose levels in ZDF rats (Castoldi *et al.*, 2014). Oshima et al., had shown that C21 treatment significantly reduced blood glucose concentration and serum insulin concentration in the fed condition as compared with the control KK-Ay mice (Ohshima *et al.*, 2012). The renal function parameters, including PAL, PCr and ALP levels were improved more significantly by C21 as compared to Telmisartan, which was further improved by the combination. These results were in line with the previous reports which have indicated C21's ability to reduce proteinuria and albuminuria and the renoprotective effect of the combination could also be due to its potential to improve these parameters as albuminuria is also a risk factor for progression of

nephropathy in diabetes (Castoldi *et al.*, 2014; Patel *et al.*, 2016). Recent reports suggest that renal expression of AT_2 receptor is higher in adult female rats as compared to the male counterparts (Yu *et al.*, 2010), which urges us to speculate that the beneficial effects obtained by the administered combination may be even better in the female rats and further studies need to be done to determine the sex specific differences in the therapeutic activity.

The RAS system is intricately associated with maintenance of hemodynamic and non-hemodynamic parameters in the physiological as well as pathological conditions and its role in secondary microvascular and macrovascular complications of diabetes is well documented (Leehey *et al.*, 2000). Type 2 diabetes has been known to activate the RAS pathway and increase the production of Ang II which in turn acts on the receptors, AT_1 receptor and AT_2 receptor to elicit the further response. AT_1 receptor activates the NOX and acts in pro-oxidative stress manner whereas the AT_2 receptor activation leads to the anti-oxidative stress response (Lakshmanan *et al.*, 2011; Matsuda *et al.*, 2014; Namsolleck *et al.*, 2014; Lu *et al.*, 2015).

The oxidative stress was assessed by estimating the TBARS, GSH and *Sod1* mRNA expression in the renal tissue. SOD1 (CuZnSOD), a cytoplasmic isoform is a major antioxidant enzyme for superoxide removal, which converts superoxide into hydrogen peroxide and molecular oxygen (Fukai *et al.*, 2011). Our results showed that oxidative stress is increased and antioxidant defence mechanism is weakened by the exposure to AT_2 receptor antagonist. The exposure to AT_1 antagonist and AT_2 agonist was found to improve the free radical stress- both oxidative as well as nitosative stress in diabetic kidney.

The increased oxidative stress leads to a depletion of the anti-oxidant enzymes in the cells which leads to an augmented nuclear translocation of Nrf2. The Keap1-Nrf2 pathway, an important regulator of cytoprotective responses to endogenous and exogenous stresses caused by reactive oxygen species and electrophiles (Kansanen *et al.*, 2013; O'Connell *et al.*, 2015). In the current study, Keap1 expression was found to be elevated in HFD+STZ treated rats as compared to the NC. The elevation in Keap1 expression was attenuated by the AT_1 receptor

antagonist and augmented by AT_2 receptor antagonist, thus indicating a rise in oxidative stress due to blockade of the protective axis of RAS, ie. AT_2 receptor. This result shows that the increase in oxidative stress and reduction in antioxidant enzyme level is associated with the increased expression of Keap1, which may be an adaptive change to overcome the cellular stress which is in accordance with the previous reports that demonstrated similar changes in spontaneously hypertensive rat kidney (Erejuwa *et al.*, 2012). This result confirms that hyperglycemia induced oxidative stress in type 2 diabetic nephropathy is associated with the activation of Ang II/AT_1 receptor axis of RAS.

A series of reactions are triggered by a high oxidative stress condition, which renders Keap1 unable to revert to protein binding conformation. This alteration in its conformation abrogates its ability to bind with IKK-β, thus promoting the activation of NF-κB, which in turn increases the pace of inflammatory cascade (Stefanson *et al.*, 2014). Thus in the current study, the increase in Keap1 expression in diabetic kidney may be correlated with the simultaneous nuclear localization and increased phosphorylation of p65 to trigger the inflammatory signaling pathway.

Hypertension is an independent risk for development of nephropathy in type 2 diabetes subjects (MacIsaac *et al.*, 2014). The current study results showed that AT_2 receptor blockade by PD123319 further increased the systolic blood pressure in diabetic rats but C21 alone could not alter it significantly as compared to the diabetic rats. The Ang II mediated vasoconstriction that leads to rise in the blood pressure is known to be enacted through Ang II/AT_1 receptor axis (Bradford *et al.*, 2010). Telmisartan treatment reduced the blood pressure and its antihypertensive effects were further improved by C21 co-administration indicating that AT_1 receptor inhibition along with AT_2 receptor stimulation might provide a better solution to hemodynamic imbalance in diabetics. In acute disease models like, 2-kidney-1-clip hypertensive rat model for ischemic kidney failure, C21 treatment lowered the levels of inflammatory mediators and cell infiltrates, and enhanced production of NO and cGMP in the ischemic kidneys, without altering the blood pressure and these effects were partially antagonised by PD123319 (Matavelli *et al.*, 2011). Recent studies have shown that endothelial dysfunction is a major culprit in pathogenesis of renal diseases. A study conducted by Rehman et al showed that

C21, either alone or in combination with Losartan abrogates the development of hypertension and vascular damage in stroke-prone spontaneously hypertensive rats (Rehman *et al.*, 2012). Castoldi et al showed that Losartan and C21 combination improved the renal function in ZDF rats but shows no blood pressure lowering effects (Castoldi *et al.*, 2014).

Since ARBs and ACE inhibitors are the only existing treatments in type 2 diabetic nephropathy and their combination shows major side effects, C21 could be a significant conjugate drug for reducing the progression of type 2 diabetic nephropathy to end stage renal failure. The increased renoprotection ushered by the combination may be attributed to the increase in expression of AT_2 receptor upon treatment with AT_1 receptor antagonist which signifies increase in the activity of the protective arm of RAS (Henrion, 2012). The SBP monitoring in our study was based on non-invasive techniques, which may not be the most accurate method to estimate the blood pressure in rats, so future investigations using invasive techniques are required to confirm our observations.

6.2. Differential regulation of angiotensin converting enzyme and angiotensin converting enzyme 2 by Ang II receptor subtypes in type 2 diabetic kidney

ACE2, a monocarboxypeptidase degrading angiotensin Ang II to Ang 1–7 is highly expressed within the kidneys. It is largely localized in tubular epithelial cells, glomerular epithelial cells and in the renal vasculature (Lely *et al.*, 2004; Bindom *et al.*, 2009; Mizuiri *et al.*, 2015; Callera *et al.*, 2016). ACE2 has been reported to possess renoprotective activity owing to its anti-oxidant, anti-fibrotic and anti-inflammatory properties which help it act in a counter-regulatory manner to that of the ACE/Ang II/AT_1 receptor axis (Bindom *et al.*, 2009; Bradford *et al.*, 2010; Varagic *et al.*, 2014; Callera *et al.*, 2016).

The elevation of ACE2 expression in diabetic kidney has been reported in *db/db* mice as well as in induced models of diabetes (Ye *et al.*, 2004b; Ye *et al.*, 2006b; Tikellis *et al.*, 2008). The role of ACE2 in diabetic nephropathy has been studied in the 8-week-old *db/db* mice, a model of early type 2 diabetes which showed an elevation in the ACE2 expression with concomitant reduction in ACE expression in

both glomeruli and cortex (Wysocki *et al.*, 2006). A recent study performed by Mori and colleagues demonstrated that renal ACE2 is elevated in 5 months old *db/db* mice, an established model for diabetic nephropathy, which was attributed to the reduction in expression of SIRT1 (Silent Information Regulator T1) , an NAD-dependent protein deacetylase (Mori *et al.*, 2014). SIRT1 binds to the promoter sites of ACE2 and leads to an increment in the production of Ang (1-7) (Clarke *et al.*, 2014). Thus, the probable reasons for ACE2 overexpression may be (1) the reduction in SIRT1 activity (2) compensatory mechanism which leads to increased ACE2 expression so as to counterbalance Ang II and increase the Ang (1-7), which has a protective role in diabetic condition.

The treatment with AT_1 receptor antagonist, Olmesartan increased ACE2 and Ang-(1–7) in both spontaneously hypertensive rats (SHR) (Ishiyama *et al.*, 2004) and balloon induced carotid artery injury in 12 week old male SHR (Igase *et al.*, 2008). It was demonstrated that under hypertensive nephropathy condition, Ang II induced upregulation of ACE and down-regulation of ACE2 in human kidney tubular cells was blocked by AT_1 receptor antagonist (Losartan), but not by an AT_2 receptor blocker (PD123319) (Koka *et al.*, 2008). Another study showed that the increase in the renal ACE2 activity in obese Zucker rats occurred due to chronic treatment of AT_2 receptor agonist (Ali *et al.*, 2013). Callera et al showed that in *db/db* mice, Candesartan at intermediate dose (1mg/kg/day) reduced renal tubular damage, albuminuria, reduced ERK1/2 phosphorylation, fibrosis and increased ACE2/AT_2/Mas expression, which was not seen with the high and ultra-high doses. The study showed that the therapeutic effect of AT_1 receptor antagonist, Candesartan at intermediate dose is related with its ability to promote protective axis activity which is hampered at ultrahigh dose (Callera *et al.*, 2016). The relation of Ang II receptor subtypes and ACE2, a component of protective axis of RAS in progression of type 2 diabetic nephropathy remains largely obscure. In the current study, administration of PD123319 leads to aggravated pathological features and oxidative stress compared to HFD+STZ treated animal. The elevation of ACE2 expression in AT_2 receptor antagonist treated type 2 diabetic kidney may be attributed to the compensatory mechanism seeking to reduce Ang II effect because AT_2 receptor blockade was found to aggravate the free radical stress and other

biochemical parameters. ACE expression was found to be elevated drastically in HFD and HFD+STZ treated rats. The beneficial effects of Telmisartan in diabetic nephropathy may be linked with its ability to reduce ACE/ACE2 ratio. Surprisingly, the treatment with PD123319 could not alter the ratio but C21 and its combination with Telmisartan significantly reduced markedly it, thus showing that the beneficial therapeutic effect of the combination is due to promote the protective axis activity. This effect of AT_2 receptor stimulation is in line with the previous reports which showed that chronic activation of AT_2 receptors using CGP42112A increased ACE2 activity and MasR levels but decreased AT_1R levels and renin activity to cause natriuresis and blood pressure reduction in obese animals (Ali *et al.*, 2013). A recent study showed that AT_2 receptor stimulation activates ACE2 and prevents TNF-α-stimulated ICAM1 expression via inhibition of NF-κB signaling (Zhu *et al.*, 2015) also its cardioprotective effects were found to be linked with increment in ACE2 and Ang (1-7) levels (Zhu *et al.*, 2016). ***Thus, the current study was the first study to show that the therapeutic effect of Telmisartan and C21 combination in diabetic nephropathy is associated with its ability to reduce the renal Ace/Ace2 ratio.***

6.3. Differential regulation of nuclear factor-κB by Ang II receptor subtypes in type 2 diabetic kidney

Ang II mediated increase in inflammatory mediators involves activation of various downstream signaling including MAPK cascade, NF-κB, Rho proteins and redox pathways (Gai *et al.*, 2015; Park *et al.*, 2015). Type 2 diabetic nephropathy is a chronic inflammatory disease involving numerous transcription factors, pro-inflammatory cytokines, chemokines and adhesion molecules (Wada *et al.*, 2013). Various *in vitro* and *in vivo* studies show that the activation of NF-κB occurs through either AT_1 receptor or AT_2 receptor or both depending upon the tissue. The mesangial cells involve both AT_1 receptor and AT_2 receptor (Ruiz-Ortega *et al.*, 2001), tubuloepithelial cells involve AT_1 receptor (Wolf *et al.*, 2002b), endothelial cells involve AT_2 receptor, and VSMCs embroil both AT_1 receptor and AT_2 receptor to activate the transcription factor (Ruiz Ortega *et al.*, 2001), NF-κB, when exposed to Ang II. In unilateral ureteral obstruction model of renal injury in mice, it was demonstrated that Ang II, *via* AT_1 and AT_2 receptors and NF-κB pathway,

participates in the regulation of renal monocyte recruitment (Esteban *et al.*, 2004). In the present study we found that the drastically increased NF-κB activation in diabetic nephropathy was partially attenuated by exposure to AT_1 receptor antagonist (Telmisartan) and further promoted by PD123319. The co-administration did not lead to any significant alteration in the expression of phosphorylated NF-κB p65 and IκB-α expression as compared to that of the HFD+STZ treated rats. This result suggests that the AT_1 receptor and AT_2 receptor act in a counter-regulatory manner to control the NF-κB mediated inflammatory pathway. The augmentation of NF-κB and proinflammatory cytokines by PD123319 treatment in HFD+STZ treated rats shows that AT_2 receptor may play a significant role in regulation of the inflammatory cascade.

MCP1 expression is regulated by NF-κB signaling pathway and according to the existing literature, the regulation of NF-κB mediated MCP1 expression by Ang II receptor subtypes varies in different tissues and different conditions. For instance an *in vitro* study using glomerular epithelial cells showed that AT_1 receptor was responsible for the regulation of pro-inflammatory genes like *Il6, Vcam, Mcp1* (Ruiz-Ortega *et al.*, 2003) whereas another study using wild type C57BL/6 mice with unilateral ureteral obstruction showed that treatment with Losartan or PD123319, partially decreased NF-κB activation, whereas only the AT_2 receptor blockade diminished monocyte infiltration, which implicates that both the receptor subtypes are involved in activating NF-κB signaling pathway while AT_2 receptor orchestrates the monocyte mediated inflammation (Esteban *et al.*, 2004). In present study, we observed a drastic upregulation of MCP1, *Rela* and *Tnfa* expression in type 2 diabetic nephropathy. Telmisartan reduced the macrophage invasion by a significant amount while PD123319 provoked the invasion, showing the involvement of both the Ang II receptor subtypes in regulation of macrophage infiltration and production of inflammatory cytokines in type 2 diabetic nephropathy.

Also, it was further observed that direct stimulation of AT_2 receptor (C21) and inhibition of AT_1 receptor curtails the expression of phosphorylated NF-κB p65 and the downstream molecules including cytokines (*Il6* and *Tnfa*), chemokines (*Mcp1*) and adhesion molecule (*Vcam1*) in type 2 diabetic kidney. C21 alone, also could

significantly reduce the *Tnfa* expression which could also be responsible for its anti-apoptotic action because TNF-α is known to act on death receptor pathway and also to directly activate caspase 8 mediated apoptosis (Chaves *et al.*, 2009). A recent study showed that TNF-α released by hyperglycemia activated macrophages promoted podocytes apoptosis via ROS-p38MAPK pathway (Guo *et al.*, 2017).

Apoptosis involves three types of caspases- initiators, including caspase 2,8,9 and 10; executioners- caspase 3,6 and 7 and processing caspases like caspase 1,4,5,11 and 12L/12S. Caspase 3, an important executioner caspase activated by both the extrinsic as well as the intrinsic pathways is considered a marker of apoptosis, superior to the TUNEL assay that also detects the necrotic cells (Ghosh *et al.*, 2009). In our study, C21 improved the effect of Telmisartan on caspase mediated apoptosis, as shown by its influence over the expression of PARP, caspase 3 and cleaved caspase 3 at protein levels and *parp1*, *caspase8* and *caspase7* at mRNA level which are closely linked with glomerulosclerosis and renal dysfunction in diabetic subjects.

It is widely known that macrophage infiltration and fibrosis are central to development of renal failure in type 2 diabetes (Kanasaki *et al.*, 2013; You *et al.*, 2013; Guo *et al.*, 2017). TGF-β1 plays pivotal role in the development of renal fibrosis by phosphorylating SMAD3/SMAD4 complex to promote its nuclear translocation and by increasing ubiquitin mediated degradation of SMAD7. The increase in recruitment of Smad3 on the promoter region of genes coding for ECM components like collagen and fibronectin leads to the increased ECM deposition in diabetes (Lan, 2012). Our results showed that hyperglycemia and hyperinsulinemia promote the expression of fibrotic markers including TGF-β1, fibronectin and *Col1a1*, which could be controlled considerably by the administration of AT_1 receptor blocker, Telmisartan but were not affected by PD123319. This shows that the activation of TGF-β1 mediated fibrosis and deposition of ECM in type 2 diabetic nephropathy may occur through AT_1 receptor instead of AT_2 receptor. The increased expression of fibrotic markers in type 2 diabetic nephropathy could be correlated with simultaneous increase in the expression of MCP1. Wolf et al had demonstrated the existence of a regulatory loop controlling MCP1 and TGF-β1 expression. This model describes the positive regulation of TGF-β production by

MCP1 and negative feedback mechanism by which TGF-β1 limits the MCP1 production (Wolf *et al.*, 2002a; Sakai *et al.*, 2006). Ang II receptor subtypes modulate the posttranslational histone modifications and their effects on inflammation in type 2 diabetic nephropathy.

6.4. Role of Ang II receptor subtypes in type 2 in modulation of posttranslational histone modifications in diabetic kidney

Renal fibrosis, inflammation, advanced glycation end product formation and oxidative stress- the main features of type 2 diabetic nephropathy are reversed significantly by the AT_1 receptor antagonists. A recent study demonstrated that losartan mediated reversal of key inflammatory and fibrotic genes in diabetic nephropathy was associated with its ability to inhibit recruitment of H3K9/14Ac and H3K36Me3, respectively, at *Rage* and *Pai1* genes promoter sites (Reddy *et al.*, 2014b). In type 2 diabetic (*db/db*) mice renal failure can increase H3K9/K14Ac in the heart, which can be a contributing factor for cardiac hypertrophy and fibrosis.

NF-κB mediated inflammatory cascade regulates the transcription of various pro inflammatory cytokines and chemokines in type 2 diabetic nephropathy and is activated by acetylation facilitated by p300/CBP and p300/CBP-associated factor (PCAF) (Park *et al.*, 2015). PCAF, a member of the GCN5-related N-acetyltransferase family plays significant roles in induction of cellular apoptosis by modulating GLI1/Bcl-2/BAX axis (Gai *et al.*, 2015) and also regulates histone H3K14 and H4K18 acetylation (Shi *et al.*, 2014). The diabetic patients showed a significant increment in H3K9/14Ac and H4 K5/8/12Ac TNF-α and COX-2 inflammatory genes promoters in human blood monocytes (Miao *et al.*, 2004).

In our study, Telmisartan was found to revert histone acetylation to certain extent while PD123319 further increased histone H3 acetylation in type 2 diabetic kidney. PCAF, a transcriptional adaptor protein and HAT functions as catalytic subunit of transcriptional co-activator complex to acetylate histone H3 and H4 (Shi *et al.*, 2014). It facilitates transcriptional activation of NF-κB by reducing its binding to IκBα enhancer and enhancing its nuclear localization (Kiernan *et al.*, 2003). In our study, PCAF was found to be significantly upregulated in type 2 diabetic kidney which could be directly correlated with increased histone H3K14Ac and C21 was

found to improve the effect of Telmisartan on PCAF and H3K14Ac. The superior anti-apoptotic action of C21 and Telmisartan combination could be partially attributed to its improved ability to regulate PCAF expression which is known to enhance DNA damage response by acetylating p53 at lysine 320 (Shi *et al.*, 2014) and to induce apoptosis in hepatocellular carcinoma by suppressing Akt1 pathway (Gai *et al.*, 2015). Recent clinical studies have demonstrated the significance of histone H3 acetylation at the promoter regions of proinflammatory genes and also have found a strong correlation between the HbA1c and histone H3K9Ac in metabolic memory responsible for persistent pathogenic properties despite achievement of normoglycemia (Miao *et al.*, 2014). Thus, the reduction in inflammation and apoptosis upon the treatment with Telmisartan, C21 or their combination and the abrupt exacerbation of these pathological pathways by PD123319 treatment may be associated with their effects on histone H3 acetylation and also on PCAF.

Histone methylation has also gained much attention as potential molecular mechanism underlying metabolic memory and diabetic nephropathy. Despite being relatively stable, histone methylation could be dynamically modified through the concerted action of HMTs and histone demethylases (HDMs). A study aimed at examining the changes in key epigenetic chromatin marks, including histone H3K9me2/3 and H3K4Me1/2/3 levels, at fibrotic gene promoters showed that these chromatin marks and SET7/9 were involved in TGF-β1 and hyperglycemia-induced up-regulation of ECM deposition associated genes in renal mesangial cells (Sun *et al.*, 2010b). Yuan et al showed that SET7 (*Setd7)* gene silencing inhibited 12(S)-hydroxyeicosatetraenoic acid [12(S) HETE] induced pro-fibrotic gene expression thus proving the role of HMT and histone methylation in fibrosis (Yuan *et al.*, 2016). Thus, the current study indicates that the permissive histone modifications increased in diabetic kidney were reduced by AT_1 receptor blockade and AT_2 receptor agonist while it was exacerbated by AT_2 receptor blockade, which can be correlated with the fibrotic and inflammation markers' expression. Histone H3 methylation occurs in close alliance with histone ubiquitination, which is a comparatively lesser explored posttranslational histone modification. Histone H2B ubiquitination is a dynamic modification that promotes methylation of histone

H3K79 and H3K4 and H2A ubiquitination is directly linked with increase in H3K27Me3 thus showing the cross-talk between these histone modifications (Kalb *et al.*, 2014; Vlaming *et al.*, 2014).

Ubiquitination of protein substrates is no longer just a 'kiss of death', its functions in regulating various biological pathways have been unravelled recently (Paul, 2008; Bonnet *et al.*, 2014). Histone ubiquitination has recently been acknowledged as vital pathway for regulation of gene transcription. The glomerular mesangial cells exposed to hyperglycemia showed elevated cell damage, induced histone H2AUb, reduced histone H2BUb accompanied by increased activation of TGF-β1 signaling pathway (Gao *et al.*, 2013). Our study establishes the fact that histone H2AK119Ub gets heightened in the insulin resistant and hyperglycaemic rats' kidney, which was well supported by the concordant changes displayed by the ubiquitination machinery. The reversal of these repressive chromatin marks by Telmisartan shows that its ability to combat type 2 diabetic nephropathy may be having an epigenetic basis and alteration in histone H2AK119Ub occupancy at *Mcp1* and *Tgfb1* promoter sites may be one of the important mechanisms involved. We observed that HFD+STZ treated rats showed an elevation in the expression of the H2A specific E3 ligases, *Rnf2* and *Rnf168* and a significant reduction in deubiquitinases, *Usp21* and *Usp16*which could be altered by reversed markedly by Telmisartan treatment and was further aggravated by PD123319. This result shows that both the Ang II receptor subtypes may elicit their response by alteration of ubiquitin proteasomal system. The ubiquitin proteasome system has a well reported role in regulation of NF-κB pathway as it mediates the degradation of IκB to initiate the nuclear translocation of p65 which attaches to the transcription factor binding site and triggers gene transcription (Gao *et al.*, 2014). However, the correlation between the inflammatory signaling and H2AK119Ub has not yet been reported. Our study presents the first report discussing the disturbance in the ubiquitination machinery i.e. elevation in H2A specific E3 ubiquitin ligase and reduction of H2A specific deubiquitinases, concomitant with elevated macrophage infiltration and renal fibrosis in the type 2 diabetic nephropathy. Thus, based on the above observations we can conclude that macrophage infiltration and renal fibrosis are regulated through both AT_1 and AT_2 receptors in type 2 diabetic nephropathy and

H2AK119Ub is an important epigenetic link between these pathological manifestations and Ang II receptors. This suggests that amelioration of inflammatory and fibrotic markers may require the co-administration of an AT_1 receptor antagonist and AT_2 receptor agonist and also that the quest to search a solution to type 2 diabetic nephropathy must consider the effect of the therapeutic intervention on ubiquitin proteasome system and histone H2AK119Ub so as to find a superior solution to the problem.

H2BK120Ub in mammals, carried out by the ubiquitin RNF20/40 and other E3 ligases such as *BAF250B, Mdm2 and BRCA1* are associated with activation of gene transcription. In yeast, several H2B DUBs have been identified, the main ones being *Ubp8* and *Ubp10*. In mammals, USP3, USP7, USP12, USP22, USP44, USP46 and USP49 have been reported to be involved in H2B deubiquitination. H2BK120 monoubiquitination correlates with transcriptional activators, H3K4Me2/Me3 and H3K79Me (Bonnet *et al.*, 2014; Fuchs *et al.*, 2014). Also, H2B monoubiquitination plays an important role in DNA damage responses and tumour suppression (Johnsen, 2012). The H2B ubiquitin specific protease-22 (USP22) is also required for both androgen receptor and oestrogen receptor-dependent transcription (Fuchs *et al.*, 2014). The present study found that the treatment with Telmisartan reversed the histone H2BK120Ub and its combination with C21 further normalized the ubiquitination level, while AT_2 receptor blockade further increased it, thus showing that modulation in activity of Ang II receptors can lead to alterations in histone ubiquitination. The increase in cleaved PARP in diabetic rat kidney may be correlated with the simultaneous increase in histone H2BK120Ub, a marker for DNA damage response.

Taken together, it can be concluded that Ang II receptors modulate the posttranslational modifications like histone H3 dimethylation, acetylation and H2A and H2B monoubiquitination in diabetic kidney and thus targeting Ang II receptors, particularly with AT_1 receptor antagonist and AT_2 receptor agonist could be an approach to treat diabetic nephropathy- its pathological cascades (including NF-κB mediated inflammation, TGF-β mediated fibrosis and caspase mediated apoptosis) as well as the underlying epigenetic mechanisms.

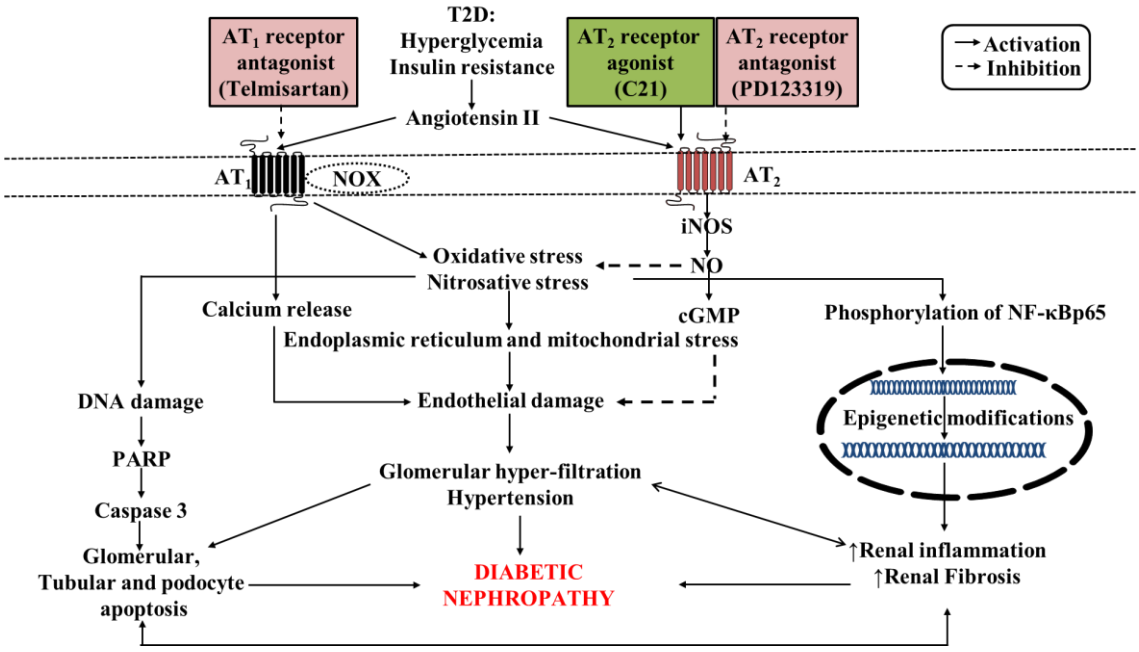


Figure 25. Summary figure.

Type 2 diabetes characterized by hyperglycemia and insulin resistance activates renin angiotensin system and subsequently elevates the levels of Ang II which acts via AT_1 and AT_2 receptors. An inhibition of AT_1 receptor, and simultaneous activation of AT_2 receptor using Telmisartan and Compound 21 reduces oxidative stress, hypertension, NF-κB mediated renal inflammation, fibrosis and caspase mediated apoptosis, which could be partly attributed to its ability to regulate PCAF and histone acetylation, methylation and ubiquitination. The AT_2 receptor antagonist, PD123319 aggravates diabetic nephropathy which could be associated with the increased oxidative stress that triggers further pathological cascades to worsen the condition.

7. Conclusions

- In type 2 diabetic kidney, ACE and ACE2 expressions are differentially regulated by AT_1 and AT_2 receptors and direct stimulation of AT_2 receptors along with AT_1 receptor blockade showed significant therapeutic effects by improving the physical and hemodynamic parameters, which may be correlated with the reduction in *Ace/Ace2* mRNA ratio indicating the promotion of protective axis of RAS by the combination therapy.

- The study demystifies the enigma related to regulation of NF-κB signaling pathway by Ang II receptor subtypes and leads to the conclusion that AT_1 and AT_2 receptors' activation leads to activation and inhibition of the inflammatory cascade mediated by NF-κB. AT_1 antagonist, Telmisartan and AT_2 receptor agonist, C21 not only reduce NF-κB mediated inflammation and renal monocyte infiltration but also reduce TGF-β1 mediated fibrosis and caspase mediated apoptosis to contribute to the renoprotective effect of the combination therapy.

- Ang II receptor subtypes, AT_1 and AT_2 receptors' activity significantly alters the posttranslational histone modifications including histone H3 lysine dimethylation, acetylation and H2A lysine 119 and H2B lysine 120 monoubiquitination in type 2 diabetic rats' kidney samples and the reversal of these alterations by Telmisartan and C21 or their combination may be the underlying mechanisms for the therapeutic benefits shown by these agents.

- It could also be suggested that the quest to search a solution to type 2 diabetic nephropathy must consider the effect of the therapeutic intervention on epigenetic factors including ubiquitin proteasome system and histone monoubiquitination so as to find a superior solution to the problem.

Therefore, a combination of AT_2 receptor agonist (Compound 21) and AT_1 receptor antagonist (Telmisartan) could be a better therapeutic regimen for the treatment of type 2 diabetes induced nephropathy as it not only controls NF-κB mediated inflammation, TGF-β1 mediated renal fibrosis, caspase mediated apoptosis, hemodynamic aberrations but also the underlying epigenetic alterations.

8. Limitations and future prospective

- The expression of AT_1 receptors, AT_2 receptors, ACE and ACE2 is different in different renal tissues. The current study involves studies on whole kidney samples, so further studies could be carried out to evaluate the effect of the administered agents in specific tissues to understand the elicited effects more clearly.

- The protective axis of RAS is constituted by various other important components which are equally important in diabetic nephropathy pathogenesis. The current study focused on ACE2 and AT_2 receptors only, other components need to be studied in depth.

- Novel therapeutic agents targeting ACE2 and AT_2 receptors could be developed and analyzed for their efficacy to treat diabetic nephropathy, which till date has not yet been treated with the existing pharmacotherapies.

- Further studies related to promoter specific occupancy of histone modifications and other epigenetic modifications like DNA methylation and microRNA could be checked in order to understand the exact underlying epigenetic mechanisms.

- Further, clinical studies should be conducted in order to check the efficacy of C21 either alone or in combination with Telmisartan in treatment of diabetes induced nephropathy as well as other associated secondary complications.

9. References

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List of Publications

Publications from thesis

1. **Pandey A**, Gaikwad AB (2017). AT_2 receptor agonist Compound 21: A silver lining for diabetic nephropathy. *Eur J Pharmacol.* 815:251-257. [Impact Factor: 2.896]

2. **Pandey A,** Gaikwad AB (2017) "Compound 21 and Telmisartan combination mitigates type 2 diabetic nephropathy through amelioration of caspase mediated apoptosis". *Biochem Biophys Res Commun.* 487(4): 827–833. [Impact Factor: 2.297]

3. **Pandey A**, Goru SK, Kadakol A, Malek V, Sharma N, Gaikwad AB (2016) "H2AK119Ub regulates angiotensin II receptor mediated macrophage infiltration and renal fibrosis in type 2 diabetic rats". *Biochimie.* 131: 68-76. [Impact Factor: 3.112]

4. **Pandey A**, Goru SK, Kadakol A, Malek V, and Gaikwad AB (2015) "Differential Regulation of ACE2 and NF-κB by Ang II Receptor Subtypes in Type 2 Diabetic Kidney". *Biochimie*. 118: 71-81. [Impact Factor: 3.112]

Other articles

5. Goru SK, Kadakol A, Malek V, **Pandey A**, Sharma N, Gaikwad AB (2017) "Diminazene aceturate prevents type 1 diabetic nephropathy through increasing glomerular ACE2 and AT2 receptor expression". *Br J Pharmacol.* 174, 3118-3130. [Impact Factor: 5.491]

6. Kadakol A, Malek V, Goru SK, **Pandey A**, Sharma N, Gaikwad AB (2017) "Esculetin ameliorates insulin resistance and type 2 diabetic nephropathy through reversal of histone H3 acetylation and H2A lysine 119 monoubiquitination". *J Funct Foods.* 35, 256-266. [Impact Factor: 3.114]

7. **Pandey A**, Priyank R, Goru SK, Kadakol A, Malek V, Sharma N, Gaikwad AB (2017) "Esculetin ameliorates hepatic fibrosis in high fat diet induced non-alcoholic fatty liver disease by regulation of FoxO1 mediated pathway". *Pharmacol Rep.* 69(4), 666-672. [Impact Factor: 2.587]

8. Goru SK, Kadakol A, **Pandey A**, Malek V, Sharma N, Gaikwad AB (2016) "Histone H2AK119 and H2BK120 Monoubiquitination Modulate SET7/9 and SUV39H1 in Type 1 Diabetes Induced Renal Fibrosis". *Biochem J* 473: 3937-3949 [Impact Factor: 3.797]

9. Kadakol A, **Pandey A**, Goru SK, Malek V and Gaikwad AB (2015) "Insulin Sensitizing and Cardioprotective Effects of Esculetin and Telmisartan combination by Attenuating Ang II Mediated Vascular Reactivity and Cardiac Fibrosis". *Eur J Pharmacol.* 765:591-7. [Impact Factor: 2.896]

10. Kadakol A, Malek V, Goru SK, **Pandey A**, and Gaikwad AB (2015) "Esculetin Reverses Histone H2A/H2B Ubiquitination, H3 Dimethylation, Acetylation and Phosphorylation in preventing Type 2 Diabetic Cardiomyopathy". *J Funct Foods.* 17:127–136. [Impact Factor: 3.114]

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12. Goru SK, **Pandey A**, Gaikwad AB. (2016) "E3 ubquitin ligases as novel therapuetic targets for inflammatory diseases". *Pharmacol Res.* 106: 1–9. [Impact Factor: 4.480]

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1. **Pandey A**, Y.A. Kulkarni, Gaikwad AB, (2016). "Curcumin: The epigenetic therapy. In: Fruits, Vegetables, and Herbs". Academic Press: Elsevier, USA. Pp.105-119.

Brief biography of the supervisor

Dr. Gaikwad Anil Bhanudas is currently working as Assistant Professor and Head of Department, Department of Pharmacy, BITS Pilani, Pilani campus, Rajasthan. He did his Masters and Ph.D. from Department of Pharmacology and Toxicology NIPER, SAS Nagar. After, completion of the doctoral studies, he worked as a Visiting Scientist at Department of Medicine/Nephrology, Albert Einstein College of Medicine, and Bronx, NY, USA (2010). He was awarded with prestigious DAAD (German Academic Exchange Services) Sandwich fellowship (2008-09) for conducting part of his Ph.D. work in the Nephrological Center, Medical Policlinic, Ludwig Maximilians-University, Munich, Germany. He has an expertise in the field of metabolic diseases including cardio-metabolic syndrome and diabetes mellitus, both type 1 and type 2 and their related renal and cardiovascular complications. He has received prestigious research grants from various government funding authorities like (i) Department of Science and Technology (DST) under fast track scheme of young scientist (ii) Department of Science and Technology (DST) under Empowerment and Equity Opportunities for Excellence in Science (iii) Council of Scientific and Industrial Research (CSIR) under Major Research Project and (iv) University Grants Commission under Major Research Project and the findings from his works have been published in more than 30 reputed international scientific journals. At present he is guiding five Ph.D. students and guided more than four M. Pharm. students for the fulfillment of their dissertation.

Brief biography of the candidate

Ms. Anuradha has graduated in Pharmacy from Kadi Sarva Vishwavidhyalaya, Gandhinagar, India in 2011 and has completed her Masters in Pharmacology and Toxicology from National Institute of Pharmaceutical Education and Research, Ahmedabad, India in 2013. She was awarded DST-INSPIRE fellowship from Department of Science and Technology, India to pursue doctoral degree studies. She joined Department of Pharmacy to pursue her doctoral degree studies. Her areas of interest include epigenetics, diabetes and its associated complications. She has published 11 research articles, 5 review aricles and one book chapter in peer reviewed journals.

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Research paper

Differential regulation of angiotensin converting enzyme 2 and nuclear factor-κB by angiotensin II receptor subtypes in type 2 diabetic kidney

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ABSTRACT

Angiotensin II (Ang II) acts through Angiotensin Converting Enzyme (ACE)/Ang II type 1 receptor (AT1R) axis to promote renal failure whereas the Ang II type 2 receptor (AT2R)/Angiotensin Converting Enzyme 2 (ACE2)/Ang1−7/Mas axis constitutes the protective arm of Renin Angiotensin System (RAS). Though Ang II has been known to activate the Nuclear Factor-κB (NF-κB) signalling pathway through different receptor subtype(s) in different tissues under various diseases, the subtype orchestrating this stimulation in type 2 diabetic kidney remains elusive. ACE2, a protective monocarboxypeptidase, responsible for conversion of Ang II to Ang1−7, opposes the deleterious effects of RAS pathway but how its expression is altered with blockade of AT1R and AT2R is not yet known. Hence, the present study was conceived to understand the regulation of NF-κB and ACE2 by using specific AT1 and AT2 receptor antagonists in non-genetic model of type 2 diabetic nephropathy. Our results show that the AT1R and AT2R antagonists lead to the repression and activation of NF-κB signalling pathway, respectively which suggests the role of AT1R in NF-κB activation. The blockade of AT2R led to an increase in ACE2 expression, which may be a compensatory response to the drastically increased inflammatory mediators and oxidative stress in the diabetic kidney. To the best of our knowledge, this is the first study showing the differential regulation of NF-κB and ACE2 by Ang II receptor subtypes and thus this study improves our understanding regarding regulation of inflammatory cascade and ACE2 by AT1R and AT2R in type 2 diabetic kidney, which may help in designing novel strategies to combat the disease in future.

1. Introduction

Type 2 diabetic nephropathy (T2DN) is one of the leading causes of end stage renal failure [1]. T2DN mainly involves inflammatory changes including alteration in levels of cytokines, chemokines and leukocyte populations, increased oxidative stress, apoptosis and tissue fibrosis [2]. Angiotensin II (Ang II), one of the most important components of Renin Angiotensin System (RAS), shows pro-inflammatory, growth stimulatory, fibrogenic and free radical promoting activities which provoke the development of end stage renal failure [3] and diabetic nephropathy [4]. Ang II shows most of its deteriorating effects like pro inflammatory, hypertrophy, proliferation, and/or apoptosis via Ang II type 1 receptor (AT1R) [5], whereas Ang II type 2 receptor (AT2R) is responsible for the protective effects like vasodilatation, anti-inflammatory and anti-apoptotic [6]. These receptors counterbalance the “ying-yang” and maintain the normal renal physiology, blood pressure, body electrolyte and fluid balance [7]. Ang II has a prominent role in activation of Nuclear Factor-κB (NF-κB) signalling pathway in diabetic nephropathy. A number of *in vivo and in vitro* studies show that it regulates the production of pro inflammatory cytokines like interleukin-1β (IL1β) [8] and chemokines such as monocyte

Abbreviations: RAS, Renin Angiotensin System; T2D, Type 2 Diabetes; T2DN, Type 2 diabetic nephropathy; NOX, NADPH oxidase; Ang II, Angiotensin II; AT1R, Angiotensin II type-1 Receptor; AT2R, Angiotensin II type-2 Receptor; ACE2, Angiotensin Converting Enzyme 2; Keap1, Kelch ECH Associating Protein1; STZ, Streptozotocin; HFD, High Fat Diet; NPD, Normal Pellet Diet; TBARS, Thiobarbituric Acid Reactive Substrate; SBP, Systolic Blood Pressure.

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chemoattractant protein-1 (MCP1), transforming growth factor-beta (TGF-β), intercellular adhesion molecule 1 (ICAM1), vascular cell adhesion molecule-1 (VCAM1), regulated on activation, normal T cell expressed and secreted (RANTES) and tumor necrosis factor alpha (TNF-α) [9]. NF-κB activity in macrophages, glomerular and tubular parenchymal cells has been linked with severity parameters of diabetic nephropathy such as proteinuria, oxidative stress or inflammation [10].

NF-κB super family consists of at least 5 genes encoding the members RelA (p65), RelB, RelC, p50, and p52. The inactive form of NF-κB is localized in the cytoplasm and consists of the DNA-binding p50 and p65 subunits and an inhibitory subunit called inhibitor of kappa B (IκB), which is bound to p65. The phosphorylation at Ser32 and Ser36 by the IκB kinase (IKK) complex releases IκB-α from the complex and unmasks the nuclear localisation sequence to promote nuclear translocation of p50/p65 complex and initiate the transcription [11]. According to various studies, Ang II mediated NF-κB activation is carried out through different receptor subtypes in different cells. In vascular smooth muscles [12] and mesangial cells [13] both AT1R and AT2R; in tubuloepithelial cells by AT1R, while in endothelial cells by AT2R [14] was reported to be associated with the NF-κB pathway activation. It has been reported that the production of cytokines involved in the process of inflammation is regulated differentially, ie, in glomerular epithelial cells, RANTES expression is regulated by AT2R [15] while expression of pro inflammatory genes like IL-6, VCAM1, MCP1 is controlled by AT1R [16]. The study performed by Esteban et al., confirmed the role of differential regulation of NF-κB by Ang II under diseased condition. In the kidney of wild type mice (C57BL/6) with unilateral ureteral obstruction, treatment with AT1R (Losartan) or AT2R antagonist (PD123319) partially decreased NF-κB activation, whereas only the AT2R blockade diminished monocyte infiltration, which implicates that both the receptor subtypes are involved in activating NF-κB signalling pathway while AT2R orchestrates the monocyte mediated inflammation [17]. However, role of Ang II receptor subtype in activation of NF-κB signalling in the kidney of type 2 diabetes (T2D) animals still remains elusive. Therefore, there is a need to identify the receptor subtype orchestrating the NF-κB signalling pathway in T2D kidney. This will give us a clear insight into the regulatory mechanism of the signalling pathway.

The increased load of inflammatory mediators and free radicals in the cell starts a feedback or compensatory mechanism through which the stress could be ameliorated and this is where the protective axis of RAS, AT2R/Angiotensin Converting Enzyme 2 (ACE2)/Mas axis comes into the picture [18]. The overexpression of ACE2 has been reported to reduce the oxidative stress by activating the anti-oxidant enzymes like superoxide dismutase and inhibiting NAD(P)H oxidase (NOX) in a dose dependent manner thus proving useful in controlling the hemodynamic parameters [19]. The Ang II receptor subtypes involved in regulation of ACE2 expression varies from tissue to tissue. A study performed by Ali et al., 2013 showed that in obese Zucker rats, a long term AT2R agonist [CGP42112A, 10 nM] treatment led to attenuation of AT1R function and increment in the of activity of ACE2/Ang-(1–7)/Mas axis [20]. Also, in the paraventricular nucleus, the centre of cardio-regulation in brain shows an overexpression of ACE2 along with reduced AT1R and Angiotensin Converting Enzyme (ACE) expression and increased AT2R and Mas expression under Ang II induced hypertensive condition [21]. The administration of AT1R antagonist, Telmisartan was found to increase the expression of ACE2 in kidney arterioles (tunica media) which in turn increases the Ang II degradation and culminates into the antihypertensive effect [22]. It was seen that both AT1R and AT2R are involved in the regulation of ACE2 expression in brain regions controlling blood pressure [23]. ACE2, an endogenous enzyme sharing 40% homology with ACE is expressed abundantly in adult kidney. It degrades Ang II to Ang 1–7 and Ang I to Ang 1–9, that is subsequently converted to Ang 1–7 by ACE. ACE2 has been found to show beneficial effects against hypertension, cardiac dysfunction, fibrosis, inflammation, atherosclerosis and diabetic complications due to its counter regulating effect on the ACE/Ang-II/AT1 receptor axis [24,25]. ACE2 contributes to the cell protective action by reducing oxidative stress, hypertrophy [26], fibrosis and inflammation through inhibition of various pathways like TGF-β, macrophage migration inhibitory factor [27], mitogen-activated protein kinases/NF-κB pathway [28]. The alteration in ACE2 expression has been known to participate in the pathogenesis of diabetes and diabetic nephropathy [24]. However, the regulation of ACE2 expression by angiotensin II receptor subtypes in type 2 diabetic kidney still remains an enigma.

In order to address these two important questions, the present study was conceived to delineate the relationship between blockade of Ang II receptors subtypes and its effect on NF-κB signalling pathway and ACE2 expression levels in the kidney of non-genetic model of T2D. The animal models used for the study of T2D should closely mimic the pathological conditions but most of the transgenic animal models do not mimic the pathological alterations [29]. Thus, the use of non-genetic model of T2D is more preferable than the transgenic animal models. Hence, in the present study we have used the non-genetic animal model of T2DN [high fat diet (HFD) feeding and administering a low dose of Streptozotocin (STZ)] which mimics the metabolic abnormalities very similar to those seen in human T2D [30,31].

2. Materials and methods

2.1. Chemicals

PD123319 was procured from Tocris Biosciences, (Bristol, UK). STZ was procured from Sigma–Aldrich (St. Louis, MO, USA). Antibodies against AT1R and AT2R were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and rest of the antibodies were purchased from Cell Signalling Technology (Danvers, MA, USA). For biochemical estimation, spectrophotometric kits purchased from Accurex (Accurex Biomedical Pvt. Ltd., Mumbai, India) and ultra-sensitive rat insulin kit obtained from Crystal Chem (Downer's Grove, IL, USA). Enhanced chemiluminescence reagent was purchased from Thermo Fisher Scientific (Waltham, MA, USA). All the other chemicals were purchased from Sigma (St. Louis, MO, USA), unless otherwise mentioned.

2.2. Animal studies

Adult *Wistar* rats (160–180 g) were procured from the central animal facility of the institute, Birla Institute of Technology and Science Pilani (BITS Pilani). The animals were maintained under standard environmental conditions and were provided with feed and water *ad libitum*. All the animals were fed on normal pellet diet (NPD) one week prior to the experimentation. Our animal protocol is in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (Ministry of Social Justice and Environment, Government of India) which follows regulations as per the guidelines of Institute of Laboratory Animal Resources, (Washington, DC, U.S.A.). A prior permission was sought from the institutional animal ethics committee, BITS Pilani, for conducting the study. All the experimental procedures had been approved by the local government authorities.

The animal model for T2DN was developed using high fat diet

(HFD) and administration of low dose STZ as described previously [32]. Briefly, the rats were divided into two groups- NPD and high fat diet (HFD) fed rats (58% fat, 25% protein and 17% carbohydrate, as a percentage of total kcal) *ad libitum* respectively for an initial period of 2 weeks. After 14 days of dietary manipulation, the HFD fed rats were treated with low dose of STZ (35 mg/kg, *intra peritoneal*.) dissolved in ice cold citrate buffer (0.1 M, pH 4.4) to induce T2D, as described by Gaikwad et al., 2007 and 2010 [31,33]. The induction of T2D was confirmed by analyzing the plasma glucose levels after 48 h of induction. After induction of diabetes, the rats were allowed to be fed on HFD diet for 24 weeks for development of renal failure.

The HFD + STZ treated rats were administered with Telmisartan (10 mg/kg, *per oral*), PD123319 (10 mg/kg, *subcutaneously*) [34] or both or vehicle (0.5% w/v sodium carboxy methyl cellulose) for 14 days prior to the biochemical studies. The dose of Telmisartan was selected based upon the literature that suggests that Telmisartan (10 mg/kg/day) reduces the pace of progression of glomerulosclerosis, and significantly improved interstitial cell infiltration, interstitial fibrosis, dilation and atrophy of renal tubules, normalized plasma lipids (total cholesterol and triglyceride), thus showing its renoprotective effect [35].

Animal body weights and kidney weights were noted and analyzed at the end of study. The animals were divided into different groups, so that the number of animals maintained in each experimental group was 6, namely: (A) Normal control (NC) (B) High fat diet fed (HFD) (C) High fat diet/STZ treated (35 mg/kg) (H + S) (D) High fat diet/STZ treated/Telmisartan treated (10 mg/kg) (H + S + T) (E) High fat diet/STZ treated/PD treated (10 mg/kg) (H + S + PD) (F) High fat diet/STZ treated/Telmisartan treated/PD (H + S + T + PD).

2.3. Assessment of type 2 diabetes and renal function

The blood samples were collected and plasma was analyzed for glucose (PGL), triglycerides (TG), total cholesterol (PTC), blood urea nitrogen (BUN), creatinine (PCr), albumin (PAL) (using commercially available kits, Accurex) as described by Gaikwad et al., 2010 [31] Insulin determination was made by ELISA kit using rat insulin as the standard (Rat ELISA kit, Crystal Chem, USA) [32].

2.4. Assessment of hemodynamic parameters

Systolic blood pressure (SBP) was recorded at end of treatment period in the pre-acclimatized animals, using a tail cuff blood pressure recorder (AD Instruments, Bella Vista, NSW, Australia) [36,37].

2.5. Estimation of lipid peroxide and reduced glutathione levels in kidney

The estimation of oxidative stress was carried out by assessing the lipid peroxide and reduced glutathione levels in the kidney. Briefly, after sacrificing the animals, the kidneys were excised, decapsulated and rinsed with normal saline and weighed. The lipid peroxide level in animal tissues was measured according to method described by Ohkawa et al., 1979. After weighing, kidney tissue was minced properly and the homogenate was prepared in 1 mL of cold phosphate buffered saline (pH 7.4) and centrifuged at 700× *g*. Supernatant was collected and used for estimation of thiobarbituric acid reacting substances (TBARS) by using spectrophotometric method at 532 nm [38]. The reduced glutathione (GSH) content was estimated according to Ellman's method. For reduced glutathione, kidney tissues were homogenized in 10 ml ice-cold homogenizing buffer combined with sulphosalicylic acid and the homogenate was centrifuged. Ellman's reagent was added to the supernatant to produce yellow colour of 5-thio-2-nitrobenzoate-SH. The absorbance was taken at 412 nm [39].

2.6. Histopathological evaluation

Histopathology was performed as per the protocol described previously [32]. Briefly, the kidney tissue was fixed in 10% (v/v) formalin in phosphate buffered saline and embedded in paraffin. 5 μm sections were stained with hematoxylin and eosin (H&E) to study glomerular damage. At least 25 kidney sections from each group (4–5 sections from each animal) were observed for any histological changes. Histopathological images were captured by using Olympus microscope (Model no. BX41, NY, USA). Glomerular damage was assessed using a semi quantitative score by a blinded observer as follows: 0 = no lesion, 1 = <25% damage, 2 = 25–49 % damage, 3 = 50–74% damage, 4 = 75–100% damage, respectively [40]. For the study of deposition of extracellular matrix (collagen), the kidney sections were stained with Picro Sirius Red. The percentage fibrotic area was analysed using Image J software and the data analysis was carried out by using GraphPad 5 Prism software [41].

2.7. Immunohistochemistry

Immunohistochemistry was performed as per the protocol described previously [32,37]. Briefly, kidney sections (5 μm) were taken from paraffin blocks and deparaffinized with xylene, followed by antigen retrieval by heating in citrate buffer (10 mmol/L). The following primary antibodies were used: anti-AT1R, anti-AT2R, anti-ACE2, anti-Ki67 (rabbit, 1:200 dilution; Santa Cruz Biotechnology), anti-Kelch-like ECH-associated protein 1 (Keap1), NF-κB p65, phosphorylated NF-κB p65 (Ser536), phosphorylated IκB-α (Ser32) (rabbit, 1:200 dilution; Cell Signaling Technology) and anti-rabbit Horse Radish Peroxidase conjugated secondary antibody was used, followed by detection with diaminobenzidine (DAB) as a chromogen. The slides were counterstained with hematoxylin, dehydrated with alcohol and xylene, and mounted in DPX (Sigma–Aldrich). All the images were analyzed using Image J software for calculating DAB positive area.

2.8. Statistical analysis

Experimental values were expressed as means ± S.E.M. Statistical comparison between different groups was performed using one way analysis of variance to detect the difference in observations between all groups. If F value was significant then multiple comparisons were done by Tukey test using Prism software (version 5.0; GraphPad, San Diego, CA) for Windows. Data was considered to be statistically significant if $p < 0.05$.

3. Results

3.1. Effect of angiotensin II receptor(s) blockade on plasma biochemical parameters

The development of T2D and its associated renal complications was confirmed by performing plasma biochemical estimations. After 24 weeks of study, HFD fed rats showed a significant rise in the plasma glucose and insulin levels whereas the HFD + STZ treated rats showed severe hyperglycemia, high cholesterol and triglyceride level along with a significant reduction in insulin level as compared to normal control rats (Table 1). These observations show the development of hyperglycemia and insulin resistance in

Table 1
Effect of Ang II receptor(s) blockade on plasma glucose (PGL), triglyceride (TG), total cholesterol (PTC) and insulin (PI) levels.

Group	PGL (mmol/L)	TG (mg/dL)	PTC (mmol/L)	PI (pmol/ml)
Normal control (NC)	6.210 ± 0.231	54.33 ± 1.703	1.190 ± 0.103	3.161 ± 0.243
High fat diet control (HFD)	7.741 ± 0.202*	72.50 ± 1.701*	2.902 ± 0.081*	6.601 ± 0.231*
High fat diet/STZ (35 mg/kg) (H + S)	31.66 ± 1.909$^{*,\#}$	166.7 ± 5.865$^{*,\#}$	15.74 ± 0.710$^{*,\#}$	0.809 ± 0.073$^{*,\#}$
High fat diet/STZ/Telmisartan (10 mg/kg) (H + S + T)	17.06 ± 0.961^{a*}	86.70 ± 4.541^{a*}	6.501 ± 0.436^{a*}	2.051 ± 0.091^{a*}
High fat diet/STZ/PD (10 mg/kg) (H + S + PD)	37.17 ± 1.253^{b*}	174.2 ± 8.423^{b*}	17.08 ± 0.951^{b*}	0.762 ± 0.086^{b*}
High fat diet/STZ/Telmisartan/PD (H + S + T + PD)	29.17 ± 2.151^{c*,b*}	158.7 ± 3.803^{b*,c*}	8.903 ± 0.743^{a*,c*}	1.201 ± 0.303^{b*,c*}

Note: Data represented in means ± SEM (n = 6). (*) p < 0.05 Vs NC; ($^{\#}$) p < 0.05 Vs HFD; (^{a*}) p < 0.05 Vs HFD + STZ; (^{b*}) p < 0.05 Vs HFD + STZ + T, (^{c*}) p < 0.05 Vs HFD + STZ + PD.

HFD fed rats and T2D in HFD + STZ treated rats. The reduced plasma insulin levels in HFD + STZ treated animals were significantly improved by the Telmisartan treatment, whereas PD123319 treatment did not make any difference. However, Telmisartan when administered with PD123319 in HFD + STZ treated animals, elevated the plasma insulin level as compared to that of the PD123319 treated animals. The plasma glucose, triglyceride and total cholesterol levels were markedly reduced by the treatment with Telmisartan whereas the treatment with PD123319 did not alter the values of these parameters, as compared to the HFD + STZ treated rats. The co-administration of AT1R and AT2R antagonists does not show any significant difference in plasma glucose and triglyceride levels as compared to HFD + STZ treated rats but is significantly higher than that of the Telmisartan treated animals (Table 1).

Blood urea nitrogen (BUN) and Plasma Creatinine (PCr), the markers of nephropathy were increased significantly and plasma albumin levels were decreased in HFD + STZ treated animals while only BUN was increased in case of HFD fed rats as compared to normal control rats (Table 2). This indicates the development of renal failure in HFD + STZ treated animals. The treatment with AT1R antagonist, Telmisartan almost normalized the levels of BUN and PCr. It was further noted that PD123319 treatment did not alter the PCr levels but elevated the BUN levels significantly as compared to the HFD + STZ treated rats. The combination of Telmisartan with PD123319 could not alter the BUN levels as compared to the HFD + STZ treated rats. However, the PCr levels were significantly reduced in H + S + T + PD group as compared to the HFD + STZ treated rats (Table 2). The reduced level of albumin was reversed by Telmisartan treatment but it was not regulated by PD123319 treatment. The simultaneous administration of both the antagonists does not show any significant difference from the HFD + STZ treated rats, but the values differ significantly from that of the Telmisartan treated animals (Table 2). These results clearly suggest that the treatment with Telmisartan controls the BUN, PCr as well as PAL levels which denote a significant amelioration of renal failure. The treatment with PD123319 was found to exacerbate only the BUN levels, whereas the co-administration of Telmisartan and PD123319 significantly could not change the levels of PAL as compared to the HFD + STZ treated rats.

3.2. Effect of angiotensin II receptor(s) blockade on morphometric and hemodynamic parameters

To further study the morphological changes associated with the development of diabetic nephropathy, the alterations in body weight (BW), kidney weight (KW) and relative kidney weight [(KW/BW) * 1000] were measured. The high fat diet (HFD) fed rats showed a significantly higher body weight and kidney weight as compared to the normal control rats whereas an increased kidney weight and relative kidney weight along with reduced body weight were seen in HFD + STZ treated rats (Table 3). In HFD + STZ treated rats, AT1R antagonist prevented body weight loss, whereas AT2R antagonist further augmented the weight loss. There was no difference between the body weights of the rats from HFD + STZ and the combination treated groups (Table 3). The significant increase in kidney weight of HFD + STZ treated rats was considerably controlled by Telmisartan treatment, but there was no difference among the kidney weights of PD123319 treated, combination treated rats and T2D rats (Table 3). The relative kidney weight showed a remarkable increase in the HFD + STZ treated rats which was ameliorated by Telmisartan treatment and augmented by PD123319. However, the administration of AT1R and AT2R blockers simultaneously, does not change the morphometric parameters as compared to the HFD + STZ treated rats (Table 3).

The micro and macrovascular complications of diabetes lead to severe hemodynamic changes. HFD fed and HFD + STZ treated rats have shown significantly elevated systolic blood pressure (SBP) when compared with normal pellet fed rats (Table 3). The Ang II mediated vasoconstriction that leads to rise in the blood pressure is known to be enacted through Ang II/AT1R axis [42]. This fact is supported by our results which show a significant decline in systolic blood pressure upon treatment with AT1R antagonist as compared to the HFD + STZ treated rats. The blockade of AT2R resulted into an elevation in SBP but the combined administration

Table 2
Effect of Ang II receptor(s) blockade on markers of renal failure-blood urea nitrogen (BUN), plasma albumin (PAL) and plasma creatinine (PCr).

Group	BUN (mmol/L)	PAL (gm/L)	PCr (mg/dL)
Normal Control (NC)	3.172 ± 0.406	30.33 ± 1.809	1.025 ± 0.175
High fat diet control (HFD)	6.003 ± 0.380*	30.20 ± 2.422	1.706 ± 0.173
High fat diet/STZ (35 mg/kg) (H + S)	9.123 ± 0.313$^{*,\#}$	19.52 ± 1.260$^{*,\#}$	4.212 ± 0.503$^{*,\#}$
High fat diet/STZ/Telmisartan (10 mg/kg) (H + S + T)	4.501 ± 0.403^{a*}	32.03 ± 2.081^{a*}	2.023 ± 0.303^{a*}
High fat diet/STZ/PD (10 mg/kg) (H + S + PD)	12.41 ± 0.732^{a*,b*}	18.06 ± 1.181^{b*}	4.303 ± 0.321^{b*}
High fat diet/STZ/Telmisartan/PD (H + S + T + PD)	8.423 ± 0.552^{b*,c*}	22.33 ± 1.055^{b*}	2.703 ± 0.212^{c*,a*}

Note: Data represented in means ± SEM (n = 6). (*) p < 0.05 Vs NC; ($^{\#}$) p < 0.05 Vs HFD; (^{a*}) p < 0.05 Vs HFD + STZ; (^{b*}) p < 0.05 Vs HFD + STZ + T, (^{c*}) p < 0.05 Vs HFD + STZ + PD.

Table 3
Effect of Ang II receptor(s) blockade on body weight (BW), kidney weight (KW), relative kidney weight [(KW/BW)*1000] and systolic blood pressure (SBP).

Group	Body weight (gm)	Kidney weight (gm)	Relative kidney weight (KW/BW)*1000	SBP (mmHg)
Normal Control (NC)	260.2 ± 4.913	1.512 ± 0.023	5.833 ± 0.120	83.03 ± 3.403
High fat diet control (HFD)	330.3 ± 3.151*	1.933 ± 0.022*	5.853 ± 0.052	103.0 ± 3.505*
High fat diet/STZ (35 mg/kg) (H + S)	185.3 ± 1.620$^{*,\#}$	2.181 ± 0.06$^{*,\#}$	11.71 ± 0.420$^{*,\#}$	125.2 ± 2.430$^{*,\#}$
High fat diet/STZ/Telmisartan (10 mg/kg) (H + S + T)	251.3 ± 2.326^{a*}	1.681 ± 0.033^{a*}	6.690 ± 0.118^{a*}	78.23 ± 4.010^{a*}
High fat diet/STZ/PD (10 mg/kg) (H + S + PD)	145.0 ± 1.768^{a*,b*}	2.281 ± 0.022^{b*}	15.72 ± 0.393^{b*}	142.3 ± 4.210^{a*,b*}
High fat diet/STZ/Telmisartan/PD (H + S + T + PD)	184.2 ± 1.08^{b*,c*}	2.105 ± 0.042^{b*}	10.26 ± 0.224^{b*,c*}	104.3 ± 4.430^{b*,c*}

Note: Data represented in mean ± SEM (n = 6). (*) p < 0.05 Vs NC; ($^{\#}$) p < 0.05 Vs HFD; (^{a*}) p < 0.05 Vs HFD + STZ; (^{b*}) p < 0.05 Vs HFD + STZ + T, (^{c*}) p < 0.05 Vs HFD + STZ + PD.

of the antagonists does not affect the blood pressure significantly, when compared with the disease control rats. The dramatically increased SBP upon PD123319 treatment could be attributed to the increase in the expression of AT1R which occurs as a consequence of AT2R blockade.

3.3. *Effect of angiotensin II receptor(s) blockade on glomerular damage and renal fibrosis*

Glomerular hypertrophy, glomerular basement membrane thickening, mesangial expansion, renal lesions and deposition of extracellular matrix (collagen) are the common features of diabetic nephropathy [43]. For the evaluation of the glomerular damage and fibrosis, Hematoxylin and Eosin (H&E) and Picro Sirius Red (PSR) staining were performed, respectively. The marker for cellular proliferation, Ki67 was checked to study the glomerular hypertrophy.

The HFD fed and HFD + STZ treated rats showed striking features of glomerular damage as compared to the normal control rats: (1) a significantly high glomerular damage as shown by microscopic changes like increase in urinary space, tubular damage, glomerular hypertrophy and vacuolations as compared to normal control, as shown by the H&E staining [Fig. 1(A)] (2) PSR staining showed a markedly high percentage of area with collagen deposition [Fig. 1(B)] (3) The Ki67 expression was found to be significantly upregulated, as shown by the increased percentage of Ki67 positive area [Fig. 1(C)]. These histopathological changes further confirm the development of renal failure in HFD + STZ treated animals. The glomerular damage and cellular proliferation were attenuated by the administration of Telmisartan and further heightened by PD123319 whereas the concurrent usage of both the antagonists significantly reduced the glomerular damage as compared to the PD123319 treated rats [Fig. 1]. The collagen deposition was significantly reduced by the Telmisartan and combination treatments, but it remained unchanged by the exposure to PD123319, when compared to HFD + STZ treated rats. These results confirmed the

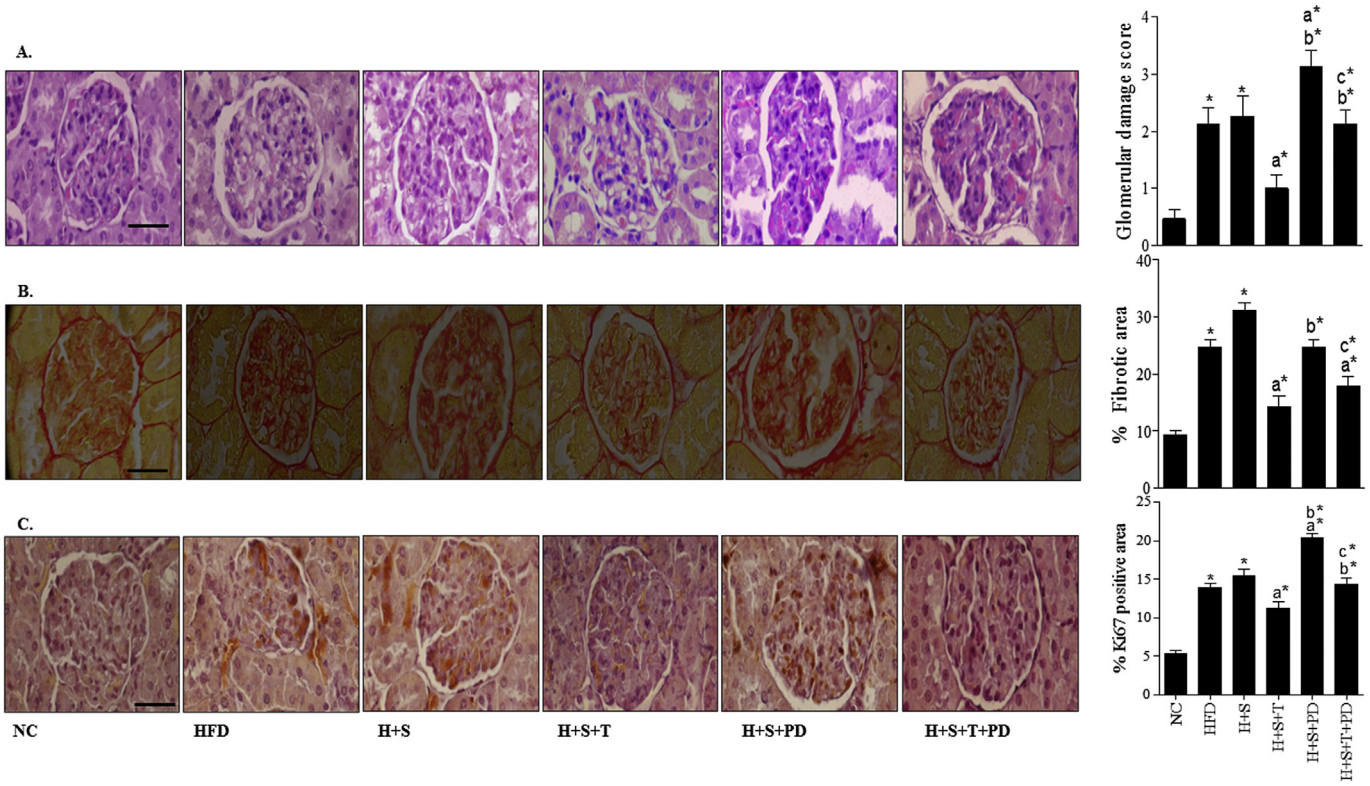

Fig. 1. The blockade of AT1R and AT2R lead to attenuation and aggravation of histopathological features in type 2 diabetic kidney. (A) Hematoxylin/eosin (H&E) staining and quantification of glomerular damage score. (B) Picro-Sirius Red (PSR) staining and quantification of % fibrotic area. (C) The light microscopic images for immunohistochemistry for Ki67 and the quantification of %Ki67 positive area. The scale bar represents 50 μm (original magnification, ×100). Note: Data represented in means ± SEM (n = 6). (*) p < 0.05 Vs NC; ($^{\#}$) p < 0.05 Vs HFD; (^{a*}) p < 0.05 Vs HFD + STZ; (^{b*}) p < 0.05 Vs HFD + STZ + T, (^{c*}) p < 0.05 Vs HFD + STZ + PD.

protective role of AT2R in diabetic nephropathy and pro-fibrotic, pro-hypertrophic face of AT1R and further show the counter-regulatory effect of AT1R and AT2R in diabetic kidney.

3.4. Effect of angiotensin II receptor(s) blockade on renal oxidative stress markers

The oxidative stress is one of the most prominent reasons for induction and development of diabetic nephropathy [43]. Oxidative stress markers like thiobarbituric acid reactive substrate (TBARS) and reduced glutathione (GSH) were measured in the kidneys of all the rats. TBARS was found to have increased remarkably in HFD fed rats and HFD + STZ treated rats [Fig. 2(A)] whereas the reduced glutathione was decreased as compared to the normal rats [Fig. 2(B)]. This heightened load of free radicals was attenuated by treatment with Telmisartan and PD123319 treatment did not change the free radical load. The simultaneous exposure to both the antagonists led to a significant reduction in oxidative stress as compared to PD123319 treated group and HFD + STZ treated group.

To further confirm the increase in the oxidative stress, Keap1 expression was studied using immunohistochemistry technique. The transcription factor NF-E2–related factor 2 (Nrf2), and its negative regulator, Keap1 maintain cellular defence mechanisms by regulating the expression of anti-oxidant genes. It was seen that the expression of Keap1 was increased significantly in both HFD fed rats and HFD + STZ treated rats as compared to that of the normal control. The treatment with Telmisartan attenuated the expression whereas PD123319 treatment further elevated it [Fig. 2(C) and 2(D)]. However, the combination of the antagonists does not change Keap1 levels as compared to the HFD + STZ treated rats. This result shows that the increase in oxidative stress and reduction in antioxidant enzyme level is associated with the increased expression of Keap1, which may be an adaptive change to overcome the cellular stress which is in accordance with the previous reports that demonstrated similar changes in spontaneously hypertensive rat kidney [44]. This result confirms that hyperglycemia induced oxidative stress in T2DN is associated with the activation of Ang II/AT1R axis of RAS.

3.5. AT1R regulates the activation of NF-κB signalling pathway in type 2 diabetic kidney

The NF-κB signalling pathway regulates the production of inflammatory cytokines and chemokines in T2DN [10]. The Ang II receptor subtype that controls this activation in T2D kidney is a mystery even today. In order to identify the Ang II receptor subtype controlling the activation of NF-κB signalling pathway in T2DN, specific receptor antagonists were used and the expression of components of NF-κB signalling pathway: NF-κB p65, phospho NF-κB p65 (Ser536), phospho IκB-α (Ser32) were studied using immunohistochemistry technique (Fig. 3).

Ang II mediated activation of NF-κB signalling pathway is marked by an increase in the phosphorylation of NF-κB p65 and IκB-α, which leads to translocation of the transcription factor to the nuclear compartment so that transcription could be initiated and related genes for inflammation, fibrosis and proliferation could be read out [11]. NF-κB p65, phosphorylated NF- κB p65 and phosphorylated IκB-α subunits were significantly elevated in the kidneys of HFD fed as well as HFD + STZ treated animals [Fig. 3(B) and (C)]. These results show that the expression of the active form was significantly enhanced under type 2 diabetic nephropathy which may be due to the increased oxidative stress. AT1R antagonist treatment significantly reduced NFκB p65, phosphorylated NFκB

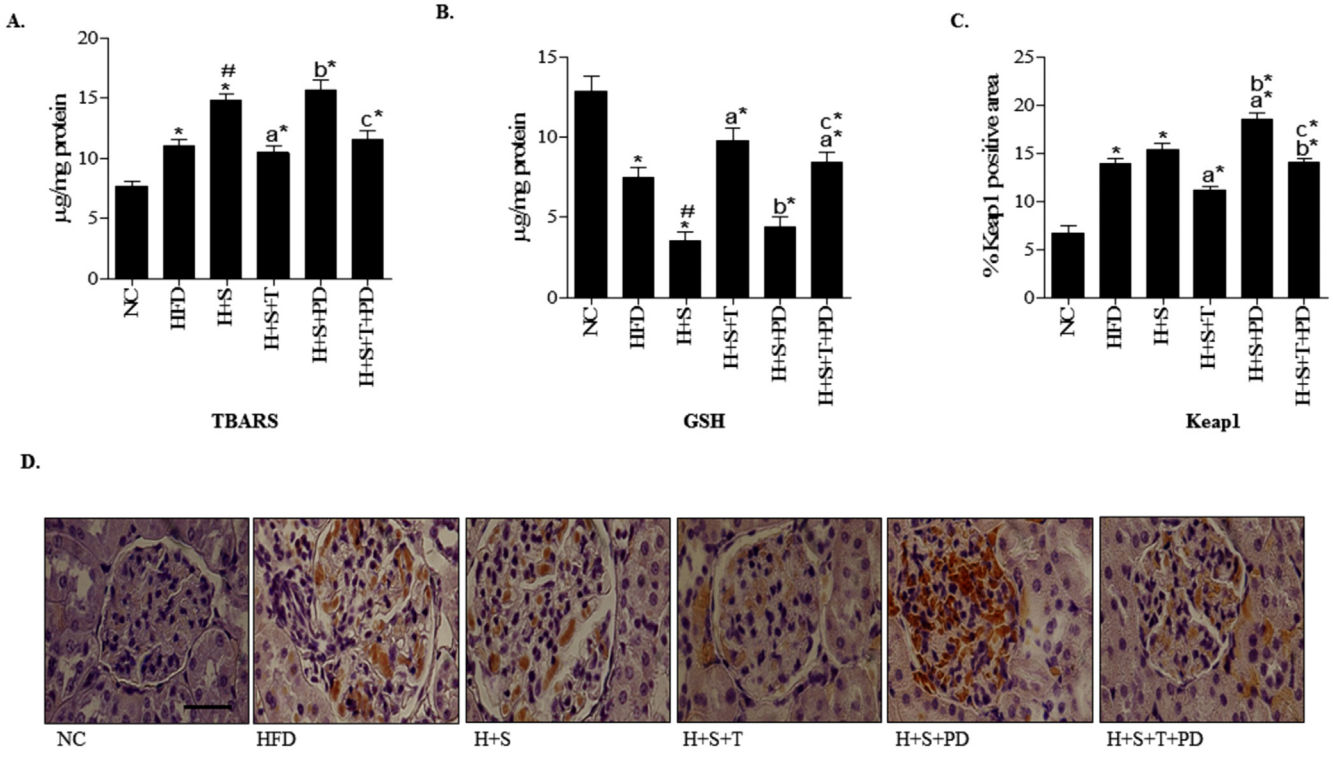

Fig. 2. Blockade of AT1R leads to a reduction in oxidative stress. Evaluation of oxidative stress markers (A) TBARS and (B) GSH. (D) The light microscopic pictures illustrating the immunostaining for Keap1 and (C) quantification of % Keap1 positive area in the kidney sections. The scale bar represents 50 μm (original magnification, ×100). Note: Data represented in means ± SEM (n = 6). (*) $p < 0.05$ Vs NC; (#) $p < 0.05$ Vs HFD; (a*) $p < 0.05$ Vs HFD + STZ; (b*) $p < 0.05$ Vs HFD + STZ + T, (c*) $p < 0.05$ Vs HFD + STZ + PD.

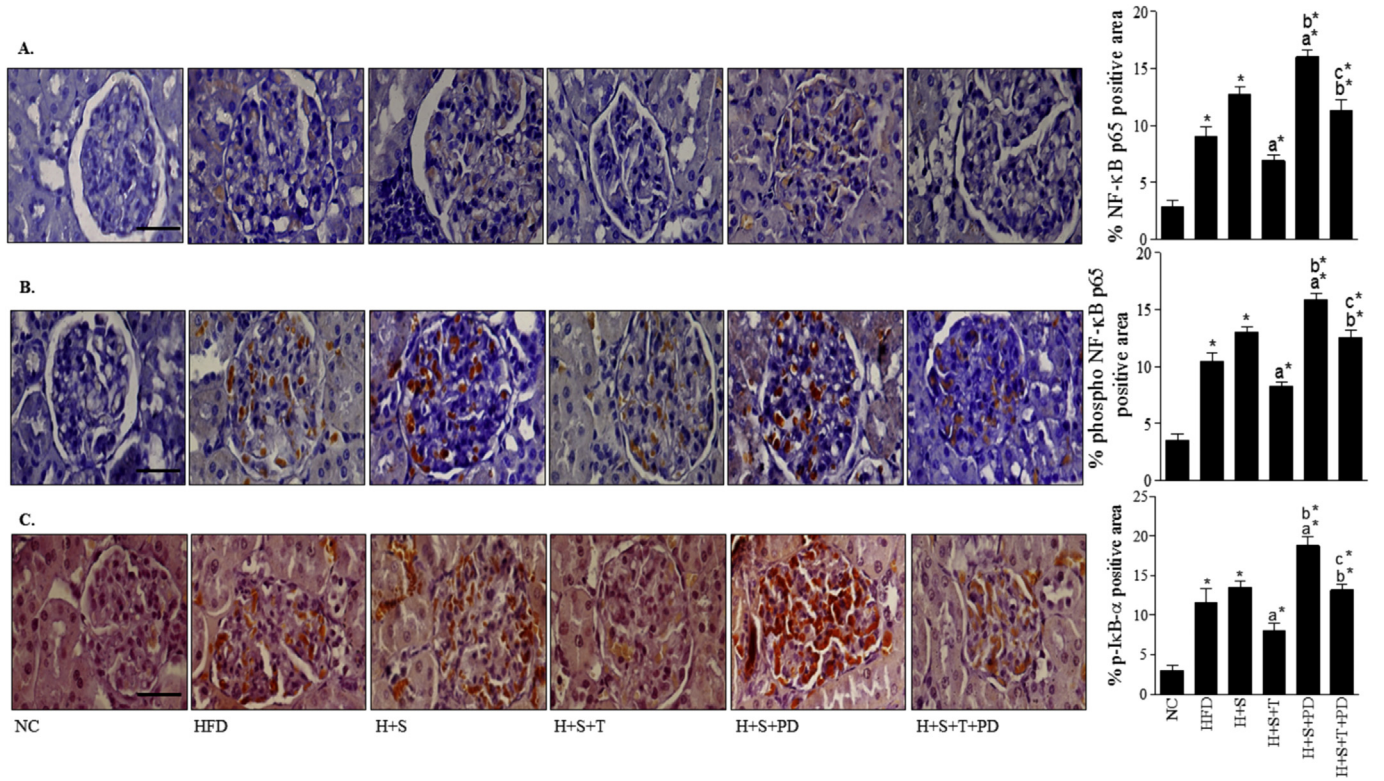

Fig. 3. AT1R antagonist reduces the activation of NF-κB signalling pathway in type 2 diabetic kidney. (A) The light microscopic pictures illustrating the immunostaining for NF-κB and quantification of % NF-κB positive area in the kidney sections. (B) The light microscopic pictures illustrating the immunostaining for phospho NF-κB p65 and quantification of % phospho NF-κB p65 positive area in the kidney sections. (C) The light microscopic pictures illustrating the immunostaining for phospho-IκB-α and quantification of % phospho-IκB-α positive area in the kidney sections. The scale bar represents 50 μm (original magnification, ×100). Note: Data represented in means ± SEM (n = 6). (*) $p < 0.05$ Vs NC; ($^{\#}$) $p < 0.05$ Vs HFD; (^{a*}) $p < 0.05$ Vs HFD + STZ; (^{b*}) $p < 0.05$ Vs HFD + STZ + T, (^{c*}) $p < 0.05$ Vs HFD + STZ + PD.

p65 and phosphorylated IκB-α level in diabetic kidney. PD123319 severely augmented the expression of the NF-κB p65, phosphorylated NF- κB p65 and IκB-α subunits. The co-administration of the two antagonists does not change the expression level as compared to the HFD + STZ treated rats but was significantly higher than Telmisartan treated group [Fig. 3(B) and (C)]. Thus, it can be concluded that the activation of NF-κB under T2DN was found to be orchestrated by AT1R. However, since the blockade of AT2R showed a significant increase in the expression of phosphorylated forms, it may be concluded that AT2R also plays a significant role in controlling this signalling pathway.

3.6. Differential regulation of renal ACE2 expression by Ang II receptor blockade

Role of Ang II receptor subtypes in type 2 diabetic kidney in regulating NF-κB and ACE signalling still remain elusive. This was addressed by performing immunohistochemistry staining of the kidney sections from each group for the receptor subtypes- AT1R and AT2R and also for ACE2. As per our results, the AT1R, which has been known to show the detrimental effects of Ang II, was drastically upregulated in HFD fed and HFD + STZ treated control rats as compared to NC. This elevation could be significantly reduced by AT1R antagonist, Telmisartan and further augmented by AT2R antagonist, PD123319 [Fig. 4 (A)]. AT2R is scarcely expressed in the normal adult tissue but upregulated by pathological conditions, like hypertension, cardiovascular complications, renal damage [45] and diabetes [46–48]. Our results demonstrated that AT2R is significantly upregulated in both HFD fed and HFD + STZ treated rats [Fig. 4 (B)]. The expression of AT2R receptor was further enhanced by the administration of the AT1R antagonist as compared with the HFD + STZ treated rats, which was in accordance with the previously reported data, which suggest that the blockade of AT1R leads to upregulation of AT2R [49]. The expression of AT2R was reduced significantly by the exposure to PD123319. On the contrary, the simultaneous exposure to both the antagonists, increased the expression of AT2R in type 2 diabetic kidney as compared to PD123319 treated rats.

Another question that still remained unanswered was the role of ACE2 in the face of blockade of Ang II receptors in type 2 diabetic kidney because ACE2 has emerged as one of the potential targets for management of cardiovascular and renal complications and thus understanding its regulation in T2DN may help us evolve novel strategies to combat the disease [50]. Thus, in order to check the effect of Ang II receptor blockade on expression of ACE2, immunohistochemistry staining was carried out. The elevated expression of ACE2 in HFD fed and HFD + STZ treated rats was normalized by Telmisartan treatment and further augmented by PD123319 administration [Fig. 4 (C)]. The simultaneous administration of both antagonists shows a reduction in the ACE2 expression when compared to that of the PD123319 treatment but does not differ remarkably from the type 2 diabetic kidney. This shows that the ACE2 expression is enhanced by diabetic nephropathy and the blockade of AT2R further signals the increase in its expression [Fig. 4(C)]. This elevation of expression of ACE2 may be the feedback mechanism of the cells to combat the aggravated pathological conditions. These results indicate that ACE2 expression gets augmented by treatment with AT2R antagonist and reduced by AT1R treatment, thus ACE2 expression could be inversely related with AT2R activity and positively correlated with the AT1R.

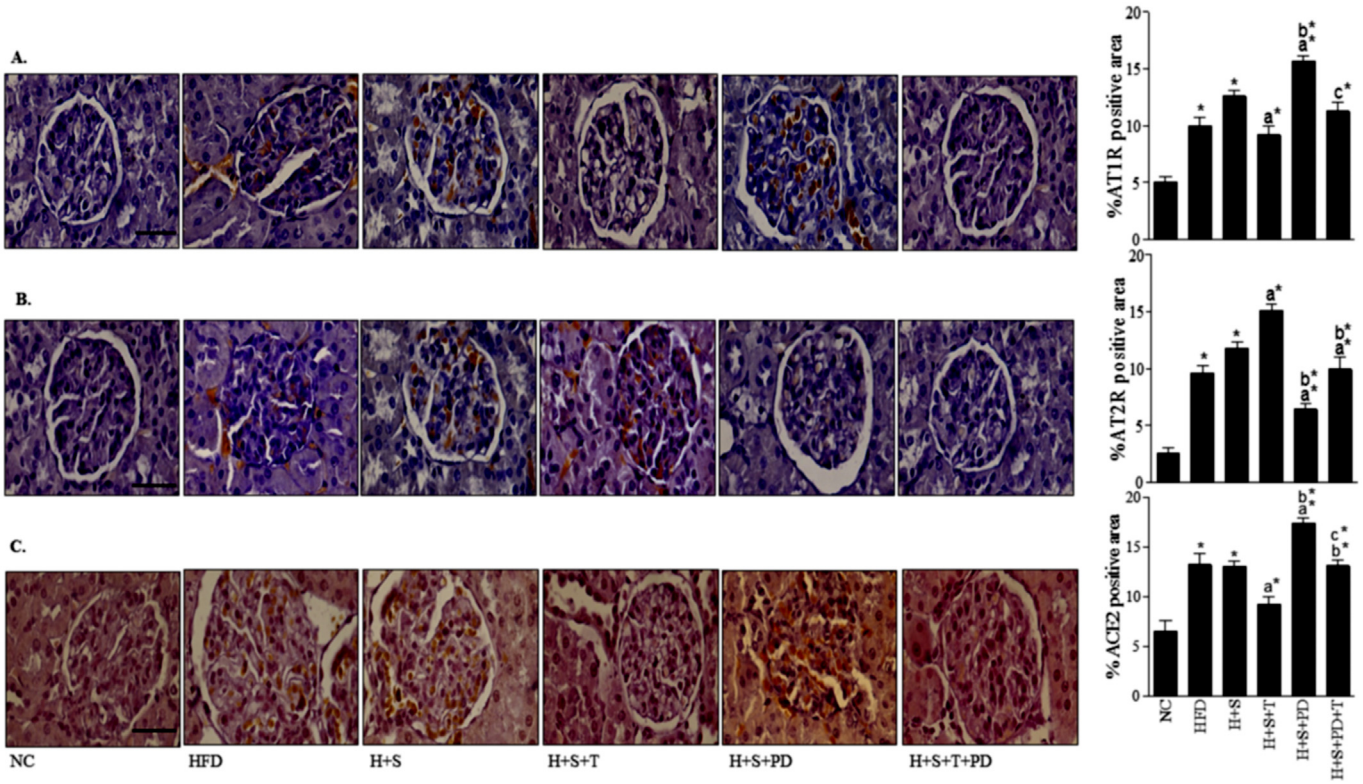

Fig. 4. Blockade of AT2R increases the ACE2 expression in type 2 diabetic kidney (A) The light microscopic pictures illustrating the immunostaining for AT1R and quantification of % AT1R positive area in the kidney sections. (B) The light microscopic pictures illustrating the immunostaining for AT2R and quantification of % AT2R positive area in the kidney sections. (C) The light microscopic pictures illustrating the immunostaining for ACE2 and quantification of % ACE2 positive area in the kidney sections. The scale bar represents 50 μm (original magnification, ×100). Note: Data represented in means ± SEM (n = 6). (*) $p < 0.05$ Vs NC; ($^{\#}$) $p < 0.05$ Vs HFD; (^{a*}) $p < 0.05$ Vs HFD + STZ; (^{b*}) $p < 0.05$ Vs HFD + STZ + T, (^{c*}) $p < 0.05$ Vs HFD + STZ + PD.

4. Discussion

In the present study, the HFD + STZ treated rats demonstrated typical characteristics of T2D, such as hyperglycaemia, elevated plasma triglyceride and total cholesterol levels. The progression of renal damage was confirmed by the increased blood urea nitrogen and serum creatinine, and reduced plasma albumin which are taken as an index of altered glomerular filtration rate in diabetic nephropathy [51]. The renal tissue damage and collagen deposition were examined by histopathological studies, including hematoxylin and eosin staining and Picro Sirius Red staining which showed an increased glomerular damage and extracellular matrix accumulation in the kidney. The administration of AT1R and AT2R antagonists has shown opposite effects by ameliorating and deteriorating the pathological features of T2DN, respectively. The co-administration of both the antagonists minorly attenuates these pathological features but does not abolish them, which confirms the counter-regulatory biological effects elicited by the two receptor subtypes.

The RAS system is intricately associated with maintenance of hemodynamic and non-hemodynamic parameters in the physiological as well as pathological conditions and its role in secondary microvascular and macrovascular complications of diabetes is well documented [52]. T2D has been known to activate the RAS pathway and increase the production of Ang II which in turn acts on the receptors, AT1R and AT2R to elicit the further response. AT1R activates the NOX and acts in pro-oxidative stress manner whereas the AT2R activation leads to the anti-oxidative stress response. The increased oxidative stress leads to a depletion of the anti-oxidant enzymes in the cells which leads to an augmented nuclear translocation of Nrf2 and NF-κB, and up-regulation of ACE2 expression (Fig. 5). The role of Ang II receptor subtypes in the regulation of ACE2 expression has largely been explored. For instance, it was demonstrated that under hypertensive nephropathy condition, AT1R mediated the Ang II induced up-regulation of ACE and down regulation of ACE2 expression [53]. Another study showed that the increase in the renal ACE2 activity in obese Zucker rats occurred due to chronic treatment of AT2R agonist [20]. However, the relation of Ang II receptor subtypes and ACE2, the protective axis in progression of T2DN remains largely obscure. In an attempt to find the missing link in the existing research, we tried to explore the impact of AT1R and AT2R blockade on ACE2 expression. ACE2, a monocarboxypeptidase degrading angiotensin Ang II to Ang 1–7 is highly expressed within the kidneys. It is largely localized in tubular epithelial cells and less prominently in glomerular epithelial cells and in the renal vasculature [19]. ACE2 has been reported to possess renoprotective activity owing to its anti-oxidant, anti-fibrotic and anti-inflammatory properties which help it act in a counter-regulatory manner to that of the ACE/Ang II/AT1R axis [19,42]. The elevation of ACE2 expression in diabetic kidney has been reported in db/db mice as well as in STZ induced models of diabetes [54–56]. The role of ACE2 in diabetic nephropathy has been studied in the 8-week-old db/db mice, a model of early T2D which showed an elevation in the ACE2 expression with concomitant reduction in ACE expression in both glomeruli and cortex [57]. A recent study performed by Mori and colleagues demonstrated that renal ACE2 is elevated in 5 months old db/db mice, an established model for diabetic nephropathy, which was attributed to the reduction in expression of SIRT1 (Silent Information Regulator T1), an NAD-dependent protein deacetylase [4].

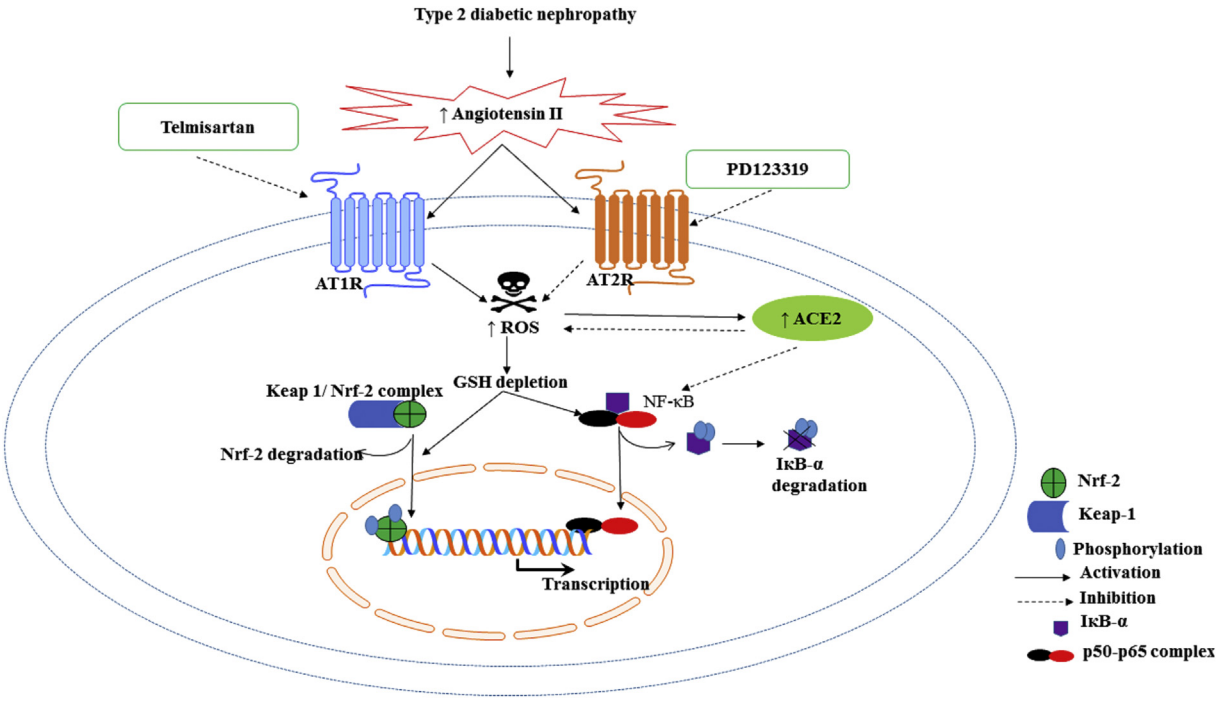

Fig. 5. Hypothesis: Differential regulation of ACE2 and NF-κB by Ang II receptor subtypes. The activation of RAS pathway by T2D is known to increase the production of Ang II which elicits its actions by acting on AT1 and AT2 receptors. AT1 increases the oxidative stress and AT2 reduces it. The role of these receptor subtypes in activation of NF-κB under T2DN is not yet known. The Ang II gets converted to Ang 1–7 due to enzymatic action of ACE2. The effect of blockade of Ang II receptors on ACE2 activity in T2DN is also not known, till date. The study aims to determine these two missing links by using the specific receptor antagonists: AT1 receptor antagonist-Telmisartan and AT2 receptor antagonist-PD123319.

SIRT1 binds to the promoter sites of ACE2 and leads to an increment in the production of Ang 1–7 [58]. Thus, the probable reasons for ACE2 overexpression may be (1) the reduction in SIRT1 activity (2) compensatory mechanism which leads to increased ACE2 expression so as to counterbalance Ang II and increase the Ang1–7, which has a protective role in diabetic condition. In the current study, administration of PD123319 leads to aggravated pathological features and oxidative stress compared to HFD + STZ treated animal. The elevation of ACE2 expression in AT2R antagonist treated type 2 diabetic kidney may be attributed to the compensatory mechanism seeking to reduce Ang II effect because AT2R blockade was found to aggravate the free radical stress and other biochemical parameters.

Hyperglycaemia induced oxidative stress enhances RAS activity which in turn increases AT1R mediated NOX cascade activation [59,60]. The oxidative stress was assessed by estimating the thiobarbituric acid reactive substrate (TBARS) and reduced glutathione (GSH) in the renal tissue. The Keap1-Nrf2 pathway, an important regulator of cytoprotective responses to endogenous and exogenous stresses caused by reactive oxygen species and electrophiles [61]. In the current study, the alteration in Keap1 expression was assessed by immunohistochemistry and it was found to be elevated in HFD + STZ treated rats as compared to the normal control. The expression of this oxidative stress marker was found to be attenuated by the AT1R antagonist and augmented by AT2R antagonist, thus indicating a rise in oxidative stress due to blockade of the protective axis of RAS, ie. AT2 receptor. A series of reactions are triggered by a high oxidative stress condition, which renders Keap1 unable to revert to protein binding conformation. This alteration in its conformation abrogates its ability to bind with IKK-β, thus promoting the activation of NF-κB, which in turn increases the pace of inflammatory cascade [62].

Various *in vitro* and *in vivo* studies show that the activation of NF-κB occurs through either AT1R or AT2R or both depending upon the tissue. The mesangial cells involve both AT1R and AT2R [13], tubuloepithelial cells involve AT1R [14], endothelial cells involve AT2R, and vascular smooth muscle cells embroil both AT1R and AT2R to activate the transcription factor [12], NF-κB, when exposed to Ang II. In unilateral ureteral obstruction model of renal injury in mice, it was demonstrated that Ang II, *via* AT1 and AT2 receptors and NF-κB pathway, participates in the regulation of renal monocyte recruitment [17]. The second aim of the study was to delineate the pathway through which NF-κB is being activated under T2DN. Based on the above observations, we thought that an understanding of the receptor subtype through which Ang II acts in the kidney to steer ahead the renal failure in type 2 diabetic rats is of immense importance, so that the targeting of drugs to treat nephropathy could be decided. The activation of NF-κB was assessed by using immunohistopathology. The drastically increased NF-κB activation in diabetic nephropathy was partially attenuated by exposure to AT1R antagonist and further promoted by PD123319. The co-administration did not lead to any significant alteration in the expression of phosphorylated NF-κB p65 and IκB-α expression as compared to that of the HFD + STZ treated rats. This result suggests that the AT1R and AT2R act in a counter-regulatory manner to control the NF-κB mediated inflammatory pathway. Thus, blockade of AT1R and not AT2R is crucial to arrest the NF-κB mediated inflammatory cascade and control the progression of renal failure. The augmentation of activation of NF-κB by PD123319 treatment in HFD + STZ treated rats shows that AT2R may play a significant role in regulation of the inflammatory cascade.

This study delineates the role of Ang II receptor subtypes in the regulation of ACE2 and NF-κB in T2DN. Both NF-κB and ACE2 have been found to be differentially regulated by AT1R and AT2R, because AT1R blockade has been correlated with reduced activation of NF-κB signalling pathway and attenuation of ACE2 expression, whereas AT2R blockade leads to activation of the inflammatory cascade mediated by NF-κB, along with accentuated ACE2 expression. The elevated ACE2 expression could be linked to

the increased inflammation and oxidative stress and impaired antioxidant enzymes in the kidney during T2D, which is exacerbated by AT2R blockade. Therefore, the current study improves our understanding of the relationship between Ang II receptor subtypes, NF-κB mediated inflammatory cascade and ACE2 expression type 2 diabetic kidney. This understanding may help the scientists to design novel strategies to treat diabetic nephropathy in a more efficient manner.

Conflict of interests

We declare that we have no conflict of interest.

Author contribution

All the authors contributed equally to this work. A.B.G. conceived the idea and designed the experiments. A.P., S.K.G., V.M. and A.K. conducted the experiments and finished the paper writing.

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Research paper

H2AK119 monoubiquitination regulates Angiotensin II receptor mediated macrophage infiltration and renal fibrosis in type 2 diabetic rats

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ABSTRACT

Monocyte chemoattractant protein (MCP-1) and transforming growth factor-β (TGF-β1)-markers of inflammation and fibrosis, are central to type 2 diabetic nephropathy (T2DN) progression. The epigenetic basis of their expression has also been explored to certain extent. H2A lysine 119 monoubiquitination (H2AK119Ub), a repressive chromatin mark regulates progression of hyperglycaemia induced fibrosis in glomerular mesangial cells. However, how H2AK119Ub affects the expression of MCP-1 and TGF-β1 and their regulation by Angiotensin II receptor subtypes remains unknown. In the current study, we aimed to study the effect of Angiotensin II receptors' blockade on the macrophage infiltration and histone modifications occurring at the promoter region of *Mcp1* and *Tgfb1*in high fat diet fed and low dose streptozotocin treated male Wistar rats. Hereby, we present the first report delineating a distinct link between H2AK119Ub and macrophage infiltration and fibrosis i.e. the enrichment of H2AUb at *Mcp1* and *Tgfb1* promoter region was found to reduce drastically in the T2DN which could be significantly reversed by Telmisartan and was further elevated by PD123319. We could conclude that the Angiotensin II mediated macrophage infiltration in T2DN is regulated at least partially by H2AK119Ub through both AT1 and AT2 receptors, which to the best of our knowledge, presents the first report for the regulation of *Mcp1* by H2AK119Ub. Thus an approach targeting AT1R blockade and AT2R activation accompanied by an epigenetic modulator may be more suitable to ameliorate the macrophage infiltration and fibrosis associated with T2DN.

1. Introduction

Type 2 diabetic nephropathy (T2DN), a chronic inflammatory condition involves an increase in macrophage infiltration which culminates into elevated production of cytokines and chemokines including CC-chemokine ligand (CCL) 2, CCL3, CCL4, CCL5 and CXC-chemokine ligand (CXCL) 2 [1,2]. CCL2, also known as monocyte chemoattractant protein-1 (MCP-1) recruits and activates monocytes/macrophages and triggers a series of reactions upregulating inflammation and fibrosis in diabetic kidney. It is known that macrophage infiltration is regulated by the renin angiotensin system through NF-κB signalling pathway and its expression has been reported to be controlled by different Angiotensin II receptor subtypes in different tissues under different conditions [3,4]. However, which of the Angiotensin II receptor subtypes is involved in its orchestration under T2DN, remains mysterious. Renal fibrosis triggered by elevated MCP-1 involves transforming growth factor-β1 (TGF-β1), which prompts the onset of reactions mediated by SMAD proteins leading to an accelerated production of extracellular matrix (ECM) [5]. Despite being biomarkers for T2DN, the epigenetic mechanisms regulating the expression of MCP-1 and TGF-β in T2DN are incompletely understood.

A myriad of signals and inputs modulate the covalent modifications of the histones to facilitate or hinder the binding of promoters to the binding site by remodelling the chromatin structure. These posttranslational histone modifications (PTHM) occur generally at the N terminal amino acid functional groups in the tail region of the histones [6–8]. The well-known PTHMs are

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Abbreviations

RAS	Renin Angiotensin System
T2D	Type 2 Diabetes
T2DN	Type 2 diabetic nephropathy
AT1R and AT2R	Angiotensin II type-1 and type-2 Receptor
STZ	Streptozotocin
HFD	High Fat Diet
NPD	Normal Pellet Diet
ECL	Enhanced Chemiluminescence
SBP	Systolic blood pressure
MCP-1	Monocyte chemoattractant protein
TGF-β1	Transforming growth factor-β
H2AK119Ub	H2A lysine 119 monoubiqutination
H2BK120Ub	H2B lysine 120 monoubiqutination
CCL	CC-chemokine ligand
ECM	Extracellular matrix

acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP-ribosylation, deimination, proline isomerization [9,10]. The part played by histone H3 acetylation, methylation and phosphorylation in alteration of gene expression in diabetic kidney has been studied [11–14]. However, histone H2A ubiquitination has not garnered much attention despite its prominent role in numerous biological pathways.

The process of addition of ubiquitin moiety, ubiquitination decides the fate of various proteins in the cells, by either directing them towards proteasomal degradation or participation in several cell signalling pathways [15]. The first protein which was found to be ubiquitinated was H2A and it was found that about 5–15% of total H2A was in ubiquitinated state in a normal mammalian cell. Though the functions of H2A ubiquitination remain unclear, abundant instances indicate its role in gene repression and deoxyribonucleic acid (DNA) damage repair and cell cycle progression [16]. Recent studies showed that hyperglycaemic state promoted monoubiquitination of histone H2A lysine 119 and reduced the monoubiquitination of histone H2B lysine 120 in glomerular mesangial cells, which in turn elevated the TGF-β mediated pathways leading to fibrotic gene expression. These alterations occurring in hyperglycaemic state were significantly controlled by exposure to a 26s proteasome inhibitor, MG132 which could partially be attributed to its ability to limit NF-κB mediated inflammatory signalling through inhibiting the IκBα sumoylation and ubiquitination [17,18]. Thus, H2AK119Ub may orchestrate the gene expression in diabetic kidney disease and it is imperative for us to understand its role in fibrosis and macrophage infiltration and elucidate the detailed mechanism linking regulators of H2A ubiquitination with inflammatory signalling in T2DN.

In our previous study, we have found that Telmisartan, an Angiotensin II type 1 receptor (AT1R) antagonist could reverse the pathological features associated with T2DN to a certain extent, whereas the PD123319, an AT2R antagonist aggravated the diseased state. Also, it was found that blockade of AT1R and AT2R leads to repression and activation of the NF-κB mediated inflammatory cascade, respectively [19]. The current study is aimed at bridging the gaps in the existing research by (i) dissecting the relationship between Angiotensin II receptor subtypes and macrophage infiltration (ii) delineating the role Angiotensin II receptor in regulation of ubiquitin proteasome system involved in H2AK119 monoubiquitination (iii) elucidating the role of epigenetics in regulation of MCP-1 and TGF-β1 expression. Our results provide a novel information about the chromatin state at key pathologic genes involved in regulation of macrophage infiltration and fibrosis in diabetic nephropathy.

2. Methodology

2.1. Chemicals

PD 123319 was procured from Tocris Biosciences, (Bristol, UK). Streptozotocin (STZ) was procured from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against MCP-1, SET7/9 and fibronectin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and rest of the antibodies were purchased from Cell Signalling Technology (Danvers, MA, USA). For biochemical estimation, spectrophotometric kits purchased from Accurex (Accurex Biomedical Pvt. Ltd., Mumbai, India) and ultra-sensitive rat insulin kit obtained from Crystal Chem (Downer's Grove, IL, USA) were used. Enhanced chemiluminescence (ECL) reagent was purchased from Thermo Fisher Scientific (Waltham, MA, USA). All the other chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA), unless otherwise mentioned.

2.2. Animal studies

Adult male Wistar rats (160–180 g), procured from the Central Animal Facility of the institute, Birla Institute of Technology and Science Pilani were maintained under standard environmental conditions and were provided with feed and water ad libitum. All the animals were fed on normal pellet diet (NPD) one week prior to the start of experimental protocol which is in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Social Justice and Environment, Government of India based on the guidelines of Institute of Laboratory Animal Resources, (Washington, DC, U.S.A.). A prior permission was sought from the institutional animal ethics committee, BITS Pilani, for conducting the study (IAEC/RES/17/04/Rev-2/19/36).

The animal model for T2DN was developed using high fat diet (HFD) and low dose STZ (35 mg/kg, *i.p.*) and the normal control was treated with vehicle (0.5% sodium carboxy methyl cellulose) as described previously [19,20]. The induction of T2D was confirmed by analyzing the plasma glucose levels after 48 h of induction. After induction of diabetes, the rats were fed HFD for 24 weeks for development of renal failure. The animals were divided into different groups, so that the number of animals maintained in each experimental group was 6, namely: (A) Normal control (NC) (B) High fat diet fed (HFD) (C) High fat diet/low dose STZ treated (35 mg/kg) (H + S) (D) High fat diet/STZ treated/Telmisartan treated (10 mg/kg, *p.o.*) (H + S + T) (E) High fat diet/STZ treated/PD123319 treated (10 mg/kg, *s.c.*) (H + S + PD) (F) High fat diet/STZ treated/Telmisartan treated/PD123319 (H + S + T + PD) [19,21]. The drug treatment was given for 14 days, after which the biochemical studies were carried out.

2.3. Assessment of type 2 diabetes and renal function

The blood samples were collected and plasma was analyzed for glucose (PGL), triglycerides (TG), total cholesterol (PTC), blood urea nitrogen (BUN), creatinine (PCr), albumin (PAL) (using commercially available kits, Accurex) as described earlier [19]. Insulin determination was made by ELISA kit using rat insulin as standard (Rat ELISA kit, Crystal Chem, USA). Systolic blood pressure (SBP) was recorded at end of treatment period in the pre-acclimatized animals, using a tail cuff blood pressure recorder (AD Instruments, Bella Vista, NSW, Australia) [19,20].

2.4. Immunohistochemistry

Immunohistochemistry was performed as per the protocol described previously [20,22]. Briefly, kidney sections (5 μm) were taken from paraffin blocks and deparaffinised with xylene, followed by antigen retrieval by heating in citrate buffer (10 mmol/L). The specific primary antibodies: anti-MCP-1, anti-fibronectin, anti-SET7/9 (rabbit, 1:200 dilution; Santa Cruz Biotechnology, CA, USA), anti-TGF-β (rabbit, 1:200 dilution; Cell Signalling Technology, Danvers, USA) and anti-rabbit Horse Radish Peroxidase (HRP) conjugated secondary antibody were used, followed by detection with diaminobenzidine (DAB) as a chromogen. The slides were counterstained with haematoxylin, dehydrated with alcohol and xylene, and mounted in DPX. At least 25 kidney sections from each group (4–5 sections from each animal) were observed and analyzed using ImageJ software for calculating percentage of DAB positive area.

2.5. Histone isolation and western blotting

Western blotting and histone isolation was performed as described previously [19]. Briefly, kidney tissues were dissected manually, and histones were isolated. Immunoblot analysis was performed by using rabbit monoclonal antibodies against: acetylation – H3K9Ac, H3K14Ac, H3K28Ac and H3K18Ac; histone H2A monoubiquitination – H2AK119Ub and total H3; all antibodies were used in 1:1000 (v/v) dilution. As secondary, anti-rabbit IgG, HRP-linked antibody was used in 1:20,000 (v/v) dilution (Cell Signalling Technology, Danvers, MA, USA). Proteins were detected by the ECL system and ECL Hyperfilm. Immunoblots were quantified by densitometric analysis using ImageJ software and the exposures were in linear dynamic range, each modification was normalized by respective total H3 blot, then data analysis was performed by using Prism software (version 5.0; GraphPad, San Diego, CA, USA) and results were expressed as fold over NC.

2.6. RNA isolation and real time-polymerase chain reaction

RNA was isolated from kidneys by using commercially available kit (Ambion™ PureLink™ RNA Mini Kit, Life Technologies, USA). 5 μg of RNA was taken and incubated with 1 μl (2U) of recombinant DNase1 for 30 min at 37 °C [Ambion™ Recombinant DNase I (RNase-free), Life Technologies, USA] to remove the single or/and double stranded DNA, chromatin and RNA-DNA hybrids present in the sample. Further, DNase1 was inactivated by heating the samples at 75 °C along with 5 mM of EDTA. cDNA was synthesized by using cDNA kit (GeneSure™ First Strand cDNA Synthesis Kit, Puregene, Genetix brand, USA). The samples were incubated at 25 °C for 5 min, 42 °C for 60 min followed by inactivation at 70 °C for 5 min. Quantitative real-time polymerase chain reaction of the samples was performed as per the protocol described by Goru et al. [23] on Light Cycler® 96 Real-Time PCR System using the Fast Start Essential DNA Green Master and results were analyzed by Light Cycler® Software (Roche, Mannheim, Germany). Primers for *Rnf2, Rnf168, Usp16, Usp21, Col1a1, Tgfb1* and *Mcp1* were designed and obtained from Eurofins (Mumbai, India) (Table 1). After amplification, a melting curve analysis was performed to verify the specificity of the reaction. Levels of mRNA were normalized to their respective *18s* contents. Experiments were carried out in triplicate ($n = 3$) for each sample and results were expressed as fold change over respective control.

2.7. Chromatin-immunoprecipitation (ChIP) assay

ChIP assay was performed as per the protocol described by Goru et al. [23] by using MAGnify™ Chromatin Immunoprecipitation System (Thermo Fisher Scientific, CA, USA) according to manufacturer's guidelines. Briefly, kidneys were chopped into small pieces, re-suspended in phosphate buffered saline (PBS) and cross linked with 1% (v/v) formaldehyde for 10 min. Cross linking reaction was stopped by adding 0.125 M glycine, washed thrice with PBS containing protease inhibitors and lysed in SDS lysis buffer. Chromatin was sonicated for 10 s and the lysate was allowed to cool for 60s over ice. This procedure was repeated for six cycles to obtain chromatin size of 0.5–1 kb. Lysates were incubated for 2 h with H2AK119Ub antibody. Before the addition of antibody, input samples were removed from the lysate and stored at −20 °C until extraction. Following incubation with antibody, protein-DNA complexes were eluted, and the cross-links were reversed using cross linking buffer. The DNA obtained was purified using magnetic beads. ChIP enriched DNA samples and input DNA samples were analysed by quantitative PCR with SYBR reagent in Light cycler 96 Real-time PCR machine (Roche, Mannheim, Germany) using promoter specific forward and reverse primers for *Mcp1* and *Tgfb1* (Table 1) (Eurofins, Mumbai, India). *Anti*-IgG antibody was used as negative control for ChIP experiment. Results were expressed as fold change over control rats.

Table 1
Primers list for qRT-PCR and ChIP experiments.

Gene Name	Primer sequence For qRT-PCR	Accession ID
Tgfb1	Forward 5′-CTGCTGACCCCCACTGATAC-3′ Reverse 5′-AGCCCTGTATTCCGTCTCCT-3′	NM_021578.2
Col1a1	Forward 5′-TGGCAACCTCAAGAAGTCCC-3′ Reverse 5′-ACAAGCGTGCTGTAGGTGAA-3′	NM_053304.1
Mcp1	Forward 5′-GTCTCAGCCAGATGCAGTTA-3′ Reverse 5′-CCTTATTGGGGTCAGCACAG-3′	NM_031530.1
Rnf2	Forward 5′-ACAGCGCACAGACCAGATACA-3′ Reverse 5′-AGACCCCACCACACCACTTG-3′	NM_001025667.1
Rnf168	Forward 5′-CCACACGCTCTGTAACCCAT-3′ Reverse 5′-CTGGCTGGTACTCATCAACGAT	NM_001127597.2
Usp16	Forward 5′-GCCGTCTCACCGGATTGTA-3′ Reverse 5′-CCCCTTTGTTCGTTTCTTTCCC-3′	NM_001100501.1
Usp21	Forward 5′-TGGAGCGAGAAGACAGCAAG-3′ Reverse 5′-CGGTCACATACTGGGGCATT-3′	NM_001127638.1
Col1a1	Forward 5′-TGGCAACCTCAAGAAGTCCC-3′ Reverse 5′-ACAAGCGTGCTGTAGGTGAA-3′	NM_053304.1
Primer sequence For ChIP-qRT-PCR		
Mcp1	Forward 5′-ACCAAGGACTCAGTGGACTA-3′ Reverse 5′-AGAGGAAGTGGCCAAGAAAC-3′	NM_031530.1
Tgfb1	Forward 5′-TTCGCGCTCTCCGAAGTT-3′ Reverse 5′-CGGGCGTCAGCACTAGAA-3′	NM_021578.2

2.8. Statistical analysis

Experimental values were expressed as means ± S.E.M. Statistical comparison between different groups was performed using one way analysis of variance to detect the difference in observations between all groups. If F value was significant then multiple comparisons were done by Tukey test using Prism software (version 5.0; GraphPad, San Diego, CA) for Windows. Data was considered to be statistically significant if $p<0.05$.

3. Results

3.1. Role of Angiotensin II receptors in development of renal fibrosis in type 2 diabetic rats

Diabetic nephropathy is a condition characterized by hyperglycaemia, progressive rise in blood pressure, declining glomerular filtration rate, and high risk of fatal or non-fatal cardiovascular events [24]. The animal model for T2DN was characterized using

the hallmark features including plasma biochemical parameters, alterations in body and kidney weight and associated haemodynamic changes. The development of T2D was validated by the elevation in plasma glucose, plasma triglycerides, total cholesterol and drastic reduction in the plasma insulin levels indicate the development of T2D in HFD + STZ treated rats (Table 2) whereas renal failure was indicated by the alterations in plasma creatinine, blood urea nitrogen and albumin levels (Table 3). The morphometric changes were checked in the form of a drastic fall in the body weight, rise in kidney and relative kidney weight which confirmed the development of renal hypertrophy in HFD + STZ treated rats. Haemodynamic alteration, a common comorbid condition in T2D was analysed by measuring the mean systolic blood pressure using non-invasive techniques (Table 3).

We found that the key pathological features were improved significantly by administration of AT1R blocker, Telmisartan while AT2R blocker, PD123319 led to further deterioration of the condition as compared to the HFD + STZ treated rats. However, the simultaneous administration of the two antagonists did not show any major differences from the HFD + STZ treated group (Table 3). These results are in line with our previous reports which had pointed towards a reno-protective role of AT1R antagonists and AT2R agonists [19].

An elevated ECM deposition and glomerular basement membrane thickening are hallmark features of renal fibrosis which correlate closely with the progressive renal dysfunction in diabetes [25]. In order to determine renal fibrosis linked with T2D, protein expression of TGF-β1 (Fig. 1A and B) and fibronectin (Fig. 1C and D) and mRNA expression of *Tgfb1* (Fig. 1E) and *Col1a1* (Fig. 1F) were checked and it was seen that the expression of these fibrotic markers was increased markedly in the HFD fed and HFD + STZ treated rats as compared to NC. The treatment with AT1R blocker could significantly revert these expression levels whereas PD123319 treatment did not show any significant difference from that of the HFD + STZ treated rats. The treatment with both the antagonists showed a marked reduction in the fibrotic markers as compared to the HFD + STZ treated rats. These results demonstrate that Angiotensin II mediated fibrotic cascade in T2DN may be activated through AT1R and not through the AT2R (Fig. 1). The progression in renal injury and fibrosis is intricately related to the accumulation and invasion of macrophages. Thus, prompting us to check the markers of macrophage infiltration in the T2DN.

3.2. Angiotensin II receptors regulate macrophage infiltration and H3 lysine 4 methyltransferase SET7/9 expression in type 2 diabetic kidney

Macrophage infiltration elevates the production of pro-inflammatory cytokines like interleukin-1 (IL-1), platelet-derived growth factor, fibroblast growth factor-2 and TGF-β1, to promote myofibroblast proliferation, ECM deposition and epithelial to myofibroblast transformation which culminate into renal fibrosis [26]. MCP-1, a marker of macrophage infiltration has been found to be an indicator of degree of severity of renal failure in T2D. In our study, we observed a significant increase in the expression of MCP-1 at both mRNA as well as protein levels in HFD fed and HFD + STZ treated rats which could be ameliorated by Telmisartan treatment to a considerable amount. The blockade of AT2R further elevated the expression in T2D rats indicating a probable role of AT2R in regulation of macrophage infiltration. The combination of both the antagonists did not lead to any major difference in MCP-1 expression from that of the HFD + STZ treated rats and HFD fed rats (Fig. 2C, D and 2E).

The increased expression of MCP-1 could be correlated with a simultaneous increase in the expression of SET7/9, a histone 3 lysine 4 methyltransferase and regulator of NF-κBp65 expression which orchestrates the production of various pro-inflammatory cytokines in the kidneys of db/db mice [27,28]. In this study, we found that *Set7/9* is upregulated in HFD fed as well as HFD + STZ treated rats as compared to the NC. The treatment with Telmisartan reduces the upsurge in SET7/9 expression, PD123319 further accelerates the expression, whereas the combination of the two does not show any significant difference in the expression from the HFD + STZ treated rats (Fig. 2A and B). Thus, it may be concluded that both AT1 and AT2 receptors are involved in regulation of macrophage infiltration under T2DN and this regulation may be associated with the epigenetic alterations. Accumulating evidences suggest that increase in expression of fibrotic and inflammatory genes in diabetic conditions may have an epigenetic link. Hence, we further investigated the PTMs of histone H3 and H2A in T2DN and the effect of angiotensin II receptor blockade on these modifications.

3.3. Angiotensin II receptors regulate the histone H3 acetylation

Histone lysine acetylation mediated by histone acetyltransferases is usually associated with chromatin opening and gene activation. This is balanced by the removal of acetyl groups by histone deacetylases which are associated with chromatin compaction and transcriptional repression [13]. Our study showed that the type 2 diabetic kidney shows an increase in the histone H3 acetylation at lysine 9, 14 and 27. This augmentation in histone H3 acetylation could be significantly reversed by the treatment with AT1R blocker, Telmisartan and further augmented by AT2R blocker, PD123319. However, their co-administration did not lead to any significant alterations in the acetylation of H3 as compared to the HFD + STZ treated group (Fig. 3). The H3 acetylation at lysine 18 was not found to show any significant elevation in HFD fed or the HFD + STZ treated rats, but Telmisartan could reduce the elevation in H3K18 acetylation as compared to HFD + STZ treated rats (Fig. 3). It has already been reported that histone H3 acetylation at lysine 9 and 23 is associated with advanced diabetic nephropathy in db/db mice [29]. Also, increased plasminogen activated inhibitor-1 and p21 expression was associated with elevated promoter H3K9/14Ac levels in glomeruli from diabetic mice [13]. Our results confirm that histone H3K9 and H3K14 acetylation get accelerated under diabetic conditions.

Table 2
Effect of Ang II receptor(s) blockade on plasma glucose (PGL), triglyceride (TG), total cholesterol (PTC) and insulin (PI) levels.

Group	PGL (mmol/L)	TG (mg/dL)	PTC (mmol/L)	PI (pmol/ml)
Normal Control (NC)	6.197 ± 0.893	55.17 ± 3.005	1.270 ± 0.093	3.332 ± 0.221
High fat diet control (HFD)	8.030 ± 0.340	77.83 ± 3.628*	4.620 ± 0.272*	6.705 ± 0.333*
High fat diet/STZ (35 mg/kg) (H+S)	30.40 ± 1.387$^{*,\$}$	165 ± 7.599$^{*,\$}$	16.23 ± 1.232$^{*,\$}$	0.859 ± 0.0912$^{*,\$}$
High fat diet/STZ/Telmisartan (10 mg/kg) (H + S+T)	16.89 ± 1.172$^{\#,\$}$	87.17 ± 4.222$^{\#,\$}$	6.882 ± 0.7338$^{\#}$	2.191 ± 0.189$^{\$,\#}$
High fat diet/STZ/PD (10 mg/kg) (H + S + PD)	40.64 ± 1.862$^{\#,@,\$}$	178.5 ± 2.850$^{@,\$}$	18.42 ± 0.1.065$^{\$,@}$	0.708 ± 0.109$^{\$,@}$
High fat diet/STZ/Telmisartan/PD (H + S + T + PD)	31.33 ± 1.455$^{\$,@,\alpha}$	154.7 ± 4.879$^{@,\$,\alpha}$	9.853 ± 0.8910$^{\$,\#,\alpha}$	1.308 ± 0.311$^{\$,@,\alpha}$

Note: Data represented in mean ± SEM (n = 6). *p < 0.001 Vs NC, #p < 0.001 Vs HFD + STZ, $p < 0.001 Vs HFD, @p < 0.001 Vs HFD + STZ + T, α p < 0.001 Vs HFD + STZ+PD.

Table 3
Effect of Ang II receptor(s) blockade on markers of renal function-Blood urea nitrogen (BUN), plasma albumin (PAL) and plasma creatinine (PCr), morphometric parameters and systolic blood pressure (SBP).

Groups	Renal failure markers			Morphometric parameters			Hemodynamic parameter
	BUN (mmol/L)	PAL (gm/L)	PCr (mg/dL)	Body weight (gm)	Kidney weight (gm)	(KW/BW)*1000	SBP (mm Hg)
Normal Control (NC)	3.291 ± 0.725	29.56 ± 1.719	1.517 ± 0.199	254 ± 8.073	1.363 ± 0.037	5.35 ± 0.004	90.24 ± 5.190
High fat diet control (HFD)	5.723 ± 0.446	31.01 ± 1.133	1.728 ± 0.266	335 ± 1.848*	1.789 ± 0.096	5.34 ± 0.051*	109.9 ± 2.987*
High fat diet/STZ (35 mg/kg) (H+S)	10.50 ± 0.771$^{*,\$}$	18.12 ± 1.627$^{*,\$}$	4.968 ± 0.239$^{*,\$}$	174 ± 3.342$^{*,\$}$	2.33 ± 0.115$^{*,\$}$	13.39 ± 0.020$^{*,\$}$	136.9 ± 4.890$^{*,\$}$
High fat diet/STZ/Telmisartan (10 mg/kg) (H + S + T)	6.330 ± 0.661$^{\#}$	31.13 ± 2.101$^{\#}$	2.800 ± 0.120$^{\#}$	232 ± 2.379$^{\#,\$}$	1.427 ± 0.027$^{\#}$	6.12 ± 0.011$^{\#}$	86.12 ± 2.002$^{\$,\#}$
High fat diet/STZ/PD (10 mg/kg) (H + S + PD)	13.64 ± 0.625$^{\$,\#,@}$	17.51 ± 1.286$^{\$,@}$	5.963 ± 0.117$^{\#,\$,@}$	156.0 ± 2.217$^{\#,\$,@}$	2.66 ± 0.032$^{\$,@}$	17.05 ± 0.014$^{\$,@}$	156.98 ± 3.980$^{\$,\#,@}$
High fat diet/STZ/Telmisartan/PD (H + S + T + PD)	11.73 ± 0.448$^{@,\$}$	23.17 ± 0.527$^{\$,@}$	3.120 ± 0.361$^{\#,\alpha}$	190.1 ± 2.272$^{\#,\$,\alpha}$	2.25 ± 0.102$^{\$,@,\alpha}$	11.83 ± 0.044$^{\$,@,\alpha}$	103.9 ± 1.467$^{\#,@,\alpha}$

Note: Data represented in mean ± SEM (n = 6). *p < 0.001 Vs NC, #p < 0.001 Vs HFD + STZ, $p < 0.001 Vs HFD, @p < 0.001 Vs HFD + STZ + T, α p < 0.001 Vs HFD + STZ+PD.

3.4. Role of Angiotensin II receptors in regulation of H2A lysine 119 monoubiquitination in T2DN

Histone H2AK119 and H2BK120 monoubiquitination generally act as transcriptional repressor and activator, respectively. An augmented H2BK120Ub may lead to increase in stable PHTMs such as histone H3K4 and H3K79 methylation [30]. We found that H2AK119Ub gets increased in the HFD + STZ treated and HFD fed rats. The treatment with Telmisartan leads to a reduction in ubiquitination, whereas PD123319 further enhanced the H2AK119Ub. The combination of the two antagonists led to a significant reduction in histone H2AK119Ub as compared to that of the PD123319 treated animals, but was slightly more than that of the Telmisartan treated animals (Fig. 4A and B). Histone ubiquitination is a tightly regulated, reversible process guided basically by two enzymes- E3 ubiquitin ligases, responsible for addition of ubiquitin to protein substrate and deubiquitinases which help in removal or detachment of ubiquitin moiety from the substrate.

We also found changes in the expression of epigenetic enzymes that catalyse some of the DN-altered histone modifications in the HFD + STZ treated rats. Notably mRNA expression of the H2A specific E3 ubiquitin ligases like *Rnf2* and *Rnf168* were elevated significantly in the HFD fed and HFD + STZ treated rats. This rise in the mRNA expression is reversed significantly by the treatment with Telmisartan and further elevated by the PD123319 exposure. The treatment with both AT1R and AT2R antagonists showed an mRNA expression level similar to that of the HFD + STZ treated rats (Fig. 4C). The H2A specific deubiquitinases, *Usp21* and *Usp16* were found to have reduced significantly in the HFD fed and HFD + STZ treated rats, thus supporting our observation that H2AK119Ub gets enhanced in pre-diabetic and type 2 diabetic kidney and this alteration could be reversed to a considerable extent by Telmisartan treatment and was adversely affected by the PD123319 exposure (Figure 4D).

3.5. Targeting AT1R ameliorates macrophage infiltration and renal fibrosis in T2DN through modulation of H2AK119 monoubiquitination

We next examined whether blocking angiotensin II receptors could alter H2AK119Ub recruitment at the promoter regions of *Mcp1* and *Tgfb1* using chromatin immunoprecipitation-QPCR assays. The results indicated that H2AK119Ub levels at *Mcp1* and *Tgfb1* promoters in the DN group were markedly lower as compared with the NC. The occupancy of H2AK119Ub was found to increase significantly by the treatment with Telmisartan whereas PD123319 treatment led to a further drop in the occupancy. These results suggest that Angiotensin II mediated induction of *Mcp1* and *Tgfb1* expression in the kidneys of type 2 diabetic rats may be attributed to reduced occupancy of H2AK119Ub at their respective promoter regions, which in turn indicates a reduction in inhibition of expression (Fig. 5A and B).

4. Discussion

It is widely known that macrophage infiltration and fibrosis are central to development of renal failure in T2D. In the current study, we aimed to delineate the role of PTHMs in regulating macrophage infiltration and renal fibrosis in the face of Angiotensin II receptor(s)' blockade under T2D. In order to fulfil our aim, we developed the non-genetic murine model of T2DN by using HFD feeding along with low dose STZ treatment and validated its development, using biochemical parameters.

TGF-β1 plays pivotal role in the development of renal fibrosis by phosphorylating SMAD3/SMAD4 complex to promote its nuclear

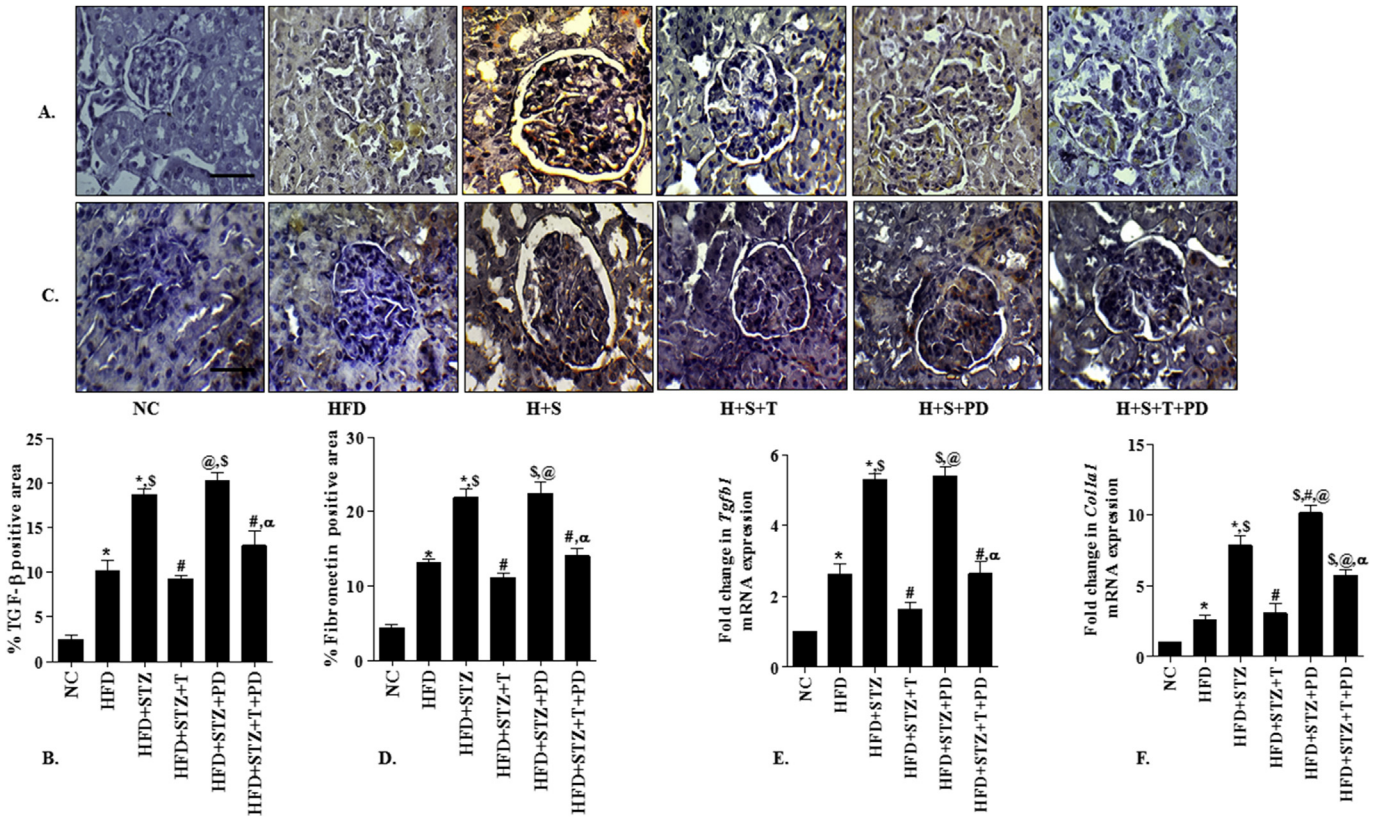

Fig. 1. Effect of Angiotensin II receptor blockade on renal fibrosis in type 2 diabetic kidney (A) Light microscopic pictures illustrating the immunostaining for TGF-β1 (40× magnification) and (B) quantification of %TGF-β1 positive area in the kidney sections (C) Light microscopic pictures illustrating the immunostaining for fibronectin (40× magnification) and (D) quantification of % fibronectin positive area in the kidney sections. All values are represented as means ± SEM from at least 25 sections per group. Fold change in the mRNA expression of (E) *Tgfb1* and (F) *Col1a1*. **Note**: Data represented in mean ± SEM (n = 6). *p < 0.001 Vs NC, #p < 0.001 Vs HFD + STZ, $p < 0.001 Vs HFD, @p < 0.001 Vs HFD + STZ + T, α p < 0.001 Vs HFD + STZ + PD.

translocation and by increasing ubiquitin mediated degradation of SMAD7. The increase in recruitment of Smad3 on the promoter region of genes coding for ECM components like collagen and fibronectin leads to the increased ECM deposition in diabetes [31]. Our results showed that hyperglycaemia and hyperinsulinemia promote the expression of fibrotic markers including TGF-β1, fibronectin and *Col1a1*, which could be controlled considerably by the administration of AT1R blocker, Telmisartan but were not affected by PD123319. This shows that the activation of TGF-β1 mediated fibrosis and deposition of ECM in T2DN may occur through AT1R instead of AT2R.

The increased expression of fibrotic markers in T2DN could be correlated with simultaneous increase in the expression of MCP-1. Wolf et al. had demonstrated the existence of a regulatory loop controlling MCP-1 and TGF-β expression. This model describes the positive regulation of TGF-β production by MCP-1 and negative feedback mechanism by which TGF-β limits the MCP-1 production [32,33]. MCP-1 expression is regulated by NF-κB signalling pathway and according to the existing literature, the regulation of NF-κB mediated MCP-1 expression by Angiotensin II receptor subtypes varies in different tissues and different conditions. For instance an in vitro study using glomerular epithelial cells showed that AT1R was responsible for the regulation of pro-inflammatory genes like *Il6, Vcam, Mcp1* [4] whereas another study using wild type C57BL/6 mice with unilateral ureteral obstruction (UUO) showed that treatment with Losartan or PD123319, partially decreased NF-κB activation, whereas only the AT2R blockade diminished monocyte infiltration, which implicates that both the receptor subtypes are involved in activating NF-κB signalling pathway while AT2R orchestrates the monocyte mediated inflammation [3]. Thus, it becomes pertinent to understand which of the Angiotensin II receptor subtypes is involved in the regulation of MCP-1 in type 2 diabetic kidney. In present study, we observed a drastic upregulation of MCP-1 expression at both protein as well as mRNA levels in T2D. Telmisartan could reduce the macrophage invasion by a significant amount while PD123319 provoked the invasion, showing the involvement of both the angiotensin II receptor subtypes in regulation of macrophage infiltration in T2DN.

Renal fibrosis, inflammation, advanced glycation end product formation and oxidative stress-the main features of T2DN are reversed significantly by the AT1R antagonists. A recent study demonstrated that losartan mediated reversal of key inflammatory and fibrotic genes in DN was associated with its ability to inhibit recruitment of H3K9/14Ac and H3K36me3, respectively, at *Rage* and *Pai1* genes promoter sites [11]. In type 2 diabetic (db/db) mice renal failure can increase H3K9/K14Ac in the heart, which can be a contributing factor for cardiac hypertrophy and fibrosis [14]. Histone methylation has also gained much attention as potential molecular mechanism underlying metabolic memory and DN. A study aimed at examining the changes in key epigenetic chromatin marks, including histone H3K9me2/3 and H3K4me1/2/3 levels, at fibrotic gene promoters showed that these chromatin marks and SET7/9 were involved in TGF-β1 and hyperglycaemia-induced up-regulation of ECM-associated genes in renal mesangial cells [12]. The current study suggests that therapeutic activity shown by Telmisartan, may be partially attributed to its ability to reduce the elevated histone H3 acetylation in diabetic nephropathy. Despite the vast studies going on to study role of PTHMs in various diseases,

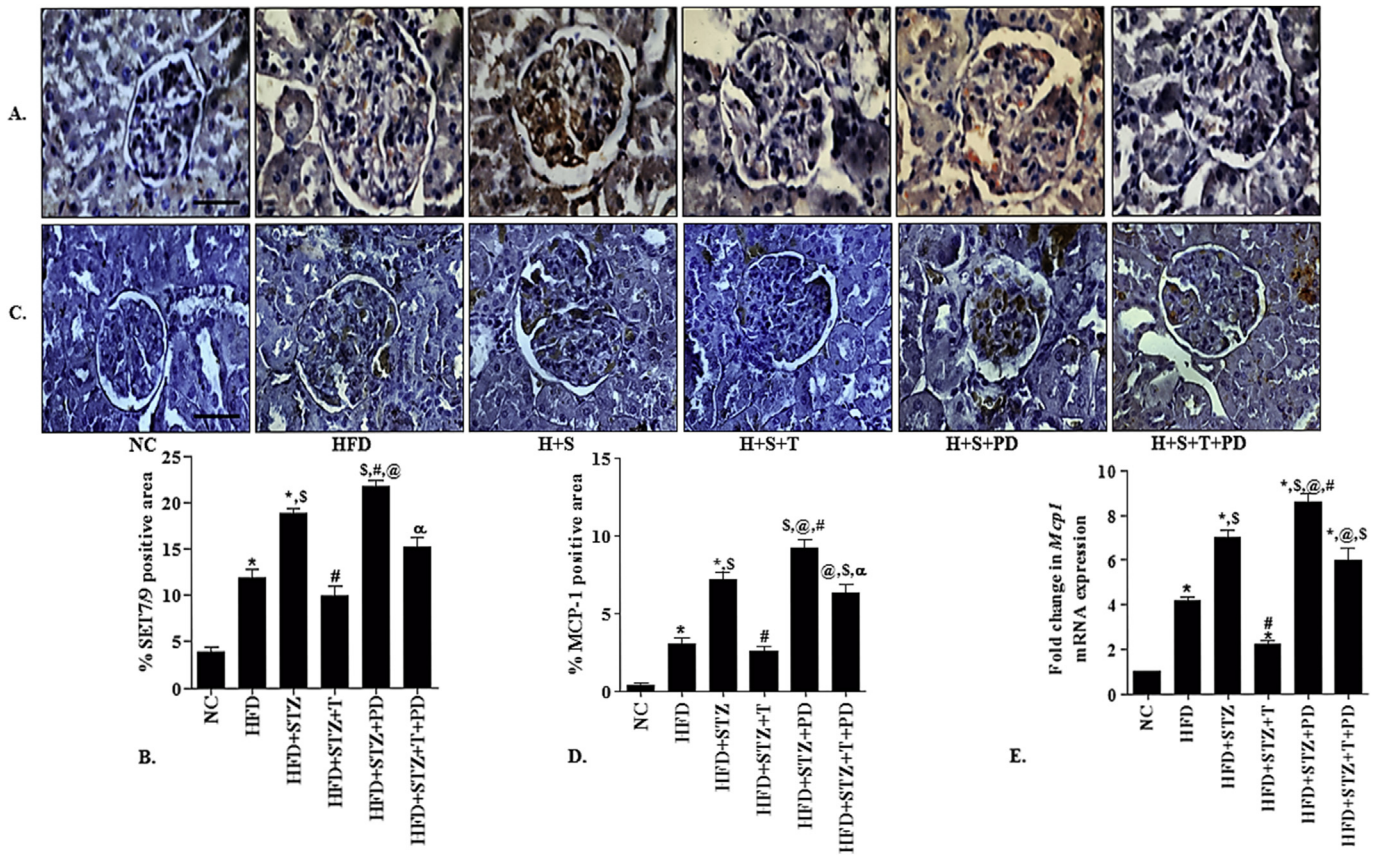

Fig. 2. Effect of Angiotensin II receptor blockade on macrophage infiltration in type 2 diabetic kidney. (A) Light microscopic pictures illustrating the immunostaining for MCP-1 (40× magnification) and (B) quantification of %MCP-1 positive area in the kidney sections. (C) Light microscopic pictures illustrating the immunostaining for SET7/9 (40× magnification) and (D) quantification of % SET7/9 positive area in the kidney sections. All values are represented as means ± SEM from at least 25 sections per group. (E) Fold change in the mRNA expression of *Mcp1* in type 2 diabetic kidney. **Note**: Data represented in mean ± SEM (n = 6). *p < 0.001 Vs NC, #p < 0.001 Vs HFD + STZ, $p < 0.001 Vs HFD, @p < 0.001 Vs HFD + STZ + T, α p < 0.001 Vs HFD + STZ + PD.

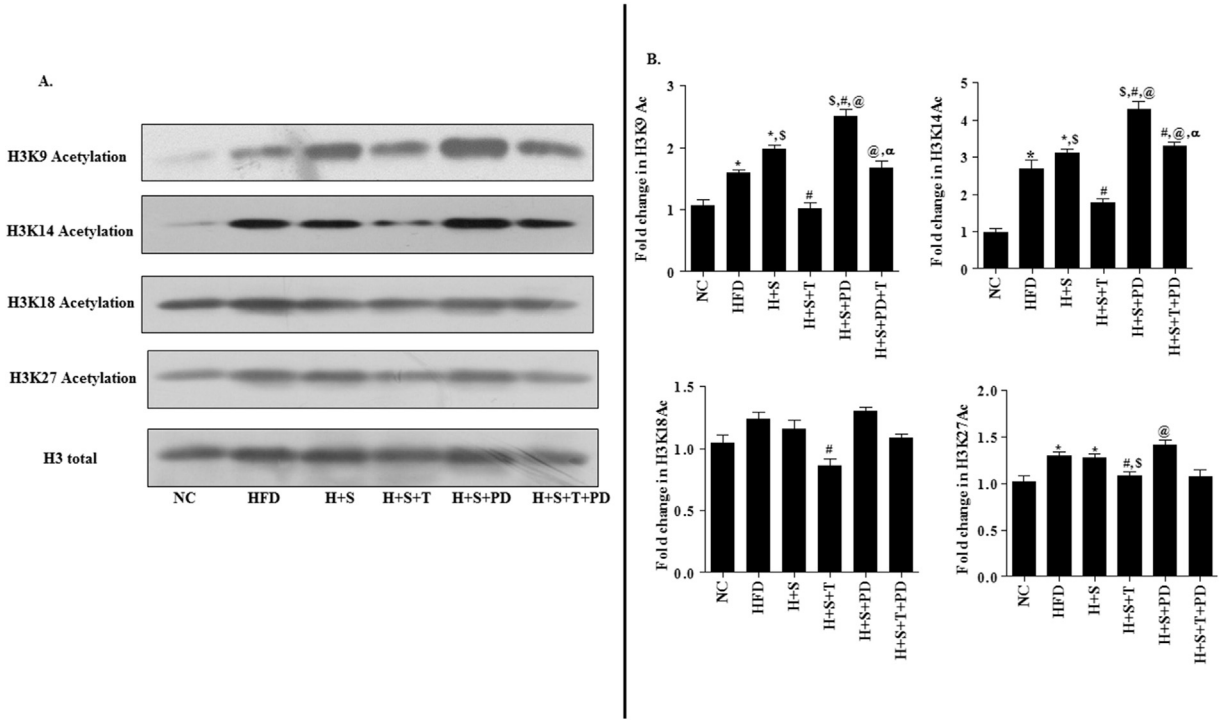

Fig. 3. Effect of Angiotensin II receptor blockade on histone H3 acetylation in type 2 diabetic kidney. (A) Western blot analysis of histone H3 acetylation at lysine 9, 14, 18 and 27 (B) quantitative analysis of respective blots using ImageJ software. **Note**: Each data point is represented as mean ± SEM, n = 3 blots/protein. *p < 0.001 Vs NC, #p < 0.001 Vs HFD + STZ, $p < 0.001 Vs HFD, @p < 0.001 Vs HFD + STZ + T, α p < 0.001 Vs HFD + STZ + PD.

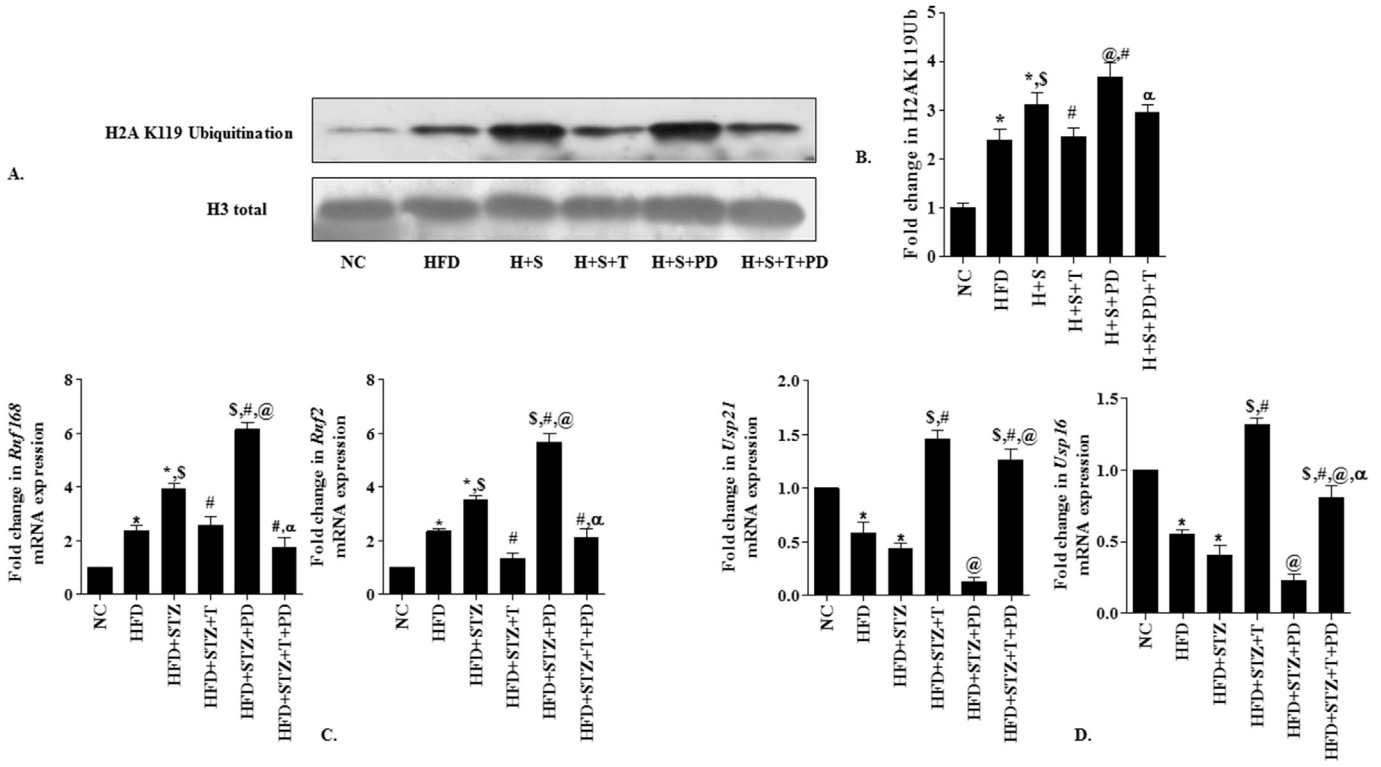

Fig. 4. Effect of Angiotensin II receptor blockade on histone H2AK119 ubiquitination in type 2 diabetic kidney. (A) western blot analysis of H2AK119Ub (B) quantitative analysis of H2AK119Ub blots using ImageJ software Each data point is represented as mean ± SEM, n = 3 blots/protein (C) Increased mRNA expression of E3 ubiquitin ligases- *Rnf2* and *Rnf168* in type 2 diabetic kidney (D) Decreased mRNA expression of deubiquitinases- *Usp21* and *Usp16* in type 2 diabetic kidney. **Note**: Data represented in mean ± SEM (n = 6). *p < 0.001 Vs NC, #p < 0.001 Vs HFD + STZ, $p < 0.001 Vs HFD, @p < 0.001 Vs HFD + STZ + T, α p < 0.001 Vs HFD + STZ + PD.

the literature regarding histone ubiquitination in diabetic nephropathy remains limited.

Ubiquitination of protein substrates is no longer just a 'kiss of death', its functions in regulating various biological pathways have been unravelled recently [34]. Histone ubiquitination has recently been acknowledged as vital pathway for regulation of gene transcription. The glomerular mesangial cells exposed to hyperglycaemia showed elevated cell damage, induced histone H2AUb, reduced histone H2BUb accompanied by increased activation of TGF-β signalling pathway [17]. Our study establishes the fact that histone H2AUb at lysine 119 gets heightened in the insulin resistant and hyperglycaemic rats' kidney, which was well supported by the concordant changes displayed by the ubiquitination machinery. The reversal of these repressive chromatin marks by Telmisartan shows that its ability to combat T2DN may be having an epigenetic basis and alteration in histone H2AUb occupancy at *Mcp1* and *Tgfb1* promoter sites may be one of the important mechanisms involved. We observed that HFD + STZ treated rats showed an elevation in the expression of the H2A specific E3 ligases, *Rnf2* and *Rnf168* and a significant reduction in deubiquitinases, *Usp21* and *Usp16*which

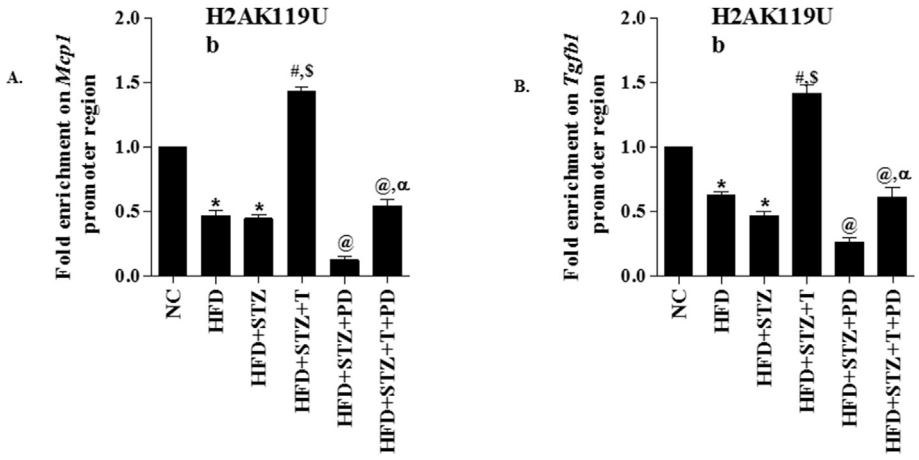

Fig. 5. Effect of Angiotensin II receptor blockade on histone H2AK119 ubiquitination occupancy at *Mcp1* and *Tgfb1* promoter region. (A) Fold change in occupancy of H2AK119Ub at *Mcp1* promoter (B) Fold change in occupancy of H2AK119Ub at *Tgfb1* promoter. **Note**: Data represented in mean ± SEM (n = 6). *p < 0.001 Vs NC, #p < 0.001 Vs HFD + STZ, $p < 0.001 Vs HFD, @p < 0.001 Vs HFD + STZ + T, α p < 0.001 Vs HFD + STZ + PD.

could be altered by reversed markedly by Telmisartan treatment and was further aggravated by PD123319. This result shows that both the Angiotensin II receptor subtypes may elicit their response by alteration of ubiquitin proteasomal system. The ubiquitin proteasome system has a well reported role in regulation of NF-κB pathway as it mediates the degradation of IκB to initiate the nuclear translocation of p65 which attaches to the transcription factor binding site and triggers gene transcription [35]. However, the correlation between the inflammatory signalling and H2AK119Ub has not yet been reported. Our study presents the first report discussing the disturbance in the ubiquitination machinery i.e. elevation in H2A specific E3 ubiquitin ligase and reduction of H2A specific deubiquitinases, concomitant with elevated macrophage infiltration and renal fibrosis in the T2DN.

Thus, based on the above observations we can conclude that macrophage infiltration and renal fibrosis are regulated through both AT1 and AT2 receptors in T2DN and H2AK119 monoubiquitination is an important epigenetic link between these pathological manifestations and Angiotensin II receptors. This suggests that amelioration of inflammatory and fibrotic markers may require the co-administration of an AT1R antagonist and AT2R agonist and also that the quest to search a solution to T2DN must consider the effect of the therapeutic intervention on ubiquitin proteasome system and histone H2AK119 monoubiquitination so as to find a superior solution to the problem.

Conflict of interests

We declare that we have no conflict of interest.

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Compound 21 and Telmisartan combination mitigates type 2 diabetic nephropathy through amelioration of caspase mediated apoptosis

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ABSTRACT

The current study aimed to understand the role of novel, highly selective, orally active, non-peptide Angiotensin II type 2 receptor (AT2R) agonist, Compound 21 and its potential additive effect with Telmisartan on apoptosis and underlying posttranslational modifications in a non-genetic murine model for type 2 diabetic nephropathy (T2DN). An experimental model for T2DN was developed by administering low dose Streptozotocin in high fat diet fed male Wistar rats, followed by their treatment with Telmisartan, C21 or their combination. Our results demonstrated that C21 and Telmisartan combination attenuated metabolic and renal dysfunction, renal morphological and micro-architectural aberrations and hemodynamic disturbances in type 2 diabetic rats. The anti-apoptotic and anti-inflammatory effects of Telmisartan were significantly accentuated by C21 indicated by expression of apoptotic markers (*Parp1, Caspase 8, Caspase 7,* cleaved PARP and cleaved Caspase 3) and NF-κB mediated inflammatory molecules like interleukin 6, tumour necrosis factor alpha; monocyte chemoattractant protein 1 and vascular cell adhesion molecule 1. C21 was found to improve Telmisartan mediated reversal of histone H3 acetylation at lysine 14 and 27 and expression of histone acetyl transferase, p300/CBP-associated factor also known to regulate NF-κB activity and DNA damage response. C21 in combination with Telmisartan markedly mitigates caspase mediated apoptosis and NF-κB signalling in T2D kidney, which could be partially attributed to its influence on PCAF mediated histone H3 acetylation. Hence further research should be done to develop this combination to treat T2DN.

1. Introduction

Type 2 diabetic nephropathy (T2DN), the leading cause of end stage renal failure is characterized by renal failure induced by chronic hyperglycemia and hyperinsulinemia [1]. In both type 1 and type 2 diabetes, hyperglycemia, free radical stress and disturbed hemodynamics promote apoptosis of kidney podocytes, epithelium, glomerular and proximal tubular cells through induction of Bcl2/Caspase/PARP pathway [2–5]. Apoptosis is triggered by dysregulated balance between two parallel pathways triggered by the same signal, TNF-α- pro-apoptotic caspase cascade and the anti-apoptotic Nuclear factor κB (NFκB)- Inhibitor κB (IκB)- inhibitor of apoptosis protein (IAP) pathway [6].

NF-κB mediated inflammatory cascade regulates the transcription of various pro inflammatory cytokines and chemokines in T2DN and is activated by acetylation facilitated by p300/CBP and p300/CBP-associated factor (PCAF) [7]. PCAF, a member of the GCN5-related N-acetyltransferase family plays significant roles in induction of cellular apoptosis by modulating GLI1/Bcl-2/BAX axis [8] and also regulates histone H3 (lysine 14) and H4 (lysine 18) acetylation [9]. Histone acetylation and deacetylation, orchestrated by histone acetyl transferases (HAT) and histone deacetylases (HDAC), respectively regulate opening and closing of the chromatin structure to guide gene expression machinery [10].

The current treatments for DN including angiotensin converting enzyme inhibitors (ACEi) or angiotensin receptor blockers (ARBs) reduce progression of albuminuria and renal failure in patients with type 2 diabetes (T2D) but don't curb nephropathy completely, thus indicating a need of novel therapeutic interventions [11,12].

AT2R, an important component of the protective axis of RAS, i.e. ACE2/MasR/Ang (1–7) axis ameliorates various pathogenic pathways like inflammation [13,14] and fibrosis [15] in different diseases including diabetes induced atherosclerosis [16], stroke [17], cardiac disorders and vascular diseases [18]. Compound 21 (C21) a

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novel, non-peptide AT2R agonist has shown renoprotective effects in renovascular hypertension by reducing inflammatory cell infiltration [13], obese Zucker diabetic fatty rats by increasing IL-10 (anti-inflammatory cytokine) production by proximal tubule epithelial cells [19]. The role of AT2R in regulation of apoptosis is incompletely understood. In spontaneous hypertensive rats, AT2R mediated vascular mass regression by stimulating smooth muscle cells' apoptosis under AT1R blockade but not during converting-enzyme inhibition [20]. C21 treatment was reported to attenuate myocardial infarction in Wistar rats due to its anti-apoptotic and anti-inflammatory actions [21]. AT2R deletion in C57/BL6 mice was found to accelerate DN, by increasing oxidative stress and ACE/ACE2 ratio in renal proximal tubules [22].

Despite the important role of apoptosis and AT2R in T2DN pathogenesis, the influence of C21 on renal apoptosis and the underlying epigenetic mechanisms in T2D remains enigmatic. Hence, the current study aims to check the effect of C21 either alone or in combination with AT1R antagonist, Telmisartan on apoptosis, NF-κB mediated inflammation, and histone H3 acetylation in T2D kidney.

2. Methodology

2.1. Chemicals

Antibodies against MCP-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and rest of the antibodies were purchased from Cell Signalling Technology (Danvers, MA, USA). All the other chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA), unless otherwise mentioned.

2.2. Animal studies

Adult male Wistar rats (160–180 g), procured from the Central Animal Facility, Birla Institute of Technology and Science Pilani were maintained under standard environmental conditions and provided feed and water ad libitum. The animals were fed on normal pellet diet (NPD) one week prior to start of experimental protocol which is in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Social Justice and Environment, Government of India based on the guidelines of Institute of Laboratory Animal Resources, (Washington, DC, U.S.A.). A prior permission was sought from the institutional animal ethics committee, BITS Pilani, for conducting the study (IAEC/RES/17/04/Rev-2/19/36).

The animal model for T2DN was developed using high fat diet (HFD) and low dose STZ (35 mg/kg, *ip*) and the normal control was treated with vehicle (0.5% sodium carboxy methyl cellulose) [23]. T2D induction was confirmed by analyzing plasma glucose levels after 48 h and diabetic rats were fed HFD for 24 weeks for development of renal failure. The animals were administered drugs for 14 days: Telmisartan (10 mg/kg/day, *po*), C21 (0.3 mg/kg/day, *po*, Vicore Pharma, Sweden), and Telmisartan plus C21 (n=6 rats/group). Plasma biochemical analysis of glucose (PGL), triglycerides (TG), total cholesterol (PTC), blood urea nitrogen (BUN), creatinine (PCr), albumin (PAL), alkaline phosphatase (ALP) and plasma insulin (PI) and non-invasive blood pressure measurement to determine systolic blood pressure (SBP) were performed as described previously [23].

2.3. Histopathological and immunohistochemistry

Histopathology and immunohistochemistry staining were performed as described previously [23–25].

2.4. Western blotting

Western blotting and histone isolation were performed as described previously [26,27].

2.5. RNA isolation and real time-polymerase chain reaction

RNA was isolated from kidneys using commercially available kit and qRT-PCR was performed using specific primers (Table 2) as described previously [26,27].

2.6. Statistical analysis

Experimental values were expressed as means ± S.E.M. Statistical comparison between different groups was performed using one way analysis of variance to detect the difference in observations between all groups. If F value was significant then multiple comparisons were done by Tukey test using Prism software (version 5.0; GraphPad, San Diego, CA) for Windows. Data was considered to be statistically significant if $p < 0.05$.

3. Results

3.1. C21 and Telmisartan combination improves biochemical, morphometric and hemodynamic parameters in type 2 diabetes

The HFD fed low dose STZ treated rats were found to show a significant increase in PGL, TG, PTC and a drastically low PI concentration as compared to the control rats. The renal function parameters, including PAL, BUN, and PCr were found remarkably disturbed in HFD fed low dose STZ treated rats. Though these alterations in plasma biochemical parameters were improved by both Telmisartan and C21, their combination showed a potential additive effect in improving the renal functioning in T2D rats. ALP, BUN and PCr, significantly elevated in T2D rats were more efficiently reduced by C21 and was further improved by its combination with Telmisartan as compared to Telmisartan alone (Table 1).

The kidney weight-body weight ratio increment indicating renal hypertrophy in T2D rats, was ameliorated more significantly by Telmisartan and the combination regimen as compared to C21 (Table 1). The microscopic analysis showed that C21 and Telmisartan ameliorated glomerular damage (thickening of basement membrane, narrowing of Bowman's capsule) (Fig. 1A), and percentage of fibrotic or collagen positive area in glomerular and tubular region (Fig. 1B) to some extent but the most prominent improvement was rendered by the combination which reduced both the glomerular and tubular alterations significantly.

SBP was found to have elevated drastically in T2D rats. Though treatment with C21 alone could not lower the SBP, it improved the effect of Telmisartan quite phenomenally, thus indicating a potential role of this combination in alleviating hypertension linked with T2D (Table 1).

3.2. C21 and Telmisartan combination reduces apoptosis in type 2 diabetic kidney

In this study, T2D kidney was found to show profound increase in protein expression of cleaved PARP, and cleaved caspase 3 (Fig. 4) whereas the expression of caspase 3 was found to have attenuated. The expression of these apoptotic markers was improved markedly by both C21 and Telmisartan treatments. C21 was found to further promote the anti-apoptotic potential of Telmisartan, as evidenced by the potent reduction in apoptotic markers in the combination treated group (Fig. 4). In order to further check the effect of treatment at mRNA levels, mRNA expression of apoptotic genes, *Casp7,*

Table 1
Effect of Angiotensin II receptor(s) manipulation on plasma parameters for metabolism and renal functioning, hemodynamic and morphometric parameters.

		NC	HS	HST	HSC	HSCT
Metabolic parameters	PGL (mmol/L)	7.922 ± 0.6947	37.62 ± 2.760*	16.89 ± 1.172#	20.58 ± 2.049#	9.169 ± 0.8346#@$
	TG (mg/dL)	63.63 ± 6.738	174.9 ± 10.37*	105.1 ± 4.221#	137.8 ± 11.05	98.61 ± 3.731#$
	PTC (mmol/L)	1.249 ± 0.2744	17.83 ± 0.9888*	6.882 ± 0.7338#	9.340 ± 0.6565#	7.672 ± 3.759#
	PI (pmol/L)	304.0 ± 4.390	107.2 ± 11.46*	158.0 ± 8.512#	176.4 ± 14.18#	291.7 ± 8.809#@$
Renal function parameters	BUN (mmol/L)	4.499 ± 0.3971	8.555 ± 0.7575*	6.418 ± 0.5741#	5.174 ± 0.3629#	4.576 ± 0.3571#@$
	PAL (gm/L)	38.78 ± 1.082	22.38 ± 1.414*	27.26 ± 1.774#	30.84 ± 1.444#	34.56 ± 1.198#@$
	PCr (mg/dL)	0.8667 ± 0.1687	3.533 ± 0.4240*	2.800 ± 0.1206#	1.533 ± 0.1308#@	0.6375 ± 0.1143#@$
	ALP (IU/L)	40.80 ± 3.218	287.5 ± 7.449*	248.7 ± 2.914#	120.8 ± 6.250#@	78.00 ± 3.185#@$
Hemodynamic parameters	SBP (mm Hg)	90.24 ± 5.190	130.9 ± 4.890*	88.12 ± 2.002#	120 ± 7.234	95.15 ± 5.54#$
Morphometric parameters	Body weight (gm)	215.5 ± 6.589	136.8 ± 3.119*	222.8 ± 5.851#	220.0 ± 3.342#	232.0 ± 6.589#
	Kidney weight (gm)	1.193 ± 0.07554	2.330 ± 0.1159*	1.420 ± 0.2041#	2.253 ± 0.06667	1.433 ± 0.02720#$
	(KW/BW)*1000	5.557 ± 0.1658	17.04 ± 1.334*	7.327 ± 0.1720#	10.30 ± 0.5776#	6.234 ± 0.1717#$

Abbreviations - Normal Control (NC), High fat diet/STZ (35 mg/kg/*ip*) (HS), High fat diet/STZ/Telmisartan (10 mg/kg/*po*) (HST), High fat diet/STZ/C21 (0.3 mg/kg/*po*) (HSC), High fat diet/STZ/C21/Telmisartan (HSCT) Plasma glucose (PGL), triglyceride (TG), total cholesterol (PTC) and insulin (PI) Blood urea nitrogen (BUN), plasma albumin (PAL) and plasma creatinine (PCr), alkaline phosphatase (ALP) and hemodynamic parameters -systolic blood pressure (SBP). Data represented in mean ± SEM (n = 6). *p < 0.001 Vs NC, #p < 0.001 Vs HS, @p < 0.001 Vs HST, $p < 0.001 Vs HSC.

Table 2
List of primers.

Gene Name	Primer Sequence	Accession ID.
Vcam1	Forward:5′-ACACCTCCCCCAAGAATAC-3′ Reverse:5′-CCAGATTCACTCCTTCACAC-3′	NM_012889.1
Il6	Forward:5′-GGATACCACCCACAACAGAC-3′ Reverse:5′-GAAACGGAACTCCAGAAGAC-3′	NM_012589.2
Nfkb p65	Forward:5′-CATCACACGGAGGGCTTC-3′ Reverse:5′-GAACGATAACCTTTGCAGGC-3′	NM_199267.2
Tnfa	Forward:5′-GATCGGTCCCAACAAGGAGG-3′ Reverse:5′-CTTGGTGGTTTGCTACGACG-3′	NM_012675.3
Mcp1	Forward 5′- GTCTCAGCCAGATGCAGTTA-3′ Reverse 5′- CCTTATTGGGGTCAGCACAG-3′	NM_031530.1
Pcaf (Kat2B)	Forward 5′- TTCCCCCTCCAGCGTGTTAG-3′ Reverse 5′- GGATTAGTGTTCTCGCTCCCAG-3′	XM_003750617.4
Casp7	Forward:5′-ACCGCTCCACCATCATCTCA-3′ Reverse:5′- CGGACATCCATACCTGTCGCT-3′	NM_022260.3
Parp1	Forward:5′-TTGGTGGAGTACGAGATTGACC-3′ Reverse:5′- TGGGGGATGAGGGTGTAGAA-3′	NM_013063.2
Casp8	Forward:5′- CGAACGATCAAGCACAGAGAG-3′ Reverse:5′- AGATCAGACAGTACCCCCGA3′	NM_022277.1

Casp8 and Parp1 was checked. Here, mRNA expression of *Casp7, Casp8 and Parp1*, elevated drastically in T2D rats was improved most significantly in combination treated group (Fig. 2A–C).

3.3. C21 and Telmisartan combination ameliorates NF-κB mediated renal inflammation in type 2 diabetes

We found that T2D kidney showed a drastic activation of the NF-κB signalling pathway, as demonstrated by the multi-fold increase in expression of phosphorylated NF-κB p65 protein and mRNA expression of *NF-κBp65* (Fig. 3A and B). NF-κB signalling was controlled most significantly by the combination treatment, followed by Telmisartan and C21. Inflammation is marked by an elevation of the assisting cytokines and chemokines which help in accelerating and propagating the cellular damage. In this study, mRNA expression of cytokines including interleukin 6 (*Il6*), tumour necrosis factor alpha (*Tnfa*); adhesion molecule, vascular cell adhesion molecule 1 (*Vcam1*) and chemokine, monocyte chemoattractant protein1 (*Mcp1*) was significantly upregulated in T2D rats' kidneys. Though, monotherapy with C21 treatment reduced only the inflammatory cytokines *Il6* and *Tnfa*, the combination improved the cytokines, adhesion molecules as well as the chemokines more efficiently than the individual treatments (Fig. 3C–F).

3.4. C21 and Telmisartan combination reverts histone H3 acetylation at lysine 14 and 27 and histone acetyltransferase expression

Here, we observed that histone H3 acetylation at both lysine 14 and 27 increases in T2D rats' kidneys. H3K14Ac was reduced more significantly by Telmisartan as compared to C21 and the combination improved the effect of Telmisartan whereas H3K27Ac was reduced equally efficiently by both Telmisartan and C21. The HAT, PCAF also called lysine acetyl transferase 2B was significantly upregulated in T2D rats' kidneys and it was reduced significantly by Telmisartan and its combination with C21 further lowered the protein expression (Fig. 4).

4. Discussion

In this study, we found that C21, a novel orally active, non-peptide AT2R agonist improves the renoprotective effects of AT1R antagonist, Telmisartan in T2D rats by reducing caspase mediated apoptosis and NF-κB activation, which could be partially attributed to its ability to regulate PCAF mediated histone H3 acetylation.

The main risk factors for diabetic nephropathy are hypertension, hyperlipidemia, and hyperglycemia [28,29]. Here, we found that though Telmisartan and C21, both reduce PGL, PTC and PI in T2D rats, their combination showed further improved carbohydrate and

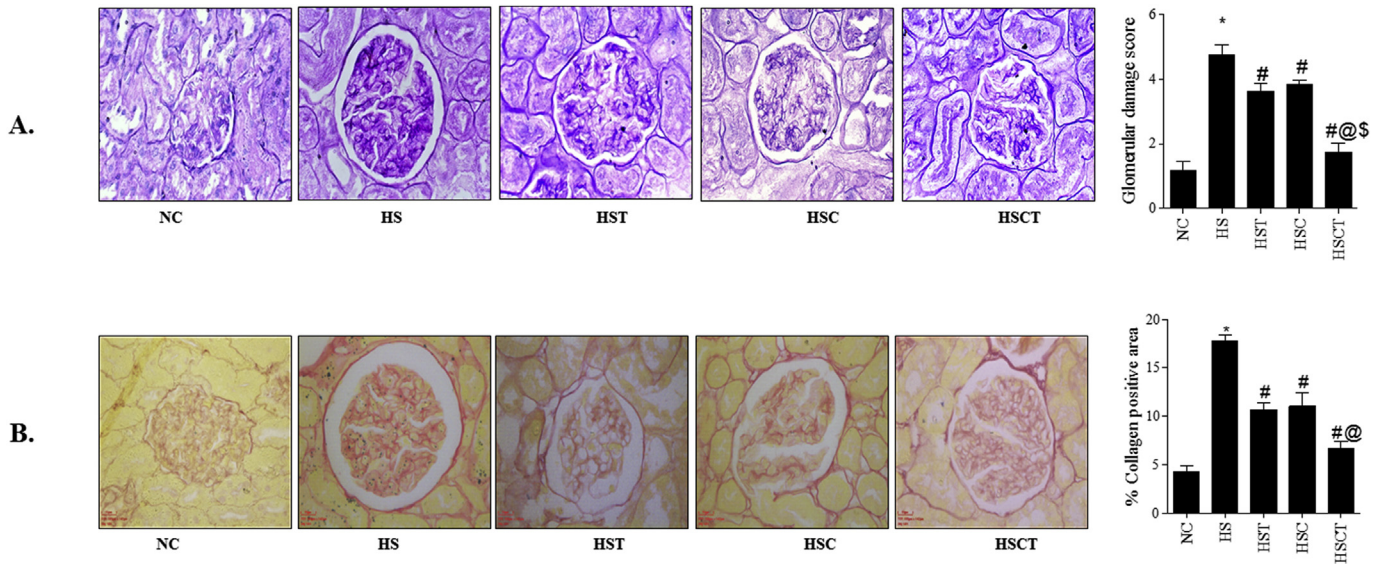

Fig. 1. Effect of Compound 21 and Telmisartan combination: renal microscopic features in type 2 diabetic rats.
Light microscopic pictures and quantification of (A) hematoxylin and eosin staining to estimate glomerular damage score (B) Picro Sirius red staining to estimate % collagen positive area. **Note**: Data represented in mean ± SEM (n = 6). *p < 0.001 Vs NC, #p < 0.001 Vs HS, @p < 0.001 Vs HST, $p < 0.001 Vs HSC.

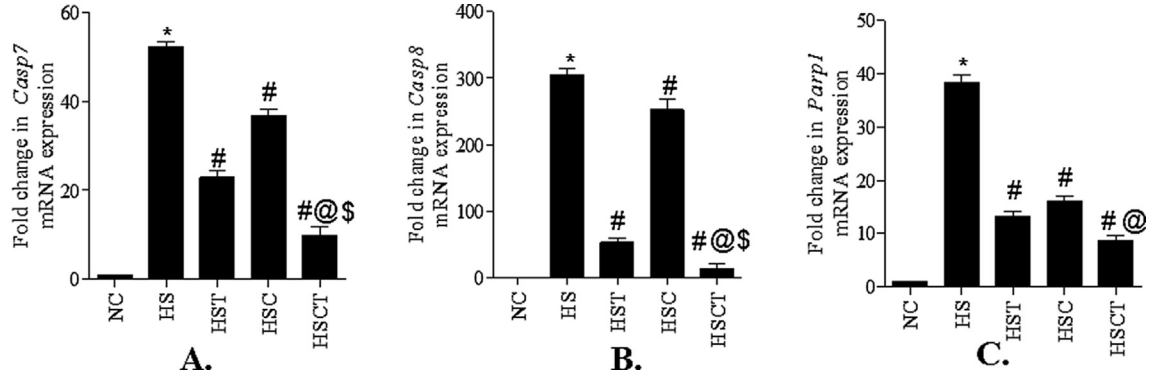

Fig. 2. Effect of Compound 21 and Telmisartan combination: apoptosis in type 2 diabetic kidney.
The graphs represent the fold change in mRNA expression of (A) Casp7 (B) Casp8 and (C) Parp1 Note: Data represented in mean ± SEM (n = 6). *p < 0.001 Vs NC, #p < 0.001 Vs HS, @p < 0.001 Vs HST, $p < 0.001 Vs HSC.

lipid metabolism which shows that it could be a better therapeutic intervention than C21 and Losartan which could not revert the elevated blood glucose levels in Zucker diabetic fatty rats [15]. Oshima et al. had shown that C21 treatment significantly reduced blood glucose concentration and serum insulin concentration in the fed condition as compared with the control KK-Ay mice [30]. The renal function parameters, including PAL, PCr and ALP levels were improved more significantly by C21 as compared to Telmisartan, which was further improved by the combination. These results were in line with the previous reports which have indicated C21's ability to reduce proteinuria and albuminuria and the renoprotective effect of the combination could also be due to its potential to improve these parameters as albuminuria is also a risk factor for progression of nephropathy in diabetes [15,31]. Recent reports suggest that renal expression of AT2R is higher in adult female rats as compared to the male counterparts [32], which urges us to speculate that the beneficial effects obtained by the administered combination may be even better in the female rats and further studies need to be done to determine the sex specific differences in the therapeutic activity.

Hypertension is an independent risk for development of nephropathy in T2D subjects [29]. SBP, markedly elevated in T2D rats was controlled significantly by Telmisartan and was further improved by C21 co-administration indicating that AT1R inhibition along with AT2R stimulation might provide a better solution to hemodynamic imbalance in diabetics. Since ARBs and ACEIs are the only existing treatments in T2DN and their combination shows major side effects, C21 could be a significant conjugate drug for reducing the progression of T2DN to end stage renal failure. The increased renoprotection ushered by the combination may be attributed to the increase in expression of AT2R upon treatment with AT1R antagonist which signifies increase in the activity of the protective arm of RAS [33]. The SBP monitoring in our study was based on non-invasive techniques, which may not be the most accurate method to estimate the blood pressure in rats, so future investigations using invasive techniques are required to confirm our observations.

T2DN is a chronic inflammatory disease involving numerous transcription factors, pro-inflammatory cytokines, chemokines and adhesion molecules [34]. The current study showed that direct stimulation of AT2R and inhibition of AT1R curtails the expression of phosphorylated NF-κB p65 and the downstream molecules

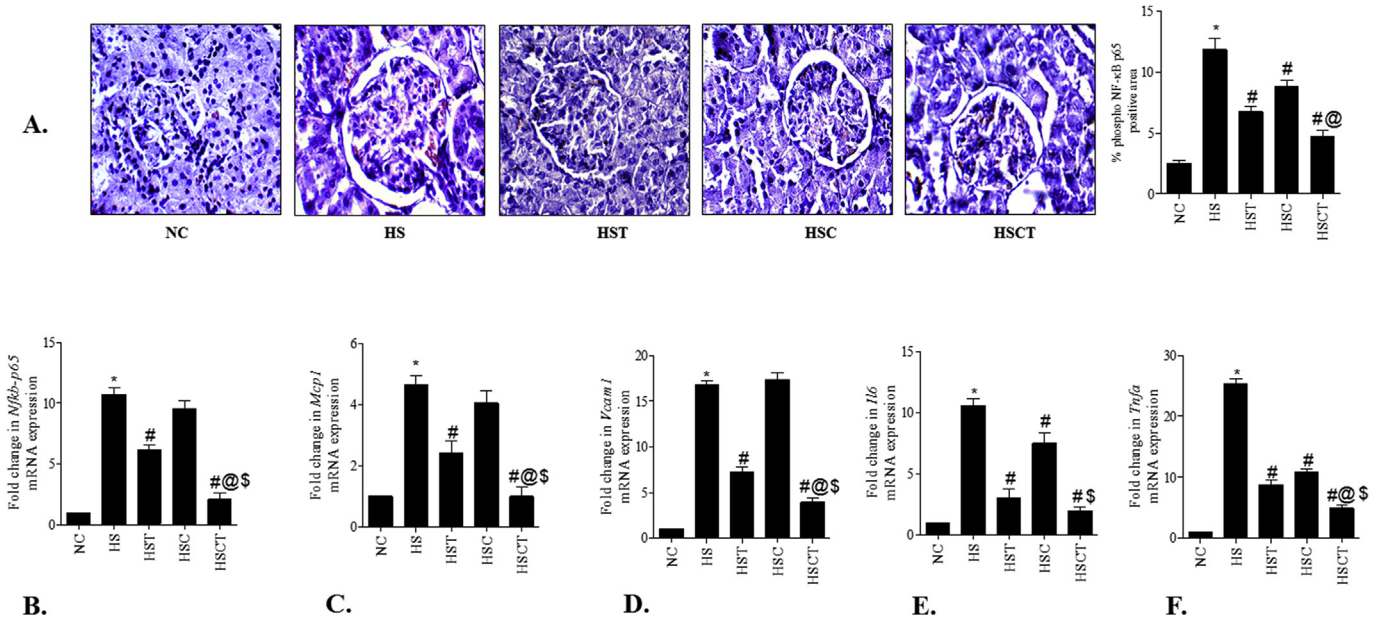

Fig. 3. Effect of Compound 21 and Telmisartan combination: inflammation in type 2 diabetic kidney.
(A) Light microscopic pictures illustrating the immunostaining for phosphorylated NF-κBp65 and quantification to determine % phosphorylated NF-κBp65 positive area and fold change in mRNA expression of inflammatory markers including (B) Nfkbp65 (C) Mcp1 (D) Vcam1 (E) Il6 (F) Tnfa **Note**: Data represented in mean ± SEM (n = 6). *p < 0.001 Vs NC, #p < 0.001 Vs HS, @p < 0.001 Vs HST, $p < 0.001 Vs HSC.

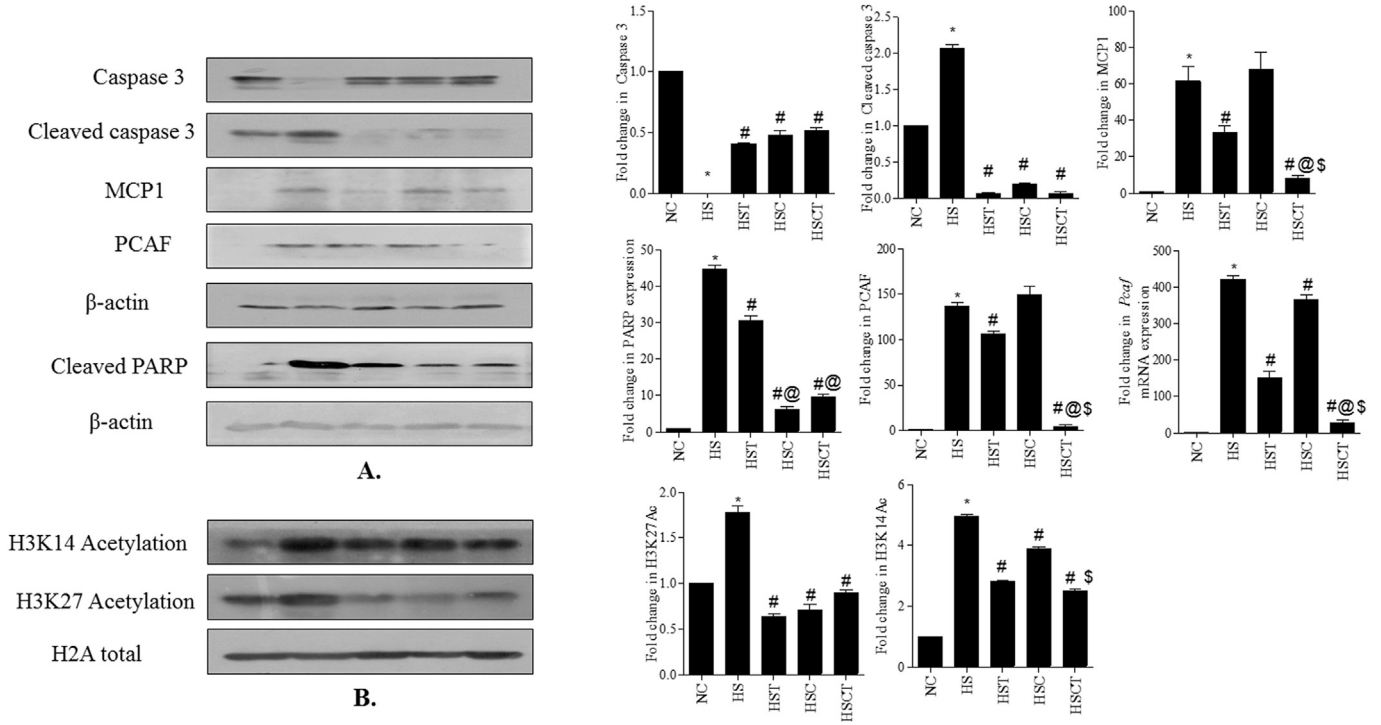

Fig. 4. Effect of AT1 receptor blocker and AT2 receptor agonist on protein expression of inflammatory, apoptotic and histone H3 acetylation in type 2 diabetic kidney. Panel A: Western blots of apoptotic marker, Caspase 3, Cleaved caspase 3, PARP; chemokine, monocyte chemoattractant protein 1 (MCP1) and histone acetyltransferase, p300/CBP-associated factor (PCAF) and normalized against β-actin. Panel B: Western blots for H3K14 and H3K27 acetylation normalized against H2A total. Note: Data represented in mean ± SEM (n = 6). *p < 0.001 Vs NC, #p < 0.001 Vs HS, @p < 0.001 Vs HST, $p < 0.001 Vs HSC.

including cytokines (*Il6* and *Tnfa*), chemokines (*Mcp1*) and adhesion molecule (*Vcam1*) in T2D kidney. C21 alone, also could significantly reduce the *Tnfa* expression which could also be responsible for its anti-apoptotic action because TNF-α is known to act on death receptor pathway and also to directly activate caspase 8 mediated apoptosis [6].

Apoptosis involves three types of caspases-initiators, including caspase 2,8,9 and 10; executioners-caspase 3,6 and 7 and processing caspases like caspase 1,4,5,11 and 12L/12S. Caspase 3, an important executioner caspase activated by both the extrinsic as

well as the intrinsic pathways is considered a marker of apoptosis, superior to the TUNEL assay that also detects the necrotic cells [35]. In our study, C21 improved the effect of Telmisartan on caspase mediated apoptosis, as shown by its influence over the expression of PARP, caspase 3 and cleaved caspase 3 at protein levels and *parp1*, *caspase8* and *caspase7* at mRNA level which are closely linked with glomerulosclerosis and renal dysfunction in diabetic subjects.

The pathogenesis of DN is linked with alterations in histone acetylation levels. The diabetic patients showed a significant increment in H3K9/14Ac and H4 K5/8/12Ac TNF-α and COX-2 inflammatory genes promoters in human blood monocytes [36]. The levels of histone acetylation are tightly controlled by HATs and HDACs [10]. PCAF, a transcriptional adaptor protein and HAT functions as catalytic subunit of transcriptional co-activator complex to acetylate histone H3 and H4 [9]. It facilitates transcriptional activation of NF-κB by reducing its binding to IκBα enhancer and enhancing its nuclear localization [37]. In our study, PCAF was found significantly upregulated in T2D kidney which could be directly correlated with increased histone H3K14Ac and C21 was found to improve the effect of Telmisartan on PCAF and H3K14Ac. The superior anti-apoptotic action of C21 and Telmisartan combination could be partially attributed to its improved ability to regulate PCAF expression which is known to enhance DNA damage response by acetylating p53 at lysine 320 [9] and to induce apoptosis in hepatocellular carcinoma by suppressing Akt1 pathway [8].

Based on our results, it may be concluded that though C21 alone ameliorates various pathological alterations in T2DN to a certain extent, a combination of C21 and Telmisartan is more efficient in reducing caspase linked apoptosis and NF-κB mediated inflammation associated with renal failure in T2D male rats. This improvement in renal dysfunction may be attributed to its ability to regulate the expression of PCAF and associated histone H3 acetylation. Hence, we suggest that Telmisartan and C21 combination may be a novel therapeutic strategy for T2DN and further studies should be undertaken to develop this combination for treatment of diabetic human subjects.

Conflict of interests

We declare that we have no conflict of interest.

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AT_2 receptor agonist Compound 21: A silver lining for diabetic nephropathy

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ABSTRACT

The currently available therapies for diabetic nephropathy, one of the leading causes of renal failure globally are based on inhibition of renin angiotensin system. However, recently, the focus has shifted towards activation of its protective arm rather than the inhibition of deteriorative axis, using specific agonists. Compound 21 (C21), a novel non-peptide Angiotensin II type 2 receptor (AT_2) agonist, recently granted orphan drug status for the treatment of a rare disease, idiopathic pulmonary fibrosis has also shown a potent anti-inflammatory, anti-fibrotic, antioxidant and anti-apoptotic potential in various diseases including heart failure, myocardial infarction, chronic inflammatory diseases, and neurological diseases such as ischemic stroke. A pool of evidences suggest that C21, either alone or in combination with angiotensin receptor blockers could be extremely beneficial in the treatment of diabetic nephropathy, a chronic inflammatory condition sharing its pathogenesis with aforementioned diseases. The review analyses the new therapeutic tool, C21, its mechanisms of action for renoprotection in diabetic nephropathy, and its future perspectives and thereby provides an insight into the potential application of C21 as a novel therapeutic tool in the eradication of diabetic nephropathy.

1. Introduction

Diabetic nephropathy (DN), characterized by a progressive decline in renal function represents the major cause of end stage renal disease throughout the world (Fineberg et al., 2013). DN is typically defined by macroalbuminuria or microalbuminuria and abnormal renal function as represented by an anomaly in serum creatinine, calculated creatinine clearance, or a rapid drop in glomerular filtration rate (Dabla, 2010). The development of DN is a multifactorial, complex process orchestrated by chronic hyperglycemia and reduced insulin sensitivity or insulin resistance. Chronic hyperglycemia is widely known to trigger rapid and drastic redox imbalance to cause an abrupt rise in the free oxidative and nitrosative radicals. Hyperglycemia has also been reported to shape various detrimental events such as structural and functional changes including glomerular hyperfiltration, glomerular and tubular epithelial hypertrophy, and microalbuminuria, followed by the development of glomerular basement membrane thickening, accumulation of mesangial matrix, overt proteinuria, finally glomerulosclerosis and end stage renal disease (Vinod, 2012). Type 2 diabetes is also closely linked with an elevated risk of hypertension, which is also an independent risk factor for development of nephropathy in type 2 diabetic subjects (MacIsaac et al., 2014). DN is also linked with endothelial dysfunction due to impaired nitric oxide release, a central pathophysiologic denominator for all cardiovascular complications of diabetes. The blockade of endothelial nitric oxide synthesis and activity has been shown to impair renal autoregulation which in turn further promotes renal dysfunction in diabetic condition (Nakagawa et al., 2007).

Recent reports suggest that rigorous research is going on globally to combat DN. A number of novel therapeutic agents have been studied in recent past which include endothelin receptor antagonists (bosentan), antioxidant therapies (vitamin analogs, probucol, xanthine oxidase inhibitors), transcription factor modulators (ruboxistaurin, genistein, imatinib), antifibrotic and anti inflammatory agents (infliximab or etanercept, pirfenidone), inhibitors of advanced glycation endproduct formation (pimagedine or pyridoxamine), urotensin-II receptor antagonist (palosuran) (Lacava et al., 2017; Quiroga et al., 2015). The transcription factor NF-E2–related factor 2 (Nrf2) negatively regulated by Kelch-like ECH-associated protein 1 (Keap1), is an important cellular defense mechanism to combat oxidative stress (Kensler et al., 2007). The small molecule activators of the Nrf2/Keap1 pathway like glutathione peroxidase-1 mimetic ebselen, sulforaphane (cruciferous vegetables), caffeic acid phenethylester (bee product propolis), cinnamaldehyde (cinnamon bark), and most recently, bardoxolone methyl are important remedies for DN (de Haan, 2011). Despite these molecules being extensively studied, a complete cure for DN has not yet been arrived at.

DN, especially in type 2 diabetes is compounded by a drastic derangement of hemodynamic balance which in turn are linked with the renin angiotensin system (RAS), also known to have prominent role in

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promotion of tissue injury in different chronic diseases including hypertension, heart failure, and kidney disease (Gurley and Coffman, 2007). According to the current understanding, RAS is composed of two arms: the pressor arm containing Angiotensin II (Ang II)/Angiotensin-converting enzyme (ACE)/(Ang II type 1 receptors (AT_1)), and the depressor arm represented by Ang-(1-7) [angiotensin-(1-7)]/ACE2/Mas receptors, which form the deteriorative and protective axes, respectively (Gironacci et al., 2014). Though the inhibition of deteriorative axis of RAS has been the mainstay of pharmacological approaches for treatment of DN since decades, as indicated by the prevalent usage of ACE inhibitors and Angiotensin receptor blockers, augmentation of the protective axis remains an underrated story despite its vast potentials. Chang et al. showed that AT_2 receptor knockout mice showed a higher degree of renal failure both in non-diabetic as well as diabetic condition due to an increased rate of extra cellular matrix deposition, tubular apoptosis and oxidative stress. An elevated ACE/ACE2 ratio in knockout animals could account for aggravation of renal injury during DN (Chang et al., 2011). These reports suggest that AT_2 receptor, an important player in protective axis has a significant role in guiding the pathogenesis of DN. This reviews aims to compile the existing literature regarding the role of Compound 21 (C21), a novel AT_2 receptor agonist in treatment of DN so as to get a better understanding of the underlying mechanisms and future prospects of the molecule.

2. AT_2 receptor agonists to the rescue

Unlike AT_1 receptors, AT_2 receptors remained undervalued despite its discovery in late 1980s due to the lack of sufficient agents which could be used as specific agonists or antagonists to the receptor (Steckelings et al., 2005). However, the design and synthesis of selective AT_2 receptor antagonists PD 123319, EMA401, and agonists like CGP42112A, M024/C21 led to an improvement in understanding of the role of this receptor and also further promoted design and synthesis of molecules possessing beneficial effects (Kellici et al., 2015). The signalling pathways through which AT_2 receptor acts involves phospholipase A2, phosphotyrosine phosphatase which in turn inhibits mitogen-activated protein kinase phosphatase-1 which inhibits ERK1/2 leading to reduction in fibrosis and proliferation. Also, AT_2 receptor is involved in controlling renal nitric oxide, guanosine cyclic 3′5′-monophosphate, and kinins levels which control vasodilation and redox balance (Fig. 1) (Recarti and Bottari, 2015). Recent research has shown that AT_2 receptor ushers a significant antioxidant, anti-inflammatory (Sabuhi et al., 2011), anti-fibrotic, anti-hypertrophic (Ma et al., 1998; Nabeshima et al., 2006) and anti-apoptotic properties (de Gasparo and Siragy, 1999; McCarthy et al., 2012), thus indicating the importance of AT_2 receptor agonists in therapeutic regimens for various diseases (Steckelings et al., 2005). The current knowledge regarding these therapeutic applications of AT_2 receptor in tissue injury has been compiled in recent reviews (McCarthy et al., 2013; Namsolleck et al., 2014; Steckelings et al., 2012). CGP42112A, a peptide AT_2 receptor agonist widely used for AT_2 receptor related studies could not translate into a clinically successful molecule owing to its poor in vivo stability and its partial antagonism (Steckelings et al., 2012). The other AT_2 receptor agonists currently available are enlisted in Table 1 (Danyel et al., 2013; Guimond et al., 2014; Jones et al., 2011; Ohinata et al., 2009; Rosenström et al., 2005; Wagenaar et al., 2013). The non-peptide, small molecule C21 (Vicore Pharma, Sweden) is the most widely used AT_2 receptor agonist available till date (Danyel et al., 2013).

3. Compound 21- novel AT_2R agonist

In 2004, the AT_2 receptor related research gained momentum with the advent of the novel non peptide AT_2 receptor agonist named C21 which was orally and systemically active (Steckelings et al., 2012). It showed an oral bioavailability of about 30% and has a plasma half-life of almost 4–6 h. C21, modelled on the C-terminal pentapeptide structure of Ang II lacks AT_1 receptor affinity and was demonstrated in human embryonic kidney cells to have 4000-fold selectivity to AT_2 receptor (Bosnyak et al., 2011; Wan et al., 2004). Owing to its vast biological activities including anti-fibrotic, anti-inflammatory, anti-apoptotic, anti-oxidant and anti-hypertensive properties, C21 showed a profound beneficial effect in heart failure, myocardial infarction, chronic inflammatory diseases, and neurological diseases such as ischemic stroke (Chow and Allen, 2016; McCarthy et al., 2012; Rehman et al., 2012). C21 has also recently been approved by European Medicines Agency (EMA) and Food and Drug Administration (FDA) for the treatment of pulmonary fibrosis, a rare disease which entitled the molecule to be granted an orphan drug status (Bruce et al., 2015; Rathinasabapathy et al., 2015). According to Vicore Pharma Annual report 2016, in clinical trials, conducted by Vicore Pharma in collaboration with the Åbo, a Finland based clinical contract research organization, increasing doses of C21 (0.3–100 mg) were given to healthy

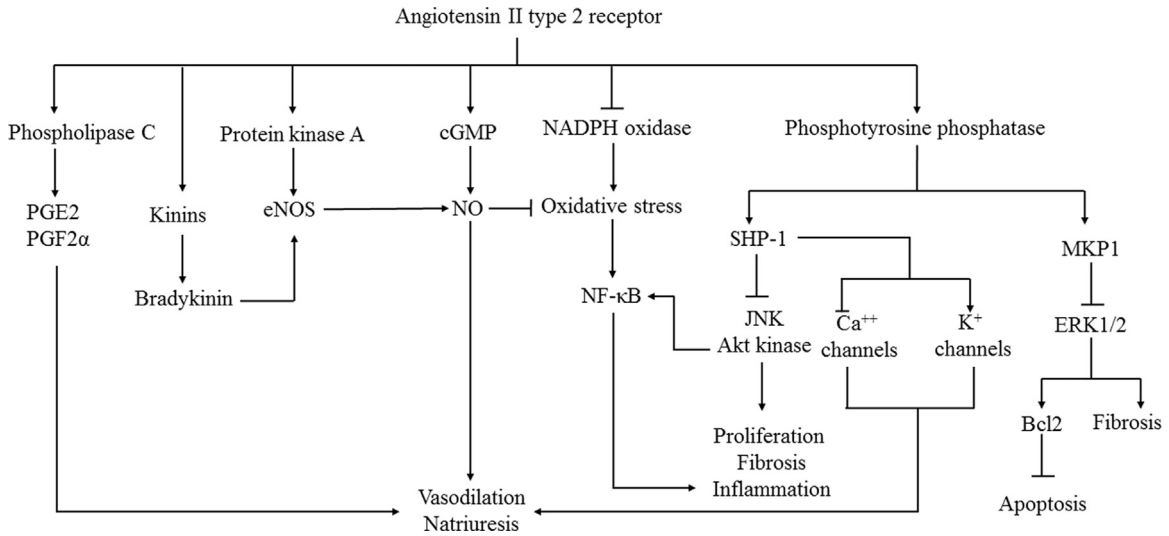

Fig. 1. Signalling pathways for AT_2 receptor. The signalling pathways through which AT_2 receptor acts involves activation of phospholipase A2, and phosphotyrosine phosphatase which in turn inhibit vasoconstriction and mitogen-activated protein kinase phosphatase-1. This leads to a reduction in ERK1/2 expression and thus controls apoptosis as well as fibrosis. AT_2 receptor is involved in controlling renal nitric oxide, guanosine cyclic 3′5′-monophosphate, and kinins levels which in turn are responsible for vasodilation and antioxidant potential. The antioxidant action is also mediated through another pathway, NADPH oxidase mediated pathway which plays a major role in activation of NF-κB signalling involved in regulation of inflammation.

Table 1
List of AT_2 receptor agonists.

#	AT_2 receptor agonists	Stage of drug development	References
1.	Compound 21 (C21) [Vicore Pharma, Sweden]	Completed phase 1 clinical trials Granted orphan drug status for treatment of idiopathic pulmonary fibrosis	(Danyel et al., 2013; Bruce et al., 2015)
2.	Lanthipeptide 2 [MOR107, formerly LP2-3, Lanthio Pharma B.V., a wholly owned subsidiary of MorphoSys AG, Germany]	Completed phase 1 clinical trials	(Wagenaar et al., 2013)
3.	β-Amino Acid Substituted Angiotensin Peptides	In vitro and in vivo studies	(Jones et al., 2011)
4.	Benzodiazepine based gamma-turn mimetics incorporated into Ang II	In vitro studies	(Rosenström et al., 2005)
5.	Novokinin (RPLKPW)	In vivo studies	(Ohinata et al., 2009)
6.	Sarile and sarlasin	In vitro studies	(Guimond et al., 2014)

Table 2
Summary of Compound 21 (C21) application in diabetic nephropathy.

Sr. No.	Animal model	Dose/route of C21 administration	Key findings	References
1.	Zucker diabetic fatty rats	C21 (0.3 mg/kg/day, *ip*) and/or Losartan (10 mg/kg/day, in drinking water) for 15 weeks	↓hemodynamic imbalance ↓glomerular, tubulointerstitial and perivascular fibrosis ↓inflammatory mediators and macrophage infiltration No change in blood glucose levels even with C21 and losartan combination	(Castoldi et al., 2014)
2.	Streptozotocin treated ApoE-/- mice	C21 (1 mg/kg/day) and/or the AT_1 receptor antagonist Candesartan (4.3 mg/L) for 20 weeks	↓albuminuria and glomerulosclerosis No significant changes in body weight, blood glucose, HbA1c or systolic blood pressure	(Mckelvey et al., 2014)
3.	Streptozotocin treated ApoE-/- mice	C21 (1 mg/kg/day) for 20 weeks	↓albuminuria, mesangial area and glomerulosclerosis ↓mRNA expression of inflammatory mediators (MCP1, CD11) ↓mRNA expression of fibrosis markers (TGF-β, Collagen and Fibronectin) No effect on diabetes induced up-regulation of AT_2 receptor expression	(Chow et al., 2014)
4.	Streptozotocin (55 mg/kg/d for 5 days) treated ApoE-/- mice	C21 (1 mg/kg/d) treatment via daily gavaging for 20-weeks	↓cystatin C, albuminuria, mesangial expansion, and glomerulosclerosis ↓oxidative stress, inflammation, and fibrosis No effect on metabolic parameters and blood pressure	(Koulis et al., 2015)
5.	Streptozotocin (65 mg/kg, *ip*) treated male Sprague Dawley rats	C21 (0.3 mg/kg/day, *sc*) for 4 weeks	↓renal inflammatory mediators in renal interstitial fluid (TNF-α, IL-6) and 8-isoprostane) and in whole kidney (TNF-α, IL-6) ↑nitric oxide and cGMP levels ↑urine albumin to creatinine ratio No alteration in body weight, blood glucose, total kidney mass index, urine output, and SBP	(Matavelli et al., 2015)
6.	Low dose Streptozotocin (35 mg/kg, *ip*) treated high fat diet fed male Wistar rats	C21 (0.3 mg/kg/day, *po*) and Telmisartan (10 mg/kg/day, *po*) treatment for 14 days	↓caspase mediated apoptosis ↓NF-κB pathway activation in type 2 diabetic kidney ↓PCAF mediated histone H3 acetylation at lysine 14 and 18	(Pandey and Gaikwad., 2017)

volunteers, first in single doses and then in repeated doses. The data from the clinical trials showed that C21 was well tolerated with no serious side-effects, an acceptable degradation profile and consistent blood levels (http://vicorepharma.com/wp-content/uploads/2017/04/Årsredovisning-2016_eng.pdf).

Though the expression of AT_2 receptor has been known to be most prominent in embryonic tissues, adult kidney tissue also shows a significant amount of expression. AT_2 receptor regulates ureteric bud morphogenesis in embryonic kidney and is expressed abundantly in various parts of the kidney including interstitial mesenchyme, capsule, inner medulla, papillary region and collecting ducts (Song et al., 2010). Under physiological conditions, the adult kidney portions including renal vessels, glomeruli, and tubules show a significant AT_2 receptor expression, which is known to increase during dietary sodium depletion, tissue healing or ischemia (Namsolleck et al., 2014; Ozono et al., 1997). A study showed that *At2* receptor mRNA and protein were widely distributed in the various tubular and vascular segments of the renal cortex and medulla while it was not readily detectable in the glomeruli and thick ascending limbs of Henle (Miyata et al., 1999). A significantly high level of AT_2 receptor expression in kidney justifies the important role of AT_2 receptor agonists in chronic as well acute kidney diseases.

3.1. C21 in kidney diseases

Accumulating evidences suggest that AT_2 receptor stimulation with C21 not only reduced the increment in inflammatory cytokines, inflammatory cell infiltration, extracellular matrix deposition, vimentin expression in kidney but also improved the brain damage and survival rate in high salt diet fed spontaneously hypertensive stroke prone rats (Gelosa et al., 2009). In acute disease models like, 2-kidney-1-clip hypertensive rat model for ischemic kidney failure, C21 treatment lowered the levels of inflammatory mediators and cell infiltrates, and enhanced production of NO and cGMP in the ischemic kidneys, without altering the blood pressure and these effects were partially antagonised by PD123319 (Matavelli et al., 2011). Another study showed that, C21 treatment in spontaneously hypertensive rats led to renal vasodilatation and increased natriuresis more prominently in females as compared to males due to higher expression of AT2 receptor in the female rats' kidney as compared to their male counterparts (Hilliard et al., 2012; Silva-Antonialli et al., 2004). Recently, Castoldi et al. demonstrated that C21 treatment in cyclosporine induced nephropathy significantly reduced the inflammatory cell infiltration and fibrosis of glomeruli and renal tubules which is the most potent predictor of renal disease progression (Castoldi et al., 2016). Recent studies have shown that

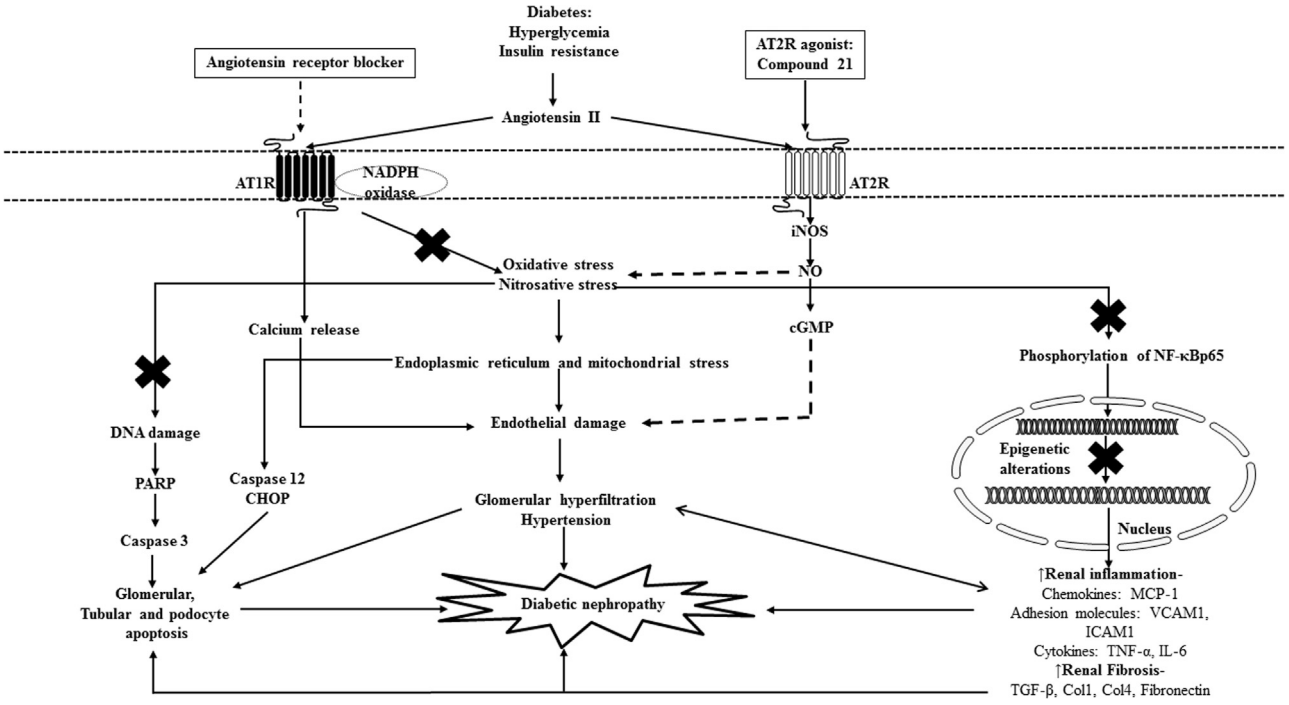

Fig. 2. Mechanism of action of Compound 21 (C21) and Angiotensin receptor blocker in diabetic nephropathy. An activation of AT_2 receptor using C21 reduces oxidative stress, NF-κB mediated inflammation, fibrosis (glomerular, tubular and perivascular) and caspase mediated apoptosis in diabetic kidney. Also, the epigenetic modifications linked with diabetic nephropathy, like histone H3 acetylation get reverted by the treatment with C21. The addition of Angiotensin receptor blocker further improve the renoprotection ushered by C21 and also improved the hemodynamic aberrations, thus indicating that AT_2 receptor agonist and AT_1 receptor antagonists could be significant drug combination for the treatment of diabetic nephropathy.

endothelial dysfunction is a major culprit in pathogenesis of renal diseases. A study conducted by Rehman et al. showed that C21, either alone or in combination with Losartan abrogates the development of hypertension and vascular damage in stroke-prone spontaneously hypertensive rats (Rehman et al., 2012). C21 alone or in combination with losartan was shown to improve endothelial function, vascular composition through amelioration of oxidative stress, fibrotic markers, and inflammatory cell infiltration (Rehman et al., 2012). Another study showed that C21 significantly controlled TNFα-induced and HFD-induced endothelial inflammation in human umbilical vein endothelial cells, as well as intact mouse aortae which thus showed an anti-atherosclerotic potential of the compound (Sampson et al., 2016). The aforementioned reports show that C21 is a potential alleviator of different pathological pathways involved in impairment of renal function in kidney diseases. These pathological pathways have also been found to be disturbed in renal failure occurring in both type 1 and type 2 diabetic patients. C21 has been shown to reduce DN in various preclinical studies which have tried to delineate the underlying mechanisms through which C21 ameliorates DN.

3.2. C21 in diabetic nephropathy

Diabetes induced hyperglycemia accelerates the pace of non-enzymatic glycosylation and polyol pathways to increase production of advanced glycosylation end products, activate protein kinase C and increase hemodynamic changes which in turn activate chronic inflammatory cascades (Wada and Makino, 2013). Inflammation though a protective mechanism, under extreme conditions can be a double edged sword, which can further deteriorate the condition rather than improving it. NF-κB activity in macrophages, glomerular and tubular parenchymal cells has been linked with severity parameters of DN such as proteinuria, oxidative stress or inflammation (Mezzano et al., 2004). According to various studies, Ang II mediated NF-κB activation is carried out through different receptor subtypes in different cells (Ruiz-Ortega et al., 2001a, 2001b, 2003; Wolf et al., 1997). We, in our study had shown that in a type 2 diabetic kidney, AT_1 receptor and AT_2 receptor antagonists lead to the repression and activation of NF-κB signalling pathway, respectively (Pandey et al., 2015) and this observation was further confirmed by our recent study in which we found that C21 in combination with Telmisartan could reduce the activation of NF-κB mediated inflammatory cascade and the production of inflammatory mediators including cytokines like TNF-α, IL6; chemokines like monocyte chemoattractant protein 1 (MCP1) and adhesion molecules like vascular cell adhesion molecule 1 (Pandey and Gaikwad, 2017). Our results also demonstrated that C21 and Telmisartan combination attenuated the aberrations in lipid and carbohydrate metabolic parameters, renal functioning, renal morphological and micro-architectural features and hemodynamic disturbances in type 2 diabetic rats (Pandey and Gaikwad, 2017).

In both type 1 and type 2 diabetes, hyperglycemia, free radical stress and disturbed hemodynamics promote apoptosis of kidney podocytes, epithelium, glomerular and proximal tubular cells through induction of Bcl2/Caspase/PARP pathway (Habib, 2013; Kumar et al., 2004; Susztak et al., 2006; Verzola et al., 2007). C21 treatment is known to attenuate myocardial infarction in Wistar rats due to its anti-apoptotic and anti-inflammatory actions (Kaschina et al., 2008). In our study, we found that anti-apoptotic effect of Telmisartan was significantly accentuated by C21 as indicated by expression of apoptotic markers including *Parp1, Caspase 7* and cleaved Caspase 3 (Pandey and Gaikwad, 2017).

Several line of evidences suggest that genetics alone is not sufficient to explain the diabetic milieu and epigenetics plays a significant role in its development (Kato and Natarajan, 2014; Pandey and Gaikwad, 2017; Pandey et al., 2016; Reddy et al., 2014; Sayyed et al., 2010). In our study, p300/CBP-associated factor (PCAF), histone H3 and H4 acetylase was found significantly upregulated in type 2 diabetic kidney which could be directly correlated with increased histone H3 lysine 14 acetylation and C21 was found to improve the effect of Telmisartan on

expression of PCAF and H3 lysine 14 acetylation. Thus, our recent study showed the anti-apoptotic and anti-inflammatory face of C21, which could be partially attributed to its influence on PCAF mediated histone H3 acetylation and thereby highlights the advantages of using it as an add on therapy with angiotensin receptor blocker (Pandey and Gaikwad, 2017).

Another study conducted by Matavelli and colleagues showed that streptozotocin treated Sprague Dawley rats showed renal function was severely disrupted, i.e. urine albumin to creatinine ratio was elevated. C21 treatment for 4 weeks was found to improve the urine albumin to creatinine ratio which showed the apparent improvement in renal function. The inflammatory mediators in renal interstitial fluid including tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), nitric oxide (NO), cyclic guanosine monophosphate (cGMP), and 8-iso-prostane, and in kidney like TNF-α, IL-6. However, the increment in blood pressure could not be improved with C21 treatment. The expressions of AT_2 receptor mRNA and protein were increased in diabetic rats as compared with the normal control rats, but this increment was not reduced by C21 treatment. The study demonstrated that C21 improved renal function by hindering the renal inflammatory cascade and improving nitric oxide and cGMP production (Matavelli et al., 2015). Though non genetic animal models showcase various advantages over the transgenic ones (Danda et al., 2005; Gaikwad et al., 2010) transgenic models also have advantages of their own, thus making them widely used and established models for diabetes and its secondary complications. The knock-out and transgenic mice have become powerful tools in elucidating the influence of specific genes in glucose metabolism on the pathogenesis of diabetes (King, 2012).

Homozygous ApoE mice are devoid of ApoE protein thus leading to a normally developed mice with a severely increased serum plasma cholesterol and spontaneous atherosclerotic plaques. Apolipoprotein-E knockout (ApoE-/-) mice treated with streptozotocin serve as appropriate murine models for diabetes and associated atherosclerotic lesions (Shen and Bornfeldt, 2007; Zaragoza et al., 2011). Chow et al., showed that streptozotocin treated apoE-/- mice showed a drastic increase in the renal dysfunction evidenced as increase in albuminuria and mesangial hypertrophy. With C21 treatment over a 20-week period albuminuria, mesangial area and glomerulosclerosis were significantly controlled as compared to that of the diabetic mice. C21 attenuated the diabetes induced upregulation of various inflammatory genes including MCP-1, CD11 which are known to further enhance the rate of renal damage (Chow et al., 2014). Sustained inflammation further enhances the albumin permeability of glomerular basement membrane and increase extracellular matrix deposition leading to proteinuria, glomerulosclerosis and finally tubulointerstitial fibrosis (Wada and Makino, 2013). The expression of fibrotic genes like TGF-β1, connective tissue growth factor, collagen type I, collagen type IV and fibronectin were also found to have been controlled by C21. These improvements in inflammation, fibrosis and overall renal functioning were not based on C21 mediated AT_2 receptor upregulation because the AT_2 receptor expression was not found to have been altered by C21 treatment, thus showing that the mechanism of action remains at the downstream level (Chow et al., 2014). Another study conducted by McKelvey et al. showed that AT_1 receptor antagonist, Candesartan further improved the renoprotection ushered by C21 in streptozotocin treated ApoE-/- mice by reducing albuminuria and glomerulosclerosis. However, there were no significant changes in body weight, blood glucose, acetylated haemoglobin or systolic blood pressure upon C21 treatment (Mckelvey et al., 2014). Koulis et al. showed that C21 treatment significantly attenuated diabetes mellitus–induced elevated levels of cystatin C, albuminuria, mesangial expansion, and glomerulosclerosis in six-week-old streptozotocin treated ApoE–/– mice. Moreover, C21 markedly inhibited the expression oxidative stress, inflammation, and fibrosis related markers. These findings validate the potential antioxidant, anti-inflammatory cum anti-fibrotic roles of C21 in diabetes induced renal failure. (Koulis et al., 2015).

Zucker diabetic fatty (ZDF) rats show a significant glomerular, tubulointerstitial and perivascular fibrosis accompanied by an upsurge of inflammatory mediators, including cytokines like TNF-α and macrophage infiltration. C21 treatment not only improved the fibrosis but also decreased albuminuria in ZDF rats only up to an age of 11 weeks but this effect vanished in 20 week old ZDF rats, as nephropathy further progressed. The treatment with Losartan decreased albuminuria (up to 15 weeks) macrophage infiltration, TNF α expression, and renal glomerular and perivascular fibrosis, restored glomerular nephrin expression, but did not reduce tubulointerstitial fibrosis, a severity marker in renal diseases. The combination of Losartan and C21 showed the best reduction in albuminuria in ZDF rats at the end of the 20 week protocol suggesting this combination as a novel pharmacological tool to slow the progression of nephropathy in type 2 diabetes (Castoldi et al., 2014).The existing literature on the role of C21 in the treatment of diabetic nephropathy has been compiled in Table 2.

4. Conclusions

These evidences suggest that C21 efficiently alleviates the development of renal failure in various genetic and non-genetic animal models for both type 1 as well as type 2 diabetes. C21 ameliorates renal dysfunction by hindering the three most significant pathological pathways like NF-κB mediated inflammatory cascade, TGF-β mediated fibrosis and caspase mediated apoptosis which are the most important orchestrators of renal damage (Fig. 2). Recent reports have also suggested a prominent role of C21 in reversing the posttranslational histone modifications like histone acetylation and its regulatory machinery including histone acetyl transferase, PCAF. This shows that C21 not only targets the genetic but also the epigenetic mechanisms involved in the pathogenesis of DN. Taken together, it could be concluded that C21, either alone or in combination with Angiotensin receptor blockers, is definitely a silver lining in the dark clouds of DN and further studies to check its clinical efficacy must be done to ascertain these speculations.

Conflict of interests

We declare that we have no conflict of interest.

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