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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