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Calorie restriction mimicking effects of roflumilast prevents diabetic nephropathy

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ABSTRACT

Little is known about role of PDE4 in the development and progression of diabetic nephropathy. Here, we investigated the effect of roflumilast, a selective PDE 4 inhibitor in type 1 diabetic nephropathy. Diabetes was induced in male Sprague–Dawley rats using streptozotocin (55 mg/kg). Diabetic rats showed elevated plasma glucose, blood urea nitrogen, creatinine and decrease in plasma albumin confirming signs of nephropathy. Roflumilast at 2 and 3 mg/kg normalized these alterations. Roflumilast also suppressed oxidative stress and deposition of an extracellular matrix protein such as fibronectin and collagen in kidney of diabetic rats. TUNEL assay revealed apoptosis in diabetic kidney than control and that roflumilast prevents this effect. We show that kidney of diabetic rats displayed a state of p-AMPK and SIRT1 deficiency and that roflumilast, interestingly, was able to restore their levels. Further, roflumilast prevented an increase in HO-1 and loss in the FoxO1 expression in diabetes. However, it did not improve the reduced NRF2 levels in diabetes. This is the first report to show that, like resveratrol and other SIRT1 activators, roflumilast also mimics calorie restriction effects through activation of AMPK/SIRT1 and protects against diabetic nephropathy. This study unveils the unexplored potential of roflumilast which can be used in treatment of metabolic disorders.

1. Introduction

Diabetes mellitus, a chronic metabolic disorder, often leads to number of micro and macro vascular complications. Among them diabetic nephropathy, is a major cause for end stage renal disease [1,2]. All renal cell types are affected by hyperglycemic injury including glomerular podocytes, mesangial and endothelial cells [3]. Glomerular basement membrane thickening, mesangial expansion, glomerular podocyte loss, tubular atrophy, interstitial fibrosis and arteriosclerosis are characteristic features involved in diabetic kidney disease [4,5].

Phosphodiesterases (PDE1–PDE11) are class of cyclic AMP or GMP degrading enzymes that are implicated in intracellular signalling of cytokines, hormones and neurotransmitters [6]. Weight of clinical data points out that PDE4 inhibition by roflumilast offer better protection than long acting bronchodilators and corticosteroids [7]. Wouters et al. for the first time, observed unexpected results that roflumilast improved fasting glucose and $HbA1_{ac}$ levels in patients with COPD and T2DM [8] but had no effect in patients with COPD without T2DM. This suggested that roflumilast has a glucose lowering potential. Further, an impressive studies in naive diabetic patients revealed the glucose lowering effects of roflumilast [9,10]. However, the precise mechanisms behind the favorable effects of roflumilast in T2DM are largely unknown.

Calorie restriction (CR) slows ageing and promotes longevity [11]. Previously, we and others have reported that CR appears to exert beneficial effect in preventing diabetic nephropathy [12,13]. The molecules through which CR confers protection are SIRT1 (silent information regulator 2) and AMPK (AMP activated protein kinase) [14]. AMPK is also required for SIRT1 activation and vice a versa. Overall, an activation of AMPK/SIRT1 leads to reduction in apoptosis, anti-inflammatory effects, regulation of glucose and lipid metabolism thus providing a therapeutic approach for diabetic nephropathy [15]. Data on whether SIRT1 or AMPK mediate the effect of PDE4 inhibition is unknown.

Multiple mechanisms of hyperglycemia induced adverse effects have emerged and of which oxidative stress is a potential cause for diabetic nephropathy. Regardless of oxidative stress, the antioxidant enzyme such as heme oxygenase-1 (HO-1) was found to be enhanced in type 1 diabetic kidney [16]. The precise mechanism of HO-1 activation is still not clear but some studies pointed out role of intra-renal angiotensin II [17]. FoxO1, is a transcription

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factor, play a role in cell survival and oxidative stress. In type 1 diabetic kidney FoxO1 expression was found to be decreased and was very well correlated with enhanced ECM proteins and oxidative stress [18]. However, little is known about crosstalk between PDE4, HO-1 and FoxO1.

Previously, the protective effect of PDE5 inhibition by vardenafil and sildenafil in kidney disease has been investigated [19,20]. But there are no reports on role of PDE4 inhibition in preventing the progression of diabetic nephropathy. Therefore we hypothesized that inhibition of PDE4 by roflumilast may impair the progression of diabetic nephropathy.

2. Materials and methods

2.1. Chemicals

All the other chemicals were purchased from Sigma (St. Louis, MO, USA), unless otherwise mentioned.

2.2. Animals

Institutional Animal Ethics Committee approved all the experiments which were performed and handling of experimental animals was done in accordance with CPCSEA guidelines. Type 1 diabetes was induced in male Sprague Dawley rats by injecting a single dose of STZ (55 mg/kg) as described previously [21] and the animals which had glucose >16.7 mmol/l were considered as diabetic. Diabetic animals were divided into three groups, namely diabetic control (DC) ($n = 6$), diabetic/treated with Roflumilast (R2) (2 mg/kg) ($n = 6$) and diabetic/treated with Roflumilast (R3) (3 mg/kg) ($n = 6$) 2 weeks after treatment with STZ. Age-matched control group ($n = 6$) was maintained along with these groups. Treatment with roflumilast was started from the third week and continued till the end of the eighth week (6-week treatment). Each animal in the control group received vehicle (4% aqueous methocel solution containing 20% polyethylene glycol) (2 ml/kg/day, per oral).

2.3. Estimation of plasma glucose, albumin, blood urea nitrogen and creatinine

Estimation were performed as described previously [12].

2.4. Assessment of renal oxidative stress markers

Activities were measured as described previously [21].

2.5. Protein isolation and western blotting

Western blotting was done as described previously [22].

2.6. Histology and Immunohistochemistry

Histology and immunohistochemistry was performed as described previously [21].

2.7. TUNEL assay

TUNEL assay was performed as described previously [22].

2.8. Real time PCR

Real time PCR was performed using SYBR master mix (Invitrogen, CA) and the specific primers (Eurofins, USA) (HMOX1 or HO-1*: Forward-TGCTGACAGAGGAACACAAA, Reverse-ACAGAG TTCACAGCCTCTGG*); (NRF2: *Forward-AGCATGATGGACTTGGAATTG, Reverse-CCTCCAAAGGATGTCAATCAA); (FoxO1: Forward-ATGGGCCCT AACTCAGTCAT, Reverse-GAAGTTTGCTGTGCATGTCC)* as described previously [23].

2.9. Statistical analysis

Experimental values were expressed as mean ± s.e.mean. Mean values of different groups were compared by one-way analysis of variance and multiple comparisons among different groups were done by Tukey's test.

3. Results

3.1. Roflumilast treatment improves kidney function and alleviates oxidative stress

In this study, the STZ-treated rats developed uncontrolled type 1 insulin-dependent diabetes mellitus as all the rats exhibited hyperglycemia, glycosuria, polyuria and increased water consumption. Diabetic rats showed significant reduction in the body weight and kidney weight and kidney weight/body weight ratio was significantly increased than control rats. This may be due to excessive deposition of extracellular matrix proteins. These observations matched with our previous reports [21,24]. Roflumilast treatment attenuated the weight loss, reduced kidney weight and kidney weight/body weight ratio in diabetic rats (Fig. 1A–C). We observed a significantly reduction in plasma glucose levels in diabetic rats after roflumilast treatment ($P < 0.01$, Fig. 1D). In addition, roflumilast reduced the elevated blood urea nitrogen (BUN) levels and creatinine levels in diabetic rats (Fig. 1E and F). Further, roflumilast was able to normalize plasma albumin levels in diabetic rats (Fig. 1G). Maintenance of these biochemical variables closer to those in control rats by roflumilast treatment suggests that PDE4 plays a role, either directly or indirectly, in providing protection against diabetic nephropathy or delay in its development. As expected, diabetic rats showed marked reduction in GSH activity and increase in MDA levels. Roflumilast treatment significantly improved GSH activity and suppressed MDA levels in diabetic rats (Fig. 1I–J).

3.2. Roflumilast prevents extracellular matrix proteins accumulation

Glomerular hypertrophy, glomerular injury, increased glomerular space and tubular vacuolations are prominent features of diabetic kidney disease. Immunohistochemistry analysis revealed an increased accumulation of extracellular matrix proteins like type IV collagen and fibronectin in kidney of diabetic rats which is a major change that occurs as a result of inflammation and oxidative stress. Diabetic rats subjected to roflumilast treatment showed markedly less accumulation of collagen and fibronectin (Fig. 2A and B). In addition specific staining for collagen by Picro-Sirius red also made evident that roflumilast treatment effectively reduced accumulation of extracellular matrix proteins (Fig. 2C).

3.3. Roflumilast repress the glomerular damage and apoptosis in diabetic kidney

Mayer's hematoxylin and eosin staining of kidney sections revealed a marked microscopic change like increased glomerular space and tubular vacuolations in diabetic rats than control rats (Fig. 3A). This incidence and intensity of tubular vacuolations and glomerular hypertrophy as well as other degenerative features were alleviated in roflumilast treated diabetic rats. Apoptosis of

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