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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.

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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

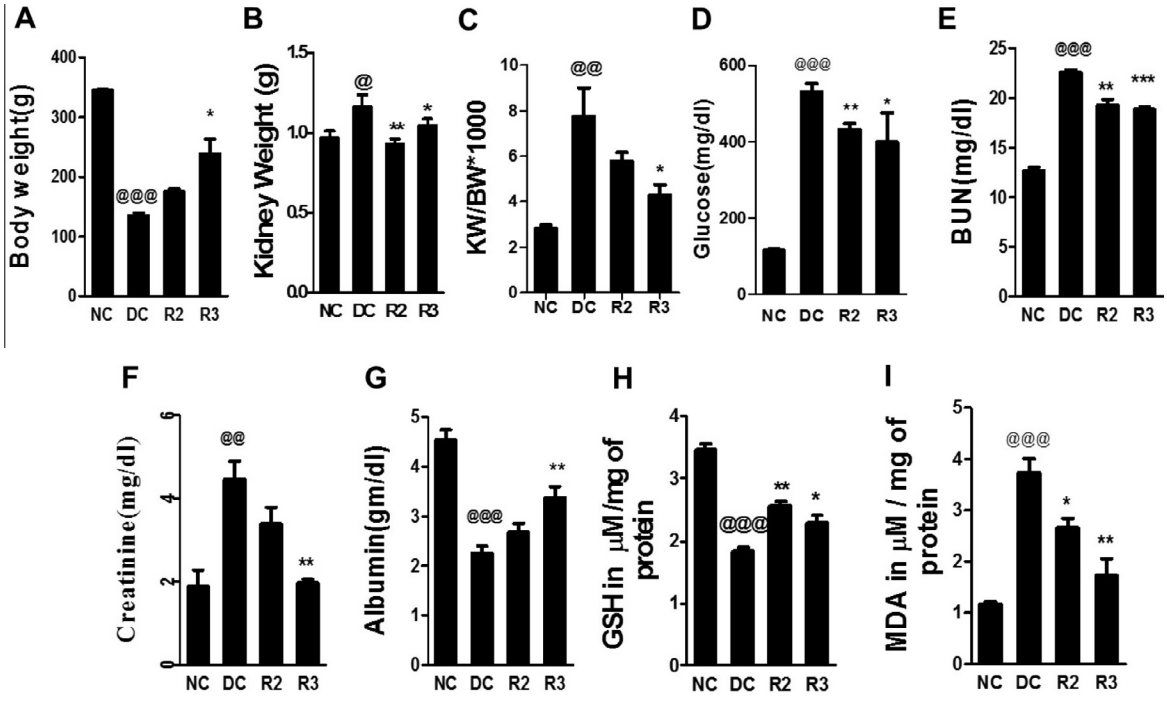

Fig. 1. Roflumilast treatment improves kidney function and alleviates oxidative stress in STZ induced diabetic nephropathy: Panel A shows body weight, kidney weight (B), kidney weight (KW)/body weight (BW) * 1000 (C) of animals measured after 6 weeks of treatment. Panel D indicates the plasma glucose levels, blood urea nitrogen (BUN) levels (E), plasma creatinine levels (F), plasma albumin levels (G), GSH (glutathione) levels (H) and MDA (malondialdehyde) (I) levels in the kidney homogenates of control, diabetic and diabetic/roflumilast treated (2 and 3 mg/kg) animals. *Abbreviations are:* NC, normal control animals; DC, streptozotocin induced diabetic rats; R2, diabetic rats treated with roflumilast 2 mg/kg; R3, diabetic rats treated with roflumilast 3 mg/kg, $^{@@@}P < 0.001$, $^{@@}P < 0.01$, $^{@}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ $^{@}$Vs NC and *Vs DC.

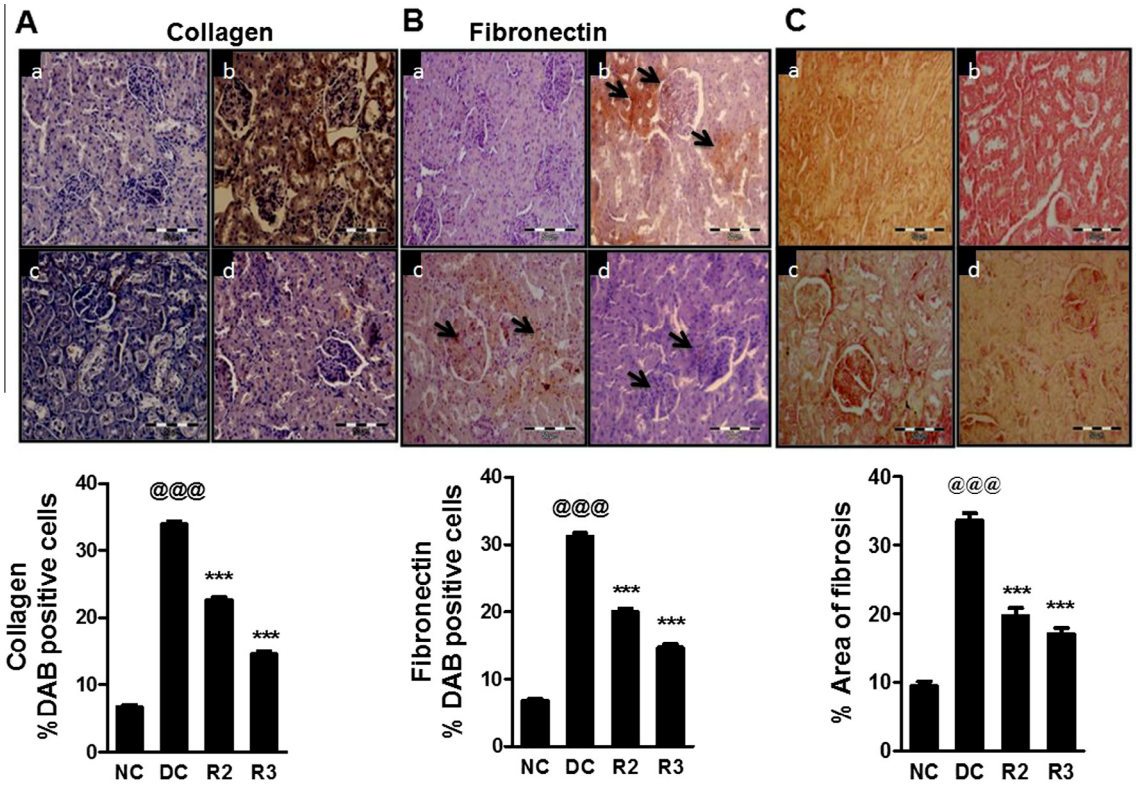

Fig. 2. Roflumilast alleviates accumulation of Extracellular matrix proteins: Panel A shows light microscopic pictures illustrating the immunostaining for collagen and quantification of collagen positive cells in the kidney sections. Panel B shows light microscopic pictures illustrating the immunostaining for fibronectin and quantification of fibronectin positive cells in the kidney sections. Panel C shows sirius red stained microscopic sections of kidney sections of (a) normal control, (b) diabetic control, (c) roflumilast treated (2 mg/kg), (d) roflumilast treated (3 mg/kg) animals respectively. All values are represented as mean ± SEM from at least 25 sections per group. $^{@@@}P < 0.001$, $^{@@}P < 0.01$, $^{@}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ $^{@}$Vs NC and *Vs DC.

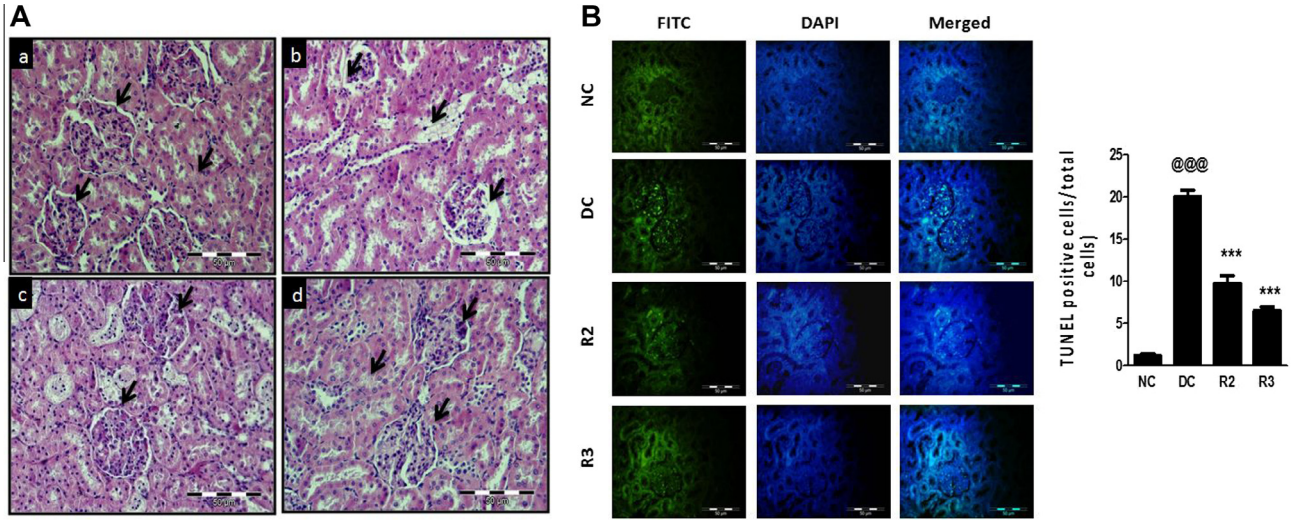

Fig. 3. Roflumilast treatment prevents kidney damage and Apoptosis in the kidney of diabetic rats: Panel A shows H & E staining of kidney sections and panel B shows TUNEL images of kidney sections showing apoptotic renal cells from (a) normal control (NC), (b) diabetic control (DC), (c) diabetic/roflumilast treated (2 mg/kg) (R2), (d) diabetic/roflumilast treated (3 mg/kg) (R3) and quantitative bar graph of mean apoptotic cells per 100 renal cells. All values are represented as mean ± SEM from at least 25 sections per group $^{@@@}P < 0.001$, $^{@@}P < 0.01$, $^{@}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ $^{@}$Vs NC and *Vs DC.

podocytes and tubular cells as a result of hyperglycemia induced oxidative stress and inflammation is one of the major degenerative process that leads to loss of kidney function. TUNEL assay was employed to assess apoptosis and green colored TUNEL positive cells were considered apoptotic. Diabetic kidney sections showed significantly high number of TUNEL positive cells than control and roflumilast treatment led to significant decrease in number of TUNEL positive cells than diabetic rats (Fig. 3B). This suggest that roflumilast treatment prevents apoptosis in the kidney of diabetic rats, thus, conferring protection against diabetic nephropathy.

3.4. Roflumilast restores AMPK/SIRT1 levels and alleviates diabetes induced stress by normalizing heme oxygenase-1 (HO-1) and FoxO1

In order to investigate the effect of PDE4 inhibition on expression of AMPK/SIRT1 we checked their expression. Previously, we have reported that diabetes is a state of AMPK and SIRT1 deficiency [22,23]. As expected, we found that the levels of p-AMPK and SIRT1 in diabetic kidney were significantly reduced. Interestingly, diabetic rats subjected to roflumilast treatment restored the levels of p-AMPK and SIRT1 (Fig. 4A–C). Further, we have checked expression of IL-1β in diabetic and roflumilast treated rats. As expected, diabetes promoted an increase in expression of IL-1β and inflammation than control rats (Fig. 4A and D). Roflumilast treatment normalized the levels of IL-1β confirming anti-inflammatory property of roflumilast. Recent studies showed that an induction of HO-1 with hemin improved glucose metabolism and glomerulosclerosis in diabetic rats [25]. We hypothesized that this physiological response may be impaired in diabetic nephropathy. Surprisingly, we observed a marked increase in an expression of HO-1 in diabetic kidneys than control ($P < 0.01$, Fig. 4E). However, roflumilast treatment prevented the HO-1 induction in diabetic rats. Next, we checked an expression of inducer of HO-1 that is nuclear factor E2 related factor 2 (NRF2). Interestingly, we found that the diabetes significantly decreased the NRF2 expression ($P < 0.01$, Fig. 4F). Further, roflumilast treatment had no effect on NRF2 expression in diabetic rats. Next, we checked an expression of Forkhead 1 transcription factor (FoxO1) in kidney of all the groups. We observed that the FoxO1 expression was significantly reduced in kidneys of diabetic rats than control rats (Fig. 4G) and roflumilast significantly increased its expression.

4. Discussion

In diabetes, a chronic hyperglycemia triggers complex pathways which elevate oxidative stress and increase the production of various inflammatory molecules, which results in various complications including kidney dysfunction which ultimately leads to end stage renal disease [12]. Gradual loss of kidney function in diabetes leads to accretion of blood urea nitrogen and creatinine. Further due to protein degradation (podocyte loss) plasma albumin levels decreases in diabetic rats. Extracellular matrix proteins like type IV collagen and fibronectin accumulate in the diabetic kidney due to inflammation and oxidative stress [21,24]. We show that roflumilast treatment ameliorated the diabetic nephropathy condition by reducing blood glucose, blood urea nitrogen, creatinine and increasing plasma albumin levels. This favorable effect of roflumilast may be due to increase in creatinine clearance or reduced kidney protein degradation. Further, we observed that deposition of extracellular matrix protein was significantly reduced by roflumilast treatment. This can be well explained if we assume that reduction in hyperglycemia by roflumilast was responsible for its beneficial effects. However the mechanism by which roflumilast exerts glucose lowering effects is largely unknown. Previous studies have observed that roflumilast increases levels of GLP-1 and fructosamine which are indicators of good glycemic control in T2DM and however, data on glucose lowering effects of PDE4 inhibition in type 1 diabetes needs an investigation [6].

Calorie restriction (CR) is reported to have robust beneficial effects in cancer, neurodegenerative disorders, cardiovascular as well as in renal diseases [26]. Several possible mechanisms responsible for such advantageous effects of CR have been reported and one of them is an increase in the activity of SIRT1 and AMPK [27]. To harness the beneficial effects of calorie restriction, an intense research has been done to develop calorie restriction mimetic agents. Resveratrol, has gained much attention as it confers beneficial effects via activation of AMPK and SIRT1 [28] A recent report suggests that resveratrol nonselectively binds to PDEs (1, 3 & 4) and increases the levels of cAMP which leads to an increase in intracellular calcium and eventually activates AMPK [29]. Therefore, it was thought that to mimic CR all PDEs, at least 1, 3 & 4 needs to be inhibited. The question arose that can a just PDE (PDE4) inhibitor would mimic all CR effects of resveratrol. In this direction evidence came from the study that

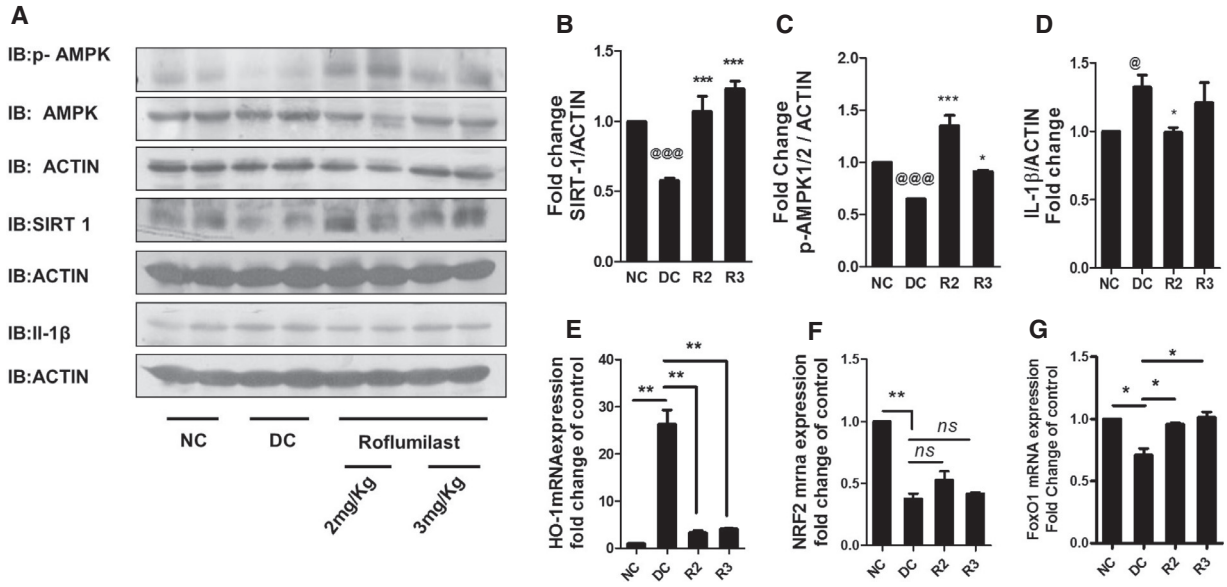

Fig. 4. Roflumilast restores p-AMPK/SIRT1, normalizes HO-1and restores FoxO1 expression in the kidney of diabetic rats: Panel A shows western blot of SIRT1, p-AMPK, AMPK and IL-1β using the whole kidney homogenates of normal control, diabetic control and diabetic/treated (2 and 3 mg/kg), and Panel B, C, and D shows quantitative bar graph of SIRT1, AMPK and IL-1β respectively using Image J software. Panel E represents changes in mRNA levels of HO-1 expression, panel F represents changes in mRNA levels of NRF2 and G represents changes in mRNA levels of FoxO1 in normal control, diabetic rats and diabetic/treated (2 mg/kg and 3 mg/kg).Each value is represented as mean ± SEM, n = at least 3 independent sets of experiments). $^{@@@}P < 0.001$, $^{@@}P < 0.01$, $^{@}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ $^{@}$Vs NC and *Vs DC.

alone PDE4 inhibition by rolipram increased the expression of AMPK/SIRT1 and was able to mimic the qualitative effects of resveratrol as well as to improve glucose tolerance in obese mice [30]. Although AMPK is abundantly expressed in all renal cell types but its action in kidney is less understood. It has been observed in an experimental models of diabetes that AMPK activity and expression were reduced [31]. Pharmacological activation of AMPK prevents tubular cellular hypertrophy *in vitro* as well as *in vivo*. SIRT1 is preferentially expressed in inner medulla and renal interstitium and has important role in renal cell survival. SIRT1 exerts antioxidant effect by up regulating catalase and cyclooxygenase-2 in mouse renal cells [32]. Further, renal specific overexpression and pharmacological activation of SIRT1 prevents renal apoptosis and fibrosis [33,34]. In our study, we observed that roflumilast prevents renal hypertrophy and this was associated with increased levels of p-AMPK and SIRT1 expression. These interesting results raise possibility that AMPK/SIRT1 might be interacting directly or indirectly with ECM proteins and play a role in preventing diabetic nephropathy.

Plethora of cytoprotective mechanisms are triggered during tissue injury and stress which includes stress proteins such as HO-1 [35,36]. HO-1 is a microsomal enzyme with inducible (HO-1) and constitutive (HO-2) forms which catalyses breakdown of heme yielding cytoprotective products like billiurubin, ferritin and carbon monoxide [37]. It has been demonstrated that the expression of HO-1 increases in kidney of type 1 diabetic rats [38]. The products of HO-1 reaction can be very toxic at high concentration but within physiological range they may have antioxidant, anti-inflammatory and anti-apoptotic properties [37]. In our study, we also observed a drastic increase in HO-1 expression which could be toxic and roflumilast treatment prevented an increase in HO-1 expression [25]. Previously, AT_1 receptor antagonist and antioxidants prevented an increase in HO-1 expression in radiation and STZ induced nephropathy [16,17]. Taken together, our data suggest that HO-1 expression is induced in diabetic nephropathy as a result of oxidative stress and roflumilast alleviates this stress hence HO-1 expression.

HO-1 expression is mediated by various intracellular cascades such as transcription factor like activator protein-1, nuclear factor kappa β (NFkβ), NRF2 and FoxO1 [37]. Several studies determined the tissue specific expression of NRF2 in diabetes [39]. Importantly, diabetes has been shown to decrease the activity of NRF2 [40,41] and this could be the reason behind increased oxidative stress and nephropathy. However, roflumilast treatment had no effect on NRF2 expression. This indicates that HO-1 was induced in diabetes independent of NRF2. Another transcription factor which induces HO-1 is FoxO1 [18]. Diabetes has been shown to decrease the FoxO1 expression which can be very well correlated with increased oxidative stress and accumulation of ECM proteins [42]. In our study we also found a similar decrease in expression of FoxO1 and roflumilast prevented this effect. This raises an interesting question that if NRF2 and FoxO1 expression is decreased in diabetes then how HO-1 was induced? What is its role? Further studies in this direction are definitely needed to unveil this interaction.

In conclusion, we propose that alone PDE4 inhibition improves progression and development of diabetic nephropathy. We also propose that, like resveratrol, PDE4 inhibitor roflumilast can be used to mimic to effects of CR and for treatment of metabolic disorders.

Author contributions

K.T. designed, supervised and approved the final version of manuscript. S.L. performed biochemical and histological experiments. P.K. performed western blotting experiments and wrote the manuscript. S.K. performed real time PCR experiments and wrote the manuscript.

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