Abstract. The authors previously reported that pigment epithelium-derived factor (PEDF) protects the diabetic kidney from fibrosis via its anti-oxidative effects. However, the underlying molecular mechanism has never been revealed. The present study aimed to investigate how PEDF protects mesangial cells from diabetes induced damage. Human mesangial cells were exposed to a diabetic environment [30 mmol/l glucose and 200 mg advanced glycation end products (AGEs)] in the absence or presence of PEDF (200 mg/l). The superoxide and peroxynitrite productions were measured by fluorescent assay. The nicotinamide adenine dinucleotide phosphate (NAPDH) oxidases (NOXs; isoforms NOX1, NOX2, and NOX4), NADPH oxidase organizer 1 (NOXO1), DHFR, endothelial nitric oxide synthase (iNOS) and phospho-p38MAPK (p-p38) protein levels were also examined to explore the possible mechanism of the PEDF anti-oxidative properties. The fibrogenesis of mesangial cells in diabetes was associated with increased superoxide generation and peroxynitrite production via iNOS induction and uncoupling. However, elevated transforming growth factor-β level, reactive oxygen species (ROS) overproduction, iNOS induction and uncoupling were all reversed by NOXO1 suppression following PEDF treatment or NOXO1 silencing. Furthermore, the p38MAPK inhibition only attenuated the ROS/peroxynitrite production partially via abolishment of iNOS induction, however had no effect on iNOS uncoupling and its regulating enzyme: DHFR suppression. PEDF prevented oxidative stress and protected mesangial cells from fibrogenesis in a diabetic environment via dual effects mediated by NOXO1 inhibitory prevention of iNOS induction through p38MAPK inactivation and effects on iNOS coupling through DHFR restoration.

Introduction
Diabetic nephropathy (DN) is a leading cause of end-stage renal failure worldwide. The pathogenetic mechanisms for DN may start with reactive oxygen species (ROS) as the common link connecting to various signaling pathways that lead to kidney impairment (1-4). The ROS are a family of molecules including superoxide anion (O2-), hydroxyl radical (HO·), hydrogen peroxide (H2O2), peroxynitrite (ONOO-), nitric oxide (NO) and lipid radicals. In mammalian cells, potential origins of ROS generation include mitochondrial respiratory chain, xanthine oxidase, NOXs, and nitric oxide synthase (NOS), with increasing evidence that NOXs appear to be the major sources or more specifically initial trigger of ROS generation in the diabetic complications including kidney (5-7).

Various subtypes of NOXs (e.g., NOX1, NOX2, NOX4) are discovered abundantly in the mesangial cells (8). Lee et al (9) showed that mitochondrial derived ROS was elevated in DN subsequent to NOX4 activation. Although NOX4 is likely implicated in the mediation of kidney fibrosis in diabetes including mesangial impairment, other subtypes such as NOX1, NOX2 and their cytosolic component NADPH oxidase organizer 1 (NOXO1) are also possible candidates involved in this pathogenesis (10). Additionally, other researches reported that NOS uncoupling instead of mitochondrial dysfunction in the mesangial cells is responsible for the early glomerular pathology in diabetes and is associated with inflammation and oxidative stress of DN (11). Thus, the downstream of NOXs activation or the main source of oxidative stress is still in debate and should be under extensive investigation. Recently, both in vivo and in vitro studies have demonstrated that nitrooxidative/nitrosative stress (ONOO-) has been actively involved in the development of DN or the hyperglycemia induced damage in mesangial cells (12). Therefore, NOS uncoupling as the most possible cause of ONOO- production should be examined. It is well known that NOS coupling status is governed by its cofactor tetrahydrobiopterin (H4B) abundance, for instance: when H4B is deficient, NOS becomes dysfunctional to produce superoxide instead of NO which changes into ONOO- on site instantly and vastly. Moreover, H4B synthesis is controlled by dihydrofolate reductase (DHFR), the key enzyme responsible for H4B salvage pathway, whose expression level would determine the abundance of H4B. Notably, DHFR suppression...
could play an important role in the development of DN via dysfunctional NOS and restoration of DHFR may well be the target for antioxidant treatment.

As an antioxidant, epithelium-derived factor (PEDF) is a glycoprotein that belongs to the superfamily of serine protease inhibitors, which is assumed to have beneficial effects on retinopathy. It was first retrieved from the culture media of human retinal pigment epithelial cells which possessed potential neuronal differentiating activity. PEDF is expressed broadly in human tissues, including the kidney. Previous studies have shown that decreased PEDF levels in the kidney are implicated in DN, especially in the early stage (13). It is reported that the injection of adenovirus expressing PEDF recombinant gene significantly reduced the albuminuria and the production of extracellular matrix (ECM) protein, and ameliorated glomerular hypertrophy in the diabetic kidney (14). Although, according to previous studies, it is assumed that PEDF protects the renal function from diabetic injury, via its anti-oxidative and anti-fibrogenic activities, the particular mechanism has not been fully illustrated (15-18). Therefore, the purpose of the present study is to investigate how PEDF protects the kidney from oxidative stress and determine whether this beneficiary effect is through rectifying dysfunctional iNOS and de-activation of NOXs including NOXO1.

Materials and methods

Cell culture. The use of human glomerular mesangial cells (HMCs; Xiangya School of Medicine, Central South University, Changsha, China) in the present study was approved by the Ethics Committee of Renmin Hospital of Wuhan University (Wuhan, China) which was in adherence with the Declaration of Helsinki. Dulbecco Modified Eagle's Medium (DMEM) were used to culture HMCs. Cells of passages 3-6 were used and after reaching nearly confluence, they were quiesced with serum starvation for 48 h, and then exposed to different conditions without elevated glucose or advanced glycation end products (AGEs) in the presence or absence of PEDF for 48 h. In some experiments, the cells were also treated with 20 μM SB203580 for p38MAPK inhibition. Bovine serum albumin (BSA; 200 mg/l) was used as a control for AGE treatment. The PEDF was purchased from Peprotech (Princeton, USA); AGES were purchased from Jiamay Biotech (Beijing, China); the rest mediums were also treated with 20 µM SB203580 for p38MAPK inhibition, whereas H2B and oxidized biopterin were measured by alkali oxidation. The ratio of H4B over H2B was calculated subjected to differential oxidation in acidic or alkalytic solutions (19). In summary, total biopterin (H4B, dihydropterin [H2B], and oxidized biopterin) were determined by acid oxidation, whereas H2B and oxidized biopterin were measured by alkali oxidation. The ratio of H4B over H2B was calculated and used as an index for DHFR activity.

HPLC-based H4B measurement. HMCs were lysed by trichloroacetic acid containing 10 mmol/l DTT. Lysates were subjected to differential oxidation in acidic or alkaline solutions (19). In summary, total biopterin (H4B, dihydropterin [H2B], and oxidized biopterin) were determined by acid oxidation, whereas H2B and oxidized biopterin were measured by alkali oxidation. The ratio of H4B over H2B was calculated and used as an index for DHFR activity.

Statistical analysis. One-way ANOVA was used to test for statistical significance, and P<0.05 was considered to indicate a statistically significant difference. All data shown in the figures were presented as means ± SD.

Results

PEDF abolishes iNOS upregulation induced by diabetic environment via p38MAPK inhibition. Both p38MAPK and iNOS protein levels were upregulated in diabetic environment (Fig. 1). However, p38MAPK and iNOS upregulations were annihilated by PEDF treatment (Fig. 1A-C). Moreover, p38MAPK inhibitor, SB203580 abolished iNOS induction completely but only canceled ONOO⁻ formation partially (Fig. 1E-H). In comparison, iNOS induction, peroxynitrite and TGF-β overproduction were resumed back to the normal level by PEDF (Fig. 1A-H).

PEDF reverses iNOS uncoupling induced by diabetic environment. H4B is the cofactor for iNOS and its abundance decides the coupling status of iNOS. DHFR is a key enzyme of H4B
salvage pathway which could modulate iNOS coupling status via regulation of H4B synthesis (Fig. 2). Interestingly, DHFR protein level was downregulated in diabetic environment and PEDF retrieved DHFR protein level back to basal level (Fig. 2A and C). As a result of DHFR reduction in diabetes environment, the H4B contents and H4B/H2B ratio were diminished (Fig. 2E-H), but PEDF restored H4B level as well as H4B/H2B ratio in accordance with upregulation of DHFR protein level (Fig. 2E, G). However, SB203580 had no effect on DHFR protein expression, H4B contents as well as H4B/H2B ratio (Fig. 2B, D, F and H).

PEDF acts independent of NOXs. Our previous study has shown that NOX1-iNOS pathway played a major role in diabetes induced oxidative stress of mesangial cells. However, this study showed that although NOX1, NOX2 and NOX4 were all upregulated in diabetes environment, PEDF had no effect on their protein levels (Fig. 3), suggesting that

Figure 1. Effects of PEDF or p38MAPK inhibition on iNOS and peroxynitrite production. Cells were cultured in 5.6 mmol/l glucose, 30 mmol/l glucose, 5.6 mmol/l glucose + 200 mg/l AGEs, 30 mmol/l glucose + 200 mg/l AGEs and 30 mmol/l glucose + 200 mg/l AGEs + 100 nmol/l PEDF for 48 h (A-D) or cells were cultured in 5.6 mmol/l glucose, 5.6 mmol/l glucose + 20 µmol/l SB203580, 30 mmol/l glucose + 200 mg/l AGEs, and 30 mmol/l glucose + 200 mg/l AGEs + 20 µmol/l SB203580 for 48 h (E-H). (A) p-p38MAPK, iNOS and β-actin protein levels were assessed by immunoblotting analysis; (B) Relative p-p38 MAPK blot density was analyzed and plotted; (C and F) The density analysis of iNOS/β-actin from immunoblots; (D and G) The peroxynitrite production was analyzed by HPF fluorescence assay; (E) p-p38MAPK, iNOS and β-actin protein levels were assessed by immunoblotting analysis in the presence or absence of SB 203580. (H) The TGF-β expression tested by ELISA. Data are expressed as the means ± standard error of the mean, n=3-5 and *P<0.05 as indicated. PEDF, pigment epithelium-derived factor; iNOS, nitric oxide synthase; AGEs, advanced glycation end products; TGF, transforming growth factor; BSA, bovine serum albumin; MAPK, mitogen activated protein kinase; p, phosphorylated.
PEDF may protect kidney at probably the downstream of NOX1 pathway. However, NOXO1, the adaptor protein for NOX1 was downregulated by PEDF suggesting that NOXO1 suppression could be responsible for the protective mechanism of PEDF.

**PEDF confers renal protection via NOXO1 inhibition.** Silencing NOXO1 with RNAi downregulated NOXO1 protein expression (Fig. 4) and reproduced the effect of PEDF on iNOS expression and coupling status. NOXO1 silencing abolished the iNOS suppression (Fig. 4A and C). Meanwhile, uncoupling of iNOS was also annihilated by NOXO1 silencing as demonstrated by measurement of DHFR expression (Fig. 4D), H4B contents, and H4B/H2B ratio (Fig. 4D and E). Consequently, in mesangial cells the protective effect of PEDF on oxidative stress (ONOO– overproduction, Fig. 4G) and kidney fibrosis (TGF-β production 4H) were also duplicated by NOXO1 silencing.

**Discussion**

The present study explored the possible mechanism or the molecular pathway of PEDF action on mesangial cells in diabetes. We found that PEDF was protective for mesangial cells in diabetic environment by reversing TGF-β overexpression, ONOO– overproduction, iNOS induction and uncoupling.
The ONOO⁻ derived from iNOS induction and uncoupling was completely abolished consequent to PEDF treatment via p38MAPK inactivation and restoration of DHFR protein level. However, those salutary effects of PEDF were mediated by NOXO1 but not individual NOXs downregulation. Taken together, our findings provided initial evidence to reveal novel mechanisms that PEDF prevented oxidative stress and protected mesangial cells from fibrogenesis in diabetic environment via dual effects converging at NOXO1 suppression: Retrivation of iNOS overexpression through p38MAPK inactivation and restoration of iNOS coupling through DHFR restoration.

Sheikpranbabu et al (21) have shown that PEDF significantly decreased NADPH oxidase and ROS generation in pericytes following AGE-BSA treatment. In the present study, we observed an attenuation of ROS production by PEDF in mesangial cells treated with high glucose and AGEs (diabetes environment). As NOX1, NOX2 and NOX4 are the three major NADPH oxidases existed in the mesangial cells and our previous study showed that the activation of NOX1 and its derived superoxide led to direct cell injury and initiated a chain of deleterious stress signaling such as iNOS induction and uncoupling. The effect of PEDF on NOXs protein level was examined (22). However, NOXs protein level was not altered by PEDF. Interestingly, NOXO1, the cytosolic regulation protein for NOXs (23), was suppressed by PEDF and downregulation of NOXO1 by RNA silencing reproduced the antioxidant effect of PEDF and its restoration effect on iNOS induction and uncoupling. All of these suggested that NOXO1 mediated the PEDF protective effect.

It has been demonstrated that p38MAPK activation is involved in the development of DN (24). However, the exact role of p38MAPK is not clear since it is traditionally considered as an anti-apoptotic signaling molecule. The p38MAPK activation is not self explanatory for the mesangial cells proliferation in the early stage of DN. Our data, among a few other researches suggested that p38MAPK is a pro-fibrogenetic rather than a pro-apoptotic molecule in high glucose induced mesangial damage (25,26).

Previously, it was suggested that p38MAPK was a possible upstream for iNOS and it has been reported that p38MAPK is activated in glomeruli isolated from streptozotocin-induced diabetic rats and glomerular mesangial cells cultured under high glucose conditions (27). Yuan et al (28) showed that high glucose (33 mM) caused activation of p38MAPK and inhibition for p38MAPK abrogated the high glucose induced iNOS expression, cell injury and levels of NO and nitrotyrosine in cultured human retinal pigmented epithelial cells. Therefore, activation of p38MAPK plays an important role in the iNOS induction and oxidative stress of DN. Recently, ATF-2 and NF-KB are suggested to be the possible downstreams or targets of p38MAPK whose activation may contribute to iNOS...
unregulation (29). In this study, we established that iNOS was induced in diabetes environment consequent to p38MAPK upregulation, but PEDF reversed the process. However, the p38MAPK inhibitor, SB203580 completely abolished iNOS induction but only partially canceled ONOO$^-$$^-$ and TGF-$\beta$ overproduction in diabetic environment. Therefore, we speculated that PEDF may protect kidney from diabetes via multiple pathways including p38MAPK and iNOS induction.

In the endothelial cells, PEDF has been shown to inhibit the cellular permeability via p38MAPK de-activation (30). While, PEDF also protected against retinal neovascularization by suppression of p38MAPK activation and dampening iNOS induction in the macrophage (31,32). Likewise, our data showed that PEDF, by targeting NOXO1, protected mesangial cells from fibrogenesis and nitroxidative/nitrosative stress, at least partly via abrogation of p38MAPK mediated iNOS induction.

It is well established that oxidative stress has a major role in the development of diabetic complications including DN. ONOO$^-$$^-$, as a notorious radical form created from the interaction between superoxide and NO, attacks various biomolecules in the tissues and induces damages in the microcirculations (33,34). Prabhakar et al (12) reported that renal ONOO$^-$$^-$ formation, which acts as a main contributor to oxidative stress of DN and accounts partly for decreased NO bioavailability of kidney, was elevated in the kidney homogenates of obese ZSF1 rats (~150% up) vs. lean ZSF rats. However, there is a lack of quantitative information of ONOO$^-$$^-$ about the formation and biological relevance because of its difficult and indirect measurements. Indeed, serum level of oxidative stress indicated by ONOO$^-$$^-$ is associated with the severity of diseases such as Parkinson’s (35).

Since iNOS is the main source for ONOO$^-$$^-$ overproduction, iNOS uncoupling could be another contributing factor for the...
nitrosative stress. Basically, when iNOS becomes uncoupled, it will fail to produce NO, and begin to produce superoxide which eventually culminates in ONOO$^-$ formation. H4B is the key co-factor for iNOS whose deficiency results in iNOS uncoupling. Indeed, DHFR (the enzyme responsible for H4B replenishment) is diminished in diabetes environment which consequently leads to H4B consumption (36). As we expected, PEDF relieved the suppression of DHFR and retrieved H4B contents (H4B/H2B ratio) in diabetes which eventually lead to iNOS recoupling. This effect is independent of p38MAPK pathway because SB203508, the p38MAPK inhibitor has no effect on DHFR suppression and H4B deficiency.

The deleterious effects of diabetes are attributed mainly to the formation of sugar-derived substances called AGEs. The AGEs formation is markedly accelerated in diabetes because of the increased availability of glucose. We always believe that the detrimental effects of diabetes are the combination of AGEs and elevated glucose, although ONOO$^-$ formation is majorly due to AGEs, not elevated glucose. Likewise, the AGE effect is more detrimental and advanced damage. On the other hand, the effect of high glucose is more of an acute damage and represents an early stage of oxidative stress such as NOX activation (37,38). Overall, AGEs represented the chronic effect of diabetic condition on renal impairment, and elevated glucose acted as an acute insult (within min). In our study, the cells were examined after 48 h during which the acute effect of elevated glucose may have faded away (so as the glucose concentration) and its extended effect may act via protein glycation which is AGEs-related. Nevertheless, it is important to expose the mesangial cells with elevated glucose and AGE together in order to mimic the in vivo diabetic condition.

NOXO1 is originally believed to be the cytosolic or inner component/subunit that solely regulates NOX1 activity/mediated superoxide generation, but is also recently found to interact with other NOXs such as NOX2, NOX4 and be required for their assembly and activation. Collectively, it is still unclear about NOXO1 regarding its function in the mesangial cells. The current data suggests that although NOX1, NOX2, NOX4 and NOXO1 are involved in the high glucose induced oxidative stress in the mesangial cells, only NOXO1 has a key role in the regulation of detrimental events. Moreover, since NOX1, NOX2 and NOX4 all possibly interact with NOXO1 (10,39), they could be linked to the effect of PEDF as a complex with NOXO1.

In summary, iNOS is the main source of ROS in diabetic kidney and PEDF protects against diabetic damage via iNOS suppression mediated by p38MAPK inactivation and iNOS recoupling via H4B restoration consequent to DHFR upregulation (Fig. 5). Therefore, we have verified the antioxidant effects of PEDF, which is consistent with previous studies. This

---

**Figure 5.** The dual mechanisms of PEDF on diabetic kidney protection. PEDF prevented oxidative stress and protected against fibrogenesis of mesangial cells in diabetic environment via dual effects mediated by NOXO1 inhibition: Annihilation of iNOS induction through p38MAPK inactivation and reactivation of iNOS coupling through DHFR restoration. PEDF, pigment epithelium-derived factor; iNOS, inducible nitric oxide synthase; NOXO1, nicotinamide adenine dinucleotide phosphate oxidase 1; AGE, advanced glycation end product; H4B, tetrahydrobiopterin; H2B, dihydropterin; DHFR, dihydrofolate reductase; TGF, transforming growth factor; NF, nuclear factor; GLUT, glucose transporter; MAPK, mitogen activated protein kinase; ATF, Activating transcription factor.
is the first time we clarify the anti-oxidation mechanism of PEDF on DN which could lead to the discovery of a potential target for PEDF and provide a theoretical basis for the clinical treatment of DN. It is noticed in this study that PEDF curbed the origination and progression of oxidative stress by NOXO1 suppression, but the mechanism of PEDF on the initial link of oxidative stress is still puzzled.

Acknowledgements

The present study is supported by National Natural Science Foundation of China (grant nos. 81170767 and 81571376 to Dr Gao) and Diabetes Study Fund from Chinese Medical Association (grant no. 13060906481 to Dr Gao) and Young Investigator Grant for diabetes study from Novo Nordisk (grant no. 2012 to Dr Gao).

References