

The PKC β -p66shc-NADPH oxidase pathway plays a crucial role in diabetic nephropathy

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Keywords

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Abstract

Objectives Oxidative stress plays a critical role in the pathogenesis of diabetic nephropathy (DN). p66shc is closely related to oxidative stress. However, the exact mechanism of its involvement in diabetic nephropathy is poorly understood. This study aimed to investigate the role of the p66shc-related pathway in diabetic nephropathy.

Methods In an in-vivo experiment, rats were injected with streptozotocin to induce early diabetic nephropathy. The treatment groups were an aminoguanidine group and an enzastaurin group. In an in-vitro experiment, human renal proximal tubule epithelial cells (HK-2 cells) were cultured and incubated with high glucose.

Key findings Upregulated protein expression of p66shc and p-p66shc was found in vivo and in vitro when cells were stimulated by high levels of glucose; this effect was accompanied by enhanced oxidative stress and damaged renal function, both of which were alleviated by p66shc siRNA. p66shc regulated NADPH oxidase, further promoting activation of oxidative stress. As an inhibitor of PKC β , enzastaurin reduced the abnormal expression of p66shc and NADPH oxidase and alleviated renal injury.

Conclusions This study demonstrated enzastaurin alleviated diabetic renal injury via modulation of the PKC β -p66shc-NADPH oxidase pathway, which provided a new perspective for the treatment of early DN.

Introduction

Diabetic nephropathy (DN) is a diabetic microvascular complication that is an important cause of death in diabetic patients. It has been reported that hyperglycaemia-induced oxidative stress plays an important role in inducing and aggravating inflammatory responses to diabetic kidney injury.^[1] Our previous studies demonstrated that reactive oxygen species (ROS) directly damaged some target proteins, aggravating inflammation of renal tissue, and inducing tubulointerstitial injury.^[2,3] Meanwhile, persistent elevated levels of oxidative stress in early diabetic nephropathy may accelerate the progression of nephropathy and even develop end-stage renal disease (ESRD).^[1] However, the mechanisms of cellular oxidative stress in the development of DN are not yet fully understood.

Migliaccio *et al.*^[4] found that p66shc gene-deficient mice had increased resistance to environmental stressors such as hydrogen peroxide and ultraviolet rays, while their life expectancy increased by 30%. Cheng YS *et al.* demonstrated that upregulation of p66shc increased the production of ROS, aggravated oxidative damage of cells and participated in myocardial injury, corpus cavernosum dysfunction and renal dysfunction.^[5–7] Diabetic nephropathy and ageing have a common mechanism, oxidative stress, and p66shc plays a key role in ageing-related diseases. Therefore, there is increasing recognition of the role of p66shc in DN. Oxidative stress, apoptosis and proliferation mediated by p66shc were involved in the onset and development of DN.^[8] However, the mechanism of p66shc regulation of oxidative stress-related cell injury and its role in DN have not been fully elucidated.

NADPH oxidase is the main source of ROS. In-vivo and in-vitro studies have confirmed that NADPH oxidase plays an important role in DN.^[9,10] Although some members of our group have reported that p66shc and oxidative stress participated in the pathological process of renal injury caused by AQP4 knockout^[5]; nevertheless, the relationship between p66shc and NADPH oxidase and the possible signalling pathways have not been clarified.

As a subtype of protein kinase C (PKC), PKC β is also involved in the pathogenesis of DN.^[11] In the present study, enzastaurin (Selleck Chemicals, Houston, TX, USA), a selective inhibitor of PKC β , was administered to rats to explore its efficacy and potential mechanism in the treatment of early DN, and aminoguanidine (Sigma-Aldrich, St. Louis, MO, USA), an inhibitor of advanced glycation end products (AGEs), served as a control drug for DN.

Based on the foregoing analysis, we proposed the following hypothesis: p66shc activates NADPH oxidase in early diabetic nephropathy, induces oxidative stress by generating ROS, then activates inflammatory factors and induces renal injury. PKC β is also involved in this pathological process.

Materials and Methods

Animal model

Male Sprague-Dawley (SD) rats were randomly divided into four groups. Except for the control group, the other animals were intraperitoneally injected with streptozotocin (STZ, 65 mg/kg, dissolved in citric acid buffer before use). Treatment started from the 5th to 8th weeks. The treatment groups included the aminoguanidine group (AG, 100 mg/kg, p.o.) and the enzastaurin group (Enza, 25 mg/kg, p.o.). The animal operating protocols were authorized by the Ethics Review Committees for Animal Experimentation of Southeast University (Permit Number: 0098).

Human renal proximal tubule epithelial cell (HK-2 cell) culture

The stable HK-2 cell line was purchased from China Infrastructure of Cell Line Resources. DMEM/F12 medium containing 10% fetal bovine serum (FBS) was used for culture. When cell proliferation occupied 80% of the bottom area of culture flask, the cells were digested and passaged with 0.25% trypsin.

p66shc siRNA transfection

HK-2 cells were passaged on 24-well plates at 1×10^5 cells per well. Then, the DNA–transfection reagent complex was

prepared. Then, the DNA–transfection reagent complex was added to the culture plate and was shaken gently. Western blot was performed to determine transfection efficiency.

Biochemical assays

Levels of serum glucose, blood urea nitrogen (BUN) and urine protein (LDH) and γ -glutamyltranspeptidase (γ -GT), malondialdehyde (MDA) and superoxide dismutase (SOD) were detected. All measurement operations were carried out strictly according to kit (Nanjing Jingcheng Bioengineering Institute, Nanjing, China) instructions.

Detection of ROS level in HK-2 cells

The cells in 24-well culture plates were synchronized for 24 h. Then, dihydroethidium (DHE; 10 μ mol/l) was added into each well and avoided light incubation for 30 min at 37 °C. After incubation, the cells were washed and observed with fluorescence microscope (Olympus IX71, Tokyo, Japan).

Immunochemical assay

Renal specimens were fixed by 10% neutral formalin and embedded by paraffin. 5 μ m thick sections of specimens were used to perform immunohistochemical assay. The slices were placed in citric acid buffer solution and boiled for 15 min to restore the antigen. And primary antibody against p66shc (1 : 150; Santa Cruz, Dallas, TX, USA) was incubated with the section at 4 °C for 12 h.

Transmission electron microscopy

Cells were fixed with 2.5% glutaraldehyde and 1% osmium. After being dehydrated with gradient concentration of alcohol and acetone, the cells were soaked and embedded with epoxy resin. The samples were stained with uranium acetate and lead citrate and were observed under a transmission electron microscope (JEM-1010, JEOL, Ltd. Tokyo, Japan).

Western blot

RIPA lysis buffer was used to extract proteins. After SDS-PAGE electrophoresis, proteins were transferred to PVDF membranes. The membranes were incubated with diluted primary antibody (β -actin, p22phox, NOX4, p-p66shc, p66shc, PKC β and ET $_A$; Santa Cruz; iNOS, IL6 and TNF- α ; Cell Signaling Technology, Danvers, MA, USA) and secondary antibody for 1–2 h at 37 °C. Signals were visualized using an ECL chemiluminescence detection kit.

Statistical analysis

All data were expressed as the mean \pm SD and were analysed with SPSS 11.5 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance was used first, and then, we performed the Student Newman–Keuls test or Games–Howell tests depending on whether the variances were equal. $P < 0.05$ was considered significant.

Results

Damaged renal function and increased oxidative stress in diabetic rats

Average blood glucose levels in the diabetic model group (HG group) increased to 29.16 mmol/l ($P < 0.01$), while enzastaurin did not effectively inhibit blood glucose levels. Simultaneously, 24-h urine protein levels increased to 504.13% in the HG group compared to those of the control group (NG group; $P < 0.01$). The blood urea nitrogen increased to 186.68% in the HG group ($P < 0.01$). The levels of LDH and γ -GT in the renal tissue of the HG group were significantly increased to 308.43% and 256.97%, respectively ($P < 0.01$), indicating that the renal function of the HG group was seriously damaged. After treatment with enzastaurin, renal function was restored ($P < 0.05$ or $P < 0.01$; Table 1).

Compared with the NG group, SOD activity in serum of the HG group decreased to 66.35%, and MDA level increased to 650.73% ($P < 0.01$). Enzastaurin treatment significantly alleviated oxidative stress levels in diabetic rats ($P < 0.05$ or $P < 0.01$; Table 1).

High levels of glucose induced cell injury and increased oxidative stress in HK-2 cells

HK2 cells were divided into the normal glucose incubating group (5.5 mM glucose, NG group) and the high levels of glucose incubating group (30 mM glucose, HG group).

Activity of γ -GT and LDH in cell supernatants was significantly higher in the HG group than those in the NG group ($P < 0.01$), indicating that the high levels of glucose stimulation caused cell damage (Figure 1a and 1b).

DHE staining was used to measure the levels of ROS in HK2 cells. The fluorescence intensity of HK2 cells in the HG group was significantly higher than that in the NG group ($P < 0.01$), indicating that a large amount of ROS was produced in the HK2 cells under the stimulation of high levels of glucose (Figure 1c). Simultaneously, the content of MDA in the HG group was also significantly higher than that in the NG group ($P < 0.01$; Figure 1d).

High levels of glucose induced upregulation of p66shc in renal tissue and HK2 cells

The expression of p66shc in renal tissue was detected by immunohistochemistry. Results showed that the region of p66shc expression was mainly located in the renal tubules. In the HG group, the ratio of positive staining of p66shc in the renal tissue was significantly higher than that in the NG group ($P < 0.01$). After treatment with enzastaurin, ratio of positive staining of p66shc was decreased ($P < 0.01$; Figure 2a). The result was consistent with protein expression of p66shc detected by Western blot (Figure 2b).

In in-vitro experiment, protein expression levels of p66shc and p-p66shc increased gradually with increasing glucose concentrations (5–45 mM) or incubation time interval (0, 12, 24, 48 h; $P < 0.01$), further demonstrating that p66shc was involved in pathological processes in HK2 cells induced by high levels of glucose (Figure 2c, 2d, 2e).

p66shc siRNA relieved cell damage and oxidative stress in HK2 cells

To further confirm the role of p66shc in HK2 cell damage induced by high levels of glucose, an RNA interference-mediated knockdown of p66shc was performed in HK2

Table 1 Streptozotocin treatment significantly caused increased levels of blood glucose, 24-h urine protein, blood urea nitrogen, malondialdehyde, as well as increases in LDH and γ -GT in the renal tissue, and reduced superoxide dismutase in serum in rats

	NG	HG	HG + AG	HG + Enza
Blood glucose (mmol/l)	7.13 \pm 1.36	29.16 \pm 2.33**	27.08 \pm 2.99	28.27 \pm 3.16
Urine protein (mg/24 h)	1.40 \pm 0.24	7.07 \pm 1.19**	5.13 \pm 0.55 [#]	5.36 \pm 0.57 [#]
BUN in serum (mmol/l)	7.96 \pm 1.02	14.86 \pm 2.95**	11.59 \pm 1.29 [#]	12.26 \pm 1.57 [#]
LDH in tissue (U/gprot)	1398.58 \pm 263.88	4313.61 \pm 657.34**	2080.15 \pm 250.60 ^{##}	2568.04 \pm 345.42 ^{##}
γ -GT in tissue (U/gprot)	21.80 \pm 3.13	56.02 \pm 6.19**	28.27 \pm 4.00 ^{##}	34.66 \pm 4.03 ^{##}
SOD in serum (U/ml)	150.39 \pm 12.58	99.79 \pm 14.55**	131.28 \pm 9.93 ^{##}	126.95 \pm 16.87 ^{##}
MDA in serum (nmol/ml)	2.72 \pm 0.31	17.70 \pm 2.72**	9.05 \pm 1.39 ^{##}	11.70 \pm 1.41 ^{##}

These abnormal changes were relieved by either aminoguanidine or enzastaurin. Mean \pm SD, $n = 8$. * $P < 0.05$, ** $P < 0.01$ vs NG; [#] $P < 0.05$, ^{##} $P < 0.01$ vs HG.

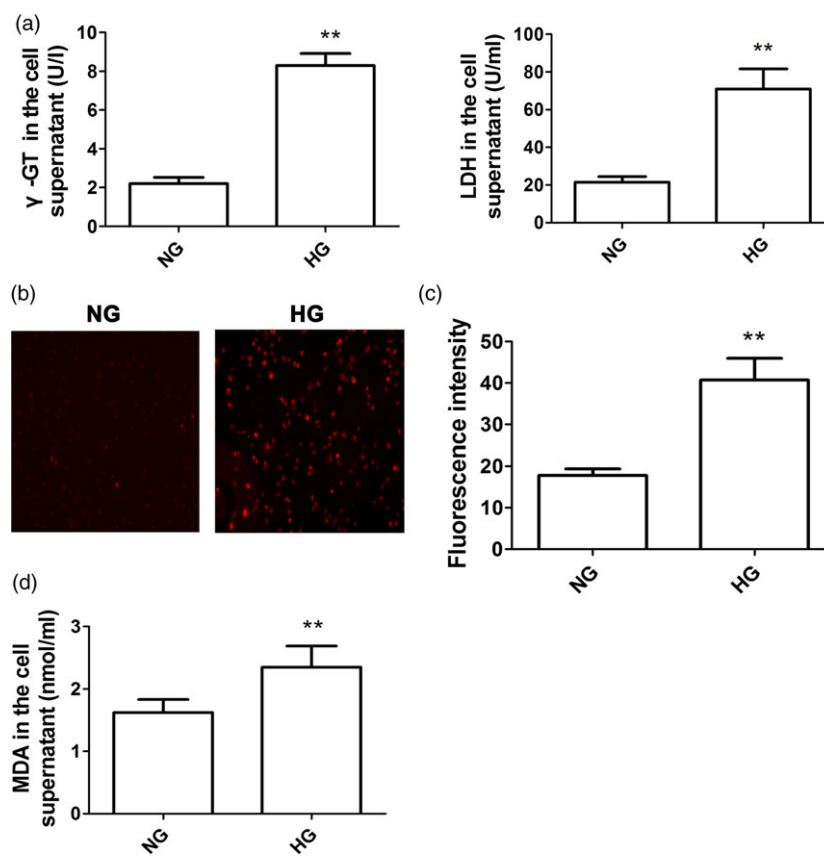


Figure 1 Decreased activities of γ -GT and LDH and increased oxidative stress were found in supernatants of HK-2 cells stimulated by high level of glucose (30 mM). (a) γ -GT activity; (b) LDH activity; (c) dihydroethidium staining and fluorescence intensity analysis; and (d) malondialdehyde level in supernatants. Mean \pm SD. * P < 0.05 vs NG, ** P < 0.01 vs NG.

cells. First, Western blot was used to evaluate the efficiency of p66shc siRNA. p66shc protein expression and p-p66shc protein expression in the p66shc siRNA group were significantly lower than the expression levels in the control group (P < 0.01; Figure 3a,b).

The levels of MDA, γ -GT and LDH in the supernatants of HK2 cells were significantly higher in the HG group than those in the NG group, but they were decreased in the HG+ p66shc siRNA treatment group compared with HG group (P < 0.05 or P < 0.01; Figure 3c, 3e, 3f). DHE staining was used to measure the level of ROS in HK2 cells. Compared with the NG group, high levels of glucose substantially increased the fluorescence intensity, while levels of ROS were significantly lower in the HG+ p66shc siRNA treatment group than in those in the NG group (P < 0.01; Figure 3d). This outcome suggested that p66shc played an important role in inducing cell damage and oxidative stress in HK2 cells.

Transmission electron microscope results showed that the endoplasmic reticulum in HK2 cells in the NG group was arranged regularly and that the morphology

of mitochondria was normal. The endoplasmic reticulum in the HG group was chaotic and swollen. Some mitochondrial vacuolization was observed. The morphology of mitochondria and endoplasmic reticulum in the HG+ p66shc siRNA treatment group was partially restored (Figure 3g).

High levels of glucose stimulation induced the upregulation of NADPH oxidase and PKC β in renal tissues and HK-2 cells

In in-vivo studies, the mRNA expression and protein expression in the renal tissue of NADPH oxidase subunit p22phox, NOX4 and PKC β in the HG group were higher than that in the NG group, all of which were alleviated by enzastaurin treatment (P < 0.01; Figure 4a,b).

In in-vitro studies, we found that the protein expression of NADPH oxidase p22phox, NOX4 and PKC β gradually increased with the increasing concentrations of glucose (5–45 mM) and incubation time interval (0, 12, 24, 48 h; P < 0.01; Figure 4c–4f).

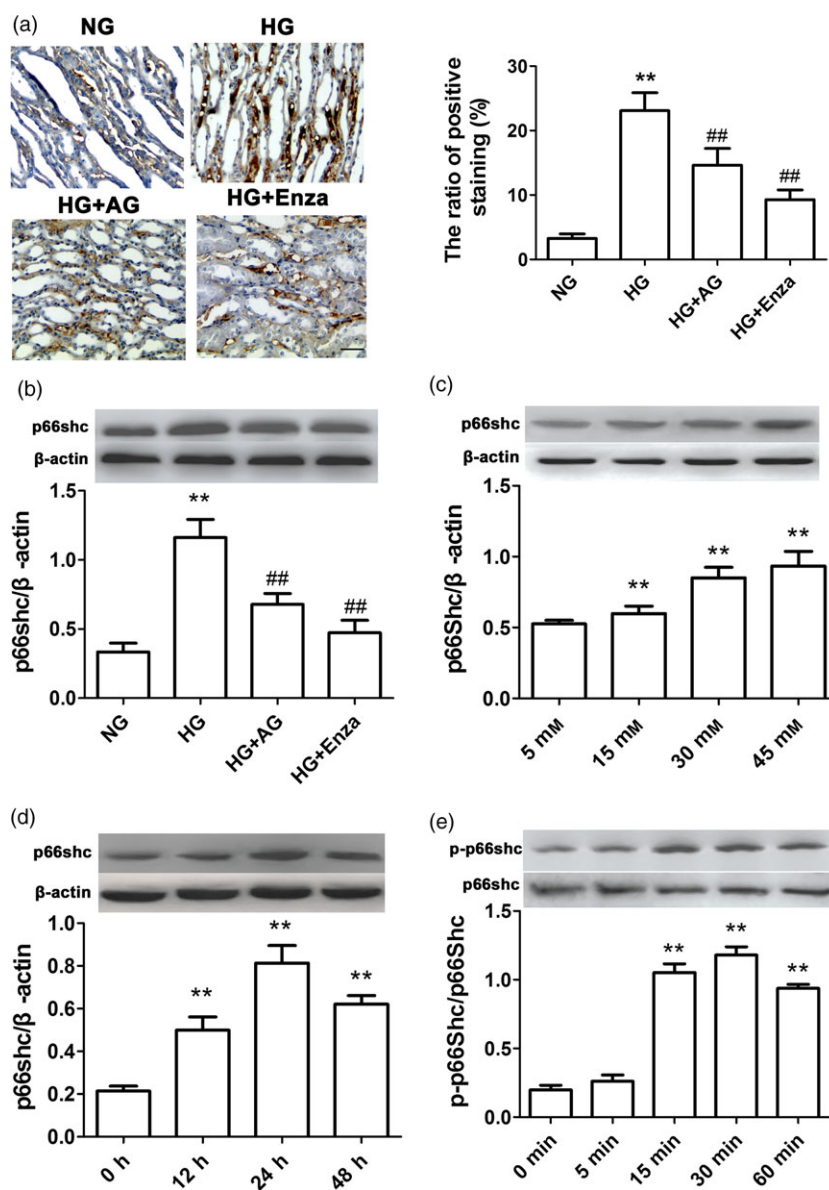


Figure 2 Upregulation of p66shc in vivo and in vitro caused by high level of glucose and was attenuated by enzastaurin. (a) Immunohistochemistry assay of p66shc (Scale bar: 50 μ m); (b) p66shc protein in renal tissue; (c) p66shc protein in HK2 cells stimulated by various concentrations of glucose; (d) p66shc protein in HK2 cells stimulated for various durations by glucose; and (e) p-p66shc/p66shc in HK2 cells stimulated for various durations by glucose. Mean \pm SD, * P < 0.05 vs NG or control, ** P < 0.01 vs NG or control; # P < 0.05 vs HG, ## P < 0.01 vs HG.

The PKC β -p66shc-NADPH oxidase pathway was activated in high glucose-stimulated HK2 cells

As an inhibitor of PKC β , enzastaurin (3×10^{-4} M) reversed the increases in p-p66shc/p66shc, NADPH oxidase p22phox and NOX4 induced by high levels of glucose in vitro (P < 0.01; Figure 5a–5d). This outcome suggested that NADPH oxidase and p66shc were downstream molecules of PKC β .

p66shc siRNA significantly reduced the expression of NADPH oxidase p22phox and NOX4 compared with that in the HG group (P < 0.01) but with no effect on the expression of PKC β (Figure 5e–5g). This outcome suggested that p66shc was an upstream molecule of NADPH oxidase but not an upstream molecule of PKC β . In combination with the previous experimental results, we demonstrated that PKC β -p66shc-NADPH oxidase signalling pathway was activated in high levels of glucose-induced HK2 cell damage (Figure 5).

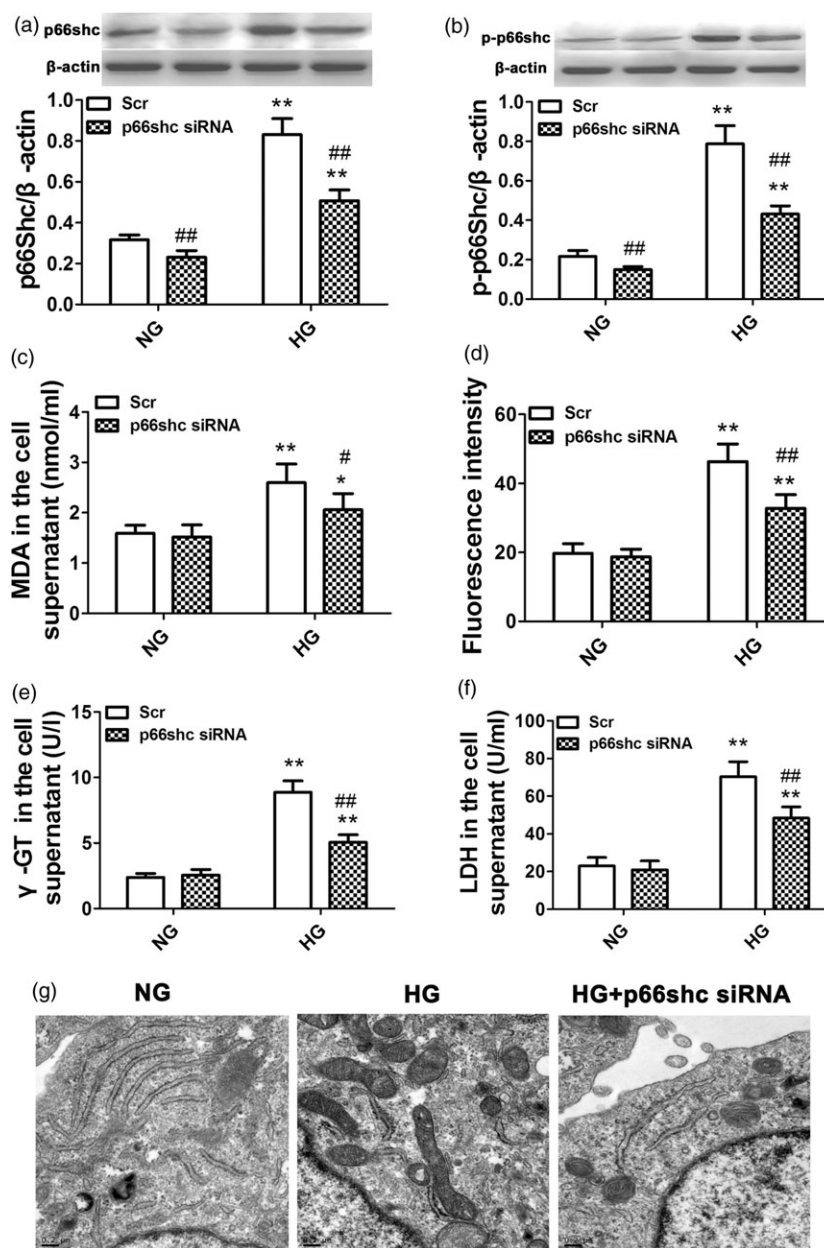


Figure 3 p66shc siRNA relieved oxidative stress and cell trauma in HK-2 cells stimulated by high level of glucose. (a) p66shc protein in HK2 cells; (b) p-p66shc protein in HK2 cells; (c) malondialdehyde content; (d) fluorescence intensity analysis of dihydroethidium staining; (e) γ -GT activity; (f) LDH activity; and (g) transmission electron microscopy depicts the changes in cellular ultrastructure. Mean \pm SD. * P < 0.05 vs NG, ** P < 0.01 vs NG. # P < 0.05 vs scr, ## P < 0.01 vs scr.

Enzastaurin inhibited the expression of proinflammatory factors in diabetic rats

In in-vivo studies, protein expression of proinflammatory factors, including iNOS, IL6, TNF- α and ET $_A$, was significantly higher in the HG group than that in the NG group (P < 0.01), indicating that proinflammatory factors were active in the pathological process of DN. The PKC β

inhibitor enzastaurin inhibited abnormal expressions of proinflammatory factors (P < 0.01; Figure 6a–6d).

Discussion

Approximately 40% of diabetic patients will develop DN.^[12] If the pathological process of early DN cannot be controlled, it will develop into ESRD. A large number of

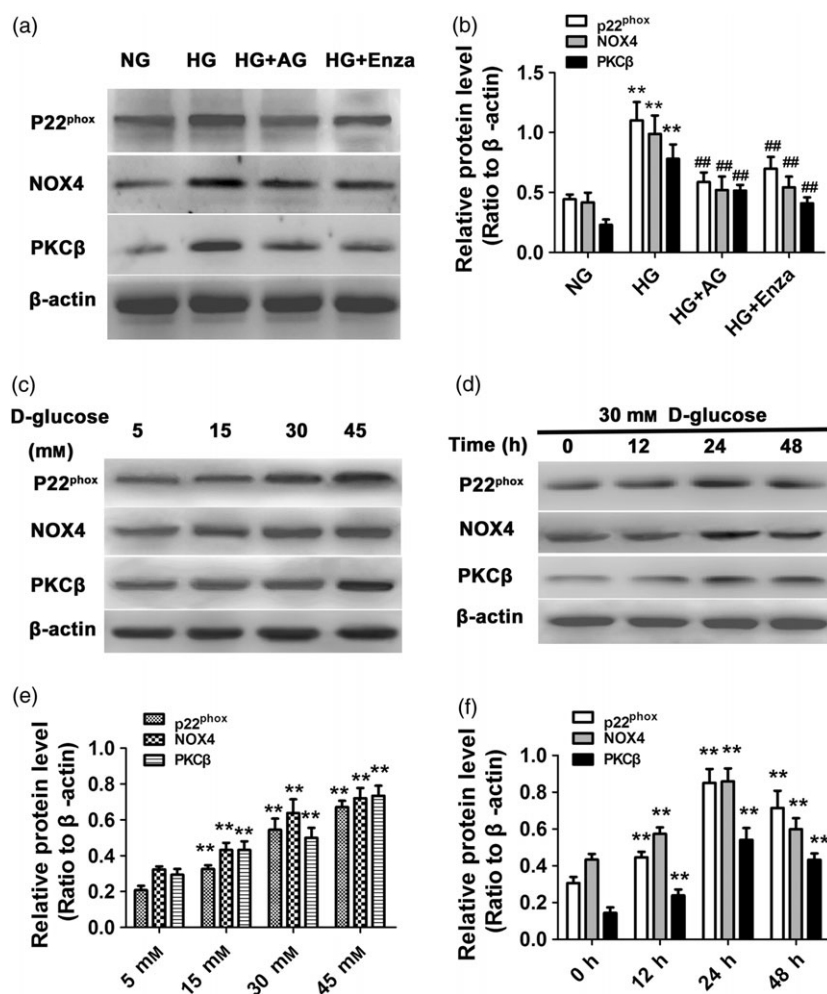


Figure 4 Effects of glucose stimulation on the expressions of NADPH oxidase p22phox, NOX4 and PKCβ in vivo and in vitro. (a, b) Protein expression of NADPH oxidase p22phox, NOX4 and PKCβ in renal tissue; (c, e) protein expression of NADPH oxidase p22phox, NOX4 and PKCβ in HK2 cells stimulated by different concentration of glucose; and (d, f) protein expression of NADPH oxidase p22phox, NOX4 and PKCβ in HK2 cells stimulated by different duration of glucose. Mean \pm SD, * P < 0.05 vs NG or control, ** P < 0.01 vs NG or control. # P < 0.05 vs HG, ## P < 0.01 vs HG.

studies have demonstrated that oxidative stress played an important role in the development of DN.^[13,14] ROS, produced by oxidative stress, caused renal impairment by activating related transcriptional proteins and cytokines.^[15,16] This study aimed to explore oxidative stress pathways associated with early DN. Injection of STZ was applied to induce early DN model. Samples were collected at week 8. Although mild morphologic changes in the renal tissue in early DN model, the changes in molecular level were obvious in this study, including activation of oxidative stress and inflammation related factors.^[1] It is meaningful to study the molecular mechanisms of DN in the early stage. Effective treatment in early diabetic nephropathy can slow down the pathological process of DN. A relief of oxidative stress in renal tissue may provide an alternative approach in treating early diabetic nephropathy in clinical setting and preventing ESRD.

As the key protein regulating oxidative stress and life cycle, p66shc played a key role in ageing-related diseases such as diabetes mellitus. In the present study, we found that p66shc was upregulated in high levels of glucose-stimulated HK2 cells and diabetic renal tissues. p66shc siRNA inhibited oxidative stress and kidney injury induced by high levels of glucose. Thus, p66shc is a key molecule involved in DN. However, it is unclear as to how p66shc mediates oxidative stress. p66shc siRNA inhibited the expression of NADPH oxidase p22phox and NOX4. The regulation of NADPH oxidase by p66shc is an important way to induce oxidative stress in DN. Therefore, inhibition of p66Shc can be an effective approach in treating abnormalities of early diabetic nephropathy related to oxidative stress.

In the present study, we found that oxidative stress levels increased in both diabetic rats and HK2 cells when stimulated by high levels of glucose. Simultaneously, protein

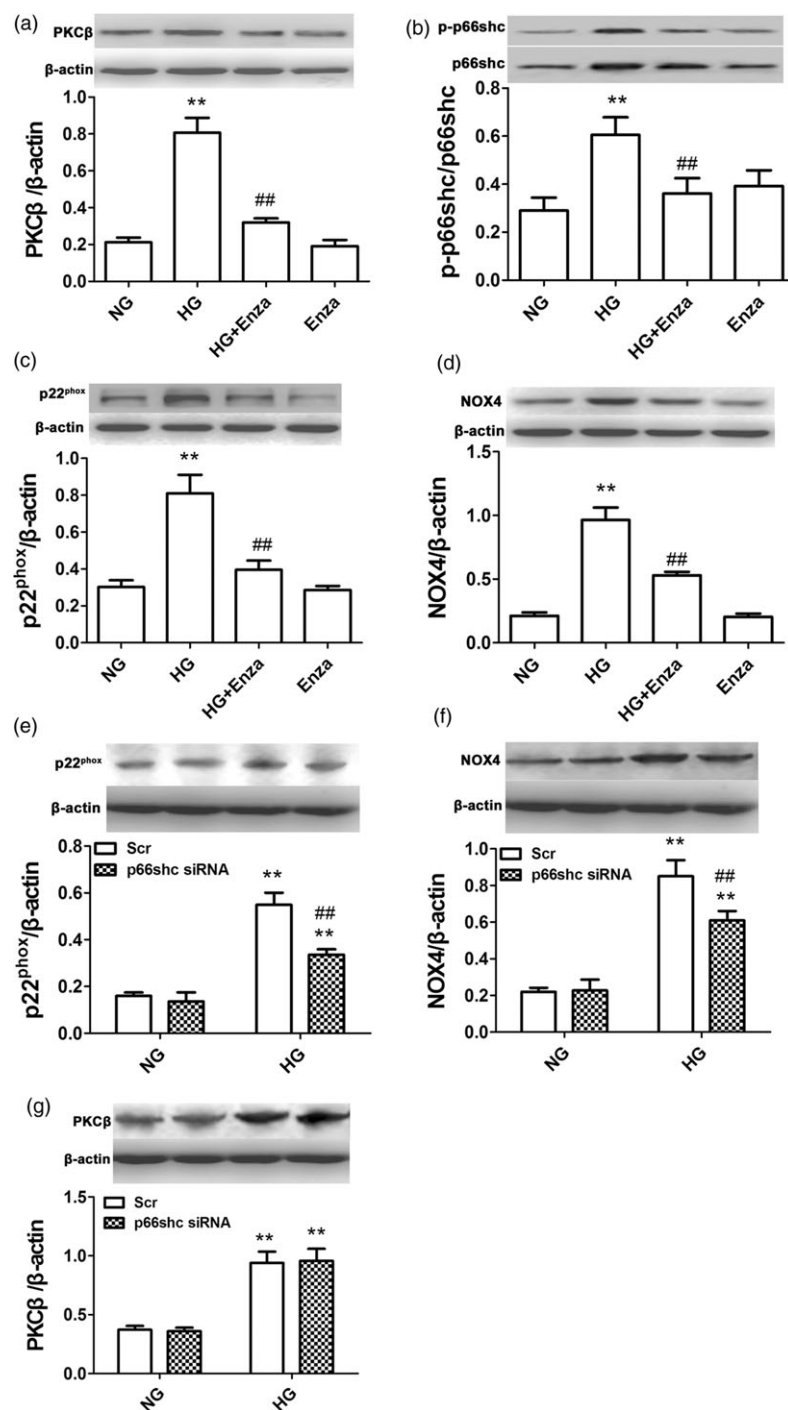


Figure 5 PKCβ-p66shc-NADPH oxidase pathway was activated in high glucose-stimulated HK2 cells. (a–d) PKCβ inhibitor enzastaurin decreased expression of PKCβ, p-p66shc/p66shc, NADPH oxidase p22^{phox} and NOX4 in HK2 cells; (e–g) p66shc siRNA downregulated NADPH oxidase p22^{phox} and NOX4, but not PKCβ in HK2 cells. Mean ± SD, * $P < 0.05$ vs NG, ** $P < 0.01$ vs NG. # $P < 0.05$ vs HG or scr, ## $P < 0.01$ vs HG or scr.

expressions of p22^{phox} and NOX4 subunits in renal tissue of diabetic rats and HK2 cells stimulated by high levels of glucose were higher than those in NG groups. This outcome suggested that NADPH oxidase was significantly

activated in DN, resulting in a large amount of ROS, consistent with previous reports.^[17]

In in-vivo experiments, we found that levels of MDA in the enzastaurin-treated group were significantly lower than

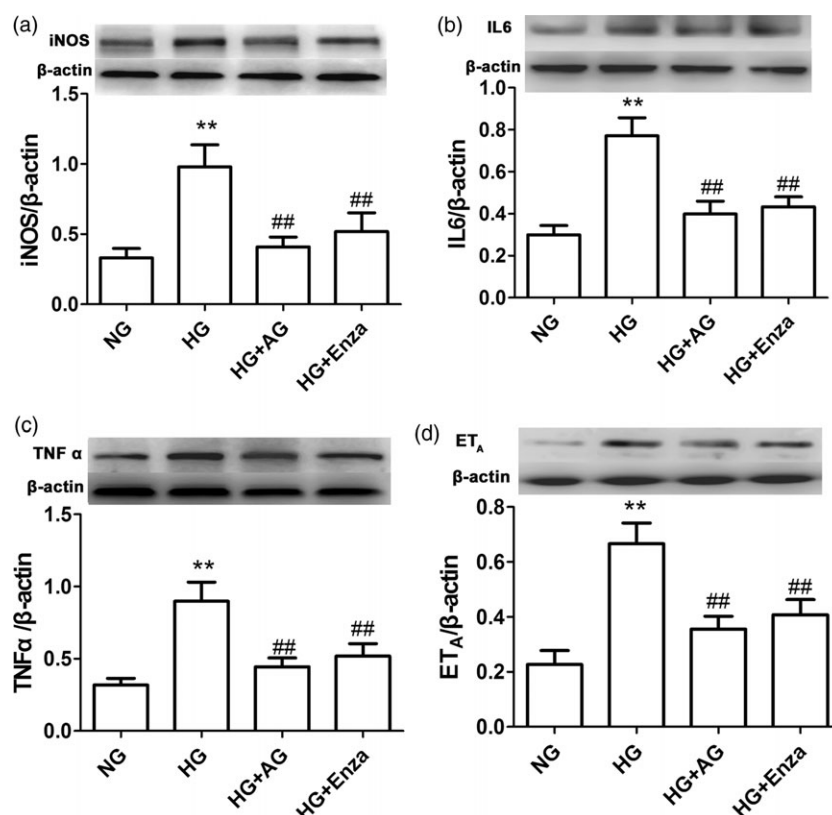


Figure 6 Abnormal protein expression of proinflammatory factors in vivo, attenuated by enzastaurin. Mean \pm SD, * P < 0.05 vs NG, ** P < 0.01 vs NG. # P < 0.05 vs HG, ## P < 0.01 vs HG.

those of HG group, while level of the antioxidant enzyme SOD was higher in the enzastaurin-treated group. Protein expressions of p66shc and NADPH oxidase were lower in the enzastaurin treatment group than those in the diabetic model group. This outcome suggested that PKC β may regulate the oxidative stress pathway related to p66shc and NADPH oxidase. In high levels of glucose-incubated HK2 cells, enzastaurin inhibited the activation and expression of p66shc, while p66shc siRNA did not affect the expression of PKC β , indicating that PKC β was upstream of p66shc. As an inhibitor of PKC β , enzastaurin not only downregulated the expression of p66shc but also reduced expression of NADPH oxidase. This further indicated that PKC β regulated expression of p66shc and NADPH oxidase, promoted the production of ROS, resulting in renal oxidative damage. A better understanding of the PKC β -p66shc-NADPH oxidase signalling pathways may lead to the development of novel therapeutic strategies.

Our previous studies demonstrated that inflammatory responses played important roles in renal injury.^[18–20] Inflammatory cytokines such as iNOS, IL6, TNF- α and ET_A played important roles in the pathological process of renal injury.^[21,22] The protein expressions of iNOS, IL6,

TNF- α and ET_A increased significantly in the renal tissue of DN model rats and were inhibited by enzastaurin. Therefore, we speculated that enzastaurin could downregulate inflammatory cytokines by PKC β -p66shc-NADPH oxidase pathway and relieve renal damage.

Conclusions

We found that high level of glucose induced abnormal expression of p66shc both in HK2 cells and in renal tissue. p66shc caused oxidative stress and cell damage through the PKC β -p66shc-NADPH oxidase signalling pathway, causing increases in the inflammatory factors iNOS, IL6, TNF- α and ET_A, aggravating diabetic renal injury. As a selective inhibitor of PKC β , enzastaurin alleviated early diabetic nephropathy via modulation of the PKC β -p66shc-NADPH oxidase pathway.

Declarations

Conflict of interest

The Authors declare that they have no conflict of interests to disclose.

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