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ORIGINAL ARTICLE

Sitagliptin improves renal function in diabetic nephropathy in male Sprague Dawley rats through upregulating heme oxygenase-1 expression

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Abstract

Purpose Oxidative stress is an important mechanism for diabetic nephropathy. Studies showed that hemo oxygenase-1 (HO-1) expression in renal tissue of patients with diabetic nephropathy has upregulated, while the HO-1 can protect the body through anti-oxidative stress. The study aimed to preliminarily explore the molecular mechanism by observing the effect of Sitagliptin on HO-1 expression in renal tissue of rats with diabetic nephropathy.

Methods The diabetic nephropathy rat model was established by STZ injection followed by intraperitoneal injection of sitagliptin with different concentrations. The mRNA expressions of HO-1 were detected by real-time PCR and Western blot and HO-1 enzyme activity change was detected by colorimetry. Human renal mesangial cell (HRMC) were cultured in vitro with high glucose concentration (30 μmol/L), phosphatidylinositol-3-kinase (PI3K) level and nuclear factor erythroid-2-related factor (Nrf2) content in cytoplasm and cell nucleus were observed before and after treatment with sitagliptin, as well as the action of in meditating HO-1 expression.

Results HO-1 mRNA, protein level, and HO-1 enzyme activity in renal tissue of rats with diabetic nephropathy were significantly increased after treatment with sitagliptin ($P < 0.05$). As comparison, the 24 h urinary microalbumin, creatinine, and boold urea nitrogen were all decreased after treatment of sitagliptin ($P < 0.05$). Similar results were observed after CoPP (an agonist of HO-1) treatment ($P < 0.05$). In contrast, ZnPP, an inhibitor of HO-1, significantly abrogated the inhibitory effect of sitagliptin ($P < 0.05$). Phosphorylation of PI3K and Nrf2 nuclear translocation under high-glucose concentration condition was induced by sitagliptin in HRMC. HO-1 expression was suppressed by pretreating HRMC with PI3K inhibitor or RNA interference.

Conclusions Sitagliptin may induce HO-1 expression via activation of PI3K and Nrf2 in rats with diabetic nephropathy; HO-1 can improve the oxidative stress of diabetic nephropathy, eventually protect from diabetic nephropathy.

Keywords Sitagliptin · Heme oxygenase-1 · Diabetic nephropathy · Phosphoinositide 3-kinase · Nuclear factor erythroid-2 related factor (Nrf2) · Phosphatidylinositol-3-kinase (PI3K)

Introduction

Diabetic nephropathy (DN) is an important cause of end-stage renal failure and one of the most common chronic complications in diabetes patients. Oxidative stress is one of the pathogeneses [1–4]. There are several defense mechanisms involved in reactive oxygen species (ROS)-induced damage for the human body, such as the anti-oxidant response element (ARE) which can regulate many protective proteins to alleviate oxidative stress under pathophysiological conditions. HO-1 is a rate-limiting enzyme which catalyzes heme metabolism to carbon monoxide (CO), biliverdin and free iron (Fe^{2+}). Recent studies

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suggested that HO-1 not only had the function of degrading heme, but also had a variety of important biological functions [5]. HO-1 can enhance the activity of catalase (Cat) and SOD, reduce the formation of superoxide anion and prevent apoptosis. Therefore, HO-1 is considered as an important defense mechanism to protect the organism from tissue damage [6]. The expression of HO-1 is low in the renal medulla of normal kidney, and may play a role in the maintenance of the renal medulla micro-circulation. The content of ROS in renal tissue of diabetic nephropathy patients is increased, which is significantly associated with upregulated HO-1 expression. HO-1 expression can also suppress TGF-β1 expression in tissue and play a role against inflammation and fibrosis [7, 8]. In addition, some studies also showed that the effect of HO-1 on high glucose-induced oxidative stress in cells may be compensatory increasing its expression for antagonistic oxidative stress, which suggests the increase of HO-1 level and its enzymes activity in vivo have important value in preventing from diabetic nephropathy.

Glucagon-like peptide-1 (GLP-1) is a hormone secreted by intestinal L cells of duodenum, ileum, and colon. The main physiological function of GLP-1 is to inhibit secretion of glucagon and growth hormone in vivo, promote the uptake of glucose into cells and play a role in stabilizing blood sugar [9]. Under physiological conditions, the plasma half-life of GLP-1 is very short and can be rapidly degraded by dipeptidyl peptidase-4 (DPP-4). Sitagliptin is a DPP-4 inhibitor that is mainly used in the treatment of type 2 diabetes mellitus [10]. Recent studies have shown that DPP-4 also possess other non-GLP-1 dependent effects on myocardial ischemia and vasodilation. Ishibashi et al.[11] reported that sitagliptin could activate cyclic adenosine monophosphate pathway, decrease the concentration of AGE, inhibit the NF-κB activity and reduce the expression of vascular endothelial cells of inflammatory factors to protect the vascular endothelial function. Our previous studies found that sitagliptin can reduce the activity of NF-κB in aorta of diabetic rats and alleviate the inflammatory response in diabetic rats. Moreover, it can reduce the expression of 8-hydroxy guanosine in renal tissue of diabetic rats, increase the expression of SOD and protect the renal function of diabetic nephropathy rats (unpublished data). It is not clear, however, whether sitagliptin can induce the expression of HO-1 in renal tissue. To investigate whether sitagliptin could affect the expression of HO-1 in kidney of diabetic rat after sitagliptin intervention, the rat model of diabetic nephropathy was established in this study by high-glucose high-fat diet and streptozotocin injection. The renal mesangial cells were cultured in vitro under high glucose condition to determine the PI3K activation and Nrf2 translocation after sitagliptin treatment as well. Our current study has provided experimental evidence in exploring the role of HO-1 in the pathogenesis of diabetic nephropathy in rats.

Materials and methods

Establishment of diabetic nephropathy rat model and sitagliptin treatment

Male Sprague Dawley(SD) rats, weighing175–225 g, were purchased from Laboratory Animal Center of University of South China [permit No. SCXK (Hunan province) 2015-0002]. After 1 weeks of adaptive feeding, the rats were randomly divided into control group (with normal saline and normal diet, $n = 20$) and model group (with high sugar and high-fat diet, $n = 80$). The rats in the model group were treated with intraperitoneally injections of streptozotocin (STZ, prepared with 0.1 mol/L citrate buffer, and the injection volume was 30 mg/kg). After 72 h, the blood glucose concentrations were measured. When three consecutive random blood glucose levels were equal to or higher than 16.7 mmol/L, it was determined as successful diabetes modeling (if the blood sugar levels in rats were lower than 16.7 mmol/L, STZ injection was repeated with injection volume of 20 mg/kg). Four weeks later, when the urine protein level was equal to or higher than 30 mg/d with optical microscopic HE staining showing glomerular basement membrane thickening and mesangial cells proliferation, it was determined as successful modeling of type 2 diabetic nephropathy rat. The rats in successful diabetic nephropathy modeling were then randomly divided into 7 groups, with 10 animals per group: model group, sitagliptin 1 mg/(kg d) group, sitagliptin 5 mg/(kg d) group, sitagliptin 10 mg/(kg d) group, CoPP 5 mg/kg group, ZnPP 20 mg/kg group and sitagliptin 10 mg/(kg d)+ ZnPP 20 mg/kg group. Sitagliptin was given by intragastric administration. CoPP group was injected intraperitoneally with 2 ml CoPP (5 mg/kg) 24 h before intragastric administration of sitagliptin. The ZnPP group was injected intraperitoneally at corresponding concentration 24 h before intragastric administration. The control group was given the same amount of normal saline at one time a day. In addition to the blank control group with normal diet, the remaining 7 groups were fed with high-fat diet, with freely eating and drinking during the whole-experimental period. The body weight and blood sugar of all animals were measured at fixed time weekly for 10 weeks. Rats with sugar level equal to or higher than 33.3 mmol/L were received subcutaneous injection of regular insulin 1~2 U to lower and maintain blood glucose level between 16.7 and 33.3 mmol/L. The proliferation of glomerular basement membrane and mesangial cells was observed under light microscopy at the end of week 10.

Biochemical analysis

At the end of week 10, rats in each group were placed in the metabolic cage and collected the 24 h urine from 6:00 to 6:00 next day. A volume of 1 ml of blood was taken from the tails of all the rats for serum creatinine (Scr) and blood urea nitrogen (BUN) levels measurements by automatic biochemical analyzer.The concentrations of microalbumin in 24 h urine samples were detected by immunoturbidimetry (Lidman biochemical Co., Ltd, China). The levels of Scr were assayed by end-point method according to Creatinine Detection Kits (Wittman biotechnology Co., Ltd, China), and levels of BUN were assayed by Urease-GLDH method according to Urea Nitrogen Assay Kits (Purebio biotechnology Co., Ltd, China). Levels of Scr and BUN were expressed in μmol/L and mmol/L, respectively.

Specimens collection and processing

The rats were fasted but with water for 12 h before weighing. The rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (250 mg/kg). The rats were processed with laparotomy to expose the left kidney for renal vein lavage with pre-cooling normal saline. The left kidney was taken out after becoming pale and removed the capsule on ice plate to obtain renal tissue for further study.

HO-1 mRNA expression detected by real-time PCR

The total RNA of obtained renal tissue was extracted with TRIzol reagent (Invitrogen, USA), and processed with reverse transcription into cDNA (Promega, Madison, WI, USA) for real-time quantitative PCR by adding forward and reverse primers and SYBR, the amplification conditions were as follows: pre-denatured 10 min at 95 °C, followed by the following 40 cycles, 95 °C for 15 s, 60 °C for 60 s, and extended 5 min at the end. HO-1 primers are as follows:

HO-1: forward 5′-ATGGCCTCCCTGTACCACATC-3′,
reverse 5′-TGTTGCGCTCAATCTCCTCCT-3′;

Beta-actin: forward 5′-CATCCTGCGTCTGGACCTG G-3′,
reverse 5′-TAATGTCACGCACGATTTCC-3′.

The result was expressed by $2^{-\Delta\Delta CT}$. [$^{\Delta\Delta CT}$ means: A (to be measured sample) = CT(target gene)–CT(internal marker gene), B (control sample) = CT(target gene)–CT(internal marker gene), $\Delta\Delta CT = A–B$]

HO-1 enzyme activity determination

Renal homogenates were collected from all experimental groups and centrifuged at 1000 rpm for 5 min and discarded supernatant. These cells were washed with washing solution provided by reagent kit (Genmed, China) before adding 100 μL lysis solution, with mixture placed on the ice for 5 min, ultrasonic crushing cells, centrifugation, discarding the precipitate and obtaining the supernatant; 20 μL reaction liquid D, 340 μL buffer solution C and 20 μL substrate were added successively to EP tube, and placed at 37 °C for incubation for 1 h. And 400 μL termination liquid was added and centrifuged at 1000 rpm for 5 min. A volume of 100 μL green phase was taken and determined the light absorption values at 464 nm and 530 nm. The activity of HO-1 was calculated according to the concentration of protein in each sample with unit as mmol bilirubin per mg protein^{-1}.

Western blot analysis of protein expression and phosphorylation

The tissue stored in liquid nitrogen was crushed completely before transferring to EP tube, and the 100 mg tissue was added with 500–1000 ml RIPA lysis buffer and lysed on the ice for 30 min; centrifuged at 24 °C for 20 min to get the supernatant, i.e., total protein. For extraction of cell total protein, the processed cells were collected and washed with pre-cooling PBS buffer twice. The cells were fully lysed by cell lysis buffer containing proteinase inhibitor and centrifuged at 4 °C 12,000 rpm for 10 min. The total protein (supernatant) was transferred to new EP tube and placed in −70 °C refrigerator for use. The nuclear protein was extracted using the kit from Pierce (Pierce, Rockford, IL). Protein of each group was separated using SDS-PAGE and transferred to nitrocellulose film. Western blot was performed using HO-1, p85 alpha and Nrf2 antibodies, respectively, as per different test purposes after sealing and washing membrane. The membrane was incubated with HRP-labeled secondary antibody and developed using by chemiluminescence and analyzed by Image J Software (National Institute of Health Bethesda MD, USA) for gray scanning.

Cell culture

Human renal mesangial cells HRMC (ATCC, Manassas, VA) were cultured in DMEM medium supplement with 10% fetal bovine serum, 1% glucose, 1% glutamine, 100 U/ml penicillin, and streptomycin 100 g/ml in thermostatic incubator containing 5% CO_2 at 37 °C. The cells were conducted with passage after the in vitro cultured renal mesangial cells covering 80% of the bottom of culture flask and the cells were incubated by intensity of 10^5 cells/ml onto six-well plates and were switched into high glucose (30 mmol/l) medium after being cultured for 48 h. They were divided into six groups as normal control group (isovolumetric without high glucose culture medium), high glucose group, sitagliptin groups at varied concentrations

Table 1 Changes of body weight, blood sugar, blood lipid, insulin, and HOMA-IR levels after 4 weeks between two groups ($\bar{x} \pm s$)

Group	*N*	Weight (g)	Blood sugar (mmol/L)	Insulin (mU/L)	HOMA-IR	TG (mmol/L)	TC (mmol/L)	LDL-C (mmol/L)
Control group	20	206.2 ± 5.29	4.8 ± 0.3	15.73 ± 0.81	3.31 ± 0.23	0.69 ± 0.08	1.69 ± 0.09	0.39 ± 0.04
Model group	80	252.4 ± 6.93*	6.7 ± 0.3*	28.84 ± 0.95*	8.60 ± 0.57*	0.98 ± 0.13*	2.62 ± 0.09*	0.49 ± 0.05*

Note: as compared with control group, *$P < 0.05$

(1 nmol/L, 10 nmol/L, and 20 nmol/L) and inhibitor group (LY294002 20 μmol/L+ sitagliptin 20 nmol/L) during the PI3K phosphorylation and inhibition test. At the RNA interference test, they were divided into five groups as blank control group, high glucose control group, high glucose + sitagliptin group, control siRNA group and Nrf2 siRNA group. Western blot was used to observe the level of p85α phosphorylation 60 min after treatment by different sitagliptin concentrations. Each experiment was independently repeated for at least three times to get the mean.

siRNA transfection experiments

The renal mesangial cells were incubated onto six-well plates (about 1×10^6) and 10 μL Nrf2 siRNA dissolved in culture medium without serum as per reagent kit instruction, making final volume as 250 μL (solution A). Take 5 μl of lipofectamine 2000 provided in the reagent kit and dilute with MEM culture medium without serum to total volume of 250 μL (solution B). After preparation, mix solution A and B and place stand at room temperature for 20 min before adding to culture plates to make final concentration of siRNA as 100 nmol/L. After incubation for 4 h, the transfection mixture was removed and replaced with complete medium. The sequences of Nrf2 siRNA were: sense chain 5′-UCCCGUUUGUAGAUGACAA-3′; antisense chain 5′-UUGUCAUCUACAAACGGGA-3′.

Statistical analysis

The statistical analyses of data were performed using SPSS 18.0 software. The results are expressed as a mean ± standard deviation ($\bar{x} \pm s$). Difference between groups and conditions were analyzed by one-way ANOVA or *t*-test, but interaction between ZnPP and sitagliptin was analyzed by two-way ANOVA. $p < 0.05$ was considered as statistically significant.

Results

Modeling of rats

After 4 weeks' treatment, the weight of rats in experimental group was significantly higher than that of normal group ($P < 0.05$). The blood glucose, insulin, HOMA-IR, TG, TC, and LDL in experimental group as compared to control group ($P < 0.05$). These results suggest that insulin resistance model had been established (Table 1). After STZ injection, six rats in the model group died, and another four rats were removed because of modeling failed. At the conclusion of experiment, a total of 70 diabetic nephropathy rats were successfully established.

The pathological findings of rats in each group were shown in Fig. 1. The renal tubules, glomerular, and basement membranes were intact in normal control rats (Fig. 1a). After STZ modeling, the rat renal epithelial cells were necrotic with thin glomerular basement membrane and matrix, suggesting that the renal damage model was established (Fig. 1b). The morphological changes and the infiltration of inflammatory cells were reduced in three groups of 1 mg/kg d (Fig. 1c), 5 mg/kg d (Fig. 1d) and 10 mg/kg d sitagliptin (Fig. 1e) after sitagliptin treatment in comparison to those of model rats induced by STZ, with 10 mg/kg d sitagliptin group (Fig. 1e) exudation and inflammatory cells showing less in rat renal tissue which suggested it can effectively improve the pathological changes of diabetic renal tissue. The heme oxygenase-1 agonist CoPP group (Fig. 1f) showed similar effect as small dose of sitagliptin. As a comparison, the heme oxygenase-1 inhibitor ZnPP could significantly suppress this effect of sitagliptin (Fig. 1g). The renal epithelial cell necrosis, glomerular basement membrane and matrix thinning were observed in group of 10 mg/kg d sitagliptin + hemo oxygenase-1 inhibitor ZnPP (Fig. 1h), similar to morphological changes of STZ model group. These results all suggested that ZnPP can reverse the protective effect of sitagliptin on diabetic renal tissue.

Expression of HO-1 mRNA in renal tissue of diabetic nephropathy rats induced by sitagliptin

The expression of HO-1 mRNA in the renal tissue of the control group was very low. There was a minimal increase in HO-1 mRNA in the rats of STZ model group. After sitagliptin treatment at varied concentration, the expression of HO-1 mRNA was increased with dose dependent manner (Fig. 1).

Expression of HO-1 protein in renal tissue of diabetic nephropathy rats induced by sitagliptin

A lower level of HO-1 protein was observed in the renal tissues of STZ rats without sitagliptin treatment. Similar to

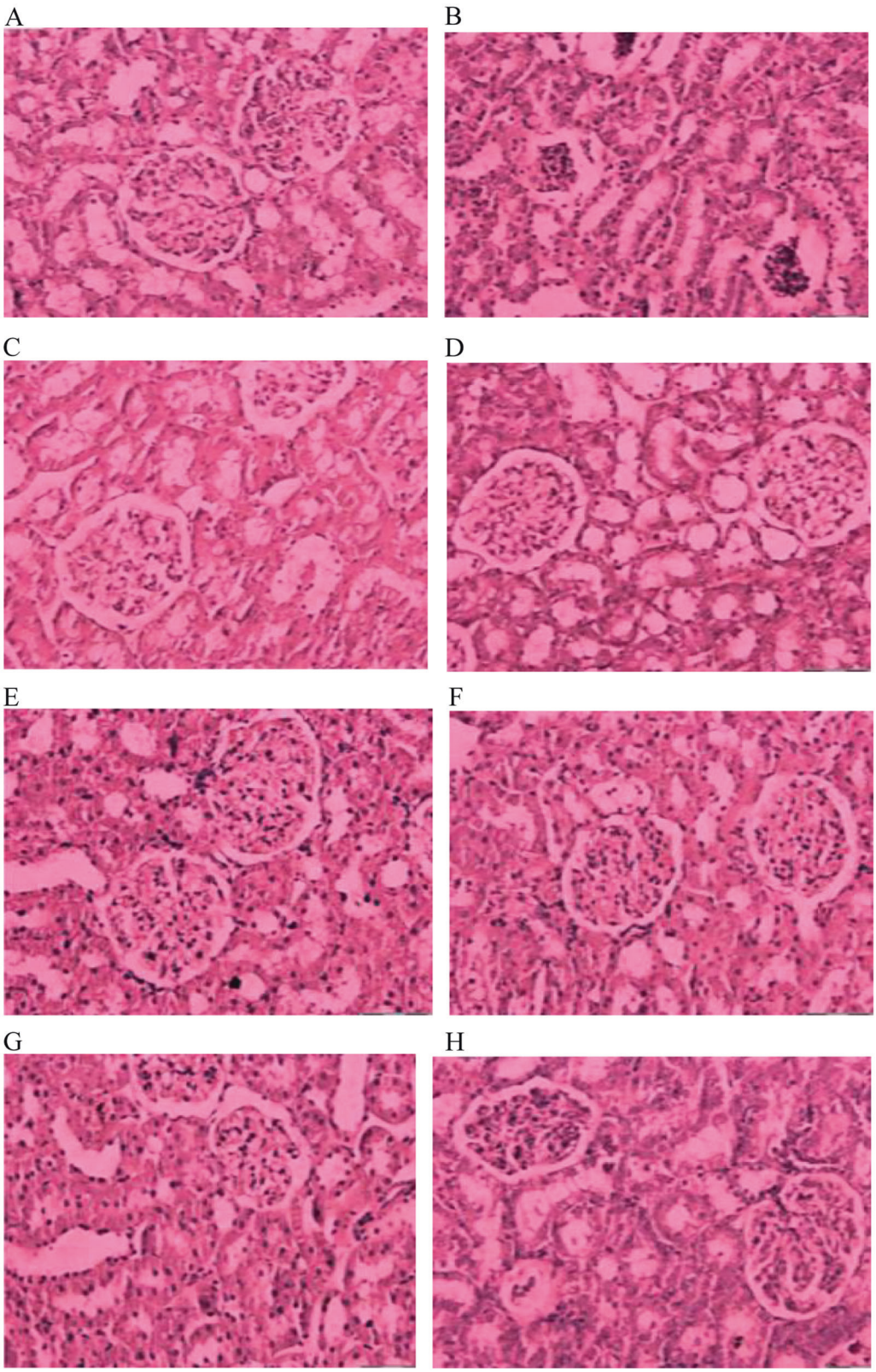

Fig. 1 STZ modeling and pathological changes in renal tissue after intervention with sitagliptin (HE staining ×400). **a** normal kidney tissue; **b** DN kidney tissue; **c**1 mg/kg d group; **d** 5 mg/kg group, **e** 10 mg/kg d group, **f** CoPP group, **g** ZnPP group, **h** ZnPP + 10 mg/kg d sitagliptin group

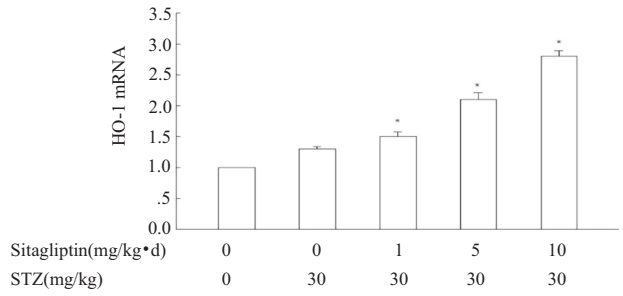

Fig. 2 Effects of sitagliptin at different concentrations on the expression of HO-1 mRNA in the renal tissue of diabetic rats. Note: as compared with control group (0 mg/kg d), $*P < 0.05$

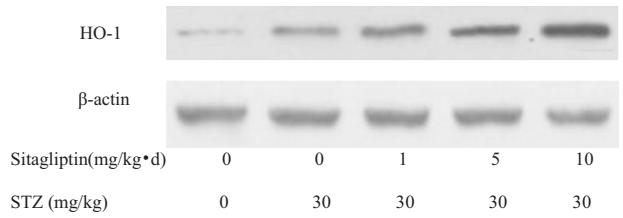

Fig. 3 Effects of sitagliptin at different concentrations on the expression of HO-1 protein in renal tissue

the results of real-time quantitative PCR, the HO-1 protein was also gradually increased after sitagliptin stimulation at different concentrations (0, 1, 5, and 10 mg/kg d) with dose dependent manner (Fig. 2). These results together suggested that sitagliptin can enhance the expression of HO-1 at mRNA and protein level in rat renal tissues (Fig. 3).

Sitaglipin upregulating the enzyme activity of HO-1 in renal tissue of diabetic nephropathy rats

In order to observe whether the expression of HO-1 increases with increase in activity, we also measured its enzyme activity indirectly. As the same trend of mRNA and protein expression, sitaglipin can also affect the enzyme activity of HO-1 in renal tissue. The enzyme activity of HO-1 in renal tissue of normal rats was low, which increased slightly after treatment with STZ, and the enzyme activity of HO-1 increased with the increase of sitaglipin concentration after combined treatment with sitaglipin (Fig. 4).

Effects of sitaglipin on renal function in diabetic nephropathy rats related to HO-1

At the end of the 14-week experiment, 24 h urinary microalbumin level was collected and blood samples were taken to determine Scr and BUN. The results showed that the urinary microalbumin, Scr, and BUN were all significantly increased. However, after treated with sitaglipin at different concentrations, these elevated biomarkers were all decreased. Similarly, when the HO-1 agonist cobalt

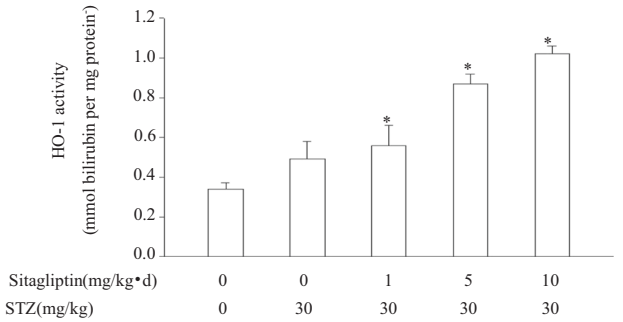

Fig. 4 Sitagliptin increasing the enzyme activity of HO-1 in renal tissue. Note: as compared with STZ group, $*P < 0.05$

protoporphyrin (CoPP) was administered, the levels of urinary microalbumin, Scr, and BUN were also effectively reduced. After treatment with HO-1 inhibitor zinc protoporphyrin (ZnPP), the protective effect of sitaglipin on renal function was further reversed (Table 2). These results suggested that sitaglipin can improve the renal function in diabetic nephropathy rats which was related to HO-1 expression.

Phosphorylation of PI3K in mesangial cells under high glucose condition induced by sitaglipin

In the negative control group, the phosphorylation levels of PI3K and p85 subunits were lower in the mesangial cells of the kidney. As comparison, the phosphorylation of p85 subunits increased after culture with 30 mmol/l high glucose. After treatment with sitaglipin at different concentrations, the phosphorylation level of p85 was further increased. No significant changes were observed in the total intracellular p85 levels (Fig. 5).

PI3K inhibitors downregulating the expression of HO-1 in renal mesangial cells induced by sitaglipin

Similar to the results of animal experiments, the level of HO-1 expression increased 24 h after sitaglipin treatment at varied concentrations in renal mesangial cells with dose dependent manner. The level of HO-1 expression was inhibited by sitaglipin after treatment with 20 μmol/l PI3K inhibitor LY294002 (Fig. 6).

Nrf2 nuclear translocation in renal mesangial cells induced by sitaglipin

As shown in Fig. 7, the expression level of Nrf2 in the cytoplasm of renal mesangial cells were decreased gradually with sitaglipin treatment at 20 μmol/l for 0–120 min. By contrast, Nrf2 content in nuclei gradually increased. The β-actin in cytoplasm and nuclear TBP in cell nucleus remained unchanged (Fig. 7).

Table 2 Effects of sitagliptin, CoPP, and ZnPP on 24 h urinary microalbumin, Scr, and BUN of diabetic rats

Group	24 h urinary microalbumin (mg/24 h)	Scr (μmol/L)	BUN (mmol/L)
Control group	9.96 ± 1.52	70.14 ± 6.81	6.87 ± 0.55
Model group	68.96 ± 7.35*	124.37 ± 5.97*	15.63 ± 0.24*
Model + sitagliptin(1 mg/kg d)	52.34 ± 4.07#	106.41 ± 7.51#	13.49 ± 0.38#
Model + sitagliptin(5 mg/kg d)	43.21 ± 2.28#	93.21 ± 4.36#	11.08 ± 0.17#
Model + sitagliptin(10 mg/kg d)	38.25 ± 4.14#	80.62 ± 6.21#	9.25 ± 0.42#
Model + CoPP	40.61 ± 5.05#	85.69 ± 6.21#	10.67 ± 0.25#
Model + ZnPP	75.63 ± 6.28	130.58 ± 4.18	16.47 ± 0.24
Model+sitagliptin(10 mg/kg d) +ZnPP	61.76 ± 4.21§	112.41 ± 3.89§	12.84 ± 0.14§

As compared with control group, *$P < 0.05$; as compared with model group, #$P < 0.05$, as compared with model group + sitagliptin (10 mg/kg d) group, §$P < 0.05$

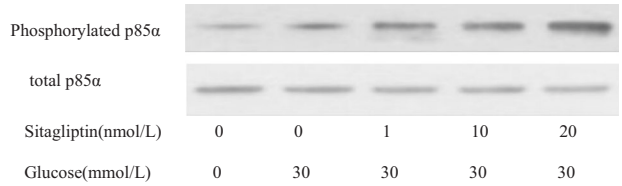

Fig. 5 Effect of sitaglipin on phosphorylation of PI3K in renal mesangial cells under high glucose condition

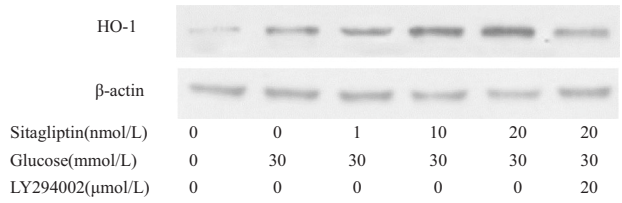

Fig. 6 Effect of PI3K inhibitors on the expression of HO-1 in mesangial cells under high glucose condition

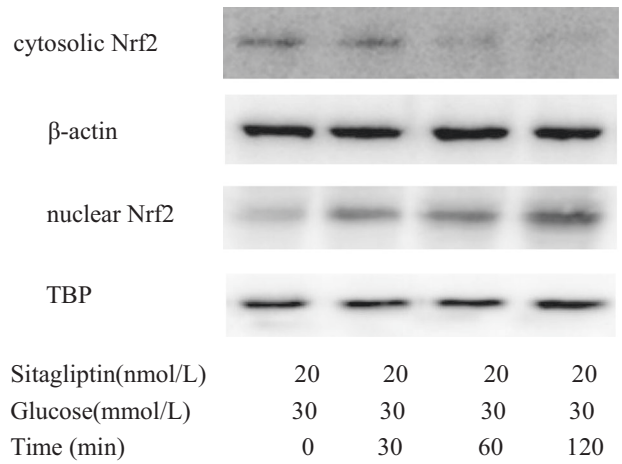

Fig. 7 Effect of sitaglipin on translocation of Nrf2 nucleus in renal mesangial cells under high glucose condition

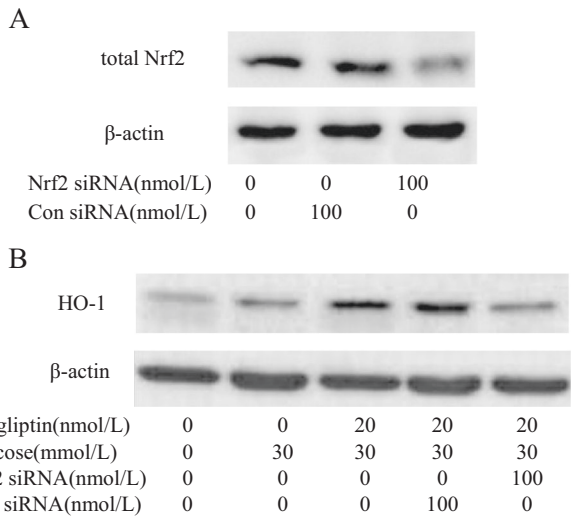

Fig. 8 Nrf2 regulating the expression of HO-1 induced by sitaglipin. **a** Nrf2 siRNA interference, **b** the expression of HO-1 protein by Nrf2 interference

Loss-of-Nrf2-expression suppressing the expression of HO-1 in renal mesangial cells induced by sitaglipin

Western blot showed that after loss expression through Nrf2 siRNA transfection (Fig. 8a), renal mesangial cells were processed with 20 nmol/L sitaglipin for 24 h, which significantly decreased HO-1 expression (Fig. 8b).

Discussion

Our pilot study suggested that sitagliptin can downregulate 8-hydroxy-deoxy-guanosine (8-OHdG) expression of the renal tissue of diabetic rats, upregulate the expression of superoxide dismutase (SOD), which had protective effect on kidney in diabetic rats [12]. In order to further investigate the potential protective role of sitagliptin on diabetic rats, we had established a model of diabetic nephropathy

induced by STZ. The results in present study confirmed that the expression of HO-1 mRNA and protein could be induced as well as enzyme activity increased in renal tissue after treatment with sitagliptin in vivo. The level of HO-1 was also increased with the increase of sitagliptin concentration. HO is a rate-limiting enzyme that catalyzing heme to CO, Fe^{2+}, and biliverdin. A total of three isozymes have been identified, namely, HO-1, HO-2, and HO-3, with HO-1 as inducible. A variety of stress states, such as shock, heavy metals, ischemia- reperfusion injury, diabetic retinopathy, can induce the expression of HO-1 [13, 14].

HO-1 can protect cells by its three metabolites (CO, bilirubin, and Fe^{2+}). CO was originally believed to be a toxic gas. Recent researches, however, have shown that it is an important neurotransmitter similar to NO. CO can act on effector target cells in an autocrine or paracrine manner and play an important role in catalyzing GTP to cGMP. cGMP can exert its corresponding biological function by activating phosphodiesterase, protein kinase or regulating ion channels [15, 16]. In addition, HO-1 and CO can also inhibit complement activation and downregulate cell adhesion molecules, E-selectin (or endothelial leukocyte adhesion molecule-1) and endothelium derived proinflammatory mediators to inhibit leukocyte adhesion and infiltration, thus regulating the inflammatory reaction intensity. Another product of heme, biliverdin, is further reduced to bilirubin.

Bilirubin is a very powerful antioxidant that has better antioxidant properties than vitamin C and vitamin E. It can directly react with free radicals, combine with singlet oxygen to prevent light oxidation of hydrocarbons [17]. Fe^{2+} can further induce the synthesis of heavy chain ferritin [17]. Ferritin can reduce the occurrence of Fenton reaction by reducing the intracellular free iron ion, and finally reduce the cell damage caused by ROS [18]. Therefore, HO-1 mainly plays antioxidant protection of these products to maintain the body's steady state [19]. The abovementioned results showed that sitagliptin can reduce the renal tissue damage caused by oxidative stress. This protective mechanism is independent of lowering blood sugar.

To further investigate the protective effect of HO-1 in diabetic nephropathy rats, we collected 24 h urinary microalbumin of rats and then evaluated renal function by measuring the levels of Scr and BUN. As expected, sitagliptin effectively reduced levels of urinary microalbumin, Scr, and BUN in model rats. At the same time, we found the HO-1 agonist CoPP could reduce the level of urine microalbumin, Scr, and BUN to certain extent, which is similar as sitagliptin. These results provide further evidence supporting protective effect of HO-1 on diabetic nephropathy rats. At the same time, we also found that HO-1 inhibitors ZnPP can significantly eliminate the inhibitory effect of sitagliptin on urinary microalbumin, Scr, and BUN, which suggests that sitagliptin plays a protective role in the kidneys through HO-1 activation.

To further investigate the molecular mechanisms of HO-1 expression induced by sitagliptin in the kidneys, we first investigated the activity of the PI3K pathway. PI3K is involved in many cellular physiological functions, such as cytoskeletal remodeling, organelle transport and the expression of multiple antioxidant kinases. Activated PI3K can reverse phosphorylation of 308th threonine and 473rd serine residues of Akt to activate downstream molecules Akt (protein kinase B). One of the mechanism is that PI3K can catalyze phosphtidyli-nositol-4,5-bisphosphate (PIP_2) to degrade to phosphatidylinositol-3-4-5-trisphosphate (PIP3), the latter can then activate Akt by combining phosphatidylinositol-dependent kinase (PDK). Another mechanism is through direct phosphorylation of Akt 473nd serine residues by mTOR followed by phosphorylation of 308th threonine residues under the mediation of the PDK1 [20]. Studies have shown that PI3K can upregulate the expression of HO-1 and participate in oxidative stress damage. To determine whether PI3K was involved in the expression of HO-1 induced by sitagliptin, we first observed whether sitagliptin could induce p85α phosphorylation of PI3K. The results showed that phosphorylated p85α was detected after sitagliptin treatment for 30 min. The expression of HO-1 was significantly decreased by PI3K inhibitor LY294002, which suggested that the expression of HO-1 was regulated by PI3K.

Nrf2 is a nuclear transcription factor involved in cell defense and regulation of endogenous antioxidant response. In the resting state, Nrf2 remains inactive in combination with its inhibitor Keap1 in the cytoplasm. When the cells were exposed to various exogenous stimuli, such as oxidative stress, Nrf2 was disassociated from Keap1, and translocated into nuclear and bound with antioxidant component element (ARE) on relevant promoter to promote antioxidant gene transcription and exert its antioxidant or anti-inflammatory action [21–23]. It was found in present study that the Nrf2 pathway was activated after treatment with sitagliptin, which was mainly characterized by an increase of intracellular Nrf2 and decrease of cytoplasm Nrf2, indicating that Nrf2 translocated to the nucleus and upregulated the expression of HO-1. The study found that the sitagliptin can upregulate Nrf2 content in nucleus, suggesting that sitagliptin can activate Nrf2. The HO-1 promoter contains several regulatory sites of Nrf2. To further determine whether Nrf2 is involved in the regulation of HO-1 expression, we used Nrf2 specific siRNA to interfere the expression of HO-1 [13]. The results showed that the expression of HO-1 induced by sitagliptin was significantly decreased by Nrf2 siRNA transfection. This suggested that the expression of HO-1 induced by sitagliptin was regulated by Nrf2.

In summary, sitagliptin can activate PI3K/Nrf2 signaling pathway and induce the expression of HO-1 in renal mesangial cells under high glucose condition. HO-1 can reduce the tissue and cell damage caused by oxidative stress injury, finally playing a protection function for the kidney and providing a new approach and ideas for the treatment of diabetic nephropathy. We only, however, preliminarily explored the expression of HO-1 induced by sitagliptin in the paper, whether there are other effect targets remains to be further studied. In addition, due to multiple regulatory sites on the upstream of *HO-1* gene, the subsequent studies will further investigate other signaling pathways beside Nrf2, which can further confirm multiple pharmacological effects of sitagliptin.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

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