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Atorvastatin inhibits hyperglycemia-induced expression of osteopontin in the diabetic rat kidney via the p38 MAPK pathway

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Abstract Osteopontin (OPN), a large phosphoglycoprotein adhesion molecule, which is up-regulated in the kidneys of humans and mice with diabetes, has emerged as a potentially key pathophysiological contributor in diabetic nephropathy. Here, we investigated the role of OPN in kidney injury caused by diabetic nephropathy and the effect of atorvastatin on the expression of OPN and on diabetic nephropathy. Diabetes was induced with streptozotocin in rats, and atorvastatin (5 mg/kg) was orally administered once a day for 8 weeks. We analyzed the expression and regulation of OPN in the kidneys of streptozotocin-induced diabetic Sprague–Dawley albino rats by immunohistochemistry and western blot analysis. The expression of OPN was increased in diabetic rat kidney, and atorvastatin inhibited this process. Atorvastatin also decreased the expression and phosphorylation of p38. In vitro, atorvastatin inhibited the high glucose-induced OPN expression in Madin-Darby canine kidney epithelial cells through the p38 MAPK signaling pathway. These results suggested that atorvastatin reduced the expression of OPN through inhibition of the p38 MAPK pathway. The expression of OPN was associated with kidney injury. These molecules may represent therapeutic targets for the prevention of acute kidney injury induced by diabetes.

Keywords Atorvastatin · Osteopontin · Diabetes mellitus · Hyperglycemia · MAPK

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Background

In 2010, the prevalence of diabetes represented 6.6 % or approximately 285 million of the adult population worldwide. Experts predicted that the number of people with diabetes will rise to 438 million by 2030. One-third of the person with diabetes would eventually develop chronic kidney disease [1, 2]. Diabetic nephropathy (DN) was a serious complication of diabetes mellitus and the most common cause of end-stage renal disease. Therefore, there was an urgent need to identify new therapeutic targets to prevent the development of DN. Atorvastatin, as a lipophilic statin, exerted the beneficial effects of in the prevention of cardiovascular disease and improvement of outcomes in patients with diabetes [3]. Several recent studies had shown that atorvastatin could also reduce renal injury by decreasing mesangial matrix expansion and hyper

cellularity [4]. In recent years, evidence supported the concept that osteopontin (OPN) [5–11] may be causally involved in the premature development of pathogenesis in patients at increased risk for DN.

Osteopontin was a secreted acidic phosphoprotein that binded to a cell-surface integrin-binding motif and was involved in many inflammatory and immune-modulating disorders [10, 11]. Takemoto and colleagues reported that high glucose concentrations stimulate OPN expression in cultured smooth muscle cells [12]. Gong and colleagues demonstrated that glucose induced a dose-dependent upregulation of OPN promoter activity after 24 h [13]. In addition, OPN protein is selectively upregulated in the serum of type 1 and type 2 diabetes mellitus, diabetic vascular walls and diabetic kidneys [14–18]. OPN was expressed in all glomerular cells: mesangial cells, podocytes and endothelial cells [19–21].

In the present study, we provided evidence that OPN was a critical mediator in DN and that the renal protective effect of atorvastatin in DN involves the suppression of OPN. In vivo, hyperglycemia induced OPN expression in kidney of rat with diabetes. Atorvastatin reduced kidney damage through the decreased expression of OPN. We also demonstrate that a high glucose condition stimulated OPN expression in MDCK cells. Atorvastatin reduced OPN expression that was induced by high glucose, and this effect was mediated by the p38 MAPK pathway. Targeting OPN could therefore provide a novel therapeutic strategy to prevent DN.

Materials and methods

Experimental animals

The experimental procedures used in this study were approved by the Animal Use Committee of Anhui Medical University. Male adult Sprague–Dawley albino rats (weighing 130–150 g) from an inbred colony were fed under well-controlled conditions of temperature (24 ± 2 °C), humidity (50–60 %) and a 12/12 h light–dark cycle with diet and water provided ad libitum.

Animal treatments

After one week of adaption period, the rats were then randomly divided two groups: normal control ($n = 9$), high fat diet control ($n = 30$). Five weeks later, the rats with high fat diet were divided into three groups: high fat diet control ($n = 6$), diabetes induced by streptozotocin ($n = 7$) (STZ: Sigma, Shanghai, China; USA) and rats with diabetes treated with atorvastatin ($n = 5$). All rats were fed with a high fat diet for 8 weeks except the normal control group. After fasting for 8 h, rats in the diabetes and atorvastatin groups were injected with STZ in 10 mM sodium citrate buffer intraperitoneally at a dosage of 45 mg/kg body weight [22]. Seven days after STZ injection, the rats were kept fasting for 12 h, blood samples extracted from the tails were collected and fasting blood sugar was measured using a commercially available kit (Zhongshan Biotechnology Limited Company, Beijing, China). Rats with a blood glucose level ≥16.7 mmol/L (300 mg/L) were considered diabetic models. Atorvastatin was dissolved in distilled water and administered orally at a dose of 5 mg kg^{-1} day^{-1} for 8 weeks with food and water ad libitum. All the animals were sacrificed by anaesthetic overdose. A portion of the kidney tissue from the animals was fixed in neutral formaline, covered with optimum cutting temperature (OCT) compound, and snap-frozen in liquid nitrogen. Samples of fresh tissue were also snap-frozen in liquid and stored in a −80 °C freezer for protein extraction.

Serum lipid and glucose level measurements

Rats were fasted for 6 h and then anesthetized with 3 % pentobarbital. Blood was collected from the retro-orbital venous plexus with a capillary tube. Serum concentrations of glucose, triglycerides, and cholesterol were determined enzymatically using kits purchased from Sigma.

Immunohistochemistry

Formalin-fixed kidney tissue was processed, and 5 μm-thick paraffin sections were stained with hematoxylin and eosin (H&E). To determine the expression and localization of OPN (Santa Cruz Biotechnology, sc21742, France), immunohistochemistry was performed as previously described [23]. Briefly, 5 μm-thick sections of paraffin-embedded kidneys were deparaffinized in xylene and rehydrated in graded alcohol baths. Glass slides with tissue section mounted were boiled in 10 mM citrate buffer (pH 6.0) for 10 min to allow antigen retrieval. The tissue then were blocked with normal sheep serum for 10 min at room temperature prior to a 12 h incubation at 4 °C with primary antibody against OPN (1:500). The slides were incubated in the biotin-labeled sheep anti-mouse IgG for 20 min at 37 °C followed by a 15 min incubation in HRP labeled strepto antibiotin at 37 °C. The tissue sections were then dyed with DAB and redyed with hematoxylin. The immunohistochemical results for OPN were classified according to the percentage of the number of positive cells as follows: −, no staining; +, weak staining (≤50 %); ++, strong staining (≥50 %). The stain was interpreted by two pathologists independently.

Cell culture

The MDCK epithelial cells (BCRC No.60004) were purchased from ATCC, maintained in Dulbecco's modified

eagle medium (Gibco-BRL, USA), and supplemented with 10 % fetal bovine serum and antibiotics. After cells reached approximately 80 % confluence, they were incubated in serum-free medium with various final concentrations of D-glucose (5.5, 10, 20, 30 or 40 mM) for 24 h. MDCK cells exposed to 5.5 mM D-glucose were used as the control. To ensure an identical osmolarity of the samples, we added 24.5 mM mannitol into the control cultures. We then treated MDCK cells with atorvastatin (50, 100, 150, 200, 250 μM), PMA (1 μM) and SB203580 (10 μM). After 24 h of incubation, the cells were washed with PBS and harvested.

Protein isolation and western blot analysis

Cells of kidney tissues from the different groups were grinded and lysed in modified RIPA lysis buffer. Meanwhile, MDCK cells treated with atorvastatin were scraped and lysed with RIPA lysis buffer. Then the protein concentrations in the supernatant were determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). Equal protein concentrations were loaded on 10 % SDS–polyacrylamide slab gels and transferred to PVDF membranes. Blotted proteins were reacted with primary mouse monoclonal OPN (1:500), β-actin (1:1,000), p38 (1:1,000) and pp38 (1:500) antibodies (diluted in Tween/PBS: 0.5 % Tween in PBS, pH 7.4; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The protein bands were visualized with enhanced chemiluminescence reagents (ECL Plus Western Blotting Detection System; Biyuntian Biosciences, China), analyzed, and intensity-quantified using Quantity One software [23].

Statistics

All data were expressed as the mean ± standard error of mean (SEM). Statistical significance between control and treated groups was assessed by one-way ANOVA. If there was a significant overall difference among groups, then Tukey's post hoc test was used to make pair-wise comparisons. Values of $P < 0.05$ were considered statistically significant.

Results

Levels of metabolic parameters

As shown in Table 1, all rats gained weight when fed with high fat diet (HFD) for 5 weeks. The level of serum glucose, triglycerides and cholesterol did not change. These results indicated that a HFD diet for 5 weeks did not cause a metabolic disturbance of glucose, triglycerides and cholesterol.

Subsequently, we induced diabetes by streptozotocin injection in SD rats. Eight weeks after injection of streptozotocin, the mean body weight decreased in diabetes rats. However, the rats regain weight after treated with atorvastatin. The animals treated with STZ showed a significant increase in serum glucose levels from 5.03 ± 1.39 (control) to 20.04 ± 6.00 mmol/L ($F = 21.971$, $P < 0.01$) that was associated with a 30 % decrease in body weight. Concomitantly, serum cholesterol levels were significantly increased in the hyperglycemic condition ($F = 12.219$, $P < 0.01$), which suggested that high fat diet resulted in the metabolic disturbance of glucose and cholesterol. In contrast, these rats treated with atorvastatin for 60 days had an increased weight compared with diabetes rats without atorvastatin, but their glucose level remained unchanged ($F = 1.938$, $P = 0.194$). Interestingly, atorvastatin was able to reduce the serum cholesterol level ($F = 17.203$, $P = 0.001$) (Table 1). Previous reports indicated that atorvastatin reduced the number of low density lipoprotein (LDL) particles [24], which was involved in the transportation of cholesterol from liver to circulation. Thus, atorvastatin might decreased the cholesterol through reduction of LDL particles.

Atorvastatin prevented the damage of the glomerulus and nephric tubules induced by diabetes

Compared with the normal rats, diabetic rats showed minor glomerular hypertrophy; there were many fissures together with a slight enlargement of the mesangial areas and diffusion of the mesangial matrix in the renal capsule. Slight hypertrophy and edema were observed in the kidney

Table 1 The Effect of atorvastatin on the diabetic rats body weight, serum glucose, triglyceride, cholesterol concentration

	n	Body weight (g)	Serum glucose (mmol/L)	Serum triglyceride (mmol/L)	Serum cholesterol (mmol/L)
Normal control	9	476.89 ± 29.85	4.16 ± 1.20	0.79 ± 0.21	2.35 ± 0.65
HCF control	6	541.67 ± 74.72	5.03 ± 1.39	0.86 ± 0.52	2.82 ± 0.98
STZ diabetes model	7	386.00 ± 20.90	20.04 ± 6.00*	0.80 ± 0.55	12.55 ± 4.82*
Atorvastatin	5	444.20 ± 57.50	18.55 ± 4.31	0.79 ± 1.76	2.83 ± 1.42**

Compare with normal control: *$P < 0.05$, compare with STZ diabetes model: **$P < 0.05$

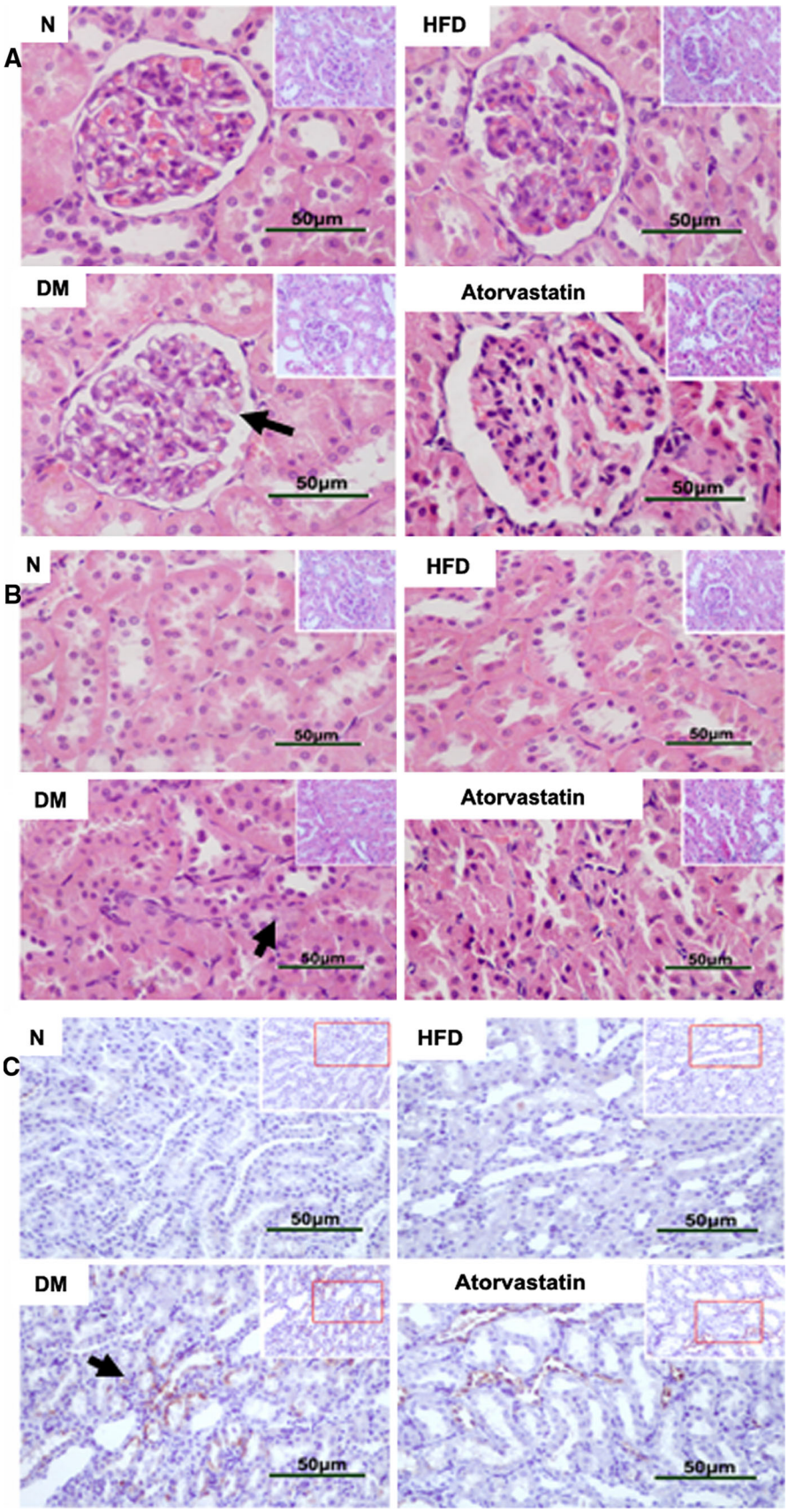


Fig. 1 Atorvastatin prevented the damage of diabetic rat renal tubules and glomerulus and decreased the expression of OPN induced by STZ. **a** Compared with the control and HFD (high fat diet) groups, the glomerulus of diabetic rats showed slight hypertrophy. The renal capsules were fissured and observed with slight mesangial expansion and mesangial matrix diffusion. After atorvastatin treatment, glomerular lesions improved significantly, as shown in **a**. **b** Compared with the control and HFD groups, the renal tubules from the diabetic group showed slight hypertrophy and edema, vacuolar degeneration, and tubulointerstitial injury with infiltration of inflammatory cells. After atorvastatin treatment, renal tubule lesions improved significantly. **c** The expression of osteopontin was minimal in control and HFD rat renal tubules but was significantly elevated in the diabetic rat renal tubules; atorvastatin significantly reduced the expression of osteopontin in diabetic rat renal tubules. Magnification: ×400

tubules followed by clear vacuoles of sample degeneration. The small tube stroma was invaded by many inflammatory cells. However, atorvastatin was able to ameliorate these changes (Fig. 1a, b).

Atorvastatin inhibits the expression of OPN in diabetic rat nephric tubules

Immunohistochemistry analysis was performed to determine the expression of OPN in rat kidney, the results indicated that the expression of OPN located in nephric tubules but not in glomerulus. The data revealed that enhanced expression of OPN was observed in the diabetes rats kidney as compared with normal rats kidney. However, the expression of OPN was decreased significantly by atorvastatin (Fig. 1c). To accurately describe the change in the expression of OPN in these groups, western blot analysis was performed, and the results indicated an identical tendency with the results from immunohistochemistry. The Western blot results were scanned and analyzed using Image-Pro 4.5 software. The data indicated that compared with normal rats, there was an obvious up-regulation in the expression of OPN in diabetic rats. ($F = 44.33$, $P < 0.01$). Meanwhile, there was a meaningful difference in OPN expression between diabetic rats and the rats treated with atorvastatin ($F = 115.337$, $P < 0.01$) (Fig. 2a).

Atorvastatin inhibits the phosphorylation of p38 in diabetic rat kidney

To elucidate the mechanism of atorvastatin action, we also determined the expression of a signal molecule involved in MAPK signal transduction. The expression of p38 showed no difference among the groups. However, the active form of p38 was increased in diabetic rat kidney compared with normal rat kidney ($F = 71.635$, $P < 0.01$). Moreover, atorvastatin could reverse this effect ($F = 63.805$, $P < 0.01$) (Fig. 2b).

Atorvastatin inhibits the expression of OPN in MDCK cells through down-regulation of the p38 pathway

The effect of high glucose on OPN expression was further characterized in cultured MDCK cells exposed to various concentrations of glucose in vitro. OPN expression in MDCK cells was increased by high glucose in a concentration-dependent manner after 24 h of incubation (Fig. 3a). However, OPN expression remained unchanged in mannitol-treated MDCK cells, which indicated that hyperosmolarity does not increase OPN levels. The increase of OPN expression by high glucose in MDCK cells was similar to that observed in diabetic rats kidneys.

To demonstrate the suppression of the p38 pathway by atorvastatin and the subsequent down-regulation of OPN

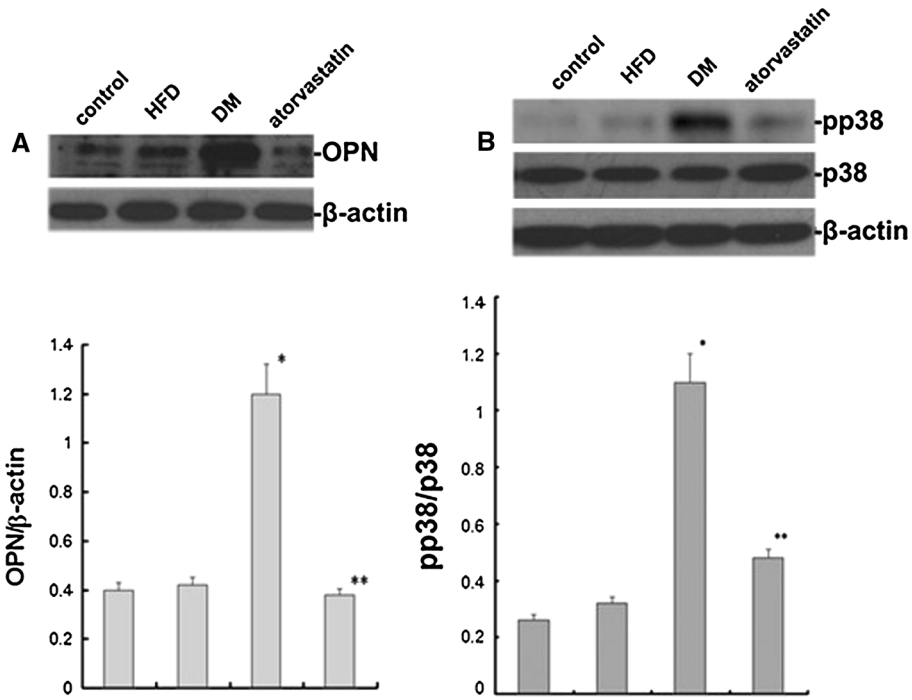
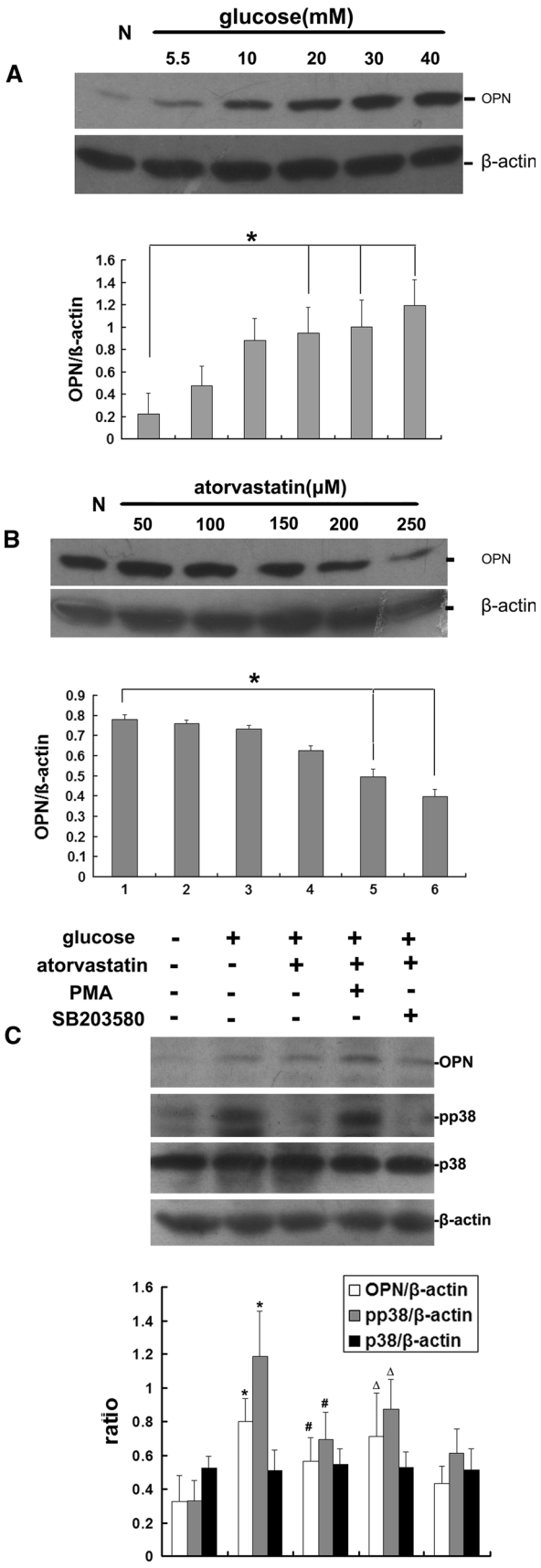


Fig. 2 Western blot detection of osteopontin expression and phosphorylation of p38 in rat kidney tissue. **a** Osteopontin was found to be few expression in the kidney of normal rat and HFD rats but highly expressed in the diabetic rat kidney, and atorvastatin significantly reduced this expression in diabetic rats. Compared with the control group, $*P < 0.05$; compared with the diabetic group, $**P < 0.05$. **b** The expression of p38 had no difference among groups. The level of phosphorylated p38 was found to be higher in the diabetic rat kidney, and atorvastatin significantly reduced p38 phosphorylation in diabetic rats. Compared with the control group, $*P < 0.05$; compared with the diabetic group, $**P < 0.05$



◀**Fig. 3** Western blot detection of the expression of osteopontin in MDCK cells. **a** The expression of OPN in MDCK cells was increased by high glucose in a concentration-dependent manner. The expression of OPN in MDCK cells cultured in 20–40 mM glucose showed a significant difference with the normal group; $^{*}P < 0.05$. **b** Atorvastatin significantly reduced the expression of OPN induced by high glucose. **c** Atorvastatin significantly reduced the expression of OPN induced by high glucose via the p38 MAPK signaling pathway. High glucose stimulated the expression of OPN; $^{*}P < 0.05$ (compared with the normal group). Atorvastatin reduced the expression of OPN induced by high glucose; $^{\#}P < 0.05$ (compared with the high glucose group). When the cells were treated with a combination of atorvastatin and PMA, the OPN expression was identical to normal MDCK cells; $^{\Delta}P < 0.05$ (compared with the PMA group). However, treatment with atorvastatin and SB203580 tremendously reduced OPN expression

protein, MDCK cells were treated with atorvastatin, and p38 phosphorylation was quantified by western blot analysis. As shown in Fig. 3b, atorvastatin inhibited high glucose-induced expression of OPN in MDCK cells.

To determine whether the p38 signaling pathway is responsible for atorvastatin-induced down-regulation of OPN, we performed a series of experiments in which MDCK cells were pretreated with pharmacological inhibitors and activator of the p38 MAPK signaling pathway, and the effect on atorvastatin-induced OPN downer-gulation was assessed by western blot analysis. As shown in Fig. 3c, pretreatment with the p38 MAPK activator PMA increased the phosphorylation of p38 and the expression of OPN ($F = 26.980$, $P < 0.01$, $F = 12.432$, $P < 0.05$), but inhibitor SB203580 inhibited glucose-induced p38 MAPK phosphorylation and correspondingly repressed OPN expression ($F = 48.341$, $P < 0.01$, $F = 28.651$, $P < 0.01$). These results suggested that atorvastatin induced the down-regulation of OPN in a p38 MAPK-mediated manner, and this was confirmed by densitometric analysis.

Discussion

In recent decades, numerous investigators have labored to identify the molecular mechanisms involved in the initiation and progression of DN to develop new therapeutic strategies. However, end-stage renal disease caused by DN continues to increase worldwide. Therefore, there is an urgent need to identify additional new therapeutic targets for the prevention of DN.

Here, we reported hyperglycemia resulted in the damage of the glomerulus and nephric tubules in diabetes rats. This event might be associated with the expression of OPN. We found that hyperglycemia stimulated OPN expression in diabetic rat kidney. These results were similar to that described in db/db mice. In these cases, OPN expression in DN mouse models enhanced glomerular damage likely

through reactive oxygen species generation, intrarenal rennin-angiotensin system activation, TGF-β expression, and PKC signaling, whereas its deletion protected against disease progression [25–29]. Several gene array studies have suggested that OPN expression strongly correlates with albuminuria and glomerular disease [30].

However, atorvastatin, which was an antilipemic agent [31], has also been demonstrated to reduce the expression of many genes, including the OPN gene [32]. In the diabetic rat model, we also detected the down-regulated expression of OPN in the kidneys of diabetic rats treated with atorvastatin. These results suggested that atorvastatin could ameliorate the glomerular damage by reducing OPN expression.

Meanwhile, in vitro studies demonstrated that under high glucose experimental conditions, the upregulated expression of OPN was detected in MDCK cells. As described previously, high glucose levels have been shown to stimulate OPN expression through protein kinase C-dependent pathways and via the hexosamine pathway in cultured rat aortic smooth muscle cells [33]. Gong et al. reported that glucose induced a dose-dependent upregulation of the OPN promoter activity after 24 h. Interestingly, atorvastatin also reduced the high glucose-induced expression of OPN in MDCK cells.

As described previously, p38 MAPK is known to upregulate specific cytokines such as IL-6, IL-8, and TNF-α in several biological contexts including kidney damage [34–36]. It has been reported that inhibition of p38 MAPK in autoimmune renal disease reduced the severity of the disease, resulting in a prolonged life span in mice [37, 38]. To elucidate the expression and regulation of OPN in diabetic rat kidney and whether atorvastatin could reduce the expression of OPN, MDCK cells were stimulated by PMA and treated with SB203580, which are a known activator and inhibitor of the p38 MAPK signaling pathway, respectively, and simultaneously treated with atorvastatin. The results indicated that activation of the p38 pathway by PMA increased the expression of OPN in MDCK cells. Meanwhile, inhibition of the p38 pathway by SB203580 significantly inhibited OPN expression in MDCK cells. Moreover, atorvastatin could inhibit the p38 pathway, although the mechanism remains unknown. OPN expression was more effectively reduced in MDCK cells treated with atorvastatin and SB303580. This protection against diabetes-induced renal injury by atorvastatin in vivo resulted from the reduced expression of OPN, which is known to promote renal injury. Atorvastatin reduced the activation of the p38 MAPK signaling pathway, which reduced the expression of OPN and in turn alleviated kidney injury, thus suggesting that inhibition of the p38 MAPK pathway may be a novel therapeutic strategy for the treatment of acute kidney injury induced by diabetes.

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