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Original Article

Saturated fatty acids bound to albumin enhance osteopontin expression and cleavage in renal proximal tubular cells

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Abstract: Osteopontin (OPN) is one of the proinflammatory cytokines upregulated in the kidneys of diabetic animals and patients with nephropathy. An increase in urinary albumin and albumin-bound fatty acids (FA) presents a proinflammatory environment to the proximal tubules in proteinuric kidney diseases including diabetic nephropathy. This study was designed to examine if FA overload could stimulate OPN expression and cleavage in renal tubule epithelial cells. OPN gene and protein expression was examined in the kidney of Zucker diabetic (ZD) rats and cultured proximal tubular cells exposed to either bovine serum albumin (BSA) or BSA conjugated with palmitic acid (PA), the most abundant saturated plasma FA. Real-time PCR analysis confirmed an upregulation of renal cortical OPN gene correlated with albuminuria and nephropathy progression in ZD rats at the age of 7-20 weeks. Immunofluorescence staining of kidney sections revealed a massive induction of OPN protein in albumin-overloaded proximal tubules of ZD rats. A significant increase in both intact and cleaved OPN proteins was further demonstrated in the diabetic kidney and urine samples, which was attenuated by antiproteinuric treatment with losartan, an angiotensin II receptor blocker. When exposed to fatty acid-free BSA, NRK-52E cells exhibited an increase in protein levels of full-length and cleaved OPN. Moreover, the increase in OPN fragments was greatly enhanced in the presence of PA (250-500 μM). Together, our results support a stimulatory effect of albumin and conjugated FA on OPN expression and cleavage in renal tubule epithelial cells. Thus, besides lowering albuminuria/proteinuria, mitigating circulating FAs may be an effective intervention for preventing and slowing down the progression of nephropathy associated with obesity and type 2 diabetes.

Keywords: Albuminuria, palmitic acid, lipotoxicity, tubular cell, diabetic kidney disease

Introduction

Obesity and Type 2 diabetes are major causes of chronic kidney disease, which can progress to end-stage renal failure. Diabetic nephropathy is characterized by persistent albuminuria, progressive decline of glomerular filtration rate, and development of tubulointerstitial inflammation, fibrosis and scarring. The presence of albuminuria is not only an early marker of kidney injury, but also has a detrimental effect on tubulointerstitial function due to exposure of the proximal tubules to excess albumin. Recently, albumin-bound free fatty acids (FAs, long chain fatty acids) have been proposed as important mediators of renal tubulointerstitial inflammation and disease progression in proteinuric kidney disease [1-3].

Plasma concentrations of FAs are increased in patients with metabolic syndrome [4], obesity [5] and type 2 diabetes mellitus [6]. Increasing evidence indicates that saturated FAs in non-adipose tissue activate inflammatory signaling pathways leading to lipotoxicity and cell dysfunction [7]. Normally, a minimum amount of albumin-bound FAs are filtered through glomeruli and reabsorbed into the proximal tubules [8]. In albuminuric/proteinuric kidney disease, excess FAs overload the proximal tubules, aggravating urinary protein-related tubulointerstitial damage. However, the exact mediator(s) responsible for FA-mediated tubulointerstitial inflammation and renal dysfunction remain largely unknown.

Osteopontin (OPN), a secreted adhesive glycoprotein, is upregulated in injured tissues and plays an important role in tissue remodeling by modulating inflammation and fibrosis [9-11]. OPN is expressed by osteoblasts, macrophages, activated T cells, and distal tubular epi-

thelial cells. Post-translationally, OPN is cleaved by thrombin and matrix metalloproteinases (MMPs) to form a C-terminal fragment (OPN-C) and an N-terminal fragment (OPN-N). Biological assays demonstrate that thrombin- or MMP-cleaved OPN has increased activity in promoting cell adhesion and migration compared with full-length OPN (OPN-FL) [12, 13]. More recently, Wolak et al. have shown that OPN-N rather than OPN-FL or its OPN-C is associated with carotid-plaque inflammation in hypertensive patients [14]. Although an upregulation of OPN expression has been demonstrated in the kidneys from diabetic animal models as well as humans with kidney injury [15-21], most of these studies evaluated the potential role of OPN in nephropathy by measuring the full-length OPN protein. Moreover, it remains unclear whether albumin-conjugated FA can modulate OPN expression and cleavage accelerating disease progression in proteinuric kidney disease.

In human aortic smooth muscle cells, palmitic acid (PA), the most abundant long-chain saturated FA in plasma, has been shown to induce gene expression for bone-related proteins including OPN [22]. Therefore, this study aimed to investigate whether PA or albumin overload could stimulate OPN expression and cleavage in renal proximal tubule epithelial cells. Our findings showed that tubular expression and secretion of intact and cleaved OPN were significantly increased in association with albuminuria and kidney injury in Zucker diabetic (ZD) rats. In vitro cell culture study further revealed a direct stimulatory effect of PA on OPN expression and cleavage in NRK-52E, a rat proximal tubular cell line.

Materials and methods

Animals

5-week-old male Zucker lean (ZL) and ZD rats were purchased from Charles River Laboratories (Wilmington, MA). Rats were housed in a temperature-controlled room with a 12:12-hour light-dark cycle and free access to Purina 5008 rat chow and water. Blood glucose was monitored weekly using the Accu-chek glucometer by tail-vein blood sampling. Urine samples were collected over a 24-hour period in metabolic cages and stored at -80°C until use. In one set of experiments, 12-week-old ZL and ZD rats were treated with angiotensin II type 1 receptor antagonist losartan (30 mg/kg/day in drinking water) for 8 weeks. The study was carried out in strict accordance with the recommendations in the Guide of the Care and Use of Laboratory Animals of the National Institutes of Health. All animal protocols were approved by the Institutional Animal Care and Use Committee of the Morehouse School of Medicine (approval number 12-27). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Urinary albumin and creatinine measurements

Urinary rat albumin was quantified using a commercially available rat albumin ELISA Kit (Exocell Inc., Philadelphia, PA, USA). Urine creatinine was measured by the modified kinetic Jaffe reaction. The urine albumin to creatinine ratio was calculated by dividing the urine albumin concentration by the urine creatinine concentration, both expressed in mg/dl.

Reagents

PA, BSA (A7030, fatty acid free and essentially globulin free), and anti-β-actin antibody were purchased from Sigma-Aldrich (St. Louis, MO). Mouse OPN monoclonal antibody was from Developmental Studies Hybridoma Bank (MPIIIB10, Iowa City, IA). Rabbit OPN polyclonal antibody against the amino-terminal and all tissue culture reagents were from Life Technologies (Carlsbad, CA).

Cell culture and FA treatment

NRK-52E cell line was purchased from the American Type Culture Collection (Monassas, VA). The cells were cultured in DMEM/F12 supplemented with 5% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were subcultured (passage number <20) once a week on reaching confluency. For FA treatment, PA was dissolved in ethanol and diluted 1:25 in serum-free DMEM/F12 medium containing 4% (w/v) BSA. Solutions were further diluted 1:4 with serum-free medium to give a final concentration of 1% BSA, 250-500 μM PA and 0.25% ethanol. Control media prepared similarly contained ethanol ± BSA in the absence of PA. 90% confluent cells were washed twice with serum-free medium and then exposed to different doses of PA for 24 hours.

Taqman and quantitative real-time PCR analysis

Total RNA was prepared from kidney cortex using ultra-pure TRIzol reagent according to the manufacturer's instructions (GIBCO-BRL, Grand Island, NY). OPN, kidney injury molecule-1 (KIM-1) and β-actin gene-specific Taqman probe and primer sets were obtained from Applied Biosystems (Foster City, CA) as Assays-on-Demand gene expression products. The Assays-on-Demand identification numbers were Rn00563571_m1 for OPN, Rn00597703_m1 for KIM-1, and 4331182 for rat β-actin endogenous control. Each sample was run in triplicate, and the comparative threshold cycle (C_t) method was used to quantify fold increase ($2^{-\Delta\Delta Ct}$) compared with lean controls.

Immunohistochemical and fluorescence staining

For immunohistochemical analysis, paraffin kidney sections were incubated with mouse anti-OPN monoclonal antibody. Slides were then incubated with ABC-HRP (peroxidase) reagents and DAB substrate (Vector, Burlingame, CA, USA). To examine the relationship between albumin overload and OPN expression, immunofluorescent staining was performed using 5-μm-thick cryostat sections of OCT-embedded kidney samples. The sections were incubated with a mixture of antibodies for mouse anti-OPN and sheep anti-rat albumin. As a negative control, the sections were exposed to nonimmune IgG (in replacement of primary antibodies) with the same secondary antibodies, and no specific staining occurred. To evaluate FA accumulation in renal tubules, BODIPY (493/503) staining of cryostat kidney sections was performed as described previously [23, 24]. The sections were observed and imaged by a Leica confocal microscope.

Immunoblot analysis

Kidney cortex homogenates, cell lysates or urine samples were separated by 10% SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. The membranes were first stained with Ponceau Red. After blocking in 5% milk for one hour, the blots were incubated with antibodies for OPN, stripped, and then reprobed with antibodies for β-actin. Detection was accomplished by enhanced chemiluminescence Western blotting (ECL, GE Healthcare, Piscataway, NJ). Relative band intensity was measured by ImageJ software. For kidney cortex or cell lysates, β-actin was used as an internal control. For urine samples, Ponceau red staining was used for loading control.

Statistical analysis

Data are expressed as mean ± SEM. Student's *t* test was used for comparison between two groups. Comparisons among multiple groups were performed by one-way ANOVA followed by Newman-Keuls post hoc test. Statistical significance was set at $P<0.05$.

Results

An upregulation of renal expression of OPN was associated with kidney damage and albuminuria in the diabetic rats

We have previously reported that blood glucose of the ZD rats began to increase at week 8, reached a peak at week 12 and remained high thereafter [25] (**Figure 1A**). A progressive increase in urinary albumin was observed in 7 to 20-week-old ZD rats (**Figure 1B**). Real-time PCR analysis was performed to determine mRNA levels of OPN and kidney injury molecule-1 (KIM-1), an early marker of tubular damage, in kidney cortex of Zucker rats at 7, 12 and 20 weeks of age. As shown in **Figure 1C**, KIM-1 transcript was significantly increased by 4-fold in 7-week-old ZD rats (prior to the development of hyperglycemia) compared to age-matched ZL littermates. Greater changes in cortical KIM-1 mRNA were observed in 12 and 20-week-old ZD rats. At the age of 7 weeks, OPN mRNA was slightly increased but did not reach statistical significance (**Figure 1D**). A significant upregulation of OPN gene was detected in ZD rats at 12 and 20 weeks of age, which was closely associated with the severity of albuminuria and kidney damage.

Next, we confirmed an induction of OPN protein in activated renal epithelial cells of ZD rats by immunohistochemical staining to the kidney sections of Zucker rats. As depicted in **Figure 1E**, massive OPN staining was observed in hyperplastic glomerular parietal epithelium and dilated renal tubules of 20-week-old ZD rats, whereas specific OPN staining was low in the ZL kidneys.

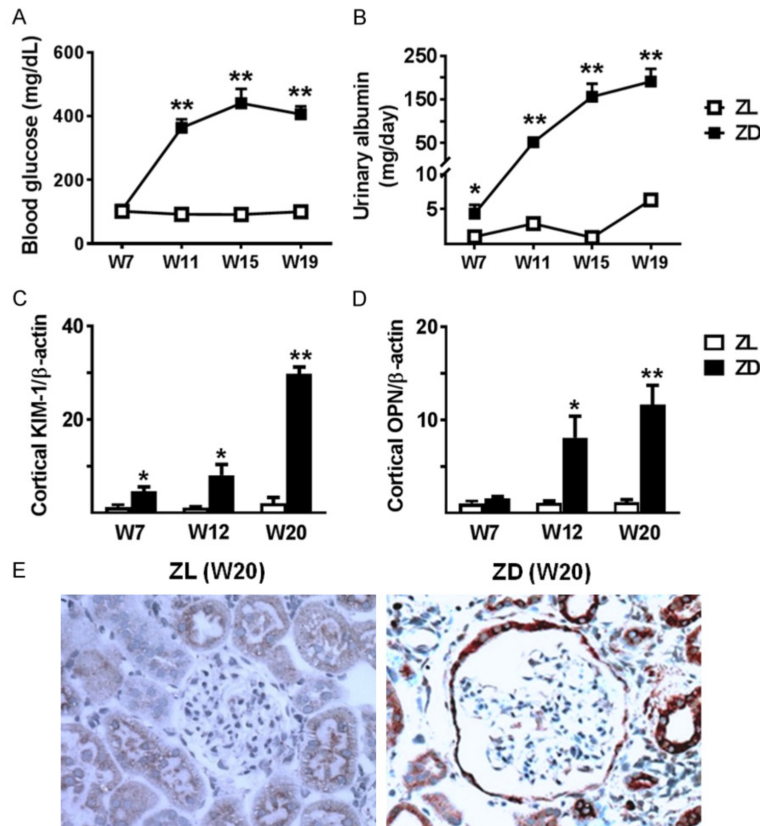

Figure 1. Diabetes-associated upregulation of kidney injury molecule-1 (KIM-1) and osteopontin (OPN) in kidney cortex of Zucker rats. (A and B) Blood glucose (A) and urinary albumin (B) levels in 7 to 19-week-old Zucker lean (ZL) and Zucker diabetic (ZD) rats. (C and D) Quantitative real-time PCR analysis of KIM-1 (C) and OPN (D) mRNA levels in the kidney cortex of ZL and ZD rats at the age of 7 weeks (W7), 12 weeks (W12) and 20 weeks (W20). Values are mean ± SEM. n = 4-6; **P*<0.05, ***P*<0.01 versus age-matched ZL rats. (E) Representative immunohistochemical images for OPN (brown) in 20-week-old ZL and ZD rat kidneys (400×).

An increase in OPN expression and cleavage was suppressed by losartan in the diabetic kidneys

As expected, losartan treatment for 8 weeks did not affect blood glucose but significantly lowered urinary albumin in ZD rats (**Figure 2A**). In ZL rats, we observed a slight but significant increase in urinary albumin following losartan administration. Using mouse anti-OPN monoclonal antibody, Western blotting detected a significant increase in OPN-FL band in kidney cortex of ZD rats (**Figure 2B**). Because cleaved OPN-N demonstrates robust inflammatory activity [14, 26, 27], we next determined the level of cleaved OPN-N fragments using rabbit OPN polyclonal antibody against the amino-terminal. Compared to ZL control group, renal cortical OPN-N fragments were significantly increased in ZD rats. This increase in both OPN-FL and OPN-N proteins was greatly attenuated by losartan treatment.

An increase in urinary OPN fragments was associated with albuminuria in ZD rats

Ponceau S staining of Western blot membranes was performed to evaluate protein contents and molecular weight in equal volumes of urine samples. As shown in **Figure 3A**, the most abundant proteins in ZL urine samples were small proteins with a molecular weight lower than 20 kDa. In contrast, a predominant albumin band was detected in untreated ZD urine, which was markedly reduced by losartan treatment. Western blot analysis of OPN-N showed high levels of three OPN-N fragments with molecular weights of 40, 32 and 25 kDa in ZD urine, whereas these bands were not detectable in ZL urine samples (**Figure 3B**). In accompany with a reduction of urinary albumin, losartan administration abolished the 40-kDa and 32-kDa bands and dramatically reduced the level of 25-kDa fragment.

OPN expression was increased in albumin-overloaded glomerular parietal and tubular epithelium of ZD rats

To further evaluate the relationship between albumin overload and OPN induction in the kidney of ZD rats, we performed double immunostaining for OPN and rat albumin. In the glomeruli of ZD rats, intensive OPN signal was observed in albumin-accumulated parietal epithelial cells (**Figure 4A**, arrow). Similarly, albumin-filled renal tubules were often OPN positive (**Figure 4A**, star), suggesting a close correlation between albumin overload and OPN induction in renal epithelium of ZD rats. Next, accumulation of fatty acids in tubular epithelial cells was

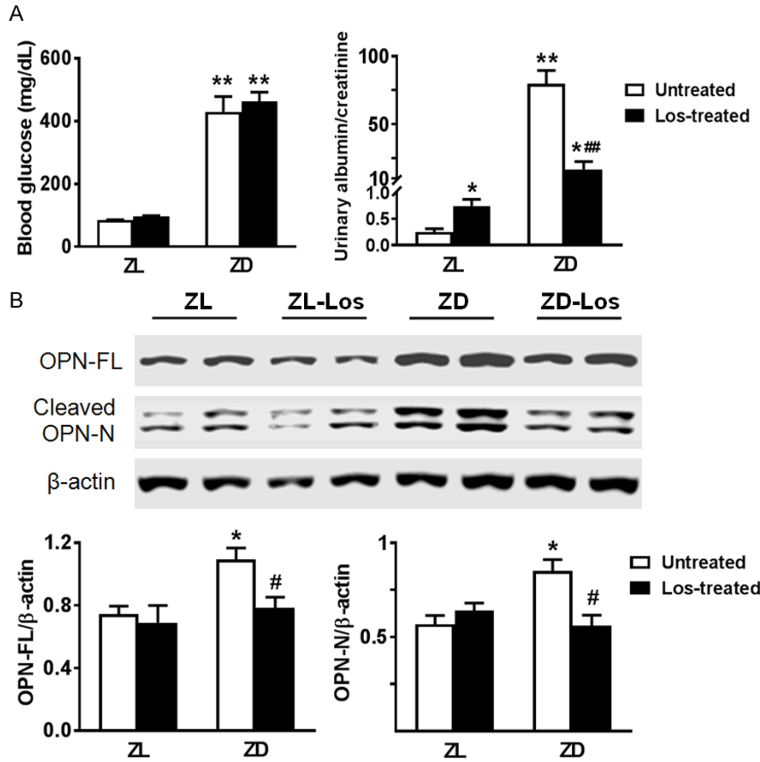

Figure 2. An upregulation of OPN protein expression and cleavage was suppressed by losartan in the diabetic kidneys. A. Losartan (Los) administration for 8 weeks did not alter blood glucose but significantly reduced urinary albumin level in ZD rats. B. Both OPN full length (OPN-FL) and cleaved N-terminal fragments (OPN-N) were significantly increased in kidney cortex of ZD rats, which was attenuated by losartan treatment. Values are mean ± SEM. n = 4-6; **P*<0.05, ***P*<0.01 versus age-matched ZL controls; #*P*<0.05, ##*P*<0.01 versus untreated ZD rats.

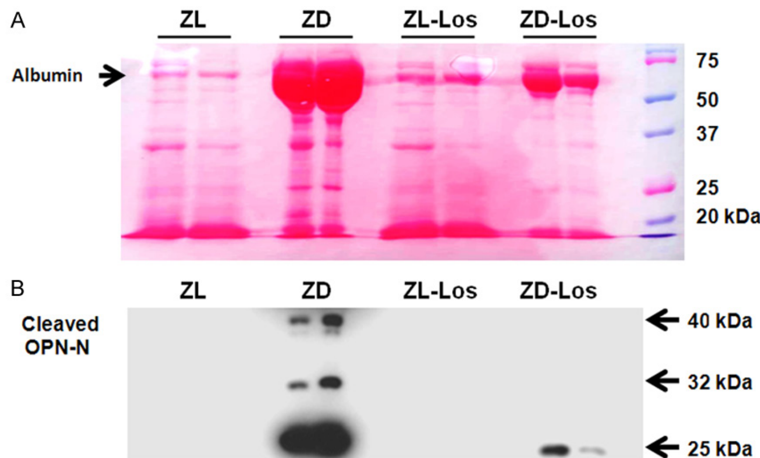

Figure 3. Increased urinary excretion of OPN fragments was mitigated by losartan in ZD rats. A. Ponceau red staining of Western blot membrane confirmed a massive increase in urinary albumin in untreated ZD rats. B. Western blot analysis detected a substantial increase in OPN-N fragments in the urine of untreated ZD rats. Chronic administration of losartan greatly mitigated urinary excretion of albumin and cleaved-OPN fragments.

determined by staining the frozen kidney sections for fat stores with BODIPY 493/503. Compared to normal controls, a substantially higher number of larger lipid droplets were found in the tubules of ZD rats, suggesting an increased accumulation of FAs in the diabetic kidneys (**Figure 4B**).

PA enhanced albumin-induced OPN protein in NRK-52E cells

To examine whether BSA-FA overload would stimulate OPN expression and cleavage in renal tubule epithelial cells, NRK-52E cells were incubated with BSA alone or BSA-conjugated PA (250 or 500 μM) for 24 hours. As shown in **Figure 5**, a predominant OPN-FL and two weak OPN-N fragments were determined in normal controls. When the cells were exposed to BSA alone, both intact and cleaved OPN were significantly increased compared to normal control group. This increase in OPN-FL and cleaved OPN-N fragments was greatly enhanced in the presence of 250 or 500 μM PA.

Discussion

In the present study, we showed an increase in OPN expression and cleavage in the kidneys of diabetic rats as well as an upregulation of OPN-FL and ONP-N in NRK-52E cells following albumin and PA stimulation. In the diabetic rats, an upregulation of OPN gene was associated with albuminuria and kidney injury progression. A close correlation between albumin overload and OPN induction was demonstrated in activated renal epi-

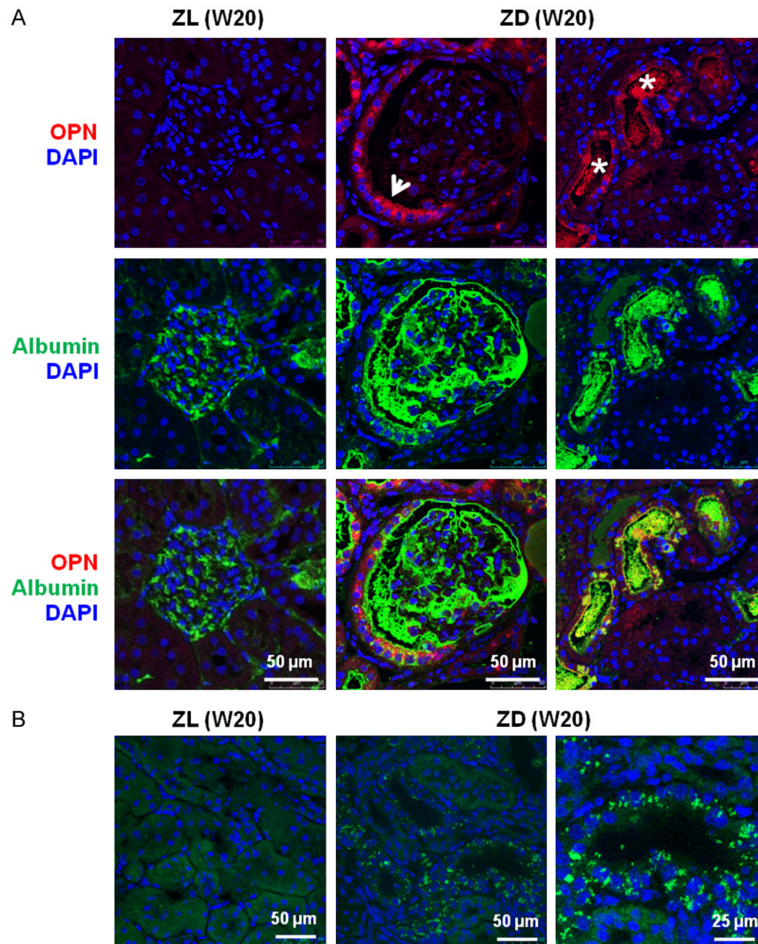

Figure 4. Immunostaining of OPN, albumin and lipids in the kidney of Zucker rats. A. Double immunofluorescence staining shows an increased OPN (red) in albumin (green)-overloaded glomerular parietal epithelial cells (arrow) and proximal tubules (star) of ZD rats. Nuclei were stained with DAPI (blue). B. Staining of neutral lipid droplets (LDs) with BODIPY (493/503) in frozen kidney sections revealed an accumulation of spherical LDs (bright green) within the tubule epithelial cells in ZD kidneys.

thelial cells of diabetic rats. Chronic administration of losartan mitigated urinary albumin and suppressed albuminuria-associated OPN expression and cleavage. Finally, albumin-induced OPN-FL and OPN-N fragments in NRK-52E cells were greatly enhanced in the presence of PA. Our in vivo and in vitro findings support a stimulatory effect of albumin and albumin-bound FA on OPN expression and cleavage in renal epithelial cells.

Overexpression of proinflammatory and profibrogenic factors and cytokines has been demonstrated in both diabetic and non-diabetic proteinuric kidney diseases. As a soluble multifunctional proinflammatory cytokine, OPN has been demonstrated to play important roles in promoting inflammation, tissue remodeling and fibrosis [28-32]. A positive correlation between OPN expression and macrophage infiltration has been shown in various experimental models of renal injury [33, 34]. For example, OPN inhibition or deficiency results in a significant decrease in macrophage infiltration and renal fibrosis in murine models of ischemia reperfusion injury [35] and diabetic nephropathy [18]. We have previously reported an upregulation of OPN in association with macrophage accumulation in the interstitial space of ZD rats [36]. Here, we further show an age-dependent upregulation of OPN transcript in proportion to the severity of albuminuria and tubular injury in ZD rat kidneys. In addition, our Western blot analysis and immunostaining confirmed a massive induction of OPN protein in albumin-overloaded glomerular parietal epithelium and proximal tubules of ZD rats. Previous studies from our lab and others evaluated the potential role of OPN in diabetic nephropathy by measuring the full-length OPN protein in the kidney or urine samples [15-21]. It is well established that after translation, OPN undergoes proteolytic cleavage by thrombin and MMPs into both OPN-C and OPN-N fragments. Importantly, proteolytic cleavage of OPN by thrombin and MMPs modulates its function. Compared to full-length OPN, in vitro [12, 37] and in vivo [34] studies have shown an enhanced chemotactic function of MMP-cleaved OPN-N fragment in different cells and tissues. Tan et al. [34] reported that reduction of macrophage infiltration in unilateral ureteral obstruction (UUO) following MMP-9 inhibition was associated with a reduction in MMP-cleaved OPN in the UUO kidneys. Moreover, they found that MMP-9-cleaved OPN significantly enhanced macrophage transwell migration [34]. In the current study, we performed Western blot analysis using an OPN

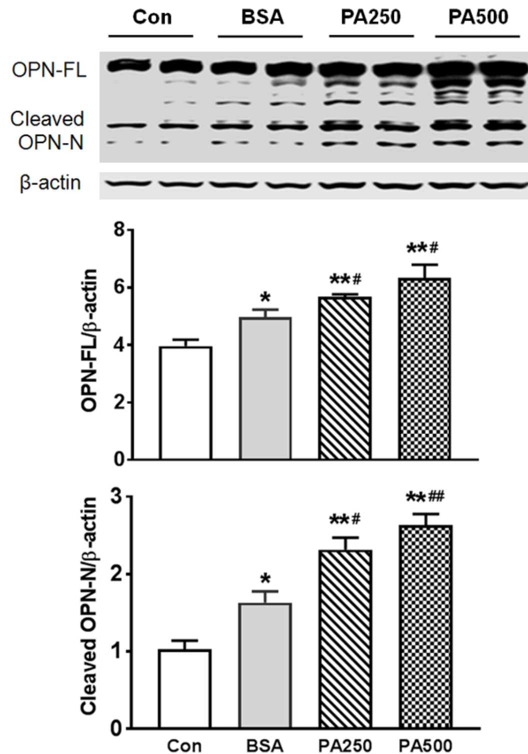

Figure 5. Effects of saturated fatty acid on OPN expression and cleavage in NRK-52E cells. NRK-52E cells were incubated with 1% bovine serum albumin (BSA) or BSA-conjugated palmitic acid 250 μM (PA250) or 500 μM (PA500) for 24 hours. Western blot analysis found that BSA alone increased both OPN-FL and cleaved OPN-N. BSA-induced OPN expression and cleavage were greatly enhanced in the presence of PA. Values are mean ± SEM. n = 3; **P*<0.05, ***P*<0.01 versus normal control group; #*P*<0.05, ##*P*<0.01 versus BSA only group.

antibody against its amino-terminal to determine the production and excretion of MMP-cleaved OPN-N fragment in diabetic rats. We found that both OPN-FL and MMP-cleaved OPN fragments were significantly increased in kidney cortex and urine samples of ZD rats. Three urinary OPN-N fragments with molecular weight around 25, 32, and 40 kDa were identified in the diabetic urine. This also supports our previous finding that MMP-9 expression and activity were massively induced in the kidney and urine of ZD rats [38]. Interestingly, a recent study has demonstrated that OPN-N terminal fragment, rather than OPN-full length, was associated with the degree of inflammation of carotid plaques in patients with hypertension, and tends to be less abundant in patients treated with ACE inhibitor or angiotensin II receptor I antagonist than in those not treated [14]. Therefore, identification of OPN-N fragments in the diabetic urine suggests that the post-translational cleavage of OPN may be a key step in exerting its inflammatory properties in diabetic nephropathy. OPN can also act as a paracrine signal, augmenting vascular pro-MMP9 activity via superoxide generation and oxylipid formation [39]. Given the abundance of both OPN and MMP-9 in the diabetic kidneys, it is possible that they function in concert to promote tubulointerstitial inflammation and accelerate nephropathy progression.

In the present study, our double immunostaining confirmed a close correlation between OPN induction and albumin overload of renal epithelial cells in ZD rats. Chronic administration of losartan significantly reduced the levels of both intact and cleaved OPN in the diabetic kidneys and urine samples. Given the fact that losartan reduced urinary albumin but had no effect on blood glucose in ZD rats, our results suggest that the inhibitory effect of losartan on renal OPN expression and cleavage may be largely attributed to its mitigation of albuminuria. Detection of lipid accumulation in renal tubules of ZD rats further supports a potential role for albumin-bound FAs in the enhancement of OPN expression and cleavage. In this study, we also observed a slight increase in urinary albumin excretion in losartan-treated ZL rats. This increase in urinary albumin is likely due to an inhibition of albumin endocytosis by renal epithelial cells since losartan has been shown to prevent angiotensin II-induced podocyte endocytosis of filtered albumin in healthy rat kidneys [40].

Hyperglycemia has been shown to upregulate OPN gene expression in kidney tissues [15, 41] and in cultures of mesangial cells [42, 43], renal tubular cells [17, 21, 44] and macrophages [45]. We and others have previously shown that albumin overload induces proinflammatory and profibrotic mediators such as monocyte chemoattractant protein-1 [46, 47], transforming growth factor-β [48, 49], and MMP-9 [38] in renal tubular cells. Here, we further demonstrated that albumin-conjugated FAs could enhance OPN expression and cleavage as evidenced by an increase in intact and cleaved OPN protein when NRK-52E cells were exposed to 250 μM and 500 μM of PA. This finding corroborates with a recent report that 250 μM PA induced gene expression for bone-

related proteins including osteopontin in human aortic smooth muscle cells [22]. Thus, our study revealed a direct stimulatory effect of FA on OPN expression and cleavage that may contribute to the aggravation of tubular injury and interstitial inflammation in proteinuric kidney disease.

There are several limitations in this study. First, our current experiments have not examined whether OPN upregulation is due to albumin uptake and intracellular release of free fatty acids. Experiments are currently underway to determine the underlying mechanisms by which PA induces OPN overexpression and cleavage in renal tubular cells. Secondly, the functional role of MMP-cleaved OPN fragments in tubular injury and renal inflammation require further exploration. Moreover, additional studies are also required to evaluate whether unsaturated FA could protect against tubulointerstitial damage by mitigating OPN activity in nephropathy associated with obesity and type 2 diabetes.

In summary, we demonstrated an albuminuria-associated increase in renal OPN expression and cleavage in a rat model of type 2 diabetes. Antiproteinuric treatment with losartan prevented renal production and urinary excretion of OPN fragments in diabetic rats. Moreover, our in vitro cell culture study further demonstrated a direct stimulatory effect of albumin and albumin-bound PA on OPN expression and cleavage. Our results suggest that mitigating circulating fatty acid levels may be an important intervention to prevent and slow down the progression of kidney disease associated with obesity and type 2 diabetes.

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Disclosure of conflict of interest

None.

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