ARTICLE
Journal of Cellular Biochemistry 109:1210–1221 (2010)

Journal of Cellular Biochemistry

# Involvement of Osteopontin Upregulation on Mesangial Cells Growth and Collagen Synthesis Induced by Intermittent High Glucose

Jiazhong Sun,* Yancheng Xu, Haohua Deng, Suxin Sun, Zhe Dai, and Yanlei Sun

*Department of Endocrinology, Zhongnan Hospital, Wuhan University, Wuhan 430071, China*

## ABSTRACT

Glucose fluctuations are strong predictor of diabetic vascular complications. We explored the effects of constant and intermittent high glucose on the proliferation and collagen synthesis of cultured rat mesangial cells. Furthermore, the possible involvement of osteopontin (OPN) was assessed. In rat mesangial cells cultured in 5, 25, or 5 mmol/L alternating with 25 mmol/L glucose in the absence or presence of neutralizing antibodies to OPN, β3 integrin receptor and β5 integrin receptor, the cell proliferation, collagen synthesis, and the expression of OPN and type IV collagen were assessed. In cultured mesangial cells, treatment with constant or intermittent high glucose significantly increased [$^3$H]thymidine incorporation in a time-dependent manner. A modest increase was observed at 12 h, and further deteriorated afterwards, and reached the maximum incorporation at 48 h. Treatment with constant high glucose for 48 h resulted in significant increases in [$^3$H]thymidine incorporation, cell number, [$^3$H]proline incorporation, mRNA, and protein levels of type IV collagen and OPN compared with mesangial cells treated with the normal glucose, which were markedly enhanced in cells exposed to intermittent high glucose medium. In addition, neutralizing antibodies to either OPN or its receptor β3 integrin but not neutralizing antibodies to β5 integrin can effectively prevented proliferation and collagen synthesis of mesangial cells induced by constant or intermittent high glucose. Intermittent high glucose exacerbates mesangial cells growth and collagen synthesis by upregulation of OPN expression, indicating that glycemic variability have important pathological effects on the development of diabetic nephropathy, which is mediated by the stimulation of OPN expression and synthesis. J. Cell. Biochem. 109: 1210–1221, 2010. 

**KEY WORDS:** INTERMITTENT HIGH GLUCOSE; OSTEOPONTIN; MESANGIAL CELLS; CELL PROLIFERATION

Diabetic nephropathy is a leading cause of morbidity and mortality in patients with diabetes mellitus. Between 20% and 40% of patients with diabetes ultimately develop nephropathy [Mauer et al., 1984; Makino et al., 1996]. Diabetic nephropathy is characterized by an expansion of glomerular mesangium, caused by mesangial cells proliferation, and excessive accumulation of extracellular matrix including type IV collagen, which eventually progresses to obliterate the capillary lumen, and leads to glomerulosclerosis and renal failure. Mesangial expansion, caused by mesangial cells growth and excessive accumulation of extracellular matrix, is a key pathologic feature of diabetic nephropathy [Klahr et al., 1988; Steffes et al., 1989; Leehey et al., 2000]. It has been widely accepted that hyperglycemia is a crucial factor in the increased deposition of matrix and the genesis of mesangial expansion and glomerulosclerosis in diabetes [Fumo et al., 1994].

Chronic hyperglycemia has been identified as a risk factor for the onset and progression of microvascular complications. Clinicians, however, often wonder why some patients under good metabolic control develop complications, while others remain free of such complications despite poor control [Klein, 1995]. One factor that can influence differing susceptibility to the appearance of microvascular complication despite similar levels of hemoglobin A1c (HbA1c) is the blood glucose control variability. Both fasting and postprandial hyperglycemia contribute to this process. However, the acute glucose fluctuations that occur in diabetes, including upward (postprandial) and downward (interprandial) fluctuations can be considered as risk factors for vascular complications and should be included in the "dysglycemia" of diabetes in combination with fasting and postprandial hyperglycemia. The sudden variations of blood glucose can have physiopathological value [Buse and Hroscikoski, 1998]. In fact, abrupt fluctuations in glucose levels are

Grant sponsor: Hubei Provincial Bureau of Health Science Foundation for Young Scholars; Grant number: QJX2008-29.

*Correspondence to: Dr. Jiazhong Sun, Department of Endocrinology, Zhongnan Hospital, Wuhan University, Wuhan 430071, China. E-mail: sjz300@163.com

Received 9 October 2009; Accepted 23 December 2009 • DOI 10.1002/jcb.22503 • © 2010 Wiley-Liss, Inc.
Published online 4 February 2010 in Wiley InterScience (www.interscience.wiley.com).

able to produce apoptosis of the retinal capillary pericytes [Li et al., 1996] and to increase collagen synthesis in tubulointerstitial cells [Jones et al., 1999]. Intermittent rather than constant hyperglycemia induces an increase in collagen production by cultured mesangial cells [Takeuchi et al., 1995]. Hence, the acute changes in plasma glucose concentrations may results in development of microangiopathy. There is increasing evidence that glycemic disorders such as rapid glucose fluctuations over a daily period might play an important role on diabetic complications [Monnier et al., 2006]. However, the mechanism and the effects of intermittent hyperglycemia on mesangial cells growth and collagen synthesis are poorly understood.

Osteopontin (OPN) is an arginine–glycine–asparatic acid (RGD)-containing protein that binds to a specific $\alpha_v\beta_3$ integrin receptor, which has been implicated in a number of cellular functions and features that are either directly or evolutionarily related to inflammation and tissue repair [Klienman et al., 1995]. OPN secreted by a variety of cells, including mesangial cells leads to cell spreading, adhesion, and proliferation [Ruoslahti and Engvall, 1997]. High levels of OPN mRNA and protein were reported in various animal models of renal fibrosis and glomerulonephritis, where OPN increases are associated with increased influx of monocytes/macrophages, suggesting a damaging role for OPN in progressive renal disease [Giachelli et al., 1994; Fischer et al., 1998]. In addition, mesangial cells in culture also exhibit substantial quantities of both OPN mRNA and protein, which can be regulated by serum and specific growth factors [Prols et al., 1998]. The OPN upregulation has also been demonstrated in the renal cortex of streptozotocin-induced diabetic rats and in the aortas of high-fat diet-induced diabetic mice, suggesting a role for OPN in the development of renal as well as vascular complications of diabetes [Fischer et al., 1998; Towler et al., 1998].

The aims of this study were to compare the effects of exposure to constant and intermittent high glucose concentrations on the proliferation and collagen synthesis of cultured rat mesangial cells. In addition, we investigated the possible involvement of OPN on cell proliferation and collagen synthesis in mesangial cells.

## MATERIALS AND METHODS

### CELL CULTURE

Rat glomerular mesangial cells were obtained from isolated, collagenase-treated rat glomeruli as previously described [Takemoto et al., 2002]. In brief, glomeruli were harvested from 100 g male SD rats by filteration with ice-cold phosphate buffer through 150, 80, and 85 mm nylon meshes. Those retained on the sieve were collected, washed by centrifugation (41°C, 800 rpm), and incubated with 250 U/ml collagenase (type I) for 30 min at 37°C under constant, gentle shaking. Mesangial cells were plated on plastic tissue culture dishes in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) containing 20% fetal bovine serum (FBS; Life Technologies), antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin), 24 mmol/L $NaHCO_3$, and 20 mmol/L HEPES. The cell medium was left untouched for 4 days and then changed every other day until confluence. These cells were characterized by typical stellate morphology, positive staining for desmin and vimentin. Cells used in these experiments were between passages 5 and 12.

### EXPERIMENTAL PROTOCOL

Mesangial cells at the logarithmic growth phase were trypsinized to a single cell suspension, adjusted to cell concentration $1 \times 10^5$/ml, and transferred to a six-well plate (the treatment volume 2.5 ml). After the cells reached confluence, the medium was replaced with serum-free DMEM and incubated for 24 h for synchronization. Cells were then incubated for a further 72 h in glucose-specific basic media. The cells were randomly divided into four groups: (1) constant normal glucose medium (5 mmol/L); (2) constant high glucose medium (25 mmol/L); (3) alternating normal and high-glucose media every 6 h; and (4) an osmotic control of 16.5 mmol/L mannose. Neutralizing antibodies to OPN, β3 integrin receptor and β5 integrin receptor (10 pg/ml; R&D Systems) were also added individually to the three media previously described. In all models, control experiments were performed in parallel, culturing cells in 5 or 25 mmol/L glucose for the whole period. All groups were otherwise subjected to identical conditions and the experiment was repeated at least five times.

### ASSESSMENT OF CELL VIABILITY AND CELL PROLIFERATION

Mesangial cells were subcultured in six-well plates as described in the Experimental Protocol Section. Quiescent cultures were then exposed to either constant or intermittent high glucose in serum-free medium for 12, 24, 48, and 72 h. Cell viability was determined at the end of treatments by trypan blue dye exclusion method.

[$^3$H]thymidine incorporation and cell number were used in the assessment of cell proliferation [Sahai et al., 1997]. Briefly, [$^3$H]thymidine (1 mCi/ml, specific activity 20 Ci/mmol) was added to one set of wells in the last 4 h of incubation. The other sets of wells were processed for cell counting. For the assessment of [$^3$H]thymidine incorporation, media was removed at the end of incubation, and cells were washed with 10% trichloroacetic acid (TCA) and digested with 0.5N NaOH. Radioactivity in the cell digest was counted in a Beckman scintillation counter. [$^3$H]thymidine incorporation is expressed as the total counts per minute per well.

### ASSESSMENT OF COLLAGEN SYNTHESIS

Measurement of total collagen synthesis was assessed by [$^3$H]proline incorporation as described Amemiya et al. [1999]. Briefly, mesangial cells were subcultured in six well plates as described in the Experimental Protocol Section. Quiescent cultures were then exposed to either constant or intermittent high glucose in serum-free medium for 48 h in the presence of 4 mCi/ml [$^3$H]proline and 50 mg/ml ascorbic acid. At the end of the incubation times, conditioned media proteins were precipitated with an equal volume of 12% TCA. The TCA-precipitated proteins were centrifuged at 1,000*g* for 10 min. The resulting pellets were washed three times with 6% TCA and then solubilized in 0.2N NaOH. Aliquots from each sample were counted in a Beckman scintillation counter. The remainder samples were adjusted to contain 100 mmol/L NaCl, 50 mmol/L HEPES, and 3 mmol/L $CaCl_2$, pH 7.0. Collagenase (type III, 100 U/ml) was then added to each sample, followed by

incubation for 16 h at room temperature. After collagenase digestion, the proteins were again precipitated as described above, washed three times with 6% TCA, solubilized in 0.2N NaOH, and subjected to liquid scintillation counting. Collagenase-sensitive [$^3$H] proline incorporation was calculated as the difference between TCA precipitable counts before and after collagenase digestion, which reflects total collagen synthesis.

### NORTHERN BLOT ANALYSIS

For the assessment of the mRNA levels of type IV collagen and OPN, mesangial cells were subcultured in 75 cm$^3$ flasks, and exposed to high glucose or intermittent high glucose as described in Experimental Protocol Section. Total RNA was isolated from the cells using TRIzol reagent (Sigma, Louis, MO). For Northern analysis, 20 μg aliquots of total RNA were separated on 1% agarose–formaldehyde gels, transferred to nylon membranes (Hybond-Nt; Amersham), and hybridized with [$^{32}$P]-labeled cDNA probe (Shanghai Sangon Biological Engineer Co. Ltd) of rat type IV collagen, rat OPN, and rat glyceraldehyde-3-phosphatedehydrogenase (GAPDH) by random priming. The cDNA probe used for type IV collagen recognizes both pro-α1 and -α2 type IV collagen. Therefore, mRNA expressions were combined to reflect total alterations in type IV collagen mRNA levels. Quantitations of Northern blots were performed by densitometric analysis by using an Eagle Eye II video system. Also, we used GAPDH as an internal control to standardize the amount of total RNA utilized for Northern blot analysis.

### WESTERN BLOT ANALYSIS

Osteopontin and type IV collagen protein levels were assessed by Western blot analysis as previously described by Sodhi et al. [2000] and Brantley et al. [2003]. Mesangial cells were subcultured in six-well plates and processed in a manner similar to Northern blot analysis. At the end of 48 h of incubation, conditioned medium was removed and centrifuged at 1,000 rpm for 5 min to remove any cell debris. Supernatants were then mixed with SDS sample buffer in a volume of total 10 μl, boiled for 5 min, and subjected to 10% SDS–PAGE. Proteins were transferred to nylon membranes and blotted for OPN [using a 1:10 dilution (5 mg/ml) of the OPN monoclonal antibody MPIIIB10; R&D Systems] and type IV collagen (using anti-Type IV collagen antibody at a 1:100 dilution; R&D Systems).The bound primary antibody was detected with a horseradish peroxidase-conjugated secondary antibody and visualized with an enhanced chemiluminescence method. Quantitations of Western blots were performed by densitometric analysis using an Eagle Eye II video system.

### STATISTICAL ANALYSIS

All experimental conditions were replicated fivefold. Protein content was expressed as a change from the control value (normal glucose) which was regarded as 100%. Results are expressed as mean ± SD. Statistical comparisons between groups were made by analysis of variance (ANOVA), with pairwise multiple comparisons made by Fisher's protected least-significant differences test. Analyses were done by the software package, SPSS 13.0. A value of $P < 0.05$ was considered significant.

## RESULTS

### EFFECT OF INTERMITTENT HIGH GLUCOSE ON MESANGIAL CELLS VIABILITY

To examine the effects of high glucose on cultured mesangial cells viability, mesangial cells were exposed to constant or intermittent high glucose in parallel for 12, 24, 48, 72 h, and at the end of the respective incubation times, cell viability was assessed by trypan blue exclusion. As shown in Table I, there was no significant difference in the percentage of viability of mesangial cells between the groups, indicating cell death was not measurably affected by high glucose conditions within 72 h of culture.

### EFFECT OF INTERMITTENT HIGH GLUCOSE ON PROLIFERATION OF MESANGIAL CELLS

As shown in Figure 1A, both intermittent and constant high glucose stimulated mesangial cells proliferation in a time-dependent manner. A modest increase was observed at 12 h, and further deteriorated afterwards, and reached the maximum incorporation at 48 h. Continuous increasing effect was not found when the cells were treated for the longer time (72 h). Therefore, 48 h was selected as the treated time in subsequent studies. Mannose (16.5 mmol/L), an enhancer of osmotic pressure did not affect the proliferation of mesangial cells, indicating that high glucose-induced mesangial cells proliferation was not due to an enhanced osmotic pressure.

Exposure to constant high glucose medium produced significant 64.4%, 96.2%, and 172.0%, 148.5% increases in [$^3$H]thymidine incorporation at 12, 24, 48, and 72 h of incubation, respectively, compared with cells exposed to normal glucose (a basic level of serum, here as a control). This effect was further enhanced when cells were exposed to intermittent high glucose medium, which caused 110.6%, 147.0%, and 242.2%, 212.4% stimulation of [$^3$H]thymidine incorporation compared with normal glucose conditions, respectively (Fig. 1A).

As shown in Figure 1B, exposure to constant high glucose medium for 48 h induced a significant 83.3% increase in cell number when compared with the results obtained under normal glucose medium ($165.7 \pm 4.21$ vs. $90.4 \pm 2.5 \times 10^3$/well, $P < 0.01$). This effect was further enhanced to 176.1% ($249.6 \pm 8.74$ vs. $90.4 \pm 2.5 \times 10^3$/well, $P < 0.01$) when cells were exposed to intermittent high glucose medium compared with normal glucose.

TABLE I. Effect of Intermittent High Glucose on Mesangial Cells Viability

| Hours of Growth | NG | OC | HG | N/HG |
|---|---|---|---|---|
| 12 | 90.6 ± 7.9 | 89.8 ± 7.7 | 90.3 ± 6.5 | 91.0 ± 8.2 |
| 24 | 91.3 ± 8.5 | 90.6 ± 6.9 | 89.8 ± 8.2 | 90.4 ± 7.1 |
| 48 | 91.1 ± 6.8 | 90.1 ± 8.6 | 88.7 ± 6.6 | 89.5 ± 7.6 |
| 72 | 88.9 ± 9.1 | 87.8 ± 7.8 | 88.1 ± 7.6 | 88.4 ± 7.4 |

Equal numbers of mesangial cells were plated in six-well plates and grown under constant or intermittent for the indicated time period. NG, constant normal glucose (5 mmol/L); OC, osmotic control (16.5 mmol/L mannose was used as a negative control); HG, constant high glucose (25 mmol/L); N/HG, 5 mmol/L alternating with 25 mmol/L glucose. After trypsinization, cell viability was assessed by trypan blue exclusion technique. Results are expressed as mean ± SD SD of at least five independent experiments.

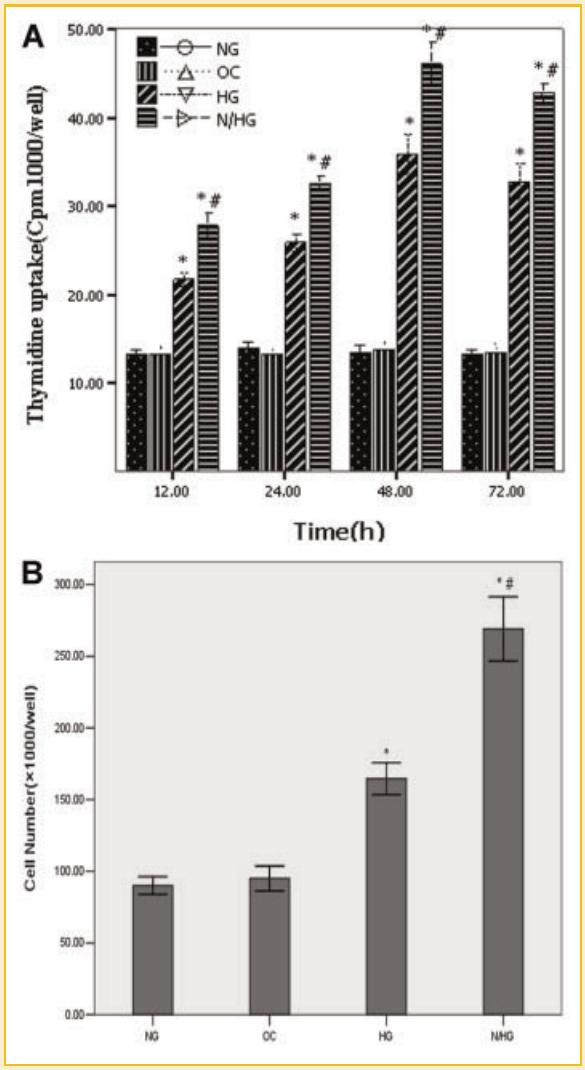


Fig. 1. High glucose induced proliferation of mesangial cells. [$^3$H]thymidine incorporation (A) and cell number (B) in cultured mesangial cells. NG, constant normal glucose (5 mmol/L); OC, osmotic control (16.5 mmol/L mannose was used as a negative control); HG, constant high glucose (25 mmol/L); N/HG, 5 mmol/L alternating with 25 mmol/L glucose. The growth-arrested mesangial cells were exposed to glucose at different concentration for indicated time and total cell lysates were harvested. Cell proliferation was evaluated by the assessment of [$^3$H]thymidine incorporation and cell number. The data were expressed as mean ± SD of at least five independent experiments. $^*P < 0.01$ versus NG; $\#P < 0.01$ versus HG.

There were also statistically significant changes in cell number after exposure to either constant or intermittent high glucose medium ($P < 0.01$).

These results indicate that intermittent high glucose appears to cause an additive stimulatory effect on proliferation of mesangial cells.

## EFFECT OF INTERMITTENT HIGH GLUCOSE ON COLLAGEN SYNTHESIS OF MESANGIAL CELLS

As shown in Figure 2, exposure to constant high glucose medium for 48 h resulted in a significant 87.4% increase in total collagen

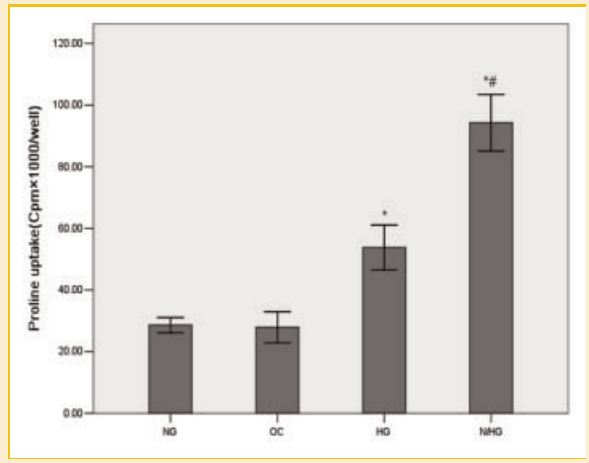


Fig. 2. Effect of high glucose on collagen synthesis in mesangial cells. NG, constant normal glucose (5 mmol/L); OC, osmotic control (16.5 mmol/L mannose was used as a negative control); HG, constant high glucose (25 mmol/L); N/HG, 5 mmol/L alternating with 25 mmol/L glucose. Collagen synthesis was determined by [$^3$H]proline incorporation. The data were expressed as mean ± SD of at least five independent experiments. $^*P < 0.01$ versus NG; $\#P < 0.01$ versus HG.

synthesis as assessed by [$^3$H] proline incorporation compared with cells exposed to normal glucose medium. However, [$^3$H] proline incorporation was significantly enhanced to 232.6% in cells incubated with intermittent high glucose.

We next examined the protein and mRNA levels of type IV collagen under the experimental conditions to determine whether intermittent high glucose alters its expression. As shown in Figure 3A, after mesangial cells were exposed to constant high glucose, there was a significant increase in type IV collagen protein levels of 97.3% (±6.1%, $P < 0.01$), compared with cells exposed to normal glucose. This effect was further enhanced to 270.0% (±9.8%, $P < 0.01$) when cells were exposed to intermittent high glucose. As shown in Figure 3B, cells exposed under constant and intermittent high glucose mediums caused 103.3% and 251.7% stimulation of mRNA levels, respectively.

These results indicate that intermittent high glucose appears to cause an additive stimulatory effect on collagen synthesis in mesangial cells.

## EFFECT OF INTERMITTENT HIGH GLUCOSE ON OPN EXPRESSION

Because OPN has been shown to play an important role in the progression of diabetic nephropathy, we examined whether constant or intermittent high glucose alters OPN expression in mesangial cells. Quiescent cultures of mesangial cells were exposed to constant or intermittent high glucose condition for 48 h. OPN protein and mRNA levels were assessed.

As shown in Figure 4A, after exposure to constant high glucose, the concentration of OPN protein was increased by 140% (±8.4%, $P < 0.01$) compared with cells exposed to normal glucose. This effect was further enhanced following exposure of cells to intermittent high concentrations of glucose with OPN protein secretion increased by 380% (±10.3%, $P < 0.01$) compared with cells exposed to

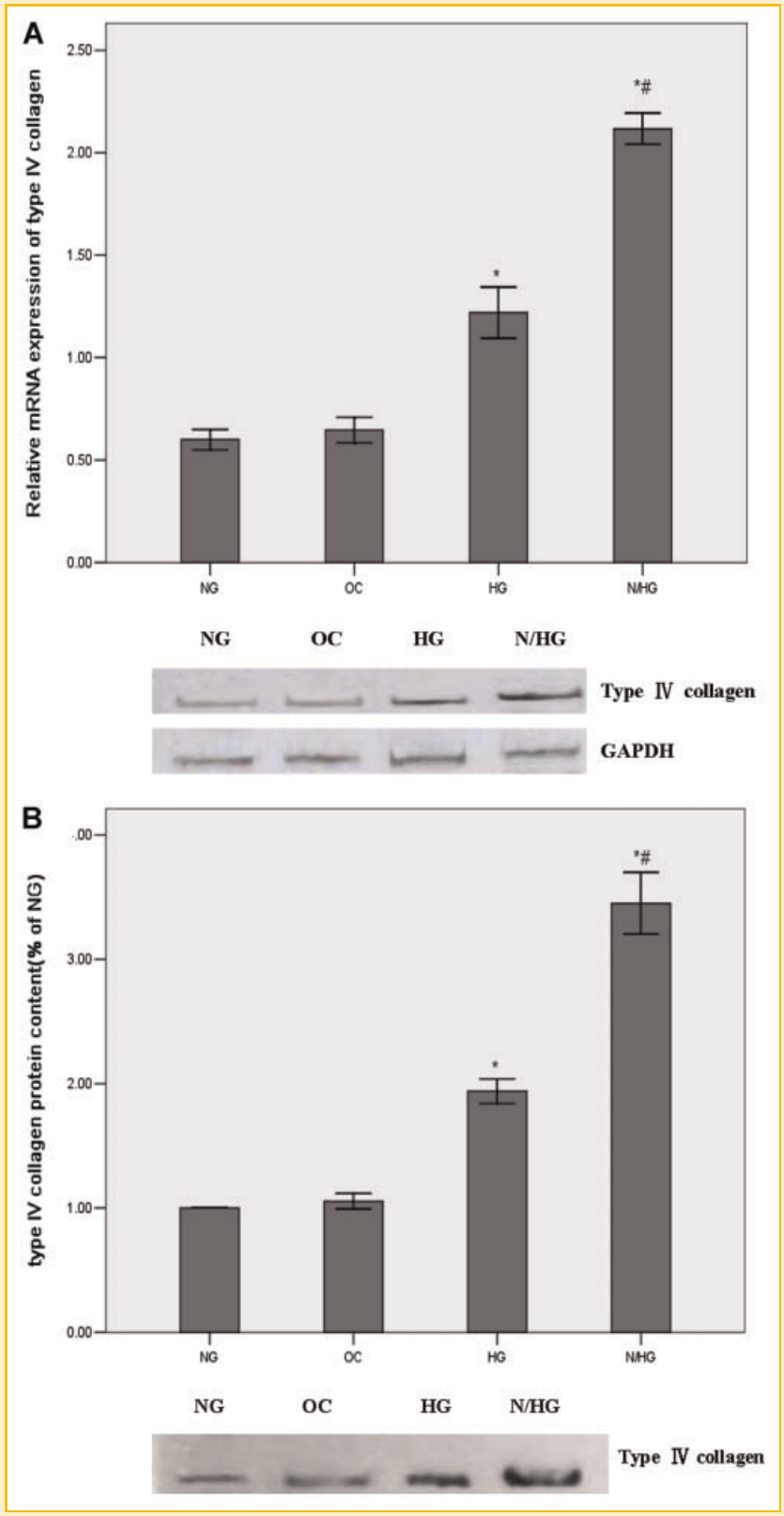


Fig. 3. The effect of high glucose on type IV collagen protein (A) and mRNA (B) levels in cultured mesangial cells. NG, constant normal glucose (5 mmol/L); OC, osmotic control (16.5 mmol/L mannose was used as a negative control); HG, constant high glucose (25 mmol/L); N/HG, 5 mmol/L alternating with 25 mmol/L glucose. Quiescent cells were exposed to glucose at different concentration for 48 h. Type IV collagen protein and mRNA expression were assessed by Western blot analysis and Northern blot analysis, respectively. Type IV collagen protein levels are expressed as a percent of the value of NG (100%). "mRNA relative expression" means the ratio of type IV collagen over GAPDH. The data were expressed as mean ± SD of at least five independent experiments. $^{*}P < 0.01$ versus NG; $\#P < 0.01$ versus HG.

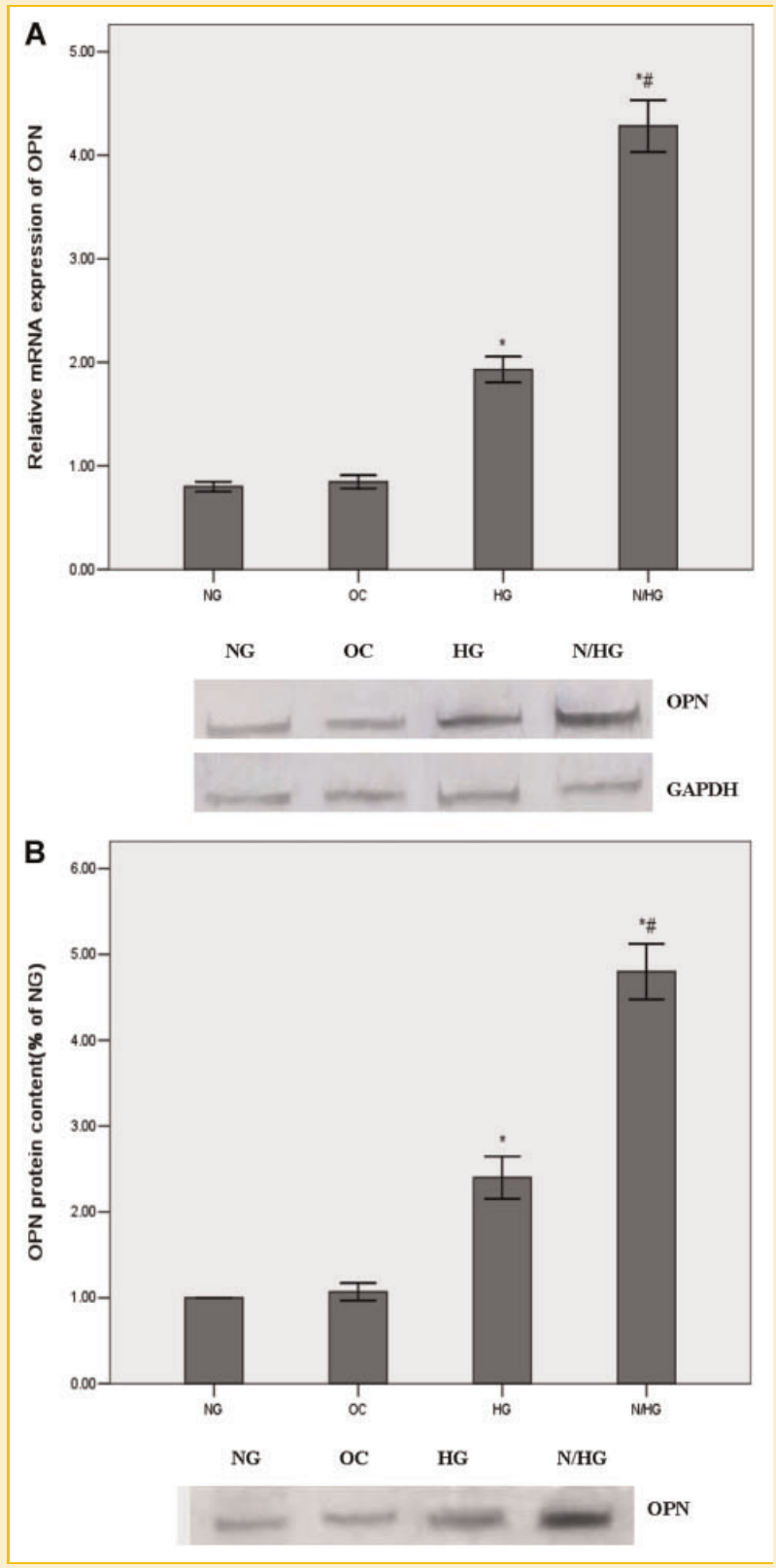


Fig. 4. The effect of high glucose on OPN protein (A) and mRNA (B) levels in cultured mesangial cells. NG, constant normal glucose (5 mmol/L); OC, osmotic control (16.5 mmol/L mannose was used as a negative control); HG, constant high glucose (25 mmol/L); N/HG, 5 mmol/L alternating with 25 mmol/L glucose. Quiescent cells were exposed to glucose at different concentration for 48 h. OPN protein and mRNA expression were assessed by Western blot analysis and Northern blot analysis, respectively. OPN protein levels are expressed as a percent of the value of NG (100%). "mRNA relative expression" means the ratio of OPN over GAPDH. The data were expressed as mean ± SD of at least five independent experiments. $^{*}P < 0.01$ versus NG; $\#P < 0.01$ versus HG.

constant normal glucose. There was a statistically significant increase in the protein levels of OPN exposed to intermittent high glucose concentrations compared with cells exposed to constant high glucose concentrations ($P < 0.01$).

Both constant and intermittent high glucose significantly increased OPN mRNA expression in mesangial cells compared with cells exposed to normal glucose (relative expression: $1.93 \pm 0.05$ vs. $0.80 \pm 0.02$, $P < 0.01$; $4.32 \pm 0.08$ vs. $0.80 \pm 0.02$, $P < 0.01$, respectively, Fig. 4B). Moreover, this effect was further enhanced in mesangial cells exposed to intermittent high concentrations than cells exposed to constant high glucose ($4.32 \pm 0.08$ vs. $1.93 \pm 0.05$, $P < 0.01$, Fig. 4B).

These results suggest that intermittent high glucose appears to cause an additive stimulatory effect on protein synthesis and secretion of OPN from mesangial cells.

## ROLE OF OPN IN PROLIFERATION OF MESANGIAL CELLS INDUCED BY INTERMITTENT HIGH GLUCOSE

To examine a role for OPN in proliferation of mesangial cells induced by intermittent high glucose, quiescent mesangial cells were exposed to constant and intermittent high glucose for 48 h in the absence or presence of neutralizing antibodies to OPN, β3 integrin receptor, β5 integrin receptor, and then [$^3$H]thymidine incorporation was assessed. As shown in Figure 5, neutralizing antibodies to OPN prevented the increase in [$^3$H]thymidine incorporation induced by constant and intermittent high glucose in a concentration-dependent manner. An inhibiting effect was observed at 5 pg/ml, and reached the maximum inhibiting effect at 10 pg/ml. Continuous inhibiting effect was not found when the cells were treated for the larger concentration (15 pg/ml). Therefore, 10 pg/ml was selected as the treated concentration in subsequent studies.

As shown in Figure 6, the blocking of OPN action with neutralizing antibody to either OPN or its β3 integrin receptor completely prevented the increase in [$^3$H]thymidine incorporation induced by constant and intermittent high glucose (Fig. 5). By contrast, the neutralizing antibody to β5 integrin receptor had no effect on reducing the increase in [$^3$H]thymidine incorporation induced by constant and intermittent high glucose. Neutralizing antibodies to OPN or β3 integrin receptor had no effect on [$^3$H]thymidine incorporation under normal glucose or 16.5 mmol/L mannose conditions (Fig. 6).

## ROLE OF OPN IN STIMULATION OF COLLAGEN SYNTHESIS IN MESANGIAL CELLS INDUCED BY INTERMITTENT HIGH GLUCOSE

The quiescent mesangial cells were exposed to constant and intermittent high glucose for 48 h in the absence or presence of neutralizing antibodies to OPN, β3 integrin receptor, β5 integrin receptor, and then [$^3$H]proline incorporation and expression of type IV collagen were assessed. The blocking of OPN action (neutralizing antibody to either OPN or its β3 integrin receptor) equally inhibited the increase of [$^3$H]proline incorporation (Fig. 7) and expression of type IV collagen (Fig. 8A,B) under the constant and intermittent high glucose condition as regards the same conditions where no inhibitor was added.

By contrast, the neutralizing antibody to β5 integrin receptor had no effect on reducing the increase of [$^3$H]proline incorporation (Fig. 7) and expression of type IV collagen induced by constant or intermittent high glucose (Fig. 8). Neutralizing antibodies to OPN or

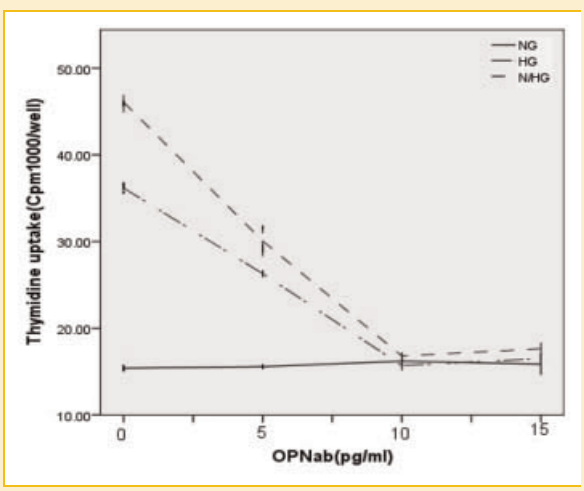


Fig. 5. Proliferation of cultured mesangial cell induced by high glucose was concentration-dependently reduced by neutralizing antibodies to OPN. NG, constant normal glucose (5 mmol/L); HG, constant high glucose (25 mmol/L); N/HG, 5 mmol/L alternating with 25 mmol/L glucose. Quiescent cells exposed to high glucose were treated with different concentration anti-OPN antibody (OPNab), and [$^3$H]thymidine incorporation was assessed. The data were expressed as mean ± SD of at least five independent experiments.

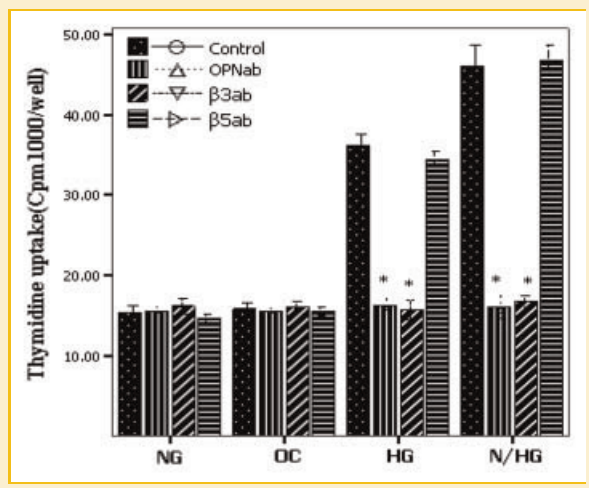


Fig. 6. Role of OPN in proliferation of cultured mesangial cell induced by high glucose. NG, constant normal glucose (5 mmol/L); OC, osmotic control (16.5 mmol/L mannose was used as a negative control); HG, constant high glucose (25 mmol/L); N/HG, 5 mmol/L alternating with 25 mmol/L glucose. Quiescent cells were exposed to glucose at different concentration for in the absence or presence of anti-OPN antibody (OPNab) or neutralizing antibody to either β3 (β3ab) or β5 (β5ab) integrin receptor, and [$^3$H]thymidine incorporation was assessed. The data were expressed as mean ± SD of at least five independent experiments. $^*P < 0.01$ versus control.

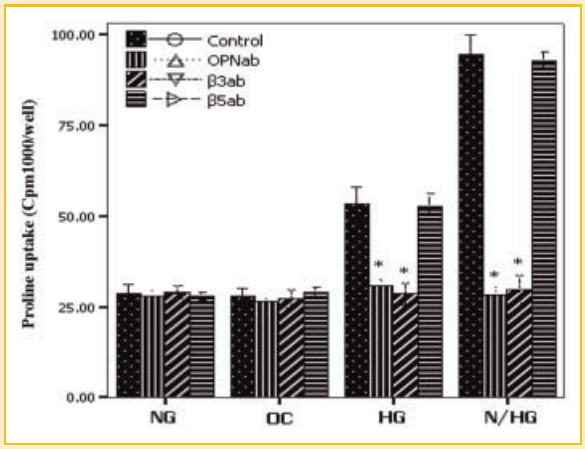


Fig. 7. Role of OPN in collagen synthesis of cultured mesangial cell induced by high glucose. NG, constant normal glucose (5 mmol/L); OC, osmotic control (16.5 mmol/L mannose was used as a negative control); HG, constant high glucose (25 mmol/L); N/HG, 5 mmol/L alternating with 25 mmol/L glucose. Quiescent cells were exposed to glucose at different concentration in the absence or presence of anti-OPN antibody (OPNab) or neutralizing antibody to either β3 (β3ab) or β5 (β5ab) integrin receptor, and [$^3$H]proline incorporation was assessed. The data were expressed as mean ± SD of at least five independent experiments. $^*P < 0.01$ versus control.

β3 integrin receptor had no effect on [$^3$H]proline incorporation (Fig. 7) and expression of type IV collagen (Fig. 8) under normal glucose or high osmotic pressure conditions.

## DISCUSSION

This study has shown that cultured rat mesangial cells exposed to high glucose concentrations have increased cell proliferation and collagen synthesis, as well as the expression of OPN and type IV collagen. Moreover, these effects were further enhanced in cells that were exposed to intermittent rather than constant high glucose concentrations. These findings suggest that variability in glycemic control could be more deleterious to the mesangial cells than constant high concentrations of glucose. Furthermore, neutralizing antibodies to either OPN or its receptor β3 integrin but not neutralizing antibodies to β5 integrin reverse the exacerbated cell proliferation and collagen synthesis induced by intermittent and constant high glucose. These findings suggest an important role of intermittent hyperglycemia in the initiation and/or development of diabetic glomerulosclerosis and demonstrate an important role for OPN in mediating this process.

Hyperglycemia is a crucial factor in the development of diabetic nephropathy because of its effects on glomerular and mesangial cells. Mesangial cells are crucial for maintenance of glomerular capillary structure and for the modulation of glomerular filtration via smooth muscle activity. Enhanced mesangial cells proliferation and an increased level of metabolism of collagen IV and fibronectin ultimately restrict glomerular filtration capacity, leading to overt nephropathy, which progresses to end-stage renal disease. In vitro studies have demonstrated that hyperglycemia is associated with increased mesangial cells matrix production [Harris et al., 1991; Heilig et al., 1995] and mesangial cells apoptosis [Lin et al., 2006]. Mesangial cells expansion seems to be mediated in part by an increase in the mesangial cells glucose concentration, since similar changes in mesangial function can be induced in a normal glucose milieu by overexpression of glucose transporters, such as GLUT1 and GLUT4, thereby increasing glucose entry into the cells [Heilig et al., 1995]. Hyperglycemia is associated with an increase in mesangial cells proliferation and collagen synthesis, as well as basement membrane thickening. Based on these findings, it has been widely accepted that hyperglycemia induced mesangial cells proliferation, and collagen synthesis is a crucial factor in diabetic glomerulosclerosis.

The effects of intermittent high glucose on proliferation and collagen synthesis in mesangial cells are not yet defined. In studying the cause of diabetic glomerulosclerosis, we have emphasized the effects of fluctuating hyperglycemia on the metabolic activity of mesangial cells. Consistent with in vitro studies by Cohen and Ziyadeh [1994], cultured mesangial cells exposed to high glucose concentrations have increased cell proliferation and collagen synthesis. More importantly, we have shown that the exposure of mesangial cells to intermittent high glucose significantly enhanced the effects of the exposure of such cells to constant high glucose concentrations, supporting a pathophysiologic link between intermittent high glucose and increased risk of microvascular complications [Gimeno-Orna et al., 2003; Kilpatrick et al., 2008, 2009]. Intermittent high glucose may be more dangerous for the cells than constant high glucose. Similarly, fluctuations of glucose display a more dangerous effect than stable high glucose on both tubulointerstitial cells and human renal cortical fibroblasts, in terms of collagen synthesis and cell growth [Takeuchi et al., 1995; Jones et al., 1999]. New diabetes therapies focused on reducing postprandial hyperglycemia have become available and may benefit glycemic control and reduce risk of microvascular complications [Gimeno-Orna et al., 2003; Kilpatrick et al., 2008]. It is now recognized that hyperglycemia at 2 h during an oral glucose challenge, as well as glucose fluctuations are strong predictor of microvascular complications [Singleton et al., 2003], and it has been suggested that "hyperglycemic spikes" may play a direct and significant role in the pathogenesis of diabetic vascular complications [Ceriello, 1998].

OPN is a cell adhesion molecule with a primitive amino acid sequence, which can combine with the cell surface integrin receptor, and facilitate cellular adhesion, proliferation, and migration [Li et al., 2007]. OPN has also been shown to be associated with the proliferation of cultured vascular smooth muscle cells and cardiac fibroblasts [Alain-Pierre et al., 1993; Asizawa et al., 1996]. A growing body of both in vivo and in vitro evidence indicated an important damaging role of OPN in the development of tubulointerstitial disease and glomerulonephritis of various initial etiologies. The increased expression of OPN has been demonstrated in animal models of angiotensin II-induced renal fibrosis [Giachelli et al., 1994], hydronephrosis [Diamond et al., 1995], acute puromycin aminonucleoside nephrosis [Magi et al., 1997], and unilateral ureteral obstruction [Kaneto et al., 1998].

Upregulation of OPN expression was found in renal cortex of streptozotocin-induced diabetic rats [Fischer et al., 1998]. Our

present study showed that proliferation and collagen synthesis of cultured mesangial cells induced by high glucose was also involved in increased expression of OPN. These results have suggested that OPN plays an important role in accelerated glomerulosclerosis in diabetes mellitus. Treatment with both constant and intermittent high glucose significantly increased OPN expression in parallel with the enhanced cell proliferation and collagen synthesis compared with mesangial cells treated with normal glucose. A neutralizing antibody to OPN or its β3 integrin receptor completely blocked the exacerbated proliferation and collagen synthesis in mesangial cells induced by high glucose, suggesting a functional role of OPN in mediating mesangial cells proliferation and collagen synthesis. Neutralizing antibody to β5 integrin receptor, which binds preferentially to vitronectin was unable to prevent the proliferation and collagen synthesis of mesangial cells induced by high glucose [Cheng et al., 2000]. Some studies demonstrated a marked

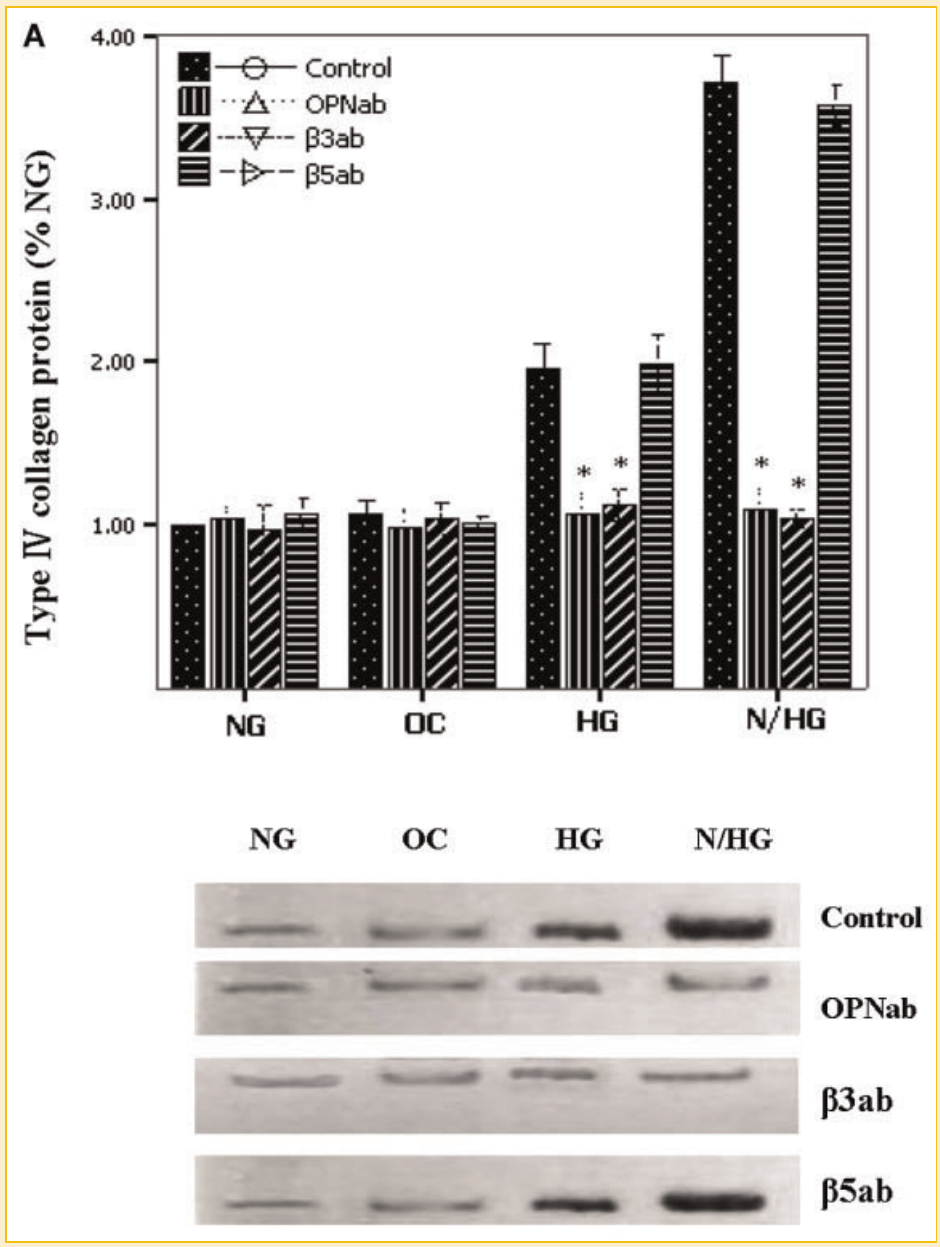


Fig. 8. Role of OPN in expression of type IV collagen protein (A) and mRNA (B) in cultured mesangial cells induced by high glucose. NG, constant normal glucose (5 mmol/L); OC, osmotic control (16.5 mmol/L mannose was used as a negative control); HG, constant high glucose (25 mmol/L); N/HG, 5 mmol/L alternating with 25 mmol/L glucose. Quiescent cells were exposed to glucose at different concentration for in the absence or presence of anti-OPN antibody (OPNab) or neutralizing antibody to either β3 (β3ab) or β5 (β5ab) integrin receptor. Type IV collagen protein and mRNA expression were assessed by Western blot analysis and Northern blot analysis, respectively. Type IV collagen protein levels are expressed as a percent of the value of NG (100%). "mRNA relative expression" means the ratio of type IV collagen over GAPDH. The data were expressed as mean ± SD of at least five independent experiments. $^{*}P < 0.01$ versus control.

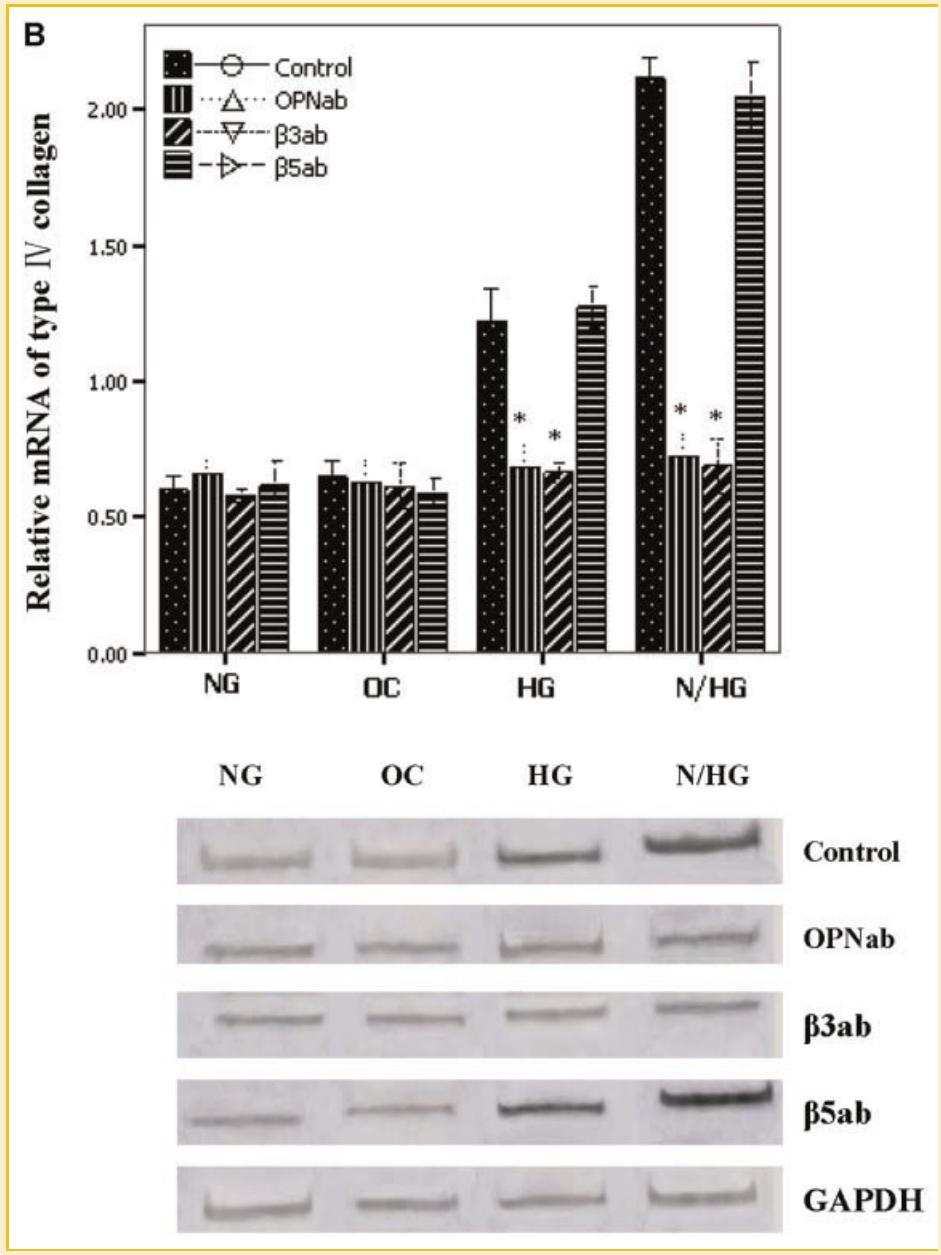


Fig. 8. (*Continued*)

reduction in renal impairment by using OPN-gene knockout mice [Ophascharoensuk et al., 1999], and antisense-treated or anti-OPN antibody treated animals [Yu et al., 1998; Panzer et al., 2001]. Taken together, these findings suggest that the high glucose and the associated marked increases in OPN expression in diabetes may be the key events responsible for accelerated mesangial cells proliferation, collagen synthesis associated with diabetic nephropathy. Intermittent high glucose may be important pathogenetic factors that contribute to progressive diabetic glomerulosclerosis by the stimulation of OPN synthesis.

In summary, we found that constant high glucose increased the proliferation and collagen synthesis in cultured mesangial cells, which is mediated by the stimulation of OPN synthesis. These effects were further enhanced in cells that were exposed to intermittent rather than constant high glucose, indicating that short lived excursions in glycemic control have important pathological effects on the development of diabetic glomerulosclerosis. In addition, experimental decrease of OPN (by a neutralizing antibody to OPN or its β3 integrin receptor) inhibited the proliferation and collagen synthesis in mesangial cells induced by constant or intermittent high glucose. Thus, our results reveal a novel function of OPN as a regulator of proliferation and collagen synthesis in mesangial cells, and provide a potential strategy of treatment for diabetic nephropathy.

## ACKNOWLEDGMENTS

This study was funded by Hubei Provincial Bureau of Health Science Foundation for Young Scholars (grants QJX2008-29).

## REFERENCES

Alain-Pierre G, Campan M, Millet D, Candresse T, Desgranges C. 1993. Osteopontin overexpression is associated with arterial smooth muscle cell proliferation in vitro. Arterioscler Thromb 13:120–125.

Amemiya T, Sasamura H, Mifune M, Kitamura Y, Hirasashi J, Hayashi M, Saruta T. 1999. Vascular endothelial growth factor activates MAP kinase and enhances collagen synthesis in human mesangial cells. Kidney Int 56:2055–2063.

Asizawa N, Graf K, Do YS, Nunohiro T, Giachelli CM, Meehan WP, Tuan TL, Hsueh WA. 1996. Osteopontin is produced by rat cardiac fibroblasts and mediates AII-induced DNA synthesis and collagen gel contraction. J Clin Invest 98:2218–2227.

Brantley JG, Sharma M, Alcalay NI, Heuvel GB. 2003. Cux-1 transgenic mice develop glomerulosclerosis and interstitial fibrosis. Kidney Int 63(4):1240–1248.

Buse JB, Hroscikoski M. 1998. The case for a role for postprandial glucose monitoring in diabetes management. J Fam Pract 47:S29–S36.

Ceriello A. 1998. The emerging role of post-prandial hyperglycaemic spikes in the pathogenesis of diabetic complications. Diabet Med 15:188–193.

Cheng SL, Lai C, Fausto A, Chellaiah M, Feng X, McHugh K, Tietelbaum S, Civitelli R, Hruska K, Ross P, Avioli LV. 2000. Regulation of alphaVbeta3 and alpha Vbeta5 integrins by dexamethasone in normal human osteoblastic cells. J Cell Biochem 77:265–276.

Cohen MP, Ziyadeh FN. 1994. Amadori glucose adducts modulate mesangial cell growth and collagen gene expression. Kidney Int 45(2):475–484.

Diamond JR, Kees-Folts D, Ricardo SD, Pruznak A, Eufemio M. 1995. Early and persistent up-regulated expression of renal cortical osteopontin in experimental hydronephrosis. Am J Pathol 146:1455–1466.

Fischer JW, Tschope C, Reinecke A, Giachelli CM, Unger T. 1998. Upregulation of osteopontin expression in renal cortex of strepto- and zotocin-induced diabetic rats is mediated by bradykinin. Diabetes 47:1512–1518.

Fumo P, Kuncio GS, Ziyadeh F. 1994. PKC and high glucose stimulate collagen alpha 1 (IV) transcriptional activity in a reporter mesangial cell line. Am J Physiol Renal Fluid Electrolyte Physiol 267:F632–F638.

Giachelli CM, Pichler RH, Lombardi D, Denhardt DT, Alpers CE, Schwartz SM, Johnson RJ. 1994. Osteopontin expression in angiotensin II-induced tubulointers titial nephritis. Kidney Int 45:515–524.

Gimeno-Orna JA, Castro-Alonso FJ, Boned-Juliani B, Lou-Arnal LM. 2003. Fasting plasma glucose variability as a risk factor of retinopathy in type 2 diabetic patients. J Diabetes Complications 17(2):78–81.

Harris RD, Steffes MW, Bilous RW, Sutherland DE, Mauer SM. 1991. Global: Glomerular sclerosis and glomerular arteriolar hyalinosis in insulin dependent diabetes. Kidney Int 40:107–114.

Heilig CW, Concepcion LA, Riser BL, Freytag SO, Zhu M, Cortes P. 1995. Over-expression of glucose transporters in rat mesangial cells cultured in a normal glucose milieu mimics the diabetic phenotype. J Clin Invest 96:1802–1814.

Jones SC, Saunders HJ, Qi W, Pollock CA. 1999. Intermittent high glucose enhances cell growth and collagen synthesis in cultured human tubulointerstitial cells. Diabetologia 42:1113–1119.

Kaneto H, Morrisey J, McCracken R, Reyes A, Klahr S. 1998. Osteopontin expression in the kidney during unilateral ureteral obstruction. Miner Electrolyte Metab 24:227–237.

Kilpatrick ES, Rigby AS, Atkin SL. 2008. A1C variability and the risk of microvascular complications in type 1 diabetes: Data from the Diabetes Control and Complications Trial. Diabetes Care 31(11):2198–2202.

Kilpatrick ES, Rigby AS, Atkin SL. 2009. The effect of glucose variability on the long-term risk of microvascular complications in type 1 diabetes. Diabetes Care 32:1901–1903.

Klahr S, Scheiner G, Ichikawa I. 1988. The progression of renal disease. N Engl J Med 318:1657–1666.

Klein R. 1995. Hyperglycemia and microvascular and macrovascular disease in diabetes. Diabetes Care 18:258–268.

Klienman JG, Beshensky A, Worcester EM, Brown D. 1995. Expression of osteopontin, a urinary inhibitor of stonechlorominеral crystal growth, in rat kidney. Kidney Int 47:1585–1596.

Leehey DJ, Singh AK, Alavi N, Singh R. 2000. Role of angiotensin II in diabetic nephropathy. Kidney Int 58:S93–S98.

Li W, Liu X, Yanoff M, Cohen S, Ye X. 1996. Cultured retinal capillary pericytes die by apoptosis after an abrupt fluctuation from high to low glucose levels. Diabetologia 39:537–547.

Li JJ, Han M, Wen JK. 2007. Osteopontin stimulates vascular smooth muscle cell migration by inducing FAK phosphorylation and ILK dephosphorylation. Biochem Biophys Res Commun 356:13–19.

Lin CL, Wang JY, Huang YT, Kuo YH, Surendran K, Wang FS. 2006. Wnt/β-catenin signaling modulates survival of high glucose-stressed mesangial cells. J Am Soc Nephrol 17:2812–2820.

Magi AB, Pichler RH, Johnson RJ. 1997. Osteopontin in chronic puromycin amino-nucleoside nephrosis. J Am Soc Nephrol 8:1383–1390.

Makino H, Kashihara N, Sugiyama H, Kanao K, Sekikawa T, Okamoto K, Maeshima Y, Ota Z, Nagai R. 1996. Phenotypic modulation of the mesangium reflected by contractile proteins in diabetes. Diabetes 45:488–495.

Mauer SM, Steffes MW, Ellis EN, Sutherland DE, Brown DM, Goetz FC. 1984. Structural–functional relationships in diabetic nephropathy. J Clin Invest 74:1143–1155.

Monnier L, Mas E, Ginet C, Michel F, Villon L, Cristol JP, Colette C. 2006. Activation of oxidative stress by acute glucose fluctuations compared with sustained chronic hyperglycemia in patients with type 2 diabetes. JAMA 295:1681–1687.

Ophascharoensuk V, Giachelli CM, Gordon K, Hughes J, Pichler R, Brown P, Liaw L, Schmidt R, Shankland SJ, Alpers CE, Couser WG, Johnson RJ. 1999. Obstructive uropathy in the mouse: Role of osteopontin in interstitial fibrosis and apoptosis. Kidney Int 56:571–580.

Panzer U, Thaiss F, Zahner G, Barth P, Reszka M, Reinking RR, Wolf G, Helmchen U, Stahl RA. 2001. Monocyte chemoattractant protein-1 and osteopon tin differentially regulate monocytes recruitment in experimental glomerulonephritis. Kidney Int 59:1762–1769.

Prols F, Heidgress D, Rupprecht HD, Marx M. 1998. Regulation of osteopontin expression in rat mesangial cells. FEBS Lett 422:15–18.

Ruoslahti E, Engvall E. 1997. Perspective series: Cell adhesion in vascular biology-integrins and vascular extracellular matrix assembly. J Clin Invest 99:1149–1152.

Sahai A, Mei C, Pattison T, Tannen RL. 1997. Chronic hypoxia induces proliferation of cultured rat mesangial cells: Role of calcium and protein kinase C. Am J Physiol 273:F954–F960.

Singleton JR, Smith AG, Russell JW, Feldman EL. 2003. Microvascular complications of impaired glucose tolerance. Diabetes 52:2867–2873.

Sodhi CP, Batlle D, Sahai A. 2000. Osteopontin mediates hypoxia-induced proliferation of cultured mesangial cells: Role of PKC and p38MAP kinase. Kidney Int 58:691–700.

Steffes MW, Osterby R, Chavers B, Mauer SM. 1989. Mesangial expansion as a central mechanism for loss of kidney function in diabetic patients. Diabetes 38:1077–1081.

Takemoto M, Asker N, Gerhardt H, Lundkvist A, Johansson BR, Saito Y, Betsholtz C. 2002. A new method for large scale isolation of kidney glomeruli from mice. Am J Pathol 161:799–805.

Takeuchi A, Throckmorton DC, Brogden AP. 1995. Periodic high extracellular glucose enhances production of collagens III and IV by mesangial cells. Am J Physiol 268:F13–F19.

Towler DA, Bidder M, Latifi T, Coleman T, Semenkovich CF. 1998. Diet-induced diabetes activates an osteogenic gene regulatory program in the aortas of low density lipoprotein receptor-deficient mice. J Biol Chem 273: 30427–30443.

Yu XQ, Nikolic-Paterson DJ, Mu W, Giachelli CM, Atkins RC, Johnson RJ, Lan HY. 1998. A functional role for osteopontin in experimental crescentic glomerulonephritis in the rat. Proc Assoc Am Physicians 110:50–64.