

*Full Length Research Paper*

# **Inhibition of inflammatory factors by parthenolide in human renal mesangial cells under hyperglycemic condition**

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**The aim of this study was to investigate the anti-inflammatory effects of parthenolide (PTN) in human renal mesangial cells (HRMCs) under high ambient glucose conditions. First we determined the non-cytotoxic concentration of PTN in HRMCs by performing the MTS assay. Enzyme-linked immunosorbent (ELISA) analysis was performed to determine the expressions of interleukin (IL)-1 $\beta$ , IL-18, tumor-necrosis factor (TNF)- $\alpha$ , transforming growth factor (TGF)- $\beta$ 1, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1 $\alpha$ , RANTES and prostaglandin (PG)E2. The total nitric oxide (NO) was determined by performing the Griess reaction. Treatment with less than 50  $\mu$ mol/L PTN concentration did not affect the viability of HRMCs, while more than 100  $\mu$ mol/L concentrations markedly altered the cell viability. In the present study, treatment with 50 mmol/L glucose markedly increased the level of IL-1 $\beta$ , IL-18, TNF- $\alpha$ , TGF- $\beta$ 1, MCP-1, MIP-1 $\alpha$ , RANTES, PGE2 and NO. PTN remarkably abolished the increase in the level of these molecules in a dose-dependent manner. Moreover, treatment with PTN concentration of 20  $\mu$ mol/L almost completely reversed IL-1 $\beta$  and TNF- $\alpha$  expression, and treatment with 50  $\mu$ mol/L reversed the expression of RANTES. In conclusion, parthenolide can inhibit the high-glucose-induced expression of inflammatory cytokines in HRMCs. Hence, PTN may be considered a promising drug with potent anti-inflammatory effect in addition to its strong anticancer, anti-angiogenesis, and antineurodegenerative effects.**

**Key words:** Parthenolide, human renal mesangial cells, inflammatory factors, diabetic nephropathy.

## **INTRODUCTION**

The understanding of the pathogenesis of diabetes mellitus has changed in the last few years due to the pivotal roles of inflammatory pathways in the development and progression of diabetic nephropathy (DN) (Navarro and Mora, 2005). The dysfunction of mesangial cells is one of the key factors causing glomerular injury in DN. When exposed to the diabetic metabolic milieu, mesangial cells

display growth abnormalities and increased expression and synthesis of cytokines, including transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) (Kanwar et al., 2008) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (Navarro and Mora, 2005; Naito et al., 2009). Moreover, several lines of evidence support the fact that many chemokines like CCL2/monocyte chemoattractant protein (MCP)-1, CCL3/macrophage inflammatory protein (MIP)-1 $\alpha$ , and CCL5/RANTES play an important role in the pathogenesis of DN or non-diabetic nephropathy (NDN) (Teramoto et al., 2008; Campbell et al., 2006; Mezzano et al., 2004). Further, activated mesangial cells produce chemokines and play a direct role in the initiation and propagation of inflam-

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matory events within the glomerulus. The release of chemokines by intrinsic renal cells may promote the influx of leukocytes into the injured tissue and contribute to the progression of inflammatory renal injury (Brown et al., 1996).

The progressive changes in the kidney of diabetic patients, such as mesangial matrix expansion and eventual glomerular sclerosis, result in a remarkable decrease in the glomerular filtration rate (GFR) until the advancement to end-stage renal disease (ESRD). Although, many therapeutic interventions delay the development or retard the progression of DN, no available intervention can stop or reverse the progression of this condition (Wang et al., 2008). The revelation that DN is an inflammatory disease triggered by altered metabolic factors has opened new and important therapeutic perspectives. Many efforts were focused on identifying novel anti-inflammatory drugs that could block the inflammatory pathway at a very early stage of gene expression. In this regard, a natural compound, namely, parthenolide (PTN), which is a sesquiterpene lactone isolated from the extracts of Mexican-Indian medicinal herb (*Tanacetum parthenium*), has attracted considerable interest. PTN-containing herbs have been commonly used for the treatment of various inflammatory conditions such as migraine, arthritis, and asthma (López-Franco et al., 2006; Heinrich et al., 1998). Several groups have reported that PTN inhibits nuclear factor-kappa B (NF- $\kappa$ B) activation in cultured cells and experimental models of diabetes (López-Franco et al., 2006; Hehner et al., 1998). However, the effect of PTN on the synthesis of inflammatory factors in renal mesangial cells under hyperglycemic condition has not yet been investigated (López-Franco et al., 2006).

In this study, we report that PTN treatment is beneficial to human renal mesangial cells (HRMCs) under hyperglycemic condition, which can be partially attributed to the inhibition of inflammatory factor expression.

## MATERIALS AND METHODS

### Materials

L-Glucose was obtained from Sigma Chemical Co., USA. HRMCs were purchased from ScienceCell, USA. PTN was obtained from Sigma, USA. PTN was dissolved in 100 mM dimethylsulfoxide (DMSO), stored at  $-30^{\circ}\text{C}$  and diluted to prepare solutions of 10, 20, and 50  $\mu\text{M}$  final concentrations. The Cell Titer 96 aqueous cell viability assay kit was purchased from Promega, USA. All other chemicals were of reagent grade. Human IL-1 $\beta$ , IL-18, TNF- $\alpha$ , monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1 $\alpha$ , RANTES, TGF- $\beta$ 1, PGE2, enzyme-linked immunosorbent assay (ELISA) kits, and NO assay kit were purchased from R&D systems, USA.

### Cell culture and treatment

To make cells better response to high-glucose stimulate, HRMCs were cultured in low-glucose Dulbecco's Modified Eagle Media (DMEM) medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA), 5.6 mmol/L glucose,

glutamine, and antibiotics (penicillin and streptomycin) and incubated at  $37^{\circ}\text{C}$  under 5%  $\text{CO}_2$  for 1 month, after which the following experiments were performed.

### Viability study

HRMCs ( $1 \times 10^4$  cells/well in 96-well plate) were treated with PTN (0, 1, 5, 10, 20, 50, 100, and 200  $\mu\text{mol/L}$ ) for 24 h and cell viability was evaluated by MTS assay. The assay included tetrazolium compound MTS (3, 4-(5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt) and an electron coupling reagent phenazine methosulfate (PMS). MTS is reduced by viable cells to formazan, which can be estimated by measuring the absorbance at 490 nm using a spectrophotometer. Formazan production was time dependent and proportional to the number of viable cells. Cultures were seeded at  $1 \times 10^4$  cells/well, and the cells were allowed to attach overnight. After incubation for the indicated time with the appropriate medium, 20  $\mu\text{l}$  MTS/PMS mixture was added to each well and the cells were incubated for 1 h; finally, the absorbance was measured at 490 nm. Background absorbance of the control samples was subtracted. Three duplicate studies were performed for each experimental condition.

### Evaluation of cytokine production

The inhibitory effect of PTN on IL-1 $\beta$ , IL-18, TNF- $\alpha$ , MCP-1, MIP-1 $\alpha$ , RANTES, and TGF- $\beta$ 1 production was determined using ELISA. HRMCs ( $3 \times 10^5$  cells/well in 6-well plate) were preincubated with or without PTN for 2 h and continuously incubated in 5.6 (normal glucose, NG) or 50 mmol/L glucose (high glucose, HG) for the next 24 h. Subsequently, the supernatants were assayed for the levels of above mentioned cytokines using ELISA kits (R&D Systems, MN, USA) according to the manufacturer's procedures.

### Assay for PGE2 production

The inhibitory effects of PTN on PGE2 production in HG-treated HRMCs were analyzed by competitive ELISA according to the manufacturer's procedures (R&D Systems, MN, USA). The lower limit of detection was 36.2 pg/ml.

### NO assay

HRMCs were preincubated with or without PTN for 2 h and then continuously activated in HG condition (50 mmol/L) for 24 h. NO levels were determined by performing the Griess reaction (R&D Systems, MN, USA). The absorbance of the product dye was measured at 540 nm using a flow-through spectrophotometer. The sensitivity of the NO assay was less than 1.35  $\mu\text{mol/L}$ .

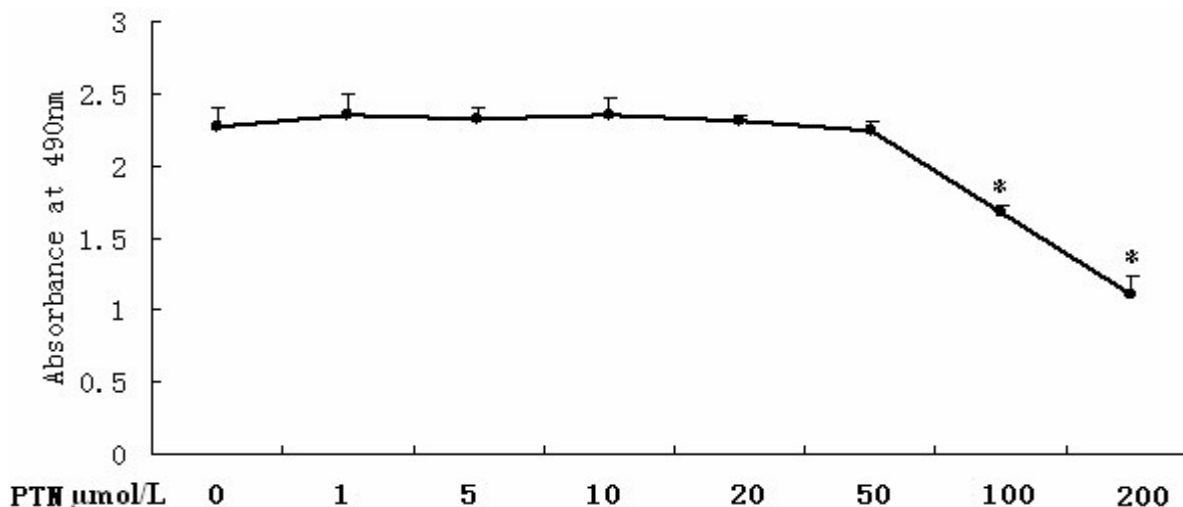
### Statistical analysis

Student's *t*-test and one-way analysis of variance (ANOVA) were used to determine the statistical significance of differences between the experimental and control groups. The P values of 0.05 or less were considered to be statistically significant.

## RESULTS

### Effect of PTN on the viability of HRMCs

We evaluated the cytotoxic effect of PTN before performing



**Figure 1.** Effect of PTN on the viability of HRMCs. HRMCs ( $1 \times 10^4$  cells / well in 96-well plate) were incubated with PTN of different concentrations for 24 h. Cell viability was determined by performing the MTS assay. Data represent mean  $\pm$  SEM (n = 3). \*P < 0.05, vs normal group.

**Table 1.** Groups in different culture conditions in this study.

Condition/group	1	2	3	4	5	6	7
Glucose (mmol/L)	5.6	50	50	50	50	50	50
PTN ( $\mu\text{mol/L}$ )	0	0	1	5	10	20	50

further studies. The viability of HRMC (Fax: +86-571-87236582s) after incubation with different PTN preparations for 24 h was determined by MTS assay. As shown in Figure 1, the viability of HRMCs did not change remarkably when incubated with PTN concentrations of 0–50  $\mu\text{mol/L}$  but the viability was markedly affected at concentrations above 100  $\mu\text{mol/L}$ . Therefore, PTN at less than 50  $\mu\text{mol/L}$  concentration was suitable to exert selective pharmacological action without any interference with normal cell function.

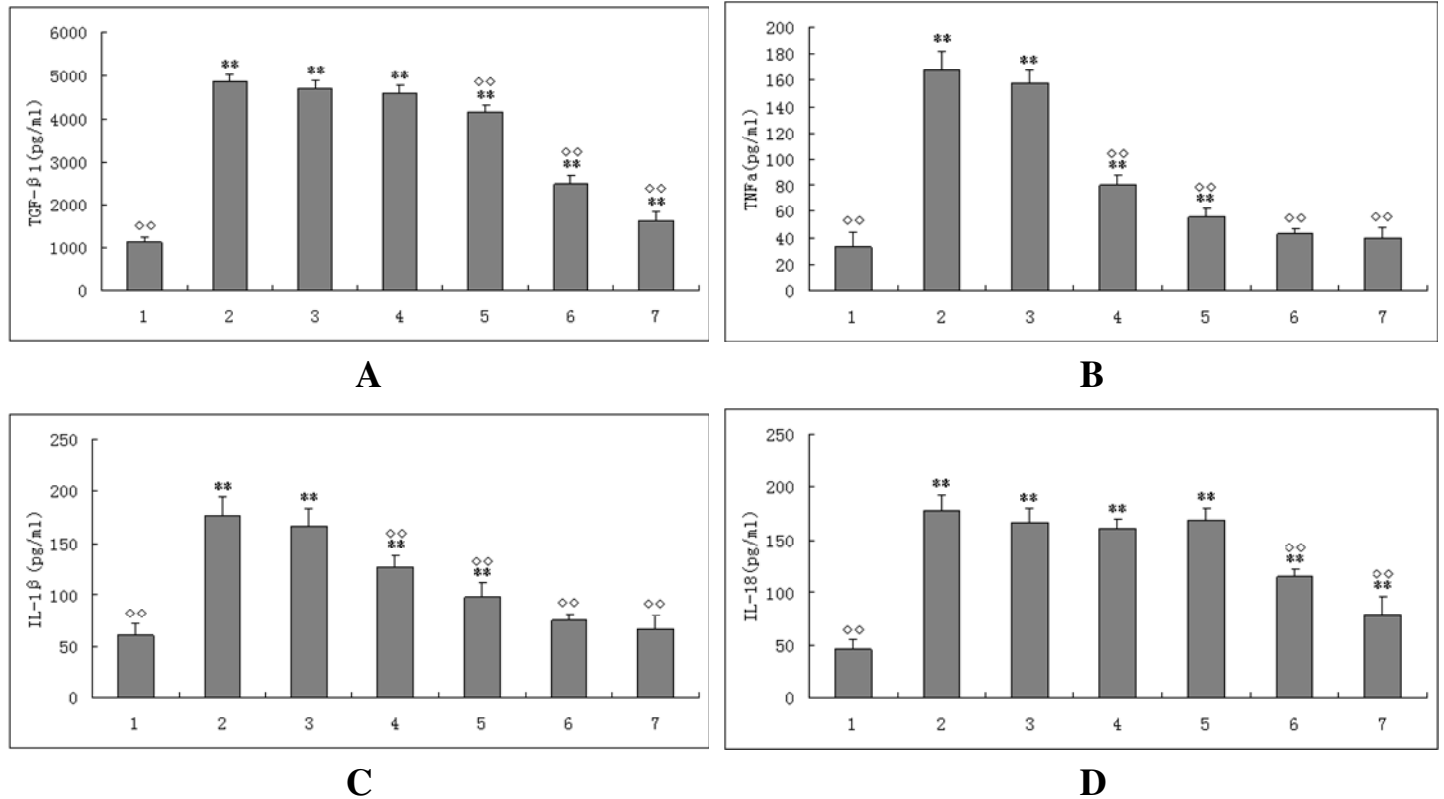
#### Inhibitory effect of PTN on HG-induced upregulation of inflammatory Factors production in HRMCs

To assess the inhibitory effect of PTN on HG-mediated production of inflammatory factors, we evaluated the levels of IL-1 $\beta$ , IL-18, TNF- $\alpha$ , MCP-1, MIP-1 $\alpha$ , RANTES, TGF- $\beta$ 1, PGE2 and NO production by performing ELISA. The samples were pretreated with PTN and then subjected to different glucose conditions. Cells were pretreated with various concentrations of PTN in the 5.6 (NG) or 50 mmol/L (HG) glucose for 24 h (Table 1). The expression of inflammatory molecules in the supernatants of HG-treated HRMCs was remarkably upregulated as compared to that in NG-treated cells. Further, PTN treatment significantly decreased the HG-induced production of the above mentioned factors (Figures 2A–I),

indicating that PTN may commonly affect HG-mediated pro-inflammatory responses of HRMCs. Furthermore, the inhibitory effect was dose dependent. In fact, PTN almost completely reversed IL-1 $\beta$  and TNF- $\alpha$  expression and NO production at 20  $\mu\text{mol/L}$  (Figures 2B, C, E), PGE2 and RANTES expression at 50  $\mu\text{mol/L}$  (Figure 2F, G).

#### DISCUSSION

DN is one of the main “microvascular” complications of diabetes (Kanwar et al., 2008). Activated innate immunity and inflammation are important factors involved in the pathogenesis of diabetes and convincing data is present to indicate that inflammatory responses are induced in type 2 diabetes (Crook, 2004; Pickup, 2004). The renal lesions in type 1 and 2 diabetes mellitus are similar (Kanwar et al., 2008). In 1991, Hasegawa et al. were the first to suggest that proinflammatory cytokines could participate in the development of DN (Hasegawa et al., 1991). There was an urgent requirement of good therapeutic modalities to block the inflammatory pathway. Recent studies revealed that PTN can be used as an efficient anti-inflammatory compound for the treatment of autoimmune diseases and inflammatory diseases, including arthritis and asthma (Nam, 2006; Yao et al., 2007). The results of our study confirm the existing data and provide new data which suggests that under high ambient glucose



**Figure 2.** Inhibitory effect of PTN on HG-induced upregulation of inflammatory factors production. HRMCs ( $3 \times 10^5$ /wells in 6-well plate) were preincubated with or without honokiol for 2 h, and then continuously incubated in 5.6 or 50 mmol/L glucose for 24 h. Levels of IL-1 $\beta$ , IL-18, TNF- $\alpha$ , MCP-1, MIP-1 $\alpha$ , RANTES, TGF- $\beta$ 1, and PGE2, and NO production were determined by ELISA and Griess reaction, respectively. Data represent mean  $\pm$  SEM (n = 3).  $\square$  P < 0.05,  $\square$  P < 0.01 vs 5.6 mmol/L glucose (group 1); \*P < 0.05, \*\*P < 0.01 vs 50 mmol/L glucose alone (group 2).

conditions human mesangial cells can induce the upregulation of the expression of several inflammatory factors and that PTN markedly suppressed the expression of inflammatory proteins activated in HG condition.

We first treated HRMCs with variable PTN concentrations (0, 1, 5, 10, 20, 50, 100, and 200  $\mu$ mol/L) for 24 h, and subsequently evaluated the cytotoxicity of PTN by performing the MTS assay. The results indicated that as compared to the control sample more than 99% HRMCs were viable after 24 h of treatment with a range of PTN concentrations below 50  $\mu$ mol/L. However, PTN concentrations above 100  $\mu$ mol/L resulted in obvious cytotoxic effects on HRMCs (Figure. 1). Thus, we used 0, 1, 5, 10, 20, and 50  $\mu$ mol/L of PTN for HRMC experiments; these PTN concentrations were non toxic and more than 99% cells were viable after 24 h incubation. The final concentration of DMSO was same in the control and PTN-containing samples.

The direct harmful effect of TNF- $\alpha$  on the glomerular protein permeability barrier is independent from its other effects such as modification of hemodynamic factors or recruitment of inflammatory cells. It is particularly relevant that TNF- $\alpha$  is suggested to be a critical factor that contributes to sodium retention and renal hypertrophy,

which are important renal alterations that occur during the initial stage of DN (Navarro and Mora, 2005; Dipetrillo et al., 2003). Moreover, multivariate analysis showed a significant and independent relationship between urinary TNF- $\alpha$  and albuminuria (Navarro and Mora, 2005). In addition, our previous study and the studies of other groups have shown that TGF- $\beta$ 1 is the key cytokine responsible for extracellular matrix (ECM) pathobiology observed in DN (Wu et al., 2007). Further renal injury results in the release of other proinflammatory cytokines such as IL-1 $\beta$  (Sánchez-López et al., 2008) and IL-18 (Shui et al., 2007) by infiltrating inflammatory cells and intrinsic mesangial cells, which may be simultaneously present in the glomerular environment. The aforementioned factors can activate mesangial cells to increase ECM production, and thereby contribute to the progression of renal damage (Sánchez-López et al., 2008). In the present study, HG treatment led to a marked increase in the level of TGF- $\beta$ 1, TNF- $\alpha$ , IL-1 $\beta$ , and IL-18 in the supernatants of treated HRMCs (Figures 2A–D). However, PTN abolished this increase in a dose-dependent manner. In fact, 20  $\mu$ mol/L of PTN almost completely reversed the expression of IL-1 $\beta$  and TNF- $\alpha$ .

Chemokines belong to a family of small and related

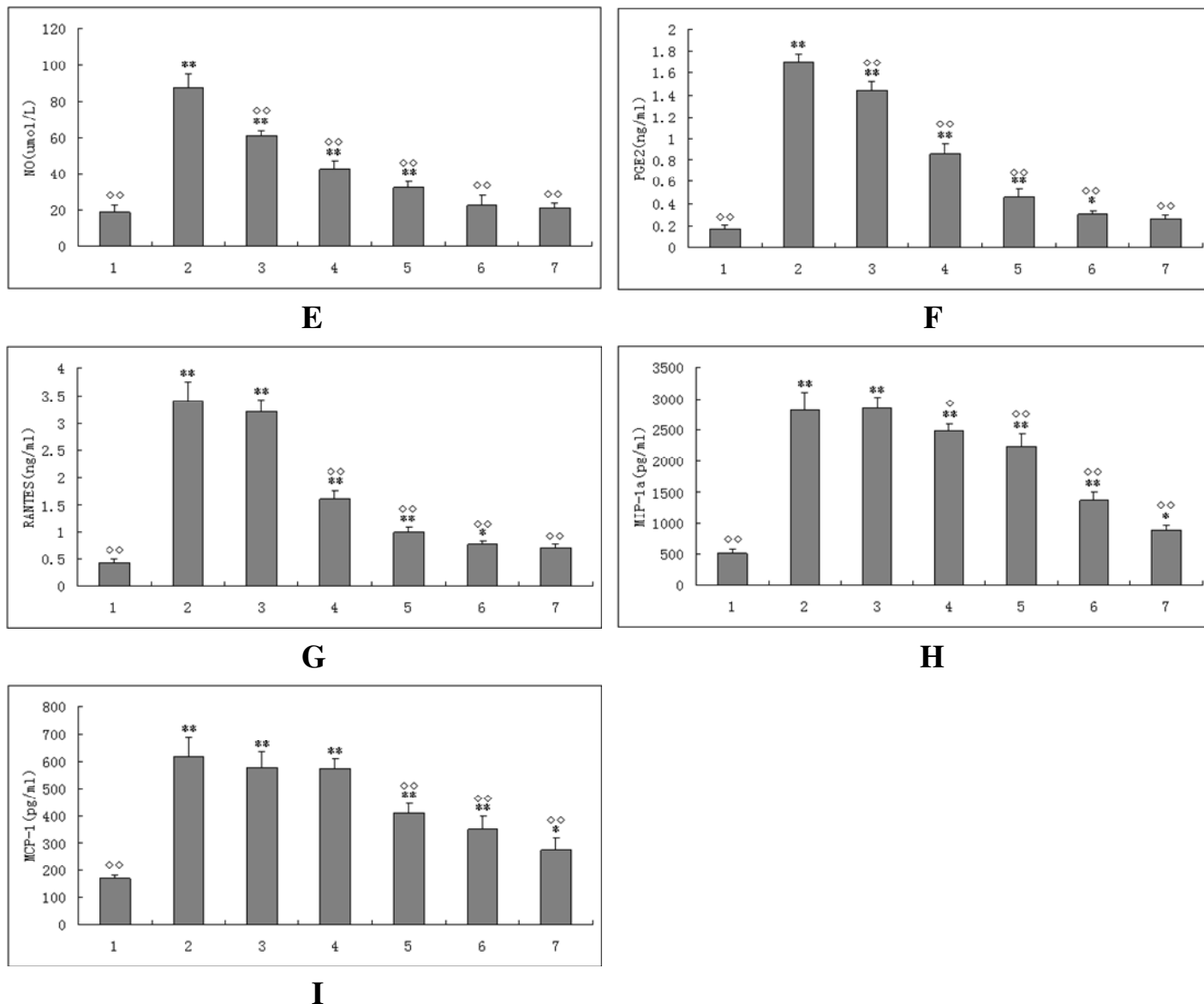


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proteins that play an important role in the selective recruitment of different leukocyte populations to the sites of inflammation. Human renal glomerular mesangial cells are potent producers of several chemokines (Schwarz et al., 1997). We examined the inhibitory effect of PTN on HG-induced mesangial cell chemokine expression, with focus on MCP-1, RANTES, and MIP-1a. As shown in Figures 2E–G, 50 µmol/L of PTN markedly reduced MCP-1, MIP-1a, and RANTES production by approximately 56, 69, and 79%, respectively (Figures 2G–I). Similar inhibitory effect was observed in the case of other inflammatory cytokines (Figures 2A–D). Furthermore, we examined biopsy specimens from 11 patients with DN; MCP-1 and RANTES expressions were strongly upregulated in all the specimens (Mezzano et al., 2004).

Hence, it appears that PTN can block a common pathway involved in the production of inflammatory cytokines and chemokines.

The GFR is elevated in the early phase of some types of nephropathies like DN. In this respect, vasodilatory prostaglandins, including PGE<sub>2</sub>, have been implicated in the glomerular hyperfiltration observed in diabetic patients (Viberti et al., 1989) and in experimental models of diabetes (DeZeeuw and Craven, 1993). In this study, we re-evaluated the inhibitory effect of PTN on PGE<sub>2</sub> production in HG-treated HRMCs. The results indicated that PTN suppressed HG-induced PGE<sub>2</sub> production in a dose-dependent manner (Figure 2F). At the same time, we also found that PTN strongly reduced HG-induced NO increase (Figure 2E). NO also serves important roles in

glomerular function, and excessive NO production has been linked to several forms of glomerular injury (Yu et al., 2003).

In conclusion, our study supports the view that inflammatory cytokine and chemokine production is upregulated in human renal mesangial cells in a hyperglycemic environment. Further, PTN treatment showed a significant anti-inflammatory effect on HRMCs in a hyperglycemic environment. Although, the mechanism of anti-inflammatory effect requires further investigation, the anti-inflammatory properties of PTN and its beneficial effects on DN can be realized as clinical treatments.

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