

Pioglitazone inhibits high glucose-induced synthesis of extracellular matrix by NF- κ B and AP-1 pathways in rat peritoneal mesothelial cells

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Received July 20, 2012; Accepted February 1, 2013

DOI: 10.3892/mmr.2013.1309

Abstract. High glucose (HG) in peritoneal dialysates has been demonstrated to induce extracellular matrix (ECM) synthesis by peritoneal mesothelial cells (PMCs) and to contribute to peritoneal fibrosis during continuous ambulatory peritoneal dialysis (CAPD). In the present study, we investigated the effects of pioglitazone, a peroxisome proliferator-activated receptor γ (PPAR γ) agonist, on HG-induced ECM accumulation and the underlying mechanism in rat PMCs (RPMCs). In cultured RPMCs, HG treatment increased the expression of fibronectin (FN), collagen I and plasminogen activation inhibitor-1 (PAI-1) at the mRNA and protein levels, while it downregulated the expression of PPAR γ in a time- and concentration-dependent manner. Pretreatment with pioglitazone not only decreased the expression of PAI-1 and matrix proteins (FN and collagen I), but prevented the downregulation of PPAR γ in RPMCs under HG conditions. HG treatment activated the nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1) pathways. In addition, the NF- κ B inhibitor, pyrrolidine dithiocarbamate (PDTC), and the AP-1 inhibitor, SP600125, decreased the protein levels of FN, collagen I and PAI-1, suggesting a role for the NF- κ B and AP-1 pathways

in the regulation of ECM accumulation induced by HG in RPMCs. Notably, we demonstrated that pretreatment with pioglitazone significantly inhibited HG-induced NF- κ B and AP-1 activation. Collectively, these results suggest that pioglitazone inhibits HG-induced ECM accumulation in RPMCs by increasing PPAR γ expression, and by inhibiting the NF- κ B and AP-1 pathways.

Introduction

Peritoneal fibrosis is one of the most serious complications in patients undergoing continuous ambulatory peritoneal dialysis (CAPD), and is a primary cause of the discontinuation of peritoneal dialysis treatment. A high concentration of glucose in the peritoneal dialysates, which is the key driving force in peritoneal dialysis, is considered to be an important initial factor in the development of peritoneal fibrosis (1). Extracellular matrix (ECM) accumulation and peritoneal thickening are the major pathological hallmarks of peritoneal fibrosis (2). Fibronectin (FN) and collagen I are the main components of the ECM; ECM accumulation results from an imbalance between the synthetic and degradative pathways of these proteins. ECM degradation is regulated by the cytokine, plasminogen activation inhibitor-1 (PAI-1). *In vitro* and *in vivo* studies have demonstrated that high glucose (HG) conditions are able to increase the synthesis of FN, collagen I and PAI-1 in peritoneal mesothelial cells (PMCs) (3,4). Downregulation of the synthesis of ECM proteins (FN and collagen I) and PAI-1 expression may be effective in preventing or delaying peritoneal fibrosis.

Peroxisome proliferator-activated receptor γ (PPAR γ), a member of the nuclear hormone receptor superfamily, has been implicated in the regulation of lipid homeostasis, energy metabolism and inflammation, as well as cellular differentiation and proliferation (5). Thiazolidinediones (TZDs), such as pioglitazone, rosiglitazone and troglitazone, are PPAR γ agonists that control hyperglycemia by improving insulin sensitivity, which has led to their use in the treatment of type 2 diabetes. In addition to glucose-lowering effects, PPAR γ agonists have also demonstrated antifibrotic effects in several experimental models. A previous study demonstrated that, in the opossum kidney model of proximal tubular cells, pioglitazone reduced

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Abbreviations: AP-1, activator protein-1; CAPD, continuous ambulatory peritoneal dialysis; ELISA, enzyme-linked immunosorbent assay; HG, high glucose; ECM, extracellular matrix; FN, fibronectin; MCP-1, monocyte chemoattractant protein-1; NF- κ B, nuclear factor- κ B; PMCs, peritoneal mesothelial cells; PPAR γ , peroxisome proliferator-activated receptor γ ; PAI-1, plasminogen activation inhibitor-1; PDTC, pyrrolidine dithiocarbamate; TZDs, thiazolidinediones; TGF- β 1, transforming growth factor β 1

Key words: pioglitazone, peritoneal mesothelial cell, nuclear factor- κ B, activator protein-1, peroxisome proliferator-activated receptor γ

production of the profibrotic and inflammatory cytokines, transforming growth factor $\beta 1$ (TGF- $\beta 1$) and monocyte chemoattractant protein-1 (MCP-1), by the tubular cells (6). Pioglitazone has also been demonstrated to reduce the expression of FN, PAI-1 and collagen IV in mesangial cells (7) and renal tubular cells (8) exposed to HG. PPAR γ agonists may further attenuate glomerulosclerosis, as suggested by studies in the 5/6-nephrectomy model (9). PMCs are the cells most likely to contribute to peritoneal fibrosis, and they exhibit constitutive expression of PPAR γ (10,11). However, the effect of PPAR γ on peritoneal fibrosis has not been investigated in detail. In rat models of encapsulating peritoneal sclerosis and dialysis fluid exposure, rosiglitazone has been demonstrated to ameliorate certain adverse peritoneal functional and morphological changes (12,13); however, the mechanisms underlying PPAR γ -mediated protection of the peritoneal membrane remain largely unknown. In the present study, we investigated the direct effects of pioglitazone on ECM accumulation and the underlying mechanism in rat PMCs (RPMCs) under HG conditions.

Materials and methods

Reagents. D-glucose, nuclear factor- κ B (NF- κ B) inhibitor [pyrrolidine dithiocarbamate (PDTCT)] and activator protein-1 (AP-1) inhibitor (SP600125) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM)/F12 cell culture medium and fetal calf serum (FCS) were obtained from Gibco-BRL (Invitrogen Life Technologies, Carlsbad, CA, USA). Rabbit polyclonal antibodies against I κ B α , NF- κ B-p65 and PPAR γ ; mouse monoclonal antibodies against c-Fos, c-Jun and β -actin; and ECL chemiluminescence reaction detection reagents were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). TRIzol, a two-step RT-PCR kit and primers were obtained from Takara Biotechnology (Dalian, China). Pioglitazone was provided by Hangzhou Huadong Medicine Group Co., Ltd. (Hangzhou, China).

Isolation and culture of RPMCs. RPMCs were isolated and cultured as described previously (14). Briefly, to harvest RPMCs, the surgically resected omentum from Sprague-Dawley rats (120 \pm 20 g) was enzymatically digested with 0.125% trypsin/0.01% ethylenediamine tetraacetic acid (EDTA) for 25 min at 37°C. Following addition of 10% FCS to stop the digestion, the tissue-free cell suspension was centrifuged at 60 x g for 5 min at 4°C. The supernatant was removed and the cell pellet was resuspended in a final volume of 4 ml DMEM/F12 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin (Invitrogen Life Technologies, Grand Island, NY, USA). Cells were then seeded into 25-cm² tissue culture flasks. Following incubation for 1-3 days at 37°C, the media were replaced for the first time. Cells were passaged when they reached 80% confluence. Passage 2 cells were observed under a phase contrast inverted microscope and subsequently identified by immunocytochemistry. Cells were positive for cytokeratin and vimentin, and negative for the factor VIII-related antigen and leukocyte CD45 antigen. Cells from passages 3-6 were used in the experiments. Cells were cultured for 24 h in serum-free DMEM/F12 medium

prior to each experiment. Control cells were treated with serum-free DMEM/F12 medium only. Cells were analyzed by reverse transcription-polymerase chain reaction (RT-PCR) and western blot analysis, and the supernatant was used for enzyme-linked immunosorbent assay (ELISA).

The RPMC experimental treatment protocol was as follows: i) untreated (control); ii) RPMCs treated with different concentrations of glucose (1.5, 2.5 and 4.25%) for 24 h; iii) RPMCs treated with HG (2.5%) for 0, 3, 6, 12, 24 and 36 h; iv) RPMCs pre-incubated with pioglitazone (10 or 20 μ M), PDTCT (25 or 50 μ M) or SP600125 (10 or 20 μ M) for 2 h, followed by HG (2.5%) for 24 h; and v) RPMCs co-transfected with 0.5 μ g pNF- κ B-Luc or pAP-1-Luc and then treated with control medium, HG or HG + pioglitazone as described above, 24 h following transfection.

Semi-quantitative RT-PCR analysis. Total RNA was extracted from RPMCs using TRIzol (Takara Biotechnology), according to the manufacturer's instructions. Total RNA (2 μ g) was subjected to DNase digestion, and total digested RNA was used for the RT reaction with the oligo-dT primer. Following cDNA synthesis, PCR amplification was performed using the cDNA template with the specific primer in the GeneAmp PCR system 9700 (Applied Biosystems, Carlsbad, CA, USA). The primer sequences used were as follows: sense, 5'-TTATGACGACGGGAAGACCT-3' and antisense, 5'-GCTGGATGGAAAGATTACTC-3' for FN (209 bp); sense, 5'-GCGAGGACATGAGGAGTAGC-3' and antisense, 5'-CCTGTGACCAGGGATGATGTCTT-3' for collagen I (308 bp); sense, 5'-TCGGCACAATCCAACAGA-3' and antisense, 5'-TGCTGAGTGAAGGCGTAG-3' for PAI-1 (383 bp); sense, 5'-TGGGACGATATGGAGAAGAT-3' and antisense, 5'-ATTGCCGATAGTGATGACCT-3' for glyceraldehyde phosphate dehydrogenase (GAPDH; 452 bp). PCR conditions included denaturation at 94°C for 2 min; 35 amplification cycles consisting of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 45 sec; and a final extension at 72°C for 10 min. Amplified products were detected by electrophoresis in a 1.5% agarose gel and visualized by ethidium bromide (EB) staining and ultraviolet transillumination. The optical densities of the PCR products were measured with ImageJ software (National Institutes of Health). RNA expression was quantified by comparison with the internal control, GAPDH.

ELISA analysis. Protein levels of FN, collagen I and PAI-1 in the supernatants of RPMC cultures were measured by ELISA according to the instructions of the manufacturer (Shanghai Sun Bio-Tech Co., Ltd., Shanghai, China). Briefly, the samples and standard were transferred to 96-well microplates, which had been pre-coated with specific polyclonal antibodies at 4°C overnight. Plates were incubated with the reaction mixture at room temperature for 2 h. After washing each well four times, biotinylated rat antibodies were added to each well and incubated at room temperature for 1 h. Following four further washes, streptavidin-peroxidase conjugate was added and incubated for 30 min. Subsequently, the chromogenic substrate was added to each well and the absorbance at 492 nm was measured using a microplate reader (Bio-Rad 3550; Bio-Rad Laboratories, Hercules, CA, USA).

Western blot analysis. Cultured RPMCs were lysed in radio-immunoprecipitation assay (RIPA) buffer. Nuclear proteins were extracted from cells using a commercial nuclear extraction kit (Active Motif, Carlsbad, CA, USA) for NF- κ B-p65 western blot analysis. Protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit (Bio-Rad Laboratories). The lysates were subjected to electrophoretic separation by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose membrane. The membrane was then blocked and incubated with antibodies specific for PPAR γ (1:400), I κ B α (1:1,000), NF- κ B-p65 (1:1,000), c-Fos (1:1,000), c-Jun (1:1,000) and β -actin (1:2,000). Membranes were subsequently washed, incubated with specific secondary horseradish peroxidase-conjugated antibodies and developed with an ECL chemiluminescence detection kit (Santa Cruz Biotechnology, Inc.). Membranes were visualized by exposure to X-ray film. The films were then scanned and quantified with ImageJ software. The results are presented as relative to β -actin.

Luciferase assay. NF- κ B and AP-1 reporter activity were measured using the Dual-Luciferase Reporter Assay system (Promega, Fitchburg, WI, USA). RPMCs were co-transfected with 0.5 μ g NF- κ B or AP-1 responsive reporter gene construct carrying two copies of the sequences linked to the luciferase gene (pNF- κ B-Luc or pAP-1-Luc; Stratagene, La Jolla, CA, USA) along with 0.01 μ g Renilla luciferase (Prl-TK; Promega), using Lipofectamine (Invitrogen, Grand Island, NY, USA). Cells were treated with or without pioglitazone for 2 h, 24 h following transfection, and then incubated with or without HG for an additional 24 h. Subsequently, the activity of luciferase and Renilla luciferase was measured using the Dual-Luciferase Reporter Assay system. Results are presented as the luciferase activity normalized to the Renilla luciferase activity.

Statistical analysis. Data are expressed as the mean \pm standard deviation. Statistical analysis of the difference among groups was performed by ANOVA followed by the Student-Newman-Keuls multiple comparisons test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Pioglitazone reduces HG-induced synthesis of FN, collagen I and PAI-1 at the mRNA and protein levels in RPMCs. To determine the effects of pioglitazone on ECM accumulation induced by HG in RPMCs, we analyzed the mRNA and protein expression of FN, collagen I and PAI-1 using RT-PCR or ELISA, as appropriate. The mRNA (Fig. 1A) and protein (Fig. 1B) expression levels of FN, collagen I and PAI-1 were significantly increased following stimulation of RPMCs with HG ($P < 0.05$ vs. control). Pretreatment with 10 μ M pioglitazone significantly inhibited the induction of FN, collagen I and PAI-1 under HG conditions ($P < 0.05$ vs. HG) and 20 μ M pioglitazone exerted an even greater inhibitory effect ($P < 0.01$ vs. HG).

Pioglitazone inhibits HG-induced downregulation of PPAR γ in RPMCs. Although pioglitazone directly activates PPAR γ , it has also been demonstrated to upregulate the expression of PPAR γ in certain tissue and cells. To explore the poten-

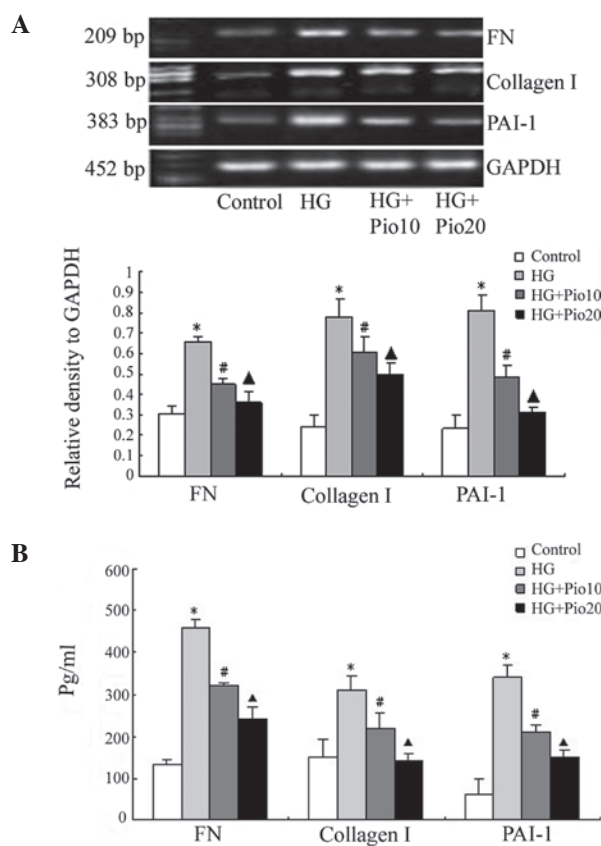


Figure 1. Pioglitazone reduced the mRNA and protein levels of fibronectin (FN), collagen I and plasminogen activation inhibitor-1 (PAI-1) induced by high glucose (HG) in rat peritoneal mesothelial cells (RPMCs). RPMCs were preincubated with pioglitazone (Pio; 10 or 20 μ M) for 2 h and then treated with HG (2.5%) for 24 h. Cells and supernatants were collected and subsequently assayed for FN, collagen I and PAI-1. (A) The mRNA expression was measured by reverse transcription-polymerase chain reaction (RT-PCR). (B) The protein levels in supernatants were measured by enzyme-linked immunosorbent assay (ELISA). * $P < 0.01$ vs. control; # $P < 0.05$ and ▲ $P < 0.01$ vs. HG; n=3 independent experiments, each performed in triplicate. GAPDH, glyceraldehyde phosphate dehydrogenase (internal control).

tial mechanism whereby pioglitazone regulates the effect of HG on RPMCs, we analyzed the PPAR γ protein expression levels of RPMCs by western blot analysis. HG significantly decreased the PPAR γ protein expression levels in a time- and concentration-dependent manner (Fig. 2A and B, respectively). Pretreatment with 10 or 20 μ M pioglitazone prevented the downregulation of PPAR γ protein (Fig. 2C; $P < 0.05$ vs. HG).

NF- κ B and AP-1 are involved in regulating the synthesis of FN, collagen I and PAI-1 in RPMCs under HG conditions. To explore the role of the NF- κ B and AP-1 signaling pathways in the regulation of FN, collagen I and PAI-1 synthesis in RPMCs under HG conditions, we investigated the effect of an NF- κ B inhibitor (PDTC) and an AP-1 specific inhibitor (SP600125) on the protein levels of FN, collagen I and PAI-1 in the cell culture supernatant. As demonstrated in Fig. 3, RPMCs secreted a limited quantity of FN, collagen I and PAI-1 protein when cultured conventionally. Exposure to HG (2.5%) significantly upregulated the levels of these proteins in the culture supernatants. Following pretreatment with PDTC or SP600125 for 2 h, the protein levels of FN and PAI-1 were significantly decreased relative to the HG group. SP600125

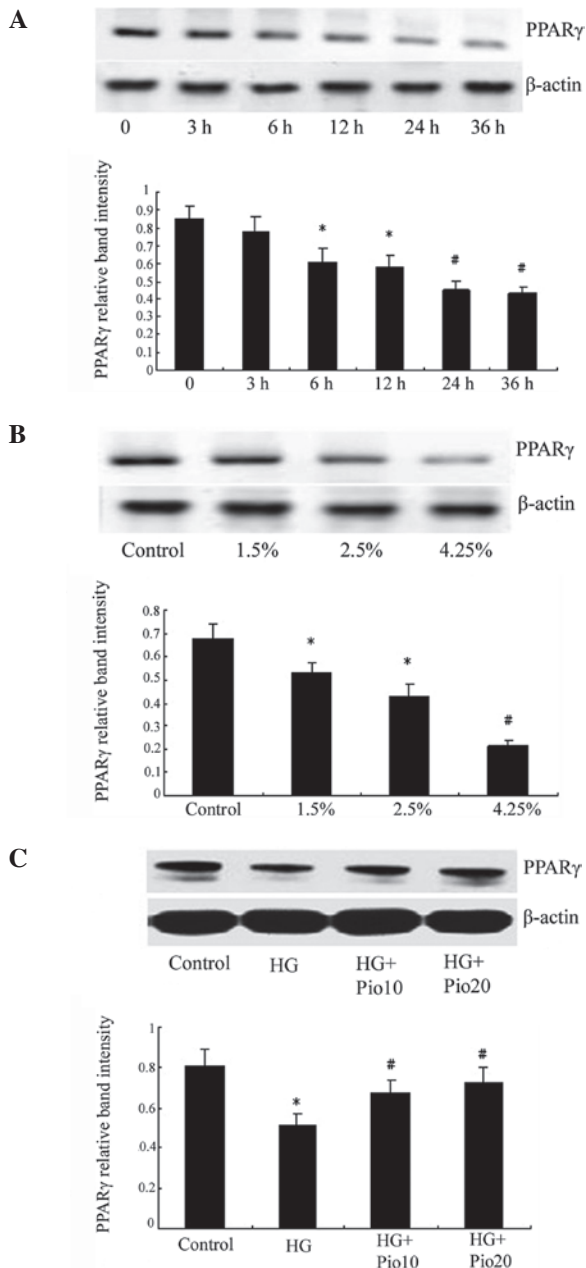


Figure 2. Effects of high glucose (HG) and pioglitazone on the protein expression of peroxisome proliferator-activated receptor γ (PPAR γ). The protein expression of PPAR γ was determined by western blot analysis as described in Materials and methods. (A) Rat peritoneal mesothelial cells (RPMCs) were treated with glucose (2.5%) for 0–36 h. * $P < 0.05$ and * $P < 0.01$ vs. 0 h. (B) RPMCs were treated with different concentrations of glucose (1.5, 2.5 and 4.25%). * $P < 0.05$ and * $P < 0.01$ vs. control. (C) RPMCs were pretreated with pioglitazone (Pio; 10 and 20 μ M) for 2 h and then exposed to HG (2.5%) for 24 h. * $P < 0.01$ vs. control; # $P < 0.05$ vs. HG. All data are expressed as the mean \pm standard deviation ($n = 3$ independent experiments, each performed in triplicate).

markedly inhibited the HG-induced upregulation of collagen I protein ($P < 0.05$ vs. HG), whereas collagen I protein was not affected by PDTC pretreatment.

Pioglitazone inhibits HG-induced activation of NF- κ B. To explore the possible mechanisms for the protective effect of pioglitazone on RPMCs, we investigated the effect of pioglitazone on the NF- κ B pathway in RPMCs. Western blot analysis results revealed that HG induced the degradation of

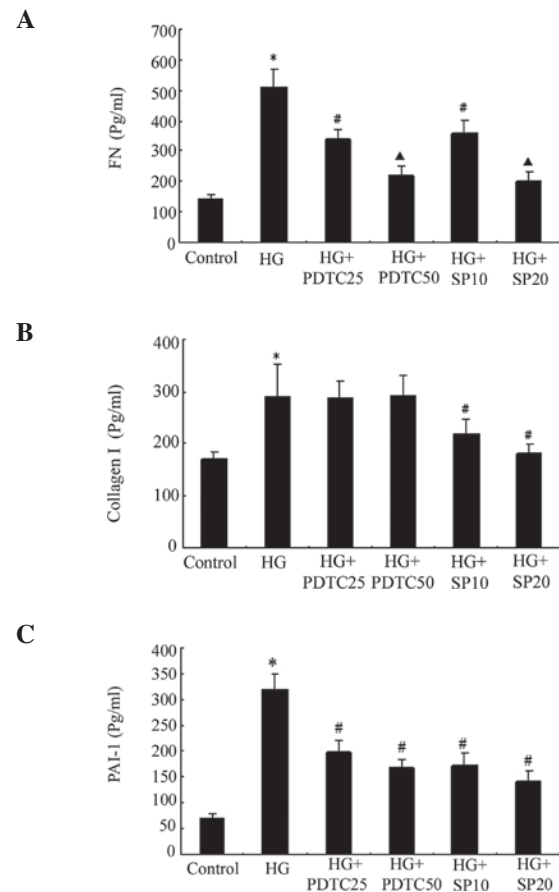


Figure 3. Effects of nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1) inhibitors on the protein secretion of fibronectin (FN), collagen I, and plasminogen activation inhibitor-1 (PAI-1) in supernatants from high glucose (HG)-stimulated rat peritoneal mesothelial cells (RPMCs). RPMCs were pre-incubated with pyrrolidine dithiocarbamate (PDTC; 25 or 50 μ M) or SP600125 (SP; 10 or 20 μ M) for 2 h, followed by HG (2.5%) for 24 h. Supernatants were analyzed for protein levels by enzyme-linked immunosorbent assay (ELISA). (A) FN, (B) collagen I and (C) PAI-1. * $P < 0.01$ vs. control; # $P < 0.05$ and * $P < 0.01$ vs. HG; $n = 3$ independent experiments, each performed in triplicate.

I κ B α and increased NF- κ B-p65 protein expression. However, these actions were attenuated by 10 and 20 μ M pioglitazone, with 20 μ M pioglitazone being the more effective (Fig. 4A). The luciferase assay indicated that NF- κ B activity was increased 7.65-fold in the HG group compared with that in the control group, and pioglitazone treatment significantly suppressed NF- κ B activity (Fig. 4B). These results indicate that pioglitazone inhibited NF- κ B transcriptional activity in RPMCs under HG conditions.

Pioglitazone inhibits HG-induced activation of AP-1. AP-1 has been demonstrated to be involved in the effect of pioglitazone in certain tissues and cells. Therefore, we investigated the effect of pioglitazone on the activation of the AP-1 pathway in RPMCs. Western blot analysis revealed that the protein expression levels of c-Fos and c-Jun (two components of AP-1) were significantly upregulated in RPMCs exposed to HG, and this upregulation was markedly suppressed by pioglitazone treatment (Fig. 5A). The luciferase assay further revealed that AP-1 activity was increased 5.16-fold in the HG-treated cells compared with that in the control cells, and pioglitazone treat-

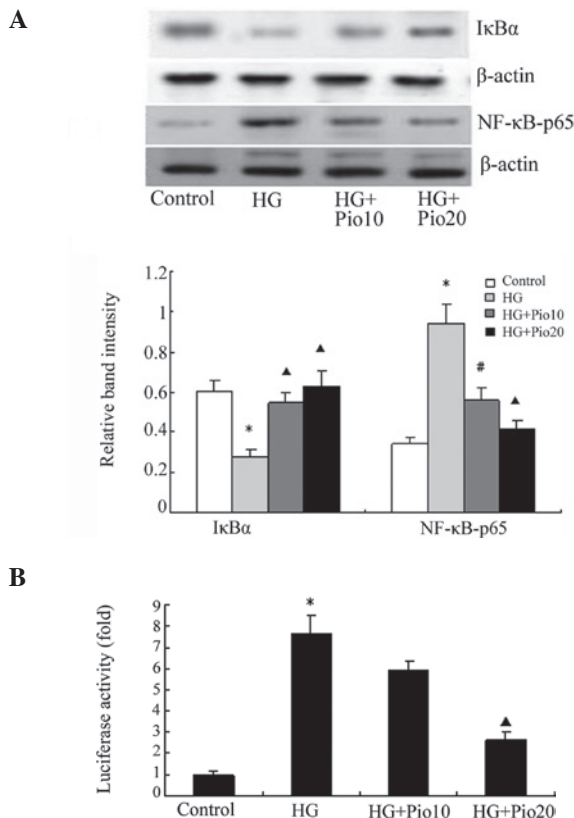


Figure 4. Effects of pioglitazone on the activation of the nuclear factor- κ B (NF- κ B) pathway induced by high glucose (HG) in rat peritoneal mesothelial cells (RPMCs). (A) RPMCs were pre-incubated with pioglitazone (Pio; 10 and 20 μ M) for 2 h and then treated with HG (2.5%) for 24 h. The protein expression levels of I κ B α (total protein) and NF- κ B-p65 (nuclear protein) were measured by western blot analysis. (B) RPMCs were co-transfected with 0.5 μ g pNF- κ B-Luc. RPMCs were treated with control medium, HG or HG + pioglitazone, 24 h following transfection. Luciferase assay was performed for NF- κ B activity. * P <0.01 vs. control; $^{\#}P$ <0.05 and $^{\Delta}P$ <0.01 vs. HG; n =3 independent experiments, each performed in triplicate.

ment significantly suppressed the increase in AP-1 activity (Fig. 5B). These results indicate that pioglitazone inhibited the HG-induced activation of the AP-1 pathway in RPMCs.

Discussion

In the present study, we demonstrated that pioglitazone reduces the HG-induced synthesis of PAI-1 and the accumulation of ECM. Furthermore, our results suggest that the effect of pioglitazone may be mediated by inhibition of HG-induced downregulation of PPAR γ expression and NF- κ B and AP-1 pathway activation.

Long-term peritoneal dialysis is limited by ultrafiltration failure due to physiological and functional alterations of the peritoneal membrane; 9-27% of patients with peritoneal dialysis cease therapy due to ultrafiltration failure (15). The main cause of ultrafiltration failure is peritoneal fibrosis. RPMCs have the capacity to produce matrix proteins and pro-fibrotic cytokines when affected by various stimuli, including HG concentrations (16), glucose degradation products (17) and pH or osmolality changes (18).

The present study focused on HG bioincompatible dialysis solutions, which are typically 1.5, 2.5 or 4.25% glucose. We

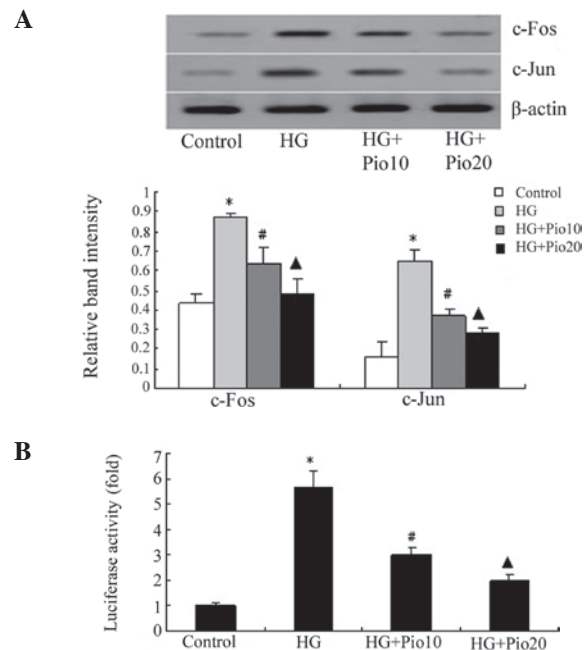


Figure 5. Effects of pioglitazone on the activation of the activator protein-1 (AP-1) pathway induced by high glucose (HG) in rat peritoneal mesothelial cells (RPMCs). (A) RPMCs were pre-incubated with pioglitazone (Pio; 10 and 20 μ M) for 2 h and then treated with HG (2.5%) for 24 h. The protein expression levels of c-Fos and c-Jun were measured by western blot analysis. (B) RPMCs were co-transfected with 0.5 μ g pAP-1-Luc. RPMCs were treated with control medium, HG or HG + pioglitazone, 24 h following transfection. Luciferase assay was performed for AP-1 activity. * P <0.01 vs. control; $^{\#}P$ <0.05 and $^{\Delta}P$ <0.01 vs. HG; n =3 independent experiments, each performed in triplicate.

used a 2.5% glucose concentration, which is most widely used in CAPD patients, to stimulate the RPMCs. Our results revealed that an HG concentration significantly increased the expression of FN, collagen and PAI-1 at the mRNA and protein levels. The increase in matrix is due to the presence of collagen and non-collagen proteins, including FN. Matrix degradation is largely inhibited by PAI-1, which prevents plasmin generation and plasmin-mediated matrix metalloproteinase (MMP) activation (19). Upregulated PAI-1 expression is correlated with the development of peritoneal fibrosis (20). In the present study, pretreatment with the PPAR γ agonist, pioglitazone, effectively reduced the HG-induced synthesis of FN, collagen I and PAI-1 in RPMCs. These data are in accordance with the results of a previous study in which troglitazone reduced the expression of ECM proteins (FN and type IV collagen) and PAI-1 in glomeruli from streptozotocin-induced diabetic rats (21). In conclusion, these results demonstrate that PPAR γ agonists have a direct effect on RPMCs, resulting in a reduced accumulation of collagen- and non-collagen-containing matrix proteins; and further, that this effect is correlated with a reduction in PAI-1, which normally inhibits matrix degradation.

In the present study, we observed that D-glucose reduces the expression of PPAR γ in a time- and concentration-dependent manner. Furthermore, we observed that pioglitazone prevented the downregulation of PPAR γ in cultured RPMCs. To the best of our knowledge, this is the first study to investigate the effects of HG and pioglitazone on PPAR γ expression in RPMCs. The results suggest that PPAR γ may be significant in PMC biology. Consistent with this finding,

a previous study demonstrated that PPAR γ expression was downregulated in the hearts of Zucker diabetic fatty rats, and its downregulation contributed to the fibrotic pathogenesis of diabetic cardiomyopathy (22). In addition, pioglitazone has been observed to inhibit the advanced glycation end products (AGE)-induced downregulation of PPAR γ in vascular smooth muscle cells (23). It should be noted that TZDs have also been demonstrated to elicit PPAR γ -independent effects. For example, in PPAR γ -deficient embryonic stem cells, TZDs have been demonstrated to suppress cell proliferation (24). Galli *et al* studied collagen deposition in a rat model of liver fibrosis using two TZD PPAR γ agonists, pioglitazone and rosiglitazone. PPAR γ -specific DNA binding was significantly impaired in the nuclear extracts of hepatic stellate cells that were isolated from rats with liver fibrosis compared with those that were isolated from control rats. Administration of either type of TZD restored PPAR-DNA binding in the hepatic stellate cell nuclei (25). TZD-induced PPAR γ activation inhibited collagen and FN synthesis induced by TGF- β 1 in subsequent *in vitro* studies using these cells (25). These findings suggest that the antifibrotic effect of pioglitazone is PPAR γ -dependent in RPMCs.

HG concentrations in cell cultures may activate transcription factors, such as NF- κ B and AP-1, and subsequently regulate the expression of various genes that mediate growth, inflammation and fibrosis. NF- κ B and AP-1 are present in numerous cell types, including endothelial cells, vascular smooth muscle cells, glomerular mesangial cells and PMCs. Normally, inactive NF- κ B resides in the cytoplasm, and is bound to the inhibitory protein I κ B. I κ B is hydrolyzed following stimulation, whereby the p50/p65 NF- κ B dimer translocates to the nucleus and initiates the transcription of various genes (26-30). Re-synthesis of I κ B, induced by NF- κ B, allows for sequestration of NF- κ B in the cytoplasm and termination of the NF- κ B response (26-28). AP-1 consists of homodimers of Jun or heterodimers of Fos and Jun (31,32), and may be regulated by cellular stress. In the present study, we observed that the activation of NF- κ B and AP-1 was stimulated by HG as demonstrated by the degradation of I κ B α , the induction of NF- κ B-p65 in the nucleus, the upregulation of c-Fos and c-Jun proteins and an increased luciferase activity in RPMCs. Our results were concordant with previous studies in cultured vascular smooth muscle cells (33,34) and glomerular mesangial cells (35). However, it remains unknown whether NF- κ B and AP-1 are involved in regulating the synthesis of FN, collagen I and PAI-1 in PMCs in an HG environment. NF- κ B and AP-1-binding sites have been identified in the promoters of FN, collagen I and PAI-1 (33-36). The NF- κ B inhibitor PDTC (34) and the transcription factor decoy for AP-1 (33) both attenuated HG-induced PAI-1 gene expression in cultured vascular smooth muscle cells. PDTC and the AP-1 inhibitor curcumin reduced FN expression in human umbilical vein endothelial cells (HUVECs) and human microvascular endothelial cells (HMECs) treated with HG (37). Our study showed that pretreatment of RPMCs with either PDTC or SP600125 (a specific inhibitor of AP-1) significantly decreased the protein levels of FN and PAI-1 in cultured RPMC supernatants stimulated by HG, suggesting that NF- κ B and AP-1 participate in the HG-induced upregulation of FN and PAI-1. Notably, SP600125, but not PDTC, inhibited the expression of

collagen I, indicating that only the AP-1 pathway is involved in collagen I regulation in RPMCs under HG conditions.

Activated PPAR γ regulates the expression of downstream cytokines via two different mechanisms: i) ligand-activated PPAR γ induced gene expression by binding with PPAR reaction elements (PPRE) in the promoter region of target genes; ii) ligand-activated PPAR γ regulated target gene expression by antagonizing other transcription factors, such as NF- κ B and AP-1. The activation of PPAR γ has been demonstrated to inhibit the expression of proinflammatory chemokines (TNF- α and MCP-1) and adhesion molecules (ICAM-1 and VCAM-1) by suppressing the activation of NF- κ B and AP-1 in both *in vitro* and *in vivo* studies (38,39). In the present study, pioglitazone pretreatment significantly decreased the I κ B α protein degradation and NF- κ B-p65 protein expression, downregulated the expression of c-Fos and c-Jun proteins, and suppressed the luciferase activity of NF- κ B and AP-1 in RPMCs in an HG environment. These findings suggest that pioglitazone directly inhibited the HG-induced activation of the NF- κ B and AP-1 pathways in the RPMCs. Our results are consistent with those of previous studies in which pioglitazone downregulated the expression of FN, collagen IV and PAI-1, by suppressing NF- κ B and AP-1 activation in cultured renal mesangial cells (7) and tubular cells (8).

In conclusion, the present study demonstrated that the PPAR γ agonist, pioglitazone, reduced ECM accumulation by inhibiting the expression of FN, collagen I and PAI-1, thereby exerting anti-fibrotic effects in RPMCs exposed to HG. The effect may be mediated by the activation of PPAR γ , and the inhibition of NF- κ B and AP-1 activation. Our results suggested that PPAR γ agonists may provide a novel therapeutic approach to the treatment of peritoneal fibrosis.

Acknowledgements

This study was supported by the Natural Science Foundation of Liaoning, China (No. 20101143) and the Scientific Research Foundation of Shengjing Hospital of China Medical University.

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