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## PPAR $\gamma$ agonists exert antifibrotic effects in renal tubular cells exposed to high glucose

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**Panchapakesan, U., S. Sumual, C. A. Pollock, and X. Chen.** PPAR $\gamma$  agonists exert antifibrotic effects in renal tubular cells exposed to high glucose. *Am J Physiol Renal Physiol* 289: F1153–F1158, 2005. First published May 10, 2005; doi:10.1152/ajprenal.00097.2005.—Peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) are ligand-activated transcription factors that regulate cell growth, inflammation, lipid metabolism, and insulin sensitivity. We recently demonstrated that PPAR $\gamma$  agonists limit high glucose-induced inflammation in a model of proximal tubular cells (PTC; Panchapakesan U, Pollock CA, and Chen XM. *Am J Physiol Renal Physiol* 287: F528–F534, 2004). However, the role of PPAR $\gamma$  in the excess extracellular matrix production is largely unknown. We evaluated the effect of 24- to 48-h 8  $\mu$ M L-805645 or 10  $\mu$ M pioglitazone on 25 mM D-glucose-induced markers of fibrosis in HK-2 cells. High D-glucose induced nuclear binding of activator protein-1 (AP-1) to  $140.8 \pm 10.9\%$  ( $P < 0.05$ ), which was attenuated with L-805645 and pioglitazone to  $82.3 \pm 14.4$  ( $P < 0.01$  vs. high D-glucose) and  $99.3 \pm 12.2\%$  ( $P < 0.05$  vs. high D-glucose), respectively. High D-glucose increased total production of transforming growth factor (TGF)- $\beta_1$   $139.6 \pm 6.5\%$  ( $P < 0.05$ ), which was reversed with L-805645 and pioglitazone to  $68.73 \pm 5.7$  ( $P < 0.01$  vs. high D-glucose) and  $112 \pm 13.6\%$  ( $P < 0.05$  vs. high D-glucose). L-805645 and pioglitazone reduced high D-glucose-induced fibronectin from  $156.0 \pm 24.9$  ( $P < 0.05$ ) to  $81.9 \pm 16.0$  and  $57.4 \pm 12.7\%$ , respectively (both  $P < 0.01$  vs. high D-glucose). Collagen IV was not induced by high D-glucose. L-805645 and pioglitazone suppressed collagen IV to  $68.0 \pm 14.5$  ( $P < 0.05$ ) and  $46.5 \pm 11.6\%$  ( $P < 0.01$ ) vs. high D-glucose, respectively. High D-glucose increased the nuclear binding of NF- $\kappa$ B to  $167 \pm 22.4\%$  ( $P < 0.05$ ), which was not modified with PPAR $\gamma$  agonists. In conclusion, PPAR $\gamma$  agonists exert antifibrotic effects in human PTC in high glucose by attenuating the increase in AP-1, TGF- $\beta_1$ , and the downstream production of the extracellular matrix protein fibronectin.

thiazolidinediones; diabetic nephropathy; proximal tubular cells

DIABETES MELLITUS (DM) is a major global health issue with up to one-third of patients suffering end-stage renal disease. The major burden of disease is derived from patients with type 2 DM (25), resulting in up to 50% of adult patients on dialysis in Westernized countries having diabetic nephropathy as their primary diagnosis.

The interventions shown to prevent the onset or attenuate the progression of diabetic nephropathy include glycemic and blood pressure control (10, 32), specific interruption of the renin-angiotensin system with either angiotensin-converting enzyme inhibitors (30) or angiotensin II receptor blockers (6, 20) and potentially lipid-lowering therapy (3, 9). Understanding the mechanisms underlying the development of diabetic nephropathy is essential in establishing novel therapeutic strat-

egies for the prevention or arrest of progressive renal failure. The search for specific molecular targets is ongoing, and PPAR $\gamma$  agonists may show promise.

PPAR $\gamma$  are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors that have a central role in regulating insulin sensitivity, adipocyte differentiation, cell growth, and inflammation (13). Synthetic agonists of PPAR $\gamma$  like the thiazolidinediones (TZDs) are widely used as insulin-sensitizing agents in patients with type 2 DM. On ligand binding, PPAR $\gamma$  form heterodimers with the retinoic acid receptor (23). This complex then binds to the PPAR response elements (PPREs) within the promoter region of target genes. Regulation of these target genes depends on the binding of ligands as well as complex interaction between resident coactivators and corepressors (36). Endogenous ligands include 15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub> (11, 17) and metabolites of oxidized low-density lipoproteins (LDL) (24, 31). Synthetic ligands include the TZDs and phenylacetic acid derivatives such as L-805645, which are specific, and selective PPAR $\gamma$  ligands (5).

There are several clinical studies demonstrating a beneficial trend, with a reduction in albuminuria in patients with type 2 DM treated with TZDs (5, 15, 18). This finding is also reflected in animal models of experimental diabetic nephropathy where treatment with TZDs reduces albuminuria and decreases glomerular matrix deposition and glomerulosclerosis (7, 8, 22, 39). These benefits appear to be independent of glycemic control. Haplo-insufficient PPAR+/- *db/db* mice exhibit more severe hyperglycemia, albuminuria, and glomerular pathology (39). The renoprotective benefit of PPAR $\gamma$  agonists is further suggested by studies in nondiabetic models of renal injury, such as the 5/6-nephrectomy model, where activation of PPAR $\gamma$  reduced glomerulosclerosis (21). In vitro studies have focused on the use of mesangial cells where PPAR $\gamma$  has been well characterized (2, 26), with specific PPAR $\gamma$  activation exerting an antiproliferative (12, 26) and antifibrotic effect, reducing type 1 collagen synthesis and secretion (29) presumed due to a transforming growth factor (TGF)- $\beta_1$ -dependent mechanism (34). In mesangial cells, pioglitazone has been shown to inhibit TGF- $\beta$ -induced fibronectin production (14). In our own study using an immortalized proximal tubular cell model (opossum kidney cell line) under normal (5 mM)-glucose conditions, we have previously demonstrated that PPAR $\gamma$  agonists stimulate tubular albumin uptake without provoking an inflammatory response (37). However, whether they protect the kidney from tubulointerstitial fibrosis, the

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hallmark of a progressive decline in renal function under hyperglycemic conditions, is unknown. Thickening of the basement membrane due to an increase in extracellular matrix production by renal proximal tubular cells is the initial pathological abnormality evident in diabetic nephropathy and central to the development of tubulointerstitial injury. Hence, our studies were designed to assess the effects of PPAR $\gamma$  agonists on the transcription factors activator protein-1 (AP-1) and NF- $\kappa$ B, the profibrotic cytokine TGF- $\beta$ <sub>1</sub>, and the extracellular matrix proteins fibronectin and collagen IV in proximal tubular cells after short-term exposure to high-glucose conditions.

## METHODS

**Cell culture.** HK-2 cells, a primary human proximal tubular cell line (a gift from Prof. J Charlesworth, Sydney, Australia), were grown in keratinocyte serum-free media (KSFM) supplemented with bovine pituitary extract (20–30  $\mu$ g/ml) and epidermal growth factor (0.1–0.2 ng/ml, GIBCO). Cell culture media was changed every 48–72 h. These cells were grown at 37°C in a humidified 5% CO<sub>2</sub> incubator and were subcultured at 50–80% confluence using 0.05% trypsin-0.02% EDTA (GIBCO).

The clinically available thiazolidinedione pioglitazone (10  $\mu$ M, Cayman Chemical) and the more selective PPAR $\gamma$  agonist L-805645 (8  $\mu$ M, Merck) were used to determine the specific effects of PPAR $\gamma$  activation in this proximal tubular model. These concentrations were chosen based on their ability to significantly upregulate PPAR $\gamma$  expression in this model and have been used in previous studies (27).

When 80% confluent, HK-2 cells were exposed to the following experimental conditions for 24 h. For TGF- $\beta$  ELISA, cells were exposed for 48 h: 1) 5 mM D-glucose (control media); 2) 30 mM D-glucose (ICN Biomedical); 3) 5 mM D-glucose and 25 mM L-glucose (osmotic control, ICN Biomedical); 4) 8  $\mu$ M L-805645 in 5 mM D-glucose; 5) 8  $\mu$ M L-805645 in 30 mM D-glucose; 6) 10  $\mu$ M pioglitazone in 5 mM D-glucose; and 7) 10  $\mu$ M pioglitazone in 30 mM D-glucose.

As L-805645 and pioglitazone were dissolved in 0.016 and 0.13% DMSO, respectively, additional controls were undertaken to evaluate independent effects of the DMSO at 0.13%.

**Nuclear extraction and EMSA for AP-1 and NF- $\kappa$ B.** After exposure to the above-mentioned experimental conditions, nuclear extracts were prepared using a NucBuster Protein Extraction Kit (Novagen, Darmstadt, Germany) as per the manufacturer's instructions. A digoxigenin (DIG) Gel Shift Kit (Roche Applied Science, Indianapolis, IN) was used in the EMSA. In brief, 25  $\mu$ g of nuclear extract were incubated with 1  $\mu$ g poly [d(I-C)] as the nonspecific competitor, 1  $\mu$ g poly L-lysine in a binding buffer [(in mM) 100 HEPES, pH 7.6, 5 EDTA, 50 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 5 DTT, and 150 KCl, as well as 1% Tween 20, wt/vol] and DIG-labeled AP-1 (5'-CGC TTG ATG AGT CAG CCG GAA-3') or NF- $\kappa$ B (5'-AGT TGA GGG GAC TTT CCC AGG C-3') consensus oligonucleotide (Promega) for 30 min at room temperature. Unlabeled AP-1 and NF- $\kappa$ B consensus oligonucleotides were used as specific competitors, respectively. The reaction mixture was electrophoresed through 6% polyacrylamide gels, transferred onto positively charged nylon membrane (Roche Applied Science), and then cross-linked using an UV transilluminator for 3 min. The membrane was subjected to immunological detection using anti-DIG-AP conjugate and chemiluminescence. Results were analyzed using Image J software, and shift bands were measured.

**TGF- $\beta$ <sub>1</sub>.** Cells were seeded at  $2 \times 10^5$  cells/well in a 24-well plate and grown in KSFM without growth factors for 24 h. At 70% confluence, cells were exposed to the experimental conditions as defined above for 48 h in quadruplicate. Supernatants were then collected, spun, and stored at -20°C until TGF- $\beta$ <sub>1</sub> levels were determined by immunoassay (Promega) as per the manufacturer's

instructions. Cell lysate protein concentration was determined (Bio-Rad), and TGF- $\beta$ <sub>1</sub> levels were corrected for protein content per well.

**Western blot analysis.** Cells were collected, and the cell pellet was resuspended in cell lysis buffer containing 50 mM Tris·HCl, 150 mM NaCl, 5 mM EDTA (pH 7.4), 0.5% Triton X-100, and protease inhibitors (Roche Diagnostics, Mannheim, Germany). Cell lysate was then sonicated to release total cell proteins, spun at 12,000 rpm at 4°C, and stored at -80°C.

Protein assay (Bio-Rad) was done to determine the protein concentration of the cell lysate. Eighty micrograms of total cell protein were mixed with 6 $\times$  Laemmli sample buffer containing mercaptoethanol and heated at 95°C for 10 min. Samples were then analyzed by SDS-PAGE in 7.5% gels and electroblotted to Hybond Nitrocellulose membranes (Amersham Pharmacia Biotech, Bucks, UK). Membranes were blocked in Tris-buffered saline containing 0.2% Tween 20 (TTBS) in 5% skim milk for 2–3 h, incubated overnight at 4°C with fibronectin (1:100, NeoMarkers), collagen IV (1:5,000, Abcam, Cambridge, UK), then reprobbed with actin (1:300, Santa Cruz) in TTBS containing 5% skim milk. Membranes were washed with TTBS and incubated with horseradish peroxidase-conjugated secondary antibody. Proteins were visualized using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). Results were corrected for actin.

**Statistical analysis.** Results are expressed as a percentage of the mean  $\pm$  SE of control values. Experiments were performed at least in triplicate or as detailed in the individual experimental protocols. Statistical comparisons between groups were made by ANOVA or unpaired *t*-tests where appropriate. Analyses were performed using the software package StatView version 5.0 (Abacus Concepts, Berkeley, CA). *P* values <0.05 were considered significant.

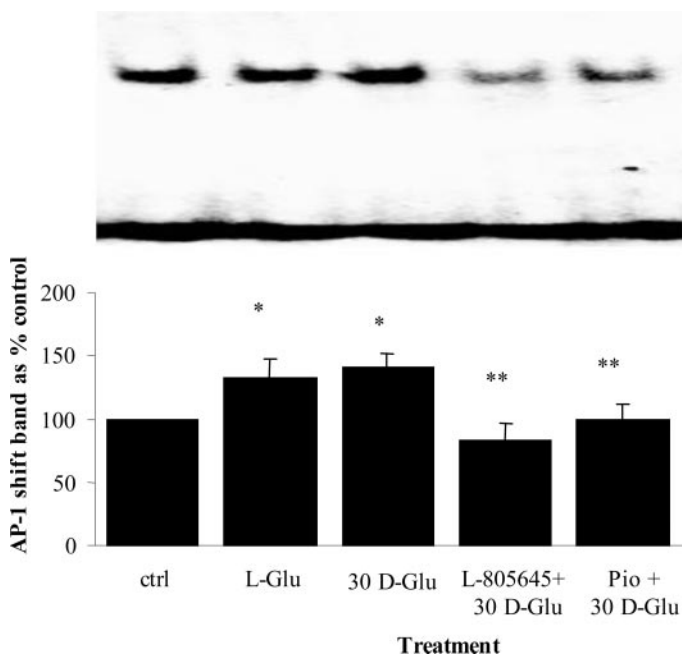


Fig. 1. L-805645 (8  $\mu$ M) and pioglitazone (10  $\mu$ M) were able to suppress high D-glucose-induced digoxigenin (DIG)-labeled activator protein-1 (AP-1) binding. HK-2 cells were incubated for 24 h with control media (ctrl), 5 mM D-glucose and 25 mM L-glucose (L-Glu), 30 mM D-glucose (30-D-Glu), 8  $\mu$ M L-805645 in 30 mM D-glucose, 10  $\mu$ M pioglitazone (Pio) in 30 mM D-glucose. Nuclear extract preparation and EMSA are as described in METHODS. Top: representative image with shift (top) and free bands (bottom). Bottom: normalized results expressed as means  $\pm$  SE; *n* = 3. \**P* < 0.05 vs. control. \*\**P* < 0.05 vs. high D-glucose.

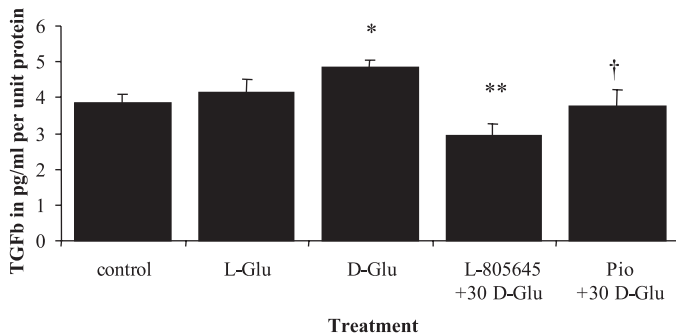


Fig. 2. L-805645 (8  $\mu$ M) and pioglitazone (10  $\mu$ M) were able to suppress high D-glucose-induced total transforming growth factor- $\beta$  (TGF- $\beta$ ) secretion. HK-2 cells were incubated for 48 h with control media, 5 mM D-glucose and 25 mM L-glucose, 30 mM D-glucose, 8  $\mu$ M L-805645 in 30 mM D-glucose, 10  $\mu$ M pioglitazone in 30 mM D-glucose. TGF- $\beta$  ELISA is as described in METHODS. Results are expressed as absolute values in pg/ml corrected for per unit protein in cell lysate  $\pm$  SE;  $n = 4$ . \* $P < 0.05$  vs. control. \*\* $P < 0.01$  vs. high D-glucose. † $P < 0.05$  vs. D-glucose.

## RESULTS

**AP-1.** High D-glucose increased the DNA binding of DIG-labeled AP-1 to  $140.8 \pm 10.9\%$  of control ( $P < 0.05$ ). Exposure to the osmotic control also increased AP-1 binding to  $133.0 \pm 14.2\%$  of control ( $P < 0.05$ ). The high glucose-induced increase in AP-1 was attenuated by concurrent exposure to L-805645 to  $82.3 \pm 14.4\%$  of control ( $P < 0.01$  vs. high D-glucose). Pioglitazone also abrogated the high glucose-induced increase in AP-1, reducing it to  $99.3 \pm 12.2\%$  of control values ( $P < 0.05$  vs. high D-glucose) (Fig. 1).

**TGF- $\beta$ .** High D-glucose increased total TGF- $\beta$ <sub>1</sub> secretion to  $4.82 \text{ pg} \cdot \text{ml}^{-1} \cdot \text{unit protein cell lysate}^{-1}$  or  $139.6 \pm 6.5\%$  of control ( $P < 0.05$ ). This increase was reduced in the presence of L-805645 to  $2.93 \text{ pg} \cdot \text{ml}^{-1} \cdot \text{unit protein cell lysate}^{-1}$  or  $68.73 \pm 5.7\%$  of control ( $P < 0.01$  vs. high D-glucose). Pioglitazone also reduced high glucose-induced TGF- $\beta$ <sub>1</sub> secretion to  $3.77 \text{ pg} \cdot \text{ml}^{-1} \cdot \text{unit protein cell lysate}^{-1}$  or  $112 \pm 13.6\%$  ( $P < 0.05$  vs. high D-glucose) (Fig. 2).

**Fibronectin.** The protein expression of fibronectin, an extracellular matrix protein downstream to TGF- $\beta$ <sub>1</sub>, was increased in the presence of high glucose to  $156.0 \pm 24.9\%$  of control ( $P < 0.05$ ). The addition of L-805645 and pioglitazone reduced high glucose-induced fibronectin expression to  $81.9 \pm 16.0$  and  $57.4 \pm 12.7\%$  (both  $P < 0.01$  vs high D-glucose), respectively (Fig. 3).

**Collagen IV.** There was no significant upregulation of collagen IV expression following short-term exposure to high glucose ( $110 \pm 23.2\%$ ;  $P =$  not significant), suggesting that the initial increase in extracellular matrix is largely as a result of deposition of noncollagen proteins. Nevertheless, both classes of PPAR $\gamma$  agonists were able to suppress collagen IV expression to levels below that observed in high-glucose conditions ( $68.0 \pm 14.5\%$ ,  $P < 0.05$  and  $46.5 \pm 11.6\%$ ,  $P < 0.01$  vs. high D-glucose following exposure to L-805645 and pioglitazone, respectively) (Fig. 4).

**NF- $\kappa$ B.** High glucose increased the binding of DIG-labeled NF- $\kappa$ B to  $167.3 \pm 22.4\%$  of control ( $P < 0.05$ ). However, neither of the PPAR $\gamma$  agonists significantly suppressed glucose-induced NF- $\kappa$ B binding, as shown in Fig. 5. Interestingly, the presence of even low concentrations of DMSO also increased the binding of DIG-labeled NF- $\kappa$ B to  $159.3 \pm 19.3\%$  of control ( $P < 0.05$ ; not shown in Fig. 5).

## DISCUSSION

TDZs are widely used as insulin-sensitizing agents in the treatment of type 2 diabetes. They appear to exert a renoprotective effect in animal models and in vitro studies, which until recently have focused on mesangial cells (2, 12, 14, 26, 34). Recent work from our laboratory has demonstrated that PPAR $\gamma$  agonists limit LDL- and albumin-induced proinflammatory responses in the human proximal tubule (37) and reduce extracellular matrix production by human cortical fibroblasts (38). However, their role and function in human proximal tubular cells under high-glucose conditions inherent in diabetic nephropathy have not been well defined. In the present study, we demonstrate that PPAR $\gamma$  ligands reverse the

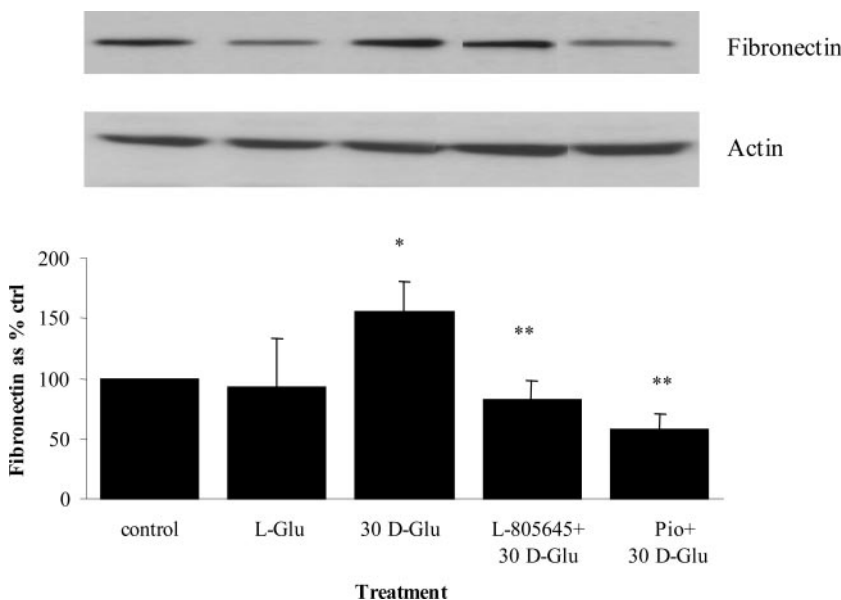
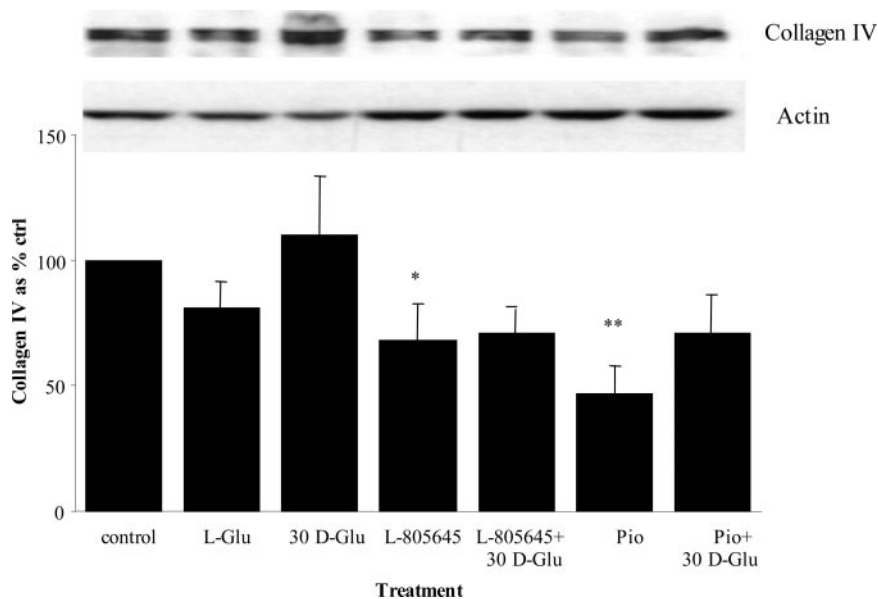


Fig. 3. L-805645 (8  $\mu$ M) and pioglitazone (10  $\mu$ M) were able to suppress high D-glucose-induced fibronectin expression. HK-2 cells were incubated for 24 h with control media, 5 mM D-glucose and 25 mM L-glucose, 30 mM D-glucose, 8  $\mu$ M L-805645 in 30 mM D-glucose, or 10  $\mu$ M pioglitazone in 30 mM D-glucose. Western blotting is as described in METHODS. Top: representative image for fibronectin and actin bands. Bottom: normalized results expressed as means  $\pm$  SE;  $n = 4$ . \* $P < 0.05$  vs. control. \*\* $P < 0.01$  vs. high D-glucose.

Fig. 4. High D-glucose did not induce collagen IV expression significantly. However, both L-805645 and pioglitazone were able to suppress collagen IV levels. HK-2 cells were incubated for 24 h with control media, 5 mM D-glucose and 25 mM L-glucose, 30 mM D-glucose, 8  $\mu$ M L-805645 in 5 mM D-glucose, 8  $\mu$ M L-805645 in 30 mM D-glucose, 10  $\mu$ M pioglitazone in 5 mM D-glucose, or 10  $\mu$ M pioglitazone in 30 mM D-glucose. Western blotting is as described in METHODS. *Top*: representative image for collagen IV and actin bands. *Bottom*: normalized results expressed as means  $\pm$  SE;  $n = 3$ . \* $P < 0.05$  vs. high D-glucose. \*\* $P$  value  $< 0.01$  vs. high D-glucose.



high glucose-induced profibrotic responses in the proximal tubule. As thickening of the basement membrane of the proximal tubule is the earliest pathological response observed in the development of diabetic nephropathy, these findings suggest that early use of PPAR $\gamma$  agonists may limit the development of diabetic nephropathy.

The results of these studies suggest that the AP-1 pathway is upregulated following exposure to high-glucose conditions, an effect that is due, at least in part, to the hyperosmolar effect. The TGF- $\beta$  gene is a known target gene for the transcription factor AP-1 (35). Furthermore, in mesangial cells TZDs prevented high-glucose induction of TGF- $\beta$  promoter activity

and elevation of nuclear *c-fos* (subunit of AP-1) protein levels. TGF- $\beta$  stimulates various extracellular matrix genes, including fibronectin. TZDs also inhibit TGF- $\beta$ -induced fibronectin expression in mesangial cells (14). Clearly, our studies demonstrated that TGF- $\beta$  was increased specifically by exposure to high glucose, suggesting downstream specificity of AP-1 activation with respect to target cytokines. Both the TZD and non-TZD PPAR $\gamma$  agonists reduced AP-1 expression and reversed the high glucose-induced increase in TGF- $\beta$ , although the effect was more marked in the presence of the TZD pioglitazone. The increased expression of both AP-1 and TGF- $\beta$  was translated into an increase in fibronectin expression, which was also reversed in the presence of both the TZD and non-TZD agonists. This effect was seen despite the increase in TGF- $\beta$  seen with DMSO, the vehicle in which L-805645 and pioglitazone were dissolved (data not shown). Hence, these results taken together strongly support the notion that these agonists exert antifibrotic effects in proximal tubular cells by attenuating AP-1, its target, i.e., TGF- $\beta$ , and the downstream fibronectin.

We previously showed that short-term exposure to high D-glucose upregulates PPAR $\gamma$  (27). This could be viewed as a protective compensatory response, which on further upregulation with the use of synthetic agonists, exerts renoprotective effects. This upregulation may be selective or sufficient in limiting the early expression of collagen IV on exposure to high glucose but not fibronectin. Alternatively, collagen IV may be deposited later in the course of fibrosis.

Monocyte chemotactic protein-1 (MCP-1) is known to be increased in diabetic nephropathy and considered to play an important role in the development of progressive tubulointerstitial fibrosis. Specifically, using immunohistochemical and in situ hybridization analyses, MCP-1-positive cells were found to be present in the advanced tubulointerstitial lesions of diabetic nephropathy and correlated with urinary MCP-1 levels (33). Part of the therapeutic benefit of angiotensin-converting enzyme inhibitors is considered to be mediated by a reduction in renal MCP-1 production (1, 16). We have previously demonstrated that a reduction in tubular production of MCP-1 is

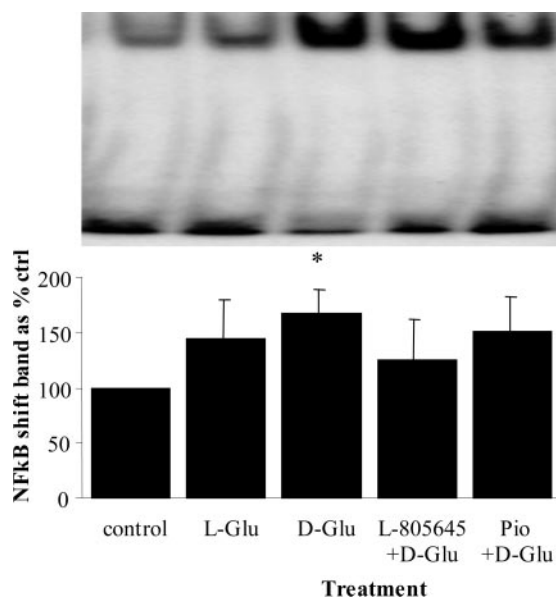


Fig. 5. D-glucose increased the binding of DIG-labeled NF- $\kappa$ B. However, neither of the PPAR $\gamma$  agonists significantly suppressed glucose-induced NF- $\kappa$ B binding. Nuclear extract preparation and EMSA are as described in METHODS. *Top*: representative image with shift (*top*) and free bands (*bottom*). *Bottom*: normalized results expressed as means  $\pm$  SE;  $n = 3$ . \* $P < 0.05$  vs. control.

associated with an upregulation of PPAR $\gamma$  (27). From our current data, this is independent of NF- $\kappa$ B regulation. This is in keeping with previous data from our group showing that PPAR $\gamma$  activation similarly reduces an LDL-induced increase in MCP-1, independently of modification of NF- $\kappa$ B transcriptional pathway (37). The signaling pathways that govern MCP-1 expression in the human kidney are unknown. Our results suggest that the AP-1 pathway, modified by PPAR $\gamma$  agonist activity, is likely to be at least in part responsible for reduction of transcription factors known to be involved in profibrotic and also proinflammatory pathways. This is consistent with the known murine MCP-1 promoter, which contains AP-1 and SP-1, in addition to NF- $\kappa$ B promoter hypermethylation and orphan sites, all of which regulate MCP-1 activity (28). Hence, its modification is of key therapeutic significance. Of note, we found that DMSO vehicle increased NF- $\kappa$ B binding (data not shown), consistent with recently reported data (19). This is important as it may be a confounding factor limiting the effects of the PPAR $\gamma$  agonists.

Therapeutic strategies to delay or attenuate the progression of diabetic nephropathy are essential to the treatment of patients with DM. These results provide new knowledge as to whether targeting PPAR $\gamma$  activation in patients with DM will ultimately reduce the burden of diabetic nephropathy.

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