PPARγ agonists exert antifibrotic effects in renal tubular cells exposed to high glucose

U. Panchapakesan, S. Sumual, C. A. Pollock, and X. Chen

Department of Medicine, University of Sydney, and Renal Research Group, Kolling Institute of Medical Research, Royal North Shore Hospital, New South Wales, Australia

Submitted 10 March 2005; accepted in final form 1 May 2005

PPARγ 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.—PPARγ 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 PPARγ in the excess extracellular matrix production is largely unknown. We evaluated the effect of 24- to 48-h D-glucose increased the nuclear binding of NF-κB (1-6) and TGF-β, and the downstream production of the extracellular matrix protein fibronectin. L-805645 and pioglitazone reduced high D-glucose-increased fibronectin from 156.0 ± 11.2% (P < 0.05) vs. high D-glucose, respectively. High D-glucose increased the nuclear binding of NF-κB to 167 ± 22.4% (P < 0.05), which was not modified with PPARγ agonists. In conclusion, PPARγ agonists exert antifibrotic effects in human PTC in high glucose by attenuating the increase in AP-1, TGF-β, and the downstream production of the extracellular matrix protein fibronectin.

Address for reprint requests and other correspondence: C. A. Pollock, Dept. of Medicine, Royal North Shore Hospital, NSW 2065, Australia (e-mail: carpol@med.usyd.edu.au).

http://www.ajprenal.org 0363-6127/05 $8.00 Copyright © 2005 the American Physiological Society

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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 PPARγ agonists on the transcription factors activator protein-1 (AP-1) and NF-κB, the profibrotic cytokine TGF-β1, 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 μg/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% CO2 incubator and were subcultured at 50–80% confluence using 0.05% trypsin-0.02% EDTA (GIBCO).

The clinically available thiazolidinedione pioglitazone (10 μM, Cayman Chemical) and the more selective PPARγ agonist L-805645 (8 μM, Merck) were used to determine the specific effects of PPARγ activation in this proximal tubular model. These concentrations were chosen based on their ability to significantly upregulate PPARγ 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-β 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 t-glucose (osmotic control, ICN Biomedical); 4) 8 μM L-805645 in 5 mM d-glucose; 5) 8 μM L-805645 in 30 mM d-glucose; 6) 10 μM pioglitazone in 5 mM d-glucose; and 7) 10 μM 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-κB. 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 μg of nuclear extract were incubated with 1 μg poly [d(I-C)] as the nonspecific competitor, 1 μg poly L-lysine in a binding buffer (in mM) 100 HEPES, pH 7.6, 5 EDTA, 50 (NH4)2SO4, 5 DTT, and 150 KCl, as well as 1% Tween 20, wt/vol] and DIG-labeled AP-1 (5’-CCG TTG ATG AGT CAG CCG GAA-3’) or NF-κB (5’-AGT TGA GGG GAC TTT CAC AGG C-3’) consensus oligonucleotide (Promega) for 30 min at room temperature. Unlabeled AP-1 and NF-κB 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-β1. Cells were seeded at 2 x 10^4 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-β1 levels were determined by immunnoassay (Promega) as per the manufacturer’s instructions. Cell lystate protein concentration was determined (Bio-Rad), and TGF-β1 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× 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 electoblotted 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 reprobed 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 ± 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.
AGONISTS EXERT ANTIFIBROTIC EFFECTS IN HK-2 CELLS

RESULTS

AP-1. High d-glucose increased the DNA binding of DIG-labeled AP-1 to 140.8 ± 10.9% of control (P < 0.05). Exposure to the osmotic control also increased AP-1 binding to 133.0 ± 14.2% of control (P < 0.05). The high glucose-induced increase in AP-1 was attenuated by concurrent exposure to L-805645 and pioglitazone (10 μM) to 82.3 ± 6.8% of control (P < 0.05 vs. high d-glucose) (Fig. 1).

TGF-β1. High d-glucose increased total TGF-β1 secretion to 4.82 pg·ml⁻¹·unit protein cell lysate⁻¹ or 139.6 ± 6.5% of control (P < 0.05). This increase was reduced in the presence of L-805645 to 2.93 pg·ml⁻¹·unit protein cell lysate⁻¹ or 68.73 ± 5.7% of control (P < 0.01 vs. high d-glucose). Pioglitazone also reduced high glucose-induced TGF-β1 secretion to 3.77 pg·ml⁻¹·unit protein cell lysate⁻¹ or 112 ± 13.6% (P < 0.05 vs. high d-glucose) (Fig. 2).

Fibronectin. The protein expression of fibronectin, an extracellular matrix protein downstream to TGF-β1, was increased in the presence of high glucose to 156.0 ± 24.9% of control (P < 0.05). The addition of L-805645 and pioglitazone reduced high glucose-induced fibronectin expression to 81.9 ± 16.0 and 57.4 ± 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 ± 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 PPARγ agonists were able to suppress collagen IV expression to levels below that observed in high-glucose conditions (68.0 ± 14.5%, P < 0.05 and 46.5 ± 11.6%, P < 0.01 vs. high d-glucose following exposure to L-805645 and pioglitazone, respectively) (Fig. 4).

NF-κB. High glucose increased the binding of DIG-labeled NF-κB to 167.3 ± 22.4% of control (P < 0.05). However, neither of the PPARγ agonists significantly suppressed glucose-induced NF-κB binding, as shown in Fig. 5. Interestingly, the presence of even low concentrations of DMSO also increased the binding of DIG-labeled NF-κB to 159.3 ± 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 PPARγ 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 PPARγ ligands reverse the
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 PPARγ 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-β gene is a known target gene for the transcription factor AP-1 (35). Furthermore, in mesangial cells TZDs prevented high-glucose induction of TGF-β1 promoter activity and elevation of nuclear c-fos (subunit of AP-1) protein levels. TGF-β1 stimulates various extracellular matrix genes, including fibronectin. TZDs also inhibit TGF-β1-induced fibronectin expression in mesangial cells (14). Clearly, our studies demonstrated that TGF-β1 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 PPARγ agonists reduced AP-1 expression and reversed the high glucose-induced increase in TGF-β1, although the effect was more marked in the presence of the TZD pioglitazone. The increased expression of both AP-1 and TGF-β1 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-β 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-β1, and the downstream fibronectin.

We previously showed that short-term exposure to high D-glucose upregulates PPARγ (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
associated with an upregulation of PPARγ (27). From our current data, this is independent of NF-κB regulation. This is in keeping with previous data from our group showing that PPARγ activation similarly reduces an LDL-induced increase in MCP-1, independently of modification of NF-κB 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 PPARγ 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-κB 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-κB 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 PPARγ 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 PPARγ activation in patients with DM will ultimately reduce the burden of diabetic nephropathy.

GRANTS

U. Panchapakesan was funded by a Jacquot Research Entry Scholarship. We also acknowledge support by the National Health and Medical Research Council of Australia, Juvenile Diabetes Research Foundation, Diabetes Australia Research Trust, and Merck Laboratories for generously providing us with L-805645.

REFERENCES


