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The effect of high glucose and PPAR-γ agonists on PPAR-γ expression and function in HK-2 cells

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Panchapakesan, U., C. A. Pollock, and X. M. Chen. The effect of high glucose and PPAR-γ agonists on PPAR-γ expression and function in HK-2 cells. *Am J Physiol Renal Physiol* 287: F528–F534, 2004. First published April 27, 2004; 10.1152/ajprenal.00445.2003.—Peroxisome proliferator-activated receptor-γ (PPAR-γ) are ligand-activated transcription factors that regulate cell growth, inflammation, lipid metabolism, and insulin sensitivity. PPAR-γ in the human kidney has been described. However, the role of PPAR-γ in proximal tubular cells with respect to cell growth and inflammation in diabetic nephropathy is largely unknown. We evaluated the effect of high (30 mM) D-glucose, thiazolidinedione pioglitazone (10 μM), and the selective PPAR-γ agonist L-805645 (8 μM) on PPAR-γ expression, growth, and inflammatory parameters in the proximal tubular model of HK-2 cells. PPAR-γ was present in HK-2 cells and upregulated with 30 mM D-glucose to 177 ± 31.2% of control ($P < 0.05$). PPAR-γ activation was induced by pioglitazone to a similar level to that observed by exposure to high glucose but maximally induced by the selective agonist L-805645. However, L-805645 reduced cell viability in both 5 and 30 mM D-glucose to 73.8 ± 3.1 and 77.6 ± 1.4% of control (both $P < 0.0001$). In parallel, thymidine incorporation was reduced with L-805645 in both 5 and 30 mM D-glucose to 33.3 ± 3.4 and 37.9 ± 2.2%, respectively (both $P < 0.0001$). Flow cytometry demonstrated increased apoptosis and G_1 phase arrest in association with an increase in $p21^{cip1/waf1}$ in cells exposed to L-805645. Exposure to 30 mM D-glucose did not significantly change AP-1 promoter activity (89.0 ± 5.5% of control); however, the addition of L-805645 significantly reduced it to 62.2 ± 2.7% of control ($P < 0.0001$). Thirty nanomolar D-glucose induced transforming growth factor-β_1 to 137.7 ± 16.9% of control ($P < 0.05$), and L-805645 was able to suppress this to 68.7 ± 5.7% of control ($P < 0.01$ vs. D-glucose). Exposure to 30 mM D-glucose reduced monocyte chemoattractant protein 1 levels to 78.6 ± 7.1% ($P < 0.05$) of control, with the reduction more marked in the presence of either pioglitazone ($P < 0.01$) or L-805645 ($P < 0.01$). In summary, high glucose upregulates PPAR-γ and when significantly induced demonstrates anti-proliferative and anti-inflammatory effects.

diabetic nephropathy; proximal tubular cells; cell cycle; thiazolidinediones

DIABETES MELLITUS IS A GLOBAL and increasing health problem. Of these, up to one-third will suffer end-stage kidney disease, resulting in significant personal morbidity and a huge cost to the community (28a). As the increasing incidence of end-stage kidney disease is largely accounted for by patients with type 2 diabetes mellitus (28a), there is a clear necessity to reduce nephropathy in patients with diabetes mellitus. The achievements offered by glycemic and blood pressure control and specific interruption of the renin-angiotensin system can only be described as modest with respect to halting the progression of diabetic nephropathy. Hence, alternative strategies to reduce the development of chronic kidney disease are required to be assessed and developed.

Peroxisome proliferator-activated receptors-γ (PPAR-γ) are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors that have central roles in regulating adipocyte differentiation, cell growth, and inflammation (13). On ligand binding, PPAR-γ form heterodimers with the retinoic acid receptor (21). This complex then binds to the peroxisome-proliferator response element within the promoter region of target genes. Regulation of these target genes depends on the binding of ligands. Endogenous ligands include 15-deoxy-delta-12,14-prostaglandin J2 (11, 17) and metabolites of oxidized low-density lipoproteins (LDL) (22, 28). The majority of the identified target genes are involved in lipid metabolism. PPAR-γ is the target of the thiazolidinedione family of compounds that are now commonly used as insulin-sensitizing agents to achieve glycemic control in patients with type 2 diabetes (9) where insulin resistance and associated metabolic abnormalities such as dyslipidemia are present.

PPAR-γ has been well characterized in mesangial cells (4, 24), with specific PPAR-γ activation exerting an antiproliferative (12, 24) and antifibrotic effect reducing type 1 collagen synthesis and secretion (27) presumed due to a transforming growth factor (TGF)-β_1-dependent mechanism (30). Clearly, mesangial proliferation and expansion with increased extracellular matrix deposition are characteristic of diabetic nephropathy. Hence, limited investigation into the renoprotective effects of PPAR-γ agonists has been undertaken.

Treatment with thiazolidinediones in animal models of experimental diabetes mellitus has been shown to reduce albuminuria and decrease glomerular matrix deposition and glomerulosclerosis (7, 8, 20, 34). Haploinsufficient PPAR+/− *db/db* mice exhibited more severe hyperglycemia, albuminuria, and glomerular changes (34). However, it is postulated that these effects are via activation of PPAR-γ rather than through its glucose-lowering effect. The renoprotective benefit of PPAR-γ agonists independent of glycemic control is further suggested by studies in nondiabetic models of renal injury, such as the 5/6-nephrectomy model, where activation of PPAR-γ reduced glomerulosclerosis (19). In a small study of humans with incipient diabetic nephropathy, a beneficial effect on microalbumin excretion has been observed (15). However, the mechanism for the observed benefit has not been eluci-

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dated. In particular, many of the studies used thiazolidinediones with both PPAR-γ and PPAR-α activity. Hence, the specificity of observed responses for the PPAR-γ activation is difficult to discern.

The research to date into the observed beneficial effects of PPAR-γ activation in the kidney has been centered on the mesangium and glomerulus. However, it is increasingly recognized that pathology within the tubulointerstitium is ultimately more predictive of the renal outcome (23). We recently clearly demonstrated that tubular albumin reabsorption is modified in the presence of high-glucose conditions (10) and tubular dysfunction is induced in the presence of pathological concentrations of albumin (18). Additionally, we showed that under "normoglycemic" conditions, PPAR-γ activation increases tubular uptake of albumin (33), which would theoretically be reflected in reduced urinary albumin excretion. However, the specific effects of PPAR-γ agonists in the presence of high glucose on the tubular expression of PPAR-γ and its functional sequelae have not been assessed. This study was designed to specifically characterize PPAR-γ in the proximal tubule under high-glucose conditions and to determine the effect of two PPAR agonists (a thiazolidinedione and a selective nonthiazolidinedione PPAR-γ agonist) on the growth and proinflammatory changes induced by high glucose.

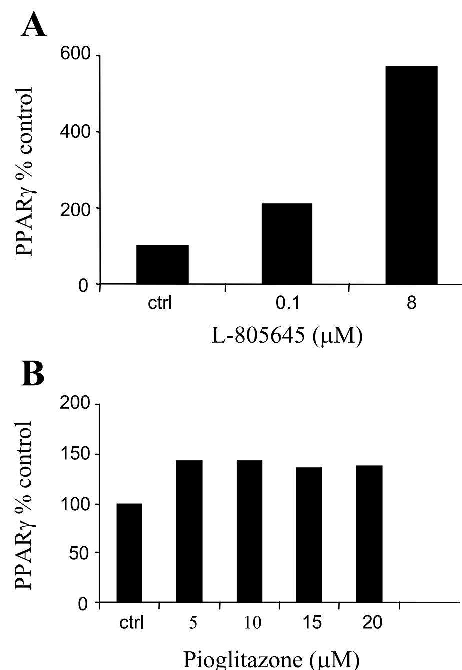

Fig. 1. *A*: dose-dependent increase in peroxisome proliferator-activated receptor-γ (PPAR-γ) expression with increasing concentration of L-805645. HK-2 cells were incubated for 24 h in keratinocyte serum-free media (KSFM) containing L-805645 at doses shown above, $n = 1$. *B*: initial dose-dependent increase in PPAR-γ expression was seen with 5 μM of pioglitazone, which then plateaued between 5 and 20 μM. HK-2 cells were incubated for 24 h in KSFM containing pioglitazone at doses shown above, $n = 1$. ctrl, Control.

MATERIALS AND 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 and epidermal growth factor (GIBCO). Cell culture media was changed every 48–72 h. These cells were grown at 37°C in a humidified 5% CO_2 incubator and were subcultured at 50–80% confluence using 0.05% trypsin-0.02% EDTA (GIBCO).

Experimental protocol. HK-2 cells were grown in 10-cm tissue culture dishes (Becton, Dickinson, NJ). The clinically available thiazolidinedione pioglitazone (Cayman Chemical) and the more selective PPAR-γ agonist L-805645 (Merck) were used to determine the specific effects of PPAR-γ activation in this proximal tubular model. Pioglitazone has a binding activity (IC_{50}) to the recombinant human PPAR-γ isoform of 3,000 nM and L-805645 of 50 nM. The binding affinity of pioglitazone to PPAR-α is 20–40 μM and L-805645 is 2,000 nM. Hence, L-805645 is more potent and more selective for PPAR-γ (6). Initial "dose-response" experiments were undertaken to determine the concentration at which pioglitazone and L-805645 maximally stimulated PPAR-γ protein expression. Ten micromolar pioglitazone maximally induced PPAR-γ protein expression and no cell toxicity was observed at this concentration. However, 10 μM L-805645 caused extensive cell death. Eight micromolar L-805645 caused significant upregulation of PPAR-γ with lesser cytotoxicity than 10 μM. Hence, 8 μM L-805645 was chosen in the experimental protocols, accepting that when using this compound, cell death was inherent in the upregulation of PPAR-γ (See Fig. 1, *A* and *B*). When 80% confluent, cells were exposed to the following experimental conditions for 24 h: *1*) 5 mM D-glucose (control media); *2*) 30 mM D-glucose (ICN Biomedicals); *3*) 30 mM L-glucose (ICN Biomedicals); *4*) 8 μM L-805645; *5*) 8 μM L-805645 plus 30 mM D-glucose; *6*) 10 μM pioglitazone; and *7*) 10 μM pioglitazone plus 30 mM D-glucose.

As L-805645 and pioglitazone were dissolved in 0.016 and 0.13% DMSO, respectively, an additional control was undertaken to exclude independent effects of the DMSO. No effects were observed. Hence, the data are not shown.

At the conclusion of the 24-h study period, the following experiments were undertaken: *1*) PPAR-γ protein expression; *2*) cell growth (cell viability, thymidine incorporation, flow cytometric analysis of progression through the cell cycle, $p21^{cip1/waf1}$ cyclin/cdk inhibitor); *3*) AP-1 transcriptional promoter activity; *4*) TGF-β_1 protein secretion (48-h treatment); and *5*) monocyte chemoattractant protein 1 (MCP-1) protein secretion.

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), 1 mM sodium orthovanadate, 0.5% Triton X-100 and protease inhibitors (Roche Diagnostics, Mannheim, Germany). Cell lysate was then sonicated to release nuclear 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 10–12% 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 and then incubated overnight at 4°C with antibodies to PPAR-γ 1:300 (Santa Cruz Biotechnology), $p21^{cip1/waf1}$ 1:1,000 (Upstate), and actin 1:300 (Santa Cruz Biotechnology) in TTBS containing 5% skim milk. Membranes were washed four times with TTBS and incubated with horseradish peroxidase-conjugated secondary antibody 1:1,600 for 2 h at room temperature and then washed four times with TTBS. The final wash was with TBS. The membranes were reprobed and results were normalized with actin. Proteins were visualized using the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech).

Cell viability. Cell proliferation was measured by an assay based on the reduction of a yellow tetrazolium 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide or MTT cell proliferation assay kit (ATCC). HK-2 cells were seeded at 1×10^4 cells/well in a 96-well plate. At 50% confluence, cells were treated for 24 h as previously described in quadruplicates. Ten microliters of MTT reagent were

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added to the wells per manufacturer's instructions for 4 h or until a purple precipitate was observed in most cells. One hundred microliters of detergent reagent were then added and the plate was left in the dark at room temperature for 20 h. The plate was read using a microplate reader at 620 nm.

[^{3}H]thymidine incorporation. HK-2 cells were seeded at 1×10^4 cells/well in a 96-well plates. At 50% confluence, cells were treated for 24 h as previously described in quadruplicates. The cells were exposed to 0.18μCi/well of methyl-[^{3}H]thymidine (Amersham) in the final 16 h of treatment. Cells were harvested using Packard FilterMate per manufacturer's instructions. The filter was left to dry overnight at 50°C. The filter was read using TopCount.NXT microplate scintillation and luminescence counter (Packard Biosciences).

Cell cycle analysis. For cell cycle analysis, supernatant was collected and cells were harvested by trypsinization at the end of the 24-h period and spun to obtain cell pellet. The cell pellet was then washed in cold PBS and fixed in 70% ethanol and kept at −20°C until staining. At the time of staining, cells were washed again in cold PBS and then resuspended in PBS containing 0.1% Triton X-100 and kept on ice for half an hour. The cell pellet was then resuspended in 1 ml of a fluorochrome solution containing 50 μg/ml propidium iodide (PI) (Sigma, St. Louis, MO), 50 μg/ml RNAse A (Sigma), 0.25 μM EDTA, and 0.001% Triton X-100 and left for at least 1 h in the dark. This suspension was filtered using a 50-μm mesh before being scanned. Flow cytometry was performed using a FACScan flow cytometer (Becton Dickinson). The PI fluorescence of individual nuclei and the forward and side scatter were all measured using identical instrument settings with a minimum of 20,000 events.

Measurement of AP-1 promoter activity. HK-2 cells were seeded at 1×10^5 cells/well in a 48-well plate using KSFM without growth factors in quadruplicates. After 24 h, when cells were 70% confluent, they were transfected with pAP-1 SEAP vector, pSEAP2-basic (negative control), pSEAP2-control (positive control) (BD Biosciences Clontech), and pC1-CMV-GFP20 (gift from Dr. K. Drumm, Institute of Physiology, University of Wuerzburg, Germany), which served as a control of transfection efficiency, using Lipofectamine 2000 transfection reagent (Invitrogen) with a DNA:Lipofectamine ratio of 0.5 μg:1 μl. After 24 h, cells were treated as previously described. The supernatant was collected and spun at 12,000 rpm for 5 min and stored at −20°C. The cell lysate was prepared and protein concentration was determined using Bio-Rad protein assay.

For SEAP chemiluminescence assay, 15 μl of supernatant samples were thawed and placed in 96-well Optiplate (Packard, BioScience) in duplicates. Placental alkaline phosphatase-positive control was assayed concurrently to ascertain appropriate assay conditions. Forty-five microliters per well of dilution buffer were added per well. Dilution buffer contained 60 mM L-homoarginine (Sigma), 0.1 M Tris·HCl, pH 9.3, 3 mM $MgCl_2$, and 0.2 mM $ZnCl_2$. The plate was incubated at 65°C after being covered for 30 min. It was then equilibrated to room temperature. One hundred microliters per well of CSPD Ready-to-Use with Sapphire Enhancer (Applied Biosystems) were added and left for 10 min. Measurement of chemiluminescence was done with a spectrofluorometer Fusion α (Packard BioScience). Results were corrected for protein content per well.

TGF-β$_1$. HK-2 cells were seeded at 2×10^5 cells/well in a 24-well plate and grown in KSFM without growth factors for 24 h. At 70% confluence, cells were treated for 48 h as previously described in triplicates. At 48 h, supernatants were collected, spun, and stored at −20°C until TGF-β$_1$ levels were determined with an immunoassay system (Promega, Madison, WI) per manufacturer's instructions and read using a microplate reader at 450 nm. This system is linear between 15.6 and 1,000 pg/ml. Samples were acid treated and then neutralized to convert the latent form to the bioactive form of TGF-β$_1$ to measure total TGF-β$_1$. This assay is designed to measure biologically active TGF-β$_1$. Cell lysate protein concentration was determined using Bio-Rad protein assay and TGF-β$_1$ levels were corrected for protein content per well.

MCP-1. HK-2 cells were seeded at 3×10^4 cells/well in a 48-well plate. At 85% confluence, cells were treated for 24 h as previously described in triplicates. Supernatants were stored at −80°C until MCP-1 levels were determined with an immunoassay kit assay (Biosource International) per manufacturer's instructions and read using a microplate reader at 450 nm. Cell lysate protein concentration was determined using Bio-Rad protein assay, and MCP-1 levels were corrected for protein content per well.

Statistical analyses. Results are expressed as a percentage of means ± SE of control values. Experiments were performed at least in triplicate or as detailed in text. 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, Berkley, CA). $P < 0.05$ was considered significant.

RESULTS

PPAR-γ agonists (L-805645) and pioglitazone both showed a dose-dependent increase in PPAR-γ protein expression (Fig. 1, *A* and *B*). For future experiments, 8 μM concentration of L-805645 and 10 μM pioglitazone were chosen.

High glucose induced increased PPAR-γ expression. Twenty-four-hour exposure to 30 mM D-glucose induced significant upregulation of PPAR-γ expression to 177.5 ± 31.2% of control values ($P < 0.05$; Fig. 2). Cells exposed to the osmotic control had an increase in PPAR-γ expression to 157 ± 21.8% of control values. However, this difference was not statistically significant to control values ($P = 0.11$). As expected, pioglitazone and the PPAR-γ agonist L-805645 increased PPAR-γ expression by 205.8 ± 33.29% ($P < 0.05$) and 444.6 ± 26.6% ($P < 0.001$), respectively, compared with control, and this value was not further increased in the presence of high glucose (Fig. 2). These results confirmed that L-805645 was a more potent PPAR-γ agonist compared with pioglitazone, and although PPAR-γ expression was induced by high glucose, it could be further induced by selective pharmacological targeting of PPAR-γ as observed in cells exposed to L-805645.

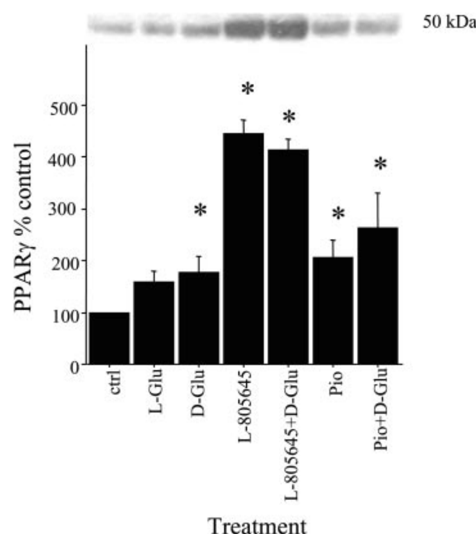

Fig. 2. PPAR-γ upregulation in the presence of 30 mM D-glucose (D-glu) and agonists L-805645 (8 μM) and pioglitazone (10 μM). HK-2 were incubated for 24 h with control media, 30 mM L-glucose (L-glu), 30 mM D-glucose, 8 μM L-805645, 8 μM L-805645 + 30 mM D-glucose, 10 μM pioglitazone (pio), and 10 μM pioglitazone + 30 mM D-Glucose. Cells were lysed and Western blot analysis for PPAR-γ was done as described in MATERIALS AND METHODS. Normalized results are expressed as means ± SE, $n = 3$ (*$P < 0.05$ vs. control). *Top*: representative Western blot.

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Cell viability in the presence of high glucose and PPAR-γ agonists. Cell viability was significantly reduced following exposure to L-805645 in both control (5 mM; 73.8 ± 3.1%; $P < 0.0001$) and high-glucose (30 mM; 77.6 ± 1.4%; $P < 0.0001$) conditions. Exposure to 30 mM glucose or pioglitazone caused no change in cell viability (Fig. 3), suggesting that more potent upregulation of PPAR-γ was associated with reduced cell viability.

[^{3}H]thymidine incorporation. Results of the thymidine uptake studies as a marker of cell proliferation paralleled the assessment of cell viability. There was a significant reduction in thymidine uptake with L-805645 to 33.2 ± 3.4% and with L-805645 plus 30 mM D-glucose to 37.9 ± 2.2% of control values ($P < 0.0001$ in both cases). There was no significant change observed following exposure to high glucose (30 mM) or pioglitazone (Fig. 4).

Flow cytometry. An assessment of progression through the cell cycle with flow cytometry showed increased cells undergoing apoptosis following exposure to L-805645 (10.9 ± 4.3%) compared with control conditions (1.6 ± 1%; $P < 0.05$) and cells exposed to pioglitazone (1.9 ± 0.3%; $P < 0.05$). G_1 phase arrest was additionally observed following exposure to L-805645 resulting in 78.2 ± 2.7% of cells in this phase compared with control at 60.7 ± 5.6% ($P < 0.05$) and with exposure to pioglitazone at 58.5 ± 4.2% ($P < 0.05$). L-805645 caused delayed progression through the S phase of the cell cycle with 14.4 ± 3.5% of cells in this phase compared with control at 23.1 ± 2.7% ($P < 0.05$) and pioglitazone at 24.4 ± 1.1% ($P < 0.05$). L-805645 caused reduced cells being in G_2 phase at 7.3 ± 1.2% compared with control at 16.0 ± 2.9% ($P < 0.05$) and pioglitazone at 16.9 ± 3.3% ($P < 0.05$; Fig. 5).

p21$^{cip1/waf1}$. The mechanism underlying the reduction in cell growth was undertaken by assessment of p21$^{cip1/waf1}$ protein, which inhibits the cyclin-dependent kinases and arrests cells in G_1 phase of cell cycle (14, 32). Hence an increase in the activity of p21$^{cip1/waf1}$ leads to delayed progression through both the S phase and G_2/M progression and reduces cell growth and proliferation. Exposure to L-805645 increased p21$^{cip1/waf1}$ levels similarly in 5 and 30 mM glucose to 436.9 ± 96.8 and

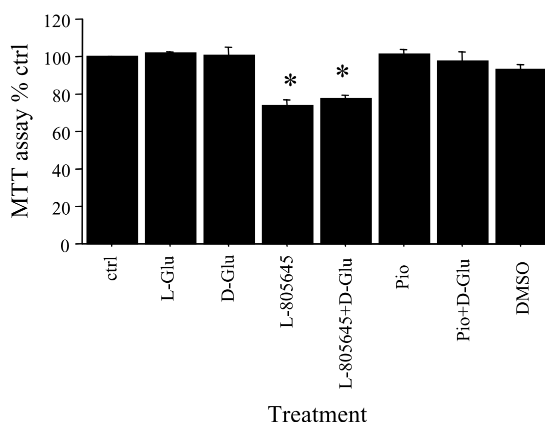

Fig. 3. Cell viability was reduced following exposure to L-805645. HK-2 were incubated for 24 h with control media, 30 mM L-glucose, 30 mM D-glucose, 8 μM L-805645, 8 μM L-805645 + 30 mM D-glucose, 10 μM pioglitazone, 10 μM pioglitazone + 30 mM D-glucose, and DMSO as vehicle control. MTT assay was used to assess cell viability as described in MATERIALS AND METHODS. Normalized results are expressed as means ± SE, $n = 4$ (*$P < 0.0001$ vs. control).

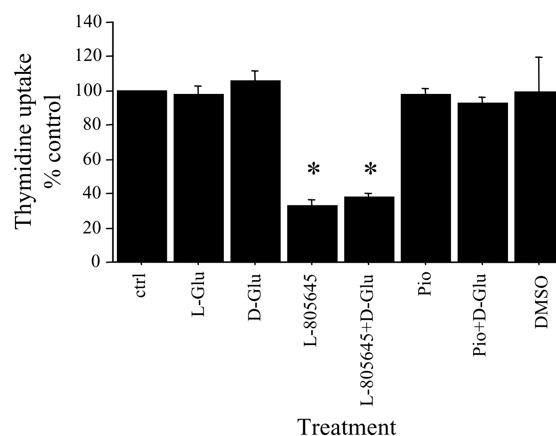

Fig. 4. Thymidine uptake as a marker of cell proliferation was markedly reduced with the addition of L-805645. HK-2 were incubated for 24 h with control media, 30 mM L-glucose, 30 mM D-glucose, 8 μM L-805645, 8 μM L-805645 + 30 mM D-glucose, 10 μM pioglitazone, and 10 μM pioglitazone + 30 mM D-glucose, and DMSO as vehicle control. Cells were exposed to thymidine in the last 16 h of treatment. Cells were harvested using Packard FilterMate and read using TopCount Counter. Normalized results are expressed as means ± SE, $n = 4$ (*$P < 0.0001$).

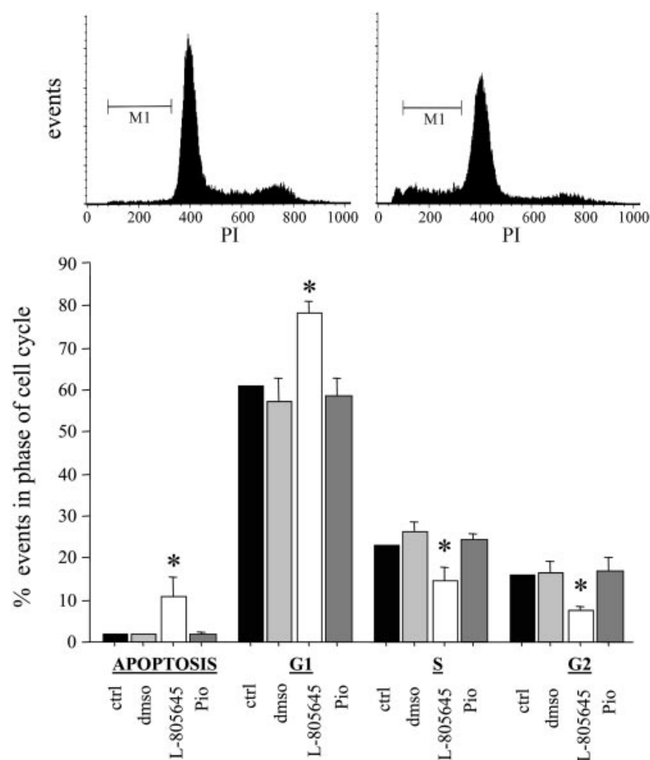

Fig. 5. Exposure to L-805645 causes apoptosis and G_1 phase arrest with a reduction in the proportion of cells entering S and G_2 phase. HK-2 cells were incubated for 24 h in control media, DMSO as vehicle control, 8 μM L-805645, and 10 μM pioglitazone. Cells were fixed in 70% ethanol and then stained with fluorochrome solution containing propidium iodide (PI). Flow cytometry was performed on the cells using a FACScan flow cytometer and 20,000 events were measured. Results are expressed as a percentage of events in each phase of cell cycle and expressed as means ± SE, $n = 3$ (*$P < 0.05$ vs. all other treatment in each phase). *Top*: diagrams show representative cell cycle profile on exposure to L-805645 (*right*) and control (*left*) with M1 outlining subG1/apoptotic events.

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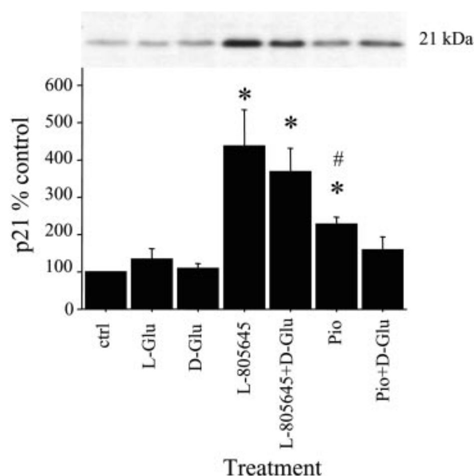

Fig. 6. Expression of p21$^{cip1/waf1}$, a cyclin/cdk complex inhibitor, was most marked in the cells exposed to L-805645 and to a lesser extent when exposed to pioglitazone. HK-2 were incubated for 24 h with control media, 30 mM L-glucose, 30 mM D-glucose, 8 μM L-805645, 8 μM L-805645 + 30 mM D-glucose, 10 μM pioglitazone, and 10 μM pioglitazone + 30 mM D-glucose. Cells were lysed and Western blot analysis for p21$^{cip1/waf1}$ was done as described in MATERIALS AND METHODS. Normalized results are expressed as means ± SE, $n = 3$ experiments (*$P < 0.005$ compared with control; #$P < 0.01$ compared with L-805645). *Top*: representative Western blot.

370.0 ± 59.8% (both $P < 0.005$). Pioglitazone also significantly induced expression of the p21$^{cip1/waf1}$ to 228.7 ± 19.5% of control ($P < 0.005$). However, the increase induced by pioglitazone was significantly less than that induced by L-805645 ($P < 0.01$; Fig. 6).

AP-1 promoter activity. As TGF-β_1 has been implicated in the genesis of diabetic nephropathy, the effect of upregulation of PPAR-γ expression on the transcriptional regulator of TGF-β_1 AP-1 was assessed. Using green fluorescent protein (GFP), visual control transfection efficiency at 48 h was ~60%. AP-1 promoter activity with exposure to 30 mM D-glucose was 89.0 ± 5.5% and this was not statistically different to control. The addition of L-805645 to 30 mM D-glucose significantly reduced AP-1 promoter activity to 62.2 ± 2.7% of control ($P < 0.0001$; Fig. 7).

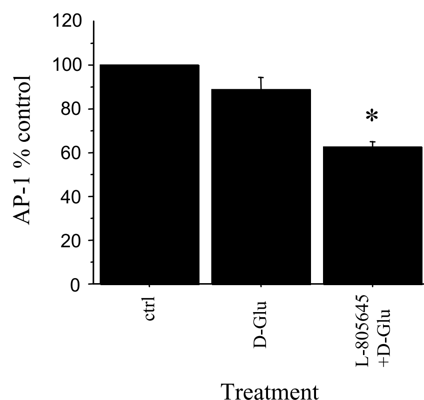

Fig. 7. L-805645 suppressed AP-1 promoter activity based on pAP-1 SEAP assay. HK-2 cells were transfected with pAP-1 SEAP using Lipofectamine 2000 as the transfecting reagent. Twenty-four-hour posttransfection cells were incubated for 24 h with control media without growth factors, 30 mM D-glucose, and 8 μM L-805645 + 30 mM D-glucose. Supernatants were assayed for pAP-1 promoter activity using SEAP chemiluminescent assay and corrected for amount of protein per well in cell lysate. Results are expressed as means ± SE of $n = 4$ (*$P < 0.0001$ vs. control).

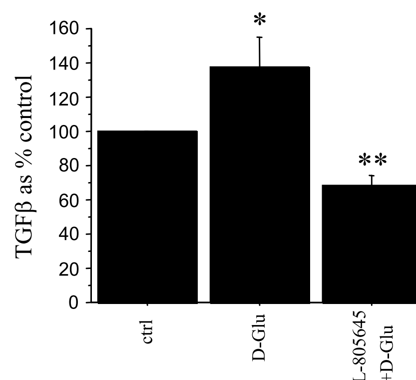

Fig. 8. L-805645 suppressed high glucose-induced transforming growth factor (TGF)-β_1. HK-2 cells were incubated in control media without growth factors, 30 mM D-glucose, and 8 μM L-805645 + 30 mM D-glucose for 48 h. Supernatants were assayed for total bioactive TGF-β_1 using ELISA. These results have been corrected for amount of protein per well in cell lysate. Results are expressed as means ± SE of $n = 3$ (*$P < 0.05$ vs. control; **$P < 0.01$ vs. D-glucose).

TGF-β_1 secretion. High D-glucose exposure increased TGF-β_1 levels in the supernatant to 137.7 ± 16.9% of control ($P < 0.05$). The addition of L-805645 suppressed D-glucose-induced TGF-β_1 levels to 68.7 ± 5.7% ($P < 0.01$ vs. D-glucose; Fig. 8).

MCP-1 secretion. A decrease in MCP-1 levels in the supernatant of cells exposed to high glucose was observed compared with cells exposed to control conditions (78.6 ± 7.1%; $P < 0.05$). There was a more significant decrease with the addition of L-805645 in control conditions to 39.9 ± 10.5% ($P < 0.01$), L-805645 plus 30 mM D-glucose to 52.2 ± 4.6% ($P < 0.01$), pioglitazone to 53.5 ± 8.9% ($P < 0.01$), and pioglitazone plus 30 mM D-glucose to 57.7 ± 8.6% ($P < 0.01$; Fig. 9).

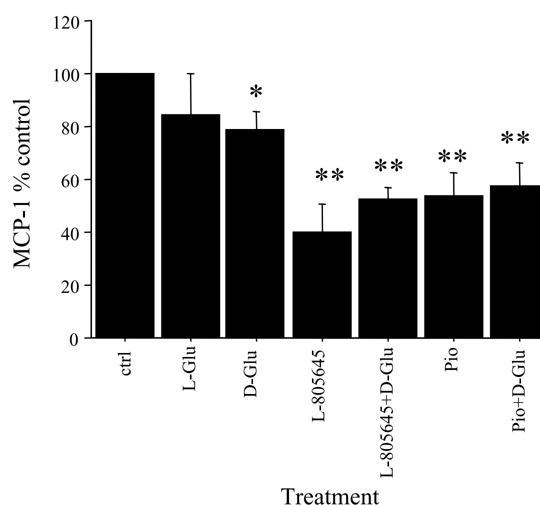

Fig. 9. Thirty millimolar D-glucose suppressed MCP-1 to a lesser extent than that observed with L-805645 and pioglitazone. HK-2 cells were incubated for 24 h with control media, 30 mM L-glucose, 30 mM D-glucose, 8 μM L-805645, 8 μM L-805645 + 30 mM D-glucose, 10 μM pioglitazone, and 10 μM pioglitazone + 30 mM D-glucose. MCP-1 levels in the supernatant were determined using ELISA. These results have been corrected for the amount of protein per well in cell lysate. Results are expressed as means ± SE of $n = 3$ (*$P < 0.05$ vs. control; **$P < 0.01$ vs. control).

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DISCUSSION

Thiazolidinediones are insulin-sensitizing agents now widely used in the treatment of type 2 diabetes. They appear to exert a renoprotective effect in animal models and in vitro studies focusing on mesangial cells. However, its role and function in human proximal tubular cells in high-glucose conditions have not been well defined. We recently demonstrated that pioglitazone promotes tubular albumin uptake and limits the proinflammatory and profibrotic response induced by tubular cell exposure to LDL (33). The effect of high glucose on PPAR-γ activity and the downstream consequences have not been defined in the proximal tubule, and yet altered proximal tubular cell growth is one of the early abnormalities seen in diabetic nephropathy. On the platform of the above findings, we sought to investigate the role of PPAR-γ in a model of human proximal tubular cells with particular focus on growth and inflammation in the context of high-glucose conditions.

Our results demonstrate that PPAR-γ is present in HK-2 cells and is upregulated in high-glucose conditions. This upregulation of PPAR-γ is likely to be a protective response as it was also associated with a downregulation of the inflammatory protein MCP-1, an effect reproduced by the pharmacological activation of PPAR-γ by both pioglitazone and L-805645. In contrast to the proapoptotic effects of intense PPAR-γ upregulation induced by L-805645, a more modest increase in PPAR-γ following exposure to high glucose and pioglitazone had no effect on cell growth parameters.

Our data with respect to the effects of PPAR-γ agonists on growth parameters are consistent with that of Arici et al. (3), who demonstrated that albumin-bound free fatty acids are tubulotoxic as a consequence of PPAR-γ activation. In their study, intense activation of PPAR-γ led to apoptosis (3). An antiproliferative and proapoptotic effect has also been described in mesangial cells and renal fibroblasts following exposure to ciglitazone (12, 25). It is unclear whether the induction of proximal tubular cell apoptosis is a desirable or deleterious consequence of PPAR-γ activation. Altered proximal tubular cell growth is one of the early abnormalities seen after the onset of diabetes with both hyperplasia and hypertrophy contributing to the overall increase in renal size early in diabetes (31). As a correlation has been found between kidney size and subsequent deterioration of renal function in type 1 diabetics (5), it is possible that limitation of early tubular cell growth is desirable. We found that cell death and cell growth arrest were related to the intensity of PPAR-γ activation, being more pronounced with L-805645 than with pioglitazone. Our results clearly show that $p21^{cip1/waf1}$ is increased by both moderate and potent upregulation of PPAR-γ. $p21^{cip1/waf1}$ Is a 21-kDa protein known to inhibit the cyclin kinases and hence an increase in the activity of $p21^{cip1/waf1}$ leads to delayed progression through both S phase and G2M. Despite the upregulation of $p21^{cip1/waf1}$ in the presence of pioglitazone, this was significantly less than that observed with L-805645 and no alteration in growth or cell cycle was observed. Taken together, these findings highlight the importance of understanding adaptive growth changes in the proximal tubular cells in diabetic nephropathy.

Our results suggest that the AP-1 pathway, known to be a key transcription factor involved in the upregulation of TGF-β in diabetic nephropathy, is modified by PPAR-γ agonist activity. L-805645 caused a reduction in AP-1 and downstream TGF-β_1. However, the high glucose-induced TGF-β_1 occurred independent of an increase in AP-1. It is likely that the upregulation of PPAR-γ seen with high-glucose exposure limits this increase in AP-1. This implies that there are other inflammatory/profibrotic pathways induced by high glucose that are independent of AP-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 (29). Part of the therapeutic benefit of angiotensin-converting enzyme inhibitors is considered to be mediated by a reduction in renal MCP-1 production (2, 16). Our data clearly demonstrate that a reduction in tubular production of MCP-1 is associated with an upregulation of PPAR-γ. We previously demonstrated that PPAR-γ activation similarly reduces LDL-induced increase in MCP-1, independent of modification of the NF-κB transcriptional pathway (33). 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-γ agonist activity, is likely to be at least in part responsible for the reduction of transcription factors known to be involved in profibrotic pathways and also proinflammatory cytokines. This is consistent with the known murine MCP-1 promoter, which contains AP-1 and SP-1, in addition to NF-κB promoter sites hypermethylation and orphan sites, all of which regulate MCP-1 activity (26). Hence, its modification is of key therapeutic significance.

In summary, we demonstrated that PPAR-γ is upregulated in the proximal tubular cell line HK-2 as a consequence of exposure to high glucose. This occurs in association with reduced production of MCP-1, which can be further decreased with PPAR-γ agonists. Further increase in PPAR-γ with L-805645, a potent agonist, is associated with a decrease in AP-1. Hence, the response is likely to be protective and mitigate against the development of diabetic nephropathy. More potent stimulation of PPAR-γ can be achieved with pharmacological agents that, if potent enough, are further associated with antiproliferative and proapoptotic effects. Whether these responses translate into a clinical benefit remains to be tested in clinical trials.

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GRANTS

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