PPARγ 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. PPARγ agonists exert antifibrotic effects in renal tubular cells exposed to high glucose. Am J Physiol Renal Physiol 289: F1153–F1158, 2005.—Peroxisome proliferator-activated receptor-γ (PPARγ) ligands 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 8 mM L-805645 or 10 mM 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) (AP-1) to 140.8 ± 10.9% (P < 0.05) which was attenuated with L-805645 and pioglitazone to 82.3 ± 14.4% (P < 0.01 vs. high D-glucose) and 99.3 ± 12.2% (P < 0.05 vs. high D-glucose), respectively. High D-glucose increased total production of transforming growth factor (TGF)-β, 139.6 ± 6.5% (P < 0.05), which was reversed with L-805645 and pioglitazone to 68.3 ± 5.7% (P < 0.01 vs. high D-glucose) and 112 ± 13.6% (P < 0.05 vs. high D-glucose). L-805645 and pioglitazone reduced high D-glucose-induced fibronectin from 156.0 ± 24.9% (P < 0.05) to 81.9 ± 16.0% and 57.4 ± 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 ± 14.5% (P < 0.05) and 46.5 ± 11.6% (P < 0.01) 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-β1, and the downstream production of the extracellular matrix protein fibronectin.

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 PPARγ agonists is further suggested by studies in nondiabetic models of renal injury, such as the 5/6-nephrectomy model, where activation of PPARγ reduces glomerulosclerosis (21). In vitro studies have focused on the use of mesangial cells where PPARγ has been well characterized (2, 26), with specific PPARγ 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)-β1-dependent mechanism (34). In mesangial cells, pioglitazone has been shown to inhibit TGF-β-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 PPARγ agonists stimulate tubular albumin uptake without provoking an inflammatory response (37). However, whether they protect the kidney from tubulointerstitial fibrosis, the...
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 (KSFN) 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.

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Nuclear extract preparation and EMSA are as described in METHODS. A representative image with shift (bottom): normalized results expressed as means ± SE; n = 3. *P < 0.05 vs. control. **P < 0.05 vs. high D-glucose.
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 to 82.3 ± 14.4% of control (P < 0.01 vs. high D-glucose). Pioglitazone also abrogated the high glucose-induced increase in AP-1 to 140.8 ± 15.1% of control (P < 0.01 vs. high D-glucose) (Fig. 2).

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. TGF-β1 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 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

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**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 μM L-805645 in 5 mM D-glucose, 8 μM L-805645 8 in 30 mM D-glucose, 10 μM pioglitazone in 5 mM D-glucose, or 10 μM 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 ± SE; n = 3. *P < 0.05 vs. high D-glucose. **P value < 0.01 vs. high D-glucose.

**Fig. 5.** D-glucose increased the binding of DIG-labeled NF-κB. However, neither of the PPARγ agonists significantly suppressed glucose-induced NF-κB 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 ± SE; n = 3. *P < 0.05 vs. control.
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

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