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PPAR-α Ligands Inhibit H_2O_2-Mediated Activation of Transforming Growth Factor-β1 in Human Mesangial Cells

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ABSTRACT

Transforming growth factor-β1 (TGF-β1) mediates the development of glomerulosclerosis by stimulating mesangial cell production of extracellular matrix (ECM) proteins. TGF-β1 and several ECM genes are regulated by promoter *O*-tetradecanoylphorbol 13-acetate-responsive elements (TREs) that are transactivated by the activator protein-1 (AP-1) transcription factor complex. AP-1–TRE interactions are regulated by redox changes. Recently, peroxisome proliferator-activated receptors (PPARs) were shown to negatively regulate several transcription factor families. In these studies, we postulated that PPAR-α could antagonize TGF-β1 expression by cultured human mesangial cells (HMC). A TGF-β1 luciferase expression plasmid was transduced into HMC via recombinant deficient adenoviral vectors. The TGF-β1 promoter activity increased twofold (209%) following 18-h treatments with H_2O_2 (1,000 μ*M*). Using RT-PCR, we demonstrated that HMC possess PPAR-α RNA, and PPAR-α protein was identified by immunohistochemistry. Pretreatment of cells with the PPAR-α ligands WY14643 (100–500 μ*M*) or clofibrate (100–500 μ*M*) dose-dependently inhibited oxidant-mediated induction of TGF-β1. This inhibition occurred without affecting the H_2O_2–mediated activation of the mitogen-activated protein kinase (MAPK) pathways extracellular regulated kinase, p38 MAPK, or Jun N-terminal kinase, which are responsible for the regulation of AP-1 phosphorylation. These studies are the first to identify PPAR-α expression by HMC. The results of these studies suggest that TGF-β1 expression mediated by oxidant stress may be suppressible by PPAR-α activation. *Antioxid. Redox Signal.* 4, 877–884.

INTRODUCTION

GLOMERULOSCLEROSIS is a final histologic feature of most glomerulopathies, including those glomerular injuries associated with systemic diseases such as diabetes and hypertension. Resident cells within the glomerulus contribute to the pathogenesis of glomerulosclerosis through changes in cell number, as well as increased synthesis and secretion of extracellular matrix (ECM) proteins.

In those glomerular injuries associated with glomerular inflammation, invading leukocytes release a variety of cytokines and growth factors that induce changes in the phenotype of the resident glomerular cells. In noninflammatory glomerulopathies, the resident glomerular cells are stimulated to secrete ECM proteins through a variety of physiologic stresses, such as cell stretch that occurs in glomerular hypertension or glucose-induced redox changes that occur in diabetes.

The mesangial cell, by virtue of its apposition to the glomerular circulation and its potential for proliferation and ECM production, maintains a pivotal role in the development of glomerulosclerosis. Both inflammatory and noninflammatory glomerular injuries can induce its production of ECM. An intermediary in such ECM production is the growth factor, transforming growth factor-β1 (TGF-β1). TGF-β1 stimulates mesangial cell and glomerular endothelial cell production of a variety of collagens, fibronectin, and laminin, which contributes to glomerulosclerosis (21, 30). TGF-β1 also

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inhibits ECM degradation via direct and indirect inhibition of matrix metalloproteinases.

TGF-β1 can be released into the glomerular environment by infiltrating leukocytes. The cytokines and other growth factors released by leukocytes can also stimulate the mesangial cell to produce additional TGF-β1 proteins. TGF-β1 proteins, made and secreted by mesangial cells, can augment additional TGF-β1 production in an autocrine fashion (2, 27). This autoinduction, as well as the production of many ECM proteins, occurs through an activation of specific cell signaling pathways, namely the mitogen-activated protein kinases (MAPKs) (22).

Downstream of MAPKs, the TGF-β1 gene and many ECM genes are regulated by the gene promoter motif *O*-tetradecanoylphorbol 13-acetate-responsive element (TRE). TREs are bound and activated by the transcription factor complex, activator protein-1 (AP-1) (7, 10, 27), which is regulated by MAPKs. AP-1 binding to TREs within the TGF-β1 promoter mediates the majority of the gene's expression following induction by phorbol esters, high glucose, and physical stresses (25, 26, 37). Similarly, the up-regulation of the genes encoding collagen I, collagen IV, collagen VI, and fibronectin occurs via AP-1–TRE interactions (10, 20, 43).

The interactions between AP-1 proteins and the TRE are altered by redox changes (1). Oxidant stress increases AP-1 protein binding to DNA and facilitates expression of TRE-dependent genes (18). This interaction explains the observations that TGF-β1 protein production, as well as the production of TRE-dependent ECM genes, is stimulated by oxidant stress (4, 5, 19). These interactions are also the basis of attempts to inhibit glomerulosclerosis by the use of dietary antioxidants (39, 42).

There exist several sources of oxidant stress in the glomerulopathies. In inflammatory glomerulopathies, infiltrating leukocytes can be an abundant direct source of exogenous oxidants. Cytokines released by infiltrating leukocytes also induce oxidant stress after binding to their mesangial cell receptors. We and others have shown within mesangial cells that oxidant stress is induced by interleukin-1β (IL-1β) binding to its receptor, and that such oxidant stress activates those MAPK pathways responsible for TGF-β1 and ECM production (46). TGF-β1 protein binding to its receptor complex can induce intracellular redox changes that alter cell function. For example, in pulmonary fibroblasts the intracellular signaling of TGF-β1 occurs through a transient production of hydrogen peroxide (H_2O_2) via NADH oxidase activation (41).

Even noninflammatory glomerulopathies are associated with reactive oxygen species (ROS) generation (49). In the case of diabetic nephropathy, high-glucose concentrations induce a systemic oxidative stress (16), and in mesangial cells, glucose excess can induce the glucose oxidase enzyme pathway, resulting in H_2O_2 formation and subsequent TGF-β1 induction (19). Glomerulosclerosis may therefore represent a state of perturbed balance between prooxidant and antioxidant potentials within glomerular cells. It has been suggested that those diabetic individuals who develop glomerulosclerosis not only experience the prooxidant effects of glucose, but fail to balance these effects, perhaps due to genetic predisposition toward deficient antioxidant capacity (6).

Given the worldwide epidemic increases in kidney failure, it is an important goal to identify endogenous inhibitors of the AP-1–TRE interactions that drive TGF-β1 and ECM gene expression. The peroxisome proliferator-activated receptors (PPARs) have recently received attention as potential endogenous regulators of AP-1–TRE interactions. PPARs are nuclear receptors that regulate gene expression through peroxisome proliferator responsive elements (PPREs), which are nested within the promoters of many genes, or through alterations of transcription factor binding to non-PPRE promoter elements. Three PPAR isoforms exist (α, γ, δ) in many cell types.

PPAR-γ is widely expressed in mammalian cells, and we have shown that cultured human mesangial cells (HMC) possess PPAR-γ (35, 48). PPAR-α is highly expressed in liver, skeletal muscle, heart, and the vascular wall, where it stimulates the β-oxidative degradation of fatty acids (8, 15, 31). Macrophages and monocytes also express PPAR-α (8). Kidney homogenates contain PPAR-α (24, 40), and Guan and colleagues observed PPAR-α RNA in proximal and medullary ascending tubule cells (17). To date, glomerular expression of PPAR-α has not been confirmed.

PPAR-α is activated by the hypolipidemic fibric acid compounds and by the structurally different compound, Wyeth 14643 (WY14643). Activated PPAR-α forms heterodimers with the 9-*cis*-retinoic acid receptor (RXR). The PPAR/RXR heterodimers regulate some genes by binding to PPREs (14). In others, they antagonize the DNA binding of other transcription factor proteins. It was recently shown that PPAR-α can suppress TRE-dependent gene activation (12, 13). As the human TGF-β1 gene is regulated by TREs, in these current studies we investigated the effects of PPAR-α ligands on oxidant-mediated activation of the human TGF-β1 promoter.

MATERIALS AND METHODS

HMC culture and conditioning

HMC were isolated from cadaver kidneys deemed anatomically unsuitable for use in transplantation. Glomeruli were isolated from the renal cortex by mincing the tissue and then passing the tissue fragments through progressively smaller sieves (Bellco Glass Inc., Vineland, NJ, U.S.A.) followed by collagenase digestion. Thereafter, the HMC were characterized as described (45). HMC were used between passages 5 and 7. They were cultured at 37°C, 5% CO_2 in RPMI 1640 medium (Life Sciences, Grand Island, NY, U.S.A.) containing 10% heat-inactivated fetal calf serum, 50 units/ml penicillin, and 50 mg/ml streptomycin (Sigma Chemical, St. Louis, MO, U.S.A.). Prior to conditioning, cells were made quiescent by conditioning in RPMI 1640 medium plus 0.25% bovine serum albumin (serum-free medium).

Activation of the TGF-β1 promoter was measured following an 18-h treatment of transduced HMC with known TGF-β1 stimuli: the phorbol ester phorbol 12-myristate 13-acetate (PMA; 25 n*M*; Sigma), TGF-β1 protein (5 ng/ml; R & D Systems, Inc., Minneapolis, MN, U.S.A.), or H_2O_2 (1,000 μ*M*; Sigma). To determine the effects of PPAR-α ligands on the

TGF-β1 promoter, cells were treated with WY14643 (100–500 μ*M*; Cayman Chemicals, Ann Arbor, MI, U.S.A.) or clofibrate [2-(4-chlorophenoxy)-2-methylpropanoic acid ethyl ester; 100–500 μ*M*; Biomol Research Laboratories, Inc., Plymouth Meeting, PA, U.S.A.], or the vehicles for each ligand, dimethyl sulfoxide (0.05%) for WY14643 and ethanol (0.05%) for clofibrate. To ensure the absence of an effect of vehicle, all control cells were treated with the maximal concentration of each vehicle.

Determination of PPAR-α expression by HMC

We recently reported that HMC express PPAR-γ, and in these current studies the same methodology to investigate PPAR-α expression was used (48). Mesangial PPAR-α expression was examined at the RNA and protein levels. Total RNA was isolated, reverse-transcribed to cDNA, and amplified with the following primers: for PPAR-γ: 5′-GGCAATTGAATGTCGTGTCTGTGGAGATAA-3′ and 5′-AGCTCCAGGGCTTGTAGCAGGTTGTCTTGA-3′ (expected size: 900 bp); and for PPAR-γ2: 5′-GCGATTCCTTCACTGATAC-3′ AND 5′-AGCTCCAGGGCTTGTAGCAGGTTGTCTTGA-3′ (expected size: 1,264 bp). For PPAR-α, the following primers were used: 5′-GACGAATGCCAAGATCTGAGAAAGC-3′ and 5′-CGTCTCCTTTGTAGTGCTGTCAGC-3′ with an expected product of 940 bp. RT-PCR products were separated on agarose gels and visualized by ethidium bromide staining.

PPAR-α protein was identified by immunohistochemistry. HMC were plated on glass chamber slides (Life Science Products, Inc.) at a density of 10,000 cells/chamber. The cells were grown to 80% confluency in RPMI 1640, 10% fetal bovine serum and then placed for 24 h in serum-free medium. The cells were fixed in 10% neutral buffered formalin and quenched of endogenous peroxidase activity (Zymed Inc., San Francisco, CA, U.S.A.). After blocking of endogenous proteins, the cells were rinsed in phosphate-buffered saline (PBS) and then incubated with rabbit polyclonal anti-PPAR-α primary antibody (clone no. PA1-822, Affinity Bioreagents, Golden, CO, U.S.A.) at 1:500 dilution for 60 min, followed by several PBS washing steps. After placing in appropriate biotinylated goat anti-rabbit secondary antibody (1:5,000), 3-amino-9-ethylcarbazole (AEC) chromogen was added to the chamber slides and development was allowed to proceed for 3 min, after which the cells were washed in PBS. As controls, cells on chamber slides were conditioned with nonimmune rabbit IgG (Sigma) at the same dilution and incubation times, followed by the same secondary antibody and AEC. Cells were visualized by microscopy to determine patterns of PPAR-α staining.

Transduction of HMC with a human TGF-β1 expression plasmid using replication-deficient recombinant adenovirus

The distal 459-bp promoter of the human TGF-β1 gene was obtained as a gift from Dr. Seong Kim (National Institutes of Health, Bethesda, MD, U.S.A.). This section of the TGF-β1 promoter contains two TRE motifs that mediate the majority of TGF-β1 gene expression following a variety of cell stimuli (*e.g.,* glucose, TGF-β1 proteins, phorbol esters) (see Fig. 2) (25). This distal 459-bp segment was fused to a firefly luciferase expression plasmid (pGL3-basic, Promega, Madison, WI, U.S.A.) and inserted into recombinant deficient adenovirus-containing expression cassettes. This TGF-β1 luciferase reporter system, rAvTGF, was provided as a generous gift by Dr. N.S. Nahman, Jr. (The Ohio State University, Columbus, OH, U.S.A.) (33). We used the adenoviral vector because this technique has allowed extremely high rates of transduction of these primary cell lines, which have historically been refractory to plasmid transfection. It was previously reported that 100% transduction of cultured HMC can be reproducibly achieved using such an adenoviral delivery system (32). To achieve transduction, mesangial cells were plated in six-well plates (Falcon, Becton–Dickinson and Co, Franklin Lakes, NJ, U.S.A.), grown in RPMI 1640 with 10% fetal calf serum to 80% confluency, and then placed in serum-free medium for 24 h. The cells were then transduced with rAvTGF (MOI of 10) in serum-free medium for 4 h. The serum-free medium was thereafter replaced, and the transduced cells were conditioned as outlined above.

Measurement of luciferase activity from HMC transduced with the rAvTGF plasmid

After overnight conditioning, transduced cells were washed with PBS, lysed with a single-step reporter lysis buffer (Promega), scraped, and exposed to a single freeze–thaw cycle. The samples were frozen at −70°C until assayed. Luciferase levels were determined by mixing 20 μl of cell lysates with 100 μl of luciferase assay substrate (Promega). Luciferase levels were measured in triplicate in a Lumat LB 9501 luminometer (Wallac, Gaithersburg, MD, U.S.A.). The cell lysates were also used to determine total protein levels, as an additional standardization of the cell number and lysis efficiency. To determine cell protein levels, lysates were assayed by a bicinchoninic system using albumin as standards.

MAPK Western blots

We previously reported in mesangial cells the strong correlation between changes in the phosphorylation of MAPK family members and the kinase activity of the pathways (46, 47). We investigated the effects of PPAR-α ligands on the phosphorylation of the three MAPK pathways: extracellular regulated kinase (ERK), p38 MAPK, and Jun N-terminal kinase (JNK), as an indicator of MAPK activity. Conditioned HMC were washed in PBS, pH 7.4, and rapidly lysed by scraping in a 4°C sodium dodecyl sulfate (SDS) lysis buffer containing 2% SDS, 1 m*M* EDTA, 1 m*M* phenylmethylsulfonyl fluoride, 20 μ*M* leupeptin, and 0.15 U/ml aprotinin. Cell lysates were briefly sonicated at 4°C, and equivalent amounts promptly separated by 10% SDS–polyacrylamide gel electrophoresis under reducing conditions. After transfer, the nitrocellulose was probed overnight at 4°C, with a rabbit polyclonal anti-phospho-p38 antibody, anti-phospho-ERK antibody, or anti-phospho-JNK antibody (New England Biolabs, Beverly, MA, U.S.A.) at a dilution of 1:1,000. Nonimmune rabbit isotype-specific IgG (Zymed Laboratories)

served as control for the primary antibodies. The nitrocellulose was washed in PBST and then incubated with appropriate biotinylated secondary antibody (Zymed Laboratories) and streptavidin-horseradish peroxidase. Bands of interest were demonstrated using an enhanced chemiluminescence technique (Amersham Life Sciences, Inc., Arlington Heights, IL, U.S.A.).

Statistical analyses

The means ± SE of the luciferase assays were compared by Student's *t* test. Statistical significance was determined by $p < 0.05$.

RESULTS

Cultured HMC possess PPAR-α

At least three PPAR isoforms exist within the mammalian cells, although not all species and not all cells within a species express all three isoforms. The presence of PPAR-γ has been shown in rodent mesangial cells (35). We recently reported that HMC possess PPAR-γ mRNA and protein, and respond to PPAR-γ ligands by altering MAPK pathway activity (48). To determine if HMC also possess PPAR-α, we performed RT-PCR and immunostaining. As demonstrated in Fig. 1, PPAR-α mRNA was detected by PCR in mesangial cells and staining for PPAR-α protein was observed by immunohistochemistry. Primers bordering a region common to PPAR-γ1 and PPAR-γ2 yielded the expected size products of 900 bp and 1,264 bp. The PPAR-α primers yielded a product with the expected size of 940 bp. Because the primers used in these experiments crossed introns, and because no product was amplified from RNA samples in which reverse transcriptase was omitted (data not shown), it is unlikely that contamination of the samples with genomic DNA occurred. Furthermore, HMC staining of PPAR-α protein was observed by immunohistochemistry. The location of the PPAR-α proteins was nuclear, a pattern of staining similar to that noted for PPAR-γ staining of endothelial cells (35).

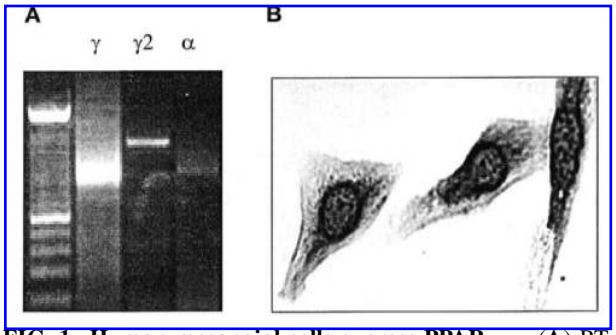

FIG. 1. Human mesangial cells express PPAR-α. (**A**) RT-PCR of the 900-bp product of PPAR-γ and the 1,264-bp product of PPAR-γ2 are shown as a control. RT-PCR of the PPAR-α product is 940 bp. $n = 5$. (**B**) Immunohistochemistry of cultured mesangial cells identified prominent nuclear staining for PPAR-α proteins. Control cells did not show staining (not shown in figure). $n = 2$.

H_2O_2 stimulates the human TGF-β1 promoter

Given the central importance of TGF-β1 expression in the development of glomerulosclerosis, our laboratory has been involved in determining the agonists and antagonists of TGF-β1 gene expression by HMC. We used a reporter system that consists of the most active human TGF-β1 promoter segment inserted into a luciferase expression plasmid. This plasmid was introduced into HMC via a recombinant deficient adenoviral vector. In these experiments, the TGF-β1 promoter activity was measured following an overnight (18 h) stimulation with H_2O_2 (1,000 μ*M*), PMA (25 n*M*), or TGF-β1 (5 ng/ml). As demonstrated in Fig. 2, H_2O_2 stimulated the TGF-β1 promoter to a level similar to the phorbol ester PMA and TGF-β1 proteins. All three agonists increased the promoter expression approximately twofold.

PPAR-α ligands inhibit H_2O_2 induction of TGF-β1

Using two structurally different PPAR-α ligands, we investigated the effects of PPAR-α activation on the TGF-β1

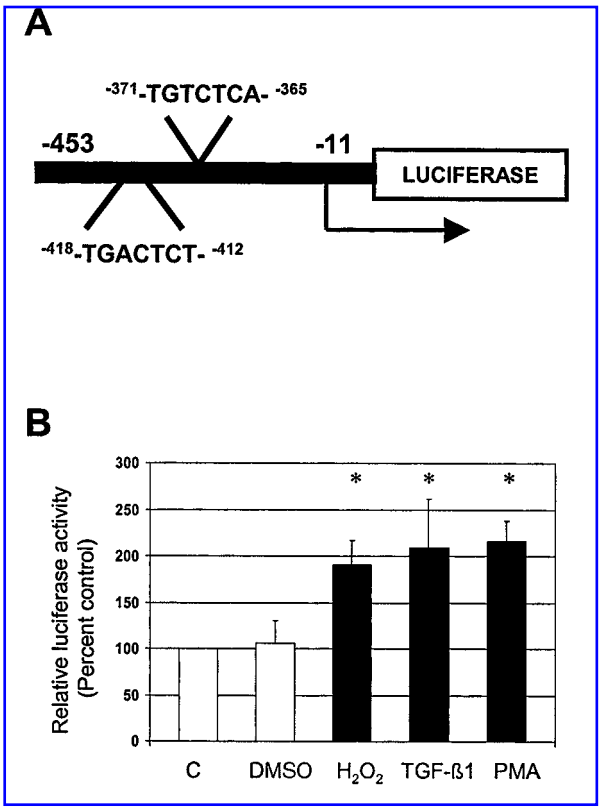

FIG. 2. H_2O_2 activates the human TGF-β1 promoter. (**A**) The 453-bp segment of the human TGF-β1 promoter was inserted into a luciferase expression plasmid (pGL3) and transduced into mesangial cells using adenoviral vectors. The schematic shows the two TRE motifs located at positions −418 to −412 and −371 to −365 relative to the transcription start site of the human TGF-β1 promoter. (**B**) HMC transduced with the expression plasmid were treated for 18 h with H_2O_2 (1,000 μ*M*), exogenous TGF-β1 protein (5 ng/ml), or PMA (25 n*M*) or the vehicle dimethyl sulfoxide (DMSO; 0.05%). The means ± SE of four sets of luciferase assays are demonstrated. *$p = 0.001$.

promoter. Following transduction with the TGF-β1 expression plasmid, cells were treated with increasing concentrations of WY14643 or increasing concentrations of the fibric acid, clofibrate. The cells were treated 2 h prior to H_2O_2 treatment. Increasing concentrations of both PPAR-α ligands decreased the H_2O_2–mediated TGF-β1 promoter expression (Fig. 3). The basal level of the TGF-β1 promoter activity in control cells not treated with H_2O_2 was minimally inhibited at the highest concentration of ligand used.

TGF-β1 promoter inhibition by PPAR-α ligands is independent of MAPK pathway inhibition

The human TGF-β1 promoter is an example of a TRE-dependent gene that can be regulated by changes in MAPK pathway activity. The MAPK superfamily includes the ERK, JNK, and the p38 MAPK pathways. We previously demonstrated in HMC that H_2O_2 can activate the MAPK kinase family members (46). We therefore investigated if pretreatment of HMC with WY14643 or clofibrate could affect these three MAPK pathways. As demonstrated in Fig. 4, H_2O_2 treatment increased the phosphorylation of each MAPK family member. However, at no concentration investigated did WY14643 or clofibrate inhibit the phosphorylation of these MAPK pathways. In the case of ERK, we observed an increase in phosphorylation by clofibrate treatment alone.

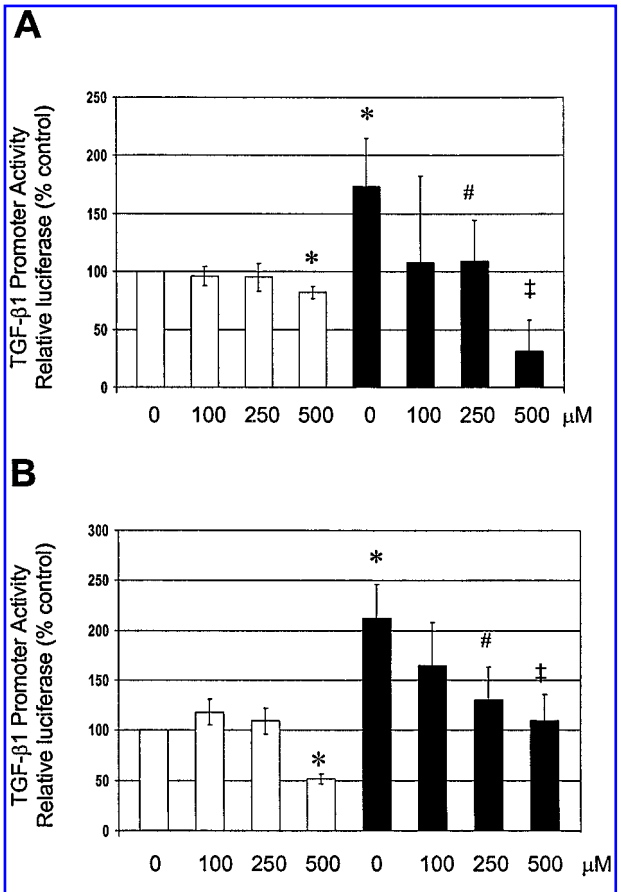

FIG. 3. WY14643 and clofibrate inhibit H_2O_2–mediated activation of the TGF-β1 promoter. Cells transduced with the TGF-β1 promoter plasmid were treated with increasing concentrations of WY14643 (**A**) or clofibrate (**B**) in the absence of H_2O_2 (open columns) or in the presence of H_2O_2 (1,000 μ*M*) (filled columns). The means ± SE of four sets of luciferase assays are demonstrated. *$p = 0.001$ versus untreated control, #$p < 0.01$ versus H_2O_2-treated cells, ‡$p = 0.001$ versus H_2O_2-treated cells; $n = 6$.

DISCUSSION

We demonstrate that HMC possess PPAR-α RNA and express PPAR-α protein. This is the first confirmation of any glomerular cell expression of this PPAR isoform. Previous work by several investigators has suggested the generalized presence of PPAR-α in kidney homogenates (40). PPAR-α RNA has been localized to tubules of the rabbit and human kidney (17). That mesangial cells, which are specialized vascular pericytes, express PPAR-α is perhaps understandable given the extensive localization of this PPAR isoform in several types of vascular cells (9, 34).

In these studies we investigated a role of PPAR-α in the control of TGF-β1 because this growth factor plays a central role in the development of glomerulosclerosis. By binding to specific receptor complexes, TGF-β1 stimulates ECM production and inhibits matrix-degrading enzymes. TGF-β1 is also able to regulate mesangial cell proliferation, and this property may indirectly affect matrix protein accumulation because the proliferation rate of mesangial cells is inversely associated with matrix production and deposition (11).

Agonists of TGF-β1 production by glomerular cells include those stimuli known to cause glomerulosclerosis. The TGF-β1 gene can be induced by several cytokines and growth factors, many of which are often present in the local environment during glomerular disease. ROS that are released from circulating leukocytes or intrinsically generated as a consequence of cytokine and growth factor effects have been shown to mediate such TGF-β1 production (39). Our current studies demonstrate that exogenous administration of H_2O_2 up-regulates the human TGF-β1 promoter to a level equivalent to that of more classical agonists, the phorbol ester PMA and TGF-β1 protein. This is an important observation given that TGF-β1 protein itself can induce ROS generation within specific cells (41). The entire cycle of TGF-β1 autoinduction, in which TGF-β1 proteins induce the transcription of the TGF-β1 gene, is therefore possibly due to ROS generation.

The hypolipidemic fibric acid compounds and the compound WY14643 are structurally different synthetic ligands for PPAR-α. We demonstrated that pretreatment of mesangial cells with the fibric acid clofibrate or WY14643 inhibited TGF-β1 promoter up-regulation in response to ROS. An ability of high concentrations of these compounds to inhibit basal TGF-β1 promoter activity was also observed. Such basal promoter activity is likely driven by the presence of low concentrations of serum in the culture media. These observations suggest that PPAR-α proteins may be natural endogenous inhibitors of TGF-β1. Recently, it was shown that PPAR-γ ligands, such as the thiazolidinedione (TZD) class of oral hypoglycemics, can prevent glomerulosclerosis that develops in the rat remnant kidney model, a noninflammatory glomerulopathy (29). Future studies using animal models of glomeru-

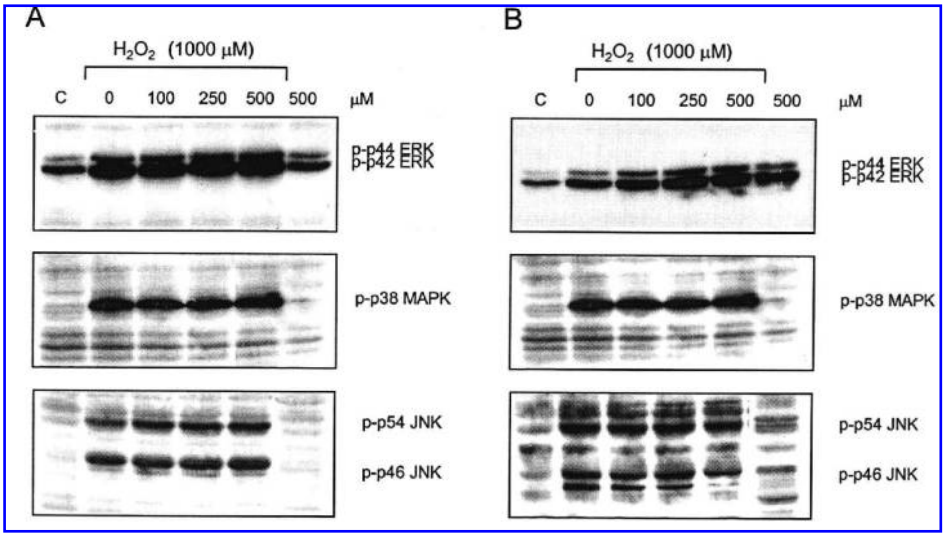

FIG. 4. WY14643 and clofibrate do not inhibit H_2O_2–mediated activation of the MAPKs ERK, p38 MAPK, and JNK. Western blots using phospho-specific primary antibodies were used to determine if the inhibition of the TGF-β1 promoter by WY14643 or clofibrate resulted from MAPK inhibition. Cells were treated with H_2O_2 (1,000 μ*M*) for 30 min, and proteins were harvested. H_2O_2 treatment caused an increase in the phosphorylation of the three MAPKs (p-p44/p-p42 ERK, p-p38 MAPK, and p-p54-/p-p46 JNK). (**A**) Increasing concentrations of WY14643 failed to reverse this phosphorylation. $n = 3$. (**B**) Similar to WY14643, increasing concentrations of clofibrate failed to inhibit MAPK phosphorylation mediated by H_2O_2. Cells treated with clofibrate demonstrated an increase in p44/p42 ERK phosphorylation, but not p38 MAPK or JNK phosphorylation. $n = 5$.

losclerosis will be needed to determine if the PPAR-α ligands similarly inhibit glomerulosclerosis *in vivo*.

The TRE promoters drive both basal TGF-β1 and ROS-mediated TGF-β1 promoter activation. Therefore, we hypothesize that PPAR-α may function as an inhibitor of TRE genes in general. TRE gene inhibition by PPAR-α activation has been shown in other cell types. For example, clofibrate treatment represses lipopolysaccharide- and IL-1β-stimulated IL-6 mRNA levels in aortic smooth muscle cells. These clofibrate effects occurred only in PPAR-α wild-type, but not of PPAR-α-null mice, arguing the effects were specific to the PPAR-α (12). This inhibition of IL-6 appeared to be due to repression of AP-1 and nuclear factor-κB (NF-κB) interactions with their cis-regulatory elements at the gene promoter. PPAR-α also inhibits expression of endothelin-1. Endothelin-1 is a vasoactive protein whose parent gene, preproendothelin, is regulated at TRE motifs (28). PPAR-α inhibits transactivation of this gene through perturbation of AP-1–TRE binding (13).

A reciprocal antagonism between PPAR-α and other nuclear proteins appears to exist, such that AP-1 activation may inhibit PPAR-α effects on PPREs and PPAR-α activation inhibits AP-1 and NF-κB effects (12). This reciprocal antagonism is perhaps understandable given experiments that show PPAR-α proteins are physically associated with AP-1 proteins (12).

Because the TGF-β1 promoter is regulated by AP-1–TRE interactions, and because the AP-1 transcription factor complex is itself regulated by MAPK pathways, we investigated the effects of PPAR-α ligands on the activation of various MAPK pathways by H_2O_2. There exists a role for each of these three MAPK pathways in AP-1–TRE regulation. The AP-1 transcription factor complex is classically comprised of Jun and Fos transcription factor dimers (3). In HMC, AP-1 consists of JunD–Fos dimers (45). The Jun component of the AP-1 complex binds DNA when it is dephosphorylated at the carboxyl terminus. Full transcription of a TRE gene by AP-1 occurs when the amino terminus of Jun is phosphorylated (44). The MAPK pathway ERK is indirectly involved in AP-1 carboxyl dephosphorylation to promote Jun binding. Both the JNK and the p38 MAPK pathways can regulate subsequent TRE transcription by amino-terminal phosphorylation. By Western blotting, we show that H_2O_2 increases the phosphorylation of the ERK, p38, and JNK pathways. We have previously demonstrated that such an increase in MAPK pathway phosphorylation correlates well with changes in kinase activity of the pathway (46, 47). Pretreatment of cells with the PPAR-α ligands at concentrations that inhibit the TGF-β1 promoter failed to reverse this oxidant-mediated activation of the MAPKs. Clofibrate had a stimulatory effect on ERK, but not on other MAPK pathways. The consequences of clofibrate-induced ERK activation remain unknown, but such ERK activation does not measurably enhance TGF-β1 promoter activity in the basal or ROS-stimulated state, and the pattern of TGF-β1 promoter inhibition by clofibrate parallels that of WY14643, which does not activate ERK. These observations collectively suggest that the inhibitory effects of the PPAR-α ligands on the TGF-β1 promoter occur downstream of the MAPK pathways. We speculate that the inhibition is due to interference of transcription factor–gene promoter interactions. In the case of clofibrate, this interference may dominate over gene promoter interactions mediated by ERK activation.

The AP-1–TRE interactions are governed by redox changes. Oxidant stress can increase AP-1 binding and stimulate the expression of TRE-dependent genes (36, 38, 45, 50). Diabetes is associated with the generation of redox changes within cells (23), and AP-1 activation in diabetes has recently been shown to occur

via a redox mechanism (18). Although the possibility exists that PPAR-α antagonizes AP-1–TRE interactions through redox changes, evidence that the PPAR-α ligands used in these studies are antioxidants is lacking at this time. However, the observations that PPAR-α disrupts the function of other transcription factors [*e.g.,* NF-κB, STATs (signal transducers and activators of transcription)] argue that manipulation of these endogenous proteins may be beneficial in the treatment of a wide variety of inflammatory and sclerosing diseases in various organs (8).

In summary, these studies suggest that PPAR-α is a naturally occurring endogenous regulator of TRE-dependent genes, such as TGF-β1. That the oxidant-mediated increases in TGF-β1 can be down-regulated when PPAR-α ligands are present suggests that glomerulosclerosis may be suppressible by the clinical use of PPAR-α ligands.

ACKNOWLEDGMENTS

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ABBREVIATIONS

AP-1, activator protein-1; clofibrate, 2-(4-chlorophenoxy)-2-methylpropanoic acid ethyl ester; ECM, extracellular matrix; ERK, extracellular regulated kinase; HMC, human mesangial cells; H_2O_2, hydrogen peroxide; IL, interleukin; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator responsive element; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; TGF-β1, transforming growth factor-β1; TRE, *O*-tetradecanoylphorbol 13-acetate-responsive element; WY14643, Wyeth 14643.

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