View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

Kidney International, Vol. 59 (2001), pp. 87–95

Hydrogen peroxide increases extracellular matrix mRNA through TGF-β in human mesangial cells

M. CARMEN IGLESIAS-DE LA CRUZ, PIEDAD RUIZ-TORRES, JOSÉ ALCAMÍ, LUISA DÍEZ-MARQUÉS, ROCÍO ORTEGA-VELÁZQUEZ, SHELDON CHEN, MANUEL RODRÍGUEZ-PUYOL, FUAD N. ZIYADEH, and DIEGO RODRÍGUEZ-PUYOL

Departments of Physiology and Medicine, Alcalá University, Madrid, Spain; Renal-Electrolyte and Hypertension Division and Penn Center for Molecular Studies of Kidney Diseases, University of Pennsylvania, Philadelphia, Pennsylvania, USA; and Centro de Biología Fundamental, Instituto de Salud Carlos III, and Nephrology Section and Research Unit, Hospital Príncipe de Asturias, Alcalá de Henares, Madrid, Spain

Hydrogen peroxide increases extracellular matrix mRNA through TGF-β in human mesangial cells.

Background. Reactive oxygen species (ROS) are excessively produced in pathologic states, including many renal diseases. Transforming growth factor-β (TGF-β) may mediate renal fibrotic injury, and ROS may act through the TGF-β pathway to exert a profibrotic effect.

Methods. The expression of TGF-β1 and extracellular matrix (ECM) components were assessed in cultured human mesangial cells (HMCs) incubated with glucose oxidase (GO), an enzyme that continuously generates hydrogen peroxide from glucose. A neutralizing anti–TGF-β antibody was added to test the hypothesis that hydrogen peroxide acts through activation of the TGF-β pathway to stimulate ECM expression.

Results. Northern blot analysis revealed significantly increased steady-state levels of TGF-β1 and ECM proteins (collagen types I, III, and IV, and fibronectin) by approximately twofold. While no significant effect on mRNA stability after treatment with GO was observed, other studies employing promoter-reporter assays, competitive-quantitative reverse transcription-polymerase chain reaction, mink lung epithelial cell proliferation assay, and TGF-β1 enzyme-linked immunosorbent assay all demonstrated significant stimulation by GO (>1.5-fold) of TGF-β1 promoter activity, mRNA level, bioactivity, and protein production, respectively. Catalase pretreatment prevented the GO-induced stimulation of TGF-β1 mRNA. When incubations were performed with a panselective neutralizing anti–TGF-β antibody, the GO-stimulated expression of ECM molecules was prevented.

Conclusions. GO-induced hydrogen peroxide production induces TGF-β1 synthesis and thereby increases ECM gene expression in cultured HMCs. These cellular responses may underlie the development and progression of renal diseases characterized by oxidative stress.

Key words: oxidative stress, renal fibrosis, glucose oxidase, reactive oxygen species, glomerulosclerosis, tubulointerstitial fibrosis, transforming growth factor-β.

Received for publication March 14, 2000
and in revised form July 21, 2000
Accepted for publication July 27, 2000

© 2001 by the International Society of Nephrology

Reactive oxygen species (ROS) are excessively produced in several disease states, and their injurious effects may contribute to the pathogenesis of many renal diseases. The ROS system plays a significant role in the pathogenesis of acute and chronic renal diseases [1–5], which are characterized by variable degrees of decreased renal function, increased proliferation of infiltrating and resident cells, and progressive accumulation of extracellular matrix (ECM) proteins. Glomerulosclerosis and tubulointerstitial fibrosis represent the final pathological features of these diseases, regardless of the inciting etiology. The relationship between increased ROS synthesis and the functional and morphological changes in the kidney has been explored using various experimental approaches. Several studies have demonstrated that ROS increase the synthesis of arachidonic acid metabolites [6], platelet-activating factor [7], endothelin [8], and nitric oxide [9] in different renal cell types. These vasoactive mediators modulate cellular proliferation and ECM synthesis in the kidney [10, 11]. Other experiments from our group have shown that hydrogen peroxide (H_2O_2) causes mesangial cell contraction [12] and induces cell proliferation [13], suggesting a pathogenic role for ROS in the development of renal diseases.

In contrast, data supporting a direct relationship between increased ROS and overproduction of ECM proteins are scarce, although indirect evidence points to this association. Transforming growth factor-β (TGF-β) is a ubiquitous fibrogenic cytokine that promotes ECM accumulation [14–16]. We hypothesize that ROS may act through the TGF-β pathway to exert a profibrotic effect. Oxygen derivatives, acting as secondary intracellular messengers [17], have been shown to activate transcription factors, such as nuclear factor-κB (NF-κB) and activated protein-1 (AP-1) [18, 19]. Since the human TGF-β1 promoter contains at least two AP-1 binding

sequences [20], ROS may stimulate the transcription and production of TGF-β1 [16]. Some investigators have recently hypothesized [21] that ROS increase the production of both TGF-β1 and ECM proteins in mesangial cells, but our current study is the first to examine whether ROS induce ECM proteins through a TGF-β–dependent pathway.

The present experiments were therefore designed to study the direct effects of H_2O_2 on the synthesis of TGF-β1 and ECM gene expression by human mesangial cells (HMCs) and to evaluate the role of TGF-β in mediating the increment in ECM synthesis by using a panselective neutralizing anti–TGF-β antibody. Our working hypothesis is that H_2O_2 increases ECM gene expression by autocrine activation of the TGF-β system.

METHODS

HMC culture

Human mesangial cells were cultured according to previously described procedures [12], with minor modifications. Portions of macroscopically normal cortical tissue were obtained from a human kidney immediately after nephrectomy for renal cell carcinoma. The cortex was cut into slices and washed twice to remove contaminating blood. The material was successively pushed through 180 and 105 μm stainless steel sieves and washed to obtain isolated glomeruli free of tubular contamination. Hank's balanced salt solution (HBSS; GIBCO BRL, Gaithersburg, MD, USA) was used in all the steps of glomerular isolation. Glomeruli were then treated with collagenase class IV (Sigma Chemicals, St. Louis, MO, USA), plated in plastic culture dishes, and maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS), L-glutamine (1 mmol/L), penicillin (0.66 mg/mL), and streptomycin sulfate (60 μg/mL; GIBCO). Cells were buffered with HEPES and bicarbonate, pH 7.4, and grown at 37°C in 5% CO_2 atmosphere. Culture medium was changed every two days. When cells reached confluence, they were subcultured at a ratio of 1:4, using the same incubation medium. The identity of HMCs was confirmed by morphological and functional criteria as previously described [12, 13]. Experiments were performed on cells in passages 3–5.

Experimental design

Studies were performed on HMCs deprived of serum for 72 hours. Rather than pulsing the culture medium with relatively high doses of exogenous H_2O_2 [21], which has a very short half-life, we added glucose oxidase (GO) to the cultured cells in order to enzymatically generate H_2O_2 from glucose and achieve a steady and continuous supply of H_2O_2 in the medium [22]. Cells were incubated for variable times (4 to 24 hours) with 1 mU/mL GO (Sigma) in fresh serum-free RPMI supplemented with L-glutamine, penicillin, and streptomycin (as described previously in this article) and buffered with HEPES and bicarbonate. This concentration of GO produces approximately 100 nmol/min H_2O_2 in the medium and permits the sustained exposure of cells to levels of H_2O_2 in the μmol/L range [23]. In some experiments, 80 U/mL catalase (Sigma) was added to the incubation medium 10 minutes before GO. Previous studies have documented that catalase addition eliminates the H_2O_2 in the medium that is generated from GO [22]. In like manner, a neutralizing, panselective, monoclonal mouse anti–TGF-β antibody (30 μg/mL; gift of Genentech, S. San Francisco, CA, USA) [14, 15] or an isotype-matched, irrelevant mouse IgG (30 μg/mL; Sigma) was added to the cells immediately prior to GO. For the last two conditions, cells were maintained in contact with GO for eight hours. Finally, to assess mRNA stability, GO was added to the cells in the presence of actinomycin D, and total RNA was extracted at different time intervals. Details regarding reagent concentrations, incubation times, and number of experiments are given in the Figure legends. Cells were lysed and denatured in solution D (4 mol/L guanidinium isothiocyanate, 25 mmol/L sodium citrate, pH 7, 0.5% sarcosyl, 0.1 mol/L 2-mercaptoethanol) to harvest total RNA. To measure the production of TGF-β1, cells were incubated with 1 mU/mL GO for 24 hours at 37°C, and supernatants were frozen at −80°C and later assayed for TGF-β1 protein by bioassay and enzyme-linked immunosorbent assay (ELISA). Cell toxicity was evaluated by the trypan blue exclusion method and by testing for lactate dehydrogenase release into the incubation media [9].

Analysis of mRNA by Northern blot analysis

Mesangial cells were homogenized in solution D. Total RNA was isolated by repeated phenol-chloroform extractions and isopropanol precipitation as described [24]. Twenty micrograms of total RNA from each experimental condition were denatured by heating in formamide/formaldehyde at 100°C for three minutes and electrophoresed through a 1% agarose gel containing 0.66 mol/L formaldehyde. The RNA was transferred to a nitrocellulose membrane (Amersham, Buckinghamshire, UK) by capillary blotting and was cross-linked by ultraviolet radiation. Uniform loading and integrity of RNA samples were assessed by methylene blue staining of the transferred RNA [25]. Hybridizations were performed in a rotating drum in a temperature-controlled oven. The membranes were prehybridized at 42°C for 24 hours in 5 × SSPE [(20 × SSPE: 3 mol/L NaCl, 0.2 mol/L NaH_2PO_4, 0.02 mol/L ethylenediaminetetraacetic acid (EDTA)], 5 × Denhardt's solution [50 × Denhardt's: 1% Ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin (BSA), 50% formamide, 0.1% sodium dodecyl sulfate (SDS)] and 0.1 mg/mL denatured salmon sperm DNA. cDNA inserts were separated from their vectors

in low-melting point agarose and radiolabeled with 5 μCi ^{32}P-deoxyadenosine 5′ triphosphate (3000 Ci/mmol; Amersham) using a radiolabeling system (Ready-to-Go; Amersham). Mouse α_1(IV) collagen cDNA was kindly provided by Dr. Kurkinen (Detroit, MI, USA). The cDNA probes encoding mouse TGF-β1, fibronectin, α_1(III) collagen, and α_1(I) collagen were synthesized by the polymerase chain reaction (PCR) using murine kidney cDNA as templates and specific oligonucleotide primers based on the published cDNA sequences [25, 26]. The PCR products were cloned into the pCRII TA cloning system (Invitrogen, La Jolla, CA, USA), and the identity of the probes was confirmed by nucleotide sequencing. Blots were hybridized for 24 hours with 10^6 cpm/mL probe at 42°C in the same buffer used for prehybridization. Afterward, the membranes were washed twice for five minutes each in 2 × standard saline citrate (SSC; 20 × SSC: 3 mol/L NaCl, 0.3 mol/L sodium citrate, pH 7.0) at room temperature and then for five minutes in 2 × SSC, 1% SDS at 65°C. Autoradiography was performed with intensifying screens (X-OMAT; Kodak, Rochester, NY, USA) at −80°C for five to six hours. Blots were stripped for 30 minutes in 1% SDS, 0.1 × SSC at 100°C and rehybridized with the remaining probes listed. Densitometric analysis of the exposed films was performed with an Apple scanner and appropriate software (NIH Image 1.55 from the National Institutes of Health).

Analysis of TGF-β_1 mRNA by RT-PCR

Competitive reverse transcription-PCR (RT-PCR) was performed on cultured HMCs in order to quantitate the GO-induced TGF-β1 mRNA expression in attomoles per μg of total RNA [27]. Total RNA was extracted from cultured control and GO-treated HMCs (1 mU/mL) as described previously in this article [24], and the quality and quantity of the RNA were verified by ethidium bromide staining of rRNA bands on an agarose minigel. The upstream and downstream TGF-β1 primers were 5′-CTT CAG CTC CAC AGA GAA GAA CTG C-3′ and 3′-CAC GAT CAT GTT GGA CAA CTG CTC C-5′, respectively. PCR amplification yielded a single band corresponding to a 298 bp cDNA fragment [28]. Sequence analysis confirmed that the fragment was identical to positions 1266 to 1564 in human TGF-β1 cDNA. One microgram of total RNA was reverse transcribed at 42°C for 30 minutes (RNA PCR kit; Perkin-Elmer, Roche Molecular Systems Inc., Branchburg, NJ, USA). For quantitation, the RT product and increasing, known amounts of a DNA mimic were amplified with the TGF-β1 primers. The DNA mimic, which resembles the TGF-β1 cDNA but competes with it for binding to the oligonucleotide primers, was synthesized using a PCR mimic construction kit (Clontech Laboratories, Palo Alto, CA, USA) according to the manufacturer's instructions. The mimic was designed to produce a 598 bp PCR product, which could easily be distinguished from the 298 bp PCR product of TGF-β1 cDNA on an agarose gel. The PCR cycler (MJ Research Inc., Watertown, MA, USA) was programmed for 35 cycles at 55°C, and the amplified cDNAs were electrophoresed through 1.5% agarose gels. The resulting ethidium bromide-stained gel was imaged with an Image-store Color One Scanner and analyzed with the NIH Image 1.55 software. The ratio of TGF-β1 cDNA to DNA mimic was plotted against the known attomolar concentrations of the DNA mimic [27]. Data were expressed as attomoles of TGF-β1 per microgram of total RNA. Contamination was ruled out by absence of a PCR product when the reverse transcription step was omitted.

Measurement of TGF-β_1 production

Active TGF-β_1 protein was measured by bioassay [29]. Mink lung epithelial cells CCL-64 were cultured at a concentration of 10^4 cells/well in 24-well plates and maintained in DMEM (GIBCO) with 10% FCS. Cells were incubated in serum-free DMEM for 24 hours to make them quiescent and then in 500 μL of serum-free DMEM plus 500 μL of conditioned medium for an additional 24 hours. Conditioned medium was acid activated with 10 μL of 6 mol/L HCl for 20 minutes to convert latent TGF-β1 to the active form and then neutralized with 20 μL of NaOH:HEPES (1:1) for 20 minutes. For the final six hours of incubation in conditioned medium, the CCL-64 cells were pulsed with 1 μCi/well ^{3}H-thymidine (5 Ci/mmol; Amersham). Afterward, the cells were washed twice in phosphate-buffered saline (PBS) and treated with 300 μL of 1 mol/L methanol at 4°C for one hour. Cells were then washed with the same buffer and maintained in 200 μL of 0.1 N NaOH at 4°C for 16 hours to lyse the cells. Finally, 15 μL of 1 N HCl were added for 15 minutes, and the cellular homogenate was collected to measure radioactivity by a scintillation counter.

Total TGF-β1 protein (latent + active) was also measured in HMC culture supernatants using a specific sandwich ELISA (Promega Corporation, Madison, WI, USA) [30] according to the manufacturer's instructions. HMCs (10^5 cells) were plated onto 24-well plates, rested in serum-free RPMI for 96 hours, and treated with a single dose of 1 mU/mL GO for the last 24 hours. Culture supernatant (250 μL) was collected, treated with 5 μL 1 N HCl for 60 minutes to activate latent TGF-β1, and neutralized with 1 N NaOH. A standard curve was constructed using serial dilutions of ultrapure human TGF-β1 (Promega). Cells released from the 24-well plate by trypsinization were counted in a Neubauer camera. All results are normalized to cell number and expressed as pg TGF-β1/10^4 cells. Each sample was measured in duplicate from eight independent experiments.

Transfection assay

Transfections were performed using TGF-β1 promoter fragments derived from either human or mouse. A 550 bp segment of the human TGF-β1 promoter [31] upstream of the transcription start site (Kpn/*Hin*dIII insert) was recloned into the *Hin*dIII site upstream of the luciferase gene as described [32]. The murine TGF-β1 promoter construct pA835, containing 835 bp 5′ from the A transcription start-site, was kindly provided by Dr. Andrew G. Geiser from the National Cancer Institute [33]. This plasmid [34] was recloned into the Hind III site upstream of the luciferase gene of the pXP2 vector [35] to yield the pLA835 reporter plasmid. The β-galactosidase–containing plasmid pCMV-βGal (Clontech) was used to control for transfection efficiency. Plasmids were purified by chromatography columns (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions, and identities were confirmed by restriction enzyme analysis.

Semiconfluent NIH-3T3 cells (10^5 cells per well) were transfected using either the Lipofectamine Reagent Plus kit (Life Technologies, Gaithersburg, MD, USA) or SuperFect Transfection Reagent (Qiagen) according to the manufacturers' instructions. The two reagents yielded comparable transfection efficiencies. After 24 hours of transfection, the media were changed, and GO was added at 1 or 2.5 mU/mL for an additional eight hours. Cells were then washed and lysed in reporter lysis buffer (Promega). Luciferase activity was measured in a luminometer and was normalized to β-galactosidase activity.

Endogenous catalase activity was measured in both HMCs and NIH-3T3 cells to rule out a significant difference in catalase activity as a cause of the ROS-induced effects on the TGF-β1 promoter. Both cell types were grown to confluence and then serum-deprived for 48 hours. Catalase activity was measured as previously described [36]. Briefly, 2 mL of cell lysate (lysis buffer: 50 mmol/L KH_2PO_4, pH 7.0, 0.2% Triton X-100) were added to 1 mL of 30 mmol/L H_2O_2, and changes in absorbance at 240 nm were measured for 30 seconds in a spectrophotometer. Catalase activity is expressed in the unit K, derived from the equation, $K = (1/t_2 - t_1) \times \ln (A_1/A_2)$, where $t_2 - t_1$ is the measured interval in seconds, and A_1 and A_2 are the initial and final absorbances at 240 nm, respectively.

Statistical analysis

Results shown are the mean ± SEM, and N = number of experiments (Figure legends). The Northern blot densitometry data were corrected for small variations in gel loading by accounting for the relative intensities of the 28S band, as assessed by methylene blue staining of the transfer membrane. When $N < 10$, nonparametric statistics were used for comparisons (Friedman's and Wilcoxon's tests). In other cases, nested analysis of variance (ANOVA) was used. $P < 0.05$ was considered statistically significant.

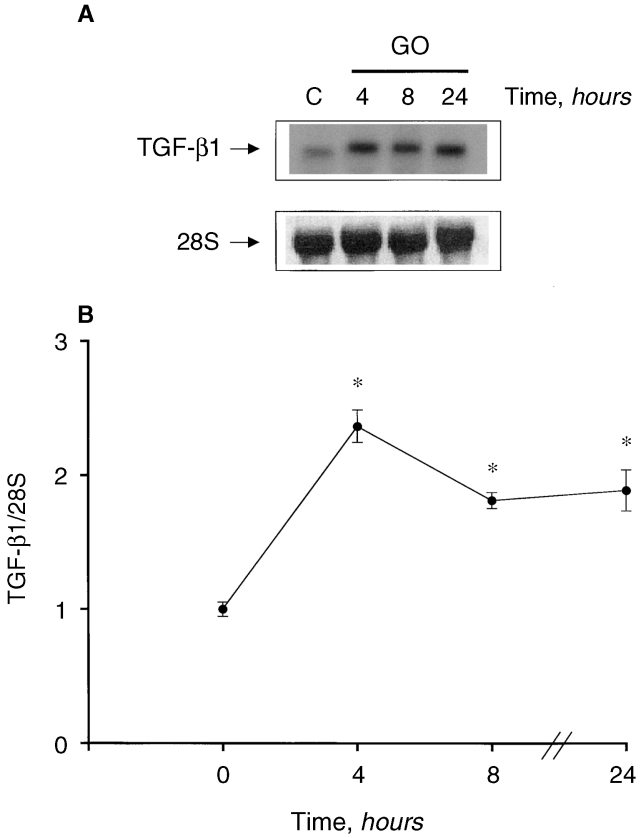

Fig. 1. Expression of transforming growth factor-β1 (TGF-β1) mRNA in cultured human mesangial cells (HMCs) treated with glucose oxidase (GO). Cells were incubated for variable times (4 to 24 h) with 1 mU/mL GO, and the TGF-β1 mRNA expression was analyzed by Northern blot. A representative experiment is shown, with the corresponding 28S signal used to control for loading (*A*). The mean ± SEM (N = 4) of the densitometric ratios of TGF-β1 mRNA to 28S are shown (*B*), with the relative ratio in control cells assigned a value of 1. *$P < 0.05$ vs. control (C).

RESULTS

Figure 1 demonstrates that incubation with GO significantly increases the expression of TGF-β1 mRNA in HMCs compared with control. Measured by Northern blot analysis, this effect was maximal after four hours of incubation and declined slightly thereafter (Fig. 1). Similar results were obtained when the TGF-β1 mRNA was quantitated by RT-PCR. The GO-induced TGF-β1 mRNA was about 2.5-fold higher than control after 24 hours of incubation (control cells, 7.88 ± 2.04 attmol/μg RNA; GO-treated cells, 20.36 ± 4.28, $P < 0.05$). Catalase abrogated the GO-induced increase in TGF-β1 mRNA, suggesting that H_2O_2 generation was the mechanism un-

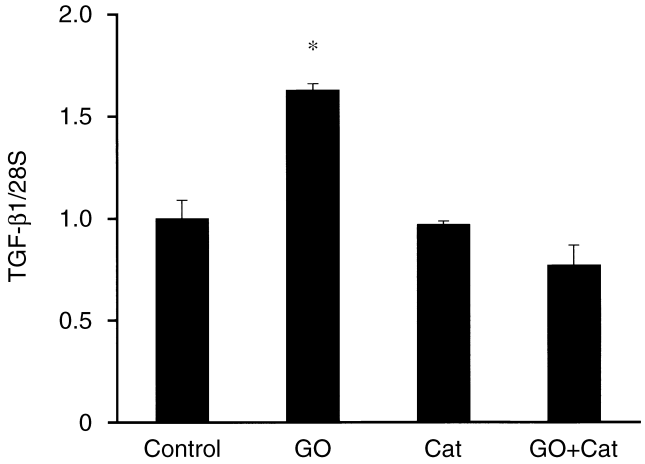

Fig. 2. Expression of TGF-β1 mRNA in cultured HMCs preincubated with catalase and treated with GO. Cells were preincubated for 10 minutes with 80 U/mL catalase (Cat) and were then incubated for eight hours with 1 mU/mL GO. TGF-β1 mRNA expression was analyzed by Northern blot with the corresponding 28S signal used to control for loading. The mean ± SEM of the densitometric ratios of TGF-β1 mRNA to 28S are shown ($N = 4$, except $N = 2$ for catalase alone), with the relative ratio in control cells assigned a value of 1. *$P < 0.05$ vs. control.

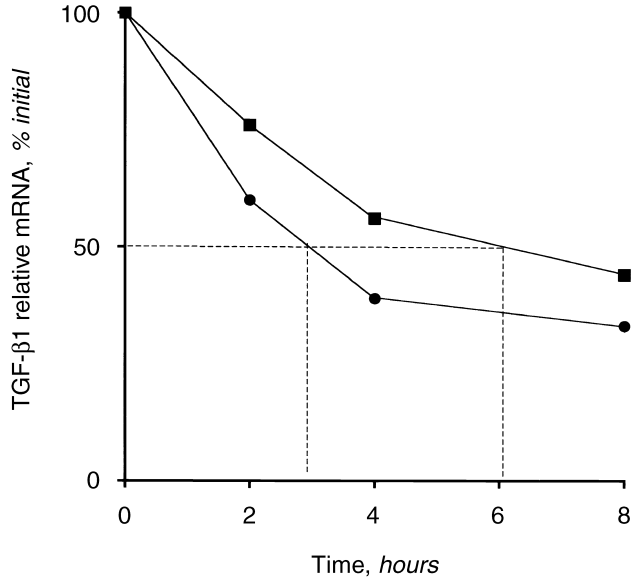

Fig. 3. Analysis of TGF-β1 mRNA stability in control (■) and GO (●)-incubated HMCs. Cultured cells were examined without or with exposure to GO (1 mU/mL) for eight hours (initial). Actinomycin D (10 μg/mL) was then added for the designated times in hours. The decay in TGF-β1 mRNA level was analyzed by Northern blot, with the corresponding 28S signal used to control for loading. The half-life under the two experimental conditions was assessed by the hatched lines. Each point represents the mean value of three independent experiments.

derlying the effect of GO (Fig. 2). At the concentrations tested, GO was not toxic to HMCs. More than 95% of the cells excluded the trypan blue dye, and lactate dehydrogenase in the incubation media was similar in control and GO-treated cells. Treatment with catalase alone did not affect basal TGF-β1 mRNA expression in HMCs (Fig. 2).

The mechanisms responsible for the increased steady-state levels of TGF-β1 mRNA in response to GO were investigated using mouse NIH-3T3 cells transfected with the TGF-β1 promoter linked to a luciferase reporter gene. NIH-3T3 cells were employed because of the relative ease of transfection of these cells as compared with HMCs. The catalase activity of NIH-3T3 cells was similar to that of HMCs (HMC, 6.31 ± 0.29 μK/10^4; NIH-3T3 cells, 5.27 ± 0.57, P = NS), indicating that H_2O_2 levels would be comparable between the two cell types. The addition of GO (1 mU/mL) increased human TGF-β1 promoter activity by nearly twofold as demonstrated by the luciferase assay (control cells, 416 ± 114 relative light units/mg protein; GO-treated cells, 817 ± 123; $N = 6$, $P < 0.05$). GO also stimulated the activity of the mouse TGF-β1 promoter construct pLA835 when this construct was transfected in NIH-3T3 cells. Transfection efficiency was controlled by cotransfection with a β-galactosidase–expressing vector. At a dose of 1 mU/mL of GO for eight hours, the relative promoter activity was increased to 138 ± 11% of control value ($N = 6$, $P < 0.05$); at a dose of 2.5 mU/mL, this activity was 146 ± 7% of control ($P < 0.05$).

The addition of GO did not appreciably affect TGF-β1 mRNA stability (Fig. 3). The slope of the mRNA degradation curve was slightly steeper initially following exposure to GO, but became almost identical to the control value after two hours. The results in Figure 3 indicate that the half-life of TGF-β1 mRNA may become shortened from approximately six hours in control cells to three hours in GO-treated cells. Taken together, these studies suggest that the increase in the steady-state level of TGF-β1 mRNA after GO treatment is most likely due to an increase in TGF-β1 gene transcription, independent of an effect on TGF-β1 mRNA stability.

To test whether increased TGF-β1 transcription translates into augmented TGF-β1 translation, TGF-β1 protein levels were measured by bioassay and ELISA in the supernatants of HMCs incubated with GO. Conditioned media of HMCs that were incubated with GO significantly inhibited the growth of mink lung epithelial cells (Fig. 4A) and contained significantly greater amounts of immunoassayable TGF-β1 as measured by ELISA (Fig. 4B).

The effect of continuous H_2O_2 exposure on the mRNA expression of ECM proteins in HMCs is summarized in Figure 5. Representative Northern blots demonstrate that H_2O_2 increases the message levels of different collagen types (I, III, and IV) and fibronectin (Fig. 5A). On average, the cells incubated with GO increased type IV collagen by 108%, type I collagen by 98%, type III collagen by 67%, and fibronectin by 96% compared with control ($N = 3$, $P < 0.05$; Fig. 5B). To assess the role

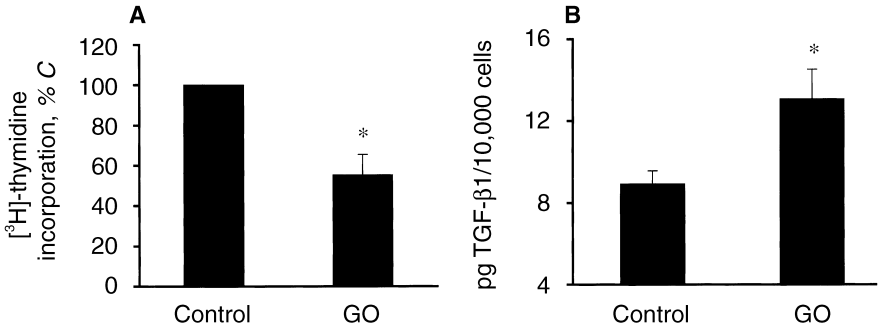

Fig. 4. Bioactivity and protein production of TGF-β1 in cultured HMCs treated with GO. Cells were incubated for 24 hours with 1 mU/mL GO, and the release of TGF-β1 into the incubation medium was assessed. (*A*) Results of the mink lung epithelial cell (CCL-64 cell) proliferation as assayed by ^{3}H-thymidine uptake (**Methods** section). The conditioned media of GO-treated cells significantly inhibited cell proliferation. Results represent the mean ± SEM (N = 10), and proliferation of control CCL-64 cells (C) is assigned a value of 100%. (*B*) TGF-β1 protein content of the incubation medium as measured by ELISA (N = 12). *$P < 0.05$ vs. control.

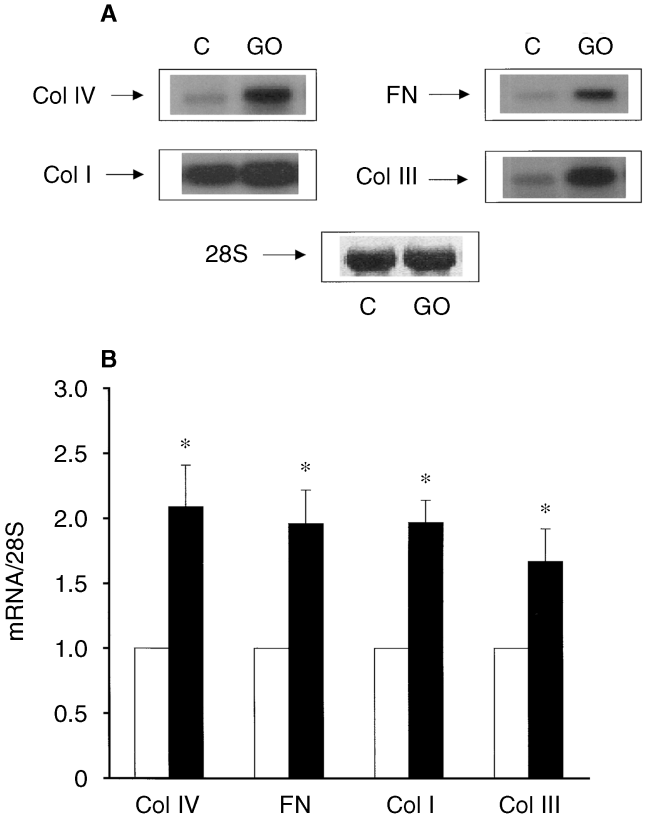

Fig. 5. Expression of extracellular matrix (ECM) mRNA in cultured HMCs treated with GO. Cells were incubated for 24 hours with 1 mU/mL GO, and the mRNA expression of α_1(IV) collagen, fibronectin, α_1(I) collagen, and α_1(III) collagen were analyzed by Northern blot with the corresponding 28S signal used to control for loading. The results from one example of the experimental studies are shown in (*A*). (*B*) The mean ± SEM (N = 3) of the densitometric ratios of different ECM mRNAs to 28S (■), where the ratio in control cells is assigned a value of 1 (□). *$P < 0.05$ vs. control.

of endogenous TGF-β1 activity in increasing ECM expression in HMCs, experiments were performed with a neutralizing anti–TGF-β antibody [37, 38]. Incubation with control IgG did not affect the GO-induced increases in the mRNA expression of collagen types I and IV, but incubation with anti–TGF-β antibody completely blocked these GO-induced changes (Fig. 6). Neither anti–TGF-β antibody nor IgG had a toxic effect on the cells (data not shown).

DISCUSSION

Previous studies from our group have shown that H_2O_2 induces mesangial cell contraction [12] and stimulates cell proliferation [13], suggesting a pathogenic role for ROS in the development of glomerular injury in many disease states. ROS may also be involved in renal hemodynamic [39] and structural changes [40] that characterize some models of renal disease. In the current study, we evaluated the direct effect of H_2O_2 on the regulation of ECM gene expression by HMCs. A sustained release of H_2O_2 into the incubation medium of HMCs increases the synthesis of TGF-β1 and stimulates the gene expression of various ECM proteins. For cell culture work, continuous low-level H_2O_2 production, such as that achieved by GO [23], is preferred to periodic high-dose pulses of H_2O_2. The former is probably less toxic and more representative of the pathophysiologic conditions that give rise to increased ROS synthesis. Cellular toxicity caused by GO was ruled out, and the specificity of GO action was confirmed by the ability of catalase to abrogate its effects.

Four different procedures—Northern blot, competitive-quantitative RT-PCR, ELISA, and bioassay—were used to evaluate the effect of H_2O_2 on TGF-β1 production by mesangial cells. The first two techniques clearly demonstrate that H_2O_2 stimulates the expression of TGF-β1 mRNA, reaching a maximum at four hours. The amount of active TGF-β1 protein in the incubation media increased significantly after 24 hours. The marked growth inhibition of mink lung epithelial cells by conditioned supernatants of GO-treated cells strongly supports the view that H_2O_2 stimulates TGF-β1 production, but perhaps significant amounts of H_2O_2 in the supernatant can also inhibit the growth of the reporter cells.

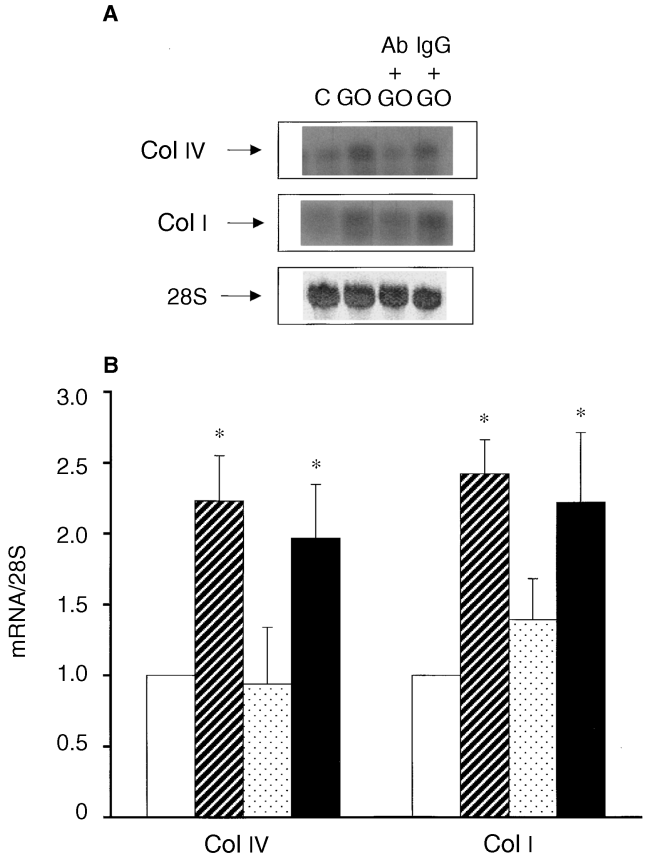

Fig. 6. Expression of collagen mRNA in cultured HMCs treated with GO in the presence of an anti–TGF-β blocking antibody. Cells were incubated for 24 hours without (C) or with 1 mU/mL GO plus 30 μg/mL anti–TGF-β antibody (Ab + GO) or 30 μg/mL nonspecific IgG (IgG + GO). (*A*) A representative Northern assay of α_1(IV) collagen and α_1(I) collagen mRNAs with the corresponding 28S signal used to control for loading. (*B*) The mean ± SEM ($N = 4$) of the densitometric ratios of collagen mRNAs to 28S, where the ratio in control cells (□) is assigned a value of 1. The stimulation of collagen mRNA levels by GO (▨) was prevented by anti–TGF-β antibody (▩), but not by IgG (■). *$P < 0.05$ vs. control.

Although the mink lung epithelial cell bioassay measures the activity of all three isoforms of TGF-β, the ELISA specific for TGF-β1 confirmed that the release of TGF-β1 isoform into the media of mesangial cells is augmented in response to H_2O_2 (Fig. 4). In a previous report by Nath et al [21], addition of a pulse of H_2O_2 was able to induce directly increases in the mRNA levels of TGF-β1, type III collagen, and type IV collagen in cultured NRK-49F fibroblasts and in rat mesangial cells. In the present studies, we demonstrate not only that TGF-β1 mRNA expression is up-regulated, but also that TGF-β1 protein synthesis is augmented in the presence of continuous exposure to H_2O_2. Moreover, we show that the effect of H_2O_2 on ECM production is mediated by increased TGF-β activity, since treatment with a panselective anti–TGF-β antibody completely abrogates the GO-induced increases in ECM gene expression. We provide further evidence that the observed effects are specifically due to H_2O_2 since pretreatment with the antioxidant catalase, which detoxifies H_2O_2, inhibits the GO-induced expression of TGF-β1.

The mechanisms responsible for the increased steady-state levels of TGF-β1 mRNA after H_2O_2 treatment were also analyzed. H_2O_2 did not stabilize the TGF-β1 mRNA level but did increase the transcription rate of the TGF-β1 gene, as evidenced by the enhanced TGF-β1 promoter activity in the transfection experiments. The level of TGF-β1 promoter activity correlated well with the quantity of TGF-β1 mRNA, as assessed by Northern blot and competitive-quantitative RT-PCR. The predominant control of TGF-β1 production in response to H_2O_2 therefore occurs at the transcriptional level.

The message expression of every ECM protein tested increased after GO incubation. Two native mesangial ECM components (type IV collagen and fibronectin) and two interstitial collagens (types I and III) were analyzed, and in every case, the mRNA levels increased after cells were exposed to GO. H_2O_2 may have stimulated ECM expression by a direct effect or an indirect effect through activation of the TGF-β system. The latter possibility seems likely because experiments employing the panselective anti–TGF-β antibody disclosed complete prevention of the GO-induced increase in ECM expression. Thus, one or more of the TGF-β isoforms can be implicated in mediating of the effect of GO on collagen or fibronectin expression. The TGF-β1 isoform is known to increase the synthesis and decrease the degradation of ECM proteins [16, 37]. The control IgG did not reverse the GO-induced increases in ECM expression, suggesting that the effect of the anti–TGF-β antibody is not due to a direct antioxidant effect.

The present results provide new insights into the pathophysiologic mechanisms that underlie certain diseases characterized by progressive renal sclerosis. Increased ROS synthesis has been implicated in some glomerulonephritides [1–5], the remnant kidney model of renal sclerosis [41], chronic renal rejection [39], cyclosporine A-related nephropathy [42], and age-related renal dysfunction [43, 44]. All of these situations are characterized by the relentless accumulation of ECM proteins in the renal parenchyma, and ROS may initiate and maintain this harmful morphological process. ROS have also been linked to fibrotic processes other than glomerulosclerosis, and they have been associated with several cytokines that up-regulate ECM proteins. In vascular smooth cells, the synthesis of insulin-like growth factor-I is increased by exposure to ROS, suggesting that the autocrine insulin-like growth factor-I system plays an important role in vascular smooth muscle cell growth in response to ROS [45]. Oxygen derivatives have been shown to produce progressive pulmonary inflammation and fibrosis through activation of MEK-ERK pathways [46]. ROS

may also activate the STAT family of transcription factors in response to platelet-derived growth factor (PDGF). These findings indicate that the JAK-STAT pathway responds to intracellular ROS and that PDGF uses ROS as a second messenger to regulate STAT activation [47].

We conclude that hydrogen peroxide stimulates extracellular matrix protein synthesis in cultured human mesangial cells, an effect that is largely dependent on activation of TGF-β1. We propose that the previously mentioned molecular changes induced by oxidative stress may have important consequences in the development and progression of many renal diseases. Furthermore, therapeutic interventions to reduce reactive oxygen species production may delay or even prevent the development of such diseases.

ACKNOWLEDGMENTS

This study was supported by the CICYT, SAF 96/0186, and FIS 98/0337. M.C. Iglesias-de la Cruz and M.P. Ruiz-Torres are postdoctoral fellows of the Ministerio de Educación y Cultura of Spain. F.N. Ziyadeh and S. Chen are supported by the Juvenile Diabetes Foundation International. The authors thank Ms. Susana Comendador for her technical support.

Reprint requests to Carmen Iglesias-de la Cruz, Ph.D., Renal-Electrolyte and Hypertension Division, 700 Clinical Research Building, University of Pennsylvania, 415 Curie Boulevard, Philadelphia, Pennsylvania 19104-6144, USA.
E-mail: ziyadeh@mail.med.upenn.edu

REFERENCES

1. Shah SV: Role of reactive oxygen metabolites in experimental glomerular disease. *Kidney Int* 35:1093–1106, 1989
2. Adler S, Baker PJ, Johnson RJ, *et al*: Complement membrane attack complex stimulates production of reactive oxygen metabolites by cultured rat mesangial cells. *J Clin Invest* 77:762–767, 1986
3. Diamond JR, Bonventre JV, Karnovsky MJ: A role for oxygen free radicals in aminonucleoside nephrosis. *Kidney Int* 29:478–483, 1986
4. McCord JM: Oxygen-derived free radicals in postischemic tissue injury. *N Engl J Med* 312:159–163, 1985
5. Paller MS, Hoidal JR, Ferris TF: Oxygen free radicals in ischemic acute renal failure in the rat. *J Clin Invest* 74:1156–1164, 1984
6. Baud L, Nivez MP, Chansel D, *et al*: Stimulation by oxygen radicals of prostaglandin production by rat renal glomeruli. *Kidney Int* 20:332–339, 1981
7. Lewis MS, Whatley RE, Cain P, *et al*: Hydrogen peroxide stimulates the synthesis of platelet-activating factor by endothelium and induces endothelial cell-dependent neutrophil adhesion. *J Clin Invest* 82:2045–2055, 1988
8. Hughes AK, Stricklett PK, Padilla E, *et al*: Effect of reactive oxygen species on endothelin-1 production by human mesangial cells. *Kidney Int* 49:181–189, 1996
9. López-Ongil S, Hernández-Perera O, Navarro-Antolín J, *et al*: Role of reactive oxygen species in the signalling cascade of cyclosporine A-mediated up-regulation of eNOS in vascular endothelial cells. *Br J Pharmacol* 124:447–454, 1998
10. Scharschmidt LA, Dunn MJ: Prostaglandin synthesis by rat glomerular mesangial cells in culture: Effects of angiotensin II and arginine vasopressin. *J Clin Invest* 71:1756–1764, 1983
11. Trachtman H, Futterweit S, Singhal P: Nitric oxide modulates the synthesis of extracellular matrix proteins in cultured rat mesangial cells. *Biochem Biophys Res Commun* 207:120–125, 1995
12. Díez-Marqués ML, García-Escribano C, Medina J, *et al*: Effects of somatostatin on cultured human mesangial cells. *Endocrinology* 136:3444–3451, 1995
13. Duque I, Rodríguez-Puyol M, Ruiz-Torres MP, *et al*: Calcium channel blockers inhibit hydrogen peroxide-induced proliferation of cultured rat mesangial cells. *J Pharmacol Exp Ther* 267:612–616, 1993
14. Sharma K, Jin Y, Guo J, *et al*: Neutralization of TGF-beta by anti-TGF-beta antibody attenuates kidney hypertrophy and the enhanced extracellular matrix gene expression in STZ-induced diabetic mice. *Diabetes* 45:522–530, 1996
15. Ziyadeh FN, Hoffman BB, Han DC, *et al*: Long-term prevention of renal insufficiency, excess matrix gene expression and glomerular mesangial matrix expression by treatment with monoclonal anti-TGF-β antibody in *db/db* diabetic mice. *Proc Natl Acad Sci USA* 97:8015–8020, 2000
16. Ketteler M, Noble NA, Border WA: Transforming growth factor-beta and angiotensin II: The missing link from glomerular hyperfiltration to glomerulosclerosis? *Annu Rev Physiol* 57:279–295, 1995
17. Sundaresan M, Yu ZX, Ferrans VJ, *et al*: Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction. *Science* 270:296–299, 1995
18. Schreck R, Rieber P, Baeuerle PA: Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *EMBO J* 10:2247–2258, 1991
19. Meyer M, Schreck R, Baeuerle PA: H_2O_2 and antioxidants have opposite effects on activation of NF-kappa B and AP-1 in intact cells: AP-1 as secondary antioxidant-responsive factor. *EMBO J* 12:2005–2015, 1993
20. Kim SJ, Angel P, Lafyatis R, *et al*: Autoinduction of transforming growth factor beta 1 is mediated by the AP-1 complex. *Mol Cell Biol* 10:1492–1497, 1990
21. Nath KA, Grande J, Croatt A, *et al*: Redox regulation of renal DNA synthesis, transforming growth factor-beta1 and collagen gene expression. *Kidney Int* 53:367–381, 1998
22. Gow AJ, Branco F, Christofidou-Salomidou M, *et al*: Immunotargeting of glucose oxidase: Intracellular production of H_2O_2 and endothelial oxidative stress. *Am J Physiol* 277:L271–L281, 1999
23. Miura K, Ishii T, Sugita Y, *et al*: Cystine uptake and glutathione level in endothelial cells exposed to oxidative stress. *Am J Physiol* 262:C50–C58, 1992
24. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159, 1987
25. Sharma K, Ziyadeh FN: Renal hypertrophy is associated with upregulation of TGF-β1 gene expression in diabetic BB rat and NOD mouse. *Am J Physiol* 267:F1094–F1101, 1994
26. Rocco MV, Neilson EG, Hoyer JR, *et al*: Attenuated expression of epithelial cell adhesion molecules in murine polycystic kidney disease. *Am J Physiol* 262:F679–F686, 1992
27. Ruiz-Torres MP, Bosch RJ, O'Valle F, *et al*: Age-related increase in expression of TGF-beta1 in the rat kidney: Relationship to morphologic changes. *J Am Soc Nephrol* 9:782–791, 1998
28. Qian SW, Kondaiah P, Roberts AB, *et al*: cDNA cloning by PCR of rat transforming growth factor beta-1. *Nucleic Acids Res* 18:3059, 1990
29. Meager A: Assays for transforming growth factor beta. *J Immunol Methods* 141:1–14, 1991
30. Wolf G, Ziyadeh FN, Zahner G, *et al*: Angiotensin II-stimulated expression of transforming growth factor beta in renal proximal tubular cells: Attenuation after stable transfection with the c-mas oncogene. *Kidney Int* 48:1818–1827, 1995
31. Kim SJ, Glick A, Sporn MB, *et al*: Characterization of the promoter region of the human transforming growth factor-beta 1 gene. *J Biol Chem* 264:402–408, 1989
32. Michelson S, Alcamí J, Kim SJ, *et al*: Human cytomegalovirus infection induces transcription and secretion of transforming growth factor beta 1. *J Virol* 68:5730–5737, 1994
33. Geiser AG, Kim S-J, Roberts AB, *et al*: Characterization of the mouse transforming growth factor-β1 promoter and activation by the Ha-ras oncogene. *Mol Cell Biol* 11:84–92, 1991
34. Hoffman BB, Sharma K, Zhu Y, *et al*: Transcriptional activation

of transforming growth factor-β1 in mesangial cell culture by high glucose concentration. *Kidney Int* 54:1107–1116, 1998
35. Nordeen SK: Luciferase reporter gene vectors for analysis of promoters and enhancers. *Biotechniques* 6:454–458, 1988
36. Aebi HE: *Catalase.* Basel, Verlag-Chemie, 1983
37. Ziyadeh FN, Sharma K, Ericksen M, *et al*: Stimulation of collagen gene expression and protein synthesis in murine mesangial cells by high glucose is mediated by autocrine activation of transforming growth factor-beta. *J Clin Invest* 93:536–542, 1994
38. Han DC, Isono M, Hoffman BB, *et al*: High glucose stimulates proliferation and collagen type I synthesis in renal cortical fibroblasts: Mediation by autocrine activation of TGF-β. *J Am Soc Nephrol* 10:1891–1899, 1999
39. Yoshioka T, Ichikawa I: Glomerular dysfunction induced by polymorphonuclear leukocyte-derived reactive oxygen species. *Am J Physiol* 257:F53–F59, 1989
40. Clayton L, Hiley C, Davies S, *et al*: Glomerular injury induced by hydrogen peroxide: Modifying influence of ACE inhibitors. *Free Radic Res Commun* 17:271–278, 1992
41. Nankivell BJ, Tay YC, Boadle RA, *et al*: Dietary protein alters tubular iron accumulation after partial nephrectomy. *Kidney Int* 45:1006–1013, 1994
42. Parra T, de Arriba G, Arribas I, *et al*: Cyclosporine A nephrotoxicity: Role of thromboxane and reactive oxygen species. *J Lab Clin Med* 131:63–70, 1998
43. Iglesias-de la Cruz MC, Ruiz-Torres MP, García-del Moral R, *et al*: Age-related progressive renal fibrosis in rats and its prevention with ACE inhibitors and taurine. *Am J Physiol* 278:F122–F129, 2000
44. Ruiz-Torres P, González-Rubio M, Lucio-Cazaña FJ, *et al*: Reactive oxygen species and platelet-activating factor synthesis in age-related glomerulosclerosis. *J Lab Clin Med* 124:489–495, 1994
45. Delafontaine P, Ku L: Reactive oxygen species stimulate insulin-like growth factor I synthesis in vascular smooth muscle cells. *Cardiovasc Res* 33:216–222, 1997
46. Cho YJ, Seo MS, Kim JK, *et al*: Silica-induced generation of reactive oxygen species in Rat2 fibroblast: Role in activation of mitogen-activated protein kinase. *Biochem Biophys Res Commun* 262:708–712, 1999
47. Simon AR, Rai U, Fanburg BL, *et al*: Activation of the JAK-STAT pathway by reactive oxygen species. *Am J Physiol* 275:C1640–C1652, 1998