

# Tretinoin Prevents Age-Related Renal Changes and Stimulates Antioxidant Defenses in Cultured Renal Mesangial Cells<sup>1</sup>

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## ABSTRACT

Age-related progressive glomerular sclerosis in the rat is associated with increased expression of tumor necrosis factor- $\beta_1$  and increased protein content in the renal cortex, enhanced production of H<sub>2</sub>O<sub>2</sub>, in both renal glomeruli and mesangial cells (MCs) cultured from them, as well as augmented glomerular oxidative damage. We have previously shown that tretinoin-treated old male Fischer 344 rats have 30% lower protein content in the renal cortex than control old rats. Here, we report that this effect may depend on the inhibition of the expression of tumor necrosis factor- $\beta_1$ , a matrigenic cytokine, and osteopontin, a protein with cell adhesive and chemotactic properties. In addition, we show that tretinoin prevents the cytotoxicity of H<sub>2</sub>O<sub>2</sub> in cultured human MCs by increasing both the catalase activity and the reduced glutathione content, which are dose- and time-dependent changes. These increases were

not dependent on each other: when these effects were previously inhibited with 3-amino-1,2,4-triazole or L-buthionine-(S,R)-sulfoximine, respectively, tretinoin still induced the increase of the other noninhibited antioxidant defense. An enhanced gene transcription is the most likely mechanism involved in the tretinoin-induced stimulation of MC antioxidant defense systems because 1) preincubation of MCs with actinomycin D or cycloheximide fully abolished it; 2) tretinoin-incubated MCs showed increased levels of catalase mRNA and  $\gamma$ -glutamyl-cysteine synthetase (catalytic subunit) mRNA, the latter being the rate-limiting step in de novo reduced glutathione synthesis; and 3) the stability of both mRNA was unchanged by tretinoin. These results show one strategy of protecting renal cells from H<sub>2</sub>O<sub>2</sub>-mediated injury based on increasing their antioxidant defenses.

Progressive glomerular sclerosis takes place in aging humans (Lindeman, 1990). Rats also exhibit an age-dependent renal deterioration, and male rats are more susceptible than female rats to age-related glomerulosclerosis (Baylis and Corman, 1998). Reactive oxygen species seem to play a role in the progression of age-related rat renal changes: there is an association between increased glomerular protein content and an augmented oxidative damage (i.e., increased levels of lipid peroxidation) in old rats (Ruiz et al., 1994). In addition, the production of H<sub>2</sub>O<sub>2</sub> in glomeruli and cultured glomerular mesangial cells (MCs) is higher in samples from old rats than in those from young animals (Ruiz et al., 1994, 1996). On the other hand, H<sub>2</sub>O<sub>2</sub> increases in cultured rat MCs, the production of transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ) (P. Ruiz, personal communication), and this effect could account, at least

in part, for the increased expression of this matrigenic cytokine (it contributes to glomerulosclerosis through promoting synthesis of extracellular matrix proteins and inducing inhibitors of metalloproteinases; reviewed by Sharma and Ziyadeh, 1994) in renal cortex of old rats (Ruiz et al., 1998). In addition to resident glomerular cells, leukocytes are other important source of reactive oxygen species and TGF- $\beta_1$ , and under appropriate conditions, these cells may infiltrate the renal glomeruli and contribute to the progression of a glomerular damage. An example of these conditions is the enhanced expression of osteopontin (OP), an arginine-glycine-aspartic acid (RGD)-containing acidic glycoprotein with cell adhesive and chemotactic properties (reviewed by Giachelli et al., 1995), which has been described in some models of renal disease, including progressive glomerulosclerosis (Narita et al., 1997) and age-related glomerulosclerosis (Floege et al., 1997).

Recently, we focused our attention on the treatment of age-related glomerulosclerosis with tretinoin (all-*trans*-retinoic acid), one of the active metabolites of vitamin A. Because it has antiactivator protein-1 activity in glomerular MCs

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**ABBREVIATIONS:** BSO, L-buthionine-(S,R)-sulfoximine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SSC, standard saline citrate;  $\gamma$ -GCS,  $\gamma$ -glutamyl-cysteine synthetase; GSH, reduced glutathione; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RT, reverse transcription; MC, mesangial cell; NAC, N-acetylcysteine; OP, osteopontin; PCR, polymerase chain reaction; TGF- $\beta_1$ , transforming growth factor- $\beta_1$ .

(Simonson, 1994), we hypothesized that it could inhibit the activator protein-dependent effects of  $H_2O_2$ , such as cell death (Ishikawa et al., 1997; Xu et al., 1997). In fact, preincubation with tretinoin abolished  $H_2O_2$ -induced MC death (Moreno et al., 1997). Although the effects of tretinoin on TGF- $\beta_1$  expression are unpredictable a priori (some authors describe that the retinoid increases it in a given cell type, others find the opposite effect in other cell types, and, finally, there are examples in the literature of unaffected TGF- $\beta_1$  expression after tretinoin treatment), we found that tretinoin slows the progression of age-related glomerular changes in male Fischer 344 rats (Moreno et al., 1997). Therefore, we expect that its net effect on the expression of the matrigenic cytokine TGF- $\beta_1$  at the renal cortex level is inhibitory. In the same way, we also expect that the retinoid will inhibit the expression of other molecules (i.e., OP) involved in the progression of age-related glomerulosclerosis.

Taking into account this background, the present work was designed with two objectives. The first objective was to study, in the context of aging, the effect of tretinoin on the renal expression of TGF- $\beta_1$  and OP, and the second objective was to examine the effect of the retinoid on antioxidant defenses of cultured glomerular MCs as a possible mechanism of tretinoin in preventing  $H_2O_2$ -induced cytotoxicity.

## Materials and Methods

Tretinoin (all-*trans*-retinoic acid) was kindly donated by Productos Roche S.A. (Spain). Unless otherwise stated, all of the biochemical reagents used in this study were purchased from Sigma Chemical Co. (St. Louis, MO). Tissue culture materials, growth media, and serum for cell culture were obtained from GIBCO (Grand Island, NY), and the RNA PCR kit was from Perkin-Elmer (Roche Molecular Systems Inc., Branchburg, NJ). All other chemicals used were of the purest grade commercially available.

### In Vivo Experiments

**Animals and Diets.** Because we were mainly interested in the early stages of spontaneous glomerulosclerosis, when it is more likely to obtain any benefit from the dietary protocol, the study was not performed in very old rats. Therefore, thirty 18-month-old male Fischer 344 rats were fed 7 days per week with standard chow (control,  $n = 15$ ) or with standard chow plus tretinoin (tretinoin-treated) for a period of 90 days. Food containing tretinoin (all-*trans*-retinoic acid) was prepared daily in the following way: a solution of tretinoin in absolute ethanol (1.5 g tretinoin/liter) was mixed in a dark, cold room with standard chow (2 ml tretinoin solution/100 g food). Once ethanol evaporated, 15 g of food (the average daily consumption of food per rat) were given to each rat every day: this renders a daily intake of tretinoin of about 1 mg/kg b.wt. The dose of tretinoin was adjusted each week depending on the body weight gain. Control animals ate food treated in the same way with 2 ml ethanol/100 g food. The physical condition of all animals was good, and no changes in the food intake of any rat were observed during the study.

After 90 days of treatment, animals received ether anesthesia. Blood taken from the lower aorta was used for hematological studies, and serum isolated from each blood sample was used for biochemical analyses. Pieces of the left kidney cortex were weighed and homogenized in phosphate buffer (pH 7.4) supplemented with 0.1% Triton X-100, 3 mM EDTA, and 2 M NaCl. Aliquots of the lysate were used for the measurement of protein and DNA (see below).

A piece of the renal cortex from the right kidney was collected in a sterile tube containing a denaturing solution for total RNA extraction (Chomczynsky and Sacchi, 1987). Both the quality and the

quantity of the RNA were verified by ethidium bromide staining of rRNA bands on an agarose minigel. RNA samples were used to study the expression of TGF- $\beta_1$  and OP. A set of RNA renal samples from 21-month-old rats was also used to further study the age-related changes in the expression of OP.

### In Vitro Experiments

**Cells.** Human MCs were obtained from adult nephrectomy specimens as we previously described (Díez et al., 1995). Culture medium was made of RPMI 1640 supplemented with 10% FCS, 200 mM L-glutamine, and antibiotics (100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 0.25  $\mu$ g/ml amphotericin B). Confluent cells between the 12th and 15th passages were used, and they were made quiescent by 48 h incubation with medium supplemented with 0.5% FCS.

**Stimulation of Antioxidant Defenses.** Fresh medium with tretinoin or its vehicle (ethanol at a final concentration of 0.09%) was added to quiescent cells. Tretinoin effects on antioxidant defenses [i.e., catalase activity and reduced glutathione (GSH) content] were first tested in dose-response experiments, with the range of retinoid concentration 0.01 to 50  $\mu$ M and an incubation period of 24 h, and then tested in time-response experiments with 10  $\mu$ M tretinoin. An approximate value for the highest tretinoin-induced increase in intracellular GSH content was obtained in other experiments, in which the synthesis of glutathione was favored through increasing the availability of cysteine by the addition of 10 mM *N*-acetylcysteine (NAC) (Deneke and Fanburg, 1989) after incubation with 10  $\mu$ M tretinoin.

Then, a set of experiments conceived to explore the mechanisms responsible for the stimulation of antioxidant defense systems was designed as described.

**Effect of dl- $\alpha$ -tocopherol on antioxidant defenses.** The contribution of antioxidant properties to the effects of tretinoin on the cellular antioxidant defenses was examined in experiments identical with those described above but cells were incubated with 10  $\mu$ M *dl*- $\alpha$ -tocopherol instead of tretinoin.

**Effect of blockade of tretinoin-induced stimulation of catalase activity on increase in intracellular GSH.** Cells were preincubated for 1 h under control conditions or with 5 mM 3-amino-1,2,4-triazole, an irreversible inhibitor of catalase activity (Aebi, 1983). Then, they were incubated for 24 h with or without 10  $\mu$ M tretinoin, and the intracellular GSH content was measured. Cells incubated in parallel in the same experimental conditions were used to assess the cellular activity of catalase.

**Effect of blockade of tretinoin-induced increase in intracellular GSH content on stimulation of catalase activity.** Cells were preincubated for 1 h in control conditions or with 0.2 mM L-buthionine-(*S,R*)-sulfoximine (BSO), an irreversible inhibitor of  $\gamma$ -glutamyl-cysteine synthetase ( $\gamma$ -GCS), the rate-limiting step of glutathione synthesis (Griffith, 1982). Then, they were incubated for 24 h with or without 10  $\mu$ M tretinoin, and the catalase activity was measured. Cells incubated in parallel in the same experimental conditions were used to assess the intracellular GSH content.

**Effect of actinomycin D and cycloheximide on tretinoin-induced stimulation of antioxidant defenses.** In experiments using these inhibitors of mRNA or protein synthesis, respectively, cells were preincubated for 30 min under three different conditions: control (no inhibitors), 2  $\mu$ g/ml actinomycin D, and 10  $\mu$ M cycloheximide. Cells were washed with fresh medium and incubated during 24 h with or without 10  $\mu$ M tretinoin. Then, catalase activity and GSH content were measured.

**Effect of tretinoin on the expression of catalase and  $\gamma$ -GCS.** In experiments of dose and time responses to tretinoin, total RNA was extracted to study the expression of catalase and of the catalytic unit of  $\gamma$ -GCS, the rate-limiting step of glutathione synthesis (Griffith, 1982).

**mRNA stability.** Cells were treated with or without 10  $\mu$ M tretinoin for 24 h. Actinomycin D (2  $\mu$ g/ml) was added to the medium, and total RNA was isolated at different time points after treatment.

Northern blot analysis for catalase as well as semiquantitative cDNA amplification  $\gamma$ -GCS(catalytic subunit), normalization, and quantification were carried out as described below

**Prevention by Tretinoin of H<sub>2</sub>O<sub>2</sub>-Induced Cell Damage.** Fresh medium with 1 to 10  $\mu$ M tretinoin or its vehicle (ethanol at a final concentration of 0.09%) was added to quiescent cells cultured in 96-well microtiter plates (typically 20,000 cells/well). After a 24-h incubation, cells were washed with fresh medium and incubated for 24 h with 0 to 150  $\mu$ M H<sub>2</sub>O<sub>2</sub>. Cytotoxicity was quantified as described below by measuring both lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells into the supernatant and the ability of cells to reduce exogenous 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. The first test is a measure of plasma membrane integrity, whereas the second test detects living but not dead cells, as the tetrazolium ring is cleaved in active cells (McGahan et al., 1995)

**Effect of Tretinoin on Expression of OP and TGF- $\beta$ <sub>1</sub>.** Fresh medium with tretinoin or its vehicle (ethanol at a final concentration of 0.09%) was added to quiescent cells. Tretinoin effects on the expression of OP and TGF- $\beta$ <sub>1</sub> were studied in dose- and time-response experiments similar to those performed to stimulate antioxidant defenses.

### Analytic Procedures

Hematological parameters (red blood cell count, hemoglobin concentration, hematocrit, white blood cell count, and platelet count) were measured in a Coulter Counter (model Ssr), and serum parameters, including triglycerides, cholesterol, creatinine, glucose, GOT, GPT, bilirubin, sodium, potassium, calcium, and uric acid concentration, were analyzed in an Hitachi 717 (Boehringer Mannheim, Mannheim, Germany).

**DNA and Protein Content in Renal Cortex.** DNA in renal cortex was measured using the DNA-binding fluorochrome H33258 (purchased from Sigma Chemical) in aliquots of the renal cortex homogenate briefly sonicated (Labarca and Paigen, 1980). In this assay, EDTA prevents DNase activity. Aliquots of the homogenate were mixed with the homogenization buffer containing compound H33258 to a final concentration of 1  $\mu$ g/ml and fluorescent measurements were made in a scanning fluorescence spectrometer (model LS-5B; Perkin-Elmer), with the excitation wavelength set at 365 nm and the emission wavelength set at 460 nm. The DNA content of the samples was calculated from a standard curve made with calf thymus DNA standards at defined concentrations and expressed as mg DNA/g renal cortex. Protein was assayed (Lowry et al., 1951) and results were expressed as mg/g renal cortex.

**Catalase Activity and GSH Content.** Catalase activity was measured as follows (Aebi, 1983): In a quartz cuvette, 2 ml of sample [previously diluted adequately in phosphate buffer (KH<sub>2</sub>PO<sub>4</sub> 50 mM, pH 7.0) containing 0.2% Triton X-100] were added to 1 ml of 30 mM H<sub>2</sub>O<sub>2</sub>. Changes in absorbance at 240 nm were measured for 30 s. The rate constant of a first order reaction ( $k$ ) was used as a unit according to 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 absorbances at initial and final measurement points, respectively.

Cells for GSH content measurement were lysed in a cold room. Cellular proteins were precipitated with 0.9 ml of perchloric acid. After neutralization with 0.3 ml 1 M KOH/KHCO<sub>3</sub> and centrifugation, 0.15 ml of supernatant was collected on plastic tubes, *o*-phthalaldehyde was added (0.15 ml of 7.46  $\mu$ M solution), and tubes were incubated at room temperature for 15 min. GSH content was measured in a scanning fluorescence spectrophotometer (model LS-5B; Perkin-Elmer) at 420 nm with excitation wavelength of 350 nm, using a standard curve (Hissin and Hilf, 1976).

Results of catalase activity and GSH content were corrected by cellular protein, which was measured according to Lowry et al. (1951).

**mRNA Expression.** *Semiquantitative cDNA amplification of TGF- $\beta$ <sub>1</sub> and  $\gamma$ -GCS.* We used semiquantitative cDNA amplification

as a sensitive method to assess the level of TGF- $\beta$ <sub>1</sub> transcripts in the rat kidney cortex (Ruiz et al., 1998). For the purposes of semiquantification, PCR components were premixed (to generate master mixes) before addition to individual PCR tubes to minimize pipetting errors, and all samples underwent PCR at the same time in the same experiment. One microgram of total RNA was reverse transcribed in a total reaction volume of 20  $\mu$ l through incubation at 42°C during 30 min. All RT reactions used oligo(dT)<sub>15</sub>-primed RNA to minimize the variations in RT efficiency seen when using specific RT primers.

The reaction product was amplified by polymerase chain reaction (PCR) using a thermal cycler (MJ Research Inc., Watertown, MA). PCR conditions were determined in the next manner. First, PCR conditions were optimized, and then comparative kinetic analyses (Noonan et al., 1990; Salomon et al., 1992) were performed to determine the phase during which there was exponential generation of PCR product before reaching plateau. It was at this point that the PCR was terminated, allowing for semiquantitative data to be obtained. In summary, semiquantitative cDNA amplification for TGF- $\beta$ <sub>1</sub> transcripts was performed as follows: it was started with 4 min of denaturation at 94°C followed by 30 PCR cycles. Each cycle consisted of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. To quantify PCR products comparatively and confirm the use of equal amounts of the RNAs, we coamplified a house-keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hughes et al., 1995). Contamination was ruled out by the fact that PCR was negative when the reaction was performed without a prior reverse transcription (RT) reaction.

The upstream and downstream TGF- $\beta$ <sub>1</sub> primers were 5'-CTTCAGCTCCACAGAGAAGAAGTGC-3' and 5'-CACGATCATGTTG-GACAAGTCTCC-3', respectively, which yielded a single band corresponding to a 298-bp cDNA fragment (Qian et al., 1990). Analysis with cycle sequencing revealed that the sequence was identical with position 1266–1564 in rat TGF- $\beta$ <sub>1</sub> cDNA (Ruiz et al., 1998). The upstream and downstream GAPDH primer sequences were 5'-GTAAAGGGTTCGGTGTCAACGGATTT-3' and 5'-CACAGTCTTCT-GAGTGGCAGTGAT-3', respectively, which yielded a single band corresponding to a 558-bp cDNA fragment (Hughes et al., 1995). Analysis with cycle sequencing revealed that the sequence was identical with position 3–561 in rat GAPDH cDNA (Ruiz et al., 1998). After amplification, 8  $\mu$ l of each PCR mix was electrophoresed through a 2% (w/v) agarose gel with ethidium bromide (0.5  $\mu$ g/ml). Gels were photographed with Polaroid 55 negative film, with the reaction products on the negative film scanned by densitometry using an Image-store Color Onescanner and analyzed with NIH Image 1.55 Software. The TGF- $\beta$ <sub>1</sub>/GAPDH product ratio (with GAPDH used as an internal standard to correct for sample-to-sample variation in RNA degradation) was calculated and considered an index of TGF- $\beta$ <sub>1</sub> mRNA expression.

To quantify  $\gamma$ -GCS mRNA expression an RT-PCR, an assay was used in the same conditions as for TGF- $\beta$ <sub>1</sub>, with the only exception that annealing was achieved at 52°C rather than at 55°C and the number of PCR cycles was 24. Oligonucleotide primers were selected from the published sequence of  $\gamma$ -GCS catalytic subunit: the upstream and downstream sequences were 5'-GTCGTAAGTCTCAC-CAGAGTGATCCT-3' and 5'-TGATCCAAGTAACTCTGGACAT-TCACA-3', respectively, which yielded a single band corresponding to a 531-bp cDNA fragment (William MacNee, personal communication). To quantify PCR products comparatively and confirm the use of equal amounts of the RNAs, we coamplified a house-keeping gene,  $\beta$ -actin, with primer sequences obtained from Stratagene (catalog no. 302110), yielding a 245-bp fragment.

**Northern blot for catalase mRNA and OP mRNA.** Catalase and OP mRNA levels were measured in total RNA extracts isolated from cultured human MCs. OP mRNA levels were also measured in RNA samples from rat renal cortex. Total RNA was electrophoresed in a denaturing 1% agarose gel, transferred to a nylon membrane, and probed either with <sup>32</sup>P-labeled pCAT10, a purified human liver catalase cDNA clone obtained from American Type Culture Collection

(ATCC 57354; the 2.4-kb insert is contained within the *EcoRI* sites of pSP65), or <sup>32</sup>P-labeled-2B7, a rat OP cDNA (Giachelli et al., 1991). The filters were washed at 45°C once with 2× standard saline citrate (SSC) (1× SSC: 0.15 M NaCl, 0.015 M Na<sub>3</sub> citrate, pH 7.0), once with 1× SSC, and twice with 0.5× SSC containing 0.1% SDS. As a loading control, blots were also probed with a GAPDH probe. The blots were exposed to film (Kodak, XOMAT) for 6 to 24 h, and autoradiograms were scanned by densitometry as above. For quantification of the changes in catalase or OP mRNA expression, catalase/GAPDH or OP/GAPDH mRNA ratios, respectively, were used after densitometric estimation of each band.

**Cytotoxicity.** Plasma membrane integrity was monitored using the cytotoxicity detection kit (LDH) supplied by Boehringer Mannheim according to the manufacturer's instructions. The MTT assay was performed in cells incubated for 6 h with 12.5 μg MTT/100 ml. The blue formazan product was solubilized with 0.04 M HCl in isopropanol, and the plates were then read on a Micro-ELISA reader using a test wavelength of 595 nm (McGahon et al., 1995).

### Statistical Analyses

Data were compared using Student's *t* test, and *P* < .05 values were considered statistically significant. When multiple mean values were involved, an ANOVA was first performed followed by post hoc comparisons performed with a Bonferroni/Dunn test.

## Results

**General Condition of Animals.** Rats under tretinoin treatment showed comparable physical conditions, weight, and food intake as those of age-matched control rats. This finding rules out the possibility that tretinoin-containing food intake, due to its poor palatability, was lower than that in control animals (Teelmann et al., 1993), with this fact being sufficient to reduce the progression of age-related glomerulosclerosis (Yu et al., 1992). Toxicity (preclinical literature shows that it is substantially toxic when given in repeated oral doses (Teelmann et al., 1993) was also reasonably excluded at the end of the treatment because blood chemistry parameters and hematological parameters were not different from those found in the age-matched control rats (results are not shown). These results confirm previous findings in which no toxic effects were present in male rats treated chronically with 1 mg tretinoin/kg/day (Kurtz et al., 1984).

Furthermore, 18-month-old tretinoin-treated rats had a lower protein content in the renal cortex than did age-matched control animals (65.5 ± 8.3 mg protein/g tissue for control rats versus 47.1 ± 7.1 mg protein/g tissue for tretinoin-treated rats; Student's *t* test *P* < .01). No differences in the DNA content of this region were observed between the two experimental groups (2.5 ± 0.2 mg DNA/g tissue for control rats versus 2.4 ± 0.2 mg DNA/g tissue for tretinoin-treated rats; Student's *t* test *P* > .05).

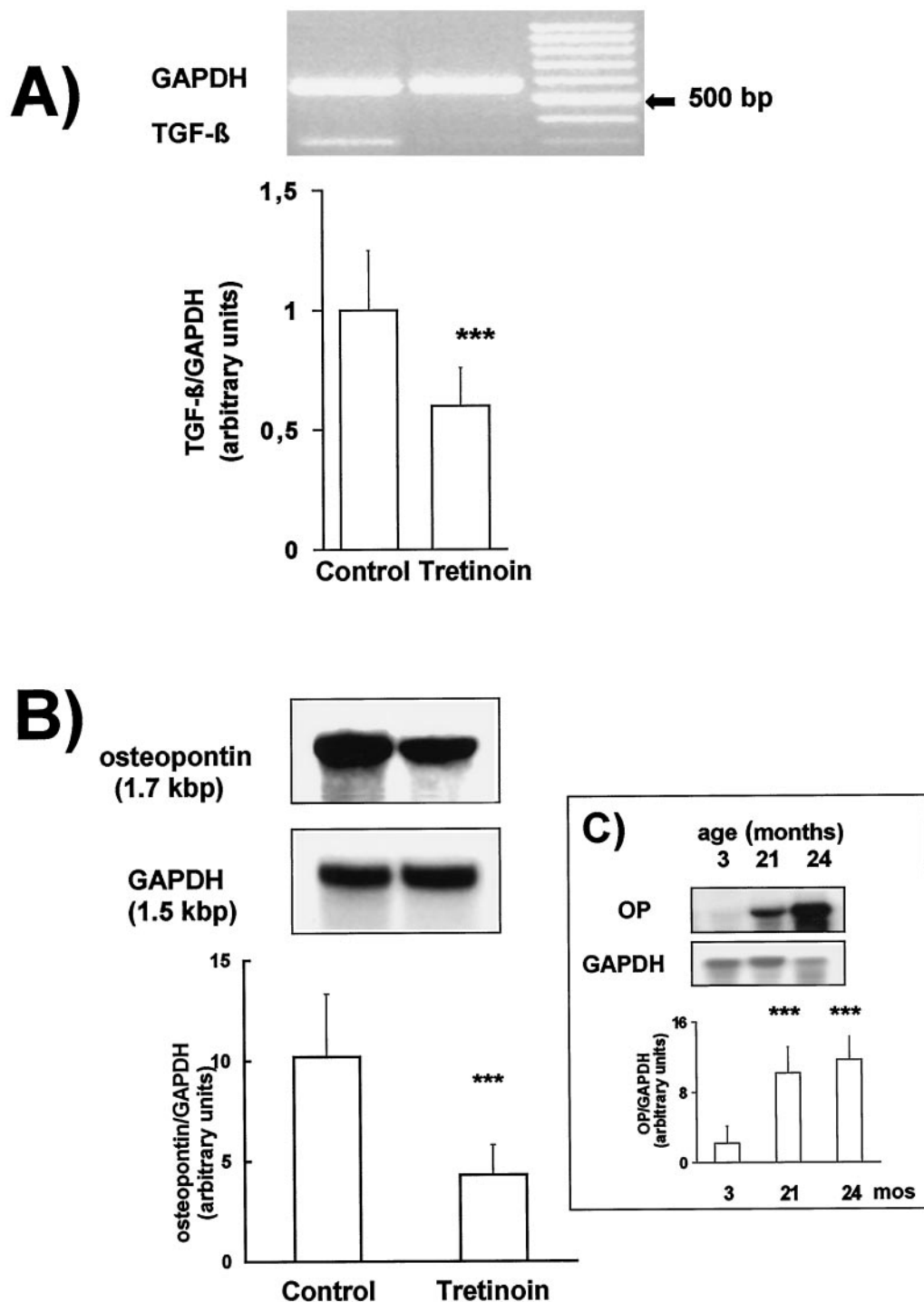
**Effect of Tretinoin on OP mRNA Expression and TGF-β<sub>1</sub> mRNA Expression.** Renal OP expression in older rats was significantly higher than that in 3-month-old animals (Fig. 1). The 18-month old rats that had been treated for 3 months with tretinoin had a statistically significant decrease in both OP mRNA expression and TGF-β<sub>1</sub> expression relative to GAPDH mRNA (Fig. 1). Interestingly, *in vitro* experiments showed that tretinoin inhibited, in a dose- and time-dependent manner, the OP expression in cultured human glomerular MCs (Fig. 2), whereas the retinoid had no effect on TGF-β<sub>1</sub> expression in these cells (results are not shown).

**Effect of Tretinoin on MC Antioxidant Defenses.** To explore the mechanism through which tretinoin prevents the effects of H<sub>2</sub>O<sub>2</sub> in cultured human renal MCs (Moreno et al., 1997), we first studied the catalase activity, and the GSH content: catalase is an obvious candidate to be involved because H<sub>2</sub>O<sub>2</sub> is its substrate. GSH is critical for another defense system against hydrogen peroxide and other peroxides: the glutathione peroxidases. Dose-response experiments (tretinoin concentrations tested were 0.1, 1, 5, 10, 25, and 50 μM) showed that these antioxidant defenses were stimulated within a narrow range of tretinoin concentrations: 1 μM tretinoin was the lowest effective concentration, whereas toxicity (in terms of LDH release, diminished reduction of MTT to formazan, and diminished cell count) appeared at 25 μM tretinoin (results are not shown). A concentration of 10 μM tretinoin was finally chosen for the time-response experiments because 1) it had the maximum stimulatory effect on the antioxidant defenses and 2) we had previously shown that 10 μM tretinoin prevents the cellular effects of H<sub>2</sub>O<sub>2</sub> on cultured human MCs (Moreno et al., 1997).

As shown in Fig. 3, 10 μM tretinoin had a stimulatory, time-dependent effect on both the activity of catalase and the intracellular GSH content of MCs. Moreover, when the availability of cysteine, one of the amino acids of the tripeptide glutathione (γ-GCS), was increased by the addition of NAC (and consequently the intracellular GSH content was 500% higher than in control cells), preincubation with 10 μM tretinoin still increased cell GSH by 1200% over control cells (Fig. 4). Overall, these results are consistent with the theory that tretinoin prevents the cellular actions of H<sub>2</sub>O<sub>2</sub> through a stimulatory effect on cell antioxidant defenses. In addition, we may reasonably exclude any contribution of retinoid intrinsic antioxidant properties because incubation of MCs with other lipid antioxidants such as *dl*-α-tocopherol did not result in any stimulatory effect on catalase activity or on GSH content (results are not shown).

**Interdependence between Tretinoin-Induced Increase in Catalase Activity and GSH Content.** Given the parallel behavior of catalase activity and GSH content after incubation with tretinoin, we tested the possibility that one of the changes was the consequence of the other. So, we first studied the possible effect of the tretinoin-induced stimulation of catalase activity on the increase of intracellular GSH content found after tretinoin incubation.

Experiments were carried out as described in Table 1: cells were first incubated with aminotriazole, an irreversible inhibitor of catalase activity (Aebi, 1983) that lowers this activity about 99% in MCs, and then with tretinoin. Under these conditions, tretinoin was still able to increase the intracellular GSH content to a similar extent as in the absence of aminotriazole. In a similar way, experiments were designed to study the possible effect of the tretinoin-induced increase of GSH content on the stimulation of catalase activity found after tretinoin incubation. Here, the inhibition of GSH synthesis was achieved by incubation with BSO, which reduces the mesangial GSH content about 60%. Under these conditions, tretinoin still retained its ability to stimulate cellular catalase activity (Table 1). In conclusion, the two effects observed in cells incubated with tretinoin (i.e., increase in both catalase activity and intracellular GSH content) do not appear to be dependent on each other but rather are directly induced by the retinoid.



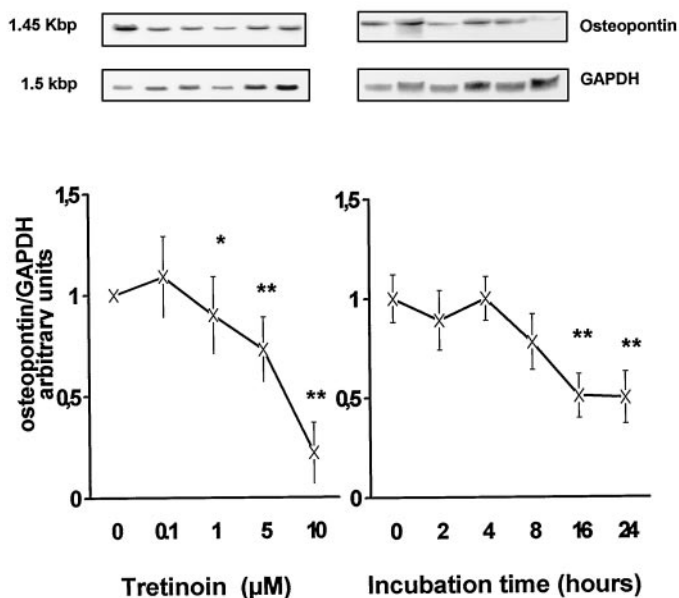
**Fig. 1.** Effect of tretinoin on the expression of TGF- $\beta_1$  and OP in the renal cortex of old rats. The 18-month-old male Fischer 344 rats were fed with standard chow (control,  $n = 15$ ) or with standard chow plus tretinoin (1 mg retinoid/kg b.wt./day; tretinoin,  $n = 15$ ) for a period of 90 days. RNA samples from the renal cortex were used to study the expression of TGF- $\beta_1$  (RT-PCR) and OP (Northern blot). A, semiquantitative cDNA amplification for TGF- $\beta_1$  transcripts. Inset, amplification product of one representative pair of samples, and the graph represents the mean  $\pm$  S.D. of the values normalized to GAPDH (whose expression was not significantly influenced by tretinoin treatment) and expressed as arbitrary units. B, Northern blot analysis of OP mRNA. C, a set of RNA renal samples from 3- and 24-month-old rats was used to further study the age-related changes in the expression of OP. The blots were stripped and rehybridized with a cDNA probe for the housekeeping gene GAPDH to adjust for small variations in RNA loading and transfer to the filter. The graphs represent the mean  $\pm$  S.D. of the values normalized to GAPDH and expressed as arbitrary units. \* $P < .001$  versus control.

It is also interesting to mention that the addition of tretinoin to the aminotriazole-treated cells or to the BSO-treated cells increased the catalase activity or the GSH content, respectively, back to levels of the untreated controls (Table 1).

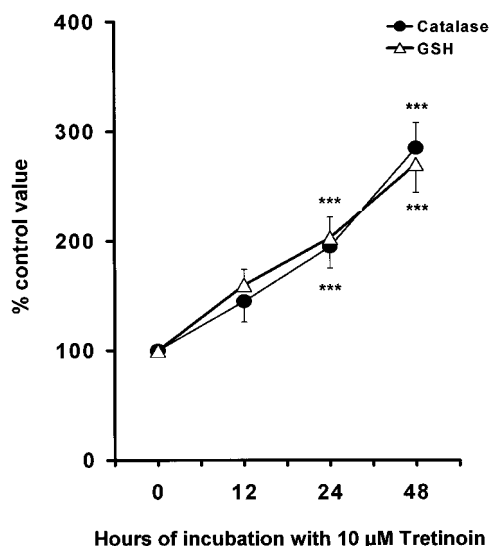
**Mechanism of Tretinoin-Induced Stimulation of Mesangial Antioxidant Defenses.** To explore the mechanism of tretinoin-induced stimulation of mesangial antioxidant defenses, we first observed the effects of RNA/protein synthesis inhibitors. We added actinomycin D for the inhibition of RNA synthesis and cycloheximide for the inhibition of

protein synthesis to the culture media. As shown in Table 2, actinomycin D and cycloheximide fully abolished the stimulatory effect of tretinoin on cell antioxidant defenses. These observations suggest that tretinoin-induced stimulation of mesangial antioxidant defenses requires de novo synthesis of responsible gene products. In fact, further experiments (Fig. 5) showed that tretinoin-treated cells had increased levels of both catalase mRNA and  $\gamma$ -GCS (catalytic subunit) mRNA, with this enzyme being the rate-limiting step in de novo GSH synthesis.

Because a change in mRNA stability could account for the

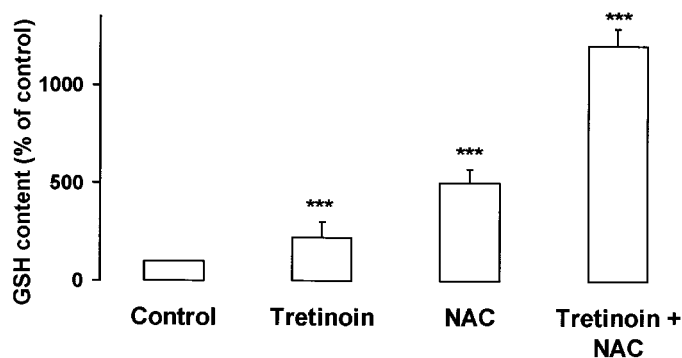


**Fig. 2.** Effect of tretinoin on OP mRNA expression in human MCs. Quiescent human MCs were incubated for 24 h with 0.1 to 10  $\mu\text{M}$  tretinoin (left) or with 10  $\mu\text{M}$  tretinoin for 0 to 24 h (right), and total RNA was harvested. Northern blotting (top) and its densitometric analysis (bottom) were carried out as described in *Materials and Methods*. The autoradiograms (top) are representative of four separate experiments. The graphs represents the mean  $\pm$  S.D. of the values normalized to GAPDH and expressed as arbitrary units. Densitometric analysis (bottom) showed a significant decrease in OP/GAPDH mRNA ratio from 1  $\mu\text{M}$  tretinoin in the dose-response experiments (left) and from 16-h incubation with 10  $\mu\text{M}$  tretinoin in the time-response experiments (right). \* $P < .05$  versus 0; \*\* $P < .01$  versus 0.



**Fig. 3.** Time-dependent effect of tretinoin on MC antioxidant defenses. Quiescent human MCs were incubated for 12 to 48 h with 10  $\mu\text{M}$  tretinoin or under control conditions. Catalase activity and GSH content were then measured and expressed as the percent of change of the respective control value (catalase activity measured in control cells at the experimental times chosen ranged between 44.3 and 50.6 mK/mg protein, whereas GSH content in these cells ranged between 21.3 and 25.4 nmol/mg protein). Results are mean  $\pm$  S.D. of four separate experiments in triplicate. \*\*\* $P < .001$  versus all the other values (within the same antioxidant defense system).

changes in steady state levels of both catalase mRNA and  $\gamma$ -GCS mRNA after retinoid treatment, we performed mRNA stability analyses in which cells were first treated with 10



**Fig. 4.** Effect of NAC on the tretinoin-induced increase in intracellular GSH content in human MCs. Quiescent human MCs were divided into four groups: two of them were incubated for 8 h, washed, and incubated for 16 h in control conditions (control) or with 10 mM NAC. The two remaining groups were incubated for 8 h with 10  $\mu\text{M}$  tretinoin, washed, and incubated for 16 h in control conditions (tretinoin) or with 10 mM NAC (tretinoin + NAC). GSH content was then measured and expressed as the percent of change of the control value ( $24.8 \pm 3.4$  nmol GSH/mg protein). Results are mean  $\pm$  S.D. of four separate experiments in triplicate. \*\*\* $P < .001$  versus all the other groups.

TABLE 1

Effect of inhibitors of catalase activity or GSH synthesis

Quiescent human MCs were preincubated for 1 h in one of these three conditions: control, 5 mM 3-amino-1,2,4-triazole, or 0.2 mM BSO. They were then incubated for 24 h with or without 10  $\mu\text{M}$  tretinoin. Results are mean  $\pm$  S.D. of four separate experiments in triplicate.

	Catalase Activity	GSH Content
	mK/mg protein	nmol/mg protein
Control	48.1 $\pm$ 4.9	23.6 $\pm$ 3.1
Aminotriazole	1.2 $\pm$ 1.1*	21.4 $\pm$ 3.2
BSO	47.2 $\pm$ 4.5	9.1 $\pm$ 2.2*
Tretinoin	102.3 $\pm$ 12.2*	52.6 $\pm$ 3.6*
Aminotriazole + tretinoin	33.6 $\pm$ 6.3	56.1 $\pm$ 6.3*
BSO + tretinoin	99.3 $\pm$ 10.6*	24.4 $\pm$ 3.5

\*  $p < .01$  versus control.

TABLE 2

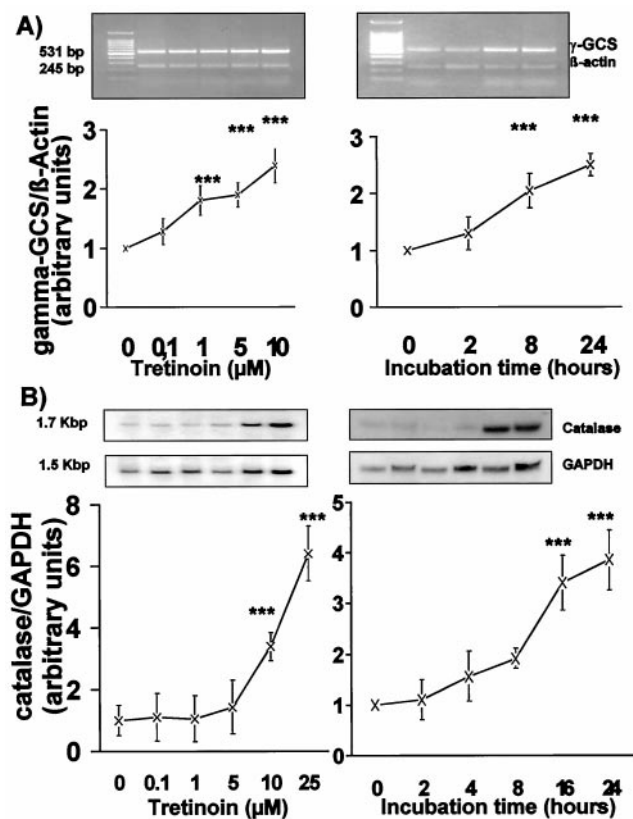
Effect of actinomycin D and cycloheximide on the tretinoin-induced stimulation of mesangial antioxidant defenses

Quiescent human MCs were preincubated for 30 min in three different conditions: control (no inhibitors), 2  $\mu\text{g/ml}$  actinomycin D, and 10  $\mu\text{M}$  cycloheximide. Cells were washed with fresh medium and incubated during 24 h with or without 10  $\mu\text{M}$  tretinoin. Then, catalase activity and GSH content were measured as described in *Materials and Methods*. Results are mean  $\pm$  S.D. of four separate experiments in triplicate.

	Catalase Activity	GSH Content
	mK/mg protein	nmol/mg protein
Control	47.2 $\pm$ 5.4	23.6 $\pm$ 3.1
Tretinoin	99.1 $\pm$ 8.2**	52.0 $\pm$ 5.6**
Actinomycin D	40.1 $\pm$ 3.8	18.1 $\pm$ 2.2
Cycloheximide	35.4 $\pm$ 3.6	16.2 $\pm$ 2.7*
Actinomycin D + tretinoin	40.4 $\pm$ 6.5	19.6 $\pm$ 1.9
Cycloheximide + tretinoin	37.7 $\pm$ 4.6	17.8 $\pm$ 2.1

\*  $p < .05$  versus control; \*\*  $p < .01$  versus control.

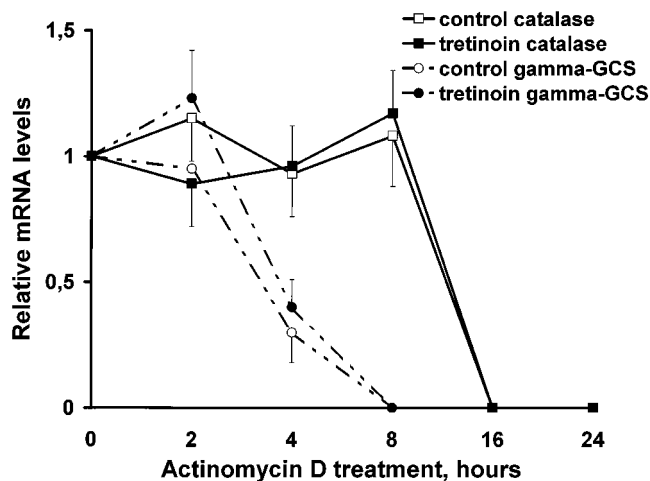
$\mu\text{M}$  tretinoin or its vehicle for 24 h and then treated with actinomycin D to block further mRNA synthesis. We then measured the decay time for catalase mRNA and  $\gamma$ -GCS mRNA by Northern blot analysis and RT-PCR, respectively. No statistically significant differences were found between the decay times in tretinoin-treated cells and their respective controls (Fig. 6). These observations suggest that an increased gene transcription, but not a change in mRNA stability, is the most likely mechanism involved in the tretinoin-induced increase in the levels of catalase mRNA and  $\gamma$ -GCS



**Fig. 5.** A, effect of tretinoin on the expression of  $\gamma$ -GCS (catalytic subunit) in human MCs. Quiescent human MCs were incubated for 24 h with 1 to 25  $\mu$ M tretinoin (left) or with 10  $\mu$ M tretinoin for 0 to 24 h (right), and total RNA was harvested. Top, RNA was reverse transcribed and used for PCR analysis of  $\gamma$ -GCS mRNA. Bottom, insets (top) show the amplification product obtained after RT-PCR assay of one representative pair of experiments, and the graphs (bottom) represent the mean  $\pm$  S.D. of the values of the densitometric analysis, obtained from four separate experiments, normalized to  $\beta$ -actin (whose expression was not significantly influenced by tretinoin treatment). The results showed a significant increase in  $\gamma$ -GCS/ $\beta$ -actin from 1  $\mu$ M tretinoin in the dose-response experiments (left) and from 8-h incubation with 10  $\mu$ M tretinoin in the time-response experiments (right). B, effect of tretinoin on catalase mRNA expression in human MCs. Quiescent human MCs were incubated for 24 h with 1 to 25  $\mu$ M tretinoin (left) or with 10  $\mu$ M tretinoin for 0 to 24 h (right) and total RNA was harvested. Northern blotting (top) and its densitometric analysis (bottom) were carried out as described in *Materials and Methods*. The autoradiograms (top) are representative of four separate experiments. The graphs represent the mean  $\pm$  S.D. of the values normalized to GAPDH and expressed as arbitrary units. Densitometric analysis (bottom) showed a significant increase in catalase/GAPDH mRNA ratio from 10  $\mu$ M tretinoin in the dose-response experiments (left) and from 16-h incubation with 10  $\mu$ M tretinoin in the time-response experiments (right). For dose-response experiments: \*\*\* $P$  < .001 versus all the other doses. For time-response experiments: \*\*\* $P$  < .001 versus 0 h.

mRNA and, therefore, in the tretinoin-induced stimulation of mesangial antioxidant defenses.

**Prevention by Tretinoin of  $H_2O_2$ -Induced Cell Damage.**  $H_2O_2$  induced a dose-dependent damage in MCs, characterized by both loss of ability to reduce MTT and plasma membrane damage, resulting in the loss of cytosolic LDH (Fig. 7A). These  $H_2O_2$  effects were clearly abolished when cells were previously incubated with 10  $\mu$ M tretinoin, with this beneficial action confirmed both by morphological examination of cells under light microscopy and by trypan blue exclusion studies (results are not shown). Protection afforded by tretinoin preincubation was directly related to the retinoid



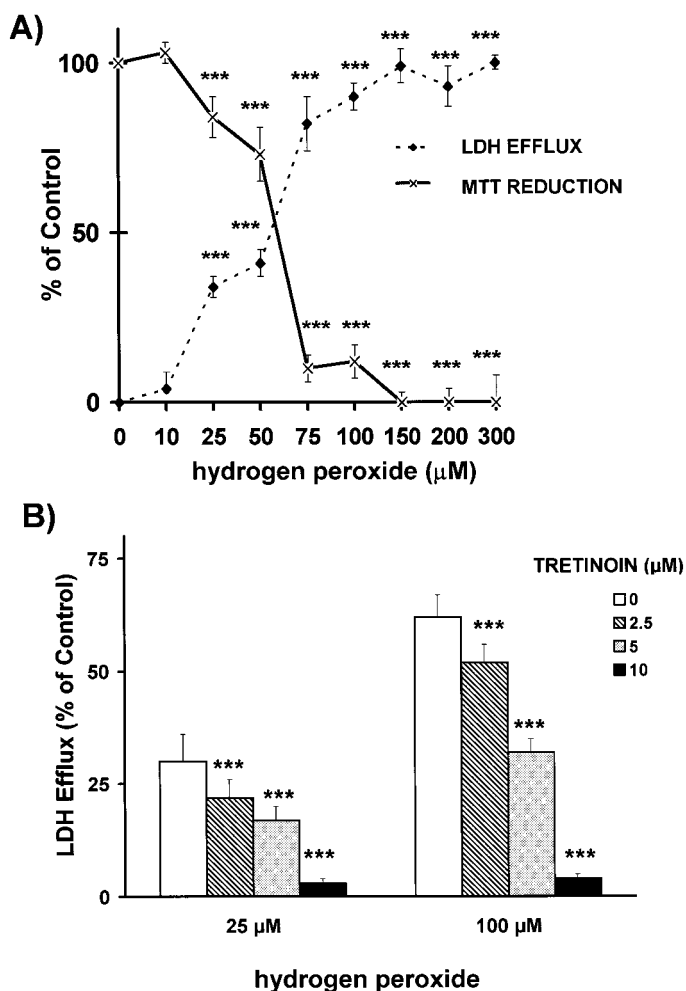
**Fig. 6.** Effects of tretinoin on catalase mRNA stability and  $\gamma$ -GCS mRNA stability. Quiescent human MCs were incubated for 24 h with 10  $\mu$ M tretinoin or under control conditions and then treated with actinomycin D (2  $\mu$ g/ml) for different time points. Total RNA was harvested and used for Northern blot analysis (catalase) or for RT-PCR assay ( $\gamma$ -GCS). Catalase RNA levels were normalized to GAPDH. In the same way,  $\gamma$ -GCS (catalytic subunit) cDNA levels were normalized with a coamplified house-keeping gene,  $\beta$ -actin. Results from the densitometric analysis were expressed as arbitrary values and plotted relative to the zero time point. Some standard deviation bars have been omitted for clarity. Catalase mRNA was quite stable in both control- and tretinoin-treated cells, with a half-life of >8 h in both cases.  $\gamma$ -GCS mRNA stability was also similar between control and tretinoin-treated cells, but the mRNA half-life was <4 h.

dose within a narrow range: 10  $\mu$ M tretinoin had the maximum protective action against  $H_2O_2$  and 2.5  $\mu$ M tretinoin was the lowest dose with a protective effect (Fig. 7B).

## Discussion

Renal function in humans declines with advancing age. The classic structural finding with aging is glomerulosclerosis, leading to complete glomerular obsolescence and glomerular dropout. This bleak outlook, however, has been challenged by the results of the Baltimore Longitudinal Study, which shows that an age-dependent fall in glomerular filtration rate is not inevitable (Lindeman, 1990).

Taken into account that the age-related renal damage may be inevitable, a number of treatments to prevent it have been tested in the rat model. We have previously shown (Moreno et al., 1997) that tretinoin slows the progression of age-related glomerular changes in male Fischer 344 rats (in 18-month-old rats of this strain, we have described that glomerular protein content is about 3-fold higher than that in 3-month-old rats (Ruiz et al., 1994). Other early age-associated changes in 18-month-old rats include increased glomerular production of  $H_2O_2$  and increased expression of TGF- $\beta_1$  in the renal cortex (Ruiz et al., 1994, 1998)). Here, we confirm these previous findings: 18-month-old rats treated with tretinoin have a lower protein content in the renal cortex than do control animals, whereas no differences in the DNA content of this region were observed between the two experimental groups (see *Results*). Because the DNA values indicate that cell numbers in the renal cortex were similar between the two groups of animals, the higher protein content of this region in the control rats could reflect an expansion of the protein in the extracellular matrix and/or a high cellular



**Fig. 7.** Prevention by tretinoin of H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity. A, effect of increasing concentrations of H<sub>2</sub>O<sub>2</sub> on the viability of MCs: Confluent, quiescent human MCs, cultured in 96-well microtiter plates, were incubated for 24 h with 0 to 300 μM H<sub>2</sub>O<sub>2</sub>. Cytotoxicity was quantified by measuring both LDH efflux from the cytosol of damaged cells to the supernatant and the ability of cells to reduce exogenous MTT to formazan. Results were expressed as the percent of change of the respective control value (LDH released by control cells incubated with 2% Triton X-100 was considered as 100% LDH efflux; formazan reduced from MTT by control cells after 6-h incubation was considered as 100% MTT reduction) and are the mean ± S.D. of four separate experiments in triplicate. As shown, H<sub>2</sub>O<sub>2</sub> significantly diminished MC viability in a dose-dependent way (ANOVA, \*\*\**P* < .001). B, prevention by tretinoin, in a dose-dependent way, of H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity. Confluent, quiescent human MCs were incubated with different concentrations of tretinoin during 24 h. Cells were then washed and incubated 24 h with different concentrations of H<sub>2</sub>O<sub>2</sub>. LDH efflux to the medium was then measured and expressed as above. Results are the mean ± S.D. of four separate experiments in triplicate. Statistical analysis showed that tretinoin significantly attenuated, in a dose-dependent way, H<sub>2</sub>O<sub>2</sub>-mediated cytotoxicity (ANOVA, \*\*\**P* < .001).

protein content (Ruiz et al., 1994). These results suggest that tretinoin may slow the rate of progression of glomerulosclerosis acting on the balance synthesis/degradation of extracellular matrix and/or on the cellular hypertrophic changes found as aging progresses.

We had hypothesized that the action of tretinoin involved an inhibitory effect on the expression of OP and TGF-β<sub>1</sub>. The expression of OP has been reported to be up-regulated in a diseased kidney before macrophage infiltration and increased fibrosis (Narita et al., 1997), and it is predominantly

expressed in chronic and progressive glomerulosclerosis (Floege et al., 1997). Increased expression of TGF-β<sub>1</sub> in the renal cortex of Fischer 344 rats during normal aging (Ruiz et al., 1998) may account for glomerulosclerosis because TGF-β<sub>1</sub> promotes synthesis of extracellular matrix proteins and induces inhibitors of metalloproteinases (Sharma and Ziyadeh, 1994). Our results indicate that the expression of OP also increases in renal cortex (Fig. 1) during normal aging (therefore, this increase may be related to the progression of age-related glomerulosclerosis) and that tretinoin inhibits this increase (Fig. 1). Moreover, this inhibition has also been specifically found in cultured human MCs from the renal cortex (Fig. 2), a fact that suggests that these cells may be one of the targets of the inhibitory effect of tretinoin in vivo. Renal cortex samples from tretinoin-treated rats also had lower TGF-β<sub>1</sub> expression than the corresponding samples from age-matched control rats (Fig. 1). The retinoid had no effect on the TGF-β<sub>1</sub> expression of cultured human MCs (see Results), this suggesting that in opposition to OP, these cells may not be the target for the inhibitory in vivo effect of tretinoin on TGF-β<sub>1</sub>. In summary, the inhibitory effect on OP expression, with OP being a protein with cell adhesive and chemotactic properties (Giachelli et al., 1995), and on TGF-β<sub>1</sub> expression, which is a matrigenic cytokine, in old rats may be an important mechanism through which the retinoid slows the progression of age-related renal cortex changes. However, immunostaining for TGF-β<sub>1</sub> and OP should be performed 1) to confirm that tretinoin inhibits not only the mRNA expression but also the production of the respective proteins and 2) to show specific localization of TGF-β<sub>1</sub> and OP in glomeruli (thus eliminating any concern about material measured in renal cortex being in tubular cells or urine). In addition, our results suggest that although tretinoin toxicity is not negligible, a rational treatment may provide significant benefits in the prevention of age-related renal changes.

Regarding the in vitro studies, we previously described that preincubation with tretinoin abolishes H<sub>2</sub>O<sub>2</sub>-dependent cytotoxicity in cultured human MCs (Moreno et al., 1997). This action could be useful in the treatment of several forms of renal damage because 1) both glomeruli and cultured glomerular MCs from 18-month-old rats produce increased amounts of H<sub>2</sub>O<sub>2</sub> and there is an augmented oxidative damage in glomeruli (Ruiz et al., 1994, 1996) during aging and 2) the production of H<sub>2</sub>O<sub>2</sub> might result in the death of glomerular cells found during the late stages of the sclerosis of the renal glomeruli (Schlondorff, 1995).

To elucidate the mechanisms involved in the prevention of H<sub>2</sub>O<sub>2</sub>-induced cell responses, we first studied catalase activity and GSH content: with H<sub>2</sub>O<sub>2</sub> being its substrate, catalase is an obvious candidate to be involved in the protective effect of tretinoin (Aebi, 1983). In addition, GSH is critical for another defense system against H<sub>2</sub>O<sub>2</sub> and other peroxides: the glutathione peroxidases (Deneke and Fanburg, 1989). Our results confirmed that tretinoin was able to induce a dose- and time-dependent increase in both the catalase activity and the GSH content in cultured human renal MCs (Fig. 3). The possibility that an antioxidant intrinsic activity of tretinoin could partially contribute to the increase in the antioxidant defenses through a "saving effect" was ruled out because cells incubated with *dl*-α-tocopherol had no changes in these defenses (results are not shown).

We tested then the possibility that one of the changes in these antioxidant systems was the consequence of the other. Experiments in cells preincubated with aminotriazole, an inhibitor of catalase, showed that tretinoin increased the GSH content to the same extent as that found in the absence of aminotriazole (Table 1). Tretinoin also retained its ability to stimulate catalase activity in cells preincubated with BSO, an inhibitor of GSH synthesis (Table 1). In conclusion, the increases in both catalase activity and intracellular GSH content do not appear to be dependent on each other but rather directly induced by the retinoid.

Interestingly, the addition of tretinoin to aminotriazole- or BSO-treated cells increased the catalase activity or the GSH content, respectively, back to the untreated control levels (Table 1). These results may be reflecting the synthesis de novo of catalase and GSH induced during 24 h by the retinoid after washing out the inhibitors and/or interference of tretinoin with the ability of aminotriazole and BSO to inhibit catalase activity or  $\gamma$ -GCS, respectively.

There is evidence suggesting that tretinoin-induced stimulation of mesangial antioxidant defenses requires de novo synthesis of responsible gene products. First, the relative levels of oxidized and GSH are regulated by a series of coupled reactions involving glutathione peroxidase, glutathione reductase, and glucose-6-phosphate dehydrogenase (Deneke and Fanburg, 1989). Under normal conditions, the balance of the equation is far in the direction of maintaining cellular glutathione in its reduced state: GSH >99% (Deneke and Fanburg, 1989). Taken into account this value, it seems unlikely that the tretinoin-induced increase in cell GSH content, which may reach 250%, could be primarily dependent on an increased activity of the enzymes directly or indirectly involved in the reduction of oxidized glutathione. In fact, when the availability of cysteine, one of the amino acids of the tripeptide glutathione ( $\gamma$ -GCS), is increased by the addition of NAC and, consequently, the intracellular GSH content is 500% higher than that in control cells, preincubation with 10  $\mu$ M tretinoin still increases cell GSH by 1200% over control cells (Fig. 4). Second, tretinoin-induced stimulation of mesangial antioxidant defenses was fully inhibited by either actinomycin D or cycloheximide (Table 2). Third, the levels of catalase mRNA and  $\gamma$ -GCS (catalytic subunit), the initial and rate-limiting enzyme in the glutathione de novo synthesis pathway (Deneke and Fanburg, 1989), were indeed found to be increased after incubation of MCs with tretinoin (Fig. 5). Fourth, stability analyses of catalase mRNA and  $\gamma$ -GCS mRNA showed that both mRNAs are no more stable in tretinoin-treated cells than they are in control cells (Fig. 6).

These observations suggest that an increased gene transcription of catalase mRNA and  $\gamma$ -GCS mRNA is directly related to the tretinoin-induced stimulation of mesangial antioxidant defenses. This mechanism is not a new one, given that retinoid signaling mechanisms are based on the intervention on the gene expression at the transcriptional level (Giguere, 1994). On the other hand, the transcription, expression, and increasing activity of catalase after ionizing radiation or oxidant stress are well known (Heintz et al., 1991; Hardmeier et al., 1997). The same is true for the catalytic subunit of  $\gamma$ -GCS (Rahman et al., 1996) The interest of our finding is that it provides one strategy by which to protect renal cells from H<sub>2</sub>O<sub>2</sub>-mediated injury: to augment cell anti-H<sub>2</sub>O<sub>2</sub> activity levels by increasing their GSH content

and their catalase activity through tretinoin treatment. In fact, there is a clear association between the dose-response stimulation of cell antioxidant defenses and the dose-dependent preventive action of tretinoin against the H<sub>2</sub>O<sub>2</sub> cytotoxicity (Fig. 7).

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