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Oxidants induce transcriptional activation of manganese superoxide dismutase in glomerular cells

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Oxidants induce transcriptional activation of manganese superoxide dismutase in glomerular cells. Cultured rat glomerular mesangial and epithelial cells and bovine glomerular endothelial cells were exposed to various concentrations of hydrogen peroxide (H_2O_2). Mesangial cells treated with 10 to 100 μM H_2O_2 for 24 hours showed a two- to ninefold increase in Mn-SOD mRNA expression associated with significantly ($P < 0.005$) increased Mn-SOD activity (22.2 ± 1.2 and 12.2 ± 0.7 μ/mg protein for H_2O_2 100 μM treated and untreated cells, respectively). In contrast, expression of Cu-Zn SOD and β-actin mRNA was not affected by H_2O_2. Induction of Mn-SOD mRNA by H_2O_2 was inhibited by actinomycin-D (4 μM) treatment. Glomerular endothelial cells also showed an increase in Mn-SOD mRNA expression following 100 μM H_2O_2 treatment, as did glomerular epithelial cells following treatment with 500 and 1000 μM H_2O_2 but not with 100 μM. Transcriptional activity of the Mn-SOD gene was assessed with a fusion reporter gene consisting of a luciferase gene (pGL2P) and a 1.2 kb fragment from the rat Mn-SOD genomic DNA (−806 to +408 bp of the transcription initiation site, −806:+408). The construct was transfected into rat glomerular mesangial and epithelial cells. Mesangial and epithelial cells transfected with pGL2P (−806:+408) and treated with H_2O_2 (100 μM and 1 mM for mesangial and epithelial cells, respectively) demonstrated some threefold increase in luciferase activity, whereas cells transfected with pGL2P lacking the Mn-SOD fragment did not show changes in luciferase activity following H_2O_2 treatment. Other oxidants, namely α- and β-naphthoflavone (50 μM to mesangial cells) and puromycin aminonucleoside (25 to 50 μg/ml to epithelial cells), also induced transcriptional activation (2- to 5-fold increase) in these cells. Thus, Mn-SOD levels in glomerular cells are enhanced when they are exposed to oxidant stress, and this up-regulation involves transcriptional activation. Further, the Mn-SOD gene contains enhancer element(s) which respond to diverse oxidant stress. The inducibility by oxidants of local Mn-SOD demonstrates that glomerular SOD may play a decisive role in the pathogenesis of glomerular injuries in which the balance between oxidants and antioxidants is critical.

Analogous to infectious diseases, the manifestation of renal diseases is determined by the dynamic balance between the pathogen and intrinsic defense mechanisms. A major cellular mechanism for eliminating reactive oxygen metabolites is the antioxidant enzyme cascade [1–3]. Among several major antioxidant enzymes, superoxide dismutases (SODs) are found mainly in the intracellular compartment. They exist as Mn-containing proteins primarily in the mitochondrial matrix and as Cu/Zn-containing enzymes primarily in the cytoplasm [1–3].

Convincing evidence for the beneficial consequence of an activating endogenous SOD was provided, although still in preliminary form, in a study of transgenic mice carrying the human SOD gene: Morphologic analysis of the kidney following experimental transient ischemia/reperfusion in these mice revealed a distinctive pattern of tissue protection from injury [4]. Moreover, both prokaryotic and eukaryotic cells appear to be endowed with the ability to exploit this benefit. Thus, following exposure to a variety of nonlethal oxidant stresses, cellular SOD activity often increases along with resistance to otherwise lethal doses of the same oxidant stress [5–7]. This phenomenon has been demonstrated recently *in vivo* [8]. Glomeruli isolated from rats subjected to ischemia/reperfusion had elevated levels of Mn-SOD, glutathione peroxidase, and catalase [8]. Rats in which glomerular antioxidant enzyme activities were raised by ischemia/reperfusion were then rechallanged later with either ischemia/reperfusion or intrarenal arterial infusion of hydrogen peroxide [8]. Whereas in normal control rats these experimental manipulations induced severe reductions in GFR and massive proteinuria, respectively, rats with elevated antioxidant enzymes were protected from these functional deteriorations.

Given the ubiquitous nature of oxidants recognized today as renal pathogens, the potential physiologic and pathophysiologic importance of intrinsic antioxidant enzymes is paramount [9]. Although it is well-known that prokaryotic cells can respond to oxidants with an induction of their endogenous antioxidant enzymes, it has yet to be established whether eukaryotic cells or mammalian cells possess a similar effective defense mechanism against oxidants. In this regard, the possibility remains that the above-described increase in renal antioxidant enzymes following ischemia/reperfusion *in vivo* may represent a response of the kidney to volume expansion (rather than oxidants *per se*), a result of the renal injury caused by the initial oxidant insult, although several enzymes or hormones unrelated to antioxidants are known to be modulated by oxygen tension or oxidant stress [10, 11].

In the present study, therefore, we investigated the capacity of the glomerular cells to respond to oxidants with induction of one of the important antioxidant enzymes, Mn-SOD, in cultured glomerular cells.

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Methods

Materials

RPMI 1640, balanced salt solutions, fetal bovine serum (FBS), penicillin/streptomycin, and amphotericin B were purchased from GIBCO (Grand Island, New York, USA); diethyl pyrocarbonate (DEPC) from Fluka Chemika (Buchs, Switzerland); restriction enzymes, Taq DNA polymerase, pGL2 promoter plasmid, pSV β-Galactosidase plasmid, agarose (free RNase) from Promega (Madison, Wisconsin, USA); Guanidinium thiocyanate/phenol solution for RNA extraction from Tel-Test (Friendwood, Texas, USA); [^{32}P] and [^{35}S] ATP from New England Nuclear (Boston, Massachusetts, USA); Dulbecco's Modified Eagle's Medium-F12 (DMEM-F12) and other reagents were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA) unless described otherwise.

Glomerular cell culture

Glomerular cell cultures for mesangial, epithelial, and endothelial cells were established as follows.

Mesangial and epithelial cells were isolated from glomeruli of rat kidneys as previously described by us [12]. Briefly, the renal cortex from male Sprague-Dawley rats was minced and passed through a series of stainless steel sieves of decreasing pore size: 180, 106 and 75 μM. The fraction accumulating on the 75 μm sieve was collected in HBSS buffered with 20 mM Hepes and washed three times with buffered HBSS. Microscopically, the glomeruli were shown to be stripped of their capsules and virtually free of tubule tissue. The glomeruli were treated with collagenase (Type I from *clostridium cristolytium,* 1 mg/ml) for 15 minutes and then plated onto 100-mm (P100) plastic tissue culture plates maintained in RPMI 1640 supplemented with 17% FBS, penicillin 100 μ/ml, streptomycin 100 μg/ml, fungizone 0.25 μg/ml at 37°C in 5% CO_2, and 95% air.

When glomerular cells were grown without selection, mesangial cells were propagated, and they were subcultured using trypsin/EDTA. The cells were characterized by their shapes and immunochemical staining of actin. Passages between 5 and 18 were used in the experiments. The cells were maintained in RPMI 1640 with 14% FBS medium all the time except during the transfection of plasmids as specified below.

Glomerular epithelial cells were established from the above-described primary culture of glomeruli [13]. Briefly, colonies of cells with polyhedral appearance were isolated with cloning cylinders, trypsinized, and propagated. Clones of epithelial cells were identified by the lack of angiotensin I converting enzyme activity and immunofluorescent staining of actin and by the presence of domes. Cells were maintained in RPMI 1640 with 14% FBS at all times except during the transfection of plasmids as specified below.

Since glomerular endothelial cells are not readily established from rat kidneys, they were isolated from a bovine kidney as previously described [14]. Briefly, fresh young calf kidneys were purchased from a local slaughterhouse (Tennessee Dressed Beef, Nashville, Tennessee, USA). Approximately 20 g of cortex were excised, minced and passed through 180 and 140 μm sieves. The glomeruli on the surface of the 140 μm sieve were collected. This glomerular preparation was routinely >98% pure, and 80 to 85% of glomeruli were free of Bowman's capsules. Glomeruli were then washed three times with HBSS and partially digested in a HBSS buffered with 20 mM Hepes and 1 mg/ml collagenase. After collagenase digestion, glomeruli were washed and suspended in a RPMI 1640 medium containing 17% FBS, 2% Nu serum, 5 U/ml penicillin, 5 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (endothelial cell medium) at 37°C in 5% CO_2, and 95% air. Cells were plated on gelatin-fibronectin-coated P100 at a density of 50 to 100 cell/ml. When colonies that morphologically resembled glomerular endothelial cells appeared, individual clones were isolated with cloning cylinders, transferred to a 24-well plate and propagated in the endothelial cell medium. For subculture, RPMI 1640/14% FBS medium was used. Cells from passages between 5 and 16 were used for the subsequent experiments. Each clone of endothelial cells was identified by expression of factor VIII and endocytosis of acetylated low density lipoprotein (LDL). To examine expression of factor VIII, cloned cells were grown to confluence on chamber slides fixed in acidic methanol and subjected to indirect immunofluorescence using rabbit anti-human factor VIII-related antigen [14]. The endocytosis of acetylated LDL was visualized by labeling the acetylated LDL with fluorescent 1.1′-dictadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate, Dil-Ac-LDL. To visualize cell-associated Dil-Ac-LDL, cells grown on chambered glass slides were incubated for three to four hours at 37°C in RPMI 1640 containing 15% FBS and 3 μg/ml Dil-Ac-LDL and examined for fluorescence.

Measurement of SOD activities in mesangial cells treated with H_2O_2

To examine whether exposure to oxidants induces alteration in cellular AOE levels, mesangial cells were treated with H_2O_2 and cellular SOD activities were determined. In this study, near confluent glomerular mesangial cells in P100 were incubated with a medium containing 100 μM H_2O_2 for 24 hours. Control cells were incubated with a fresh medium for the same duration. At the end of incubation, plates were washed three times with ice-cold phosphate-buffered saline (PBS), removed from plates by a rubber policeman, and suspended in 1 ml per plate of PBS. Cells from three P100 plates were combined into one tube to obtain a sufficient amount of sample for the subsequent assays. The cell suspensions thus obtained were sonicated (Output power 7, 5 sec × 3, W-225 Sonicator, Heat Systems-Ultrasonics, Inc., Farmingdale, New York, USA), centrifuged (800 g, 15 min, 4°C), and the supernatants were immediately frozen and stored at −70°C until assay.

Activities of superoxide dismutases (SODs) were measured based on the inhibition of a superoxide-mediated reduction of cytochrome c by SOD, as previously described [15, 16]. Cyanide-uninhibitable activity (activity measured with 1 mM potassium cyanide) was regarded as Mn-SOD activity, whereas total SOD was determined by a reaction mixture with a low concentration of cyanide (10 μM), and Cu/Zn-SOD activity was calculated by total minus Mn-SOD activity [16]. The amount of SOD required to inhibit the rate of reduction of cytochrome c by 50% was defined as 1 unit of activity.

mRNA expression study

To examine whether oxidants alter Mn-SOD mRNA expression in glomerular cells, Northern analysis was performed. In this study, near confluent cells in P100-dishes were incubated with H_2O_2 (1 to 100 μM for mesangial and endothelial cells; 100 to

1000 μM for epithelial cells) for 24 hours in an identical manner to that described above for the study of enzyme activities. In some experiments with mesangial cells, a transcriptional inhibitor, actinomycin D (4 μM), was added to the culture medium during incubation with H_2O_2. This concentration of actinomycin D has been shown to inhibit endotoxin-induced Mn-SOD mRNA transcription but not basal expression in pulmonary epithelial cells [17]. After the completion of incubation, cells were washed twice with ice-cold PBS, and total RNA was isolated by the guanidinium thiocyanate/phenol method as previously described [18]. The concentration of RNA was estimated by absorption at 260 nm, and the samples were stored at −70°C until the assay.

For Northern analysis, 20 μg of total RNA were denatured in 25 μl of 50% formamide, 17.5% formaldehyde, 20 mM MOPS, pH 7.0, 5 mM sodium acetate, 0.5 mM EDTA, and 0.3% ethidium bromide at 65°C for 10 minutes. The RNA was electrophoresed on a 1% agarose, 6% formaldehyde, MOPS buffer gel [17], transferred to a noncharged nylon membrane (Genescreen, Dupont, Albany, Massachusetts, USA) and covalently cross-linked to the membrane with UV light [17]. The membrane was prehybridized in 0.45 M sodium phosphate, 6% sodium dodecyl sulfate, 1 mM EDTA, and 1% bovine serum albumin for four hours, and then hybridized (16 to 18 hr, 65°C) with a ^{32}P-labeled cDNA of rat Mn-SOD, Cu-Zn SOD for rat mesangial and epithelial cell mRNA, and bovine Mn-SOD cDNA for bovine glomerular endothelial cells. Rat Mn-SOD cDNA (1.6 kb) and Cu/Zn-SOD cDNA (0.6 kb) have been previously isolated and sequenced and were provided to us by Dr. Nick of the University of Florida [17, 19]. Bovine Mn-SOD cDNA was cloned by us and is described elsewhere [20]. The membranes were also hybridized with human β-actin cDNA (Clontech, Palo Alto, California, USA) as an index of RNA loading consistency. All probes were labeled by a random primer extension method (Bethesda Research Laboratory, Gaithersberg, Maryland, USA). Following hybridization, the membranes were washed in 0.04 M sodium phosphate in 1% SDS at 65°C (30 min, 3 times) and subjected to autoradiography using an intensifying screen at −70°C (1 to 4 days). The blots obtained were semiquantitated by densitometry using Bio-Rad video densitometry Model 620 (Hercules, California, USA).

Construction of reporter genes fused with rat Mn-SOD 5′-regulatory region

To evaluate the transcriptional control of the Mn-SOD gene by hydrogen peroxide, a fusion gene was constructed, containing a luciferase expression vector and a 5′ fragment of a Mn-SOD genomic DNA from a Sprague-Dawley rat. Thus, based on the previously published genomic sequence [21], primers for PCR (TGGAGAATTCAGTGGCAGAGGAAAGCTGC for sense and AGGTGAATTCGTGGTACTTCTCCTCGGTG for antisense) were prepared and a 1.2 kb fragment of a rat Mn-SOD genomic segment (−806 to +408 bp of transcription initiation site) was amplified with Taq DNA polymerase using a thermal cycler (PTC100, MJ Research, Inc., Watertown, Massachusetts, USA). Following determination of the 1.2 kb band by agarose gel electrophoresis, the amplified 1.2 kb DNA fragment was cloned into pCRII® cloning vector (Invitrogen, San Diego, California, USA), and the nucleotide sequence of the fragment was verified using a sequence kit (Sequenase, U.S. Biochem. Corp., Cleveland, Ohio, USA). The fragment was then excised with restriction enzymes, Sac I and Xho I, and ligated with pGL2-Promoter (pGL2P) vector.

Luciferase reporter gene study for transcriptional activation of the rat Mn-SOD gene by oxidants

To investigate the transcriptional activation of the Mn-SOD gene by oxidants, the above-described rat Mn-SOD (−806:+408 or −151:+23) fusion construct was transfected into mesangial or epithelial cells, and the level of luciferase expression was studied in those cells following various treatments.

Transfection was performed using the calcium phosphate method previously described [22]. Briefly, 10 μg per P60-dish of test plasmid (pGL2P or fusion genes) and 10 μg of pSV β-galactosidase (pSV βGAL) were dissolved in 256 μl of 0.1× TE, pH 8 (1 mM Tris Cl, pH 8, 0.1 mM EDTA) with 250 mM $CaCl_2$, and mixed with an equal volume of 2× Hepes-buffered saline (20 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 12 mM Dextrose, 50 mM Hepes) for 30 minutes at room temperature. The pSV βGAL contained both a SV40 early promoter and an enhancer, and β-galactosidase activity was used as an index for transcription efficiency. Cells were plated on P60 dishes at 72 hours before the transfection, and the medium was replaced from RPMI 1640/14% FBS to DMEM-F12/10% FBS at 12 to 16 hours before. Before transfection, the medium was washed and cells were incubated with CMFH for 15 minutes. The HBSS was then replaced with the Ca-P solution containing plasmid, incubated for 30 minutes at 37°C, and 3 ml of DMEM-F12/10% FBS were added. Five hours after the transfection, cells were shocked with a 10% glycerol in HBSS, and the medium was replaced with culture medium (RPMI/14% FBS). Forty-eight hours after the transfection, cells were treated with various concentrations of H_2O_2, 50 μM α-, β-naphthoflavone, or puromycin aminonucleoside (5 to 50 μg/ml).

Two, 12 or 24 hours after the treatment, cells were harvested and lysed in 55 mM Tris-phosphate, pH 7.8, 2 mM DTT, 2 mM 1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid, 10% glycerol, 1% Triton X-100; cellular extracts were stored at −70°C until assayed for protein concentration, luciferase, and β-galactosidase activities.

Protein concentration of the cellular extract was determined spectrophotometrically by the bicinchoninic acid method [23]. Fifty micrograms of cellular protein were used for each luciferase and β-galactosidase assay. The luminescence produced by luciferase was determined by a luminometer (Model ILA911, Tropix, Bedford, Massachusetts, USA) using coenzyme A as a substrate. Our pilot study demonstrated that photon emission by this reaction remains stable from 10 seconds to 5 minutes after the initiation of reaction. A linear relationship between standard luciferase and the relative light unit of the luminescence was obtained between 10^{-16} and 10^{-12} M. β-galactosidase activity was determined spectrophotometrically using *o*-nitrophenyl-β-D-galactopyranoside as a substrate [24]. To minimize variability due to the difference in transfection efficiency among plates, the activity of luciferase from each sample was corrected based on the activity of β-galactosidase [25, 26].

Statistics

All values are expressed as the mean ± SE. Data from two groups were compared by unpaired *t*-test. Data from multiple groups were compared by one way analysis of variance with a

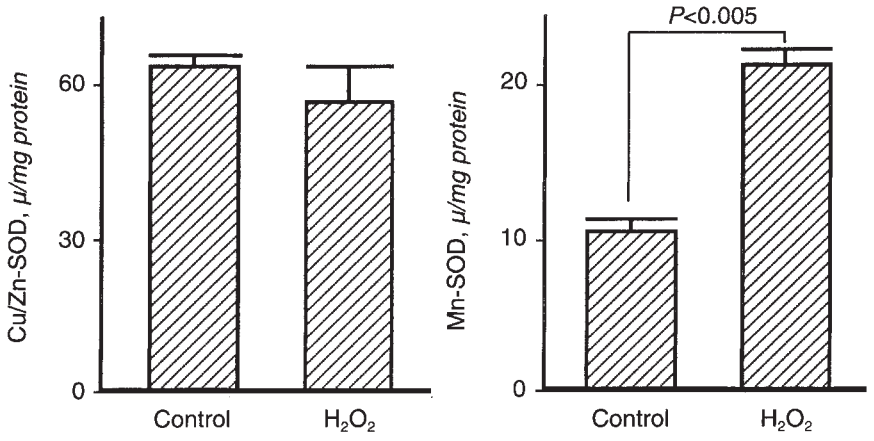

Fig. 1. *Changes in SOD activities in mesangial cells treated with hydrogen peroxide (H_2O_2).* Semiconfluent mesangial cells grown in P100 dishes were treated with 100 μM H_2O_2. Control cells underwent exchange of medium not containing H_2O_2. Twenty-four hours after treatment, cells were harvested and SOD activities were determined. While Cu/Zn-SOD activities were not different between control and H_2O_2-treated cells, Mn-SOD activity was significantly elevated in H_2O_2-treated cells. Values are mean ± SE. $N = 6$ each.

Scheffe's F-test [27]. $P < 0.05$ was considered to be statistically significant.

Results

SOD activities following hydrogen peroxide treatment in mesangial cells

The activities of SODs in glomerular mesangial cells treated with hydrogen peroxide are shown in Figure 1. On average, the activity of cyanide-uninhibitable SOD, or Mn-SOD, in cells treated with 100 μM H_2O_2 for 24 hours was 22.2 ± 1.2 μ/mg protein, a value significantly higher than that of control cells (12.2 ± 0.7 μ/mg protein). Although not shown, there were no detectable changes in Mn-SOD activity between 0 and 24 hours of incubation in control cells. The activity of cyanide-inhibitable SOD (Cu/Zn-SOD) was not statistically different between H_2O_2-treated and control cells following the 24 hours of incubation. Therefore, the H_2O_2-induced increase in enzyme activity was specific for Mn-SOD.

Changes in SOD mRNA expressions following hydrogen peroxide treatment in mesangial cells

To examine whether H_2O_2-induced changes in Mn-SOD activity involve changes in gene expression, Northern blot analysis was performed using total RNA extracted from mesangial cells which were treated with H_2O_2. Figure 2 shows the Northern blots hybridized with rat Mn-SOD cDNA (bottom), rat Cu/Zn SOD cDNA (middle) and human β-actin (top). As previously described, Mn-SOD was demonstrated in 3 to 5 bands (1.3 to 4.1 kb in size), depending on the intensity of the blot, and Cu/Zn SOD was demonstrated as a single band [19, 21]. When mesangial cells were treated with H_2O_2 for 24 hours, there was an increase in Mn-SOD mRNA expression in those treated with 10 and 100 μM H_2O_2, whereas those treated with 1 μM H_2O_2 had low constitutive expression, which was at a level similar to that of control cells (left 4 lanes in Fig. 2). β-actin and Cu/Zn SOD mRNA expressions were not appreciably different among groups.

The increase in Mn-SOD mRNA expression by H_2O_2 was abolished by a transcriptional inhibitor. Thus, when mesangial cells were treated with actinomycin D, H_2O_2-induced increase in Mn-SOD expression was no longer observed (Lanes 6 vs. 8). Cu/Zn SOD and β-actin mRNA expressions were unaffected by either H_2O_2 or actinomycin D.

Semiquantitative analysis of the H_2O_2-induced increase in Mn-SOD mRNA was performed for the time course of Cu/Zn- and Mn-SOD mRNA response to H_2O_2. As shown in Figure 3, while Cu/Zn-SOD mRNA expression remained unchanged between 2 and 24 hours after incubation with 100 μM H_2O_2, there was a significant increase in Mn-SOD mRNA at six hours and some ninefold increase at 24 hours.

The Northern blot analyses indicated that H_2O_2-treatment of mesangial cells led to a dose- and time-dependent increase in Mn-SOD activity and mRNA expression.

SOD mRNA expressions following hydrogen peroxide treatment in bovine glomerular endothelial and rat glomerular epithelial cells

Hydrogen peroxide also induced Mn-SOD mRNA expression in other glomerular cells. As shown in Figure 4, glomerular endothelial and epithelial cells treated with H_2O_2 showed enhancement of Mn-SOD mRNA expression. The enhancement in the endothelial cells was demonstrated at 100 μM but not at 10 μM (Lanes 1 to 3 in Fig. 4). The threshold of Mn-SOD induction was higher in glomerular epithelial cells. Thus, an increase of Mn-SOD mRNA expression was observed at 500 and 1000 μM H_2O_2, but not at 100 μM H_2O_2, and the magnitude of increase was less than that seen in other cells (Lanes 4 to 7 in Fig. 4).

Oxidant-induced transcriptional activation of the reporter gene

Transcriptional regulation of the Mn-SOD gene by hydrogen peroxide was studied using a fusion gene construct containing a rat Mn-SOD 5′ fragment, SV40 early promoter, and luciferase reporter gene. As presented in Table 1, mesangial cells transfected with pGL2P (−806:+408) had more than threefold higher transcriptional activity when exposed to 100 μM H_2O_2 for 24 hours than unstimulated cells. Moreover, H_2O_2-induced change in transcriptional activity was dose dependent (Table 1). Transfection with pGL2P alone, that is, the reporter gene without Mn-SOD DNA fragment, demonstrated no enhancement of transcription. Therefore, the 1.2 kb fragment of rat Mn-SOD DNA activated transcription of the reporter gene in response to hydrogen peroxide. Figure 5 depicts the time-course of changes in luciferase transcriptional activity following treatment with 100 μM H_2O_2. The increase in luciferase activity became detectable by six hours after treatment, and the activity remained high between 12 and 24 hours.

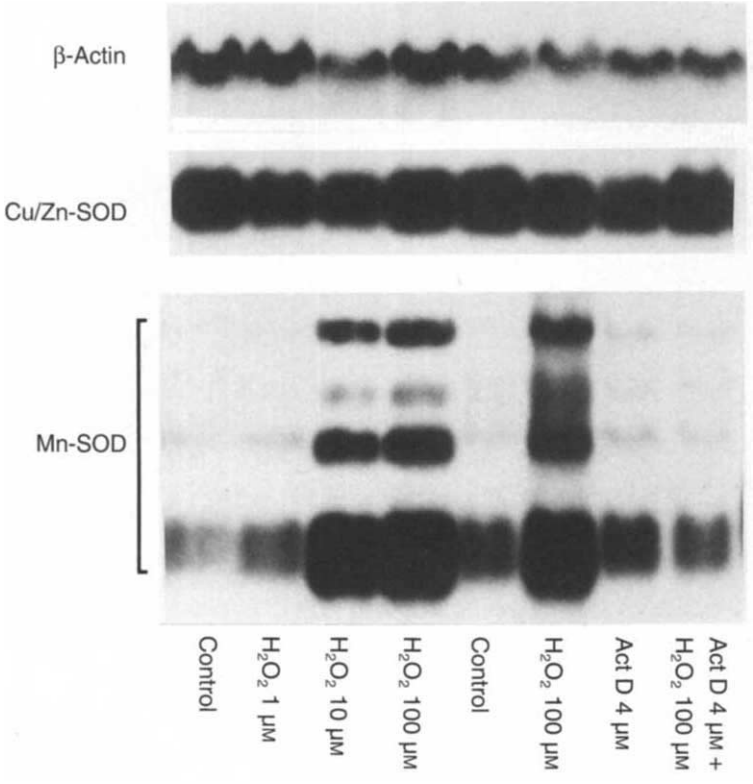

Fig. 2. *Northern analysis of rat SOD mRNA expression in mesangial cells.* Twenty micrograms of total RNA isolated from mesangial cells were electrophoresed in an agarose gel, transferred to a nylon membrane, and hybridized with rat Cu/Zn-SOD or Mn-SOD cDNAs. Lane 1 (extreme left): control cells; lane-2: H_2O_2 1 μM for 24 hours; lane 3: H_2O_2 10 μM for 24 hours; lane 4: H_2O_2 100 μM for 24 hours; lane 5: control cells; lane-6: H_2O_2 100 μM for 24 hours; lane 7: Actinomycin D (Act D) 4 μM for 24 hours; lane 8: Actinomycin D 4 μM and H_2O_2 100 μM for 24 hours. Treatment with 10 to 100 μM H_2O_2 resulted in a marked increase in Mn-SOD mRNA (five bands ranging from 1.3 to 4.1 kb) as shown in lanes 3 and 4. β-actin and Cu/Zn-SOD expressions were not affected by H_2O_2. Right 4 lanes examine the effect of Act D. Thus, lanes 5 and 6 repeat the effect of 100 μM H_2O_2. The enhancement of Mn-SOD expression shown in lane 6 is abolished by cotreatment with Act D. Act D did not affect basal expression of Mn-SOD mRNA (lane 7). Again, β-actin and Cu/Zn-SOD expressions were not affected by H_2O_2 or by Act D.

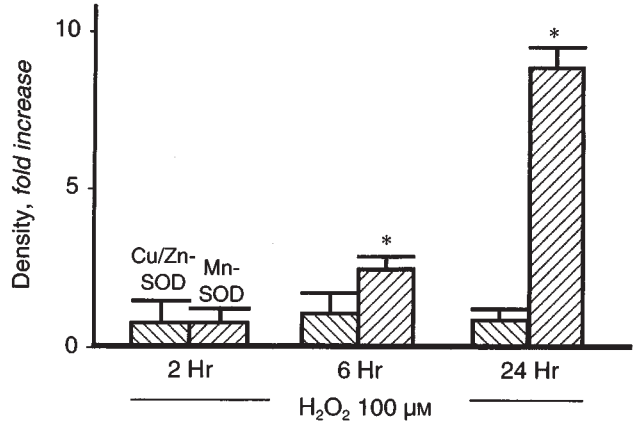

Fig. 3. *Densitometric analysis of rat SOD mRNA expression in mesangial cells treated with hydrogen peroxide.* Densities of SOD mRNAs in the autoradiograph were semiquantitated with a densitometer. Obtained densities were corrected with those of β-actin, compared with control cells of the same autoradiograph (that is, same membrane), and ratio to the average values of untreated cells (fold-increase) was calculated. Values are mean ± SE. $N = 6$ for Control, 3 for 2 Hr, 5 for 6 Hr, and 7 for 24 Hr.

We tested whether the transcription of the Mn-SOD gene is sensitive to oxidants of diverse origins, as demonstrated in several other genes [6, 7, 11]. As shown in Figure 5, cells transfected with pGL2P (−806:+408) responded markedly to 50 μM α- or β-naphthoflavone to increase luciferase activity. Therefore, the 1.2 kb fragment can induce transcriptional activation in response to cellular exposure to oxidants of diverse origins.

Hydrogen peroxide-dependent transcriptional activation of Mn-SOD gene pGL2P is summarized in Table 2. Epithelial cells transfected with pGL2P (−806:+408) showed a higher threshold for transcriptional activation of the reporter gene by H_2O_2. Thus, the luciferase activity was enhanced in those cells treated with 500 and 1000 μM H_2O_2, but not in cells treated with 100 μM H_2O_2. In epithelial cells, treatment with puromycin aminonucleoside (PAN) also led to an increase in transcriptional activity of the reporter gene (Fig. 6). A previous study by others demonstrated an increase in superoxide production by epithelial cells treated with >25 μg/ml PAN [28]. In the present study, PAN 25 and 50 μg/ml, but not 5 μg/ml, demonstrated an enhancement in luciferase activity in epithelial cells transfected with pGL2P (+806:+408).

Discussion

The present study was conducted to examine (1) whether oxidants can induce Mn-SOD in renal cells locally; if so, (2) whether the induction involves a transcriptional activation of the Mn-SOD gene.

A series of studies was first conducted by measuring Mn-SOD activity in mesangial cells exposed to hydrogen peroxide. While the initial concentration of hydrogen peroxide in the medium was 100 μM, the actual concentration of hydrogen peroxide achieved within the cell is likely to have been much lower, since other antioxidants capable of metabolizing the hydrogen peroxide were present in the cell preparation. In response to this stimulation, a marked increase in Mn-SOD activity was observed, contrasting to the absence of induction of Cu/Zn-SOD activity. In some mammalian cells, the same pattern of induction has been recognized

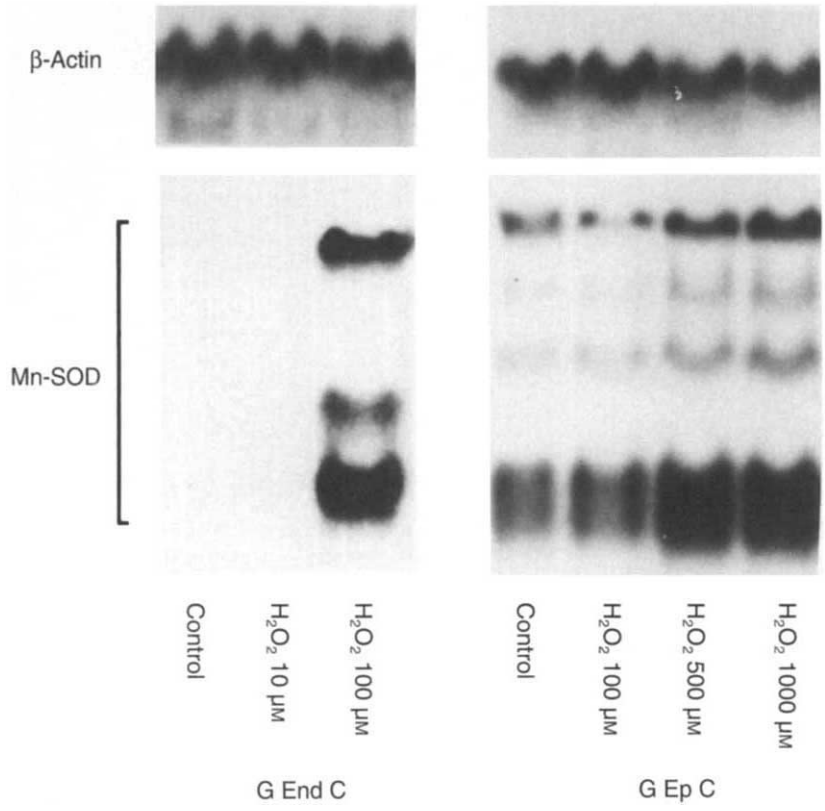

Fig. 4. *Northern analysis of SOD mRNA expressions in bovine glomerular endothelial cells (G End C; left 3 lanes) and rat glomerular epithelial cells (G Ep C; right 4 lanes).* For lanes 1 to 3, total RNA extracted from bovine glomerular endothelial cells was hybridized with bovine Mn-SOD cDNA (bottom) and with human β-actin (top). Lane 1 (extreme left): control cells; lane 2: H_2O_2 10 μM for 24 hours; lane 3: H_2O_2 100 μM for 24 hours. In endothelial cells treated with 100 μM H_2O_2, expression of Mn-SOD was enhanced whereas those of β-actin and Cu/Zn-SOD were not affected. For lanes 4 to 7, total RNA extracted from rat glomerular epithelial cells was hybridized with rat cDNA probes and with human β-actin. Lane 4: control cells; lane 5: H_2O_2 100 μM for 24 hours; lane 6: H_2O_2 500 μM for 24 hours; lane 7: H_2O_2 1,000 μM for 24 hours.

Table 1. Transcriptional activation of the rat Mn-SOD gene by hydrogen peroxide in mesangial cells transfected with a luciferase reporter gene containing a 5′ fragment of the gene

Treatment	*N*	Relative change to control[a]
pGL2P (+806:−408)		
H_2O_2 1 μM	6	1.01 ± 0.04
H_2O_2 10 μM	6	1.56 ± 0.06[a]
H_2O_2 100 μM	6	3.17 ± 0.37[ab]
pGL2P		
H_2O_2 100 μM	4	0.98 ± 0.10

Cultured rat glomerular mesangial cells were transfected with 10 μg of a luciferase reporter gene which contains a SV40 early promoter alone (pGL2P), or pGL2P containing the −806:+408 fragment of rat Mn-SOD DNA. The cells were cotransfected with 10 μg of plasmid that expresses β-galactosidase as a control for transfection efficiency. Forty-eight hours after the transfection, cells were treated with hydrogen peroxide (H_2O_2 1 to 100 μM) for 24 hours; luciferase activity of cellular extract was then determined.

[a] Ratio of luciferase activity in H_2O_2-treated cells vs. untreated control cells

[b] and [c] $P < 0.05$ vs. H_2O_2 1 μM and vs. H_2O_2 10 μM, respectively

when cells were exposed to certain cytokines, such as tumor necrosis factor (TNF), interleukin (IL)-1, or endotoxins [17, 29]. Although measurement was not made at the enzyme activity level, our mRNA assays revealed that other types of glomerular cells, that is, epithelial and endothelial cells, also have the potential to induce their intrinsic Mn-SOD in response to hydrogen peroxide. It is further noted that, while the baseline level of mRNA in rat renal glomeruli was higher in epithelial than in mesangial cells, epithelial cells were less sensitive to hydrogen peroxide. However, the biologic significance of the observed difference in the Mn-SOD inducibility among cell types remains unknown since, in real *in vivo* settings, the degree of oxidant stress *per se* is likely to be variable within the kidney and influenced largely by the anatomical localization of the cells. Moreover, as discussed above, the local level of oxidants is affected by the availability of other antioxidant enzymes, which may be different between the two cell types.

Several methodologies are available to test the transcriptional activation of the Mn-SOD gene by hydrogen peroxide. In our study, a transcriptional inhibitor, actinomycin D, inhibited the hydrogen peroxide-induced Mn-SOD mRNA. This result, however, cannot rule out the possibility that the hydrogen peroxide's effect may still be via attenuation of the degradation of a (possibly) highly unstable Mn-SOD mRNA. Indeed, while oxidants have been shown to up-regulate Mn-SOD mRNA in other mammalian cells, inhibitable by actinomycin D [30], they were demonstrated to increase the stability of the mRNA [31]. For this reason, we chose to use a reporter gene system. The results showed that the patterns of transcriptional activation assessed by luciferase activity closely parallel those of Mn-SOD induction measured as an absolute mRNA amount. Thus, in mesangial cells, a maximum luciferase response was observed with 100 μM hydrogen peroxide, with a peak at 24 hours. Moreover, in epithelial cells, an increase in luciferase activity was detected in response to 500 μM but not 100 μM hydrogen peroxide, a pattern identical to Mn-SOD mRNA. Collectively, the results indicate that the induction of Mn-SOD by hydrogen peroxide in glomerular epithelial

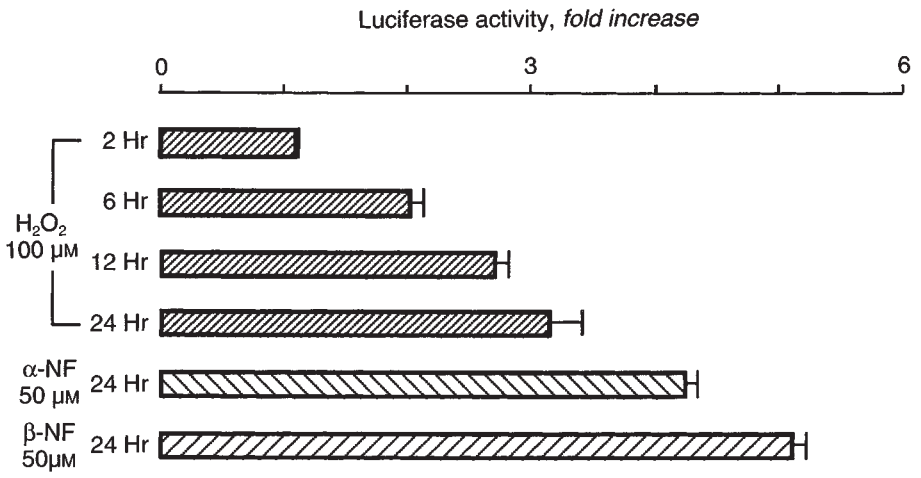

Fig. 5. *Time course of hydrogen peroxide-induced changes in luciferase activities in mesangial cells transfected with pGL2P (−806:+406) and pSV β-GAL.* Cultured mesangial cells were transfected with 10 μg of the pGL2P (−806:+408) together with 10 μg of pSV β-GAL. Forty-eight hours after transfection, the cells were treated with 100 μM H_2O_2 and harvested for luciferase and β-galactosidase assays at 2, 6, 12, and 24 hours later (N = 4, 5, 5, 6, respectively). Some cells were treated with oxidants known to induce transcription in another gene, namely, α- and β-naphthoflavone (NF: 50 μM) for 24 hours (N = 3 each) [11]. Luciferase activity was corrected with transfection efficiency calculated from β-galactosidase activity. The ratio of luciferase activity in treated cells to unstimulated control cells was calculated and expressed as fold increase. Values are mean ± SE.

Table 2. Transcriptional activation of the rat Mn-SOD gene by hydrogen peroxide in glomerular epithelial cells transfected with a luciferase reporter gene containing a 5′ fragment of the gene

Treatment	N	Relative change to control[a]
pGL2P +806:−408		
H_2O_2 100 μM	6	1.13 ± 0.15
H_2O_2 500 μM	6	3.19 ± 0.32[b]
H_2O_2 1000 μM	6	2.86 ± 0.29[b]

Cultured rat glomerular epithelial cells were transfected with a luciferase reporter gene (pGL2P −806:+408) in an identical manner to that shown in Table 1. Forty-eight hours after the transfection, cells were treated with hydrogen peroxide (H_2O_2 100 to 1000 μM) for 24 hours and luciferase activity of the cellular extract was determined.

[a] Ratio of luciferase activity in treated cells vs. untreated control cells

[b] $P < 0.05$ vs. H_2O_2 100 μM

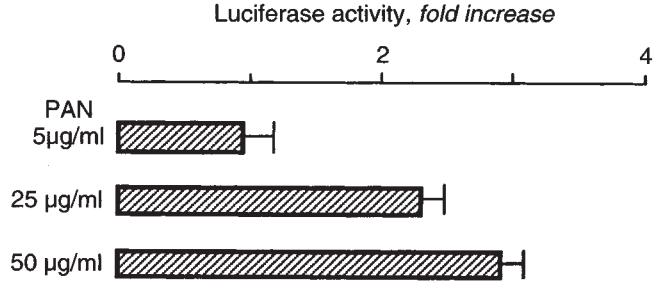

Fig. 6. *Transcriptional activation of Mn-SOD DNA by puromycin aminonucleoside (PAN) in rat epithelial cells transfected with pGL2P (−806:+406) and pSV β-GAL.* Cultured epithelial cells were transfected with 10 μg of the pGL2P (−806:+406) together with 10 μg of pSV β-GAL in an identical manner to that shown in Tables 1 and 2 and Figure 5. The transfected cells were treated with 5 μg/ml, 25, or 50 PAN for 24 hours and luciferase activity and fold-increase of transcriptional activity were determined in an identical manner as described earlier. Values are mean ± SE.

and mesangial (and presumably endothelial, as well) cells involves, and is largely attributable to, transcriptional activation of the gene. Since hydrogen peroxide is a product of the enzymatic reaction of SOD, it appears paradoxical that the product (hydrogen peroxide) up-regulates the enzyme (Mn-SOD). However, it should be noted that, of the several forms of reactive oxygen metabolites, hydrogen peroxide is relatively low in toxicity. Moreover, for hydrogen peroxide to give rise to the extremely toxic hydroxyl radical requires superoxide anion (which is metabolized to hydrogen peroxide under the catalysis of SOD). Thus, activation of SOD can reduce the highly toxic hydroxyl radical at the expense of an increased amount of less toxic hydrogen peroxide. Of note, our additional observation, that agents capable of producing diverse cellular oxidant stress (α- and β-naphthoflavone and puromycin aminonucleoside [11, 28]) were also capable of transcriptionally activating the Mn-SOD gene, attests to the notion that the response of the Mn-SOD gene is not limited to hydrogen peroxide but sensitive to a broad range of reactive oxygen metabolites. Furthermore, a recent preliminary study by Gologorsky et al demonstrated that mesangial cells exposed to hydrogen peroxide chronically had higher catalase activity [32]. Thus, oxidants may induce antioxidant enzymes other than Mn-SOD, as observed in prokaryotic cells [5].

Our observations with the luciferase reporter gene also indicate that the element(s) responsive to reactive oxygen metabolites resides within the 1.2 kb 5′ fragment tested in the study. Since this fragment contains the promoter region of the Mn-SOD gene, it is not readily apparent whether the element sensitive to oxidants is an enhancer or a promoter in nature.

In this regard, studies by others have identified NF-κB, a multisubunit transcription factor protein which, in collaboration with cell-specific factors, can activate the expression of TNF-α, known to be capable of inducing SOD mRNA [33]. Since TNF-α fails to activate NF-κB when cells are concurrently treated with antioxidants [25], reactive oxygen metabolites appear to be a secondary messenger for cytokine activation of NF-κB and to act as its direct activator. Moreover, the rat Mn-SOD gene contains, in its upstream region, a consensus decanucleotide sequence which has been determined in other genes to be the binding site for NF-κB [34], although, in mesangial cells, a NF-κB binding site of certain genes was found to be sensitive to superoxide but not to hydrogen peroxide [35]. The possibility exists, therefore, that activation of NF-κB, followed by its binding to its recognition site,

is involved in the transcriptional up-regulation of the Mn-SOD gene by oxidants.

Alternatively, activation of the Mn-SOD gene may involve a separate set of unknown binding protein and nucleotide elements responsive to reactive oxygen metabolites. Of interest in this regard, recent deletion mutation studies have identified a nucleotide consensus sequence (GTACNNNGC) linked to an enhancement in transcription of the rat glutathione-S-transferase Ya subunit gene in response to hydrogen peroxide [11]. This consensus sequence is present in the first intron within the 1.2 kb Mn-SOD DNA fragment. It should also be noted, however, given our findings, that since the Mn-SODs of glomerular mesangial versus epithelial cells differ in their sensitivity to hydrogen peroxide, the possibility exists that different proteins and transcriptional control elements are involved in the regulation by oxidants of this gene in the two cell types. Moreover, since the present study does not test a potential intermediatory role of cytokine(s) in the oxidant-induced Mn-SOD gene activation, the possibility exists that the observed difference in the two cell types may represent a difference in their autocrine function to release and/or respond to oxidant-sensitive cytokine(s).

The functional significance of Mn-SOD induction was not tested in the present study; however, data available in the literature are in favor of its significance. When Mn-SOD activity was augmented by other experimental maneuvers in endothelial cells to a degree similar to that achieved in the present study by hydrogen peroxide, those cells were protected from the cytotoxic effect of oxidants, as demonstrated by reduction in both cellular lipid peroxidation and in release of labeled chromium from cells [36]. As mentioned earlier, other antioxidant enzymes were also activated in those experiments and in the present study; thus, it is also possible that oxidants can induce antioxidant enzymes other than Mn-SOD. In such cases, a protective mechanism against oxidants cannot be attributed solely to the Mn-SOD induction. In this regard, more compelling evidence for the functional significance of Mn-SOD induction was provided by Wispe et al in a model of the transgenic mouse in which pulmonary epithelial cells with increased levels of Mn-SOD (similar to those found in the present study) were protected from the toxic effect of oxygen [37]. Together with the recent demonstration of induction of SOD by ischemia/reperfusion in the kidney *in vivo* [8], these studies support the notion that Mn-SOD can be activated in the kidney in response to oxidant stress and that the induced Mn-SOD, among other defense mechanisms, plays an important protective role in determining the renal tissue injury caused by oxidants. In the present study, puromycin aminonucleoside, an agent that causes minimal change nephrotic syndrome through oxidant stress *in vivo* [38], also induced Mn-SOD gene transcription. These data further suggest that the disease process takes place within an ongoing, dynamic interaction between oxidants and antioxidants (both Mn-SOD and non-Mn-SOD enzymes not measured in the present study) which are, in turn, induced by the oxidants.

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