# Induction of Metallothionein-I Protects Glomeruli from Superoxide-Mediated Increase in Albumin Permeability

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Metallothioneins (MT) are low-molecular-weight, heat-stable, cysteine-rich proteins with four isoforms. MT-I and MT-II are ubiquitous and are induced by oxidative, physical, and chemical stress. MT-I is an efficient scavenger of superoxide ($^{\cdot}O_2$) and hydroxyl ion ($OH^-$). We have demonstrated that $^{\cdot}O_2$ and hypohalous acid can cause an increase in glomerular albumin permeability ($P_{alb}$) *in vitro*. The purpose of this study was to document the protective effect of MT gene product on the $^{\cdot}O_2$-mediated increase in $P_{alb}$. Glomeruli from Sprague-Dawley rats in 4% BSA medium were incubated for 4 hr at 37°C in duplicate tubes. Each set contained glomeruli alone or with 5 μM $Cd^{++}$, 0.3 mM Spermine-NONOate (NO donor), 0.3 mM Sulfo-NONOate (nitrous oxide donor), 0.6 mM SNP (nonspecific NO donor) and SNP + carboxy-PTIO (10 mg/ml). After incubation, one set of tubes was used to isolate total RNA for the measurement of the mRNA levels of MT-I by reverse transcriptase polymerase chain reaction (RT-PCR). Duplicate tubes were incubated for an additional 10 min with 10 nM of $^{\cdot}O_2$, and $P_{alb}$ was measured using video microscopy. RT-PCR of total RNA from $Cd^{++}$ and Spermine-NONOate treated glomeruli revealed a 2-fold induction of MT-I expression at the mRNA level. $^{\cdot}O_2$ caused a significant increase in $P_{alb}$ (0.8 ± 0.06 vs. control 0.0 ± 0.12, $P < 0.05$) and induction of MT-I in glomeruli by $Cd^{++}$ or by Spermine-NONOate blocked this effect (0.21 ± 0.12 and 0.24 ± 0.19, respectively, $P < 0.05$ vs. $^{\cdot}O_2$). In contrast, Sulfo-NONOate and SNP did not induce mRNA for MT-I in glomeruli and did not provide protection against $^{\cdot}O_2$-mediated increase in $P_{alb}$. We conclude that MT-I gene products may play an important role in protecting the glomerular filtration barrier from the injury induced by reactive oxygen species in immune and/or nonimmune renal diseases.

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**Key words:** metallothioneins; glomerular albumin permeability; reactive oxygen species

In 1957, Margoshes and Vallee discovered metallothioneins (MT) in mammalian kidney (1). Since then, their presence in almost all mammalian tissues (2) and in certain microorganisms has been documented (3). MT are 61-amino acid-long, low-molecular weight (6–7 kD), heat-stable (85°C for 10 min), cysteine-rich (20 residues) proteins without aromatic amino acids. All cysteines in MT are reduced and highly conserved. MT have affinity for metals (silver>mercury>copper>cadmium>zinc) and contain 7 g/M of metal (4).

In mammals, four isoforms of MT have been reported (MT-I, II, III, and IV). MT-I and II are ubiquitous. MT-III has been detected in mouse and human brain, and MT-IV has been found in certain stratified epithelia (5). Several types of MT-1 exist in human kidney (MT-1e, MT-1g, and MT-1k), and changes in their relative amounts have been associated with tumors (6). To study the synthesis and biological functions of MT-I and MT-II, the genes have been characterized (7), and transgenic (MT-I) and knockout (MT-I and II) mouse models have been developed (8). Role of metal response elements and metal transcription factor-1 in oxidative stress-induced MT gene expression has been documented (9).

Proteinuria is a nonspecific manifestation of glomerular injury that results from an increase in the permeability of albumin and other plasma proteins from the glomerular permeability barrier (10). The glomerular permeability barrier consists of endothelial cells, the glomerular basement membrane, and the slit pore junctions of the glomerular epithelial cells. Alterations in either cells or matrix of glomerular permeability barrier by inflammatory and noninflammatory molecules lead to increase in glomerular permeability and proteinuria. We have developed a highly sensitive *in vitro* assay system to study the alterations in glomerular permeability barrier (11). This assay permits us to study the direct

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immediate effects of a single agent on glomerular albumin permeability under controlled conditions free from secondary hemodynamic and inflammatory responses that might play a role *in vivo*.

Reactive oxygen species (ROS) generated by infiltrating cells or by resident glomerular cells including superoxide, hydroxyl radicals, and hypohalous acid have been implicated in renal dysfunction (12). Concentration of ROS is controlled by enzymatic degradation by superoxide dismutase (SOD) and catalase and by nonenzymatic intracellular scavenging by MT, a family of stress-induced proteins with diverse physiological functions, including protection against metal toxicity and oxidants. *In vivo* studies have shown that both MT-I and MT-II are capable of scavenging free radicals, but the MT-I appeared to be a superior scavenger of superoxide (13).

Using our *in vitro* method, we have shown that incubations of isolated rat glomeruli with ROS generated from enzymatic reaction (14) or from phorbol myristate acetate (PMA)-activated macrophages and neutrophils caused a significant increase in glomerular albumin permeability within 10 min (14, 15). The purpose of this study was to test the hypothesis that induction of MT-I will protect glomeruli from superoxide-mediated increase in glomerular albumin permeability *in vitro*.

## Materials and Methods

**Chemicals and Supplies.** All commonly used supplies and analytical grade chemicals for buffers and bovine serum albumin (BSA, 35% solution) were obtained from Sigma (St. Louis, MO). Spermine-NONOate, sulfo-NONOate, carboxy-PTIO [2-(4 carboxyphenyl) 4,4,5,5 tetra-methyl imidazoline-3-oxide-1-oxyl], and sodium nitroprusside (SNP) were obtained from Alexis Biochemicals (San Diego, CA).

**Experimental Animals.** Normal male Sprague-Dawley rats (180–250 g) maintained on Purina rat chow and water *ad libitum* were used in all experiments. In all cases, kidneys were removed via abdominal incision after the animals were properly anesthetized using metofane (Pitman-Moore Inc., Titusville,, NJ). After removal of the kidneys, rats were sacrificed under anesthesia.

**Isolation of Glomeruli.** After removal of the kidney capsule, the outer 1–2 mm of renal cortex was excised, and cortical slices were cut into fine fragments and were passed through consecutive stainless steel screens of 80, 120, and 200 mesh size (11). Glomeruli were retained by the 200 screen and were collected in a modified Ringer's buffer. The modified buffer (pH 7.4) contained: sodium chloride, 115 mM; potassium chloride, 5 mM; sodium acetate, 10 mM; dibasic sodium phosphate, 1.2 mM; sodium bicarbonate, 25 mM; magnesium sulfate, 1.2 mM; calcium chloride 1 mM; glucose, 5.5 mM; L-alanine, 6 mM; sodium citrate, 1 mM; sodium lactate, 4 mM, and 4 g/dl of BSA as an oncotic agent. The oncotic pressure of the medium was measured using membrane colloid osmometer (Model 4100, Wescor Inc., Logan, UT).

**Treatment of Glomeruli.** Glomeruli in medium containing 4 g/dl BSA were incubated with or without experimental reagents that are known to induce MT-I (16, 17) in duplicate tubes for 4 hr at 37°C. Each set of tubes contained glomeruli alone, glomeruli + cadmium (5 μM), glomeruli + SNP (0.6 mM), glomeruli + SNP + carboxy-PTIO (c-PTIO, 10 mg/ml), glomeruli + spermine-NONOate (0.3 mM), and glomeruli + sulfo-NONOate (0.3 mM). After incubation, RNA was isolated from one set of tubes to document the induction of MT-I by RT-PCR. Glomeruli in other set of tubes were washed with fresh 4% BSA and were then incubated with 10 nM potassium superoxide for an additional 10 min at 37°C prior to measurement of glomerular albumin permeability ($P_{alb}$) using video microscopy.

**Isolation of Glomerular RNA.** After 4 hr of incubation with the agents outlined above, total glomerular RNA was extracted using Tri-reagent (Sigma). Briefly, the glomeruli were transferred to microcentrifuge tubes, pelleted by centrifugation, and resuspended in 0.5 ml of Tri-reagent and were then incubated at room temperature for 5 min. Fifty microliters of chloroform was then added and the suspension was mixed vigorously. The tubes were then incubated at room temperature for 5 min and the phases were separated by centrifugation at 14,000*g* for 15 min. The aqueous phase was then transferred to fresh microcentrifuge tubes and RNA was precipitated by addition of isopropanol, followed by centrifugation at 14,000*g* for 15 min. The RNA pellet was then resuspended in diethylpolycarbonate (DEPC)-treated water. RNA concentration and quality were assessed spectrophotometrically at wavelengths 260 and 280 nm.

**Quantification of Glomerular MT-1 mRNA Levels by RT-PCR.** As per the manufacturer's instructions (Perkin Elmer, Foster City, CA), 200 ng of total glomerular RNA was reverse transcribed using 2.5 U/μl of MuLV reverse transcriptase in a total volume of 10 μl. To the total RNA in 5 mM $MgCl_2$, 1× PCR buffer II (50 mM KCl and 10 mM Tris-HCl, pH 8.3) 2.5 μM of random hexamer primers, 1 mM each of dATP, dTTP, dCTP, dGTP, and RNase inhibitor (1U/μl) were added. Primers for the MT-I and GAPDH were synthesized by Operon (Alameda, CA). The sequences of the primers are as follows: MT-I (forward, 5′-GCTTGCTCCAGATTCAGATC-3′; reverse 5′-TCACATGCTCGGTAGAAAA CGG-3′, PCR product size of 312 bp) and GAPDH primers (forward, 5′-GTGCTGAGTATGTCGTGGA-3′; reverse, 5′-TCACAT GCTCGGTAGAAA ACGG-3′, PCR product of 298 bp). One micromole of each pair of primer was used in a final reaction volume of 50μl containing 1× PCR buffer II and 0.05 U/μl of AmpliTaq DNA polymerase (Perkin Elmer).

The PCR cycling profile was 1 min at 95°C, 1 min at 55°C, and 3 min at 72°C. Thirty cycles were carried out and followed by a final extension at 72°C for 10 min. Ten microliters of PCR products was then separated on a 2% aga-

rose gel, stained with ethidium bromide, and photographed. For the quantification of the MT-I and GAPDH PCR products, an image of the gel was captured using Kodak Digital Sciences Image Station 440 CF (NEN, Boston, MA). The intensity of the products was quantitated using Kodak Digital Sciences 1D Image Analysis software (NEN). The ratio of the mean intensities of MT-I /GAPDH were used to calculate fold induction of MT-I mRNA.

**Calculation of $P_{alb}$.** A detailed protocol and rationale for the measurement of $P_{alb}$ using isolated rat glomeruli has been outlined earlier (11). In short, the volume response of glomerular capillaries to an oncotic gradient was measured in the following manner. Incubated glomeruli in 4% BSA medium were affixed to a glass coverslip coated with poly-L-lysine (1 mg/ml) and were observed using video microscopy before and 2–3 min after the medium was replaced by a lower oncotic medium containing 1 gm/dl BSA. This exchange of medium from 4% to 1% BSA produced an oncotic gradient across the capillary wall, net influx of fluid, and an increase in glomerular volume. Relative increase in glomerular volume ($\Delta V$) was calculated using the formula: $\Delta V = [(V_{final} - V_{initial})/V_{initial}] \times 100\%$. Increase in $\Delta V$ is directly proportional to the applied oncotic gradient ($\Delta\pi$) across the capillary wall. We used this principle to calculate the reflection coefficient for albumin ($\sigma_{albumin}$) using the ratio of $\Delta V$ of experimental to $\Delta V$ of control glomeruli in response to identical oncotic gradients: $\sigma_{albumin} = \Delta V_{experimental}/\Delta V_{control}$. Convectional albumin permeability ($P_{alb}$) was defined as: $1 - \sigma_{albumin}$ (12). When $\sigma_{albumin}$ is zero, albumin moves at the same rate as water and $P_{alb}$ is 1.0. Alternatively, when $\sigma_{albumin}$ is 1.0, albumin cannot cross the membrane with water and $P_{alb}$ is zero.

**Statistical Analysis.** Albumin permeability values are expressed as mean ± SD as indicated. *n* represents the total number of glomeruli for $P_{alb}$ studies. Values among various groups were compared using one-way analysis of variance (ANOVA) and significance was defined as $P < 0.05$.

## Results

**Induction of MT-I by Cadmium and NO Donor.** RT-PCR using MT-I and GAPDH primers of total RNA from glomeruli incubated with $Cd^{++}$, spermine-NONOate, sulfo-NONOate, SNP, and SNP + CPTO was carried out as outlined in "Materials and Methods." Results are shown in Figure 1. Incubation of glomeruli with both $Cd^{++}$ and spermine-NONOate (NO donor) caused a significant (2-fold) increase in the mRNA levels of MT-I as compared with glomeruli incubated with sulfo-NONOate (nitrous oxide donor) and control medium (Fig. 1, compare lanes 5 and 7 with lanes 6 and 4, respectively). Surprisingly, SNP, a nonspecific donor of NO, did not induce expression of MT-I in the glomeruli (Fig. 1, compare lane 2 with lane 1).

**Effect of $Cd^{++}$ Preincubation on $^{\cdot}O_2$-Mediated Increase in $P_{alb}$.** Glomeruli were incubated with or with out $Cd^{++}$ (5 μM) for 4 hr at 37°C. After 4 hr, glomeruli were

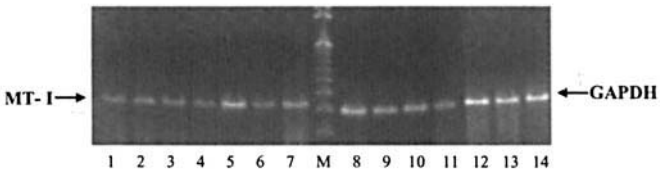


**Figure 1.** Documentation of MT-1 message expression by RT-PCR. As outlined in "Materials and Methods," glomeruli were incubated with various agents. Total RNA was prepared, and mRNA for MT-I and GAPDH was detected and quantified by RT-PCR. Lanes 1 and 12 are from control glomeruli. Lanes 2 and 13 are from glomeruli incubated with SNP (0.6 mM). Lanes 3 and 14 are from glomeruli incubated with SNP (0.6 mM) + carboxy-PTIO (NO scavenger). Lanes 4 and 8 are from control glomeruli. Lanes 5 and 9 are from glomeruli incubated with cadmium (5 μM). Lanes 6 and 10 are from glomeruli incubated with sulfo-NONOate (0.3 mM). Lanes 7 and 11 are from glomeruli incubated with spermine-NONOate (0.3 mM).

incubated with 10 nM of $^{\cdot}O_2$ for an additional 10 min at 37°C. $P_{alb}$ was measured as detailed in "Materials and Methods." Results are shown in Figure 2. Ten nanomoles $^{\cdot}O_2$ caused an increase in $P_{alb}$ ($0.8 \pm 0.06$, $n = 15$) as compared with control glomeruli ($0.0 \pm 0.12$, $n = 13$). The effect of $^{\cdot}O_2$ on $P_{alb}$ was significantly blocked when glomeruli were preincubated with 5 μM of $Cd^{++}$ ($0.21 \pm 0.12$, $n = 10$). These results suggest that upregulation of MT-1 by $Cd^{++}$ in glomeruli protected them from the effect of $^{\cdot}O_2$.

**Effect of NO and Nitrous Oxide Donor on $^{\cdot}O_2$-Mediated Increase in $P_{alb}$.** Glomeruli were incubated with or without NO, spermine-NONOate (0.3 mM), and nitrous oxide, sulfo-NONOate (0.3 mM) donor for 4 hr at 37°C. After 4 hr, glomeruli were incubated with 10 nM of $^{\cdot}O_2$ for an additional 10 min at 37°C. $P_{alb}$ was measured as detailed in "Materials and Methods." Results are shown in Figure 3. Ten nanomolar $^{\cdot}O_2$ caused an increase in $P_{alb}$ ($0.8 \pm 0.06$, $n = 15$) as compared with control glomeruli ($0.0 \pm 0.12$, $n = 13$). The effect of $^{\cdot}O_2$ on $P_{alb}$ was significantly blocked when glomeruli were preincubated with NO donor, spermine-NONOate ($0.24 \pm 0.19$, $n = 15$ vs. $^{\cdot}O_2$), whereas preincubation of glomeruli with nitrous oxide donor, sulfo-NONOate, did not block this effect ($0.54 \pm 0.19$,

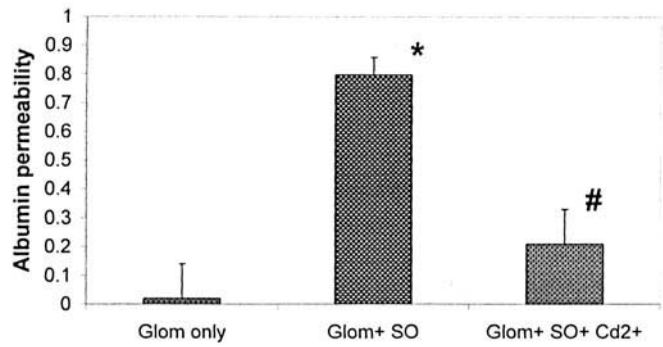


**Figure 2.** Effect of preincubation with $Cd^{++}$ on $^{\cdot}O_2$-mediated increase in $P_{alb}$. Glomeruli were preincubated with or with out $Cd^{++}$ (5 μM) for 4 hr and then with $^{\cdot}O_2$ (10 nM) for an additional 10 min. $^{\cdot}O_2$ ($n = 15$) significantly increased ($P < 0.01$) $P_{alb}$ of glomeruli as compared with control ($n = 13$), as shown by an asterisk. Preincubation with $Cd^{++}$ protected glomeruli ($P < 0.01$) from the effect of $^{\cdot}O_2$ ($n = 10$) as shown by #.

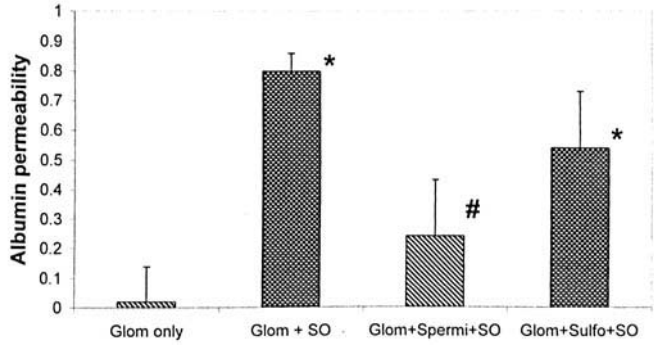


**Figure 3.** Effect of preincubation with nitric and nitrous oxide on $^{\cdot}O_2$-mediated increase in $P_{alb}$. Glomeruli were preincubated with or with out spermine-NONOate (0.3 mM) and sulfo-NONOate (0.3 mM) for 4 hr and then with $^{\cdot}O_2$ (10 nM) for an additional 10 min. $^{\cdot}O_2$ ($n$ = 15) significantly increased ($P < 0.01$) $P_{alb}$ of glomeruli as compared with control ($n$ = 13), as shown by an asterisk. Preincubation with spermine-NONOate ($n$ = 15) significantly protected ($P < 0.01$) glomeruli (#), where as sulfo-NONOate ($n$ = 15) did not protect (*) them from the effect of $^{\cdot}O_2$.

$n$ = 10 vs. $^{\cdot}O_2$). These results suggest that only those agents that upregulate MT-1 expression in glomeruli provide protection to glomeruli from the $^{\cdot}O_2$-mediated increase in $P_{alb}$.

**Effect of a Nonspecific Donor of NO on $^{\cdot}O_2$-Mediated Increase in $P_{alb}$.** Glomeruli were incubated alone or with nonspecific donor of NO, SNP (0.6 mM), in presence or absence of NO scavenger carboxy-PTIO (2-(4-carboxyphenyl) 4,4,5,5 tetra-methylimidazoline-3-oxide-1-oxyl) for 4 hr at 37°C. After 4 hr of incubation, glomeruli were washed with fresh 4% BSA medium and then incubated with 10 nM of $^{\cdot}O_2$ for an additional 10 min at 37°C. $P_{alb}$ was measured as detailed in "Materials and Methods." Results are shown in Figure 4. Ten nanomolar $^{\cdot}O_2$ caused an increase in $P_{alb}$ (0.8 ± 0.06, $n$ = 15) as compared with control glomeruli (0.02 ± 0.12, $n$ = 13). SNP alone (0.44 ± 0.19, $n$ = 10) or SNP with carboxy-PTIO (0.48 ± 0.21, $n$ = 15) did not block the $^{\cdot}O_2$-mediated increase in $P_{alb}$.

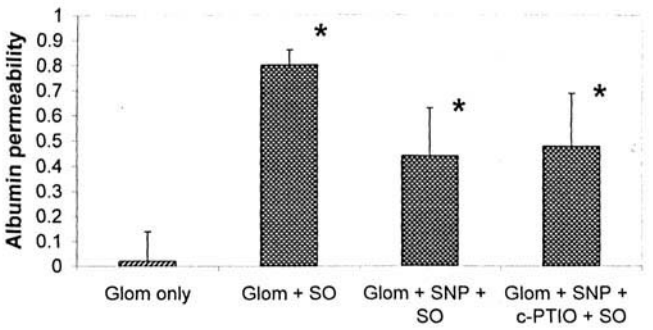


**Figure 4.** Effect of preincubation with nonspecific NO donor on $^{\cdot}O_2$-mediated increase in $P_{alb}$. Glomeruli were preincubated with or without SNP (0.6 mM) and SNP + carboxy-PTIO (5 µl of 10 mg/ml) for 4 hr and then with $^{\cdot}O_2$ (10 nM) for 10 min. $^{\cdot}O_2$ ($n$ = 15) significantly ($P < 0.01$) increased $P_{alb}$ of glomeruli as compared with control ($n$ = 13), as shown by an asterisk. Preincubation of glomeruli with SNP ($n$ = 15) or SNP + carboxy-PTIO ($n$ = 15) did not protect them from the effect of $^{\cdot}O_2$.

## Discussion

Our results indicate that $^{\cdot}O_2$ increased $P_{alb}$ *in vitro*. Pre-incubation of glomeruli with $Cd^{++}$ or spermine-NONOate (NO donor) increased the glomerular mRNA levels of MT-I and protected them from the effect of $^{\cdot}O_2$. Our results further document that a nonspecific NO donor (SNP) and nitrous oxide donor (sulfo-NONOate) did not induce glomerular MT-I expression and did not protect glomeruli from $^{\cdot}O_2$-mediated increase in $P_{alb}$.

The assay for measurement of $P_{alb}$ was developed and standardized in our laboratory 12 years ago to study the direct and immediate effect of an agent on glomerular permeability barrier (11). Since then we, along with other investigators, have used this assay extensively to study the alterations in the glomerular filtration barrier (11, 18, 19). In this method, isolated glomeruli are observed as isolation medium is replaced by medium of lower oncotic concentration. Under constant experimental conditions, the resulting expansion of capillaries and increase in glomerular size are proportional to the reflection coefficient of albumin ($\sigma_{alb}$). For convenience, we have calculated convectional albumin permeability, $P_{alb}$, as $(1 - \sigma_{alb})$. Alternative causes for diminished capillary distention, including altered exchangeable volume due to cell swelling and diminished capillary compliance, have been excluded using solutions of high molecular weight dextran. Capillary responses to these gradients assure that alterations in volumetric responses to an albumin gradient are due to a change in permeability rather than other changes. We have used our $P_{alb}$ assay to demonstrate the effects of anti-Fx1A (20), antibodies to $\beta_1$ integrin (21), TNF-α (22), TGF$\beta_1$ (23), protamine (11), platelet activating factor (24), and matrix metalloproteinase-3 (25) on the glomerular permeability barrier. In addition, we have used the assay to permit us to follow the permeability activity of FSGS sera through a series of biochemical steps to achieve partial purification of the FSGS factor (26).

As reviewed by Dr. Shah, there is ample evidence in literature that ROS are crucial mediators in inflammatory and noninflammatory glomerular disease (12). It has been shown that glomerular epithelial cells in response to toxin doxorubicin and puromycin aminonucleoside, (PAN; 27, 28) and mesangial cells in response to immune complex (29), phorbol myristate acetate (30), platelet activating factor (31), and TNF-α (32) are capable of producing ROS. Production of ROS is associated with increased albumin permeability in several animal models (33), and the blocking of the effects of these mediators with scavengers is associated with improvement of proteinuria (33). Wang *et al.* (34) showed that treatment with cyclosporine A decreased proteinuria in PAN-nephrosis rats; treated rats also showed higher activities of glomerular SOD and catalase and attenuation of foot process effacement. Ricardo *et al.* (35) showed that administration of SOD to rats with PAN nephrosis not only decreased proteinuria but also pro-

tected podocyte foot processes as examined with electron microscopy.

Using *in vitro* assay, we have demonstrated that $^{\cdot}O_2$ generated by either xanthine/xanthine oxidase system or by PMA-activated macrophages increased $P_{alb}$ of isolated glomeruli, and this effect was abrogated by SOD but not by catalase. These results indicate that $^{\cdot}O_2$ is the mediator of proteinuria (14). In other studies, we have also shown that incubation of glomeruli with PMA-activated rat PMN cells increased $P_{alb}$ and this increase was prevented by catalase, SOD, taurine, or sodium azide, implicating hypohalous acid as the mediator of proteinuria (15).

Functionally, MT is involved not only in metal detoxification and homeostasis, but also in scavenging free radicals during oxidative damage (36). Induction and synthesis of MT-I and II by oxidative and chemical stress and protection from radiation-induced injuries in human central nervous system cells (37) has been documented. The protective role of MT induction in rats against anticancer agent-mediated nephrotoxicity (38), allogeneic stimuli-mediated oxidative testicular damage (39), and *N*-nitrosodimethylamine-mediated oxidative and hepatotoxic effects (40) have been documented. Similarly, induction of MT in kidneys after injections of cadmium and zinc provided protection against cadmium nephrotoxicity (40, 41) and renal ischemia-reperfusion injury (42).

Results of this study show that $^{\cdot}O_2$ causes an immediate increase in $P_{alb}$, and induction of MT-1 genes with $Cd^{++}$ or spermine-NONOate protects glomeruli from the effect of $^{\cdot}O_2$. The type(s) of MT-1 gene product(s) and their localization in various glomerular cells that play a role in protection of glomerular filtration barrier function against oxidative injury remain to be studied. We conclude that induction of M-1 by oxidative stress may play an important role in protecting the glomeruli from immune and/or nonimmune renal injury caused by ROS.

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