Reactive Oxygen Species and Antioxidant Defense in Puromycin Aminonucleoside Glomerulopathy

WILFRIED GWINNER,* ULF LANDMESSER,* RALF P. BRANDES,† BIRGIT KUBAT,‡ JENS PLASGER,* OLIVER EBERHARD,* KARL-MARTIN KOCH,* and CHRISTOPH J. OLBRICHT*

*Division of Nephrology, †Division of Cardiology, Department of Internal Medicine, and ‡Department of Anatomy, Medical School Hannover, Hannover, Germany.

Abstract. Results from several radical scavenger studies indirectly suggested an involvement of reactive oxygen species in the pathogenesis of puromycin aminonucleoside glomerulopathy. In this study, generation of reactive oxygen species was examined directly in glomeruli isolated from rats in the acute phase of puromycin aminonucleoside nephrosis and related to the changes in the glomerular antioxidant defense. Five and nine days after puromycin aminonucleoside injection, gross proteinuria, reduced creatinine clearances, and typical changes of glomerular morphology were present. Levels of reactive oxygen species were increased eightfold in glomeruli isolated 15 min after puromycin aminonucleoside injection, returned to baseline levels on days 1 and 5 after injection, and rose again to 14-fold on day 9 after injection, as determined by chemiluminescence with luminol. Further analysis of increased glomerular radical generation, using the chemiluminescence enhancer lucigenin and different radical scavengers, suggested a predominant involvement of hydroxyl radical and hydrogen peroxide in the initial increase in reactive oxygen species 15 min after puromycin aminonucleoside. Nine days after induction of nephrosis, primarily superoxide anion and hydroxyl radical were found to contribute to increased reactive oxygen species. Despite oxidative stress, antioxidant enzymes were not induced in the course of nephrosis. On the contrary, catalase and glutathione peroxidase activities declined 9 d after puromycin aminonucleoside injection. The results indicate that a transient increase in glomerular reactive oxygen species is sufficient to induce the oxidative glomerular injury observed in this model and that the glomerulus may not necessarily respond to oxidative stress with an induction of antioxidant enzymes. (J Am Soc Nephrol 8: 1722–1731, 1997)

There is indirect evidence that reactive oxygen species (ROS) could mediate the glomerular injury in the acute phase of puromycin aminonucleoside (PAN) nephrosis in rats, a model of the human minimal change nephropathy. Metabolites of the reaction of ROS with lipids were increased in the course of nephrosis (1–7), and treatment with various oxygen radical scavengers reduced proteinuria and mitigated the pathomorphological alterations (2,8–14). However, glomerular ROS have not been determined directly in PAN nephrosis, and several important issues remain unsolved. First, the time course of increased glomerular ROS generation is unknown. In the available studies (2,8–14), antioxidant treatment was initiated before induction of nephrosis and was maintained for various time intervals thereafter, allowing no clear-cut conclusion of which times ROS were increased. Furthermore, measurements of lipid peroxidation as a correlate of increased ROS are difficult to interpret, because the kinetics of lipoperoxide metabolism in PAN nephrosis are unknown. Second, it has not been determined yet which radical is involved in the pathogenesis of the glomerulopathy. Whereas some investigators found that scavengers of superoxide anion radical, hydrogen peroxide, and hydroxyl radical were protective with different efficiencies (8,10), Diamond et al. (9) demonstrated a protective effect with superoxide anion scavengers only. A third, unsolved question is whether elevated glomerular ROS levels are the result of an increased radical generation alone or are also due to an impaired antioxidant defense of the glomeruli.

In the clinical setting, where in most instances therapy would be implemented after the onset of symptoms of renal disease, knowledge of the course of increased glomerular ROS in relation to the antioxidant defense in PAN nephrosis could help to establish specific antioxidant therapeutic strategies as a true interventional measure.

Therefore, the levels of glomerular ROS in the course of PAN nephrosis were determined using chemiluminescence with luminol, which detects superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals (15), and chemiluminescence with lucigenin, an enhancer that is relatively specific for superoxide anion radicals (15,16). In addition, specific radical scavengers were used in the chemiluminescence measurements for a more detailed analysis of increased radical levels. To assess the glomerular antioxidant defense, enzyme activities of the superoxide dismutases, catalase, and glutathione peroxidase were examined, and the observed changes were analyzed further by the determination of mRNA and protein levels of the
enzymes. In addition, to identify potential sources of ROS, the number of macrophages were examined in the glomeruli.

**Materials and Methods**

**Animals and Basic Procedures**

Male Sprague Dawley rats (8- to 10-wk old) were injected with PAN (Sigma, St. Louis, MO) dissolved in 0.9% NaCl at a dose of 15 mg/100 g body wt via the tail vein. Control rats received a similar volume of 0.9% NaCl. ROS were determined in glomeruli isolated 15 min, and 1, 5, and 9 d after PAN injection, and enzymes and histology were examined in glomeruli isolated 1, 5, and 9 d after PAN injection. Before the isolation of glomeruli 1, 5, and 9 d after PAN injection, 24-h urine collections were made for the determination of protein excretion and creatinine. In addition, 6-h urine samples were obtained directly after PAN injection and compared with the individual protein excretion of these animals before injection. Serum creatinine measurements were performed using an autoanalyzer (Beckman Instruments, Munich, Germany). Protein concentration in urinary samples was determined according to Lowry et al. (17). All procedures on animals were conducted in accord with National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Isolation of Glomeruli**

Animals were killed by cervical dislocation. Kidneys were flushed with 20 ml of ice-cold Krebs-Henseleit-saline buffer (KHS) via an aortal catheter. Minc ed cortices were passed through three consecutive sieves (300, 150, and 75 μm) using 2000 ml of ice-cold KHS. Glomeruli were washed twice with KHS and resuspended in 15 ml of KHS. Quantitation of glomeruli was performed in aliquots of the glomerular suspension by light microscopy. The purity of the glomerular suspension was more than 95% in all preparations.

**Glomerular Generation of ROS**

Generation of ROS was determined in a multichannel luminometer (Biolumat LB 9505, Berthold Corp., Wildbad, Germany). Measurements were performed at 37°C in the presence of 0.23 mM luminol or lucigenin (Sigma) in 500 μl of Dulbecco’s minimal essential medium for chemiluminescence without phenol red, and bicarbonate, containing 30 mM Hepes (Boehringer-Mannheim, Mannheim, Germany). After recording the background activity for 10 min, freshly prepared suspensions of 1000 glomeruli were added and allowed to equilibrate for 1 min. Subsequently, chemiluminescence (CL) activity was recorded over 30 min. In some of the measurements with luminal-enhanced CL, superoxide dismutase (SOD; 30 U) or dimethyl thiourea (0.1 M) was added to the glomeruli thereafter to detect specifically superoxide anion and hydroxyl radical. Hydrogen peroxide was detected by addition of horseradish peroxidase (1 U) and subsequent scavenging of the obtained signal by catalase (200 U) (18). In each case, a second dose of the radical scavenger was added after 10 min and showed no further decrease in CL activity, thus confirming that the antioxidant had been added in a sufficient dosage. All antioxidants were obtained from Sigma Chemicals. Glomerular generation of ROS was expressed as the average counts/min minus background activity and related to the protein concentration (Bio-Rad protein assay based on the Bradford procedure, Bio-Rad Laboratories, Munich, Germany) determined in the glomerular suspensions. Preliminary experiments confirmed that the CL activity of fresh glomeruli remains constant over more than 60 min and that glomeruli are still able to respond to the stimulus 12-O-tetradecanoylphorbol 13-acetate (Sigma) with an increased ROS generation after this time. Also, measurements were shown to be linear, in the range of 500 to 2500 glomeruli per vial.

**Glomerular Antioxidant Enzymes**

For the determination of enzymatic activities, pellets of 10,000 glomeruli were lysed at 4°C for 1 h in 1 ml of hypo-osmolar buffer containing 50 mM potassium phosphate, 0.1 mM ethylenediamine tetra-acetic acid (EDTA), and 1% Triton X-100, pH 7.8. From the resulting homogenate, an aliquot was drawn to determine protein concentration, and the remainder was centrifuged at 12,000 × g at 4°C for 20 min. The supernatant was stored at −70°C until assay of each enzyme activity. Preliminary experiments with different Triton X-100 concentrations confirmed that this isolation procedure resulted in a maximal release of enzymatic activities, leaving no measurable activity in the cellular debris. Sonication was not used because this led to a severe loss of catalase activity (>50%) even at very low energy levels.

SOD activities were determined by the cytochrome c assay, according to Crapo (19). Total SOD activity, i.e., copper/zinc-SOD (CuZn-SOD) plus manganese SOD (MnSOD), was assayed in the presence of 10 μM potassium cyanide to avoid interference by cytochrome c oxidase. MnSOD activity was measured separately after inhibition of CuZnSOD activity by 1 mM potassium cyanide. Preliminary experiments confirmed that 1 mM potassium cyanide was sufficient to inhibit CuZnSOD activity in glomerular samples by more than 90%. One unit of SOD was defined as the amount of SOD activity necessary to inhibit the reduction of cytochrome c by 50%.

Catalase activities were assayed spectrophotometrically by the conversion rate of 10 mM hydrogen peroxide in 50 mM potassium phosphate, 0.1 mM EDTA, pH 7.0. Calculation of activities was based on the extinction coefficient of hydrogen peroxide at 240 nm, 0.0425 mM⁻¹cm⁻¹, and the change in absorbance during the first 60 s of the reaction (20). Measurements were performed only in the linear range of the reaction.

Glutathione peroxidase (GSH-Px) activities were determined by the method described by Beutler (21), with minor modifications. tert-Butyl hydroperoxide (70 μM) served as a substrate for GSH-Px, and the reaction was followed spectrophotometrically by the consumption of NADPH by glutathione reductase, the enzyme that reduces the glutathione oxidized by GSH-Px. Reactions were performed in a buffer containing 0.1 M Tris-Cl, 0.5 mM EDTA, 20 mM glutathione, 2 mM NADPH, and 0.5 U of glutathione reductase, pH 7.7 (Sigma). Activities were calculated using the extinction coefficient of NADPH at 340 nm, 6.22 mM⁻¹cm⁻¹ (21), and the change in absorbance during the first 2 min of the reaction. Measurements were performed only in the linear range of the reaction.

Bovine enzyme standards of erythrocyte CuZnSOD, GSH-Px, and liver catalase (Sigma) were included in each assay to control interassay variation. Analysis of the protein concentration in glomerular homogenates containing Triton-X 100 was performed with a modified Lowry’s method (17). Protein concentrations relative to the number of glomeruli revealed no differences between PAN-treated animals and controls. Therefore, all enzyme activities were expressed relative to the glomerular protein concentration.

Detection of antioxidant enzymes by Western blotting was performed as described previously (22). Samples of 80 μg of glomerular protein were separated on a 1.5-mm-thick sodium dodecyl sulfate/12% polyacrylamide gel and blotted to nitrocellulose. Catalase was detected with a polyclonal antibody from rabbit against human erythrocyte catalase (Calbiochem, San Diego, CA). GSH-Px was detected
with a polyclonal antibody from rabbit against human erythrocyte GSH-Px, kindly provided by Dr. Nelly Avissar (23). The bound primary antibody was reacted with an anti-IgG rabbit antibody from donkey coupled with horseradish peroxidase (Amersham Corp., Paisley, United Kingdom). Protein bands were detected by using a CL kit (Amersham Corp.) and exposure of blots to x-ray film (Kodak X-OMAT AR, Rochester, NY). The intensity of the detected bands was analyzed by densitometry, and readings of PAN-treated animals were expressed as percentage of the controls. In preliminary experiments with different amounts of glomerular protein and enzyme standards loaded on the gel, the linear range of densitometry readings was established, enabling a quantitative evaluation of antioxidant enzymes.

mRNA levels of antioxidant enzymes were quantitatively determined according to established protocols described elsewhere (22). Rat catalase cDNA was a gift from Dr. Shuichi Furuta (24), and rat GSH-Px cDNA was a gift from Dr. Ambati P. Reddy (25). As an internal control for loading variances, the constitutively expressed mRNA levels of β-actin were used (26).

**Morphological Studies**

After perfusion of the kidneys with KHS, a tissue slice was cut from the cortex, fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate HCl buffer, post-fixed in 2% osmium tetroxide, and embedded in Epon 812 (Serva, Heidelberg, Germany). For light microscopy, 1-μm sections were stained with toluidine blue. For electron microscopy, 60-nm sections were cut and stained with uranyl acetate and lead citrate. Tissue for staining with a murine monoclonal antibody to ED-1 (Serotec, Oxford, United Kingdom), a cytoplasmic antigen present in monocytes and macrophages (27), was fixed in methyl Carnoy’s fixative (28) and embedded in paraffin. Staining of 4-μm sections was performed using an immunoperoxidase system. The number of positive cells per glomerular cross-section of 30 glomeruli was determined.

**Statistical Analyses**

All values are expressed as means ± SD. For the results of antioxidant enzyme activities and mRNA levels; protein excretion; and creatinine clearance, statistical comparison among the groups was performed by one-way ANOVA followed by t-test. All controls from days 1, 5, and 9 were combined in one group because there was no statistical difference among the control groups. Protein excretion of the 6-h period directly after PAN injection was compared with the individual protein excretion of these animals before injection by Wilcoxon’s U test for paired data. The results on glomerular generation of ROS and ED-1-positive cells were tested with the nonparametric Kruskal-Wallis test and post-tested with Dunn’s multiple comparisons test. Data on Western blot analysis and CL radical scavenger experiments were tested with Wilcoxon’s U test. A P value <0.05 was considered statistically significant.

**Results**

**Protein Excretion, Creatinine Clearance, and Kidney Morphology**

Protein excretion in the first 6 h after PAN administration was not different from the value before injection, regardless of whether protein excretion was expressed per hour or was related to creatinine in urine (before PAN: 4.58 ± 1.52 mg/h or 133 ± 47 μg/μmol creatinine; after PAN: 5.02 ± 1.35 mg/h or 172 ± 44 μg/μmol creatinine). A dramatic increase in protein excretion was noted 5 and 9 d after PAN treatment (Figure 1). At the same time, creatinine clearances declined progressively by 40 and 50%, respectively. Glomerular morphology was unchanged 1 d after PAN injection (not shown). Five days after induction of nephrosis, all glomeruli were found to be affected at the site of the visceral epithelial cells (shown in Figure 2). Nine days after PAN, the changes in epithelial cells were more pronounced than 5 d after PAN. In addition, focal detachment of epithelial cells from the glomerular basement membrane and occasional adhesions between visceral epithelial cells and the Bowman’s capsule were observed (not shown).

**Glomerular Levels of ROS**

By CL with luminol, a general radical detector (15), an eightfold increase in ROS was observed in glomeruli isolated.

**Figure 1.** Protein excretion and creatinine clearance of controls (C) and puromycin aminonucleoside (PAN)-treated animals 1, 5, and 9 d after PAN administration. *P < 0.01.
Figure 2. Glomerular morphology of controls (2a and 2b) and nephrotic animals 5 d after PAN injection (c through e). By light microscopy, visceral epithelial cells of nephrotic animals showed marked vacuolization (2c, asterisks). Electron microscopy (2d and 2e) revealed loss of the normal architecture of epithelial foot processes and flattened epithelial cells with multiple cytoplasmatic electron-lucent or electron-dense (asterisk) vacuoles. No changes were observed in the glomerular basement membrane and in mesangial and endothelial cells. Occasionally, ladder-like structures could be detected in the the slits between adjacent visceral epithelial cells (arrows). Magnification: ×460 in 2a and 2c, ×6250 in 2b, ×6800 in 2d, and ×40,000 in 2e.

15 min after PAN injection. However, with lucigenin as a relatively specific probe for superoxide anion radicals (15,16), no increase in CL was detected at this time. Nine days after PAN treatment, glomerular ROS levels, as detected with luminol, were elevated 14-fold. With lucigenin, a fivefold increase in CL activity was noted at the same time (Figure 3a).
In additional experiments, it was shown that PAN did not cause a CL signal in the absence of glomeruli (data not shown).

Increased glomerular ROS levels were examined further by specific radical scavengers. The contribution of superoxide anion radicals to increased total CL was examined by the addition of SOD, and results were expressed as SOD-inhibitable CL. As shown in Figure 3b, superoxide anion did not contribute significantly to the initial rise in glomerular CL 15 min after PAN injection, but was found to account for a considerable portion of the increased CL observed at day 9 after PAN. The hydroxyl radical scavenger dimethyl thiourea was used to identify an involvement of hydroxyl radical in increased ROS observed in the glomeruli. Dimethyl thiourea-inhibitable CL accounted for the major portion of total increased CL in glomeruli of PAN-treated animals at both time points (15 min and 9 d after PAN), indicating that hydroxyl radical was mainly responsible for increased ROS after PAN. Low concentrations of hydrogen peroxide are difficult to detect by CL. Therefore, a horseradish peroxidase-based CL assay was used (18), taking advantage of the fact that the reaction of peroxidase with hydrogen peroxide results in a strong CL signal. The specificity of the obtained signal for hydrogen peroxide was confirmed by scavenging with catalase, and hydrogen peroxide levels were expressed as peroxidase-elicited CL that is inhibitable by catalase. Compared with the controls, glomeruli isolated 15 min after PAN injection had a small, twofold increase in catalase-inhibitable CL activity, whereas glomeruli from animals 9 d after PAN injection showed no changes.

**Glomerular Antioxidant Enzymes**

The activities of the superoxide anion scavenging enzymes CuZnSOD and MnSOD were unchanged in the course of PAN nephrosis. The activities of catalase, a specific hydrogen peroxide scavenger, declined to 75% on day 9 after PAN injection. GSH-Px, the enzyme that metabolizes different peroxides, including hydrogen peroxide, was also reduced significantly 9 d after PAN (Figure 4).

The cause of reduced catalase and GSH-Px activities was analyzed further by Northern blotting, revealing no changes in steady-state mRNA levels of the enzymes in PAN-treated animals at any time point compared with the controls (Figure 5). Analysis of the enzyme protein levels by Western blotting in glomerular samples from animals 9 d after PAN treatment showed a reduction of catalase by almost 50% compared with the controls, whereas the 20% reduction in GSH-Px levels was statistically insignificant (Figure 5).

**Potential Source of ROS**

Macrophages have been identified as potential mediators of glomerular injury in PAN nephrosis (29) and could also be a source of ROS (30). Using an antibody to an antigen of macrophages and monocytes, ED-1, the number of positive cells per glomerular cross-section was unchanged 1 d after PAN (0.62 ± 0.14) compared with controls (0.53 ± 0.14), and more than doubled on day 5 (1.11 ± 0.38; \( P = 0.055 \)) and day 9 (1.32 ± 0.36; \( P < 0.01 \)) after PAN administration.

**Discussion**

On the basis of several studies with ROS scavengers (2.8–14), there is no doubt that ROS are involved in the pathogenesis of acute PAN nephrosis. However, an unexpected finding of this study was that glomerular ROS were increased at specific time points only, and not during the entire course of acute nephrosis. The observed immediate and transient rise of ROS in glomeruli isolated 15 min after PAN injection is in accordance with *in vitro* results on kidney slices from Ricardo *et al.* (31), who showed that ROS increased within 1 h after incubation with PAN and returned to baseline levels within 4 h. Earlier studies confirm that PAN rapidly induces renal injury.
In the transplantation experiments of Hoyer et al. (32), PAN was shown to require less than 15 min to cause subsequent nephrosis.

Despite the observed rapid increase in ROS, no rise in protein excretion was found immediately after PAN injection, as compared to a previous study by Yoshioka et al. (33), in which direct injection of hydrogen peroxide into the renal artery led to an immediate increase in protein excretion. An explanation for this may be that ROS levels in our experiments were much lower than in their study, in which hydrogen peroxide was used in the micromolar range. Even more remarkable, despite normal glomerular ROS levels 1 and 5 d after PAN injection, animals developed nephrotic syndrome and pathomorphological alterations within this time, indicating that sustained elevated ROS levels were not required in the progress of renal injury. Of note, this conclusion does not contradict the available studies on the protective effect of antioxidants in PAN nephrosis (2,8–14). Antioxidant therapy was always started before PAN administration, and, although the duration of treatment varied greatly, the protective effect was not related to the length of antioxidant therapy. For instance, Diamond et al. (9) observed a 60% reduction in proteinuria in rats treated with SOD 30 min before and 30 min after PAN injection. This is not different from the results of Ricardo et al. (10), in which SOD treatment 3 h before, and 3 and 24 h after PAN injection led to a reduction of proteinuria by 60%. In another study (11), dimethyl thiourea or deferoxamine was given before PAN injection and subsequently continued for either 2 or 7 d; however, the observed protection was independent of the duration of treatment with these antioxidants. On the other hand, there is no study demonstrating a protective effect in the acute phase of PAN nephrosis when antioxidant therapy is implemented subsequent to PAN administration.

Seemingly in contrast to our results that glomerular ROS were not elevated on days 1 and 5 after PAN injection, increases in malondialdehyde (MDA) levels in renal cortex or glomeruli and in urine have been found at various time points in acute PAN nephrosis (1–3,5,7), together suggesting sustained lipid peroxidation due to continuously elevated renal ROS levels during the entire course of acute PAN nephrosis. However, it has also been shown that PAN causes increased MDA levels in plasma (6) and in several organs, including the liver, lung, and heart, that peak at different times during the acute phase of PAN nephrosis (3). Because the kinetics of MDA metabolism and the redistribution of MDA between the different organs and plasma are unknown, we propose that MDA may generally serve as an indicator of increased radical
Figure 5. Glomerular mRNA and enzyme protein levels of catalase and glutathione peroxidase in PAN-treated animals. Values are expressed as the percentage of controls (mRNA levels: n = 9 each group; protein levels: n = 11 each group).

generation. However, MDA measurements may be of limited value to exactly determine the time course of increased ROS levels in the glomerulus in PAN nephrosis.

Using different probes for the CL measurements on glomeruli, superoxide anion radicals were not found to contribute to increased ROS in glomeruli isolated 15 min after PAN injection, suggesting that radicals other than superoxide anion, such as hydrogen peroxide or hydroxyl radical, were primarily responsible for the induction of nephrosis. Different from this early time point, at 9 d after PAN administration, increased CL with lucigenin indicated an involvement of superoxide anion radicals. To examine this phenomenon more closely, increased CL activities were analyzed further by specific radical scavengers. First, the results of these studies may be taken as evidence that the observed increases in CL activity were actually due to increased levels of radicals. Second, similar to the results with luminol- and lucigenin-enhanced CL, the results indicate differences in the radical species involved in the induction phase of nephrosis compared to 9 d after PAN.

In the glomeruli isolated 15 min after PAN injection, the major portion of increased CL was, as shown by the effect of dimethyl thiourea, attributable to hydroxyl radical, which is considered one of the most toxic radicals (34). Furthermore, hydrogen peroxide, as detected with horseradish peroxidase and catalase, was increased twofold, whereas SOD-inhibitable CL as a measure of superoxide anion was not increased. Interestingly, studies mentioned earlier (8–10) could demonstrate partial protection by superoxide anion scavengers in the induction phase of PAN nephrosis. In light of our findings, this protective effect could be related to a removal of the superoxide anions physiologically present at low levels in glomeruli (as shown in Figure 3b) that otherwise could react with hydrogen peroxide in the Haber-Weiss reaction (34) to yield hydroxyl radicals. In agreement with this hypothesis and with our observations that hydrogen peroxide and hydroxyl radical are increased during the induction of PAN nephrosis, are reports that showed maximal antioxidant protection in PAN nephrosis using hydroxyl radical scavengers and iron chelators that inhibit the Haber-Weiss reaction (2,11,12). Similarly, catalase and an iron chelator, but not SOD, was cytoprotective in PAN-treated glomerular epithelial cells in culture (35).

Nine days after PAN administration, SOD-inhibitable CL was found to contribute significantly to total CL, indicating a role for superoxide anion radicals in this phase, whereas no change in hydrogen peroxide was observed. As with the glomeruli isolated 15 min after PAN, hydroxyl radical appeared to account for the major portion of increased CL activity.

However, caution must be exercised in interpreting these CL data. First, an exact quantitative assessment of the contribution of a particular radical is certainly not possible with this
method. Second, although the use of enzymes such as SOD and catalase is the most specific approach in identifying superoxide anion radicals and hydrogen peroxide, it is limited by the fact that these enzymes can only scavenge ROS released extracellularly. Because superoxide anion and hydrogen peroxide can cross cell membranes (36), the results obtained with the enzymes may be taken as an indirect measure of the excess intracellular ROS that permeate extracellularly, in addition to ROS potentially generated by membrane-bound oxidases and primarily released extracellularly. Taken together, our results point to an important role of hydroxyl radical, but also to qualitatively different oxidant mechanisms in the initiation phase and the subsequent course of PAN nephrosis.

As pointed out by Ichikawa et al. (37), the level of glomerular ROS is precisely balanced by a system of antioxidant enzymes, and oxidative stress can induce these enzymes in the glomerulus (38) and in glomerular cell culture (39). Therefore, our aim was to relate the findings on glomerular ROS to the regulation of the glomerular antioxidant enzymes. An important result of these studies is that antioxidant enzymes were not induced. Instead, a decrease in enzyme activities of catalase and GSH-Px was noted 9 d after PAN administration. Similar results on catalase in PAN nephrosis have been obtained by Kawamura et al. (5). Further analysis of the mechanism leading to decreased catalase and GSH-Px activities showed that mRNA levels of these enzymes were unchanged, indicating a posttranscriptional alteration. Using a quantitative Western blot system, we found unchanged glomerular GSH-Px enzyme levels, suggesting that inactivation of the enzyme caused decreased GSH-Px activity. In contrast, we could demonstrate a significant decrease in catalase protein levels as the probable cause for the decreased activity. It remains uncertain whether reduced catalase protein levels were due to decreased synthesis or increased degradation of the enzyme. In this respect, it has been shown that PAN can decrease protein synthesis in various organs, including the kidney, as determined by glycine incorporation into proteins, whereas mRNA synthesis remains unaffected (40).

The mechanism(s) leading to increased glomerular ROS levels in PAN nephrosis remain largely unknown. Because antioxidant enzymes were unchanged 1 d after PAN injection, the initial increase in ROS in all likelihood resulted from an increased generation of ROS induced by PAN by a hitherto unknown pathway (14,41).

With regard to the increased ROS levels 9 d after PAN, different mechanisms must be considered. Increased generation of ROS may be primarily attributed to the increased numbers of macrophages that were found in the glomerulus 5 and 9 d after PAN administration. Potential mechanisms include direct generation of superoxide anions by macrophages (30) and macrophage-mediated release of the cytokines tumor necrosis factor-α and interleukin-1 in PAN nephrosis (42) that in turn can induce ROS in glomerular cells (43). Because increased glomerular macrophages were already found on day 5 after PAN, whereas ROS levels were still unchanged, a lag phase in the activation of radical-generating pathways may be assumed.

In addition to increased generation of ROS, decreased detoxification of ROS due to the observed impaired glomerular antioxidant defense must be considered as a contributing factor that led to increased ROS levels at day 9 after PAN administration. Because activities of enzymes involved in hydrogen peroxide catabolism were decreased, one might have expected increased hydrogen peroxide levels in the CL measurements. However, based on the observed high levels of superoxide anion and hydroxyl radical, it is also conceivable that superoxide anion reacted via the Haber-Weiss reaction with the hydrogen peroxide not detoxified by catalase or glutathione peroxidase, thereby lowering hydrogen peroxide levels and increasing the levels of hydroxyl radical.

In summary, the oxidative injury that induces PAN nephrosis is mediated by a rapid and transient generation of ROS. However, progression to nephrotic syndrome does not require continuously elevated glomerular ROS. Increased ROS 9 d after PAN treatment, when nephrotic syndrome is already present, are associated with increased numbers of glomerular macrophages and with an impaired antioxidant glomerular defense. Although not within the scope of this study, this late increase in glomerular ROS might be one of the mechanisms leading to the chronic picture of focal glomerular sclerosis observed in PAN nephrosis (29). In this respect, evidence for a role of ROS in the chronic phase of PAN nephrosis has been demonstrated by ongoing lipid peroxidation and decreased glomerular antioxidant enzyme activities 20 d after PAN administration (4).

Our findings are intriguing with respect to the clinical problem of glomerulonephritis. If induction of glomerulonephritis requires only one transient increase in radicals, timely antioxidant therapy will be a major problem. Also of importance is that the glomerulus apparently does not always respond to oxidative stress with an increase in the intrinsic antioxidant defense, as observed in other models of renal disease (38). It remains to be evaluated whether stimulation or supplementation of antioxidant enzymes is beneficial in the later course of glomerulopathy when the intrinsic antioxidant defense is impaired, as in PAN nephrosis.

Acknowledgments

This work was supported by Grant GW 4/3-1 from the Deutsche Forschungsgemeinschaft to Dr. Gwinner. The authors acknowledge the help of Dr. J. Floege (Medical School Hannover, Hannover, Germany) on the immune histochemistry and the technical assistance of E. Gutjahr, H. Lindemann, and G. Schweitzer. We thank Dr. Nelly Avisar (University of Rochester, Rochester, NY) for the GSH-Px antibody, Dr. Shuichi Furuta (Shinsu University School of Medicine, Matsumoto, Japan) for the catalase cDNA, and Dr. Ambati P. Reddy (University of Michigan, Detroit, MI) for the GSH-Px cDNA.

References


