Glomerular Oxidative and Antioxidative Systems in Experimental Mesangioproliferative Glomerulonephritis

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Abstract. Increased mesangial cell proliferation is a hallmark of many glomerulopathies in humans. Whereas the pathogenic role of reactive oxygen species (ROS) in the development of experimental mesangioproliferative glomerulonephritis (GN) is well established, very little is known about the mechanisms leading to increased ROS concentrations in the glomerulus. This study therefore examined glomerular ROS and the activities of oxidative and antioxidative enzymes during the early course of mesangioproliferative anti-Thy 1.1 GN. Anti-Thy 1.1 GN was induced in male Wistar rats, and glomeruli were isolated 2, 24, and 120 h after disease induction to examine ROS levels as well as oxidative and antioxidative enzyme expression in comparison to non-nephritic controls. At all time points, increased glomerular ROS levels, particularly of hydrogen peroxide and superoxide anions, were observed. Activities of NADH-dependent and NADPH-dependent oxidative enzymes were also increased during the course of GN, whereas no increased activity of xanthine oxidase, another potential source of ROS, was detectable. Despite glomerular oxidative stress, no compensatory increase of antioxidative enzyme activities occurred. On the contrary, catalase, superoxide dismutase, and glutathione peroxidase activities even decreased during the course of disease. In tubulointerstitial samples, no increase in oxidative activity was observed in the course of disease, thus confirming that detected ROS were of glomerular origin. Our data document for the first time a pronounced increase in oxidative activity was observed in the course of disease.

Proliferation of mesangial cells and extracellular matrix protein accumulation is a hallmark of several types of glomerulonephritis (GN) in humans, such as membranoproliferative GN, poststreptococcal GN, variants of focal glomerulosclerosis, lupus nephritis, diabetic nephropathy, and IgA-nephropathy (1,2). Anti-Thy 1.1 GN in rats is a particularly well-characterized model of mesangioproliferative GN (1–3) that is induced by a single intravenous injection of an anti–mesangial cell antibody leading to acute mesangial cell lysis followed by a phase of intense mesangial cell proliferation and extracellular matrix accumulation (1,2,4). Several studies have provided definitive evidence for both increased glomerular reactive oxygen species (ROS) as well as a pathogenic role of ROS during the acute phase of anti-Thy 1.1 GN (5–8). Despite these studies, several important issues are still poorly understood. First, our knowledge about the sources of ROS generation in the course of anti-Thy 1.1 GN is incomplete. Second, the time course of the in vivo production of glomerular ROS and the predominant species of radicals generated are unknown. Thus, in the available studies, analyses of ROS were performed only in a very early phase of the disease (6,8,9) or by using isolated macrophages and nonadherent cells obtained from enzymatically digested glomeruli (7). The third and most important question, which has not been addressed so far, is whether increased levels of glomerular ROS are the consequence of augmented oxidative enzyme activities or of an inadequate function of antioxidative systems in the glomeruli. Therefore, the aim of this study was to perform a comprehensive assessment of glomerular oxidative and antioxidative systems in the acute and chronic phase of anti-Thy 1.1 mesangioproliferative GN.

Materials and Methods

Experimental Model and Experimental Design

Anti-Thy 1.1 mesangioproliferative GN was induced in 18 male Wistar rats (Charles River, Salzfeld, Germany), weighing 160 to 200 g at the beginning of the experiment, by a single injection of 1 mg/kg anti-Thy 1.1 IgG (clone OX7; European Collection of Animal Cell Culture, Salisbury, UK) into a tail vein. Seven control animals received an equal volume of 0.9% NaCl solution. Animals were kept at 25°C with free access to tap water and to a standard rat chow. The animals were sacrificed at 2 h and on days 1 and 5 after disease induction. A renal cortical section from each rat was taken for immunohistochemistry. Glomeruli and the tubulointerstitial fraction were prepared by sequential sieving (see below). Determination of ROS was performed immediately after the isolation of glomeruli, and the remaining glomerular samples were stored for analysis of the activities, protein, and mRNA levels of oxidative and antioxidative en-

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zymes. To rule out a tubulointerstitial contribution to oxidative activities observed in anti-Thy 1.1 nephritis, the tubulointerstitial generation of ROS was determined in the course of disease by determination of lucigenin-mediated chemiluminescence. Before the isolation of glomeruli at 2 and 5 d after disease induction, 12-h overnight urine collections were performed to determine proteinuria. All animal experiments were conducted in accord with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the institutional and governmental review boards.

Isolation of Renal Tissues

Animals were sacrificed by cervical dislocation, and the kidneys were perfused immediately with 10 ml of ice-cold Krebs-Henseleit-Saline buffer (KHS) (10) via an intraaortal catheter. The glomeruli and the tubulointerstitial fraction were prepared by differential sieving of minced cortices as described previously (6) and stored in ice-cold KHS until further use. All steps were performed at 4°C within 15 to 20 min to preserve functional and structural integrity of the tissue. The purity of the glomerular preparations was higher than 95% in all samples, and tubulointerstitial samples were free of glomeruli, as determined by light microscopy, respectively.

Renal Morphology

Tissue for light microscopy and immunoperoxidase staining was fixed in methyl Carnoy solution (4) and embedded in paraffin. Four-micrometer sections were stained with the periodic acid-Schiff (PAS) reagent and counterstained with hematoxylin. In these PAS-stained sections, the degree of mesangiolysis was graded semiquantiitatively on a scale from 0 to 4 as described previously (2). Mesangial cell proliferation was determined by the number of glomerular cells showing mitotic figures. The quantification of glomerular polymorphonuclear leukocytes (PMN) was performed by light microscopy at a 1000-fold magnification. In addition, 4-μm sections of methyl-Carnoy–fixed and paraffin-embedded renal tissue were processed by an indirect immunoperoxidase technique (4). To detect monocytes, macrophages, and dendritic cells, the ED1-antibody was used, a murine monoclonal IgG against a cytoplasmic antigen present in those cell populations (11) (Bioproducts for Science, Indianapolis, IN). To assess mesangial cell activation, immunostaining for α-smooth muscle actin was performed (2) and evaluated using a semiquantiitative score from 0 to 4 as described previously (12). Detection of catalase was performed using methyl-Carnoy–fixed tissue and a polyclonal rabbit antibody to human erythrocyte catalase. Renal copper/zinc-SOD was measured separately after inhibition of copper/zinc-SOD by 1 mM potassium cyanide. One unit of SOD was defined as the amount of SOD activity necessary to inhibit the reduction of cytochrome c by 50%.

Glomerular Antioxidative Enzymes

The activities of glomerular antioxidative enzymes were determined as described previously (15). In brief, enzymes were extracted from glomerular cells by hypo-osmolar lysis in 1% Triton, and an aliquot of the homogenate was kept for determination of the protein content. The remainder was centrifuged, and the supernatant was stored at −70°C until the assay of each enzyme activity.

SOD activities were determined as total SOD (i.e., copper/zinc and manganese SOD) by the cytochrome c reduction assay according to Crapo et al. (16) in the presence of 10 μM potassium cyanide to avoid interference by cytochrome c oxidase. Manganese SOD activity was measured separately after inhibition of copper/zinc-SOD by 1 mM potassium cyanide. 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 hydrogen peroxide. Calculation of enzyme activities was based on the extinction coefficient of hydrogen peroxide at 240 nm, 0.0425 mM−1·cm−1, and the change in absorption during the first 60 s of the reaction (15).

Glutathione peroxidase activities were determined according to the method described by Beutler (17), with minor modifications. Briefly, tert-butyl hydroperoxide served as a substrate for glutathione peroxidase, and the reaction was followed spectrophotometrically by the consumption of NADPH by glutathione reductase. Activities were calculated using the extinction coefficient of NADPH at 340 nm, 6.22 mM−1·cm−1 (17), and the change of absorption during the first 2 min of the reaction.

Measurements for catalase and glutathione peroxidase were only performed in the linear range of the reaction. Bovine enzyme standards of erythrocyte copper/zinc–SOD, glutathione peroxidase, and liver catalase were included in each assay to control intra-assay variation.

Analysis of the protein concentration in glomerular homogenates was performed with a modified Lowry method (18), and enzyme
activities were expressed relative to the glomerular protein concentration. There was no detectable difference in protein concentrations per glomerulus between nephritic and control animals.

Glomerular Oxidative Enzymes

Glomeruli stored at −70°C were thawed, stored on ice, and homogenized with a potter. From the resulting homogenate, an aliquot was taken for protein determination; the remainder was subjected to another freeze-thaw cycle at −70°C to ensure complete release of enzyme activities. Samples were kept on ice until assay of each enzyme activity.

NADH- and NADPH-dependent oxidative activities were determined by detection of superoxide anion–dependent, lucigenin-mediated chemiluminescence as described previously by our group (15). Briefly, glomerular samples containing 5 μg of protein were preincubated for 5 min in the presence of 0.23 mM lucigenin and background chemiluminescence was subsequently recorded. After addition of the respective substrate (100 μM NADH or NADPH), the resulting chemiluminescence was recorded during the following 2.5 min. The results were expressed as average counts · min⁻¹ · mg⁻¹ of protein after subtraction of the background chemiluminescence. The measurements were done in the linear range of the reaction.

Xanthine oxidase activity was determined in a similar manner by a procedure described previously by Mohazzab and Wolin (19) with modifications (15). The assay is based on the enzyme-catalyzed conversion of xanthine to uric acid with the simultaneous generation of superoxide anion detectable by lucigenin chemiluminescence. Glomerular homogenates containing 50 μg of protein were preincubated in the presence of 0.23 mM lucigenin at 37°C in the luminometer. After a 2-min equilibration and recording of background chemiluminescence, the reaction was started by addition of 0.0338 mM xanthine; the resulting chemiluminescence signal was subsequently recorded for a period of 2.5 min. After subtracting the background chemiluminescence signal, results were expressed as average counts · min⁻¹ · mg⁻¹ of protein.

Detection of Antioxidative Enzymes by Western Blot Analysis

Western blotting was performed as described previously (20). Samples of 80 μg of glomerular protein were separated on a sodium dodecyl sulfate/12% polyacrylamide gel and blotted to nitrocellulose. Catalase was detected with a polyclonal rabbit antibody to human erythrocyte catalase, copper/zinc-SOD was detected by incubation with a polyclonal sheep antibody to human erythrocyte SOD (both obtained from Calbiochem), and manganese SOD was detected with a polyclonal rabbit antiserum (prepared as described in reference 15) to rat manganese SOD. All primary antibodies were used in a dilution of 1:1000 in phosphate-buffered saline (PBS) followed by incubation with an anti-rabbit IgG or anti-sheep IgG antibody (1:10,000) from donkey coupled with horseradish peroxidase (Amersham Corp., Paisley, UK). Protein bands were detected with a chemiluminescence kit (Amersham Corp.) and exposure of the blots to x-ray film (X-OMAT AR; Kodak, Rochester, NY). The intensity of the bands detected was analyzed by densitometry, and readings obtained in anti-Thy 1.1 nephritic animals were expressed as percentage of the controls.

Preparation of Glomerular RNA and Northern Blot Analysis

Glomerular manganese SOD, copper/zinc SOD, catalase, and glutathione peroxidase-I mRNA levels were detected after electrophoresis of 10 μg of total RNA and Northern blotting according to established protocols described elsewhere (20). Rat catalase cDNA was a kind gift from Dr. Shuichi Furuta (Shinshu University School of Medicine, Matsumoto, Japan), and rat glutathione peroxidase-I cDNA was a gift from Dr. Ambati P. Reddy (University of Michigan, Detroit, MI). All probes were successively hybridized on the same blot. As an internal control for loading variances, the constitutively expressed mRNA-levels of 28 S rRNA were used.

Miscellaneous Measurements

Urinary protein excretion was determined according to the method of Lowry et al. (18).

Statistical Analyses

Results were derived from at least five independent experiments. All values were expressed as mean ± SD. Comparisons were performed with the Wilcoxon U test. P < 0.05 was considered statistically significant.

Results

Course of Acute Anti-Thy 1.1 GN

We first confirmed that anti-Thy 1.1 nephritis followed the typical course of the disease, and we paid particular attention to activated mesangial cells and infiltrating leukocytes as potential sources of ROS.

### Table 1. Proteinuria, mesangiolysis, mitotic activity, glomerular α-smooth muscle actin expression, glomerular PMNL, and monocytes/macrophages in rats with anti-Thy 1.1 mesangioproliferative glomerulonephritis at 2 h and on days 1 and 5 after disease induction and in NaCl-treated control animals

<table>
<thead>
<tr>
<th></th>
<th>Proteinuria (mg/d)</th>
<th>Mesangiolysis (score)</th>
<th>Mitotic Figures/100 Glomerular Cross-Sections</th>
<th>Glomerular α-Smooth Muscle Actin Expression (score)</th>
<th>PMNL per 100 Glomerular Cross-Sections</th>
<th>Monocytes/Macrophages per 100 Glomerular Cross-Sections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>21 ± 19</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0.03 ± 0.05</td>
<td>2.7 ± 2.6</td>
<td>57 ± 25</td>
</tr>
<tr>
<td>2 h</td>
<td>NDb</td>
<td>0.17 ± 0.22</td>
<td>0.26 ± 0.89</td>
<td>0.05 ± 0.07</td>
<td>114.8 ± 44.3a</td>
<td>199 ± 82a</td>
</tr>
<tr>
<td>Day 1</td>
<td>143 ± 116a</td>
<td>0.37 ± 0.28</td>
<td>0.37 ± 1.29</td>
<td>0.12 ± 0.17a</td>
<td>16.2 ± 10.6a</td>
<td>523 ± 166a</td>
</tr>
<tr>
<td>Day 5</td>
<td>141 ± 12a</td>
<td>0.64 ± 0.26a</td>
<td>8.19 ± 4.74a</td>
<td>1.94 ± 0.52a</td>
<td>4.3 ± 2.6a</td>
<td>447 ± 153a</td>
</tr>
</tbody>
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a P < 0.01 versus controls.
b ND, not determined.
As shown in Table 1, following induction of nephritis, proteinuria increased nearly sevenfold on days 1 and 5 as compared with non-nephritic control rats. Mesangiolysis and formation of microaneurysms, indicating glomerular damage was observed in PAS-stained tissue sections in all nephritic animals (Table 1). On day 5, pronounced glomerular cell proliferation (as assessed by mitotic figures) occurred, which was paralleled by glomerular de novo expression of α-smooth muscle actin, indicating mesangial cell activation (Table 1). In agreement with previous studies (21), glomerular infiltration with PMNL peaked 2 h after disease induction, with a 42-fold increase in the number of glomerular PMNL, and declined to normal values thereafter (Table 1). Increased glomerular monocyte/macrophage infiltration was also detected as early as 2 h after injection of the antibody with a 3.5-fold increase, rising to a maximum ninefold increase on day 1 and still being eightfold increased on day 5 after disease induction (Table 1). Immunolocalization of catalase and copper/zinc SOD showed only a nonsignificant increase in activity on day 5, whereas we failed to detect any activity one day after disease induction (Figure 4). Xanthine oxidase, another potential source of glomerular ROS, showed only a nonsignificant increase in activity on day 5, whereas we failed to detect any activity one day after disease induction (Figure 4).

**Glomerular Oxidative Enzymes**

Copper/zinc SOD and manganese SOD are superoxide anion metabolizing enzymes (23) that accelerate the spontaneous dismutation of superoxide anions to hydrogen peroxide. Consequently, hydrogen peroxide is disintegrated into water and molecular oxygen due to catalase and glutathione peroxidase.

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metabolism. During the early course of anti-Thy 1.1 nephritis the glomerular activity of total SOD (copper/zinc SOD and manganese SOD) decreased significantly at all three time points investigated in comparison with the control animals (Figure 5A). The activity of manganese SOD, the mitochondrial SOD isozyme, also decreased in the course of the disease and was significantly reduced at 2 h and on day 5 after disease induction (Figure 5B). Analysis of glutathione peroxidase, an enzyme that metabolizes different peroxide-species including hydrogen peroxide (29), showed a significant decrease in activity on days 1 and 5 after disease induction (Figure 5C). Finally, the activity of catalase, the most important cellular hydrogen peroxide scavenger (24), progressively decreased to 51% on day 5 as compared with the control value (Figure 5D).

To assess how the reduced glomerular activities of catalase, SOD, and glutathione peroxidase in anti-Thy 1.1 nephritis related to changes in their mRNA and protein levels, Northern and Western blot analyses were performed. After the induction of disease, we observed an initial increase in glomerular mRNA levels of copper/zinc SOD, glutathione peroxidase-I and catalase followed by decreased mRNA levels on day 5 for both SOD isoenzymes and catalase, whereas glutathione per-

![Figure 3](image-url)  
**Figure 3.** Glomerular NADH-dependent (A) and NADPH-dependent (B) oxidative activities in anti-Thy 1.1 GN at 2 h and on days 1 and 5 after induction of the disease and in NaCl solution–treated control animals. *P < 0.05 versus controls.

![Figure 4](image-url)  
**Figure 4.** Glomerular xanthine oxidase activity in nephritic animals at 2 h and on days 1 and 5 after induction of anti-Thy 1.1 GN and in control animals receiving NaCl solution. n.d., not detectable.

oxidase mRNA levels declined to the control values (Figure 6). By Western blot analysis, we observed a decrease in glomerular protein levels of manganese SOD on days 1 and 5 after injection of the antibody (Figure 7).

**Discussion**

In the present study, we analyzed the different glomerular ROS and the activities of oxidative and anti-oxidative enzymes in the course of anti-Thy 1.1 mesangio proliferative GN. Injection of anti-Thy 1.1 IgG led to mesangiolysis, glomerular infiltration with monocytes and PMNL, and to a marked proliferation and activation of mesangial cells, thus confirming various prior descriptions of the disease (1–3,21). To determine glomerular ROS, luminol-enhanced chemiluminescence was used, which allows a very sensitive and immediate detection of intracellular and extracellular ROS (25–28). In contrast with other studies (5–7), we investigated ROS in freshly isolated glomeruli, because the use of glomerular cell suspensions may lead to spurious results due to an alteration of ROS generation by the isolation procedure and because immunohistochemical assays do not allow immediate quantitation and differentiation of the oxidative species. We decided to use glomeruli instead of whole cortex or even kidney specimens because the latter would have yielded misleading results, given the considerable differences in oxidative and antioxidative enzyme expression between the glomerulus and the proximal tubule (15).

In our study, major changes in glomerular ROS levels were observed at all time points investigated during the course of anti-Thy 1.1 nephritis. The increased glomerular ROS levels detected at 2 h after disease induction are in agreement with previous findings of Mosley et al. (8), who observed an increased SOD-inhibitable luminol chemiluminescence in the first hour after induction of the disease. Our studies extend these findings, showing that ROS are elevated over the entire course of 5 d in anti-Thy 1.1 nephritis and that different species of oxidative metabolites are involved. Specific testing using
scavengers, i.e., SOD-inhibitable and DMTU-inhibitable chemiluminescence, revealed that superoxide anion and hydroxyl radical contributed to increased ROS levels in the course of anti-Thy 1.1 GN. Further analysis of ROS using the peroxidase-based chemiluminescence assay showed that hydrogen peroxide was also elevated in the nephritic glomeruli. The absence of tubulointerstitial oxidative activities confirmed glomerular specificity of increased ROS-dependent chemiluminescence determined in the course of anti-Thy 1.1 nephritis.

Important cellular sources of ROS are cell membrane–bound, mitochondrial, and microsomal enzymes, and many of

Figure 5. Glomerular antioxidative activities in nephritic animals at 2 h and on days 1 and 5 after induction of anti-Thy 1.1 GN and in NaCl solution–treated control animals. (A) Total superoxide dismutase (SOD) (manganese + copper/zinc SOD); (B) manganese SOD; (C) glutathione peroxidase; (D) catalase. * P < 0.05 and ** P < 0.01 versus saline-treated control animals.

Figure 6. mRNA levels of glomerular antioxidative enzymes in the course of anti-Thy 1.1 GN on days 1 and 5 as compared with results obtained in NaCl solution–treated control animals. For manganese SOD, all five transcripts present in rat tissue are shown (49); densitometry was performed on the largest transcript. Numbers represent the results of densitometry relative to 28s rRNA signal as compared with results from control animals.

Figure 7. Enzyme protein levels of catalase, manganese superoxide dismutase (SOD), and copper/zinc-SOD in the course of anti-Thy 1.1 GN as detected by Western blotting. M_r, relative molecular weight in kD.
these depend on NAD(P)H as a substrate (29). Such NAD(P)H-dependent oxidative enzymes are present in both infiltrating leukocytes and resident mesangial cells (30,31) and have been identified as a major source of ROS in other experimental models of GN (5,32). In our study, increased ROS after induction of the nephritis were associated with glomerular infiltration of PMNL and monocytes and increased NAD(P)H-dependent oxidative activities. Therefore, NAD(P)H oxidase in leukocytes may be the major source of ROS in anti-Thy 1.1 GN, besides a contribution of resident glomerular cells, such as mesangial cells. This oxidative stress could be one of the major causes for the increased proliferation of mesangial cells that was particularly pronounced on day 5 after induction of anti-Thy 1.1 GN. In this regard, it has been shown that ROS can stimulate cell proliferation (33). Besides NAD(P)H oxidases, xanthine oxidase has been identified as a potent source of ROS in other models of experimental renal disease (15,34,35). However, our studies demonstrate that this oxidative enzyme is not involved in the pathogenesis of anti-Thy 1.1 mesangioproliferative GN.

Increased generation of ROS by oxidative enzymes may not be the sole cause of oxidative injury in GN, as ROS levels are determined not only by oxidative mechanisms but also by alterations of the antioxidative defense (36). Compared with other organs, the activity of antioxidative enzymes in the kidney is rather high (37,38). However, this high activity is mostly confined to the renal tubules, whereas the glomerulus is less well equipped with antioxidative enzymes (15). These findings were confirmed by immunolocalization of renal antioxidative enzymes, showing strong staining of proximal tubules, whereas glomeruli showed only weak, if any, staining. Although induction of antioxidative enzyme expression by ROS and glucocorticoids has been reported as a mechanism to increase the glomerular antioxidative defense (39–41), no compensatory increase in glomerular antioxidative enzyme activities was observed in our study, despite increased glomerular ROS. The initial increase in mRNA levels of copper/zinc SOD, catalase, and glutathione peroxidase may reflect an attempt to enhance the glomerular antioxidative defense. However, initially increased mRNA levels were ineffective to maintain the antioxidative defense, as demonstrated by decreased protein levels in the Western blot analysis, decreased mRNA levels on day 5, and by the decreased activities of the enzymes during the course of nephritis. These observations are reminiscent of findings in lung and tubulointerstitial renal damage, demonstrating upregulation and downregulation of antioxidative mRNA expression, respectively (42,43). In addition, an inactivation of antioxidative enzymes by ROS was demonstrated earlier (44–47), and the underlying mechanism has been described previously as an inactivation of the enzymes followed by augmented proteolytic disintegration (44–48). Given the high superoxide anion and hydrogen peroxide levels in anti-Thy 1.1 nephritis, this mechanism might be also operative in the glomeruli.

In summary, the results of our study demonstrate for the first time that various ROS, namely hydrogen peroxide, superoxide anions, and hydroxyl radicals, are increased in the glomeruli throughout the acute phase of anti-Thy 1.1 GN. NAD(P)H-dependent oxidative activity was identified as a major source of glomerular ROS, most likely resulting to a substantial proportion from infiltrating monocytes and PMNL. We also show that increased glomerular ROS levels are associated with decreased antioxidative enzyme activities, thus revealing a profound impairment in the oxidative-antioxidative balance in this model of GN. These data may contribute to the development of rational new therapeutic strategies in the treatment of mesangioproliferative GN in humans.

Acknowledgments

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