Superoxide dismutase mimetic drug tempol aggravates anti-GBM antibody-induced glomerulonephritis in mice

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1Department of Pathology and 2Division of Rheumatology, Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas; 3Department of Pathology, Shandong University School of Medicine, Jinan; 4Department of Nephrology, Shanghai Tenth People’s Hospital of Tongji University, Shanghai; 5Department of Nephrology, First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China; and 6Division of Nephrology and Hypertension, University of California, Irvine, California

Submitted 13 October 2009; accepted in final form 24 May 2010

Lu H, Zhen J, Wu T, Peng A, Ye T, Wang T, Yu X, Vaziri ND, Mohan C, Zhou XJ. Superoxide dismutase mimetic drug tempol aggravates anti-GBM antibody-induced glomerulonephritis in mice. Am J Physiol Renal Physiol 299: F445–F452, 2010. First published May 26, 2010; doi:10.1152/ajprenal.00583.2009.—Oxidative stress plays an important role in the pathogenesis of anti-glomerular basement membrane antibody-induced glomerulonephritis (anti-GBM-GN). Superoxide dismutase (SOD) is the first line of defense against oxidative stress by converting superoxide to hydrogen peroxide (H2O2). We investigated the effect of the SOD mimetic drug tempol on anti-GBM-GN in mice. 129/svJ mice were challenged with rabbit anti-mouse-GBM sera to induce GN and subsequently divided into tempol (200 mg·kg−1·day−1, orally) and vehicle-treated groups. Routine histology, SOD and catalase activities, malondialdehyde (MDA), and immunohistochemical staining for neutrophils, lymphocytes, macrophages, p65-NF-κB, and osteopontin were performed. Mice with anti-GBM-GN had significantly reduced renal SOD and catalase activities and increased H2O2 and MDA levels. Unexpectedly, tempol administration exacerbated anti-GBM-GN as evidenced by intensification of proteinuria, the presence of severe crescentic GN with leukocyte influx, and accelerated mortality in the treated group. Tempol treatment raised SOD activity and H2O2 level in urine, upregulated p65-NF-κB and osteopontin in the kidney, but had no effect on renal catalase activity. Thus tempol aggravates anti-GBM-GN by increasing production of H2O2 which is a potent NF-κB activator and as such can intensify inflammation and renal injury. This supposition is supported by increases seen in p65-NF-κB, osteopontin, and leukocyte influx in the kidneys of the tempol-treated group.

Oxidative stress; catalase; hydrogen peroxide; crescentic glomerulonephritis; NF-κB

ANTI-GLOMERULAR BASEMENT MEMBRANE antibody-induced glomerulonephritis (anti-GBM-GN) is a rare autoimmune disorder in which circulating antibodies are directed to the GBM component, the α-3 chain of type IV collagen, leading to an inflammatory reaction in the glomerular capillaries (2, 13, 21). If the disease also affects the lung, it is then termed Goodpasture syndrome. Anti-GBM-GN is pathologically and clinically the most severe form of glomerulonephritis, characterized by crescent formation and linear glomerular deposits of IgG. Clinically, patients most often present with rapidly progressive glomerulonephritis, hematuria, and subnephrotic-range proteinuria. The mainstays of therapy usually consist of high-dose corticosteroids, cytotoxic drugs, and plasmapheresis. However, end-stage renal disease develops in 40–70% of patients (16, 21).

Reactive oxygen species (ROS) are products of normal cellular metabolism that modulate various physiological functions (30). In addition, ROS serve as the main weapon of innate immunity in the course of infectious and noninfectious inflammation. Overproduction of ROS, however, can induce cell damage and cause inflammation, contributing to pathogenesis of many diseases including anti-GBM-GN (20, 25). Superoxide dismutase (SOD) and catalase (CAT) are two main antioxidant enzymes. SOD catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide (H2O2), which is subsequently degraded to H2O and molecular oxygen by catalase. Failure of sufficient dismantling of superoxide by SOD may result in increased glomerular permeability and worsening of the disease (6). Administration of SOD to attenuate glomerular injury by ROS has proven unsuccessful in various experimental models (25). This might be due to the short half-life, negative charge, and cell impermeability of native SOD, which prevents its distribution to the intracellular space and mitochondria where most of the superoxide is generated (6). Tempol (4-hydroxy-2,2,6,6-tetramethyl-piperidine-N-oxyl) is a membrane-permeable, SOD-mimetic compound that has been reported to ameliorate glomerular injury and lower arterial blood pressure in various animal models (1, 9, 18, 24, 32) and reduce oxidant stress-mediated renal dysfunction and injury in the rats (3). The effect of tempol on the course of anti-GBM-GN has not been investigated.

In the present study, we tested the hypothesis that the antioxidant properties of tempol may improve the course of anti-GBM-GN in a mouse model. Contrary to our expectations, tempol administration exacerbated anti-GBM-GN in 129/svJ mice.

MATERIALS AND METHODS

Anti-GBM rabbit sera. Anti-GBM serum was generated by Lampire Laboratories (Pipersville, PA). Essentially, renal cortices of B6 mouse kidneys were minced and then pressed through a series of sieves of decreasing pore size (250-, 150-, and 75-μm mesh), and the glomeruli were collected on the finest sieve, washed with cold PBS, and sonicated for 7 min. The glomerular sonicates were used to immunize rabbits (2 mg/rabbit, 3 injections administered 21 days apart) for generation of anti-GBM sera in rabbits. Sera obtained from these rabbits 50 days following the primary immunization stained the

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TEMPOL AGGRAVATES ANTI-GBM-INDUCED GN

Detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. For renal tissue SOD activity, tissue samples were first rinsed with PBS (pH 7.4, containing 0.16 mg/ml heparin) to remove any red blood cells and clots. Then, samples were homogenized in 5–10 ml of cold 20 mM HEPES buffer (pH 7.2, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose) per gram tissue. The tissue suspension was centrifuged at 1,500 g for 5 min at 4°C. The resulting supernatant was used for the assay.

Catalase in renal tissue was measured by using the Cayman Chemical catalase assay kit according to the procedures suggested by the manufacturer. Hydrogen peroxide (H2O2) was determined in urine and renal tissue using a BioAssay System peroxide assay kit (Hayward, CA), which utilizes the chromogenic Fe3+-xylenol orange reaction, in which a purple complex is formed when Fe3+ is oxidized to Fe3+ by H2O2. The intensity of the color (measured at 540–610 nm) is an accurate measure of the H2O2 level in the sample. Lipoperoxides in serum, urine, and renal tissue were determined by measurement of malondialdehyde-thiobarbituric acid (MDA-TBA) using a TBARS assay kit from Cayman Chemical according to the procedures suggested by the manufacturer.

Renal histopathology and immunohistochemistry. Three-micrometer sections of formalin-fixed, paraffin-embedded kidney tissues were cut and stained with hematoxylin and eosin and periodic acid-Schiff (PAS) reaction. These sections were examined in a blinded fashion for any evidence of pathology in the glomeruli, tubules, or interstitial areas, as described below (38). The glomeruli were screened for evidence of hypertrophy, proliferative changes, crescent formation, hyaline deposits, sclerosis, and basement membrane thickening. The severity of glomerulonephritis was graded on a 0–4 scale as follows: 0, normal; 1, mild increase in mesangial cellularity and matrix; 2, moderate increase in mesangial cellularity and matrix, with thickening of the GBM; 3, focal endocapillary hypercellularity with obliteration of capillary lumina and a substantial increase in the thickness and irregularity of the GBM; and 4, diffuse endocapillary hypercellularity, segmental necrosis, crescents, and hyaliniﬁzed end-stage glomeruli. Similarly, the severity of tubulointerstitial injury was graded on a 0–4 scale, based on the extent of tubular atrophy, inﬂammatory inﬁltrates, and interstitial ﬁbrosis, as detailed previously (41).

Table 2. Renal morphological features in tempol- and vehicle-treated mice with anti-GBM glomerulonephritis and in normal controls

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Anti-GBM/Vehicle</th>
<th>Anti-GBM/Tempol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomerulonephritis score (0–4)</td>
<td>0.2 ± 0.2</td>
<td>2 ± 0.4*</td>
<td>3.4 ± 0.6†</td>
</tr>
<tr>
<td>Crescents, %</td>
<td>0 ± 0</td>
<td>5 ± 4.6*</td>
<td>30.9 ± 19.1†‡</td>
</tr>
<tr>
<td>Tubulointerstitial injury score (0–4)</td>
<td>0.1 ± 0.2</td>
<td>0.6 ± 0.3*</td>
<td>2.4 ± 0.8‡</td>
</tr>
<tr>
<td>Neutrophils</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glomeruli (no./50 glomeruli)</td>
<td>1.6 ± 0.7</td>
<td>4.9 ± 1.1*</td>
<td>8.9 ± 2.7*</td>
</tr>
<tr>
<td>Intersitial (no./20 HPF)</td>
<td>2.7 ± 2.2</td>
<td>5.8 ± 1.8*</td>
<td>17.6 ± 2.6†‡</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glomeruli (no./50 glomeruli)</td>
<td>1.3 ± 0.5</td>
<td>6.6 ± 3.1*</td>
<td>7.7 ± 2.5*</td>
</tr>
<tr>
<td>Intersitial (no./20 HPF)</td>
<td>12.6 ± 2.7</td>
<td>67.8 ± 19.5*</td>
<td>140 ± 33.5†‡</td>
</tr>
<tr>
<td>Macrophages</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glomeruli (no./50 glomeruli)</td>
<td>0 ± 0</td>
<td>1.0 ± 0.4*</td>
<td>2.1 ± 0.4†</td>
</tr>
<tr>
<td>Intersitial (no./20 HPF)</td>
<td>4.1 ± 1.2</td>
<td>14.5 ± 3.5*</td>
<td>22.1 ± 6.5*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 7–9/group. HPF, high-power field. *P < 0.05 vs. normal. †P < 0.01 vs. anti-GBM/Vehicle.
ogy) in mice kidneys were also evaluated. The standard ABC method was applied for immunohistochemical staining following the manufacturer’s instructions. The intensity of immunohistochemical signal for various antibodies was estimated semiquantitatively using the following scales as described previously (41): 0, none; 1+, mild; 2+, moderate; and 3+, marked. In addition, intrarenal neutrophils were stained with the naphthol AS-D chloroacetate esterase method using a kit purchased from Sigma.

Statistical analysis. Data are presented as means ± SE. ANOVA and Student’s t-test were used in statistical evaluation of the data as appropriate. P values ≤0.05 were considered significant.

RESULTS

General data and biochemical measurements. Data are shown in Table 1. As expected, at the end of the 2-wk observation period, both groups of anti-GBM antibody-injected mice developed azotemia and proteinuria compared with the normal control group. No significant difference was found in either body weight or BUN between the tempol- or vehicle-treated groups. However, the tempol-treated mice showed significantly more proteinuria than the vehicle-treated group. In addition, tempol treatment led to an accelerated mortality.

Renal histology and immunohistochemistry. Kidneys from mice 2 wk after induction of anti-GBM-GN with or without tempol treatment and normal control mice were evaluated by light microscopy. As illustrated in Table 2 and Fig. 1, vehicle-treated mice exhibited mild to moderate renal injury characterized by mild to moderate mesangial proliferation with slightly increased mesangial matrix and rare crescent formation. Focal mild tubulointerstitial injury was noted. In contrast, tempol treatment aggravated renal injury characterized by

![Fig. 1. Renal histology and immunohistochemical analysis of tempol- or vehicle-treated 129/svJ mice with anti-glomerular basement membrane (GBM) glomerulonephritis.](image)

- **PAS**
- **Lymphocytes**
- **Macrophage**
- **Neutrophils**
significant crescent formation (30%) with marked intracapillary hypercellularity with obliterated capillary lumens and thickened capillary walls. Tubular atrophy and dilation with hyaline casts and interstitial fibrosis were also noted. No significant glomerular or tubulointerstitial lesions were seen in the normal control group (Table 2). In addition, interstitial infiltration of neutrophils and lymphocytes was markedly increased in the tempol-treated group compared with both normal controls and vehicle-treated mice. The infiltration of interstitial macrophages was markedly increased in both anti-GBM groups compared with normal controls. Although more interstitial macrophages were seen in the tempol-treated group compared with the vehicle-treated mice, the differences did not reach statistical significance. The glomerular infiltration of neutrophils and lymphocytes was comparable between the tempol- and vehicle-treated groups but significantly increased compared with the normal controls. In addition, increased glomerular macrophage infiltration was noted in the tempol-treated group compared with the vehicle-treated group.

OPN is a secreted phosphoprotein with diverse biological functions including promoting the formation and progression of crescentic glomerulonephritis in human and animal models. Therefore, we decided to determine whether OPN played any role in the exacerbation of anti-GBM-GN in tempol-treated animals. In the vehicle-treated group, mild OPN expression was noted in occasional glomerular parietal epithelial cells (1+), distal tubules (1+), and occasional interstitial infiltrating cells (1+). In contrast, the tempol-treated animals showed diffuse and strong OPN expression in the renal cortex in virtually all the proximal and distal tubules (3+). (Fig. 2). The glomeruli revealed positive staining in parietal epithelial cells (2+), scattered podocytes (2+), and infiltrating leukocytes (3+). Numerous interstitial macrophages were strongly positive for OPN (3+) (Fig. 2). No significant glomerular or tubulointerstitial staining was seen in the normal control group (Fig. 2). Similarly, in the vehicle-treated group, there was focal, mild total p65-NF-κB expression in occasional glomerular and interstitial infiltrating leukocytes (1+) and scattered tubular epithelial cells (1+). The p65 reactivity was primarily localized in the cytoplasm. In contrast, the tempol-treated animals showed diffuse and strong total p65-NF-κB expression in the renal cortex in virtually all the proximal and distal tubules (3+). In addition, many glomerular infiltrating leukocytes and resident cells were positive for p65-NF-κB (2+) (Fig. 2). Numerous interstitial macrophages were strongly positive for p65-NF-κB (3+) (Fig. 2). The p65 reactivity was localized in both the cytoplasm and nuclei, the latter signifying NF-κB activation. No significant glomerular or tubulointerstitial staining was seen in the normal control group.

Oxidative stress and antioxidant system. The total SOD activity data in serum, urine, and renal cortical tissue are shown in Fig. 3. SOD activity was significantly reduced in mice with anti-GBM-GN in renal tissue compared with normal control mice. In contrast, serum SOD activity was comparable among the three groups. The SOD activity of the urine was increased in tempol-treated mice with anti-GBM-GN compared with the vehicle-treated and normal control groups. Renal tissue catalase activity was markedly reduced in both tempol- and vehicle-treated mice with anti-GBM-GN. Tempol treatment had no effect on catalase activity (Fig. 4).

Oxidative stress was determined by measurement of MDA and H2O2 levels. As illustrated in Fig. 5, MDA levels in serum, urine, and renal tissue were markedly elevated in mice with anti-GBM-GN compared with the normal control group, indicating oxidative stress. Tempol treatment lowered urine and renal MDA levels compared with vehicle-treated mice with anti-GBM-GN. Urinary H2O2 excretion was moderately elevated in mice with anti-GBM-GN compared with normal control mice. Tempol treatment resulted in a nearly eightfold increase in urinary H2O2 excretion compared with the vehicle-
Fig. 3. SOD activity in 129/svJ mice with anti-GBM glomerulonephritis. Serum SOD activities were comparable among the 3 groups. Renal SOD levels were significantly reduced in both vehicle- and tempol-treated groups compared with the normal control group. Urinary SOD activities were elevated in the tempol-treated group compared with the vehicle-treated mice and comparable with that of the normal control group. Values are means ± SE; n = 6–8.

Fig. 4. Hydrogen peroxide (H$_2$O$_2$) levels in urine and renal tissue and catalase (CAT) activity in renal tissues of 129/svJ mice with anti-GBM glomerulonephritis. Urinary and renal tissue H$_2$O$_2$ levels were markedly elevated in mice with anti-GBM glomerulonephritis compared with the normal control group. Tempol treatment was associated with a marked elevation of H$_2$O$_2$ level in the urine but not in renal tissue. The lack of significant renal tissue accumulation of H$_2$O$_2$ after tempol administration is perhaps due to its rapid clearance from urine. In contrast, the renal catalase activities were markedly and equally reduced in both tempol- and vehicle-treated groups. Values are means ± SE; n = 6–8.
treated group (Fig. 4). In addition, the renal tissue H2O2 level was markedly elevated in mice with anti-GBM disease compared with the normal control group. Tempol treatment resulted in a slight elevation of tissue H2O2 level compared with that found in the vehicle-treated group. However, the difference did not reach statistical significance most likely due to rapid urinary clearance of H2O2, which must have blunted its retention in the renal tissue (Fig. 4).

**DISCUSSION**

The SOD mimetic tempol has been shown to have a beneficial effect in animal models for several diseases including diabetes, hypertension, endothelial cell dysfunction, inflammation, ischemia-reperfusion, and shock (4–6, 29). To date, its role in anti-GBM-GN has not been elucidated. Our data showed unexpectedly that instead of reducing glomerular injury, tempol aggravated anti-GBM-GN in mice, characterized by significant proteinuria and crescent formation as well as accelerated mortality. Although it is possible that tempol could have direct nephrotoxic effects, numerous studies have shown that oral tempol administration in both short-term (<5 wk) (23, 24, 27, 35) and long-term studies (up to 15 wk) (31) is not associated with significant effects on blood pressure, renal function, or structure in healthy normotensive rats. Moreover, accentuation of glomerular crescent formation and proteinuria with tempol administration in this model reflects its adverse impact on the course of underlying glomerular disease as opposed to the typical drug toxicity.

As expected, anti-GBM-GN was associated with oxidative stress as evidenced by elevated MDA levels in serum, urine, and renal tissue. The SOD-mimetic effects of tempol were confirmed by the findings that urine and renal tissue MDA were lowered by tempol treatment and by the fact that urinary SOD activity was elevated in the tempol-treated group. It has been shown that high levels of uncontained superoxide can promote glomerular injury through degradation of GBM by metalloproteinases (26), decreased synthesis of proteoglycans (14), and enhanced synthesis of gelatinase by glomerular cells (12). Therefore, SOD and tempol should exert protection against tissue damage by promoting dismutation of superoxide. However, this is not always the case, and it has been shown that GBM degradation caused by activation of metalloproteinases was not inhibited by SOD, whereas catalase, a scavenger of H2O2 caused marked inhibition (26). In a recent study, Quiroz et al. (19) showed that although tempol administration reduced plasma MDA and enhanced superoxide dismutation in the remnant kidney of rats with renal mass reduction, it was ineffective in reducing tissue oxidative stress or improving renal function or structure. In the present study, we found that anti-GBM-GN was associated with reduced catalase and tempol treatment had no effect on catalase. We further showed that anti-GBM-GN was associated with elevated urinary and renal tissue H2O2 levels. Tempol treatment further elevated urine H2O2 level by nearly eightfold compared with that observed in the vehicle-treated group. The renal tissue H2O2 level was also elevated by tempol treatment although it was not statistically significant compared with the vehicle-treated group. This is not surprising because rapid urinary clearance of H2O2 must have blunted its accumulation in the renal tissue. Hence, in the absence of a concomitant increase in catalase, the imbalance in the rates of production and conversion of H2O2 to H2O can lead to its accumulation. It should be noted that the current findings are consistent with the observations of Makino et al. (17), who showed that in the presence of a high level of superoxide in the renal medulla, tempol administration leads to the accumulation of H2O2, which, in turn, causes hypertension in rats. It is known that H2O2 can raise renal vascular resistance and blood pressure by raising cytosolic...
Ca\(^2+\) concentration (22, 28) and inducing renal vasoconstriction (7, 8). In addition, H\(_2\)O\(_2\) may contribute to proteinuria (11, 39) and cause GBM degradation (33) and endothelial damage (34).

We showed an increase in p65-NF-\(\kappa\)B and OPN expression and neutrophil, lymphocyte, and macrophage infiltration in the kidneys of tempol-treated mice. These events were associated with and most likely mediated by overproduction of H\(_2\)O\(_2\). Among ROS, H\(_2\)O\(_2\) is a potent activator of NF-\(\kappa\)B, which is the general transcription factor for proinflammatory cytokines, chemokines, and adhesion molecules that drive the inflammatory process. Similarly, we observed a strong, diffuse expression of OPN, which is a secreted acidic glycoprotein that acts as a potent monocyte chemoattractant to promote macrophage infiltration in anti-GBM-GN (10, 15, 40).

In summary, the current study contradicts the belief that the effects of the SOD mimetic tempol are mainly beneficial in kidney injury. The lack of protection might be due to the nature of anti-GBM-GN, which differs from other glomerular diseases. However, the more likely scenario is that the heightened superoxide dismutation alone in the absence of a concomitant increase in catalase activity can result in amplification of oxidative stress by augmenting H\(_2\)O\(_2\) production. The latter can, in turn, heighten NF-\(\kappa\)B activation and intensify the underlying inflammation and renal injury in tempol-treated animals.

ACKNOWLEDGMENTS
The authors thank Dr. Agata Bogusz for helpful discussions. Some of the results were presented in poster form at the 2008 annual meeting of the United States and Canadian Academy of Pathology, Denver, CO.

GRANTS
This work was supported by grants from the Drs. George and Anne Race Distinguished Professorship of Pathology and from the Lupus Research Institute.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

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