Addition of the Antioxidant Probucol to Angiotensin II Type I Receptor Antagonist Arrests Progressive Mesangioproliferative Glomerulonephritis in the Rat

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Angiotensin II (Ang II) and reactive oxidative species (ROS) that are produced by NADPH oxidase have been implicated in the progression of glomerulonephritis (GN). This study examined the effect of simultaneously interrupting Ang II and ROS with an Ang II receptor blocker (ARB), candesartan, and a free radical scavenger, probucol, in a model of progressive mesangioproliferative GN induced by the injection of anti–Thy-1 antibody into uninephrectomized rats. Nephritic rats were divided into four groups and given daily oral doses of the following: Vehicle, 1% probucol diet, 70 mg/L candesartan in drinking water, and probucol plus candesartan. These treatments lasted until day 56. Vehicle-treated nephritic rats developed progressively elevated proteinuria and glomerulosclerosis. Candesartan kept proteinuria significantly lower than vehicle or probucol. The addition of probucol to candesartan normalized urinary protein excretion. Increases in BP in nephritic rats were lowered by these treatments, except with probucol. It is interesting that both glomerular cell number and glomerulosclerosis were significantly decreased by candesartan and normalized by the addition of probucol. Immunohistochemical studies for TGF-β1, collagen type I, and fibronectin revealed that the combined treatment abolished glomerular fibrotic findings compared with candesartan. In addition, glomerular expression of NADPH oxidase components and superoxide production suggested that the combined treatment completely eliminated NADPH oxidase–associated ROS production. In conclusion, our study provides the first evidence that the antioxidant probucol, when added to an Ang II receptor blockade, fully arrests proteinuria and disease progression in GN. Furthermore, the data suggest that NADPH oxidase–associated ROS production may play a pivotal role in the progression of GN. The combination of probucol and candesartan may represent a novel route of therapy for patients with progressive GN.

The marked accumulation of extracellular matrix (ECM), including collagens and fibronectin, in glomeruli is a hallmark in the development of human and experimental progressive glomerulonephritis (GN) (1–4). TGF-β1, a prime fibrogenic factor, stimulates the deposition of ECM by mesangial cells and thereby may induce glomerulosclerosis (2,5).

Recently, the interventions with angiotensin II (Ang II) type I receptor blocker (ARB) and/or angiotensin-converting enzyme inhibitor (ACEI) have shown their protective potential against human nephropathies, including IgA nephropathy and diabetic nephropathy, and progressive experimental models such as mesangioproliferative GN and nephrectomized rats (6–10). These clinical and experimental in vivo studies have strongly suggested that the renoprotective effect of these antihypertensive agents results from not simply the inhibition of its hemodynamic action but also blockade of its hemodynamic-independent actions. In fact, many studies, including our previous in vitro studies, have disclosed the mechanisms and the role of Ang II–induced ECM deposition through mesangial cells (11–13). Ang II stimulates ECM synthesis through the induction of TGF-β1 expression in cultured rat glomerular mesangial cells (11) and affects the plasminogen activator/plasminogen activator inhibitor-1 system to result in accelerated deposition of ECM (12), which constitutes evidence that Ang II can act as a potent fibrotic molecule independent of its effects on BP. Although the current therapy by inhibition of Ang II retards renal fibrosis, it is not enough to prevent completely the progression of GN. This suggests that other fibrogenic factors such as PDGF-BB, TNF-α, and IL-1β might also be

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involved in the progression of GN (3,14). For maximizing the therapeutic potential of antifibrotic treatment, additional therapy that is aimed at suppression of these fibrogenic factors and their related signaling pathways may be required.

Recently, reactive oxygen species (ROS) that are produced by NADPH oxidase has been implicated in the development and progression of GN (15–17). The administration of various natural or synthetic antioxidants has been shown to be beneficial in the prevention and attenuation of renal scarring in numerous animal models of renal disease (18). These compounds include vitamins, N-acetylcysteine, α-lipoic acid, lipid-lowering agents, and many others. Probucol is a lipid-soluble, cholesterol-lowering drug with potent antioxidant properties that has been shown to scavenge lipid peroxidation (19). This agent seems to play a role in eliminating ROS produced by NADPH oxidase (20).

It is interesting that Ang II is considered a representative stimulator of NADPH oxidase activity that leads to the enhanced production of superoxide (O$_2^-$) in several cell lines, including mesangial cells and vascular smooth muscle cells. These studies have also demonstrated that NADPH oxidases are composed of gp91phox-homolog and produce ROS including O$_2^-$ not only in phagocytes but also in nonphagocytic cells (21–25). In addition, these studies indicated that NADPH oxidase plays an important role as a cell-signaling molecule in addition to being a classical mediator of tissue injury (21,22). Whereas gp91phox, which has been called Nox2 (for NADPH oxidase), is generally expressed in inflammatory cells such as leukocytes, the Nox family is also composed of various members from Nox1 to Nox5, which are expressed in noninflammatory cells, including mesangial cells and podocytes. In particular, Nox1, Nox2, and Nox4 have been investigated in Ang II–induced ROS production in renal and cardiovascular diseases (21,22,25). Besides, the levels of these Nox proteins are associated with the activity and O$_2^-$ production (21,22). Other fibrogenic factors, such as PDGF-BB, TNF-α, and IL-1β, all of which are involved in the progression of GN, also stimulate ROS production in mesangial cells, although the detailed mechanism of their production remain unknown (15,23,24). Therefore, we hypothesized that the blockade of both the action of Ang II and ROS production associated with NADPH oxidase activation might be a novel strategy for preventing the progression of glomerulosclerosis. We examined whether simultaneously interrupting Ang II and ROS with the ARB candesartan and probucol could reduce proteinuria and disease progression in a rat model of irreversible mesangioproliferative GN.

Materials and Methods

**Antibodies and Reagents**

Previously characterized mouse mAb that specifically recognized Nox2 (clone 54.1) and rabbit polyclonal antibody against Nox4 were used in this study (26,27). Horseradish peroxidase–conjugated goat anti-mouse IgG (H+L; Bio-Rad, Hercules, CA) and horse anti-rabbit IgG (H+L; Cell Signaling Technology Inc., Beverly, MA) were used for Western blot analysis. For immunohistochemistry, previously characterized rabbit polyclonal anti–TGF-β1 antibody was used, and rabbit antibodies against collagen type I (COL I) and fibronectin (FN) were obtained from Chemicon International Inc. (Temecula, CA) (8).

**Experimental Design**

All experimental procedures were performed according to the guidelines for the care and use of laboratory animals established by National Institutes of Health and the Institute for Animal Experimentation, the University of Tokushima Graduate School. A progressive model of mesangioproliferative GN was induced as described previously, with some modifications (8). Briefly, rats were uninephrectomized and 1 wk later received an intravenous injection of 1.0 ml of PBS that contained 2 mg of the nephritogenic anti–Thy-1 mAb 1-22-3. Nephritic rats were divided into four groups (n = 6) and given daily oral doses as follows: (1) Vehicle, (2) 1% probucol diet, (3) 70 mg/L candesartan in drinking water, or (4) probucol plus candesartan. The dose concentration of probucol in the rat diet and candesartan in drinking water were selected on the basis of previous reports (28–31). The therapeutic regimen that was used in our study was pharmacologically valid and efficient according to the manufacturer’s information for probucol. For achieving a therapeutically adequate concentration of probucol, this reagent was given 7 d before the injection of mAb 1-22-3. The dose of candesartan (70 mg/L) that was used in this experiment was high enough (10 mg/kg per d) to inhibit Ang II receptor binding to kidney tissue (28,29).

Candesartan was started on the same day as disease induction (day 0). Six rats in each group were killed at 7 d and 56 d after the injection of mAb 1-22-3. As controls, six rats received injections of PBS 1 wk after unilateral nephrectomy and were killed on days 7 and 56.

**Urinary Protein Excretion, Blood Parameters, and BP**

Urine was periodically collected from the rats in a metabolic cage during the course of GN. The amount of urinary protein excretion was measured by the Bradford method (Bio-Rad, Oakland, CA). The amount of food consumed and water intake were measured and calculated on day 56. At the end of the study period, serum concentrations of blood urea nitrogen, creatinine, total cholesterol, and nonesterified fatty acid were measured using the reagents that were manufactured by WAKO Chemical Industries (Osaka, Japan). Serum total protein was measured by a BCA protein assay (Pierce, Rockford, IL). The serum level of triglyceride was assessed with a Lipidos Kit (TOYOBO, Osaka, Japan). Systolic BP (SBP) was measured noninvasively by the tail-cuff method (32). Concentrations of serum probucol were measured by HPLC at the laboratories of Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan).

**Histology and Immunohistochemistry**

The right kidney of each rat was removed immediately, fixed in 10% buffered formalin and embedded in paraffin, and 4-μm sections were stained with periodic acid–Schiff reagent. Glomerular cell number was assessed on the basis of total glomerular cell count per glomerular cross-section. It was counted in 30 glomeruli per kidney in a periodic acid–Schiff–stained section, and the mean number of cells per glomerulus was calculated. A pathologist who was blinded to other findings semiquantitatively analyzed the glomerulosclerosis score. The percentage of each glomerulus that was occupied by mesangial matrix was estimated and assigned a code as follows: 0 = absent, 0.5 = 1 to 5%; 1 = 5 to 25%; 2 = 25 to 50%; 3 = 50 to 75%; or 4 = >75%.

Frozen sections (3 μm) were incubated with anti–COL I antibodies, anti–FN antibodies, and anti–TGF-β1 Ab for 1 h and then with FITC-conjugated secondary antibodies. To evaluate the level of glomerular staining with each antibody, we performed semiquantitative analysis as follows: 0 = diffuse, very weak, or absent mesangial staining; 1+ = 1 to 25% of focally increased mesangial staining; 2+ = 25 to 50% of glomerular tuft demonstrating strong mesangial staining; 3+ = 50 to 75% of glomerular tuft demonstrating strong mesangial staining; and
Table 1. Total food intake, total water intake, body weight, and the level of serum probucol in rats that had GN on day 56 and had been treated with probucol and/or ARB (candesartan)\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>GN</th>
<th>GN + Probucol</th>
<th>GN + ARB</th>
<th>GN + Probucol + ARB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total food intake (g/d)</td>
<td>22.7 ± 0.8</td>
<td>21.2 ± 1.0</td>
<td>23.8 ± 1.4</td>
<td>23.3 ± 2.9</td>
<td>22.8 ± 1.9</td>
</tr>
<tr>
<td>Total water intake (ml/d)</td>
<td>37.8 ± 2.8</td>
<td>41.7 ± 4.0</td>
<td>39.2 ± 3.7</td>
<td>35.8 ± 3.0</td>
<td>41.5 ± 3.0</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>449 ± 6</td>
<td>442 ± 12</td>
<td>429 ± 14</td>
<td>420 ± 22</td>
<td>433 ± 12</td>
</tr>
<tr>
<td>Serum probucol (μg/ml)</td>
<td>ND</td>
<td>ND</td>
<td>11.2 ± 3.8</td>
<td>ND</td>
<td>4.5 ± 0.9</td>
</tr>
</tbody>
</table>

\(^a\)The values are expressed as mean ± SEM (n = 6). ARB, angiotensin II receptor blocker; GN, glomerulonephritis; ND, not detected, indicating the level of serum probucol is below the measurement limit.

4+ = >75% of glomerular tuft stained strongly. For each kidney section, 30 glomeruli were selected at random and evaluated by the same blinded pathologist; the mean value per section was calculated (33).

Formalin-fixed tissue sections (3 μm) were deparaffinized with xylene and rehydrated with a graded series of ethanol. Endogenous peroxidase was blocked with hydrogen peroxide, and the samples were heated at 121°C for 15 min in 0.01 mol/L citrate buffer (pH 6.0). The sections were incubated with anti–ED-1 mAb (Serotec, Kinglington, UK) diluted in PBS that contained 1% BSA at 4°C for 24 h. After being washed with PBS, the sections were incubated with biotinylated secondary antibody, avidin-biotin-peroxidase complex (ABC Elite; Vector Laboratories, Burlingame, CA), and then 3,3′-diaminobenzidine (Djodjino, Kumamoto, Japan). Each section was counterstained with Mayer’s hematoxylin (Wako, Tokyo, Japan), dehydrated, and coverslipped. The number of ED-1-positive cells was counted and calculated in 30 full-size glomeruli (34).

Western Blotting

Western blotting was performed as described previously (35). Total protein was extracted from glomeruli that were isolated by the sieving method (11). Protein samples (30 μg) were separated by 12.5% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with mouse monoclonal anti-Nox2 antibodies, rabbit polyclonal anti-Nox4 antibodies, and mouse monoclonal anti–β-actin antibodies (Sigma Chemical Co., St. Louis, MO) as a standard and then incubated with an horseradish peroxidase–conjugated secondary antibodies. Immunoreactive proteins were detected with an enhanced chemiluminescence detection system (Amersham Corp., Arlington Heights, IL). Densitometric analysis was performed using an LKB UltraScan XL apparatus (Pharmacia LKB Biotechnology, Uppsala, Sweden).

In Situ Superoxide Production

As described elsewhere, in situ production of O$_2^-$ was determined using 30-μm sections of frozen tissue that were incubated with dihydroethidium (DHE; 10 μmol/L) in PBS for 30 min at 37°C in a humidified chamber that was protected from light. DHE is oxidized upon reacting with O$_2^-$ to ethidium bromide, which binds to DNA in the nucleus and fluoresces red. To detect ethidium bromide, we used a 543-nm He-Ne laser combined with a 560-nm long-pass filter. Glomerular O$_2^-$ content was estimated by analyzing the mean fluorescence intensity in glomeruli using National Institutes of Health imaging software (27). To identify O$_2^-$-producing cells in glomeruli, we performed immunofluorescent staining combined with DHE assay (red) and FITC labeling (green) using anti–ED-1 mAb and anti–Thy-1 mAb as markers of macrophages and mesangial cells, respectively.

Statistical Analyses

The values are expressed as mean ± SEM. The differences were evaluated with the Stat Mate III software package (ATMS Co., Ltd., Tokyo, Japan). For urinary protein excretion, blood parameters, and BP, statistical significance was evaluated using a t test (8,33). The data for histology and immunohistochemistry were analyzed using the non-parametric Kruskal-Wallis test for multiple comparisons (8). The statistical significance level was defined as \( P < 0.05 \).

Table 2. Time course of urinary protein excretion (mg/d) in rats that had GN on day 56 and had been treated with probucol and/or ARB (candesartan)\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 28</th>
<th>Day 56</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20 ± 1.1</td>
<td>23 ± 2.4</td>
<td>18 ± 2.6</td>
<td>23 ± 2.4</td>
<td>16 ± 1.7</td>
</tr>
<tr>
<td>GN</td>
<td>20 ± 2.1</td>
<td>74 ± 12.9b</td>
<td>78 ± 17.1b</td>
<td>170 ± 64.5c</td>
<td>273 ± 100.6c</td>
</tr>
<tr>
<td>GN + probucol</td>
<td>19 ± 2.1</td>
<td>90 ± 5.6b</td>
<td>88 ± 20.6b</td>
<td>117 ± 17.3b</td>
<td>140 ± 33.6b</td>
</tr>
<tr>
<td>GN + ARB</td>
<td>20 ± 3.5</td>
<td>88 ± 6.3b</td>
<td>37 ± 2.5b,de</td>
<td>33 ± 5.3f</td>
<td>23 ± 5.0d,f</td>
</tr>
<tr>
<td>GN + probucol + ARB</td>
<td>20 ± 2.8</td>
<td>56 ± 8.0b,fg</td>
<td>33 ± 1.7b,de</td>
<td>22 ± 2.5d,f</td>
<td>10 ± 2.6d,h</td>
</tr>
</tbody>
</table>

\(^a\)The values are expressed as mean ± SEM (n = 6).
\(^b\)\( P < 0.01 \) versus control.
\(^c\)\( P < 0.05 \) versus control.
\(^d\)\( P < 0.05 \) versus GN.
\(^e\)\( P < 0.05 \) versus GN + probucol.
\(^f\)\( P < 0.01 \) versus GN + probucol.
Figure 1. Systolic BP in rats that had glomerulonephritis (GN) on day 56 and had been treated with probucol and/or angiotensin II receptor blocker (ARB; candesartan). □, nephrectomized control rats; ■, vehicle-treated GN rats; ■, probucol-treated GN rats; □, ARB-treated GN rats; □, probucol + ARB-treated GN rats. Data are mean ± SEM. *P < 0.01 versus control; †P < 0.01 versus GN; ‡P < 0.01 versus GN + probucol.

Results

Effect of Probucol and/or ARB on Urinary Protein Excretion, BP, and Blood Parameters

As shown in Table 1, total food intake, total water intake, and body weight were comparable among all groups. The serum level of probucol was 11.2 ± 3.8 μg/ml in probucol-treated nephritic rats, 4.5 ± 0.9 μg/ml in probucol plus ARB-treated nephritic rats, and below the measurement limit in rats that were not treated with probucol, indicating that the serum level of probucol was significantly elevated by a 1% probucol diet.

There was no difference in 24-h urinary protein excretion among all groups before the induction of GN. As shown in Table 2, proteinuria was significantly increased in all nephritic rats on day 7 after disease induction. In vehicle-treated nephritic rats, proteinuria gradually increased until the end of the experiment. Treatment with ARB or probucol plus ARB reduced proteinuria compared with vehicle treatment from 2 wk after the induction of nephritis. Conversely, treatment with probucol did not significantly reduce proteinuria. Treatment with probucol plus ARB significantly prevented proteinuria compared with treatment with probucol or ARB on days 7 and 56.

SBP of vehicle-treated nephritic rats on day 56 was elevated, although not significantly. Treatment with ARB or probucol plus ARB significantly reduced SBP compared with vehicle treatment. Conversely, treatment with probucol did not lower SBP (Figure 1).

Blood parameters on day 56 are shown in Table 3. Remarkable hypoproteinemia was not detected in any of the groups. A significant elevation of serum total protein was observed in probucol plus ARB-treated nephritic rats. The level of blood urea nitrogen in vehicle-treated nephritic rats was elevated, whereas the level in probucol plus ARB-treated nephritic rats was significantly lowered to the level in the control. The serum level of creatinine in probucol plus ARB-treated nephritic rats was slightly lower than that in vehicle-treated nephritic rats, although the difference was not statistically significant. Hyperlipidemia was observed in vehicle-treated nephritic rats, as shown in Table 3. A significant improvement of hyperlipidemia was not observed in rats with probucol or ARB treatment. Combination treatment with probucol and ARB significantly decreased the levels of triglyceride and nonesterified fatty acid, respectively.

| Table 3. Serum levels of total protein, BUN, creatinine, T-CHO, TG, and NEFA in rats that had GN on day 56 and had been treated with probucol and/or ARB (candesartan)† |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Control                         | GN              | GN + probucol   | GN + ARB        | GN + probucol + ARB |
| Total protein (g/dl)            | 6.3 ± 0.1       | 6.0 ± 0.2       | 6.0 ± 0.2       | 6.6 ± 0.1       |
| BUN (mg/dl)                     | 21 ± 1.6        | 32 ± 3.3        | 26 ± 2.3        | 23 ± 2.4        |
| Creatinine (mg/dl)              | 0.88 ± 0.04     | 1.16 ± 0.15     | 0.97 ± 0.04     | 0.89 ± 0.03     |
| T-CHO (mg/dl)                   | 71.0 ± 3.3      | 171.3 ± 42.0    | 124.5 ± 18.6    | 98.1 ± 3.5      |
| TG (mEq/L)                      | 147.2 ± 34.5    | 281.3 ± 47.7    | 283.6 ± 64.5    | 216.9 ± 39.2    |
| NEFA (μg/ml)                    | 0.35 ± 0.01     | 0.63 ± 0.08     | 0.63 ± 0.04     | 0.57 ± 0.08     |

†The values are expressed as mean ± SEM (n = 6). BUN, blood urea nitrogen; T-CHO, total cholesterol; TG, triglyceride; NEFA, nonesterified fatty acid.

‡P < 0.05 versus GN + probucol.
†P < 0.05 versus GN + ARB.
§P < 0.05 versus control.
†P < 0.05 versus GN.
*P < 0.01 versus GN + probucol.
†P < 0.01 versus GN.
‡P < 0.01 versus GN + ARB.

0.01 versus GN + ARB.
Figure 2. Light micrographs showing the effects of probucol and/or ARB (candesartan) on glomerular structural changes in rats with GN on day 56. (A) Control. (B) Vehicle-treated GN. (C) Probucol-treated GN. (D) ARB-treated GN. (E) Probucol + ARB–treated GN. Periodic acid-Schiff–stained sections. Magnification, ×200.

Figure 3. The glomerular cell number (A and D), the glomerulosclerosis score (B and E), and the number of ED-1–positive macrophages (C and F) in rats that had GN on days 7 (A through C) and 56 (D through F) and had been treated with probucol and/or ARB (candesartan). □, nephrectomized control rats; ■, vehicle-treated GN rats; ■, probucol-treated GN rats; □, ARB-treated GN rats; ■, probucol + ARB–treated GN rats. Data are mean ± SEM. *P < 0.01 versus control; †P < 0.01 versus GN; ‡P < 0.01 versus GN + ARB.

Renal Histology

The effects of treatment with probucol and/or ARB on renal histology were examined on days 7 and 56 after disease induction. None of the treatments significantly affected morphologic changes such as mesangial cell proliferation accompanied by mild mesangial matrix accumulation and segmental mesangio-lytic lesions seen on day 7 in vehicle-treated nephritic rats (data not shown). Representative light micrographs of glomerular lesions on day 56 after each treatment are shown in Figure 2. The most striking glomerular changes that were observed in vehicle-treated nephritic rats were severe mesangial proliferation and marked ECM accumulation, which were accompanied by adhesion to Bowman’s capsule, glomerulosclerosis, and tubulointerstitial fibrosis (Figure 2B). Treatment with ARB improved these pathologic findings (Figure 2D). Furthermore, glomerular injury could not be detected in probucol plus ARB-treated nephritic rats (Figure 2E).

To examine the effects of each treatment on nephritic glomeruli, we performed histologic and histochemical analyses on days 7 and 56 of the study period (Figure 3). On day 7, the glomerular cell number and the glomerulosclerosis score were significantly increased to similar levels in all nephritic rats that received each treatment (Figure 3, A and B). Conversely, the increased number of ED-1–positive macrophages seen in day 7

Figure 4. Representative immunohistochemical micrographs showing the effects of probucol and/or ARB (candesartan) on the expression of TGF-β1, collagen type I (COL I), and fibronectin (FN) in rats with GN on day 56. Magnification, ×200.

Figure 5. Semiquantitative assessment of the expression of TGF-β1, COL I, and FN in rats that had GN on days 7 (A) and 56 (B) and had been treated with probucol and/or ARB. □, nephrectomized control rats; ■, vehicle-treated GN rats; ★, probucol-treated GN rats; □, ARB-treated GN rats; ★, probucol + ARB–treated GN rats. Data are mean ± SEM. *P < 0.01 versus control; †P < 0.05 versus control; ‡P < 0.01 versus GN; §P < 0.05 versus GN + ARB.
vehicle-treated nephritic rats was significantly reduced in rats that were given probucol and/or ARB (Figure 3C). On day 56, a striking increase in both glomerular cell number and the glomerulosclerosis score was observed in vehicle-treated nephritic rats (Figure 3, D and E). Treatment with ARB but not probucol led to a considerable reduction in both parameters. Furthermore, a remarkable reduction of these parameters to the control level was achieved in probucol plus ARB-treated nephritic rats. There were no significant changes in the number of ED-1–positive macrophages in any of the groups (Figure 3F).

Renal Expression of TGF-β1 and ECM Proteins

Representative immunostaining micrographs on day 56 after disease induction showed that a marked increases in the expression of TGF-β1, COL I, and FN were observed in the mesangial area in >90% of the glomeruli from vehicle-treated nephritic rats (Figure 4). Treatment with ARB significantly reduced glomerular immunofluorescence staining for TGF-β1, COL I, and FN, whereas treatment with probucol did not. The administration of probucol plus ARB improved staining to the control level. Glomerular staining scores for TGF-β1, COL I, and FN are shown in Figure 5. A semiquantitative analysis on day 7 revealed that the glomerular expression of COL I and FN but not TGF-β1 was upregulated in all nephritic rats regardless of treatment (Figure 5A). Conversely, on day 56, the levels of immunofluorescence for these factors indicated that combined treatment with probucol plus ARB normalized immunofluorescence intensity for the expression of TGF-β1 and ECM proteins to the control level, in parallel with histologic improvement (Figure 5B).

**Figure 6.** Expression of Nox2 and Nox4 in rats that had GN on days 7 (A through C) and 56 (D through F) and had been treated with probucol and/or ARB (candesartan). Total protein was extracted from glomeruli that were isolated by the sieving method. The samples were adjusted to a standard content (30 μg) and analyzed by Western blotting using the mouse monoclonal anti-Nox2 antibody and rabbit polyclonal anti-Nox4 antibody. (A and D) Representative Western blotting is shown. (B, C, E, and F) Results of densitometric analysis. The results represent the mean ± SEM. □, nephrectomized control rats; ▪, vehicle-treated GN rats; □, probucol-treated GN rats; □, ARB-treated GN rats; □, probucol + ARB–treated GN rats. *P < 0.01 versus control; †P < 0.05 versus GN; ‡P < 0.01 versus GN.

Glomerular Expression of NADPH Oxidase Components and In Situ Production of Superoxide

To examine whether probucol and/or ARB affected the levels of Nox2 and Nox4 protein, the major component of NADPH oxidase, we performed Western blotting on days 7 and 56 after disease induction (Figure 6). On day 7, a significant increase in Nox2 protein was observed in only vehicle-treated nephritic rats, whereas Nox4 protein was significantly increased in all nephritic rats regardless of treatment (Figure 6, A through C). The increased level of Nox2 protein in vehicle-treated nephritic rats seemed to be slightly suppressed by probucol and/or ARB administration even though this effect was NS (Figure 6, A and B). On day 56, the levels of Nox2 and Nox4 protein in vehicle-treated nephritic rats were upregulated 4.6- and 1.6-fold, re-
spectively (Figure 6, D through F). This increased level of Nox2 protein was significantly decreased in probucol-treated nephritic rats and was attenuated by approximately 50% in ARB-or probucol plus ARB-treated nephritic rats (Figure 6, D and E). In addition, ARB or probucol plus ARB treatment tended to decrease the level of Nox4 in nephritic rats, although these changes were not statistically significant (Figure 6, D and F).

For evaluation of the effect of probucol and/or ARB on glomerular \( \text{O}_2^- \) content, in situ superoxide production of days 7 and 56 was detected by DHE (Figure 7). A significant increase in glomerular \( \text{O}_2^- \) production was observed in only vehicle-treated nephritic rats on day 7 (Figure 7F). Treatment of nephritic rats with probucol or ARB seemed to decrease its production, although it was numerically NS. Treatment with probucol plus ARB significantly attenuated glomerular \( \text{O}_2^- \) production to near the control level. On day 56, \( \text{O}_2^- \) production was strikingly enhanced in vehicle-treated nephritic rats, and this was significantly reduced to the control level in probucol- or ARB-treated nephritic rats (Figure 7, B through D and G). Of note, combined treatment completely eliminated the glomerular \( \text{O}_2^- \) content from day 56 nephritic glomeruli (Figure 7, E and G).

Next, double-immunofluorescent staining of mesangial cells or macrophages combined with the detection of \( \text{O}_2^- \) by DHE on frozen sections was performed to identify the \( \text{O}_2^- \)-producing cells in day 56 nephritic glomeruli. Figure 8, A through C, shows that immunostaining of Thy-1–positive mesangial cells was co-localized with most of the nuclear staining of \( \text{O}_2^- \)-producing cells. Staining of \( \text{O}_2^- \) was also detected on a few ED-1–positive macrophages that had infiltrated nephritic glomeruli (Figure 8, D through F). These results suggest that mesangial cell \( \text{O}_2^- \) production, possibly via NADPH oxidase activation, is involved mainly in the level of glomerular injury, including morphologic parameters in this rat model of GN.

**Discussion**

In this study, we demonstrated that the combined administration of probucol and ARB fully arrests proteinuria and the progression of severe mesangioproliferative GN in the rat. It seems likely that the beneficial effect of such combined treatment is due to the synergistic action of Ang II inhibition with a receptor antagonist and the elimination of ROS with a radical scavenger.

At present, blocking of Ang II action is one of the most useful strategies for treating chronic progressive mesangioproliferative GN, including IgA nephropathy. ARB and/or ACEI limits proteinuria and reduces the decline in GFR and the risk for ESRD more effectively than other antihypertensive treatments (6,36–38). Several in vivo animal experiments have indicated that the effect of ARB and/or ACEI in preventing the progression to end-stage renal failure is due to the ability of ARB and ACEI to modulate the effects of Ang II on the production of TGF-\( \beta \)1 and ECM as well as on glomerular hypertension (8,10,39). In vitro studies have demonstrated that Ang II stimulates TGF-\( \beta \)1 expression, which promotes the synthesis of ECM proteins and inhibits ECM turnover by decreasing pro-

![Figure 7](image-url)
tease activity and increasing the production of protease inhibitors (11–13). Our findings that an ARB, candesartan, considerably reduced proteinuria, the level of TGF-β1, and ECM accumulation at the late stage in this rat model of GN and finally inhibited the progression of glomerulosclerosis are consistent with the above concept.

ROS has been implicated in the development and progression of GN (15–17,40,41). NADPH oxidase is a major source of ROS in phagocytes as well as non-phagocytic cells, including fibroblasts, vascular smooth muscle cells, and glomerular cells (21–25). NADPH oxidases are composed of gp91phox-homolog, called Nox protein and several subunits that contain p22phox, p47phox, p67phox, and small GTPase rac. Nox2 indicates that gp91phox, the main subunit of the phagocyte NADPH oxidase, and Nox4, which seems to share the same overall structure as Nox2, are abundant in the vascular system, kidney cortex, and mesangial cells (21,22,25). In fact, an increase in glomerular ROS and NADPH-dependent oxidase activity has been reported in an acute model of Thy-1 GN that was induced by a single injection of anti–Thy-1 antibody (15). In addition, our data clearly showed that an increase in glomerular O$_2^•$ content and Nox2 and 4 proteins was detected in the early and advanced stages of chronic progressive GN in rats (17). Recently, it was shown that ROS that is produced by NADPH oxidase activates intracellular signal molecules, such as mitogen-activated protein kinases (MAPK) and activator protein-1, and thereby might contribute to cell proliferation, ECM production, and ECM remodeling involved in the progression of GN (42). Indeed, Budisavljevic et al. (16) suggested that ROS might mediate glomerular injury by activating extracellular signal–regulated kinase that could induce mesangial cell proliferation and increase of glomerular TGF-β1 expression in acute Thy-1 GN. We have also reported in renal injury in Dahl salt-sensitive hypertensive rats that the renal expression of p22phox and Nox1 and phosphorylation of MAPK are associated with the level of progressive sclerotic and proliferative glomerular changes (43). Tempol, a superoxide dismutase, ameliorated not only those glomerular changes but also the expression of p22phox, Nox1, and MAPK activities, suggesting that ROS, generated by NADPH oxidase, contributes to the progression of renal injury through MAPK activation in Dahl salt-sensitive hypertensive rats (43).

Of note, Ang II as well as other fibrogenic factors, such as PDGF-BB, TNF-α, and IL-1β, are involved in ROS production (3,14,44). We showed in this study that ARB significantly decreased the level of glomerular O$_2^•$ production on day 56. Western blot experiments indicated that this reduction of glomerular O$_2^•$ content is probably due to the decreased expres-

Figure 8. Representative double-fluorescence study with immunofluorescence staining of Thy-1–positive mesangial cells or ED-1–positive macrophages and dye DHE fluorescence in vehicle-treated nephritic rats on day 56. In A and D, green indicates Thy-1–positive mesangial cells and ED-1–positive macrophages, respectively. In B and E, red indicates nuclear staining of superoxide (O$_2^•$–)-producing cells. In C and F, merged images show O$_2^•$–-producing mesangial cells and macrophages, respectively. Magnification, ×400.
sion of glomerular Nox2 protein by ARB, because several reports based on cell culture studies have demonstrated that Ang II stimulated the protein and mRNA expression of Nox2 (45,46). Therefore, the reduction of glomerular $\text{O}_2^-$ content in GN by ARB might be linked to some of the renoprotective effects that are induced by Ang II blockade.

Probucol, a cholesterol-lowering drug with potent antioxidant properties, reduced glomerular $\text{O}_2^-$ production in GN to almost the normal level, although a significant amelioration of proteinuria, glomerular pathology, and expression of TGF-$\beta 1$ and ECM was not seen in probucol-treated nephritic rats. Probucol seems to act mainly as an antioxidant in nephritic rats because the level of serum cholesterol was not significantly reduced. The level of Nox2 protein was slightly decreased on day 7 and significantly downregulated on day 56 by probucol. This suppressive effect on Nox2 might lead to significant $\text{O}_2^-$ reduction in long-term probucol-treated nephritic rats via coordination with the action as a radical scavenger.

The combination of probucol and ARB not only completely eliminated $\text{O}_2^-$ production but also normalized urinary protein excretion and the expression of TGF-$\beta 1$ and ECM seen in GN, resulting in full prevention of the progression of GN. Long-term combination therapy with ARB and probucol may confer a strong, synergistic antioxidative action on nephritic glomeruli by inhibiting Nox2 protein expression associated with NADPH oxidase activity and by scavenging glomerular $\text{O}_2^-$ content and thereby enhance the renoprotective effect of ARB against the progressive nature of the disease in this rat model of GN. It is interesting that HMG-CoA reductase inhibitors or statins, which are cholesterol-lowering agents, also show strong antioxidant ability involved in the inhibition of Rac1 geranylgeranylation and enhance the beneficial effects of RAS inhibition on several types of organ damage (47,48). Nie et al. (49) reported in a model of CCl$_4$-induced liver fibrosis that pitavastatin enhances the antifibrogenic effects of candesartan. Pitavastatin significantly enhanced the suppressive effects of ARB on TGF-$\beta 1$ expression and fibrogenesis, whereas it alone did not affect liver fibrogenesis or TGF-$\beta 1$ expression. Regarding the effect of statins on glomerular injury, Zojia et al. (39) reported that therapy with either ACEI and ARB or cerivastatin had limited effects, but combination therapy with these agents further reduced glomerulosclerosis and urinary protein excretion in a rat model of Heymann nephritis. The expression of TGF-$\beta 1$ was significantly decreased by triple combined therapy, whereas ACEI, ARB, and cerivastatin did not significantly affect TGF-$\beta 1$ expression in GN. Although neither study mentioned substantial changes in $\text{O}_2^-$ production in damaged tissues by statins, several studies have demonstrated that statins reduced oxidative stress in damaged tissues (47,48). Therefore, the combination of an antioxidant and Ang II blockade might be a useful strategy for preventing the progression of chronic GN. Further investigations, including in vitro studies, are needed to explore the molecular mechanisms that are responsible for the inhibitory effects of combined therapy on the progression of GN by especially focusing on the signal pathways that lead to the suppression of the expression of TGF-$\beta 1$.

Probucol is a well-established pharmacologic agent that has been shown to reduce restenosis after coronary balloon angioplasty by improving vascular remodeling in humans (50). Furthermore, Haas et al. (51) reported that probucol also had favorable renal effects in membranous nephropathy in a clinical study. Therefore, further studies on rat models of progressive GN and patients with chronic GN, such as IgA nephropathy and diabetic nephropathy, will be necessary to address whether combined therapy with probucol and ARB could have clinical significance.

### Conclusion

Our study provides important evidence that the antioxidant probucol when added to ARB fully arrests proteinuria and the progression of GN. The data suggest that the beneficial effects of this combined therapy might be mediated through the elimination of ROS that is produced mainly by NADPH oxidase in GN. The combination of probucol and ARB, both of which are frequently used in daily clinical practice, may represent a novel route of therapy for patients with progressive GN.

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