Renoprotective and antihypertensive effects of S-allylcysteine in 5/6 nephrectomized rats

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reduce oxidative and nitrosative stress, progression of structural kidney injury, and systemic hypertension in 5/6NX rats. To establish a potential mechanism of action of SAC and AG in this model, we evaluated the following markers: 1) iNOS, which synthesizes copious amounts of NO (14), 2) 3-NT formation as a marker of ONOO− generation and nitrosative stress (14, 34, 51), 3) poly(ADP-ribose) (pADPR), a marker of poly(ADP-ribose) polymerase (PARP) activation, a nuclear enzyme activated by oxidative and nitrosative stress (24), and 4) gp91phox and p22phox, subunits of NADPH oxidase, an enzyme that synthesizes O2− (13).

MATERIALS AND METHODS

Reagents. SAC was synthesized by the reaction of L-cysteine with allyl bromide and purified by recrystallization from ethanol-water, as previously described (27, 33). AG (Kyolic) was obtained from Wakunaga (Mission Viejo, CA). Xanthine, xanthine oxidase, and nitro blue tetrazolium (NBT) were purchased from Sigma Chemical (St. Louis, MO). Blood urea nitrogen (BUN) and creatinine concentrations were measured using commercial kits (Sera-Pak Plus Creatinine and Urea, Bayer, Tarrytown, NY). H2O2 was purchased from Mallinkrodt Baker (Xalostoc, México). Pentobarbital sodium was obtained from Pfizer. Mouse monoclonal antibodies against 3-NT (catalog no. 189542) were purchased from Cayman Chemical (Ann Arbor, MI), mouse monoclonal antibodies against pADPR (catalog no. SA-216) from Biomol (Plymouth Meeting, PA), and goat polyclonal antibodies against p22phox (catalog no. sc-11712) and gp91phox (catalog no. sc-5827), mouse monoclonal antibodies against iNOS (catalog no. sc-7271), and rabbit polyclonal antibodies against endothelial NOS (eNOS; catalog no. sc-654) from Santa Cruz Biotechnology (Santa Cruz, CA). The following secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA): biotin-streptavidin peroxidase (SP)-conjugated AffiniPure donkey anti-mouse IgG (catalog no. 715-065-151), biotin-SP-conjugated AffiniPure goat anti-rabbit IgG (catalog no. 111-065-003), and biotin-SP-conjugated AffiniPure donkey anti-goat IgG (catalog no. sc-633C) were obtained from Cell Marque (Hot Springs, AR). ABC Elite Vectastain kit (catalog no. PK-6101) from Vector Laboratories (Orton Southgate, Peterborough, UK), and diaminobenzidine substrate (catalog no. K3466) and Mayer’s hematoxylin (Lillie’s modification; catalog no. S3309) from DakoCytomation (Carpentaria, CA). All other chemicals were reagent grade.

Animals and procedures. The experimental protocol was approved by the local animal care committee (CINVA, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán). Male Wistar rats (280–300 g body wt) were anesthetized with pentobarbital sodium (50 mg/kg ip), and a ventral laparotomy was performed under aseptic...
conditions. The right kidney was removed, and a portion (two-thirds) of the left kidney was acutely infarcted by ligation of two first-order branches of the main renal artery (50). Recovery from anesthesia and from the surgical procedure was complete within 24 h. Rats were divided into three groups as follows: 5/6NX, 5/6NX + AG (1.2 ml/kg ip, every other day) (31), and 5/6NX + SAC (200 mg/kg ip, every other day) (25). Rats were maintained in stainless steel metabolic cages for collection of urine samples. Samples of venous blood were taken from the tail of the rats 5, 10, 15, and 30 days after 5/6NX for serum creatinine and BUN determinations. Systolic blood pressure (SBP) was measured by a noninvasive tail-cuff method (model 179 Blood Pressure Analyzer, IITC, Woodland Hills, CA) on days 0, 15, and 30. Sham-operated (sham) rats were also studied on days 0, 15, and 30. At the end of the study, rats were anesthetized by an injection of pentobarbital sodium (30 mg/kg ip), and blood samples were taken from the aorta. The kidneys were excised and frozen in liquid nitrogen at −80°C. The experimental protocol was approved by the Local Animal Care Committee (CINVA) of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán.

Biochemical markers of renal injury. Serum creatinine and BUN concentrations were measured with an autoanalyzer (Technicon RA-1000, Bayer). Urine protein concentration was measured by a previously described method (30).

Activity of antioxidant enzymes. SOD activity in kidney homogenates was assayed by a previously reported method (42) based on NBT reduction to formazan. The amount of protein that inhibits NBT reduction to 50% of maximum was defined as 1 unit of SOD activity. Results are expressed as units per milligram of protein. Catalase activity was measured by a previously described method, with H₂O₂ used as substrate (41).

Histological and immunohistochemical analyses. For light microscopy, kidney tissue was fixed by immersion in buffered formalin (pH 7.4) and embedded in paraffin. For histological analysis, kidney sections (3 μm) were stained with hematoxylin and eosin and with Masson’s trichrome to stain collagen as an indicator of fibrosis. Interstitial fibrosis was determined in five random fields of the cortical area at ×200 magnification (total area 4.1 × 10⁵ μm²) with use of a computer-assisted color image analyzer (Qwin-500, Leica, Milton Keynes, Cambridge, UK). The same instrument was used to determine glomerulosclerosis by measurement of the extent of fibrosis identified by Masson’s trichrome in 20 randomly selected glomeruli from each animal. The fraction of fibrosis was obtained by normalization of the area of the Masson’s trichrome-positive material in each glomerulus to the whole glomerular area.

For immunohistochemistry, paraffin was removed from the kidney sections (3 μm), which were then boiled in Declere to unmask antigen sites; the endogenous activity of peroxidase was quenched with 0.03% H₂O₂ in absolute methanol. Kidney sections were incubated overnight at 4°C with a 1:100 dilution of anti-iNOS, a 1:250 dilution of anti-p22phox, a 1:70 dilution of anti-eNOS, a 1:50 dilution of anti-3-NT, a 1:50 dilution of anti-iNOS, and a 1:200 dilution of anti-pADPR antibodies in PBS. Bound antibodies were detected with
an avidin-biotinylated peroxidase complex ABC kit (Vectastain) and diaminobenzidine substrate. After they were appropriately washed in PBS, the slides were counterstained with hematoxylin. All specimens were examined by light microscopy (Axiovert 200M, Carl Zeiss). For automated morphometry analysis, the percentage of positive cells (stained brown) was determined with a computerized image analyzer (model KS-300 3.0, Carl Zeiss), which automatically detects positive cells and determines the number of positive cells per field. Five random fields per kidney were studied at ×100 magnification (total area $1 \times 10^4 \mu m^2$) for comparison of the different groups. All sections were incubated under the same conditions and in the same run, so immunostaining was comparable among the different experimental groups. For the negative control, preimmune goat serum, instead of the primary antibodies, was used (40, 47).

Statistical analysis. Values are means ± SE. Data were analyzed with Prism (version 3.02, GraphPad, San Diego, CA) by one- or two-way analysis of variance followed by Dunnett’s post test or Bonferroni’s multiple comparisons method, as appropriate. Pearson’s correlation coefficient ($r$) was used for testing variables of interest. $P < 0.05$ was considered significant.

### RESULTS

Serum creatinine and BUN concentrations were increased in 5/6NX compared with sham rats. Both markers reached a peak value on day 5 and then decreased but remained high until the end of the study (day 30) in 5/6NX, 5/6NX + SAC, and 5/6NX + AG rats. SAC and AG ameliorated the increase in serum creatinine and BUN concentrations on days 5, 10, 15, and 30 (Fig. 1).

Proteinuria increased in 5/6NX compared with sham rats. Proteinuria increased by day 10, reached a peak value on day 15, and decreased but remained high on day 30 in 5/6NX rats. This increase was significantly less in SAC- and AG-treated rats on days 10, 15, and 30 (Fig. 2A). The increase in SBP in 5/6NX rats on days 15 and 30 was significantly prevented by AG and SAC (Fig. 2B).

Kidneys from the sham-operated rats showed normal structure (Fig. 3). The kidney histology of remnant rats of all groups on day 30 showed extensive fibrosis, mild chronic tubulointerstitial inflammatory infiltrate, and thickening of the muscular layer and decrease in luminal diameter of many intermediate-sized arteries and arterioles. Numerous convoluted proximal tubules showed extensive tubular damage characterized by atrophy and detachment of epithelial cells, with active epithelial regeneration and luminal proteinaceous casts. Multiple glomeruli showed different grades of fibrosis and mesangial cell hyperplasia (Fig. 3). The immunohistochemical detection of 3-NT showed strong immunostaining in the tubular epithelium, mesangial and visceral epithelial glomerular cells, endothelium, and smooth muscle cells from the vascular walls (Fig. 3). 3-NT immunostaining was weak in kidneys from sham rats.

In contrast to the untreated 5/6NX rats, the morphometry study showed that AG or SAC significantly improved renal damage, reducing the area affected by fibrosis sixfold in 5/6NX compared with untreated 5/6NX rats (Figs. 3 and 4, Table 1).

### Statistical analysis

Values are means ± SE, expressed as percentage ($n = 6$). 5/6NX, 5/6 nephrectomy; AG, aged garlic extract; SAC, 5-allylcysteine; 3-NT, 3-nitrotyrosine; iNOS, inducible nitric oxide synthase; pADPR, poly(ADP-ribose).

$\star P < 0.001$; $\dagger P < 0.05$; $\ddagger P < 0.01$ vs. 5/6NX. § $P < 0.001$ vs. sham.

### Correlation studies

As expected, SBP showed a significant correlation with glomerulosclerosis ($r = 0.86$, $P = 0.0004$) and tubulointerstitial damage ($r = 0.91$, $P = 0.0001$). SBP also displayed a positive correlation with 3-NT ($r = 0.806$, $P = 0.0002$), iNOS ($r = 0.68$, $P = 0.0036$), gp91phox ($r = 0.775$, $P = 0.0004$), p22phox ($r = 0.889$, $P < 0.0001$), and pADPR ($r = 0.57$, $P = 0.02$) and a negative correlation with SOD ($r = -0.786$, $P = 0.0002$; Fig. 7).

Glomerulosclerosis correlated positively with tubulointerstitial injury ($r = 0.91$, $P < 0.0001$), 3-NT ($r = 0.92$, $P < 0.0001$).

### Table 1. Quantitative data from immunohistochemistry studies

<table>
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<tr>
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<th>Sham</th>
<th>5/6NX</th>
<th>5/6NX + AG</th>
<th>5/6NX + SAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-NT</td>
<td>0.23 ± 0.14</td>
<td>6.5 ± 0.58§</td>
<td>1.5 ± 0.17*</td>
<td>2.2 ± 0.035*</td>
</tr>
<tr>
<td>iNOS</td>
<td>0.70 ± 0.20</td>
<td>3.2 ± 0.58§</td>
<td>1.5 ± 0.25†</td>
<td>1.9 ± 0.33</td>
</tr>
<tr>
<td>gp91phox</td>
<td>16.26 ± 1.53</td>
<td>35.5 ± 2.68</td>
<td>15.7 ± 1.08*</td>
<td>20.1 ± 1.41*</td>
</tr>
<tr>
<td>p22phox</td>
<td>18.5 ± 1.95</td>
<td>40.7 ± 2.27§</td>
<td>19.8 ± 1.61*</td>
<td>25.1 ± 3.5*</td>
</tr>
<tr>
<td>pADPR</td>
<td>22.39 ± 2.10</td>
<td>50.1 ± 1.72§</td>
<td>36.6 ± 1.78§</td>
<td>40.2 ± 2.20+</td>
</tr>
</tbody>
</table>

$\star P < 0.001$; $\dagger P < 0.05$; $\ddagger P < 0.01$ vs. 5/6NX. § $P < 0.001$ vs. sham.

**Fig. 5.** Representative immunohistochemical detection of inducible nitric oxide synthase (iNOS) and p22phox in kidney sections after 30 days of 5/6NX and effect of SAC and AG. iNOS immunostaining is strong in epithelial tubular cells (arrows), mesangial and visceral epithelial cells from damaged glomeruli (+), and interstitial cells in 5/6NX rats, weaker in 5/6NX + SAC and 5/6NX + AG rats, and scarce in sham rats. p22phox immunostaining in interstitial cells (arrows) and mesangial cells from glomerulus (+) is strong in 5/6NX rats, less intense in 5/6NX + SAC and 5/6NX + AG rats, and scarce in sham rats. Magnification ×400.
0.0001), gp91phox ($r = 0.78, P = 0.0029$), p22phox ($r = 0.83, P = 0.0008$), and pADPR ($r = 0.81, P = 0.0014$) and negatively with SOD ($r = -0.82, P = 0.001$; Fig. 8). Tubulointerstitial injury displayed a positive correlation with 3-NT ($r = 0.91, P < 0.0001$), iNOS ($r = 0.75, P = 0.0048$), gp91phox ($r = 0.75, P = 0.0046$), p22phox ($r = 0.81, P = 0.0013$), and pADPR ($r = 0.68, P = 0.015$) and a negative correlation with SOD ($r = -0.68, P < 0.015$).

3-NT immunostaining correlated positively with p22phox ($r = 0.89, P < 0.0001$), gp91phox ($r = 0.86, P < 0.0001$), iNOS ($r = 0.75, P = 0.0003$), and pADPR ($r = 0.75, P = 0.0003$) and negatively with SOD ($r = -0.8072, P < 0.0001$; Fig. 9). iNOS immunostaining positively correlated with gp91phox ($r = 0.55, P = 0.021$) and pADPR ($r = 0.66, P = 0.0039$) and negatively with SOD ($r = -0.54, P = 0.02$). Finally, gp91phox, p22phox, and pADPR correlated negatively with SOD ($r = -0.83, P < 0.0001$; $r = -0.73, P = 0.0005$; and $r = -0.61, P = 0.0074$, respectively).

**DISCUSSION**

Renal damage in 5/6NX rats has been clearly associated with oxidative and nitrosative stress, characterized by 1) enhanced 3-NT formation, 2) downregulation of CuZn SOD and Mn SOD, 3) upregulation of gp91phox, 4) enhanced urinary excretion of NO−metabolites (51), and 5) enhanced iNOS expression (3). Our results suggest an imbalance in $O_2^{•−}$ and NO−metabolism favoring their production. The above-mentioned alterations may lead to an enhanced ONOO−production and 3-NT formation. Interestingly, the scavenging of $O_2^{•−}$ with tempol (51) reduced hypertension in these rats, suggesting a role of $O_2^{•−}$ not only in renal injury, but also in the development of hypertension. In fact, oxidative and nitrosative stress has been.

![Fig. 6. SOD (A) and catalase (B) activity in renal cortex on day 30 in sham, 5/6NX, 5/6NX + SAC, and 5/6NX + AG rats. Values are means ± SE (n = 6). #P < 0.001 vs. sham. *P < 0.001 vs. 5/6NX.](#)

![Fig. 7. Correlation analysis between SBP and 3-NT (A), gp91phox (B), p22phox (C), and SOD (D) in 5/6NX (●), 5/6NX + AG (○), and 5/6NX + SAC (□) rats.](#)
involved in the pathogenesis of hypertension (10, 53). Considering the previously discussed background, we explored the effect of SAC and AG on rats with remnant kidneys. SAC and AG have demonstrated an antioxidant capacity, as well as tissue protective effects, in several experimental conditions (9, 16, 21, 22, 31, 32, 38, 43). In the present study, the following properties of AG and SAC are remarkable and support their use in rats with 5/6NX: 1) SAC and AG scavenge $O_2^{-•}$ (23, 31–33), 2) SAC scavenges ONOO$^{-}$ (22, 33) and prevents

Fig. 8. Correlation analysis between glomerulosclerosis and 3-NT (A), gp91$^{phox}$ (B), p22$^{phox}$ (C), and SOD (D) in 5/6NX (○), 5/6NX + AG (●), and 5/6NX + SAC (■) rats.

Fig. 9. Correlation analysis between 3-NT and p22$^{phox}$ (A), gp91$^{phox}$ (B), iNOS (C), and SOD (D) in 5/6NX (○), 5/6NX + AG (●), and 5/6NX + SAC (■) rats.
hemolysis induced by this anion (35), 3) SAC inhibits iNOS expression and enhances NO\(^+\) production from endothelial cells (23), 4) SAC prevents NF-\(\kappa\)B activation (12), and 5) AG ameliorates high blood pressure in hypertensive rats (15) and increases NO\(^+\) production (37). AG, one of the best-known garlic preparations, is formed during garlic aging (up to 20 mo). During this time, unstable and highly odorous compounds in fresh garlic are converted to more stable and much less odorous compounds (1). The chemical composition of AG is different from that of whole garlic (26): the most abundant compounds in AG are alliin, cycloalliin, SAC, and \(S\)-allymercaptocysteine, and the most abundant compounds in whole garlic are alliin and \(\gamma\)-glutamylcysteines (26).

In the present work, the development of oxidative and nitrosative stress in rats with remnant kidney was confirmed by the formation of 3-NT and pADPR, the upregulation of iNOS, p22\(^{\text{phox}}\), and gp91\(^{\text{phox}}\), and the decrease in SOD and catalase activities.

Our data clearly show that SAC and AG dramatically reduced renal injury and hypertension in 5/6NX rats. The renoprotective effect was clearly and significantly associated with a decrease in abundance of 3-NT, gp91\(^{\text{phox}}\), p22\(^{\text{phox}}\), and pADPR and an increase in SOD activity. 3-NT is a marker of ONOO\(^-\), a powerful oxidant and nitrating agent, and nitrosative stress (7, 34, 51); pADPR is a marker of PARP activation secondary to oxidative and nitrosative stress (24). These data strongly suggest that the protective effect of SAC and AG in 5/6NX rats is associated with their antioxidant properties.

The decrease in 3-NT abundance may be secondary to the SAC- and AG-induced decrease in iNOS, gp91\(^{\text{phox}}\), and p22\(^{\text{phox}}\) abundance and the increase in SOD activity. These combined effects should reduce the availability of NO\(^-\) and O\(_2\)\(^{\cdot}\), the substrates for ONOO\(^-\) formation. 3-NT abundance showed a positive correlation with p22\(^{\text{phox}}\), gp91\(^{\text{phox}}\), and iNOS and a negative correlation with SOD activity.

The mechanism(s) by which SAC and AG decrease the abundance of iNOS, gp91\(^{\text{phox}}\), and p22\(^{\text{phox}}\) and the increase in SOD activity is not clear. Our data suggest that iNOS activation is involved in renal injury in this experimental model, as reported by Bautista-Garcia et al. (3). In addition, our results are also in good agreement with those of Vaziri et al. (52), who suggested that NADPH oxidase is a source of O\(_2\)\(^{\cdot}\) in 5/6NX rats. The activation of NADPH oxidase has also been observed and implicated in the pathogenesis of several renal diseases (13, 20, 29). It has also been suggested that PARP activation is involved in the pathogenesis of 5/6NX. In fact, PARP activation has been implicated in several nephropathies (6, 8, 24, 39, 45). The use of PARP inhibitors may help clarify the role of PARP in 5/6NX rats.

The antihypertensive effect of AG in 5/6NX rats is consistent with the hypotensive effect of AG in spontaneously hypertensive rats (15). The SAC- and AG-induced reduction in oxidative and nitrosative stress may be associated with this effect. Our results clearly show that hypertension correlates positively not only with renal structural damage, but also with the immunostaining of iNOS, 3-NT, gp91\(^{\text{phox}}, \) and p22\(^{\text{phox}}\), and negatively with SOD activity. Our data are consistent with the fact that SAC and AG modulate NO\(^+\) production (21, 37). To our knowledge, this is the first report of the antihypertensive effect of SAC. Kim et al. (21) found a protective effect of SAC in stroke-prone spontaneously hypertensive rats, despite persistent high blood pressure.

In summary, SAC and AG showed marked antihypertensive and renoprotective effects in 5/6NX rats. These protective effects were clearly associated with the correction of the imbalance between O\(_2\)\(^{\cdot}\) production and the decrease in oxidative and nitrosative stress markers. These data suggest that SAC and AG may be used to ameliorate hypertension and delay the progression of renal injury.

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GRANTS

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SAC DECREASES RENAL DAMAGE AND BLOOD PRESSURE IN 5/6NX