Atorvastatin Improves the Course of Ischemic Acute Renal Failure in Aging Rats

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Abstract. Statins increase the production of nitric oxide (NO) and have beneficial effects on the course of acute renal failure (ARF) in young rats. The effects of a short-term treatment with atorvastatin (ATO) on ischemic ARF in old rats, characterized by a great susceptibility to ischemia, was tested. No difference was found in renal dynamics between young (Y, 3 mo old) and old (O, 18 mo old) rats in normal conditions (CON) or after ATO treatment (12 mg/kg/d for 14 d). Twenty-four hours after clamping of both renal arteries, a more pronounced decrease in GFR was observed in O rats versus Y rats after a greater renal vasoconstriction and hypoperfusion of aging animals. Pretreatment with ATO mitigated renal vasoconstriction in O rats and restored GFR values to Y rats. Nitrate excretion was enhanced in Y rats after ARF but was not further modified by ATO; in O rats, ARF did not increase nitrate excretion, which was raised after ATO treatment. This reflected the increase in endothelial NO synthase (eNOS)–mRNA expression and eNOS protein observed in old ATO-treated animals with ARF. ATO treatment had also a significant protective effect against the cell injury at tubular level in O, but not Y, rats. The Ras system was not influenced by ATO in O rats, whereas the activation of Rho proteins was partially inhibited by ATO. Low-dose treatment with ATO enhances NO availability in aging rats, improving renal dynamics and conferring a peculiar histologic protection at tubular level after ischemia.

In most animal species, the aging process is associated with a peculiar predisposition to renal damage in response to drugs, altered salt metabolism, and ischemia (1,2). A previous study from our laboratory has shown that ischemic injury after renal arteries clamping determines a more pronounced decrease of renal plasma flow (RPF) and GFR in old (O) rats when compared with young (Y) rats (3). Renal impairment in aging rats, however, was partially blunted by the administration of oral supplements of either arginine, the precursor of nitric oxide (NO), or SOD, a scavenger of reactive oxygen species (ROS), clearly suggesting that an endothelial dysfunction, secondary to NO deficiency and increased ROS production, was the main factor responsible for the intense renal vasoconstriction of aging animals (3). Although that study raised the theoretical possibility that some unfavorable effects due to renal hypoperfusion in the elderly could be prevented, to date, there is no definitive evidence that a specific prophylactic treatment can reduce the occurrence of these events in aging, because the use of arginine as precursor of NO does not seem to be indicated in the elderly on the basis of the better knowledge of the metabolism of this amino acid and its possible side effects after oral administration (4).

A recent study has shown that a 3-d treatment with a high dose of cerivastatin had beneficial effects in mitigating the course of ischemic acute renal failure (ARF) in young rats independent of their cholesterol levels (5). On this basis, we have hypothesized that statins could play a role in preventing the susceptibility to ischemic damage of aging rats, given the ability of these drugs to increase NO synthesis (6) and to reduce both inflammation (7) and ROS production (8), factors involved in the pathogenesis of the endothelial dysfunction of the elderly (3,9).

Therefore, the aim of the study presented here was to investigate whether the prolonged treatment with low doses of atorvastatin (ATO) could improve the course of ARF after ischemia-reperfusion injury in aging rats compared with untreated age-matched rats. To exclude the notion that the morphologic changes of aging (glomerular sclerosis, tubular atrophy, and arteriolosclerosis) (1) would have conditioned the degree of postischemic damage, only O rats without age-related nephropathy were used in this study (3). Because the positive effects of statins in endothelial protection seem to be mediated in vitro by the changes in the activity of the small G-proteins (Ras and Rho) and by the increased expression of endothelial NO synthase (eNOS) (10), we have also evaluated the modifications induced by ATO treatment on these proteins in the aging rat, a model in which these systems have never been investigated.
Materials and Methods

The study was carried out in 69 male Sprague-Dawley rats (Morini, Italy) with an age of either 3 to 4 mo (young rats, Y) or 18 mo (old rats, O). Both Y and O rats were fed a moderately hypoproteic diet from birth (protein content, 14% as casein); previous studies in this laboratory have shown that such a diet delays the occurrence of age-related glomerulosclerosis while permitting a normal growth (3).

After arriving at our facility, the rats were administered either the standard diet or a diet supplemented with ATO with free access to tap water for at least 14 d. The amount of ATO in the diet was calculated according to daily food intake of the rats to deliver about 15 mg/kg/d in Y rats and 12 mg/kg/d in O rats.

Young (Y) and old (O) rats were divided into the following experimental groups: group CON, sham-operated rats with normal diet (n = 6 and n = 7 in Y and O, respectively); group CON + ATO, sham-operated rats fed a diet supplemented with ATO (n = 6 and n = 8 in Y and O, respectively); group ARF, rats observed 24 h after the induction of ARF (n = 7 and n = 9 in Y and O, respectively); group ARF-ATO, rats with ARF, fed the diet supplemented with ATO (n = 7 and n = 8 in Y and O, respectively).

ARF was induced by clamping both renal arteries for 30 min with a sterile surgical procedure under light Nembutal anesthesia (35 to 50 mg/kg intraperitoneally) (3). Twenty-four hours after the ischemia-reperfusion injury, the rats were prepared for the hemodynamic study under Inactin anesthesia (80 to 100 mg/kg). The details of these procedures are described elsewhere (3,4). Briefly, the rats underwent tracheostomy and catheterization of jugular and femoral veins (for sustaining infusions and inulin administration), femoral artery (for BP monitoring and arterial blood sampling), and bladder (for timed urine collection).

After an adequate stabilization period, three to five clearance periods were performed. For determination of renal extraction of inulin, the left renal pedicle was exposed and blood was withdrawn from the renal vein in conjunction with an arterial blood sample inulin, the left renal pedicle was exposed and blood was withdrawn for evaluation of renal dynam-
ics), both kidneys were excised and stored for a quantitative evaluation of Ras proteins activation. An arterial blood sample was also withdrawn for determination of total cholesterol, triglycerides, aspar-
tate aminotransferase, alanine aminotransferase, and creatine kinase. In 12 additional O rats (4 CON, 4 O-ARF, and 4 O-ARF-ATO), prepared for the experiment (but with no evaluation of renal dynam-
ics), both kidneys were excised and stored for a quantitative evaluation of the isoforms of NO synthase (NOS) and of the activity of Rho-A system.

Analytical Determinations

Urinary volume was measured gravimetrically in preweighed vials. The concentrations of inulin were measured by diphenylamine method (11). The urine for determination of urinary nitrate concentra-
tions was collected in preweighed sterile vials under ice (4°C), filtered through a 0.2-μm filter (Acrodisc, Gelman), and frozen until the time of assay (−80°C). The dose was carried out by a total NO assay utilizing the nitrate reductase and the Griess reagent (RD Systems). Plasma parameters were determined by an autoanalyzer.

GFR was calculated according to standard formulas. Filtration fraction (FF) was estimated by inulin renal extraction \([\frac{\text{A IN} - \text{V IN}}{\text{A IN}}] \times 100\). RPF was measured by the ratio between GFR and FF. The correction of RPF by the percentage of hematocrit was used as estimate of renal blood flow. Renal vascular resistances (RVR) were determined by the ratio between mean BP and renal blood flow.

Histology

For studying the effects of the different regimens on renal pathology, kidney samples were evaluated in a blinded fashion. Plastic-embedded 3-μm sections were cut and stained with hematoxylin-eosin, periodic acid–Schiff, and Jones stains. Histologic analysis was performed separately for chronic and acute changes. Chronic changes were evaluated grading (0 to 3) glomerulosclerosis, arteriolarclerosis, and interstitial fibrosis/tubular atrophy according to morphologic criteria (12). The score for arteriolar sclerosis was assigned on the basis of severity of the fibrointimal thickening of arteries. Because ischemic acute tubular damage is most evident in the outer stripe of the outer medulla (13), 50 proximal tubules of this area were examined in each rats at ×400 magnification and assigned to three categories, according to the following criteria: (0) tubules with normal appearance; (1) tubules with signs of sublateral injury (loss of apical brush border); and (2) tubules with signs of acute tubular necrosis (from a few sloughed epithelial cells to tubules with a complete naked basal membrane) (14). Proximal tubules were distinguished from distal tubules on the basis of morphologic criteria (12,15).

eNOS-mRNA Extraction

For extraction of total mRNA, 100 mg of renal tissue, collected under sterile conditions and stored in Eurozol at −80°C until time of assay, were homogenized at 4°C; after addition of chloroform (1:10), the samples were mixed, incubated for 5 min on ice, and then centrifuged at 12,000 × g for 15 min at 4°C. Equal amounts of isopropanol were added to the upper phase of the solution, which was stored in melting ice for 15 min and then further centrifuged at 12,000 × g for 15 min. The pellet was firstly washed with 1 ml of 75% ethanol, centrifuged, dried, and then dissolved in 30 μl of sterile water. Aliquots of 1 μg of total RNA were electrophoresed in agarose gel (1.2%) with 2.2 M formaldehyde and stained with ethidium bromide to verify the quality and quantity of the RNA. Further aliquots were then treated with deoxyribonuclease I (Invitrogen) to eliminate DNA before RNA-PCR amplification. One microgram of these samples was used to perform reverse transcriptase (RT) of RNA to cDNA and its amplification through PCR (Perkin-Elmer, Roche).

Thirty-eight cycles were performed at the following conditions: 94°C for 30 s, 55°C for 60 s, and 72°C for 30 s. Sense and antisense primers for nitric oxide synthase cDNA and glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA were used, respectively. The GAPDH band intensities were used to standardize loading conditions. Equal amounts of RT-PCR products were loaded on a 2% agarose gel and stained with ethidium bromide, and the optical densities of ethidium bromide–stained DNA were quantified through a computed system.

Western Blot Analysis

For Western blot test, the frozen renal samples were brought to 4°C, washed, and homogenized in Teflon-potter with a specific buffer (0.25 M sucrose; 5 mM imidazole; 0.5 M mDTT) and a cocktail of protease inhibitors (Boehringer), then centrifuged at 3000 rpm for 10 min. Protein amounts were determined by the Lowry assay (15).

For evaluation of NOS isoforms (eNOS; neuronal NOS [nNOS]; inducible NOS [iNOS]), 50 μg of total protein (100 μg for iNOS)
from each sample was suspended in loading buffer, separated on 7.5% polyacrylamide gel, and transferred to a PVDF membrane. Immunoblotting was performed with monoclonal antibodies to eNOS, iNOS, and nNOS (Transduction Lab), diluted 1:2500 in 5% Tris-buffered saline (TBS)–milk and Tween 0.1%. Immunodetection was accomplished by the appropriate anti-mouse horseradish peroxidase–linked secondary antibody (dilution 1:2000 in 5% TBS-milk and 0.1% Tween) and the enhanced chemiluminescence kit (Amersham).

To separate the active, membrane-bound fractions of Ras and Rho-A proteins from the inactive (cytosol-bound) ones, the supernatant of renal homogenates was ultrafiltraged again at 100,000 x g for 60 min at 4°C. The membrane pellet was resuspended in 200 μl of RIPA buffer supplemented with a cocktail of protease inhibitors (Boehringer), and both fractions were charged on polyacrylamide SDS-PAGE and then transferred on a PVDF filter. The filters were blocked in 5% TBS-milk and 0.05% Tween for 20 min and then incubated over night with an anti–pan-Ras antibody, diluted 1:1000 at 4°C (Upstate), washed, and incubated with a horseradish peroxidase–linked anti-mouse immunoglobulin (Amersham) diluted 1:2000 for 90 min, and evidenced by enhanced chemiluminescence (16). For Rho-A determinations, different samples were incubated overnight with an anti–Rho-A antibody (Santa Cruz Biotechnology), diluted 1:100, and the same procedure followed.

Statistical Analyses
One-way ANOVA was used to compare the different mean values in the three experimental groups of both Y and O rats. Bonferroni’s test was used to find significant differences among the groups under study. Histologic data were analyzed by the Kruskall-Wallis one-way ANOVA followed by the Dunn’s multiple comparison test. P < 0.05 was considered statistically significant. The data are expressed as mean ± 1 SD.

Results
The body weight (BW) of Y and O rats averaged 348 ± 52 and 496 ± 44 g, respectively, and there was no difference among the single groups of Y and O rats; therefore, the data of GFR and other parameters of renal hemodynamics were factored for 100 g of BW. Old rats had values of proteinuria comparable to those of Y rats (13.7 ± 1.4 mg/24 h versus 10.4 ± 1.5 mg/24 h, O-CON versus Y-CON), and also similar were the degrees of glomerulosclerosis, arteriolosclerosis, and interstitial fibrosis. Data of blood parameters were in the normal range and were not modified by ATO treatment in any of the groups under study. In particular, cholesterol levels were not modified to a significant extent in either Y (41.9 ± 19.7 mg/dl versus 35.0 ± 13.1 after ATO) or O rats (54.2 ± 11.8 versus 43.6 ± 15.3 after ATO).

Effects of Atorvastatin in Normal Rats
The effects of ATO administration in normal Y and O rats are shown in Table 1. There was no significant difference in renal hemodynamics between Y and O rats both in normal conditions and after ATO administration. Only the urinary excretion of nitrates of aging rats showed a significant increase after ATO (+70%, P < 0.002 versus O-CON), but these rats had basal values lower than in Y rats.

ARF Studies
BP was similar in all groups of rats in the different experimental conditions (Table 2). The onset of ARF was characterized by a slight decrease in hematocrit values and a significant decrease in FF in all of the groups. In Y rats, ARF was associated with a consistent decrease in GFR (−60% versus Y-CON, P < 0.001), mediated by the significant increase in RVR (+91% versus Y-CON, P < 0.001) and the consequent decrement in renal plasma and blood flow (RPF, −44%, P < 0.05 versus Y-CON). Prolonged administration of ATO (group Y-ARF-ATO) did not modify consistently the pattern of renal hemodynamics. The weight of both kidneys (KW) was slightly increased after ARF in both groups of Y rats (Y-ARF and Y-ARF-ATO), as did the ratio KW/BW; this was due to renal interstitial edema and medullary blood congestion.

In aging rats, the induction of ARF (group O-ARF) was associated with a sharper decrease in GFR (−76% versus O-CON, P < 0.001); in fact, these rats responded to the ischemic insult with a more pronounced increase in RVR (+203% versus O-CON, P < 0.001), which caused a sharp decrease in RPF (−62.5%, P < 0.001) and FF compared with control rats (−36%, P < 0.001). Pretreatment with ATO greatly blunted the harmful effects of ischemia on renal function of aging rats; the increase observed in RVR was, in fact, less pronounced (+75% versus O-CON, P < 0.05) and significantly lower than in O-ARF rats (P < 0.05). This resulted in a better preservation of RPF (−38% versus O-CON, P < 0.001) and GFR (−55% versus O-CON, P < 0.001), both values being significantly higher than in O-ARF rats (P < 0.05). Aging rats with ARF showed a significant increase in kidney weight (+34% versus O-CON, P < 0.05); such an increase was less pronounced in rats in group O-ARF-ATO (+20% versus O-CON, NS). The ratio KW/BW, lower than in Y rats, was significantly increased by induction of ARF in both groups of O rats with ARF.

The urinary excretion of nitrates is represented in Figure 1. In Y rats, renal ischemia caused a consistent increase in nitrate excretion that showed no further change in rats treated with ATO. Aging rats, which started from lower values of urinary nitrates in control conditions, did not show any increase in their nitrate excretion after ischemia; treatment with ATO, however, significantly increased urinary nitrate excretion (+165% versus O-CON and +105 versus O-ARF, both P < 0.001), with numerical values similar to those observed in Y rats with ARF.

Histologic Data
Chronic Lesions. As anticipated, the degree of glomerular sclerosis, arteriosclerosis, and interstitial fibrosis/tubular atrophy was mild and comparable between Y and O rats in control conditions. The score for glomerular sclerosis averaged 0.43 ± 0.62 in Y-CON rats and 0.34 ± 0.50 in O-CON rats; the scores of arteriosclerosis were similar between Y and O rats (0.18 ± 0.47 and 0.11 ± 0.28, respectively), as well as the grading of interstitial fibrosis/tubular atrophy (0.14 ± 0.36 and 0.11 ± 0.39, respectively). These data confirmed the validity of our experimental model of the aging rat. The onset of ARF did not modify the score of the chronic lesions in any group.
Table 1. Effects of administration of atorvastatin on renal hemodynamics in normal rats

<table>
<thead>
<tr>
<th>Group</th>
<th>GFR/BW</th>
<th>FF</th>
<th>RVR</th>
<th>RPF</th>
<th>Ht</th>
<th>RBF</th>
<th>BP</th>
<th>KW</th>
<th>NO₂/NO₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y-CON (n = 6)</td>
<td>0.75 ± 0.03</td>
<td>26.2 ± 1.7</td>
<td>21.46 ± 1.58</td>
<td>2.89 ± 0.29</td>
<td>46.3 ± 2.2</td>
<td>5.41 ± 0.67</td>
<td>115.5 ± 7.4</td>
<td>2.47 ± 0.19</td>
<td>12.61 ± 2.8</td>
</tr>
<tr>
<td>Y-ATO (n = 6)</td>
<td>0.83 ± 0.12</td>
<td>26.5 ± 1.3</td>
<td>19.69 ± 2.95</td>
<td>3.23 ± 0.88</td>
<td>45.2 ± 1.1</td>
<td>5.93 ± 1.35</td>
<td>116.4 ± 6.1</td>
<td>2.62 ± 0.14</td>
<td>15.35 ± 3.96</td>
</tr>
<tr>
<td>O-CON (n = 7)</td>
<td>0.80 ± 0.13</td>
<td>25.7 ± 2.5</td>
<td>21.08 ± 4.17</td>
<td>3.10 ± 0.46</td>
<td>45.0 ± 1.9</td>
<td>5.67 ± 0.95</td>
<td>116.4 ± 8.1</td>
<td>2.90 ± 0.26</td>
<td>8.11 ± 1.27</td>
</tr>
<tr>
<td>O-ATO (n = 8)</td>
<td>0.81 ± 0.11</td>
<td>24.3 ± 1.5</td>
<td>20.59 ± 2.74</td>
<td>3.32 ± 0.53</td>
<td>43.1 ± 2.5</td>
<td>5.87 ± 1.77</td>
<td>118.6 ± 7.5</td>
<td>3.01 ± 0.41</td>
<td>13.8 ± 3.4</td>
</tr>
</tbody>
</table>

Y, young; O, old; CON, free diet; ATO, diet supplemented with atorvastatin; BW, body weight (g); GFR/BW, glomerular filtration rate factored for body weight (ml/min/100 g BW); FF, filtration fraction; RPF, renal plasma flow (ml/min); Ht, hematocrit (%); RBF, renal blood flow (ml/min); BP, blood pressure (mmHg); RVR, renal vascular resistances (dyne/s/cm²); KW, total kidney weight (g); NO₂/NO₃, urinary nitrate excretion (nmol/min).

Table 2. Renal hemodynamics in young (Y) and old (O) rats in normal conditions (CON) and 24 hours after acute renal failure (ARF), without and with ATO treatment (ATO)

<table>
<thead>
<tr>
<th>Group</th>
<th>GFR/BW</th>
<th>FF</th>
<th>RVR</th>
<th>RPF</th>
<th>Ht</th>
<th>RBF</th>
<th>BP</th>
<th>KW</th>
<th>KW/BW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y-CON (n = 6)</td>
<td>0.75 ± 0.03</td>
<td>26.2 ± 1.7</td>
<td>21.46 ± 1.58</td>
<td>2.89 ± 0.29</td>
<td>46.3 ± 2.2</td>
<td>5.41 ± 0.67</td>
<td>115.5 ± 7.4</td>
<td>2.47 ± 0.19</td>
<td>0.73 ± 0.01</td>
</tr>
<tr>
<td>Y-ARF (n = 7)</td>
<td>0.30b ± 0.04</td>
<td>18.8b ± 1.6</td>
<td>40.94b ± 5.51</td>
<td>1.59c ± 0.14</td>
<td>43.2 ± 1.2</td>
<td>2.86c ± 0.30</td>
<td>116.2 ± 9.4</td>
<td>2.86 ± 0.55</td>
<td>0.82 ± 0.11</td>
</tr>
<tr>
<td>Y-ARF-ATO (n = 7)</td>
<td>0.33b ± 0.09</td>
<td>19.2b ± 0.9</td>
<td>39.95b ± 9.75</td>
<td>1.73c ± 0.46</td>
<td>42.3 ± 4.1</td>
<td>2.99c ± 0.73</td>
<td>115.4 ± 14.0</td>
<td>2.81 ± 0.49</td>
<td>0.78 ± 0.08</td>
</tr>
<tr>
<td>O-CON (n = 7)</td>
<td>0.80 ± 0.13</td>
<td>25.7 ± 2.5</td>
<td>21.08 ± 4.17</td>
<td>3.10 ± 0.46</td>
<td>45.0 ± 1.9</td>
<td>5.67 ± 0.95</td>
<td>116.4 ± 8.1</td>
<td>2.90 ± 0.26</td>
<td>0.59 ± 0.04</td>
</tr>
<tr>
<td>O-ARF (n = 8)</td>
<td>0.19b ± 0.06</td>
<td>16.3b ± 0.9</td>
<td>64.06b ± 14.99</td>
<td>1.16b ± 0.39</td>
<td>41.0 ± 2.9</td>
<td>1.96b ± 0.7</td>
<td>115.7 ± 7.8</td>
<td>3.90c ± 0.78</td>
<td>0.80b ± 0.09</td>
</tr>
<tr>
<td>O-ARF-ATO (n = 8)</td>
<td>0.36bd ± 0.07</td>
<td>18.8bd ± 1.9</td>
<td>37.05cd ± 6.03</td>
<td>1.91bd ± 0.32</td>
<td>40.1c ± 3.7</td>
<td>3.21bd ± 0.74</td>
<td>116.2 ± 14.8</td>
<td>3.53 ± 0.63</td>
<td>0.71c ± 0.09</td>
</tr>
</tbody>
</table>

BW, body weight (g); GFR/BW, glomerular filtration rate factored for body weight (ml/min/100 g BW); FF, filtration fraction; RPF, renal plasma flow (ml/min); Ht, hematocrit (%); RBF, renal blood flow (ml/min); BP, blood pressure (mmHg); RVR, renal vascular resistances (dyne/s/cm²); KW, kidney weight (g). Data are expressed as means ± SD.

b P < 0.001 versus respective Group CON.
c P < 0.05 versus respective Group CON.
d Significantly different from Group O-ARF (P < 0.05 minimum value).
Acute Postischemic Changes. Nearly 90% of the proximal tubular segments in the outer stripe of the outer medulla resulted normal in both Y and O control rats (89/11006 12% and 91/11006 15%, respectively). Ischemic tubular damage was more pronounced in aging animals because tubular damage in Y rats did not differ significantly from their normal control (75/11006 20% normal, and 25/11006 6.7% sublethally injured, NS versus Y-CON), whereas the ischemic kidneys of the O rats showed a significantly greater number of sublethally injured or necrotic cells (45/11006 20% normal, 45/11006 16.3% sublethally injured, P 0.001 versus group O-CON and all of the groups of Y rats; and 10/11006 2.2% necrotic cells). ATO treatment, in agreement with the hemodynamic results, did not modify the morphologic pattern in Y rats (Y-ARF-ATO: 80/11006 25% normal, and 20/11006 6.4% sublethally injured cells, NS versus other Y rats) but significantly attenuated the ischemic lesions in O animals (O-ARF-ATO, 70/11006 21% normal, and 30/11006 8.2% sublethally injured cells, P 0.05 versus O-ARF) (Figure 2).

Evaluation of NOS Expression and Ras and Rho-A Activity

In some O rats, eNOS and GAPDH mRNA expressions were assessed via a semiquantitative RT-PCR. A representative agarose gel is shown in Figure 3A, and the densitometric results of the experiments are reported in Figure 3B. The expression of eNOS-mRNA, which did not change in rats of group O-ARF compared with controls, was increased in rats pretreated with ATO (O-ARF-ATO, +18.8% versus O-CON, and + 34.0% versus O-ARF). The densitometric value of phosphate dehydrogenase was constant in all of the experimental conditions.

The quantitative analysis of the isoforms of NOS by Western blot test is represented in Figure 4. In rats O-ARF-ATO, the changes in protein level of eNOS paralleled those of their respective mRNA, showing a significant increase compared with control rats (+27%, P < 0.05) and O-ARF rats (+25.5%, P < 0.05) (Figure 4). It is interesting to note that in the latter group, no change in eNOS protein was observed with respect to control. nNOS expression was not modified after the ischemic insult in rats with ARF compared with control (Figure 4).

The expression of iNOS protein level was very low in all of the groups under study; their values, however, were comparable among the groups (n = 4 in each group).

No difference was detected in the activation of Ras proteins in the different groups of aging rats under study (expressed by the amount of protein bound to cellular membrane with respect to that remaining in the cytoplasm; Figure 5, top). In O rats with ARF, in fact, administration of ATO caused no evident change compared with other groups (n = 4). Different results were obtained with Rho-A. In control conditions, as well as after administration of ARF, most of Rho was detected in the active, membrane-bound form (53.5% and 56.3%, respectively). ATO treatment induced a discrete change in O-ATO-ARF rats because the membrane-bound Rho-A was decreased...
to 44.7%, with the consequent increase of the inactive cytosol-bound form to 55.3%, in agreement with a decreased activity of this system (Figure 5, bottom).

Discussion

The results of our study demonstrate that in aging rats, pretreatment with ATO mitigates the course of ischemic ARF by blunting the more pronounced negative response to renal ischemia through a mechanism independent of cholesterol levels. Our data, moreover, suggest that the partial inhibition of Rho, the activation of eNOS, and the higher availability of NO after statin treatment play a major role in this protection, obtained in rats free of age-related nephropathy.

Because old animals are particularly prone to develop ARF in response to even low degrees of renal hypoperfusion (2), we studied a mild model of ARF characterized by the complete survival of aging rats to surgical procedures (3) and by the absence of significant tubular back leakage of filtrate (17). This allowed the accurate determination of both renal function and renal extraction of inulin to calculate the parameters regulating renal dynamics; the handling of para-aminohippurate, in fact, is altered in aging rats (18).

Our data show that the positive modifications in renal function in ATO-treated O rats with ARF are associated with improved renal hemodynamics; the correction of the huge increase in RVR, in fact, caused the increase in RPF and the consequent partial increase of GFR up to the values of Y rats. Our results clearly indicate that the beneficial effects of ATO on aging animals are mediated by the higher production and availability of NO, as witnessed by the higher levels of both eNOS-mRNA and its protein level and the higher excretion of urinary nitrates. It is interesting to note that the significant increase in urinary nitrates in control O rats after ATO (group O-A TO) is associated with no change in renal dynamics. This suggests that in normal conditions the relative deficiency of NO is counterbalanced by local hemodynamic adjustments, and low doses of ATO, even increasing nitrate excretion, do not modify this steady state. Conversely, when the severe vasoconstriction of renal ischemia occurs, aging animals (group O-ARF) cannot recruit enough NO to blunt the increase in RVR, as Y rats do; in these latter animals, in fact, a sharp increase in nitrate excretion is observed (group Y-ARF).

This is a very important point: the strict dependence of eNOS up-regulation on the administration of statins has been
clearly demonstrated in Y rats (6), but no study has shown that ATO increases the availability of NO in aging animals. On the contrary, previous studies have described a decreased eNOS expression in aging (19), failing to demonstrate any change of eNOS in old cells even after stimulation by shear stress (20).

Our in vivo data clearly show that after ischemia, ATO increases both eNOS-mRNA and the level of eNOS without administering an excess of arginine, which stimulates eNOS but mostly activates arginase, thus enhancing interstitial fibrosis and iNOS up-regulation (4).

Because both nNOS and iNOS proteins are not modified in the different experimental conditions, the higher nitrate excretion observed after ATO necessarily derives from eNOS; such an increase is much more pronounced than the increase in eNOS expression, but this is an apparent discrepancy. Although the limited increase in eNOS may be due to the low doses of ATO that we have used, the higher availability of NO could be accounted for by the reduced oxidative stress observed with statins (8). The ischemia-reperfusion injury, in fact, is characterized by a sharp production of ROS at time of reperfusion, which avidly binds to NO, reducing its level (3); any maneuver that reduces ROS production (like statins (8)) determines a greater availability of NO (3).

Although a direct contribution of statins in decreasing ROS through a reduced oxidation of LDL cannot be excluded, other lipid-independent effects are probably more important, like a downregulation of vascular AT₁ receptor, demonstrated by Wassmann et al. (8), or a possible inhibition of the small G-proteins (Ras, Rho), which may regulate ROS production in stressed cells (16). Statins, in fact, may interfere with the activation process of these systems through a lower production of some isoprenoid intermediates of the cholesterol biosynthetic pathway, like farnesyl-pyrophosphate and geranylgeranyl-pyrophosphate (essential for Ras and Rho activation, respectively) (21); the reduced activation of the mitogen-activated protein kinase ERK1/2 by statins after ARF reported by Gueler et al. (5) appears to confirm these findings.

The exact relationship between the use of statins and Ras inhibition, however, is not clear, particularly in aging cells or animals. Weiss et al. (22) have shown that high doses of pravastatin mediate vascular cell apoptosis through a Ras-independent mechanism after a short incubation period. Others have shown that lovastatin can inhibit the process of farnesylation that is necessary for inhibition of Ras (23). It is possible that the concentration of the drugs and the length of the treatment (or exposure to the drug) may affect the different activation rate of the small G-proteins. Our data suggest that low doses of ATO do not influence the activation of Ras. It should be noted, however, that we have used a “pan-Ras” antibody, which does not detect the two isoforms of such protein, namely Ha-Ras and Ki-Ras, which have opposite actions on ROS production (the former enhances ROS production, and the latter stimulates mitochondrial SOD) (24).

Conversely, ATO inhibits the activity of Rho-A compared with both control rats and untreated rats of group O-ARF favoring its translocation in the cytosol (+20.6% versus O-ARF); this difference, although not statistically significant, is not trivial considering the very low dosage of the drug and, mostly, that this was an in vivo study, carried out in conditions quite different from those requested and obtained on cells (9).

Thus, our data confirm previous findings in vitro of Rho inhibition by ATO (26,27). This inhibition is probably the main determinant of the increase in eNOS. The activity of Rho induces peculiar modifications in the cell cytoskeleton (28) able to modify the half-life of eNOSmRNA and influence its protein level; a reduced activity determines an increase in eNOS expression (10), as evidenced in our study.

But this signaling system is also involved in the regulation of

Figure 5. Effects of atorvastatin (ATO) on pan-Ras (top) and Rho-A (bottom) proteins in kidney homogenates of aging rats. (A) Immunoblots (50 µg protein per lane) showing the effects of ATO on cytosolic (C)– and membrane (M)–associated protein. (B) Densitometric analysis of the immunoblot; the white part of the column represents the membrane-bound protein (M), and the gray part represents the cytosol-bound protein. O-CON, untreated rats; O-ARF, rats with acute renal failure; O-ARF-ATO, rats with ARF supplemented with ATO. This blot is representative of four different experiments.
apoptosis. The significant histologic protection observed in aging kidneys of our study after ATO administration but not with administration of arginine (3), suggests that a peculiar remodeling has occurred in old animals treated with ATO—that is, a new balance between cell growth and death or apoptosis (29). A recent article by Hoffman et al. (20) has demonstrated that the sensitivity of endothelial cells toward apoptotic stimuli is augmented in aging cells because of an inappropriate activation of caspases. NO donors determine a better cell preservation through the process of nitrosilation of caspase-3 and its inactivation. The enhanced availability of NO after ATO, because of reduced Rho activation and enhanced eNOS expression, could be another important and novel mechanism of statins in preserving renal histology in old animals, one associated with the better tissue perfusion.

Despite the beneficial effects observed in aging animals, no modification of renal function or of histology was observed in Y rats after a 14-d treatment with ATO, either in normal conditions or after ischemia-reperfusion. This observation is in contrast with the recent study by Gueler et al. (5), who have shown positive effects after a 3-d treatment with cerivastatin (0.5 mg/kg). In addition to a different experimental model (45 min of ischemia and concomitant contralateral nephrectomy), in the study of Gueler et al., cerivastatin was used at a dosage of 0.5 mg/kg, i.e. a dosage pharmacologically 2.5- to 3-fold higher than our 12 mg/kg of ATO, as assessed by clinical and experimental studies (30,31). Our model of ARF was milder and characterized in Y animals by the predominance of functional hemodynamic changes in determining renal dysfunction. Our aim, then, was to use a low dose of drug: a future clinical trial will require the administration of ATO for prolonged periods to old patients, who are more prone to drug toxicity and side effects. Our dose was probably too low to elicit a positive hemodynamic response in Y animals, as suggested by the unchanged excretion of nitrates after ATO.

Most experimental studies on animals and cells have used doses of statins much higher than those potentially requested. Thus, Wassmann et al. (8) have used dosages of 50 mg/kg of ATO for 30 d in spontaneously hypertensive rats, characterized by a deep alteration of the angiotensin-NO axis. These doses are justified by the genetic resistance of rats to developing atherosclerosis (6), and by the attempt to reach serum levels of the drug comparable to those observed in human studies. But statins metabolism in the rat is characterized by the fast clearance of the drug from blood and its prompt entrance into the cells, where it reaches high concentrations that may be toxic in prolonged treatments (6), particularly when high doses are used. In our study, low but sustained doses of statins have exerted beneficial effects in aging animals while avoiding side effects. The potential clinical implications of our results are evident.

In conclusion, our study demonstrates that ATO reduces NO deficiency in aging rats through small but biologically significant effects on eNOS and Rho-A proteins. These beneficial actions of statins in aging animals may also be mediated by changes in lipid oxidation and by decreased ROS production and cell turnover, which are all altered in the elderly. Our data have important clinical implications in treating elderly patients with ischemic ARF and in preventing renal dysfunction due to renal hypoperfusion.

References


