Endothelial dysfunction and hypertension in 5/6 nephrectomized rats are mediated by vascular superoxide

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**Endothelial dysfunction and hypertension in 5/6 nephrectomized rats are mediated by vascular superoxide.**

**Background.** Nitric oxide inactivation by superoxide impairs endothelium-dependent vasodilation and plays a role in various forms of hypertension. Almost no data exist regarding hypertension secondary to chronic renal failure. Previous studies have shown that endothelium-related relaxations, secondary to decreased nitric oxide bioactivity, are impaired in resistance vessels from rats 3 to 10 days after renal mass reduction (RMR).

**Methods.** The membrane-permeable superoxide dismutase (SOD)-mimetic (tempol) was administered IP (1.5 mmol/kg/day for 10 days) to RMR rats and sham-operated controls (SN). Systolic blood pressure (SBP) was measured by tail cuff manometry at days 0, 3, 6 and 10. The increase of flow induced by acetylcholine (10⁻⁶ mol/L) was measured in isolated perfused mesenteric arteries from RMR and SN rats pre-contracted with noradrenaline (1 to 5 µmol/L), with or without exogenous SOD. Plasma levels of advanced oxidative protein products (AOPPs; chloramine-T equivalents) were measured in SN and RMR rats.

**Results.** Tempol prevented the increase of SBP: 118 ± 2.2 mm Hg at baseline and 122 ± 1.6 mm Hg at 10 days in tempol-treated vs 118.14 ± 1.65 mm Hg at baseline and 145 ± 7.69 mm Hg at 10 days in untreated RMR rats (P < 0.01). Responsiveness to acetylcholine was reduced in RMR rats (peak flow increase: 139 ± 7.8% vs. 176 ± 11% in SN, P = 0.028 at 3 days and 140 ± 6.4% vs. 187 ± 16.9% in SN at 10 days, P = 0.007). In arteries pre-incubated with SOD (200 U/mL) the peak flows were 175 ± 9.4% at 3 days and 157 ± 5.8% at 10 days (P = 0.007 and P = 0.051, respectively, vs. control RMR vessels). AOPP values were significantly increased in plasma from RMR rats 3 days after 5/6 nephrectomy (747 ± 107 vs. 481 ± 77 µmol/L, P < 0.05) but returned to normal by day 10. AOPP levels were not significantly reduced by tempol.

**Conclusions.** Increased vascular superoxide production plays a central role in the development of vascular endothelial dysfunction and hypertension early after 5/6 nephrectomy.

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**Key words:** blood pressure, nitric oxide, endothelium, renal mass reduction, SOD, vasodilation, chronic renal failure.

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ing 185 to 400 g) were allowed to acclimatize to their environment for one week. The animals received water and food ad libitum. The rats were anesthetized with pentobarbital (35 mg/kg body weight, IP) and underwent either RMR, (right nephrectomy and ligature of two of the major branches of the left renal artery) or a sham operation (S; consisting of exposure of kidneys and removal of perirenal fat).

**Protocols**

**Effect of tempol on blood pressure and plasma levels of advanced oxidative protein products.** Systolic blood pressure (SBP) was measured by tail cuff manometry (Narco Bio-Systems, Austin, TX, USA) before surgery and on postoperative days 3, 6 and 10. Some of the animals were given tempol (4-hydroxy-TEMPO) 1.5 mmol/kg body weight/day IP [11] beginning immediately after the end of the operation. On the last day of the study, a 24-hour urine collection was obtained in metabolic cages for measurement of creatinine clearance. At the end of the study animals were anesthetized with pentobarbital (50 mg/kg body weight) and blood obtained by cannulation of the abdominal aorta.

**Spectral analysis of advanced oxidative protein products.** Advanced oxidative protein products (AOPPs) were determined in the plasma using the semiautomated method previously described [19]. In brief, acetic acid (100 μL) was added to 100 μL of plasma, diluted 1:10 in phosphate-buffered saline (PBS; final volume 1 mL), or chloramine-T standard solutions (0 to 100 μmol/L). Then, 50 μL of potassium iodide 1.16 mol/L were added, followed by 100 μL of acetic acid. The absorbance of the reaction mixture was immediately read at 340 nm in an UV-260 Shimadzu spectrophotometer (Shimadzu, Japan). Chloramine-T absorbance at 340 nm was linear within the range of 0 to 100 μmol/L. AOPP concentrations were expressed in micromol/liter of chloramine-T equivalents.

**Effect of SOD on the response to acetylcholine in isolated mesenteric arteries.** Mesenteric vessels from SN and RMR rats were obtained 3 and 10 days after surgery and prepared by a modification of a previously described method [20]. In brief, the superior mesenteric artery was cannulated and perfused at a rate of 1.5 mL/min. Several fourth order arterioles were excised at the intestinal border and their proximal ends lightly cleaned of surrounding fat. The proximal ends of two or three arteries (external diameters ~200 μm) were cannulated with glass micropipettes, using a micromanipulator and a stereomicroscope. They were then mounted into separate chambers, each of which was connected to a syringe pump (GF-SP4/IPX-D; Foures S.A., France). The vessels were perfused at a rate of 300 μL/min with a buffer solution comprised of (in mmol/L): NaCl 130, KCl 4.7, MgSO4 1.15, NaHCO3 15, Na2HPO4 1.15, KH2PO4 1.25, CaCl2 1.25, glucose 5, pH 7.4, and paO2 200 mm Hg, at 37°C. In some experiments, 200 U/mL SOD were added to the buffer. The inflow line was connected via a lateral line to a 40 mm Hg column, allowing the perfusate to overflow when vessel contraction increased resistance and intravascular pressure exceeded that of the column. The perfusate was collected and its volume was measured gravimetrically. After allowing 60 minutes for equilibration, basal outflow was measured and vessels were constricted by means of noradrenaline (NOR; 1 to 5 μmol/L) until the flow rate was approximately 60% of basal. Outflow was measured after stabilization of contraction and NOR administration was continued throughout the experiment at the same concentration. Acetylcholine (ACh; 10^-6 mol/L) was infused for 28 minutes, while the outflowing perfusate was collected for a two minute period every seven minutes. Outflow rates were expressed relative to the value measured for each artery before ACh. The results of several arteries obtained from each rat were pooled and average values used for statistical analysis.

**Drugs**

Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl) and SOD (bovine erythrocyte No. 2515) were from Sigma Chemical Co. (St. Louis, MO, USA). Drug solutions were freshly prepared immediately prior to use.

**Statistics**

Results are expressed as mean ± SEM. Inter-group differences were analyzed by the Kruskall-Wallis analysis of variance and the Mann-Whitney U test.

**RESULTS**

Blood pressure was already slightly elevated in RMR rats at day 3 after surgery and increased further at days 6 and 10. By contrast, SBP did not change in RMR rats receiving tempol. Tempol had no effect on blood pressure of Sham-operated rats (Fig. 1).

Serum creatinine was elevated and creatinine clearance reduced in all RMR animals. There were no statistically significant differences between RMR groups. Plasma AOPP values were elevated in RMR rats at day 3 but returned to normal by day 10. Tempol-treated animals tended to have lower levels, but the differences were not statistically significant (Table 1). There was no correlation between the values of serum creatinine, creatinine clearance and AOPP levels.

Figure 2 shows the effect of exogenous SOD on acetylcholine response. This was blunted in isolated mesenteric arteries of RMR rats as compared with SN, both at day 3 and 10 (peak flow increase: 139 ± 7.8% vs. 176 ± 11% in SN, P = 0.028 at 3 days and 140 ± 6.4% vs. 187 ± 16.9% in SN at 10 days, P = 0.007). Preincubation with exogenous SOD completely restored the response to
ACh in vessels from RMR rats 3 days after surgery (peak flow 175 ± 9.4%, P = 0.007 vs. control RMR vessels). The response to ACh in vessels from RMR rats at day 10 also was significantly augmented by SOD (peak flow: 157 ± 5.8%, P = 0.051 vs. control RMR vessels), but remained lower than observed in arteries from Sham-operated animals.

**DISCUSSION**

The pathogenesis of hypertension, which is a common complication in renal insufficiency, is not well understood [1]. Different mechanisms have been proposed including reduced NO bioactivity leading to endothelial dysfunction [21], as demonstrated by experimental [22, 23] and human [24, 25] studies. However, these investigations often were performed in the presence of established hypertension. Because hypertension itself alters vascular morphology and behavior [26], the abnormal findings reported may reflect a secondary phenomenon. In a previous study performed in rats 3 and 10 days after 5/6 nephrectomy, at a stage characterized by normal or mildly elevated blood pressure, we observed a reduced vasodilator response to acetylcholine in vessels from RMR rats. In contrast with the normal behavior of vessels from Sham-operated animals, the abnormal endothelium-related relaxation in the RMR group was not significantly affected by L-NAME, suggesting decreased NO bioactivity (abstract; Benche- et al, *Proc XXXVII Congr EDTA-ERA*, p 84, 2000). Because superoxide levels in vascular tissue are impor-

Fig. 1. Effect of tempol (1.5 mmol/kg/day IP) on systolic blood pressure in renal mass reduction (RMR) and Sham operated rats. Values are mean ± SEM; *P < 0.05 comparing untreated RMR rats with the other groups. Symbols are: (○) normal Sham (SN; N = 13), (●) RMR (N = 14); (□) SN + tempol (N = 5), (■) RMR + tempol (N = 10).

tant determinants of NO bioavailability and endothelial function [2], the present studies were aimed at assessing the possible role of vascular superoxide production in RMR.

Recent evidence supports the concept that O$_2^-$ plays a role in various forms of hypertension. O$_2^-$ is increased in venules and arterioles of SHR [7] and in different tissues from stroke prone SHR [8] and Dahl hypertensive rats [9]. SOD activity is lower in the blood of ISIAH (stress-sensitive) rats compared with that of Wistar rats [10]. The functional importance of reactive oxygen species (ROS) in leading to hypertension has been suggested by the fact that tempol [11] or heparin-binding SOD, which localizes within the vessel wall [12], normalizes blood pressure in SHR. Similar findings have been demonstrated in deoxycorticosterone acetate (DOCA)-salt hypertension [13] and in renovascular hypertensive rats [14]. In the model of chronic infusion of angiotensin II in rats, the nicotinamide adenine dinucleotide phosphate [NAD(P)H] subunit p22phox mRNA is up-regulated and NAD(P)H oxidase-derived O$_2^-$ increases. Both hypertension and the increase in p22phox mRNA are prevented by pretreatment with SOD [27]. Evidence also exists for a role of O$_2^-$ in human essential hypertension: erythrocyte SOD activity is reduced [3] and antioxidant therapy improves endothelial function [6].

Very little is known about the role played by superoxide in the endothelial dysfunction and hypertension of CRF. Oxidative stress is considered an important cause of cardiovascular disease, the major factor of morbidity and mortality in CRF patients [15]. There is evidence that oxidative stress occurs early during the evolution of CRF. Parameters of oxidative stress and antioxidant enzyme activities are altered in patients with various degrees of CRF [15–17]. The activity of antioxidant enzymes decreases in the renal cortex of rats after RMR [28, 29], and the associated increase in oxidative stress seems to play a role in the development of renal fibrosis, since antioxidant treatments (vitamin E [30] or magnesium lithosper- mate B [28]) may hinder its progression. Only one study so far, however, has addressed the possible role of reactive oxygen species in the hypertension of chronic renal failure (CRF) [18].

To assess the presence of increased free oxygen radicals in RMR rats, we measured plasma levels of oxidized proteins. Such changes have been demonstrated recently in uremic patients and are considered to be not only a marker of oxidative stress, but also a potential mediator of inflammation [19, 31]. We found that plasma AOPP levels are increased by 3 days in RMR rats, and return to normal values by day 10. Thus, this acute increase of AOPP may be related to the acute renal infarctions caused by the procedure and/or the acute decrease of renal function. Of note, tempol, at the dosage preventing the increase of blood pressure [11] caused only a minor,
Table 1. Creatinine clearance (C\textsubscript{Cr}) and plasma levels of advanced oxidative protein products (AOPP)

<table>
<thead>
<tr>
<th></th>
<th>SN Control</th>
<th>SN Tempol</th>
<th>NX Control</th>
<th>NX Tempol</th>
<th>Day 3</th>
<th>Day 10</th>
</tr>
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<tbody>
<tr>
<td>N</td>
<td>9</td>
<td>5</td>
<td>13</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C\textsubscript{Cr} \text{mL/min/100 g body wt}</td>
<td>0.44 ± 0.03</td>
<td>0.57 ± 0.04</td>
<td>0.23 ± 0.04\textsuperscript{a}</td>
<td>0.23 ± 0.02\textsuperscript{a}</td>
<td>0.42 ± 0.02</td>
<td>0.39 ± 0.04</td>
</tr>
<tr>
<td>Plasma AOPP \text{pmol/L}</td>
<td>481 ± 77</td>
<td>483 ± 24</td>
<td>747 ± 107\textsuperscript{a}</td>
<td>630 ± 39</td>
<td>455 ± 111</td>
<td>397 ± 28</td>
</tr>
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\textsuperscript{a} P < 0.05 vs. the respective SN group

Fig. 2. Effect of bovine erythrocyte superoxide dismutase (SOD; 200 U/mL) on the vasodilator response to acetylcholine (ACh) in noradrenaline-contracted isolated mesenteric arteries at day 3 (A) and 10 (B). * P < 0.05, comparing SN and RMR+SOD vessels with those of RMR. Symbols are: (○) SN (day 3, N = 8; day 10, N = 7); (●) RMR (day 3 N = 8, day 10, N = 7); (■) RMR+SOD (day 3, N = 8; day 10, N = 5).

not significant, reduction of AOPP. This argues in favor of a specific effect at the level of the vasculature, where the extracellular SOD activity is considered of paramount importance in the balance between O\textsubscript{2}\textsuperscript{-} and NO [32]. In addition, preincubation with exogenous SOD significantly increased the acetylcholine-induced relaxation in mesenteric vessels. This effect also has been observed in isolated vessels from rats with endothelial dysfunction caused by angiotensin II [33] and argues in favor of an extracellular site of action, as Cu/Zn SOD is unable to pass cell membranes.

Taken together, our results suggest that superoxide production is increased in the vascular wall in the early days after RMR, and is an important pathogenetic factor in the development of high blood pressure and endothelial dysfunction in resistance vessels. The possible mechanisms leading to increased vascular O\textsubscript{2}\textsuperscript{-} include increased production by enzymes such as NAD(P)H oxidase, xanthine-oxidase or uncoupled endothelial nitric oxide synthase, or lower superoxide dismutase activity. Finally, because our study was specifically performed early after nephrectomy, the results do not necessarily explain the mechanisms that contribute to the maintenance of chronic long-term hypertension in this model. However, Vaziri et al have observed that high blood pressure was partially controlled by lazaroid (an O\textsubscript{2}\textsuperscript{-} scavenger) in hypertensive rats, two to six weeks after RMR [18].

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