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Acute antihypertensive action of nitroxides in the spontaneously hypertensive rat

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1Division of Nephrology and Hypertension, Georgetown University, Washington, DC; 2Anesthesiology Research Division, University of Alabama, Birmingham, Alabama; and 3Radiation Oncology Branch, Clinical Oncology Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Maryland

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Patel, Kinjal, Yifan Chen, Kathryn Dennehy, Jonathan Blau, Stephanie Connors, Margarida Mendonca, Margaret Tarpey, Murali Krishna, James B. Mitchell, William J. Welch, and Christopher S. Wilcox. Acute antihypertensive action of nitroxides in the spontaneously hypertensive rat. Am J Physiol Regul Integr Comp Physiol 290: R37–R43, 2006. First published September 22, 2005; doi:10.1152/ajpregu.00469.2005.—Tempol is an amphipathic radical nitroxide (N) that acutely reduces blood pressure (BP) and heart rate (HR) in the spontaneously hypertensive rat (SHR). We investigated the hypothesis that the response to nitroxides is determined by SOD mimetic activity or lipophilicity. Groups (n = 6–10) of anesthetized SHRs received graded intravenous doses of Ns: tempol (T), 4-amino-tempol (AT), 4-oxo-tempo (OT), 4-trimethylammonium-2,2,6,6-tetramethylpiperidine-1-oxyl iodide (CAT-1), 3-carbamoyl-proxyl (3-CP), or 3-carboxyproxyl (3-CTPY). Others received native or liposomal (L) Cu/Zn SOD. T and AT are unchanged, AT is positively charged and cell-permeable, and CAT-1 is positively charged and cell-impermeable. 3-CP and 3-CTPY have five-member pyrrolidine rings, whereas T, AT, OT, and CAT-1 have six-member piperidine rings. T and AT reduced mean arterial pressure (MAP) similarly (P > 0.05) than OT and CAT-1. 3-CP and 3-CTPY were ineffective. The group mean change in MAP with piperidine Ns correlated with SOD activity (r = −0.94), whereas their ED50 correlated with lipophilicity (r = 0.89). SOD and L-SOD did not lower BP acutely but reduced it after 90 min (−32 ± 5 and −31 ± 6 mmHg; P < 0.05 vs. vehicle). Pyrrolidine nitroxides are ineffective antihypertensive agents. The antihypertensive response to piperidine Ns is predicted by SOD mimetic action, and the sensitivity of response is by hydrophilicity. SOD exerts a delayed hypotensive action that is not enhanced by liposome encapsulation, suggesting it must diffuse to an extravascular site.

tempol; superoxide dismutase; blood pressure; nitric oxide; hypertension.

OXIDATIVE STRESS IMPLIES THAT reactive oxygen species, including superoxide anion (O2−·), are produced in excess of their metabolism (8). Defense is provided primarily by SOD that metabolizes O2−· to H2O2 and by catalase and glutathione peroxidase that metabolize H2O2 to water and oxygen. O2−· enhances the contractility of blood vessels during stimulation with agonists, such as ANG II (16, 31, 35, 36). O2−· may cause hypertension by many mechanisms (35), including bioinactivation of nitric oxide (NO) (27), by central actions (39), by enhancing the peripheral sympathetic nervous system (37, 38), or by enhancing renal tubular NaCl reabsorption (17, 22, 23).

Oxidative stress accompanies hypertension in many models of hypertension, including the spontaneously hypertensive rat (SHR) (25). Mitchell, Krishna, and colleagues (15, 18, 24) have shown that tempol (T) is a permeant amphipathic radical nitroxide (N) that detoxifies oxygen metabolites by redox cycling through one-electron transfer reactions. The nitroxide/oxygenonation cation pair form an efficient redox coupling that mimics the enzymatic action of SOD and confers catalase-like action to heme proteins (14, 15). Although T lowers blood pressure (BP) in many animal models of hypertension accompanied by oxidative stress, including the SHR (9, 21, 26, 27, 29, 36–38), the mechanisms of its in vivo action are not clearly established.

Fink and colleagues (38) have shown that T given to deoxycorticosterone acetate-salt rats reduces BP before it has dissipated O2−· in the aorta. This acute antihypertensive response is accompanied by reduced renal sympathetic nervous system activity. It is unclear whether these neural actions of T depend on SOD mimetic action. Nevertheless, intravenous injection of liposomal (L), polyethylene-glycol, or heparin-bonded SOD lowers BP in SHR (20) or ANG-II-infused hypertensive rats (19) or restores ACh-induced relaxation in blood vessels from atherosclerotic rabbits (34).

We investigated the hypothesis that the acute antihypertensive response to radical Ns is determined by their chemical class, SOD mimetic activity, or lipophilicity. These studies were conducted in anesthetized SHR because this model has a robust acute antihypertensive response to T (27). The acute in vivo dose-response relationships for a family of radical Ns were related to in vitro measurements of SOD mimetic activity and lipid solubility and were compared with native and L-Cu/Zn SOD (16).

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MATERIALS AND METHODS

In vivo studies. Experiments were approved by the Georgetown University Animal Care and Use Committee. Male SHR weighing 250–350 g (Taconic, Germantown, NY) were anesthetized with thiobutabarbital (Inactin; Sigma, St. Louis, MO) (10 mg/100 g) after halothane (Halocarbon Laboratories, River Edge, NJ) induction and prepared as described previously (27). Rats received 0.9% NaCl at 2 ml/h iv up to the time of receiving drugs to maintain euvolemia. A femoral artery was cannulated with polyethylene (PE)-50 tubing connected to a digital blood pressure analyzer (Micro-Med, Louisville, KY). Animals were equilibrated for 45 min. Thereafter, a nitroxide (17 mol/kg) was infused intravenously over 10 s. The mean arterial pressure (MAP) and heart rate (HR) were recorded over the first 5 min and at 10 and 15 min. This was followed by doses of 54, 72, 174, and 270 mol/kg under similar conditions. The same protocol was followed with each nitroxide: T, 4-oxo-tempo (OT), 4-amino-tempo (AT), 3-carboxy-peroxyl (3-CTPY) (Aldrich, Milwaukee, WI), 3-carbomyl-proxyl (3-CP) (Sigma), and 4-trimethylammonium-2,2,6,6-tetramethylpiperidine-1-oxyl iodide (CAT-1) (Molecular Probes, Eugene, OR) (Fig. 1). Doses of bovine Cu/Zn SOD (MW 32,500; Oxis Research, Portland, OR) were selected that had equivalent SOD mimetic activity in vitro to the doses of T used for intravenous studies: 34, 110, 140, 260, 350, and 540 U/kg. SOD injection followed a similar protocol to the nitroxides and was compared with vehicle-injected rats. Additional rats received injections of previously boiled SOD as a further control group. Six rats were studied in each group.

In vitro studies. The SOD activities of Ns were evaluated in vitro for their efficacy in dampening O2·− generated by xanthine (25 mol/l) plus xanthine oxidase (9 IU/ml), as described (13, 32). Their lipid solubility was assessed by shaking the Ns in a 50:50 mixture of PBS (Ambion, Austin, TX) and chloroform (CHCl3; EM Science, Gibbstown, NJ), taking an aliquot of the PBS phase, evaporating the CHCl3 phase to dryness, reconstituting it in PBS, and assessing the SOD activities in the two solutions. O2·− was assessed by chemiluminescence with 5 μM lucigenin (AutoLumat Plus LB 953; EG&G, Berthold, Germany). The reduction in the stable peak value for chemiluminescence for each N, relative to vehicle, defined the SOD mimetic activity.

Statistics. The mean ± SE changes in MAP and HR were calculated from the individual dose-response relations. The responsiveness was the maximum effect, and the sensitivity was the expected dose for ED50. Data were analyzed by ANOVA with post hoc testing by the Dunnett test, where appropriate. Statistical significance was taken as P < 0.05.

Table 1. Basal data for body weight, mean arterial pressure, and heart rate

<table>
<thead>
<tr>
<th>Compound</th>
<th>Number of Rats</th>
<th>Body Weight, g</th>
<th>MAP, mmHg</th>
<th>HR, min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>6</td>
<td>268 ± 6</td>
<td>159 ± 6</td>
<td>369 ± 10</td>
</tr>
<tr>
<td>AT</td>
<td>6</td>
<td>279 ± 19</td>
<td>167 ± 7</td>
<td>363 ± 15</td>
</tr>
<tr>
<td>OT</td>
<td>6</td>
<td>241 ± 17</td>
<td>156 ± 5</td>
<td>351 ± 14</td>
</tr>
<tr>
<td>CAT-1</td>
<td>6</td>
<td>251 ± 20</td>
<td>153 ± 11</td>
<td>357 ± 14</td>
</tr>
<tr>
<td>3-CP</td>
<td>6</td>
<td>256 ± 7</td>
<td>157 ± 3</td>
<td>382 ± 9</td>
</tr>
<tr>
<td>SOD</td>
<td>6</td>
<td>275 ± 13</td>
<td>164 ± 9</td>
<td>357 ± 14</td>
</tr>
<tr>
<td>L-SOD</td>
<td>6</td>
<td>272 ± 12</td>
<td>166 ± 7</td>
<td>380 ± 9</td>
</tr>
<tr>
<td>Liposomes alone</td>
<td>6</td>
<td>270 ± 10</td>
<td>156 ± 4</td>
<td>368 ± 7</td>
</tr>
<tr>
<td>Boiled SOD</td>
<td>6</td>
<td>276 ± 10</td>
<td>172 ± 9</td>
<td>384 ± 10</td>
</tr>
<tr>
<td>Vehicle</td>
<td>18</td>
<td>265 ± 7</td>
<td>155 ± 5</td>
<td>368 ± 4</td>
</tr>
</tbody>
</table>

Values are presented as means ± SE. Data were obtained under anesthesia before drug administration. T, tempol; AT, 4-amino-tempo; OT, 4-oxo-tempo CAT-1, 4-trimethylammonium-2,2,6,6-tetramethylpiperidine-1-oxyl iodide; 3-CP, 3-carbomyl-proxyl; L-SOD, liposomal-SOD.

Fig. 1. Chemical formulas of the nitroxides used.
RESULTS

The body weight and baseline values for MAP and HR did not differ significantly between groups (Table 1). Figure 2 displays the time course of MAP and HR during graded intravenous T. The maximum reductions in MAP and HR were apparent within 1 min. This was followed, at lower doses, by a return to baseline over 15 min or an incomplete return at higher doses. AT, OT, and CAT-1 also caused graded reductions in MAP and HR, whereas 3-CP, 3-CTPY, and vehicle were ineffective. The dose-response relationships for T and CAT-1 relative to vehicle are shown in Fig. 3. There was a greater maximal fall in MAP with T than CAT-1.

Figure 4 depicts the time course of changes in MAP during graded intravenous doses of native Cu/Zn SOD. Unlike T, there was no acute effect with SOD. However, the MAP became lower in SHR given Cu/Zn SOD than rats given vehicle, but this was delayed 90–110 min. The response to liposomal Cu/Zn SOD was strictly comparable to native Cu/Zn SOD (Fig. 5) and followed a similar time course. BP responses to previously boiled SOD did not differ from vehicle.

Table 2 summarizes the maximum changes (potency) and the ED50 (inverse of sensitivity) of the agents tested. The piperidines, T, AT, OT, and CAT-1 decreased MAP and HR acutely, whereas the pyrrolidines, 3-CP and 3-CTPY, and SOD and L-SOD did not. T and AT caused the greatest reductions in MAP, whereas OT and CAT-1 caused significantly more modest reductions. T, AT, and CAT-1 caused similar reductions in HR, whereas the reduction with OT was smaller. In contrast, the charged cationic CAT-1 had the lowest ED50 (greatest sensitivity) for changes in both MAP and HR, whereas the basic, lipophilic AT and OT had the highest ED50s.

Fig. 2. Means ± SE values for mean arterial pressure (MAP) (A) or heart rate (B) of spontaneously hypertensive rats (SHR) given graded intravenous doses of Tempol (○ with dashed lines; n = 6) or vehicle (● with continuous lines; n = 6). BPM, beats/min. Compared with vehicle: *P < 0.05; **P < 0.01; ***P < 0.005.
The in vitro assessment of SOD activity by the nitroxides is shown in Fig. 6 and Table 3. When tested in PBS (Fig. 6A), T and AT at 10^{-4} M extinguished 92 and 88%, respectively, of O_{2}^{*•} generated by a xanthine-xanthine oxidase reaction, whereas 3-CP, OT, CAT-1, and 3-CTPY extinguished a significantly lower fraction. The ratio of the activity that partitioned into CHCl_{3} compared with PBS is shown in Fig. 6B. OT demonstrated the greatest partition ratio, indicating strong lipophilicity. CAT-1 and 3-CP demonstrated ratios < 1, indicating strong hydrophilicity.

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DISCUSSION

These findings confirm that T reduces BP and HR in the SHR (28). The main new findings are that the acute group mean antihypertensive response to piperidine nitroxides is predicted by their SOD mimetic activity, whereas the group mean ED_{50} is predicted by their lipophilicity. Pyrrolidine nitroxides do not reduce MAP, despite possessing in vitro SOD activity. Neither native nor liposomal SOD reduces MAP acutely but both cause similar falls in MAP, albeit less than T, that are delayed 90–110 min.

Highly polar compounds that do not penetrate cells have a relatively restricted peak volume of distribution, leading to higher initial plasma levels. This can explain the dependence of the antihypertensive sensitivity of the piperidine nitroxides on their hydrophilicity (Fig. 8).

Both five-member ring pyrrolidine nitroxides and six-member ring piperidine nitroxides acted as SOD mimetics in our in vitro assay. Although 3-CP is a very effective SOD mimetic in vitro, we confirm that it does not reduce BP in vivo (6, 7). The data in this study are consistent with redox chemistry of nitroxides. Electron paramagnetic resonance spectrometry, cyclic voltammetry, and bulk electrolysis has been used to characterize and quantitate the redox midpoint potential of nitroxides. Among the six-member ring nitroxides, their antihypertensive effect in this study follows their redox midpoint potentials (E_{1/2}) measured previously (14, 15). Thus the lower E_{1/2} of the six-member ring nitroxide, the better its SOD
mimetic capability and the more effective it was in reducing the BP. For example, T and 4-aminotempol have E_{1/2} values of 800 and 820 mV, respectively, whereas oxotempos has a higher E_{1/2} of 913 mV, and CAT-1, although not estimated in the prior published studies (14, 15), has significantly higher values still (Krishna, KC, personal communication, 2005). Thus the E_{1/2} values follow the rank order of antihypertensive activities of the four components tested (Table 1). It is established that six-member ring nitroxides participate in redox reactions more effectively and rapidly than five-member rings because their reversible conformational transformation between “boat” and “chair” structures facilitates access to reactants, making them kinetically more effective, in addition to thermodynamic considerations. It is during interconversion between “boat” and “chair” configuration that the NO’ site on the six-member nitroxide is exposed for catalysis. In contrast, five-member rings are always planar and thus less reactive (14, 15).

![Graphs A and B](image)

**Table 2. Changes in MAP and HR with intravenous nitroxides and SOD**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Maximum Response, mmHg</th>
<th>ED_{50}, μmol/kg</th>
<th>Maximum Response, beats/min</th>
<th>ED_{50}, μmol/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>-48 ± 2</td>
<td>70 ± 8^a</td>
<td>-42 ± 3</td>
<td>63 ± 9</td>
</tr>
<tr>
<td>AT</td>
<td>-55 ± 8</td>
<td>114 ± 8^a</td>
<td>-44 ± 8</td>
<td>80 ± 6^a</td>
</tr>
<tr>
<td>OT</td>
<td>-34 ± 4^b</td>
<td>112 ± 12^a^c</td>
<td>-28 ± 3^b</td>
<td>81 ± 6^c</td>
</tr>
<tr>
<td>CAT-1</td>
<td>-28 ± 4^c</td>
<td>48 ± 5^c</td>
<td>-39 ± 8</td>
<td>48 ± 11</td>
</tr>
<tr>
<td>3-CP</td>
<td>-3 ± 2^c</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>3-CTPY</td>
<td>-2 ± 3^c</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>SOD</td>
<td>-2 ± 10^d</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>L-SOD</td>
<td>-2 ± 9^e</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Boiled SOD</td>
<td>4 ± 9^f</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Vehicle</td>
<td>2 ± 1^g</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

Values are presented as means ± SE. NT, not tested. Compared to T: *P < 0.05; **P < 0.01; ***P < 0.005. ED_{50} data compared to CAT-1: *P < 0.05; **P < 0.005.

The antihypertensive response to the piperidine nitroxides was independent of their lipophilicity. CAT-1 is a highly polar tetramethylammonium compound that is as effective (relative to SOD activity) in lowering MAP in vivo as the highly lipophilic compounds, 4-oxo-tempo or T. Studies in mice with gene deletions of Cu/Zn or extracellular (EC)-SOD or given SOD inhibitors have concluded that vascular NO is protected by SOD from bioinactivation by O_2•, both intracellularly (1, 30) and extracellularly (5, 11). Diffusional cellular uptake depends on lipophilicity. Therefore, the finding that the acute antihypertensive response to nitroxides is independent of lipophilicity suggests that the initial effects are extracellular. Indeed, CAT-1 was effective in reducing BP acutely. CAT-1 does not permeate cells but can react with cell membrane components (3, 4).

Therefore, we investigated the hypothesis that the acute antihypertensive response to SOD does not require cellular uptake by the use of native and liposome-encapsulated Cu/Zn SOD. Neither had any acute antihypertensive action caused a similar, moderate, and delayed reduction in BP over 110 min. Our findings are consistent with previous reports. Recombinant EC-SOD injected into wild-type mice infused with ANG II has no immediate effect, although there is a fall in BP in EC-SOD knockout mice (12). Liposomal Cu/Zn SOD injected intravenously over 5 days into cholesterol-fed rabbits with atherosclerosis is taken up in both endothelial and vascular smooth muscle cells, where it increases SOD activity and partially restores endothelium-dependent relaxation to ACh (34). Liposomal Cu/Zn SOD given over 5 days reduces the MAP and improves ACh-induced vascular relaxations in rats infused with ANG II, but not with norepinephrine (16). Heparin-bonded SOD binds to endothelial cells, penetrates extravascularly, and causes a delayed lowering of MAP after intravenous injection, whereas native SOD is excluded and does not acutely reduce the MAP (20). Injection of polyethylene-glycolated SOD for 1 wk into cholesterol-fed, atherosclerotic rabbits increases blood vessel SOD activity and partially restores...
endothelium-dependent relaxation to ACh (19). Because liposomal SOD is taken up into endothelial cells (34), the similar MAP responses to native and liposomal Cu/Zn SOD in the present study suggest that the antihypertensive action of SOD is not exerted in the vascular endothelium. The slower response to SOD may relate to its larger molecular size compared with nitroxides. This suggests that SOD must diffuse from the vascular space into the interstitium to lower BP. Native SOD has a plasma half time of 6 min after intravenous administration, with 10% of injected SOD being associated with the kidney 45 min after injection (10).

Perspectives

Piperidine nitroxides exert an acute combination of a rapid, substantial, and reversible reduction in MAP, accompanied by bradycardia. These characteristics could make piperidine nitroxides ideal agents for the treatment of hypertensive crises. The reduction in HR and sympathetic nerve tone (38) after intravenous nitroxides could give these compounds an advantage over sodium nitroprusside, which causes reflex tachycardia and cardiac stimulation (1). The finding that the effectiveness of nitroxides is predicted by their SOD mimetic activity provides a rational basis for selection of T or AT for this indication. T is also effective as an oral antihypertensive antioxidant agent in the SHR model (33).

GRANTS

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REFERENCES


Fig. 7. Group mean ± SE data relating maximal fall in MAP by piperidine nitroxides in vivo to SOD mimetic activity in vitro. ○, T; ■, 4-amino-tempo; □, 4-oxo-tempo; A, CAT-1. There was a significant correlation (r = −0.94; P < 0.02).
Log ratio of SOD activity in CHCl₃: PBS in vitro

Fig. 8. Group means ± SE data relating ED₅₀ for changes in MAP after intravenous administration of piperidine nitroxides in vivo to the log of the partition coefficient of SOD activity into chloroform relative to saline in vitro. ○, tempol; □, 4-oxo-tempol; ●, CAT-1. There was a significant correlation (r = 0.89; P < 0.05).