Nitric oxide-independent effects of tempol on sympathetic nerve activity and blood pressure in normotensive rats

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Xu, Hui, Gregory D. Fink, Alex Chen, Stephanie Watts, and James J. Galligan. Nitric oxide-independent effects of tempol on sympathetic nerve activity and blood pressure in normotensive rats. Am J Physiol Heart Circ Physiol 281: H975–H980, 2001.—The role of the sympathetic nervous system in 4-hydroxy-2,2,6,6-tetramethyl piperidinoxyl (tempol)-induced cardiovascular responses in urethane-anesthetized, normotensive rats was evaluated. Tempol caused dose-dependent (30–300 μmol/kg iv) decreases in renal sympathetic nerve activity (RSNA), mean arterial blood pressure (MAP), and heart rate (HR). Similar responses were obtained after sinoaortic denervation and cervical vagotomy. These responses were not blocked following treatment with the nitric oxide synthase inhibitor N^G-nitro-L-arginine (2.6 mg·kg^-1·min^-1 iv for 5 min) or the α_2-adrenergic receptor antagonist idazoxan (0.3 mg/kg iv bolus). Idazoxan blocked the effects of clonidine (10 μg/kg iv) on HR, MAP, and RSNA. Hexamethonium (30 mg/kg iv) inhibited RSNA, and tempol did not decrease RSNA after hexamethonium. The effects of tempol on HR and MAP were reduced by hexamethonium. In conclusion, depressor responses caused by tempol are mediated, partly, by sympathoinhibition in urethane-anesthetized, normotensive rats. Nitric oxide does not contribute to this response, and the sympathoinhibitory effect of tempol is not mediated via α_2-adrenergic receptors. Finally, tempol directly decreases HR, which may contribute to the MAP decrease.

sympathetic nervous system; N^G-nitro-L-arginine

TEMPOL, a superoxide dismutase (SOD) mimic, has been used to reduce superoxide-related tissue injury in ischemia-reperfusion protocols (4) and in inflammation assays (7). Tempol also lowers blood pressure in spontaneously hypertensive, deoxycorticosterone acetate salt, and angiotensin II-treated hypertensive rats (1, 11, 13, 14). Schnackenberg and colleagues (13) also found that tempol reduces blood pressure in normotensive rats, but they did not show that this effect was NOS dependent. Furthermore, these authors did not study changes in heart rate (HR) during the blood pressure decrease. This measurement would have been important because tempol can directly decrease HR in isolated heart preparations (4).

SOD injected into the rostral ventrolateral medulla inhibits renal sympathetic nerve activity (RSNA) and decreases blood pressure and HR, but the depressor response could not be completely accounted for by an interaction with NO (23). Therefore, all of the mechanisms mediating tempol-induced hemodynamic changes have not been identified. The present study evaluated the role of the sympathetic nervous system and NO availability in tempol-induced cardiovascular responses in urethane-anesthetized normotensive rats. These studies examined for the first time the effects of tempol on sympathetic nerve activity. Because SOD (23) and the α_2-adrenergic receptor agonist clonidine (16) can lower blood pressure and inhibit sympathetic nerve activity via a central mechanism, the role of α_2-adrenergic receptors in mediating the effects of tempol was also investigated. RSNA was used as a measure of sympathetic nerve activity.

METHODS

Surgical Procedures

Animal use protocols were approved by the All University Committee for Animal Use and Care at Michigan State University. Male Sprague-Dawley rats (n = 20, 300–350 g; Charles River; Portage, MI) were used. Rats were anesthetized with urethane (1.5 g/kg ip). Body temperature was maintained at 36–37°C by a heating pad. After tracheostomy, respiration was maintained by positive pressure ven-
tilation with room air (60 cycles/min, 3 ml cycle volume). Animals were paralyzed (4 mg·kg⁻¹·h⁻¹ iv gallamine triethiodide) during periods of data collection to maintain stable recording conditions. The depth of anesthesia was monitored continuously and supplement doses of urethane (25–50 mg iv) were given as required. Depth of anesthesia was assessed as stability of HR, blood pressure, and respiratory movement and pupil size and paw-pinch reflexes. Before periods of paralysis, anesthesia was assessed as described and during periods of paralysis by monitoring HR, blood pressure, and RSNA.

A polyethylene catheter was placed into a femoral artery and two femoral veins for measurement of blood pressure and for administration of fluids and drugs. A left flank incision was made and a retroperitoneal dissection was used to expose the renal artery and nerves. Renal sympathetic nerves were identified, and a branch was dissected free of connective tissue and placed on a bipolar stainless steel electrode. When stable recording conditions were established, the renal nerve and electrode were covered with silicone rubber, and the rats were placed in the right lateral decubitus position (20–22).

Sinoaortic denervation and cervical vagotomy (SADV) were accomplished by cutting the cervical sympathetic trunks, the aortic depressor nerve, the superior laryngeal nerve, and vagal nerves. Carotid baroreceptors were denervated by cutting the carotid sinus nerve and stripping the area of the carotid sinus (12). After denervation, blood pressure was elevated for 30–40 min, but hemodynamics and RSNA gradually returned to predenervation levels. Completeness of SADV was confirmed by the absence of HR and RSNA responses to sodium nitroprusside (SNP)-induced decreases and phenylephrine-induced increases in blood pressure.

Data Acquisition

The arterial catheter was connected to a Statham pressure transducer (P23D; Oxnard, CA) to measure arterial blood pressure. An electronic resistance-capacitance filter with a 0.5-s time constant was used to derive mean arterial pressure (MAP). HR was determined electronically from the blood pressure signal using a cardiacotachograph (model 7P4FG, Grass Instruments; Quincy, MA). RSNA was amplified (PS11, Grass Instruments) with the use of a band-pass filter (low pass, 100 Hz; high pass, 1,000 Hz). The amplified and filtered signal was displayed with a digital oscilloscope (model 1425, Gould Instruments; Cleveland, OH) and monitored by an audiospeaker (Grass Instruments). Raw nerve activity was full-wave rectified and integrated using a polygraph integrator (model 7P10F, Grass Instruments). Analog signals for HR, MAP, and RSNA were digitized at 633 Hz (Digidata 1200, Axon Instruments; Foster City, CA) and were displayed using Clampex 8 software (Axon Instruments). Data were stored on a computer hard drive. RSNA was standardized between animals by setting resting nerve discharges as 100% and by expressing RSNA after various treatments as a percentage of the resting level. The level of activity obtained after the death of each animal was recorded and set as a zero level of nerve activity. The zero level of activity was digitally subtracted from recordings obtained from each animal. RSNA was measured at 0, 2, 5, 10, and 20 min after each tempol treatment. RSNA was quantitated as the root mean square of nerve activity during a 1-min interval at the time points described above. Root mean square was determined using a fast Fourier transform (Clampfit 8, Axon Instruments).

Experimental Protocols

After surgical preparation, 30–40 min were allowed for stabilization of all variables. Tempol, hexamethonium, SNP, idazoxan, or clonidine (Sigma; St. Louis, MO) were dissolved in saline. Nω-nitro-L-arginine (L-NNA, Sigma) was dissolved in sodium phosphate buffer (pH 7.2). A volume of 0.4 ml of saline or sodium phosphate buffer injected in 1 min did not change HR, MAP, or RSNA. HR, MAP, and RSNA were monitored for 20 min after drug treatments.

Effects of tempol on HR, MAP, and RSNA with or without L-NNA. Tempol was administered in increasing doses (30, 100, and 300 μmol/kg iv bolus) with an interdose interval of 30 min. When HR, MAP, and RSNA recovered to control levels, the NOS inhibitor L-NNA was administered by infusion (2.6 mg·kg⁻¹·min⁻¹) for 5 min for a total L-NNA dose of 13 mg·kg⁻¹. This dose of L-NNA was chosen because it inhibits NOS activity in vivo by >70% for >2 h in the periphery and in the central nervous system (8, 15). Beginning at 20 min after L-NNA infusion, tempol was injected again as described above.

Effects of tempol on HR, MAP, and RSNA in SADV rats. Hemodynamic measurements were made after SADV. One hour after the effectiveness of SADV was tested, tempol was administered as described above.

Effects of tempol on HR, MAP, and RSNA after ganglion block. Tempol was injected as described above before and after hexamethonium (30 mg/kg iv). Because hexamethonium decreases MAP, it may not be possible for tempol to produce further decreases in MAP after ganglion blockade.

To verify that MAP could be further decreased after ganglionic blockade, depressor responses to SNP (5 μg/kg) were examined before and after hexamethonium.

Effects of tempol on HR, MAP, and RSNA after α₂-adrenergic receptor blockade. To determine whether the responses to tempol were mediated by an α₂-adrenergic receptor-dependant pathway, tempol was given before and after idazoxan (an α₂-adrenergic receptor antagonist) treatment (0.3 mg/kg iv) (17). Clonidine, an α₂-adrenergic receptor agonist, was used to verify the effectiveness of α₂-adrenergic receptor blockade (16).

Statistics

Data are means ± SE and n values are the number of animals from which the data were obtained. The overall effects of tempol were evaluated using one-way analysis of variance with repeated measures. Differences among levels of MAP, HR, and RSNA before and after tempol were evaluated using Student’s paired t-test by comparing control responses to those obtained after various treatments. Group differences in baseline values were analyzed using Mann-Whitney U-tests. P < 0.05 was taken as the level of statistical significance.

RESULTS

Effects of Tempol on HR, MAP, and RSNA With or Without L-NNA Treatment

The effects of tempol on MAP, HR, and RSNA before and after L-NNA treatment were studied in six rats. Tempol alone at 100 and 300 μmol/kg but not at 30 μmol/kg transiently decreased HR, MAP, and RSNA (Figs. 1 and 2). Peak responses occurred 2–4 min after tempol administration. L-NNA treatment increased MAP by ~20 mmHg (P < 0.05) without significantly
changing HR or RSNA (Table 1). However, the effects of tempol on MAP after L-NNA treatment were not different from those obtained before L-NNA treatment ($P < 0.05$) (Figs. 1 and 2). By 20 min after tempol administration, all parameters returned to pretreatment levels (Fig. 1).

Effects of Tempol on HR, MAP, and RSNA in SADV Rats

Tempol produced dose-dependent decreases in MAP, RSNA, and HR in SADV rats ($n = 5$) (Fig. 2). Tempol-induced changes in MAP and RSNA in SADV rats were different from control rats only at the 30 μmol/kg dose (Fig. 2). Baseline levels of each parameter before tempol injection in SADV rats did not differ from levels recorded in control rats (Table 1).

Effects of Hexamethonium on Tempol-Induced Changes in HR, MAP, and RSNA

The effects of tempol before and after hexamethonium treatment were studied in five rats. Hexamethonium (30 mg/kg iv) significantly decreased MAP and HR and completely inhibited baseline RSNA (Table 1). As shown in Fig. 2, the effects of tempol on MAP and HR were inhibited following hexamethonium treatment. To determine whether hexamethonium-induced blockade of the depressor response caused by tempol was due to a decreased baseline MAP level, SNP (5 μg/kg), a direct-acting vasodilator, was administered before and after hexamethonium treatment. Before hexamethonium, SNP reduced MAP by 45 ± 5% and after hexamethonium SNP reduced MAP by 39 ± 6%;
these values were not significantly different \( (P > 0.05) \).

SNP-induced decreases in MAP were associated with a reflex increase in HR \((6.5 \pm 1.5\%) \) that was blocked by hexamethonium.

**Effects of \( \alpha_2 \)-Adrenoceptor Blockade on Tempol-Induced Changes in HR, MAP, and RSNA**

The effects of tempol and clonidine on MAP, HR, and RSNA before and after idazoxan treatment were studied in four rats. These studies were done as the decreases in MAP, HR, and RSNA activity caused by tempol were similar to effects caused by centrally acting \( \alpha_2 \)-adrenergic receptor agonists on these variables \((16, 17, 20) \). Figure 3 shows changes in HR, MAP, and RSNA caused by clonidine \((10 \mu g/kg) \) before and after idazoxan treatment \((0.3 \, \text{mg/kg iv}) \). Clonidine caused a transient increase followed by a decrease in MAP that was maintained for up to 2 h. Clonidine also inhibited HR and RSNA (Fig. 3). The effects of clonidine on MAP, HR, and RSNA were inhibited by idazoxan pretreatment (Figs. 3 and 4). To determine whether the depressor response caused by tempol was mediated via an \( \alpha_2 \)-adrenoceptor pathway, tempol \((300 \, \mu g/kg) \) was administered to rats before and after idazoxan treatment. Idazoxan transiently decreased MAP and increased RSNA and HR, but after 10 min, these parameters returned to baseline levels. Tempol-induced changes in MAP, HR, and RSNA were unaffected by idazoxan pretreatment (Fig. 4).

**DISCUSSION**

The data presented here indicate that in urethane-anesthetized, normotensive rats, acute tempol treatment causes a depressor response that is independent of NOS activity and is associated with an inhibition of RSNA. RSNA was used as an index of global sympathetic nerve activity because changes in RSNA can, under some conditions, be correlated with changes in sympathetic nerve activity in other vascular beds \((19) \). However, it is also recognized that responses of the renal nerve to physiological or pathophysiological stimuli can differ from those of sympathetic nerves supplying other vascular beds. For example, hemorrhagic shock in anesthetized rats is associated with a decrease in RSNA but an increase in adrenal sympathetic nerve activity \((18) \). Therefore, it is possible that the sympathoinhibitory effects of tempol may be restricted to the kidney, and further studies are needed to establish

![Fig. 3. Effect of idazoxan on depressor and sympathoinhibitory responses caused by clonidine. A: clonidine caused a biphasic change in MAP (in mmHg) and a decrease in HR (in beats/min) and RSNA (in μV). B: idazoxan (0.3 mg/kg iv) blocked the effects of clonidine on HR, MAP, and RSNA.](image-url)
whether tempol has a more general sympathoinhibitory effect. Finally, data from the present study indicate that tempol-induced inhibition of RSNA is not mediated through α₂-adrenergic receptors and tempol directly inhibits HR, an effect that may contribute to the depressor response.

Superoxide anions quench NO, and therefore superoxide anions can inhibit responses mediated by endogenously released NO. As tempol chelates superoxide anions, it can potentiate NO-mediated responses. Increased oxidative stress occurs in some forms of experimental and human hypertension and antioxidants, including tempol, can lower blood pressure. For example, in spontaneously hypertensive rats and angiotensin II-infused rats, the antihypertensive action of tempol is blocked following NOS inhibition presumably because superoxide anions inactivate NO and diminish its vasodilatory action (11, 13, 14). Human subjects receiving high-dose ascorbic acid also showed reduced blood pressure levels; however, the relationship between the depressor effect of ascorbic acid and NO was not investigated (2). In the present study, tempol-induced depressor responses were unaffected by L-NNA treatment. However, L-NNA treatment was effective at inhibiting NOS as blood pressure was increased by ~20 mmHg in rats receiving L-NNA infusions. These data suggest that tempol can lower blood pressure via an NO-independent mechanism. It is unlikely that, in urethane-anesthetized normotensive rats, tempol lowers blood pressure only by causing direct vasodilation. Additional effects may include direct inhibition of sympathetic nerve activity and HR. It is important to note that these data were obtained in anesthetized rats and that hemodynamic control mechanisms are altered under anesthesia. The proposed direct sympathoinhibitory effect of tempol needs to be confirmed in studies done in conscious animals.

Tempol lowered MAP, HR, and RSNA in SADV rats. This result indicates that tempol-induced decreases in RSNA, MAP, and HR do not require intact baroreceptor reflex pathways. However, after hexamethonium-treatment, the tempol-induced depressor response was reduced and the sympathoinhibitory response was completely blocked. Hexamethonium lowered MAP, and it may not have been possible for tempol to lower MAP any further. This is unlikely because SNP lowered MAP to the same degree before and after hexamethonium treatment. If tempol was acting only as a vasodilator in normotensive rats, it also should have lowered MAP to a similar degree before and after hexamethonium treatment. Therefore, inhibition of sympathetic ganglionic transmission accounts for the blockade of the tempol-induced depressor response by hexamethonium. Tempol produced a small decrease in MAP and HR after hexamethonium treatment. The residual depressor and HR responses may be due, in part, to a direct action of tempol on the heart because tempol slows HR in isolated heart preparations (4). Direct vasodilation caused by tempol could also contribute to the residual depressor response.

SOD injected directly into the rostral ventrolateral medulla of pigs potentiates tonic inhibition of sympathetic nerve activity and decreases RSNA, blood pressure, and HR (23). The depressor effect of SOD was most prominent in animals under oxidative stress when superoxide levels would be high (23). The depressor and sympathoinhibitory effects of SOD were blocked by NOS inhibition, suggesting that NO inhibits central sympathetic nerve activity and that superoxide ions inactivate endogenous NO. Tempol is a membrane-permeable SOD mimetic (5, 10) that freely crosses the blood-brain barrier after peripheral administration (9). Therefore, it is possible that superoxide scavengers such as SOD and tempol can lower blood pressure by causing central sympathoinhibition. However, this central mechanism may not be applicable to all models of hypertension (6). L-NNA readily crosses the blood-brain barrier, and, at the dose used in the present study, it produces a prolonged inhibition of NOS activity (15). Because L-NNA did not alter the
depressor response caused by tempol, our data indicate that, if tempol is acting as a central superoxide scavenger in normotensive rats, then superoxide ions are not interacting with endogenous NO to alter RSNA. Alternatively, there may be sufficient endogenous SOD available to scavenge superoxide ions and therefore tempol would not be expected to have an effect. Activation of central α2-adrenoceptors inhibits sympathetic nerve activity (16, 20). In the present study, a potential α2-adrenergic receptor-mediated inhibition of RSNA by tempol was investigated. However, inhibition of RSNA, HR, and MAP caused by tempol was not affected following idazoxan treatment to block α2-adrenergic receptors. Idazoxan blocked clonidine-induced decreases in RSNA, HR, and MAP, indicating that it was an effective antagonist of α2-adrenergic receptors. These data indicate that the inhibition of RSNA caused by tempol is independent of α2-adrenergic receptor activation.

In summary, this study has shown for the first time that tempol can lower blood pressure in normotensive rats via a sympathoinhibitory mechanism. In contrast to responses in hypertensive animals, the effects of tempol on RSNA, HR, and MAP are unaffected following idazoxan treatment to block α2-adrenergic receptors. These data indicate that tempol is independent of α2-adrenergic receptor activation. This work was supported by National Heart, Lung, and Blood Institute Grants HL-63973 and HL-24111.

REFERENCES