Effects of Local Administrations of Tempol and Diethyldithio-Carbamic on Peripheral Nerve Activity

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Abstract—We have recently shown that systemic administration of a superoxide dismutase mimetic, tempol, resulted in decreases in mean arterial pressure and heart rate along with a reduction in renal sympathetic nerve activity (RSNA). It has also been shown that these parameters are significantly increased by systemic administration of a superoxide dismutase inhibitor, diethyldithio-carbamic (DETC), indicating a potential role of reactive oxygen species in the regulation of RSNA. In this study, we examined the effects of local administrations of 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (tempol) and DETC on RSNA in anesthetized rats. Either tempol or DETC was directly administered onto the renal sympathetic nerves located between the electrode and ganglion. Local application of tempol (10 μL, 0.17 to 1.7 mol/L, n=6) resulted in dose-dependent decreases in integrated RSNA (by −81 ± 6% at 1.7 mol/L) without alterations in mean arterial pressure and heart rate. In contrast, DETC (10 μL, 0.17 to 1.7 mol/L, n=6) increased RSNA dose dependently. The responses of RSNA to tempol and DETC were significantly greater in spontaneously hypertensive rats than in normotensive rats (n=6, respectively). Local application of sodium nitroprusside (1 mmol/L) or N\textsuperscript{G}-nitro-L-arginine methyl ester (0.11 mol/L) altered neither basal RSNA nor tempol-induced reductions in RSNA (n=6 and 5, respectively). A voltage-gated potassium channel blocker, 4-aminopyridine (0.1 mol/L), significantly decreased basal RSNA (by −81 ± 1%) and completely prevented DETC-induced increases in RSNA (n=5). These results suggest that reactive oxygen species play a role in the regulation of sympathetic nerve activity, and that at least part of this mechanism is mediated through voltage-gated potassium channels. (Hypertension. 2004;44:236-243.)

Key Words: nitric oxide ■ oxidative stress ■ potassium channels ■ rats, spontaneously hypertensive ■ sympathetic nervous system

Superoxide anion (O\textsubscript{2}\textsuperscript{-}) and other reactive oxygen species (ROS) play a critical role in the pathogenesis of hypertension.\textsuperscript{1} It has been shown that administration of a cell membrane permeable superoxide dismutase (SOD) mimic, 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (tempol) lowers vascular O\textsubscript{2}\textsuperscript{-} levels and blood pressure in hypertensive animals.\textsuperscript{2-4} Furthermore, administration of a SOD inhibitor, diethyldithio-carbamic (DETC), increased renal medullary tissue O\textsubscript{2}\textsuperscript{-} levels and vascular resistance.\textsuperscript{5,6} Several in vitro studies also demonstrated that tempol and DETC alter acetylcholine-induced vasodilation under some experimental conditions,\textsuperscript{7-9} suggesting that ROS production contributes to altered control of vasomotor tone. Xu et al\textsuperscript{10} reported for the first time that tempol had the ability to reduce renal sympathetic activity (RSNA) in anesthetized Sprague-Dawley (SD) rats. We have also shown that in anesthetized Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR), systemic administration of tempol results in decreases in mean arterial pressure (MAP) and heart rate (HR) along with a significant reduction in RSNA.\textsuperscript{11} Furthermore, RSNA is not affected following administration of another nitro oxide compound, 3-carbamoyl proxyl (3-CP), which has minimal superoxide scavenging activity.\textsuperscript{12} In addition, MAP, HR, and RSNA are significantly increased by systemic administration of DETC.\textsuperscript{11} These observations suggest that ROS play a role in the regulation of sympathetic nerve activity.

Kagiyama et al\textsuperscript{13} showed that chronic administration of tempol into the rostral ventrolateral medulla does not alter blood pressure in SHR. Similarly, it was shown that intracebroventricular administration of tempol does not alter either RSNA or systemic hemodynamics.\textsuperscript{11} Furthermore, sinoaortic denervation and cervical vagotomy do not alter the responses of RSNA and systemic hemodynamics to tempol.\textsuperscript{10} Although these findings suggest that the sympathoinhibitory effects of tempol are mediated through mechanisms that are independent of the central nervous system and do not require intact baroreceptor reflex pathways, the action sites of tempol on the sympathetic nervous system have not yet been clarified.
In SHR and angiotensin II–infused hypertensive rats, the tempol-induced reductions in vascular resistance were markedly attenuated by NO synthase inhibition, indicating that tempol decreases vascular resistance through the enhancement of NO activity in the vasculature. However, Xu et al. reported that the responses of RSNA to systemic administration of tempol were not affected following treatment with an NO synthase inhibitor. Thus, the mechanisms responsible for tempol-induced reduction in RSNA are still not clear.

In this study, we examined the effects of local administrations of tempol and DETC on RSNA. Tempol and DETC were administered directly onto the renal sympathetic nerves (postganglion). To evaluate the contribution of NO to tempol-induced alterations in sympathetic nerve activity, we compared the effects of systemic and local administrations of an NO donor, sodium nitroprusside (SNP), and tempol on systemic hemodynamics and RSNA. Additional studies were performed to examine the effects of local administration of tempol on (1) RSNA during treatment with SNP and (2) RSNA during treatment with an NO synthase inhibitor, N\(^-\)nitro-L-arginine methyl ester (L-NAME). The effects of local administration of DETC on RSNA were also examined during treatment with a voltage-gated potassium channel blocker, 4-aminopyridine (4-AP).

Previously, we have shown that systemic administration of tempol decreased MAP, HR, and RSNA to a greater extent in SHR than in WKY rats, suggesting that augmented ROS contributes to the development of hypertension through activation of the sympathetic nerve system. To investigate the possible role of ROS in the activation of peripheral sympathetic nerves in hypertensive animals, the effects of local administrations of tempol and DETC were examined in SHR.

Materials and Methods

Animal Preparation

The experiments were performed on 13- to 14-week-old male SD rats (Clea Japan). All surgical and experimental procedures were performed according to the guidelines and practices established by the Animal Care and Use Committee of Kagawa Medical University. Rats were anesthetized with sodium pentobarbital (50 mg/kg, IP) and given additional doses as required. The surgical preparation of the animals and basic experimental techniques were identical to those previously described. The renal nerve branch was then isolated from the ganglion and the electrode. Silicon rubber was made by mixing semicross 932A and B gel (Wacker Asahikasei Silicone Co Ltd). To administer the drug locally and to prevent drug solutions spillover, the cavity was formed within the silicon rubber. A harmless material was put onto the renal nerve when the mixed gel was poured, and then it was removed before the gel set completely. The drug solutions were then inserted directly into the cavity and onto the renal nerve. The renal nerve was also enclosed with silicon rubber at the point of electrode-nerve contact. The distance between the sealed electrode and the sealed point of drug administration was approximately 1 cm. Thus, drug solutions were unable to come into contact with the electrode. Tempol was dissolved in an isotonic saline (37°C) and 10 \( \mu \)L of solution was administered. Preliminary experiments showed that local application of an isotonic saline solution (10 \( \mu \)L) does not affect MAP, HR, or RSNA (data not shown). Tempol was applied for 1 minute and then washed out with isotonic saline solution. Each dose of application was separated by at least 15 minutes. The peak responses to tempol were evaluated for each parameter during each period. In a separate group of experiments, the time-dependent responses of RSNA to tempol (1.7 mol/L) without washing out the nerves were determined in SD rats (n=6). 3-CP (1.7 mol/L) was also administered onto the renal sympathetic nerves in the other 5 rats. Similarly, SNP (1 mmol/L) was administered onto the renal sympathetic nerves (n=5). 3-CP and SNP were also dissolved in isotonic saline (37°C), and 10 \( \mu \)L of solution was administered. The dose of SNP was chosen on the basis of the results from previous studies performed in vitro.

Effects of Local Administration of Tempol on RSNA During Treatment With SNP and L-NAME

SNP (1 mmol/L) was administered directly onto the renal sympathetic nerves in SD rats (n=6). Fifteen minutes after the administration of SNP, a saline solution containing SNP (1 mmol/L) and tempol (1.7 mol/L) was administered. In a separate group of experiments, L-NAME (0.11 mol/L, Sigma Chemical Co) was administered directly onto the renal sympathetic nerves in SD rats (n=5). L-NAME was dissolved in isotonic saline (37°C) and 10 \( \mu \)L of solution was administered. Fifteen minutes after the administration of L-NAME, a saline solution containing L-NAME (0.11 mol/L) and tempol (1.7 mol/L) was administered. The dose of L-NAME was chosen on the basis of the results from previous studies performed in vitro.

Effects of Local Administration of DETC on RSNA and Systemic Hemodynamics

DETC (Sigma Chemical Co) was administered directly onto the renal sympathetic nerves in SD rats (n=6). DETC was dissolved in an isotonic saline (37°C) and 10 \( \mu \)L of solution was administered. DETC (0.17, 0.57, and 1.7 mol/L) was applied for 1 minute and washed out with isotonic saline solution. Each dose of application was separated by at least 15 minutes, and the peak responses to DETC were evaluated for each parameter during each period. In a separate group of experiments, the time-dependent responses of RSNA to DETC (1.7 mol/L) without washing out the nerves were determined (n=5).
Effects of Local Administration of DETC on RSNA After Blockade of Kv Channels

After recording the basal MAP, HR, and RSNA, a Kv channel blocker, 4-AP (0.1 mol/L, Sigma Chemical Co), was administered onto the renal sympathetic nerves in SD rats (n=5). 4-AP was dissolved in isotonic saline (37°C) and 10 μL of solution was administered. Fifteen minutes after the administration of 4-AP, a saline solution containing 4-AP (0.1 mol/L) and DETC (1.7 mol/L) was administered to these animals. Preliminary experiments were performed to determine the effective dose of 4-AP (n=5). The results showed that local administration of 4-AP (0.01, 0.05, and 0.1 mol/L) reduced RSNA by 10±1%, 39±3%, and 90±3%, respectively. Based on these data, responses of DETC on RSNA were investigated during treatment with 0.1 mol/L of 4-AP. We also examined the effects of systemic administration of DETC after local application of 4-AP. Fifteen minutes after the administration of 4-AP (0.1 mol/L) onto the renal nerves, DETC (173 μmol/kg) was infused intravenously (n=5).

Effects of Local Administrations of Tempol and DETC on RSNA in Hypertensive Rats

Effects of local administrations of tempol and DETC on RSNA were examined in 13- to 15-week-old male WKY rats and SHR (n=6, each) instead of in SD rats. The surgical preparations and experimental protocols in this study were identical to those described earlier.

Statistical Analysis

The values are presented as means±SE. Statistical comparisons of the differences were performed using the 1-way or 2-way ANOVA for repeated measures combined with Newman-Keuls post hoc test. P<0.05 was considered statistically significant.

Results

Responses of RSNA and Systemic Hemodynamics to Intravenous Administrations of Tempol, 3-CP, and SNP

The typical responses of RSNA and systemic hemodynamics to intravenous administrations of tempol (173 μmol/kg) and SNP (0.17 μmol/kg) in SD rats are shown in Figure 1A. Similarly to the results previously obtained in WKY rats,11 intravenous administration of tempol decreased MAP, HR, and integrated RSNA by 19±2, 9±1, and 37±5%, respectively, in SD rats (Figure 1B). These parameters returned to their respective control levels within 20 minutes. Intravenous administration of SNP caused a similar reduction in MAP (by 24±3%). However, SNP significantly increased HR and integrated RSNA by 13±3 and 44±7%, respectively (Figure 1B). The second administration of tempol resulted in changes in MAP, HR, and integrated RSNA similar to those observed after the first administration (Figure 1B). On the other hand, intravenous administration of 3-CP (173 μmol/kg) did not alter MAP (from 97±3 to 92±3 mm Hg), HR (from 283±3 to 278±4 bpm), or integrated RSNA (by 6±4%).

Responses of RSNA and Systemic Hemodynamics to Local Applications of Tempol, 3-CP, and SNP

Typical responses of RSNA and systemic hemodynamics to local administrations of tempol (1.7 mol/L) and SNP (1 mmol/L) are shown in Figure 2A. Local application of tempol (0.17, 0.57, and 1.7 mol/L) resulted in dose-dependent decreases in integrated RSNA (by −11±6, −38±6, and −81±6%, respectively) without alterations in MAP and HR. In all cases, the tempol-induced reductions in integrated RSNA returned to the basal levels immediately after being washed out with an isotonic saline solution. On the other hand, local application of SNP did not cause any changes in integrated RSNA, MAP, or HR (Figure 2A and 2B). Similarly, local application of 3-CP (1.7 mol/L) did not alter integrated RSNA (by 1±2%), MAP (from 94±2 to 97±1 mm Hg), or HR (from 333±19 to 330±20 bpm). Figure 2C shows the time-dependent responses of RSNA to local application of tempol without washing out the nerves. Tempol-induced reductions in RSNA were sustained for 15 minutes, then gradually returned to basal levels.
Responses of RSNA and Systemic Hemodynamics to Local Application of Tempol During Treatment With SNP or L-NAME

Local application of SNP (1 mmol/L) did not alter basal integrated RSNA (by 5±5%), MAP (from 101±2 to 103±3 mm Hg), or HR (from 326±7 to 327±7 bpm). After adding tempol (1.7 mol/L) to SNP on the renal sympathetic nerves, integrated RSNA was decreased by 80±4% (Figure 3), but MAP and HR did not change significantly (from 104±3 to 104±2 mm Hg and from 326±7 to 326±7 bpm, respectively). On the basis of group comparisons, the tempol-induced reductions of integrated RSNA in SNP-treated animals were not significantly different from those observed in untreated animals. Similarly, local application of L-NAME (0.11 mol/L) did not alter basal integrated RSNA (by ±2±2%), MAP (from 104±2 to 104±2 mm Hg), or HR (from 350±5 to 347±5 bpm). Furthermore, L-NAME did not modify the tempol-induced reductions in integrated RSNA (by 74±5%, Figure 3).

Responses of RSNA and Systemic Hemodynamics to Local Application of DETC

The typical responses of RSNA and systemic hemodynamics to local application of DETC (1.7 mol/L) are shown in Figure 4A. DETC (0.17, 0.57, and 1.7 mol/L) increased integrated RSNA in a dose-dependent manner (by 33±6, 73±11, and 133±25%, respectively). The DETC-induced increases in integrated RSNA returned to the basal levels immediately after being washed out with an isotonic saline solution. At doses of 0.17 and 0.57 mol/L, DETC did not significantly alter MAP or HR. On the other hand, DETC at a dose of 1.7 mol/L significantly increased MAP from 99±3 to 112±6 mm Hg and HR from 318±7 to 337±5 bpm. Similar responses of RSNA to DETC (1.7 mol/L) were observed in the case where DETC solution was not washed out. DETC increased RSNA by 146±25, 48±26, and 12±5% at 1, 5, and 15 minutes, respectively.

Basal activities of sectioned renal sympathetic nerves were nearly undetectable, as shown in Figure 4B. However, ad-
ministration of DETC onto the sectioned renal sympathetic nerves markedly increased RSNA (by 16±2-fold). In contrast to the results observed in intact nerves, administration of DETC (1.7 mol/L) onto the sectioned renal sympathetic nerves affected neither MAP nor HR (from 105±6 to 104±5 mm Hg and from 306±9 to 312±7 bpm, respectively).

Responses of RSNA and Systemic Hemodynamics to Local or Systemic Administration of DETC During Blockade of Kv Channels
Local application of 4-AP (0.1 mol/L) decreased basal integrated RSNA by −81±1% but did not alter basal MAP (from 104±4 to 108±8 mm Hg) or HR (from 337±8 to 341±12 bpm). Preliminary experiments (n=4) showed that 4-AP–induced reduction in RSNA is reversed by removing 4-AP solution (data not shown), indicating that the sympathetic nerves were not injured by local application of 4-AP. In the presence of 4-AP, DETC (1.7 mol/L) did not cause any changes in integrated RSNA (Figure 5A). Similarly, systemic administration of DETC (173 μmol/kg) did not alter RSNA in the presence of 4-AP (Figure 5B). In both cases, MAP and HR were also not altered (data not shown).

Responses of RSNA and Systemic Hemodynamics to Local Applications of Tempol and DETC in WKY Rats and SHR
Local application of tempol (0.17, 0.57, and 1.7 mol/L) resulted in dose-dependent decreases in integrated RSNA (Figure 6A) without alterations in MAP and HR (data not shown) in both WKY rats and SHR. Based on group comparisons, the magnitude of the DETC-induced increases in RSNA were significantly greater in SHR than in WKY rats (P<0.05 for each). In WKY rats, DETC at 0.17 and 0.57 mol/L did not significantly alter MAP or HR (data not shown), whereas DETC at a dose of 1.7 mol/L significantly increased MAP from 99±2 to 108±2 mm Hg and HR from 304±6 to 324±5 bpm, respectively. On the other hand, local application of DETC (0.17, 0.57, and 1.7 mol/L) resulted in dose-dependent increases in MAP (from 153±3 to 159±4, 163±3 and 169±3 mm Hg, respectively) and HR (from 359±2 to 371±4, 374±4, and 379±4 bpm, respectively) in SHR.

Discussion
The sympathetic nervous system plays a critical role in the control of arterial blood pressure and renal function.21,22 We previously showed that systemic administration of tempol resulted in decreases in MAP and HR along with a reduction in RSNA. We also observed that intracerebroventricular administration of tempol did not alter either RSNA or systemic hemodynamics,11 indicating that tempol reduces sympathetic nerve activity through mechanisms that are independent of the central nervous system. The present study demonstrates that administration of tempol directly onto the renal sympathetic nerves markedly reduces RSNA without affecting MAP. Furthermore, the time-dependent responses of RSNA to systemic and local administration of tempol showed similar trends. These data suggest that reductions in RSNA induced by systemic administration of tempol are at least partially mediated through its sympathoinhibitory effects at peripheral sites. Because local administration of another nitroxide compound, 3-CP, did not affect RSNA, it seems likely that these effects of tempol are caused by the reductions in ROS rather than to some nonspecific effects of the compound. Recently, DiBona and Sawin22 showed that acute renal denervation increased renal blood flow but did not change MAP in SHR. Therefore, it is possible that local administration of tempol increases renal blood flow. Further studies will be needed to determine the effect of local...
administration of tempol on renal hemodynamics and function.

Recent studies showed that polyethylene glycol-SOD or SOD altered neither blood pressure nor RSNA, suggesting that the sympathoinhibitory actions of tempol are not accompanied by its SOD-mimetic action. However, Nakazono et al. showed that HB-SOD, which is a fusion protein consisting of human Cu/Zn-type SOD and a C-terminal basic peptide with high affinity for heparan sulfate on endothelial cells, significantly decreased blood pressure in SHR. From these observations, it seems likely that membrane permeability, a half-life, and/or distribution of these drugs or tempol might be critical for their effects on blood pressure and RSNA. Nevertheless, to what extent the antioxidant actions of tempol mediate its sympathoinhibitory effects cannot be directly addressed by the current study.

Many studies indicate that tempol decreases vascular resistance by increasing NO availability in the vasculature. However, Xu et al. showed that the responses of RSNA to systemic administration of tempol were not affected by NO synthase inhibition, suggesting that tempol decreases sympathetic nerve activity predominantly by mechanisms other than by increasing NO availability. In the present study, systemic administrations of tempol and an NO donor, SNP, resulted in similar decreases in MAP. However, HR and RSNA were significantly decreased by systemic administration of tempol, whereas SNP markedly increased HR and RSNA. Thus, the responses of sympathetic nerve activity to systemic administration of tempol are clearly different from those induced by SNP. We also observed that RSNA was markedly decreased by a local application of tempol, but not by SNP. In addition, local application of SNP or L-NAME altered neither the basal RSNA nor the tempol-induced reductions in RSNA. These results suggest that tempol reduces peripheral sympathetic nerve activity via NO-independent mechanisms.

Similar to the results obtained by systemic administration of DETC, local application of DETC onto the sympathetic nerves markedly increased RSNA. These data further support the concept that ROS can influence peripheral sympathetic nerve activity. Previous studies showed that electrical stimu-

Figure 5. A, Effects of local applications 4-AP (10 μL, 0.1 mol/L) and 4-AP plus DETC (10 μL, 1.7 mol/L) on integrated RSNA (%) in SD rats (n=5). B, Effects of systemic administration of DETC (173 μmol/kg, IV) on integrated RSNA (%) in the presence of local application of 4-AP (10 μL, 0.1 mol/L, n=5).

Figure 6. Effects of local application of tempol (10 μL, 0.17 to 1.7 mol/L; A) or DETC (10 μL, 0.17 to 1.7 mol/L; B) on integrated RSNA (%) in WKY rats and SHR. *P<0.05 vs the baseline; †P<0.05; WKY rats vs SHR. n=6, each.
lation of renal nerves increased afferent nerve activity and blood pressure,\textsuperscript{25} suggesting that afferent nervous stimulation increases systemic blood pressure. In the present study, MAP and HR were significantly increased following local administration of a high dose of DETC (1.7 mol/L). It was also observed that DETC administration onto sectioned renal nerves resulted in a marked increase in RSA, indicating that DETC may have effects on silent nerve fibers. However, DETC-induced increases in MAP and HR were abolished by cutting the nerves. These data suggest that systemic hemodynamic changes induced by local administration of DETC are mediated through increases in the afferent nerve activity.

Recent studies have indicated that O$_2^-$ inhibits Kv channel activity in a variety of cells.\textsuperscript{26} It has also been indicated that voltage-gated potassium channels determine the frequency of action potentials and the shape of the action potential waveform.\textsuperscript{15} Furthermore, Kv channels may play a role in setting the resting membrane potential of a neuron and in regulating the excitability of individual neurons.\textsuperscript{15,16,27} Therefore, we examined the effects of a Kv channel blocker, 4-AP, on DETC-induced increases in RSA. The results showed that basal RSA is significantly reduced by local application of 4-AP. Interestingly, we also observed that 4-AP completely prevented increases in RSA induced by the local or systemic administration of DETC. These data suggest that at least part of the DETC-induced increases in peripheral sympathetic nerve activity are mediated through 4-AP–sensitive Kv channels at peripheral sites. It should be noted, however, that high doses of 4-AP may also have effects on other potassium channels, such as ATP-sensitive or Ca$^{2+}$-activated potassium channels.\textsuperscript{15} Therefore, further studies will be required to examine the effects of more specific Kv channel inhibitors.

Local administration of tempol reduced RSA to a greater extent in SHR than in normotensive WKY rats. Although ROS levels in the peripheral sympathetic nerves cannot be measured in the present in vivo experimental settings, these data suggest the possibility that augmented ROS levels participate in the activation of the sympathetic nervous system, which may contribute to the pathogenesis of hypertension. We also observed that increases in integrated RSA induced by local application of DETC were significantly greater in SHR than in WKY rats. These data are consistent with previous studies that SHR showed larger responses of RSA to systemic administration of DETC than WKY rats.\textsuperscript{11} Although these results suggest that in hypertensive animals RSA is more influenced in responses to further increases in ROS levels at peripheral sympathetic nerves, the precise mechanisms by which SHR show larger responses of RSA to local administration of DETC are not clear. Recently, studies have indicated functional differences of Kv channel–dependent membrane potentials between SHR and WKY rats.\textsuperscript{28,29} Thus, it is interesting to speculate that the larger responses of RSA to local administration of DETC in hypertensive animals are because of the different functions (or sensitivity) of Kv channel–dependent membrane potential activities at peripheral sympathetic nerves. Other possibilities cannot be ruled out and need to be examined further.

**Perspectives**

The present data support the hypothesis based on the results from previous studies\textsuperscript{10,11,13,14} that ROS play a role in the regulation of sympathetic nerve activity at peripheral sites. These data may also provide important information regarding mechanisms underlying the activation of the sympathetic nervous system in the conditions of high ROS state, such as hypertension or diabetes mellitus. However, the mechanisms of ROS generation at the peripheral nerves are still not clear and need to be investigated further. Future studies using the patch-clamp technique will be performed to determine the cellular mechanisms by which ROS influence sympathetic nervous signals. The role of ROS in other nervous systems will also be investigated.

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