Renal Sympathetic Nerve Responses to Tempol in Spontaneously Hypertensive Rats

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Abstract—Recent studies have implicated a contribution of oxidative stress to the development of hypertension. Studies were performed to determine the effects of the superoxide dismutase (SOD) mimetic 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (Tempol) on vascular superoxide production and renal sympathetic nerve activity (RSNA) in anesthetized Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR). Compared with WKY rats (n=6), SHR showed a doubled vascular superoxide production, which was normalized by treatment with Tempol (3 mmol/L, n=7). In WKY rats (n=6), Tempol (30 mg/kg IV) significantly decreased mean arterial pressure (MAP) from 108±5 to 88±6 mm Hg and HR from 304±9 to 282±6 beats/min. In SHR (n=6), Tempol significantly decreased MAP from 166±4 to 123±9 mm Hg and HR from 380±7 to 329±12 beats/min. Furthermore, Tempol significantly decreased RSNA in both WKY rats and SHR. On the basis of group comparisons, the percentage decreases in MAP (~28±4%), HR (~16±3%) and integrated RSNA (~63±6%) in SHR were significantly greater than in WKY rats (~17±3%, −9±2%, and −30±4%, respectively). In SHR, changes in integrated RSNA were highly correlated with changes in MAP (r=0.85, P<0.0001) during administration of Tempol (3, 10, and 30 mg/kg IV). In both WKY rats and SHR (n=4, respectively), intracerebroventricular injection of Tempol (300 μg/1 μL) did not alter MAP, HR, or RSNA. Intravenous administration of a SOD inhibitor, diethyldithio-carbamic acid (30 mg/kg), significantly increased MAP, HR, and integrated RSNA in both WKY rats and SHR (n=6, respectively). These results suggest that augmented superoxide production contributes to the development of hypertension through activation of the sympathetic nervous system.

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Key Words: nervous system, sympathetic renal ■ rats, spontaneously hypertensive ■ rats, inbred WKY ■ Tempol ■ arterial pressure

A growing body of evidence indicates that superoxide and other reactive oxygen species (ROS) contribute to the development of hypertension.6-5 Spontaneously hypertensive rats (SHR),6,7 stroke-prone SHR,8 deoxycorticosterone acetate (DOCA)-salt hypertensive rats,7 and angiotensin II–induced hypertensive rats9 have been shown to have elevated superoxide production in aortic vessels. Schnackenberg and Wilcox10 showed that a cell membrane–permeable superoxide dismutase (SOD) mimetic, 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (Tempol), decreased mean arterial pressure (MAP) and renal vascular resistance in SHR and that blockade of nitric oxide (NO) synthase by intravenous administration of an NO synthase inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME), abolished the effects of Tempol. The authors also showed that Tempol significantly decreased urinary excretion of 8-isoprostaglandin F2α,11 which is a marker of oxidative stress.12 Beswick et al13 showed that long-term treatment with Tempol decreased systolic blood pressure (BP) and attenuated renal injury in DOCA-salt hypertensive rats. Similarly, chronic treatment with Tempol prevented the progression of hypertension and vascular remodeling in salt-loaded stroke-prone SHR.14 We have also demonstrated that administration of Tempol normalized vascular superoxide production and decreased MAP in angiotensin II–infused hypertensive rats.9 It was also observed that Tempol significantly decreased vascular resistance in the kidney, brain, heart, liver, and intestine. Furthermore, these effects of Tempol were significantly attenuated by treatment with L-NAME.9 These results suggest that Tempol-induced reduction in superoxide production decreases vascular resistance through the enhancement of vascular NO activity in hypertensive animals.

The sympathetic nervous system plays a critical role in the control of arterial BP.15,16 Recently, Xu et al17 performed studies with anesthetized normotensive rats and found that Tempol decreased MAP, heart rate (HR), and sympathetic nerve activity, indicating that Tempol-induced decreases in BP are accompanied by a reduction in sympathetic nerve
activity. Interestingly, the authors also showed that these responses to Tempol were not altered by pretreatment with an NO synthase inhibitor, N\textsuperscript{\textdegree} - nitro-L-arginine. Thus, these results suggest that Tempol reduces sympathetic nerve activity through mechanisms that are independent of NO activity. However, the mechanisms responsible for Tempol-induced reductions in sympathetic nerve activity have not yet been clarified. In addition, the effects of Tempol on sympathetic nerve activity in SHR remain undetermined.

The primary objective of the present study was to investigate the participation of superoxide in the regulation of the sympathetic nervous system in SHR. Accordingly, the effects of systemic administration of Tempol on renal sympathetic nerve activity (RSNA) and systemic hemodynamics were determined in SHR. To further explore the mechanisms responsible for Tempol-induced alterations in RSNA, the effects of intracerebroventricular administration of Tempol were also examined. Additional studies were performed to determine the effects of another nitrooxide compound, 3-carbamoyl proxyl (3-CP), which is structurally similar to Tempol but has minimal superoxide scavenging activity. We also examined the effects of a SOD inhibitor, diethyldithio-carbamic acid (DETC), on RSNA and systemic hemodynamics. Recent studies have indicated that DETC increases superoxide production in vivo. In a separate group of animals, the effects of Tempol and DETC on vascular superoxide anion production were determined.

Methods

Animal Preparation

The experiments were performed in 13- to 14- week-old male Wistar-Kyoto (WKY) rats and SHR. 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. For the measurement of RSNA, 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. A polyethylene catheter was inserted into the abdominal aorta through the right femoral artery, and MAP and HR were continuously monitored with a pressure transducer (NEC-San-et, 361). A catheter was also inserted into the inferior vena cava through the right femoral vein, and an isotonic saline solution was infused at a rate of 2 mL/h for the duration of the stabilization and experimental periods. The left kidney was exposed through a retroperitoneal flank incision. The renal nerve branch was then isolated near the aortic-renal arterial junction and placed on a Teflon-coated stainless steel bipolar electrode. Thereafter, the renal nerve and electrode were covered with silicone rubber. The renal nerve discharge was amplified with a differential amplifier (Nihon Kohden, AVB-10) with a band-pass filter. The amplified and filtered signal was visualized on a dual-beam oscilloscope (Nihon Kohden, VC-10) and monitored by an audio speaker. The output from the amplifier was integrated by an amplifier and displayed by a dual-beam oscilloscope (Nihon Kohden, VC-10) with a differential amplifier. After the stabilization period of 60 minutes after the completion of surgery, the experimental protocol was started by recording the basal levels, Tempol (30 mg/kg IV) was administered to these animals. 3-CP was dissolved in 1 mL of isotonic saline solution and administered intravenously to WKY rats (n=6) and SHR (n=6). Tempol was dissolved in 1 mL of an isotonic saline solution and administered by slow infusion over a period of 1 minute at doses of 3, 10, and 30 mg/kg. Each dose of infusion was separated by at least 15 minutes. The peak responses of all the parameters to Tempol were evaluated during each period. Preliminary experiments showed that 1 mL of an isotonic saline solution did not alter MAP, HR, or RSNA (data not shown). In WKY rats (n=4) and SHR (n=4), 3-CP (Sigma Chemical Co) was administered intravenously at a dose of 30 mg/kg. 3-CP was dissolved in 1 mL of isotonic saline solution and administered by slow infusion over a period of 1 minute.

Effects of Systemic Administration of Tempol and 3-CP on RSNA and Systemic Hemodynamics After Ganglion Blockade

After recording the basal MAP, HR, and RSNA, rats were anesthetized with sodium pentobarbital (50 mg/kg IP), and MAP and HR were continuously monitored with a pressure transducer (NEC-San-et, 361). A catheter was also inserted into the inferior vena cava through the right femoral vein, and an isotonic saline solution was infused at a rate of 2 mL/h for the duration of the stabilization and experimental periods. The left kidney was exposed through a retroperitoneal flank incision. The renal nerve branch was then isolated near the aortic-renal arterial junction and placed on a Teflon-coated stainless steel bipolar electrode. Thereafter, the renal nerve and electrode were covered with silicone rubber. The renal nerve discharge was amplified with a differential amplifier (Nihon Kohden, AVB-10) with a band-pass filter. The amplified and filtered signal was visualized on a dual-beam oscilloscope (Nihon Kohden, VC-10) and monitored by an audio speaker. The output from the amplifier was integrated by an amplifier and displayed by a dual-beam oscilloscope (Nihon Kohden, VC-10) with a differential amplifier. After the stabilization period of 60 minutes after the completion of surgery, the experimental protocol was started by recording the basal MAP, HR, and RSNA. Tempol (Sigma Chemical Co) was then administered intravenously to WKY rats (n=6) and SHR (n=6). Tempol was dissolved in 1 mL of an isotonic saline solution and administered by slow infusion over a period of 1 minute at doses of 3, 10, and 30 mg/kg. Each dose of infusion was separated by at least 15 minutes. The peak responses of all the parameters to Tempol were evaluated during each period. Preliminary experiments showed that 1 mL of an isotonic saline solution did not alter MAP, HR, or RSNA (data not shown). In WKY rats (n=4) and SHR (n=4), 3-CP (Sigma Chemical Co) was administered intravenously at a dose of 30 mg/kg.

Effects of Intracerebroventricular Administration of Tempol on RSNA and Systemic Hemodynamics

In this series of experiments, WKY rats (n=4) and SHR (n=4) were placed in a stereotaxic instrument in a prone position. According to the methods of Paxinos et al., a stainless steel needle (gas-tight syringe; Hamilton Co) was implanted stereotaxically with the following coordinates: 1.5 mm posterior to the bregma, 1.8 mm lateral to the mid line, and 3.6 mm below the surface of the left cortex. Tempol (300 μg/1 μL) was administered by slow infusion over a period of 1 minute. In this study, we could not apply higher concentrations of Tempol intracerebroventricularly because of the limitations of osmolality and solubility. Twenty minutes after the injection of Tempol, angiotensin II (2 ng/1 μL) was administered by slow infusion over a period of 1 minute, and MAP, HR, and RSNA were determined.
Effects of DETC and Tempol on Vascular Superoxide Anion Production

The lucigenin chemiluminescence from aortic segments of WKY rats averaged $12\pm1\times10^3$ counts/min per milligram of dry tissue weight. In SHR, the lucigenin chemiluminescence was $\approx$2-fold higher than that in WKY rats ($27\pm4\times10^3$ counts/min per milligram of dry tissue weight; $P<0.05$, Figure 1). Tempol (3 mmol/L) did not significantly alter the lucigenin chemiluminescence from aortic segments of WKY rats ($10\pm1\times10^3$ counts/min per milligram of dry tissue weight). However, Tempol significantly decreased the lucigenin chemiluminescence from aortic segments of SHR to levels that were the same as those of WKY rats ($12\pm2\times10^3$ counts/min per milligram of dry tissue weight, Figure 1). Treatment with DETC (10 mmol/L) significantly increased the lucigenin

Statistical Analysis

The values are presented as mean±SEM. Statistical comparisons of the differences were performed by using 1-way or 2-way ANOVA for repeated measures combined with Newman-Keuls post hoc test. Correlations of the responses were made by the Spearman test. A value of $P<0.05$ was considered statistically significant.

Results

Effects of DETC and Tempol on Vascular Superoxide Anion Production

The lucigenin chemiluminescence from aortic segments of WKY rats averaged $12\pm1\times10^3$ counts/min per milligram of dry tissue weight. However, Tempol (3 mmol/L) did not significantly alter the lucigenin chemiluminescence from aortic segments of WKY rats ($10\pm1\times10^3$ counts/min per milligram of dry tissue weight; $P<0.05$, Figure 1). Tempol (3 mmol/L) did not significantly alter the lucigenin chemiluminescence from aortic segments of WKY rats ($10\pm1\times10^3$ counts/min per milligram of dry tissue weight). However, Tempol significantly decreased the lucigenin chemiluminescence from aortic segments of SHR to levels that were the same as those of WKY rats ($12\pm2\times10^3$ counts/min per milligram of dry tissue weight, Figure 1). Treatment with DETC (10 mmol/L) significantly increased the lucigenin
307±13 to 293±10 beats/min. Hexamethonium also significantly decreased MAP from 173±9 to 128±5 mm Hg and HR from 357±16 to 308±9 beats/min in SHR. In both WKY rats and SHR, hexamethonium abolished basal RSNA. Tem- pol (30 mg/kg IV) slightly but significantly decreased MAP (to 79±6 mm Hg in WKY rats, and 113±3 mm Hg in SHR). On the basis of group comparison, Tempol-induced reductions in MAP and HR in hexamethonium-treated animals were significantly smaller than those observed in untreated animals (P<0.05, respectively). In hexamethonium-treated animals, Tempol did not alter HR significantly (to 286±10 in WKY rats and 292±10 beats/min in SHR).

Responses of RSNA and Systemic Hemodynamics to Intracerebroventricular Administration of Tempol
Intracerebroventricular injection of Tempol (300 μg/1 μL) did not alter either MAP (from 108±2 to 108±1 mm Hg) or HR (from 315±10 to 317±2 beats/min) in WKY rats. Integrated RSNA was also unchanged by intracerebroventricular administration of Tempol (1±3% of baseline) in WKY rats. Similarly, intracerebroventricular injection of Tempol did not alter MAP (from 162±8 to 163±9 mm Hg), HR (from 381±6 to 386±8 beats/min), or integrated RSNA (2±3% of baseline) in SHR. In contrast, intracerebroventric-
cular injection of angiotensin II (2 ng/μL) significantly increased MAP, HR, and integrated RSNA in both WKY rats and SHR (data not shown).

**Responses of RSNA and Systemic Hemodynamics to Intravenous Administration of DETC**

The typical responses of systemic hemodynamics and RSNA to intravenous administration of DETC (30 mg/kg) in WKY rats are shown in Figure 5. In contrast to the responses to Tempol, DETC (30 mg/kg) rapidly increased MAP, HR, and integrated RSNA by 20±2%, 13±2%, and 58±3%, respectively, in WKY rats (Table and Figure 6A). These parameters returned to the control levels within 10 minutes. DETC at lower doses (3 and 10 mg/kg) tended to increase integrated RSNA in WKY rats, although these changes were not statistically significant (Figure 6A). On the other hand, DETC caused dose-dependent increases in integrated RSNA in SHR (Figure 6B). In SHR, DETC (30 mg/kg) increased MAP, HR, and integrated RSNA by 23±3%, 28±6%, and 50±9%, respectively (Table and Figure 6B).

**Discussion**

In agreement with previous studies, SHR showed higher vascular superoxide production from aortic segments compared with normotensive rats. Furthermore, the present study demonstrated that administration of Tempol normalized vascular superoxide production in SHR. Consistent with the results from previous studies, the present results confirm that systemic administration of Tempol results in significant decreases in MAP and HR along with a reduction in RSNA in normotensive rats, suggesting that at least part of the Tempol-induced alterations in systemic hemodynamics are accompanied by a reduction in sympathetic nerve activity. The present study also showed that Tempol reduced MAP, HR, and RSNA to a greater extent in SHR than in normotensive rats. In addition, the Tempol-induced changes in MAP were positively correlated with the changes in RSNA in SHR. These data suggest that augmented superoxide production contributes to the development of hypertension through activation of the sympathetic nervous system.

In this study, we also examined the effects of another nitroxide compound, 3-CP, which has minimal superoxide scavenging activity. The results showed that MAP, HR, and RSNA were not affected after systemic administration of 3-CP. These data support the concept that Tempol reduces sympathetic nerve activity by scavenging superoxide anions. Recent studies have shown that sinoaortic denervation and cervical vagotomy did not alter the responses of RSNA and systemic hemodynamics to Tempol, suggesting that the Tempol-induced changes in RSNA do not require intact baroreceptor reflex pathways. In this study, further investigations were performed to determine whether the Tempol-
induced alterations in RSNA and systemic hemodynamics are mediated through inhibition of the central nervous system. We observed that intracerebroventricular administration of Tempol did not alter either RSNA or systemic hemodynamics in both WKY rats and SHR. These observations are in accordance with those of Kagiyama et al, who reported that chronic administration of Tempol into the rostral ventrolateral medulla did not alter BP in SHR. These data suggest that the Tempol-induced alterations in systemic hemodynamics are not mediated through inhibition of the central nervous system.

Although the present results suggest that part of the hypotensive effects of Tempol are mediated by a reduction in sympathetic nerve activity, the extent to which this process regulates the hypotensive effects of Tempol remains unclear. In agreement with recent studies, we observed that intravenous administration of hexamethonium abolished basal RSNA and significantly attenuated Tempol-induced reduction in arterial pressure. However, hexamethonium failed to block the hypotensive effects of Tempol completely. These results suggest that the depressor responses caused by Tempol are not solely mediated through inhibition of the sympathetic nervous system. Schnackenberg and Wilcox showed that the Tempol-induced reductions in arterial pressure were markedly attenuated by treatment with an NO synthase inhibitor, suggesting that superoxide production may have contributed to the alterations in systemic BP through inactivation of NO in SHR. The authors also showed that the vasoconstrictor response to the thromboxane A2/prostaglandin H2 receptor agonist is abolished by Tempol and enhanced by NO synthase blockade in isolated renal afferent arterioles. More recently, it has also been shown that acetylcholine-induced renal afferent arteriolar vasodilation is impaired in diabetic rabbits and that this response is restored by Tempol administration. Collectively, these data suggest that Tempol causes direct vasodilation through an NO-dependent mechanism in some experimental conditions. In contrast, Xu et al showed that the responses of MAP, HR, and RSNA to Tempol were not affected after treatment with an NO synthase inhibitor in normotensive rats and DOCA-salt hypertensive rats. Clearly, further studies are needed to determine the contribution of NO to the effects of Tempol on systemic hemodynamics and RSNA in SHR.

This study showed that vascular superoxide production was significantly increased by treatment with DETC. In vivo studies, Zou et al showed that renal interstitial infusion of DETC decreased medullar blood flow and that Tempol had the opposite effect. Majid and Nishiyama also showed that intra-arterial infusion of DETC significantly decreased renal blood flow and urinary sodium excretion in dogs. Recently, it was shown that systemic administration of DETC significantly increased tissue superoxide concentrations and urinary 8-isoprostane excretion. Thus, these data suggest that superoxide generation is augmented by treatment with DETC in vivo. In the present study, we observed that RSNA was significantly increased by DETC administration in both WKY rats and SHR. These results further support the hypothesis that superoxide production participates in the regulation of sympathetic nerve activity. As shown in Figure 6, we observed that the responses of RSNA to lower doses of
DETC (3 and 10 mg/kg) were significantly larger in SHR compared with WKY rats. However, the responses of MAP and HR to DETC were not statistically different between these animals. We presently have no satisfactory explanation why SHR did not show larger responses in systemic hemodynamics to DETC. We also observed that the effects of Tempol on systemic hemodynamics and RSNA appeared to be long-acting compared with those of DETC. It is possible that these differing time courses of action are due to differences in the half-life of these compounds. Therefore, the effects of a longer infusion of Tempol and DETC on systemic hemodynamics and RSNA need to be investigated.

We previously reported that in normotensive rats, intravenous administration of Tempol slightly decreased MAP but that these changes were not statistically significant.9 In the present study, however, it was observed that Tempol administration resulted in significant decreases in MAP and HR, along with a significant reduction in RSNA in normotensive rats. The reason for the discrepancy between these results is not clear, although it might be due to different experimental settings. The previous studies were performed in conscious animals, whereas the present experiments were carried out in rats anesthetized with sodium pentobarbital. Since studies indicate that sodium pentobarbital increases basal sympathetic nerve activity,30,31 it is possible that Tempol-induced reductions in sympathetic nerve activity and MAP are enhanced in the present experimental settings.

**Perspectives**

This study showed that changes in systemic hemodynamics and RSNA induced by intravenous administration of Tempol were significantly augmented in hypertensive rats. Furthermore, these changes in MAP were positively correlated with the changes in RSNA in hypertensive rats. These data suggest that in hypertensive rats, the hypotensive effect of Tempol is accompanied by a reduction in sympathetic nerve activity and support the hypothesis that augmented superoxide production contributes to the development of hypertension through activation of the sympathetic nervous system. The present study also showed that intracerebroventricular administration of Tempol did not alter either RSNA or systemic hemodynamics. Although these data indicate that the Tempol-induced alterations in systemic hemodynamics are not mediated through inhibition of the central nervous system, further studies are required to determine the precise mechanisms responsible for the Tempol-induced changes in systemic hemodynamics and sympathetic nervous system. Additional studies are under way to determine the effects of local application of Tempol in peripheral nerves and nerve chains on sympathetic nerve activity as well as superoxide production in nerve regions.

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