Tempol Attenuates Excitatory Actions of Angiotensin II in the Rostral Ventrolateral Medulla During Emotional Stress

Dmitry N. Mayorov, Geoffrey A. Head, Robert De Matteo

Abstract—Superoxide has been shown to be an important intracellular mediator of actions of angiotensin II. Recently, we found that blockade of angiotensin II type-1 receptors in the rostral ventrolateral medulla (RVLM) abrogated the pressor effect of emotional stress in rabbits. In the present study, we examined the influence of superoxide dismutase mimetics, tempol and tiron, in RVLM on cardiovascular stress response in conscious rabbits. Air-jet stress evoked a sustained increase in blood pressure (+14±2 mm Hg), tachycardia (+52±7 bpm), and renal sympathoactivation (+58±8%). Bilateral microinjections of tempol or tiron (20 nmol) into RVLM did not alter resting cardiovascular parameters, but attenuated the pressor, sympathetic, and tachycardiac response to stress by 40% to 55%. By contrast, 3-carbamoylpropyl, which is structurally close to tempol but has a lower superoxide scavenging activity, did not alter the stress response. Neither tempol nor tiron altered the sympathoexcitatory response to glutamate microinjections into RVLM or to baroreceptor unloading. Microinjections of nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (l-NAME; 10 nmol) into RVLM did not affect the stress response. Coinjections of tempol and l-NAME decreased the pressor response to stress by 35±3%. Tempol attenuated the pressor response to microinjection of angiotensin II into RVLM by 59±15%, whereas l-NAME did not alter this response. These results suggest that superoxide dismutase mimetics in RVLM attenuate, partially via a nitric oxide-independent mechanism, the pressor effect of emotional stress in rabbits. Together with our previous studies, these results also indicate that superoxide is a key mediator of excitatory actions of angiotensin II in RVLM during acute stress. (Hypertension. 2004;44:101-106.)

Key Words: angiotensin II • nitric oxide • stress • blood pressure • brain • rabbits

Strong evidence is accumulating to indicate that the ligand-stimulated, low-level production of reactive oxygen species (ROS) plays a role in the normal regulation of cell function.1 In particular, superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) appear to be important intracellular messengers of many of the effects of circulating angiotensin II (Ang II),2 including Ang II-induced vasoconstriction.3,4 It is likely that ROS are also involved in the actions of brain-borne Ang II, because the adenosinergic vector-mediated overexpression of superoxide dismutase (SOD) abolishes the cardiovascular and dipsogenic effects of intracerebroventricularly injected Ang II in mice.5 In the same study, Ang II-induced superoxide generation was prevented by losartan in primary neuronal cell culture, indicating the role of Ang II type-1 (AT$_1$) receptors in activating redox-sensitive pathways in neurons. Although these studies have demonstrated the importance of ROS in mediating the acute effects of exogenously applied Ang II, the role of ROS in signal transduction initiated by Ang II, endogenously released under normal physiological conditions, remains to be determined.

One brain region where endogenous Ang II is thought to be important in cardiovascular regulation is the rostral ventrolateral medulla (RVLM), which maintains sympathetic vaso-motor outflow and also plays a key role in controlling baroreceptor and other reflexes.6 The RVLM has a high density of AT$_1$ receptors7 and is a major site of the tonic sympathoexcitatory action of endogenous Ang II in hypertensive animals.8,9 We found recently that endogenous Ang II in the RVLM is also important in mediating the hypertensive response to acute emotional stress, because local microinjections of AT$_1$ antagonists abrogated the maintenance of this response in rabbits.10 In the same preparation, blockade of Ang II receptors did not affect the sympathoexcitatory response to baroreceptor unloading or to glutamate microinjections,10,11 indicating that the acute excitatory action of Ang II in the RVLM may be specifically linked to stress exposure in normotensive animals. These findings are in line with growing evidence that brain Ang II is critical in mediating the cardiovascular effects of various physicoemotional and psychomotional stressors.12

In the current study, we sought to determine whether acute excitatory action of endogenous Ang II in the RVLM during emotional stress requires activating a redox-sensitive signaling pathway. We tested whether microinjections of the
membrane-permeable, stable SOD mimetics, tempol and tiron, alter the cardiovascular response to emotional stress in conscious rabbits. As a control, we used microinjections of 3-carbamoylproxyl (3-CP), which is structurally similar to tempol but has a much lower superoxide scavenging capacity. We also determined the role of local nitric oxide (NO) in actions of tempol on the stress response. In additional experiments, we evaluated whether SOD mimetics in the RVLM change the hypertensive effect of exogenously applied Ang II or glutamate. As a control, we determined the influence of SOD mimetics, in the RVLM, on sympathetic and cardiac baroreflexes in conscious rabbits.

Methods

General Procedures

The experiments were performed in 17 conscious multicolored and New Zealand White rabbits, weighing 2.6 to 3.2 kg, and bred and housed at the Baker Heart Research Institute. All procedures were approved by the Alfred Medical Research and Education Precinct Animal Ethics Committee. Two weeks before the experiments, all rabbits were implanted with guide cannulae for bilateral microinjections into the RVLM. One week later, in some rabbits, a bipolar electrode was implanted for recording renal sympathetic nerve activity (RSNA). On the day of the experiment, the animal was placed in a standard rabbit box (15×40×18 cm, width×length×height) and the central ear artery and marginal ear vein were catheterized under local anesthesia.

During the experiment, pulsatile arterial pressure and integrated RSNA were continuously monitored and sampled at 500 Hz using an analog-to-digital data acquisition card as described previously. The beat-to-beat mean arterial pressure (MAP), heart rate (HR), and RSNA were continuously monitored and sampled at 500 Hz using an analog-to-digital data acquisition card as described previously. The RSNA were continuously monitored and sampled at 500 Hz using an analog-to-digital data acquisition card as described previously.

Experimental Protocol

In each rabbit, (1) the cardiovascular response to emotional stress; (2) baroreflexes; or (3) the pressor response to microinjections of Ang II or glutamate into the RVLM were evaluated before and 10 to 20 minutes after local microinjections of equimolar doses of SOD mimetics, tempol, tiron, and 3-CP (20 nmol, bilaterally). In additional experiments, tempol and NO synthase (NOS) inhibitor N0 nitro-L-arginine methyl ester (L-NAME, 10 nmol) were coinjected into the RVLM to evaluate the role of NO in the observed effects of tempol. The selected doses of the drugs were based on our preliminary experiments and on previous studies from other laboratories. Each rabbit was subjected to 1 to 2 treatments per experiment in 3 to 4 experiments 2 days apart. In the case of 2 treatments during the same experiment, a 2- to 3-hour period was allowed between treatments and full recovery of the stress response was observed before proceeding to the next treatment. The order of treatments was randomized between and within experiments. All drugs, except Ang II (Auspep), were obtained from Sigma, dissolved in Ringer’s solution (Baxter), and injected in a volume of 100 nL using a microsyringe system described previously.

Statistical Analysis

All values are expressed as mean ± SEM. All data were tested for normality and equal variance and analyzed by a 2-way repeated measures ANOVA followed by the Bonferroni post-hoc test. The tests were considered significant when P<0.05.

Results

Effects of Tempol on the Cardiovascular and Respiratory Response to Emotional Stress

Resting hemodynamic and sympathetic parameters were not different before bilateral microinjections of equimolar doses (20 nmol) of tempol, tiron, and 3-CP into the RVLM (Table). Air-jet stress evoked a rapid increase in MAP, HR, and RSNA, which typically reached a plateau within the first 2 minutes and did not change thereafter (Figures 1 and 2). Therefore, the mean change over the past 5 minutes of stress exposure was used to estimate the air-jet response. Before microinjections of SOD mimetics there was no difference between groups in the air-jet–induced changes in MAP (F1,40<0.44), HR (F1,40<1.03), and RSNA (F1,40<0.23), with the overall average response being +14±2 mm Hg, +52±7 bpm, and +15±2 normalized units, respectively (Figure 2). Bilateral microinjections of tempol, tiron, and 3-CP did not alter resting MAP (−0±2 mm Hg, +3±1 mm Hg, and −1±1 mm Hg, respectively), HR, or RSNA (Figure 1). By contrast, tempol and tiron attenuated the pressor response to

<table>
<thead>
<tr>
<th>Compound</th>
<th>MAP, mm Hg</th>
<th>HR, bpm</th>
<th>RSNA, nu</th>
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<tr>
<td>Control</td>
<td>∆</td>
<td>Control</td>
<td>∆</td>
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<tr>
<td>Tempol</td>
<td>78±2 (10)</td>
<td>+1±1</td>
<td>170±8</td>
</tr>
<tr>
<td>Tiron</td>
<td>73±2 (9)</td>
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<td>166±9</td>
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<tr>
<td>3-CP</td>
<td>72±2 (6)</td>
<td>+0±1</td>
<td>168±6</td>
</tr>
<tr>
<td>Tempol+L-NAME</td>
<td>72±2 (6)</td>
<td>+2±2</td>
<td>168±11</td>
</tr>
<tr>
<td>L-NAME</td>
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</tbody>
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Values are mean±SEM. All parameters represent values averaged over 2 minutes immediately before air-jet. ∆ indicates change from control before air-jet; numbers in parentheses, number of experiments; nu, normalized units.
air-jet by 52±9% and 55±8%, respectively (P<0.001; Figure 2). The stress-induced tachycardia was decreased after injections of tempol and tiron by 38±13% and 47±9%, whereas the RSNA response was attenuated by 46±7% and 40±15%, respectively (P<0.05). However, 3-CP, which is structurally similar to tempol but has a lower superoxide scavenging activity,13 did not alter the MAP, HR, or RSNA responses to air-jet stress (Figure 2). The cardiovascular stress response recovered 2 hours after microinjections of SOD mimetics. At this time, air-jet stress increased MAP by 13±2 mm Hg, 15±3 mm Hg, and 12±2 mm Hg in tempol, tiron, and 3-CP groups, respectively.

Air-jet stress increased respiratory rate from 93±11 breaths/min to 120±12 breaths/min (P<0.05). Microinjections of tempol into the RVLM did not alter respiratory rate either at rest (91±11 breaths/min) or during stress exposure (122±11 breaths/min). Similarly, respiratory rate remained unchanged after microinjections of tiron and 3-CP (data not shown).

Effects of Tempol on Sympathetic and Cardiac Baroreflexes
Bilateral microinjections of tempol into the RVLM did not change the upper plateau (which is an index of the maximal sympathoexcitatory response to baroreceptor unloading), the lower plateau, or the gain of the RSNA baroreflex, and neither did microinjections of tiron or 3-CP (Figure 3). Similarly, the HR baroreflex parameters remained unaffected by bilateral microinjections of the superoxide scavengers into the RVLM of conscious rabbits (data not shown).

Effects of l-NAME on the Sympathoinhibitory Action of Tempol During Stress
There was no difference in hemodynamic and sympathetic parameters either at rest or during stress exposure before microinjections of l-NAME (10 nmol) or comicroinjections of tempol (20 nmol) and l-NAME (10 nmol) into the RVLM (F2,20<0.86; Table, Figure 4).

Bilateral microinjections of l-NAME or comicroinjections of tempol and l-NAME did not alter resting MAP (+0±1 mm Hg and −2±1 mm Hg, respectively), HR, or RSNA (not shown). Microinjections of l-NAME did not affect the hemodynamic and sympathetic response to air-jet stress (Figure 4). By contrast, comicroinjections of tempol and l-NAME attenuated the MAP and RSNA responses to air-jet stress by 35±3% (P<0.01) and 57±22% (P<0.05), respectively, whereas the HR response remained unaltered (Figure 4). Bilateral microinjections of N(G)-nitro-d-arginine methyl ester (d-NAME; 10 nmol) into the RVLM did not alter hemodynamic or sympathetic parameters either at rest or during stress exposure (data not shown).

Effects of Tempol on the Pressor Response to Exogenous Ang II
To confirm that a superoxide-sensitive pathway mediates actions of Ang II in the RVLM, the cardiovascular response to exogenously applied Ang II was tested before and after microinjections of superoxide scavengers into this region of conscious rabbits. Unilateral microinjections of Ang II (100
pmol) into the RVLM increased MAP by 12±3 mm Hg (Figure 5). Preinjections of tempol (n=6) or tiron (n=5) attenuated the pressor response to Ang II by 59±15% and 58±12%, respectively (P<0.05). By contrast, pretreatment with L-NAME did not change the pressor response to Ang II (n=6; Figure 5). The pressor response to unilateral microinjections of another excitatory agent, glutamate (3 to 5 nmol), into the RVLM was not different before and after local administration of tempol, tiron, L-NAME (Figure 5), or 3-CP (n=4 to 6; not shown).

Discussion

The major finding of the present study is that superoxide scavenging in the RVLM attenuates the acute pressor response to emotional stress in conscious rabbits. Together with our previous finding that this response is critically dependent on AT1 receptors in the RVLM,10 these results suggest that superoxide plays an essential role in the excitatory action of Ang II in the RVLM during acute stress. The current results also extend the recent finding that superoxide mediates the cardiovascular response to exogenous Ang II in the brain,3 indicating that superoxide is also important in signaling activated by Ang II, endogenously released under normal physiological conditions in freely behaving animals.

To determine the role of superoxide in the RVLM, we used the cell-permeable, stable SOD mimetics, tempol and tiron, which have been shown to scavenge, in the millimolar concentration range, superoxide in vitro.15,16 In addition, tempol has been reported to normalize blood pressure and vascular superoxide production in animal models of hypertension.18–20 The ability of tempol to react with superoxide is likely to be critical for its antihypertensive action during emotional stress, as 3-CP, which is structurally close to tempol but reacts with superoxide less effectively,13 did not alter the stress response. However, tiron, which does not chemically relate to tempol but has a similar superoxide scavenging activity,15 mirrored the effect of tempol on the stress response. By contrast, the interaction of tempol with NO appears to be less important in modulating the pressor stress response in the current preparation, because coinjections of tempol and L-NAME were only slightly less effective in attenuating this response than tempol alone. Accordingly, earlier studies failed to ascribe the depressor effect of systemically given tempol to a direct interaction with NO.21,22 Nevertheless, NO may play a role in modulating the cardiac stress response, because coinjections of tempol and L-NAME into the RVLM did not attenuate the stress-induced tachycardia as did tempol alone. It is noteworthy that in our previous study, microinjections of AT1 receptor antagonists into the RVLM decreased the pressor, but not tachycardic, response to air-jet stress.10 Together, these findings suggest that the cardiac component of the stress response may be specifically regulated in the RVLM by a separate NO-sensitive signaling mechanism, which is independent of AT1 receptor stimulation.

Our findings do not exclude a potential role of hydrogen peroxide or other downstream products of superoxide dismutation by SOD mimetics (which lack catalase activity) in the effects observed. Nevertheless, there is good evidence that...
the use of SOD mimetics does not lead to a toxic condition by generating more hydrogen peroxide. In fact, tempol has been shown to attenuate the cytotoxic effects of hydrogen peroxide, which are essentially mediated by hydroxyl radicals. Additionally, preservation of the respiratory response to stress after tempol and tiron strongly indicates that attenuation of the cardiovascular response was not caused by a toxic condition, compromising the alertness of the animal.

The current data show that microinjections of SOD mimetics into the RVLM did not change resting hemodynamic or sympathetic parameters. Previous studies have also demonstrated only moderate inhibitory effects of SOD, microinjected into the RVLM, on baseline arterial pressure and sympathoexcitatory reflexes by the RVLM during acute stress. Nevertheless, superoxide has been shown to be important in the tonic support of blood pressure under conditions of chronic oxidative stress, because microinjections of SOD into the RVLM evoked depressor responses in nitrate-treated anesthetized pigs. The present results extend these findings, indicating that local ROS are also involved in mediating the acute hypertensive response evoked by an emotional stressor in conscious animals.

In this study, microinjections of SOD mimetics into the RVLM did not alter the sympathoexcitatory response to baroreceptor unloading, which is principally mediated by disinhibition of local GABAergic inputs, or to glutamate receptor stimulation. Similarly, previous studies found that the transmission of sympathoexcitatory reflexes by the RVLM, as evoked by sciatic nerve stimulation, was not affected by SOD microinjections in pigs. By contrast, tempol and tiron attenuated the pressor response to exogenously applied Ang II. Together with our previous findings, these results indicate that the air-jet-induced increase in superoxide production in the RVLM is not a general phenomenon caused by neuronal excitation, per se, but may specifically relate to the activation of Ang II signaling pathways. In the same way, the earlier reports that Ang II, but not other pressor agents evoked acute vasoconstriction via redox-sensitive mechanisms, suggested a specific link between Ang II and ROS formation in vascular cells. However, in contrast to vascular cells, the Ang II–superoxide signaling in the RVLM may be essentially independent of NO, because preinjections of L-NAME into this region did not alter the pressor effect of exogenous Ang II and only moderately attenuated inhibitory effect of tempol on the stress response.

The current results suggest that SOD mimetics in the RVLM attenuate, in part via NO-independent mechanism, the acute cardiovascular response to emotional stress in conscious rabbits. In conjunction with our previous studies, these results may also indicate that superoxide is a key intracellular signaling molecule in the excitatory action of endogenous Ang II in the RVLM during acute stress. The lack of effects of SOD mimetics on resting cardiovascular parameters and on baroreflexes suggests that activation of the Ang II–superoxide signaling cascade in the RVLM may be intrinsically linked to stress exposure.

**Perspectives**

The intracellular pathways mediating the Ang II–dependent superoxide generation in the RVLM during stress and, specifically, the source of superoxide, remain to be determined. Little effect of local microinjections of L-NAME or another NOS inhibitor, 7-nitroindazole (unpublished observations), on the pressor response to air-jet stress suggests that one potential enzymatic source of superoxide, uncoupled NOS, is not critical in the generation of superoxide in the RVLM during acute stress. By contrast, in our preliminary experiments, microinjections of a low dose (1 nmol) of specific NAD(P)H oxidase inhibitor, apocynin, into the RVLM attenuated the pressor response to air-jet, without altering resting hemodynamic parameters in rabbits. This indicates that the NAD(P)H oxidase may be important in the stress-induced superoxide production in the RVLM, similar to its role in the Ang II–dependent superoxide generation in vascular tissues. Nevertheless, the contribution of other major sources of superoxide production in the brain, such as xanthine oxidase and mitochondrial respiration, also needs to be determined to further understand how ROS, in the RVLM, modulate cardiovascular stress responses.
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References
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