Activation of Vascular BK Channel by Tempol in DOCA-Salt Hypertensive Rats

Hui Xu, Xiaochun Bian, Stephanie W. Watts, Alexandra Hlavacova

Abstract—Large-conductance Ca\(^{2+}\)/H11001-activated potassium (BK) channels modulate vascular smooth muscle tone. Tempol, a superoxide dismutase (SOD) mimetic, lowers blood pressure and inhibits sympathetic nerve activity in normotensive and hypertensive rats. In the present study, we tested the hypotheses depressor responses caused by tempol are partly mediated by vasodilation. It was found that tempol, but not tiron (a superoxide scavenger), dose-dependently relaxed mesenteric arteries (MA) in anesthetized sham and deoxycorticosterone acetate (DOCA)-salt hypertensive rats. Tempol also reduced perfusion pressure in isolated, norepinephrine (NE) preconstricted MA from sham and DOCA-salt hypertensive rats. Maximal responses in DOCA-salt rats were twice as large as those in sham rats. The vasodilation caused by tempol was blocked by iberiotoxin (IBTX, BK channel antagonist, 0.1 \(\mu\)mol/L) and tetraethylammonium chloride (TEA) (1 mmol/L). Tempol did not relax KCl preconstricted arteries in sham or DOCA-salt rats, and \(N^\omega\)-nitro-L-arginine methyl ester (L-NAME), apamin, or glibenclamide did not alter tempol-induced vasodilation. IBTX constricted MA and this response was larger in DOCA-salt compared with sham rats. Western blots and immunohistochemical analysis revealed increased expression of BK channel \(\alpha\) subunit protein in DOCA-salt arteries compared with sham arteries. Whole-cell patch clamp studies revealed that tempol enhanced BK channel currents in HEK-293 cells transiently transfected with \(mslo\), the murine BK channel \(\alpha\) subunit. These currents were blocked by IBTX. The data indicate that tempol activates BK channels and this effect contributes to depressor responses caused by tempol. Upregulation of the BK channel \(\alpha\) subunit contributes to the enhanced depressor response caused by tempol in DOCA-salt hypertension. (Hypertension. 2005;46:1154-1162.)

Key Words: antioxidants ■ hypertension ■ vasodilation

Superoxide anion (O\(_2^-\)) increases blood pressure in hypertensive animals and humans in part by reducing the vascular bioavailability of nitric oxide (NO).

Acute tempol treatment of normotensive, DOCA-salt and spontaneously hypertensive rats (SHRs) inhibits sympathetic nervous activity and this effect is not prevented by nitric oxide synthase (NOS) inhibition. Local application of tempol onto renal sympathetic nerves decreased nerve activity without changing blood pressure (BP) or heart rate (HR). The results suggested that changes in K\(^+\) channel activity might contribute to sympatho-inhibition caused by tempol. Central administration of tempol in normotensive rats reduced sympathetic nerve discharge and attenuated sympathetic excitation caused by central angiotensin II administration. Therefore, it is possible that tempol-induced vasodilation in vivo is masked by its sympa-tho-inhibitory effects.

Chronic tempol treatment attenuates blood pressure increases in hypertensive animals, an effect attributed to improvement in endothelium function and/or a reduction of oxidative stress. However, data obtained in salt-induced or corticotropin (ACTH)-induced hypertension in rats indicate tempol-induced depressor effects are independent of endothelial function and oxidative stress. Therefore, tempol-induced depressor effects may not be caused entirely by removal of O\(_2^-\) and/or increased NO availability.

The present studies were performed to determine whether tempol directly causes vasodilation via an increased NO availability in DOCA-salt hypertensive rats. Furthermore, the role of K\(^+\) channel activation in tempol-induced vasodilation was also studied. Finally, the effects of tempol on BK channels were determined by whole cell patch clamp in HEK-293 cells transfected with \(mslo\), the mouse BK channel \(\alpha\) subunit.

Materials and Methods

Animals
Animal use protocols were approved by the All University Committee for Animal Use and Care at Michigan State University. Sham operated and DOCA-salt hypertensive rats (male, 170 to 225 grams; Charles River Laboratories) were prepared as previously de-
scribed. Blood pressure was measured in conscious animals by tail-cuff plethysmography 4 weeks after DOCA implantation.

**In Situ Measurement of Mesenteric Artery Diameter in Anesthetized Rats**

HR, BP, and mean arterial blood pressure (MAP) were recorded as reported. An ileal loop was drawn out and fixed to a silastic chamber perfused with Krebs solution (37°C). The arterial tree was perfused with Krebs’ solution (4% CO₂-21% O₂-74% N₂). Mesenteric artery (MA; outer diameter 20%). Control cells were transfected with GFP only.

**Whole-Cell Recording of BK Channel Currents**

Whole-cell current recordings were performed between 1 and 4 days after transfection. The bath solution contained (mmol/L): NaCl 117.

**Expression of BK Channel A-Subunits (m slo) in HEK-293 Cells**

The BK channel m slo α-subunit cDNA plasmid (in the expression vector pcDNA3Zeo, Invitrogen) was a gift from Dr Christopher Lingle, (Department of Anesthesiology, Washington University, St. Louis, Mo). HEK-293 cells were cultured in Dulbecco’s F-12 medium (Invitrogen) supplemented with 10% FCS (Sigma) and 2 mmol/L glutamine (Invitrogen). Cells were transfected with 2 μg of m slo plasmid and green fluorescence protein (GFP) (0.1 μg) using Lipofectamine 2000 (Invitrogen). Transfection efficiency was ~20%. Control cells were transfected with GFP only.

**Drugs**

Tempol, IBTX, apamin, TEA and tiron were purchased from Sigma Chemical Co. Glibenclamide was purchased from Tocris.

**Statistics**

Paired and unpaired t tests were used to make single point comparisons. Comparison of multiple points generated in dose-response curves were tested by 2-way ANOVA with repeated measures, followed by Duncan’s multiple range test. Significance was accepted at P<0.05.

**Results**

In anesthetized rats, MAP was 160±5 mm Hg in DOCA-salt (n=8), and 110±4 mm Hg in sham rats (n=8) (P<0.05). HR in DOCA-salt rats did not differ from sham rats. Average systolic blood pressure was 195±4 mm Hg in DOCA-salt (n=44) and 120±5 mm Hg in sham rats (n=43) (P<0.05) in vitro studies. The body weight of sham rats was 420±8 grams and 320±7 grams in DOCA-salt rats (P<0.05).

**Tempol Relaxed MA In Situ in Anesthetized Sham and DOCA-Salt Rats**

Tempol was applied by superfusion cumulatively (0.001 to 3 mmol/L). The resting MA-OD in DOCA-salt rats did not differ from sham rats (265±20 μm, n=4, versus 270±15 μm, n=4; P>0.05). Tempol increased MA-OD without changing HR or MAP (Figure 1A and 1B). Dose-response curves in DOCA-salt rats were left-shifted (Figure 1C). The EC₅₀ for tempol was 40±2 μmol/L in DOCA-salt, and 170±12 μmol/L in sham rats (P<0.05). The maximal response was larger in DOCA-salt MA (31±4% versus 19±5%), but this did not reach statistical significance (P=0.057) (Figure 1C).

**Topical L-NAME treatment (0.3 mmol/L; 20 minutes) decreased MA-OD by 10% in sham and 5% in DOCA-salt rats without changing HR or MAP. L-NAME inhibited vasodilation caused by Ach (0.1 μmol/L) by >70% without changing SNP (0.1 μmol/L) responses in sham or DOCA-salt MA (not shown). L-NAME treatment did not affect the vasodilation caused by tempol in sham or DOCA-salt rats (Figure 1C).**

**Tempol, But Not Tiron, Reduced MAPP in Isolated Perfused MA**

MAPP increased to 120±15 mm Hg by NE (1 to 2 μmol/L) and 120±13 mm Hg by KC (60 to 80 mmol/L) in sham (n=8), and 140±15 mm Hg by NE and 127±15 mm Hg by KC in DOCA-salt MA (n=9) (P<0.05).

Increased MAPP caused by NE was stable for >20 minutes in sham and DOCA-salt MA (Figure 2A, 2B).
2C and 2D show tempol (3 mmol/L) responses in sham and DOCA-salt MA. In DOCA-salt MA, tempol caused a larger decrease in MAPP (−87±7% versus −45±6% at 3 mmol/L, P<0.05) and the left-shifted dose-response curve (Figure 2F). Tempol did not change MAPP in KCl-preconstricted sham or DOCA-salt MA (−7±4% and −10±5%, n=5; respectively, P>0.05) (Figure 2E, 2F).

To determine whether depressor responses caused by tempol could be mimicked by another antioxidant, tiron (3 mmol/L) was applied to NE-preconstricted MA. Tiron did
not change MAPP in DOCA-salt (−10±4%, n=4, P=0.05) or sham arteries (−7±3%, n=4, P>0.05).  

**BK Channel Antagonists Block Depressor Responses Caused by Tempol**

We used IBTX (BK channel blocker, 0.1 μmol/L), glibenclamide (ATP-sensitive K⁺ channel blocker, 1 μmol/L), or apamin (small-conductance Ca²⁺-activated K⁺ (SK) channel blocker, 1 μmol/L), and a BK channel selective concentration of TEA (1 mmol/L). IBTX and TEA inhibited tempol-induced depressor responses in sham and DOCA-salt MA (Figure 3A, 3C). IBTX did not affect depressor responses of SNP (1 μmol/L) (Figure 3B, 3C). Apamin and glibenclamide did not affect depressor responses of tempol (Figure 3C).

**Increased BK Channel Function and α-Subunit Expression in MA from DOCA-Salt Rats**

In anesthetized sham and DOCA-salt rats, the MA constriction caused by IBTX (0.1 μmol/L, 15 minutes) was larger in DOCA-salt than in sham rats (−18±4% versus −8±5%, n=4, P<0.05) (Figure 4A, 4B, 4C). IBTX caused a higher frequency of phasic constrictions in sham than in DOCA-salt MA. Constrictions caused by KCl (80 mmol/L) were similar between DOCA-salt and sham rats (−54±6% versus −50±4%, n=4, P>0.05). The resting MA-OD in sham and DOCA-salt rats was 265±10 μm (n=4) and 275±15 μm (n=4) (P>0.05), respectively.

In 5 separate comparisons using different protein isolations, immunoblot density corresponding to the BK channel α-subunit (100-kDa bands; Figure 5A) were increased by 77±4% in DOCA-salt aorta and 136±7% in DOCA-salt MA compared with levels observed in sham MA (Figure 5B).

Figure 6A, 6B, and 6C show brightfield images to illustrate isolated VSMC morphology. Immunocytochemical studies in single MA VSMC from 3 sham and DOCA-salt rats showed increased fluorescence intensity in cells from DOCA-salt rats (Figure 6E) compared with those from sham rats (Figure 6D). Immunofluorescence was absent in cells exposed to the anti Kca²⁺ 1.1 antibody that had been pre-absorbed the competing peptide (Figure 6F).

**Tempol Increased BK Currents in Transfected HEK 293 Cells**

Outward currents (Iₒ) were observed in 147 mslo transfected HEK-293 cells from 24 separate transfections. (Figure 7A, 7C). The activation threshold was ≈0 mV. Iₒ was inhibited by IBTX (0.1 μmol/L, n=4) (Figure 7C). In 12 cells transfected with GFP only, Iₒ was not detected with or without tempol treatment (1 mmol/L) (Figure 7D).

Tempol (1 mmol/L) reversibly increased peak currents at +80 mV by 30±5% from control levels in mslo transfected HEK-293 cells (P<0.05, n=7, Figure 8A, 8B). Time control studies showed that there were no differences in consecutive current-voltage curves. IBTX (0.1 μmol/L) completely blocked Iₒ activated by tempol (Figure 8A, 8C, n=3).

When the free Ca²⁺ level in the pipette solution was reduced (5 nmol/L), the peak Iₒ amplitude recorded at +80 mV decreased from 2.1±0.6 to 1.1±0.3 nA (n=3). The peak Iₒ amplitude recorded under reduced Ca²⁺ conditions was increased to 1.3±0.3 nA (+16%) (P>0.01, n=3) by tempol (1 mmol/L).
Discussion
Tempol is a SOD mimetic that quenches O$_2^-$, In the present study, tempol caused an NO-independent arterial dilation in sham and DOCA-salt hypertensive rats in situ and in vitro. BK channel antagonists inhibited tempol responses and the data indicate that vascular BK channels contribute to depressor responses caused by tempol. It is also important to note that vasodilation caused by tempol in DOCA-salt rats was larger than in sham rats. This is the first study showing that arterial BK channel activity and expression are increased in DOCA-salt rats and that BK channel upregulation likely contributes to the larger vasodilation caused by tempol in DOCA-salt rats in situ and in vitro. Furthermore, vasodilation caused by tempol was not mimicked by tiron, suggesting that the vasodilation caused by tempol is independent of an SOD mimetic mechanism. This suggestion is supported by the observation that tempol increases $I_o$ in msl0 transfected HEK-293 cells. These data indicate that tempol may directly activate BK channels.

Function of BK Channels in Arteries
Ca$^{2+}$ influx through voltage-gated Ca$^{2+}$ channels regulates vascular tone. Graded increases in intravascular pressure depolarize VSMC activating L-type Ca$^{2+}$ channels increasing intracellular Ca$^{2+}$. Increased cytoplasmic Ca$^{2+}$ activates ryanodine receptors resulting in Ca$^{2+}$ sparks which activate BK channels. This causes hyperpolarization of the membrane potential ($E_m$), closure of voltage-gated Ca$^{2+}$ channels, and reducing depolarization. Blocking BK channels causes $E_m$ depolarization, an elevation of intracellular Ca$^{2+}$ and vasoconstriction.

In the present study, tempol relaxed MA in anesthetized rats in situ, and in isolated, perfused NE pre-constricted but not KCl pre-constricted MA. These initial data suggested that K$^+$ channels contributed to tempol-induced vasodilation. The vasodilation caused by tempol in vitro was blocked by IBTX, a selective BK channel blocker, but not by SK or ATP-sensitive K$^+$ channel blockers. Therefore, the data presented indicate that activation of vascular BK channels is involved in the vasodilation caused by tempol. This effect likely contributes to the depressor responses caused by tempol in vivo. However, K$^+$ channels on VSMC are not the only target for depressor responses caused by tempol. Tempol is known to activate inhibitory mechanisms in the central and peripheral nervous systems that decrease sympathetic nerve activity. Shokoji et al$^7$ reported that 4-aminopyridine (4-AP)
(0.1 mmol/L) blocked reduction of nerve activity by local application of tempol onto renal sympathetic nerves. They concluded that tempol may activate voltage-gated K⁺ (Kv) channels. Thus, it is possible activation of neuronal K⁺ channels by tempol may also contribute to depressor responses caused by tempol in vivo.

Upregulation of BK Channels in Arteries from DOCA-Salt Rats

BK channels are composed of pore-forming α and accessory β1 subunits.22,23 Upregulation of BK channel α-subunit expression and function occurs in VSMC in SHR and aldosterone-salt hypertensive rats,24–28 suggesting that there is a dynamic relationship between BP and BK channels.25,28 Pressure-induced upregulation of BK channel α-subunit protein levels and channel function in VSMC provides an important counter-regulatory mechanism to prevent further increases in vascular tone. BK channel activity contributes to resting Em in arteries from hypertensive animals. Arteries from normotensive rats constrict only mildly in response to BK channel inhibition, indicating that K⁺ efflux through BK channel makes a smaller contribution to resting Em. This scheme is supported by data from studies showing that inhibition of BK channels constricts MA and cerebral arteries in SHRs and aldosterone-salt hypertensive rats.24–29

In the present study, tempol caused larger vasodilations in anesthetized DOCA-salt rats in situ and in isolated and perfused DOCA-salt MA in vitro compared with sham rats. In addition, IBTX caused larger MA constrictions in anesthetized DOCA-salt rats in situ, indicating that BK channel expression or activity was increased. This conclusion is supported by data from studies showing that inhibition of BK channels constricts MA and cerebral arteries in SHRs and aldosterone-salt hypertensive rats.24–29

Figure 6. Upregulation of BK channel α-subunit in isolated VSMC from DOCA-salt MA A, B, and C, brightfield images (BF) of sham and DOCA-salt MA smooth muscle cells. D, E, same smooth muscle cells labeled with anti KCa²⁺ 1.1 antibody (P) and visualized with Cy3 (red). Immunofluorescence was increased in DOCA-salt smooth muscle cells. DAPI staining (blue) indicates cell nucleus. F, Pre-absorption of anti KCa²⁺ 1.1 with competing peptide (CP) blocked staining. Images are typical of 3 separate experiments. Images were obtained and displayed using the same exposure, brightness and contrast settings.

Figure 7. Iₒ recording from m slo transfected HEK 293 cells. A, Iₒ recorded from m slo/GFP transfected HEK-293 cells. (B), I-V relationship of the Iₒ. Data points are mean ± S.E. (n=8). (C), Iₒ recorded from m slo transfected cell after cell membrane was depolarized from a holding potential of -70 mV and at a test potential of +80 mV. IBTX (0.1 μmol/L) blocked the Iₒ. D, Iₒ recorded from a HEK-transfected with GFP only with or without tempol treatment.

The Role of O₂⁻ in Regulating Vascular BK Channel Function

O₂⁻ is elevated in the aorta, and MA from DOCA-salt rats,2,30 scavenging O₂⁻ or inhibiting its synthesis will increase the bioavailability of NO and will reduce the activity of the
signaling mechanisms activated by $\text{O}_2^{-}$ to reduce vascular tone. $\text{O}_2^{-}$ directly inhibits BK channel activity making the channel less sensitive to $\text{Ca}^{2+}$. Direct application of peroxynitrite to skeletal muscle resistance and human coronary arteries inhibits BK channel activity and causes vasoconstriction. Antioxidant treatment restores impaired BK channel function. NO increases BK channel activity in VSMC and cslo-transfected HEK-293 cells. This response is mediated by cGMP-dependent channel phosphorylation. Previous reports indicate that NOS inhibitors do not block tempol effects on BP and RSNA. It should be noted that acute treatment with antioxidants (ascorbic acid and tiron), other than tempol, does not lower blood pressure in hypertensive patients or in DOCA-salt hypertensive rats, although SOD and tiron quench $\text{O}_2^{-}$ in human saphenous vein and internal mammary artery by >70%. Similarly we found that tiron did not mimic the effects of tempol on isolated, perfused NE preconstricted MA, this suggests that reduction of $\text{O}_2^{-}$ is not the only mechanism by which tempol causes vasodilatation. Our results indicated that tempol might directly activate BK channels. This possibility is supported by data obtained from studies of HEK-293 cells transiently transfected with mslo channels. Tempol increased $I_o$ in a reversible and IBTX-sensitive manner in these cells. However, it is unclear if tempol is activating mslo by quenching $\text{O}_2^{-}$ in HEK-293 cells or if the effects of tempol are related to the spin trap properties of tempol. Thus, we cannot completely exclude a role for $\text{O}_2^{-}$ and NO in tempol caused vasodilation. Therefore, the signaling mediating BK activation channel caused by tempol needs further study. It will also be important to using whole cell and single channel recording techniques to tempol activates BK channels occurs in myocytes.

Tempol converts $\text{O}_2^{-}$ to hydrogen peroxide ($\text{H}_2\text{O}_2$) which is acted on by endogenous catalase to produce $\text{O}_2$ and $\text{H}_2\text{O}$. Tempol could increase levels of $\text{H}_2\text{O}_2$ over those that can be handled by $\text{H}_2\text{O}_2$-scavenging systems. This pathway is relevant to our data as $\text{H}_2\text{O}_2$ relaxes porcine coronary arteries by stimulating BK channel activity via a phospholipase A2-coupled signaling cascade. However, in MA, $\text{H}_2\text{O}_2$ induced vasodilation was largely independent of BK channel activity and exogenous $\text{H}_2\text{O}_2$ inhibits BK channels in skeletal muscle and hslo-transfected HEK-293 cells. Endogenous $\text{O}_2^{-}$ in MA and mslo-transfected HEK-293 cells could be converted to $\text{H}_2\text{O}_2$ by tempol and $\text{H}_2\text{O}_2$ could be the activator of BK channels as occurs in coronary arteries. It is also anticipated that this pathway would be unaffected by NOS inhibition accounting for our observation that tempol-induced vasodilation is resistant to L-NAME treatment.

**Conclusion**

Tempol activates vascular BK channel to cause vasodilation which is larger in DOCA-salt than normotensive rats. Increased BK channel $\alpha$-subunit protein may provide the fundamental explanation for increased channel activity. Finally, tempol increases $I_o$ in that in mslo-transfected HEK 293 cells and could be blocked by IBTX, providing direct evidence that activation of BK channel contributes to the vasodilation caused by tempol. Tempol causes arterial vasodilation suggesting that the antihypertensive effects of tempol may be mediated by direct activation of BK channel in cardiovascular system, but the role of $\text{O}_2^{-}$ in regulation of BK channel function in DOCA-salt hypertension is still unclear.
Perspectives

BK channels are expressed in VSMCs, where they are negative-feedback regulators of vascular tone. Oxidative stress is prominent in human hypertension and antioxidants have potential therapeutic applications for the treatment of hypertension and associated pathologies. Tempol, a drug that can scavenge $O_2^-$, lowers blood pressure after acute treatment of hypertensive rats. Tempol activates vascular BK channel and inhibits sympathetic nerve activity suggesting that the antihypertensive effects of tempol may be mediated by direct dilation of vasculature and inhibition of sympathetic input to the cardiovascular system. Our results indicate that activation of BK channel could reduce blood pressure in hypertensive subjects. These data suggest that drugs, like tempol, that have antioxidant and vascular BK channel activator properties would be novel treatments for the prevention or treatment of hypertension.

Acknowledgments

We thank Dr. Christopher J. Lingle, (Department of Anesthesiology, Washington University School of Medicine, St. Louis, Mo) for a gift of the nsls plasmid. We thank Drs James J Galligan and Gregory D Fink for advice. We also thank Catherine M. Rondelli for technical assistance. This work was supported by National Heart, Lung, and Blood Institute grant numbers: HL-63973, HL-24111, and PO1 HL70687, and by postdoctoral fellowship grant from American Heart Association Mid-West Affiliate for Dr. Xu (0325510Z).

References


Activation of Vascular BK Channel by Tempol in DOCA-Salt Hypertensive Rats
Hui Xu, Xiaochun Bian, Stephanie W. Watts and Alexandra Hlavacova

Hypertension. 2005;46:1154-1162; originally published online October 10, 2005;
doi: 10.1161/01.HYP.0000186278.50275.fa

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/46/5/1154

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at: http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at: http://hyper.ahajournals.org/subscriptions/