Activation of Potassium Channels by Tempol in Arterial Smooth Muscle Cells From Normotensive and Deoxycorticosterone Acetate-Salt Hypertensive Rats

Hui Xu, William F. Jackson, Gregory D. Fink, James J. Galligan

Abstract—Large-conductance Ca$^{2+}$-activated potassium (BK) channels modulate vascular tone. Tempol, an O$_2^-$ dismutase mimic, causes vasodilation via activation of vascular BK channels. In this study, we investigated the mechanisms underlying tempol-induced activation of BK channels in mesenteric arterial (MA) myocytes from sham and deoxycorticosterone acetate (DOCA)-salt hypertensive rats. In sham myocytes, whole-cell patch clamp studies showed that tempol enhanced peak outward currents ($I_o$). This effect was larger in DOCA-salt myocytes. Tempol caused a leftward shift in the activation curve for $I_o$ in sham and DOCA-salt myocytes. In DOCA-salt myocytes, the peak $I_o$ at +80 mV did not differ from sham myocytes, but iberiotoxin (BK channel blocker) caused a larger reduction of $I_o$ in DOCA-salt compared with sham myocytes. Iberiotoxin but not 4-aminopyridine blocked the $I_o$ activated by tempol. Tiron, another O$_2^-$ scavenger, had no effect on $I_o$. Using inside-out patches, we found that tempol caused a 4-fold increase in open probability ($P_o$) of BK channels but did not change the mean channel open time in sham and DOCA-salt myocytes. Tempol did not change single channel conductance in sham or DOCA-salt myocytes. Western blot and immunocytochemical studies revealed that BK channel $\alpha$-subunit expression was increased in DOCA-salt MA compared with sham MA. The data indicate that tempol directly activates BK channels by increasing channel $P_o$. We conclude that upregulation of the BK channel $\alpha$-subunit protein and tempol-induced increases in BK channel $P_o$ contribute to the enhanced depressor response caused by tempol in DOCA-salt hypertensive rats. (Hypertension. 2006; 48:1080-1087.)

Key Words: tempol $\bullet$ O$_2^-$ $\bullet$ vascular BK channel $\bullet$ myocytes $\bullet$ hypertension

O$^-$ is produced in the vasculature of hypertensive animals and humans, and O$_2^-$ increases blood pressure in part by reducing the bioavailability of NO. Tempol, an O$_2^-$ dismutase (SOD) mimic, lowers blood pressure in normotensive and hypertensive rats by multiple mechanisms. Acute tempol treatment of normotensive, deoxycorticosterone acetate (DOCA)-salt and spontaneously hypertensive rats (SHRs) inhibits sympathetic nerve activity, and this effect is not prevented by NO synthase inhibition. Studies on local application of tempol onto renal sympathetic nerves decreased nerve activity. These investigators suggested that changes in K$^+$ channel activity might mediate sympathoinhibition caused by tempol. In our recent studies, tempol but not tiron (another SOD mimetic) dose-dependently relaxed the mesenteric artery (MA) in situ and in isolated, perfused and preconstricted MA from sham and DOCA-salt hypertensive rats. The responses were twice as large in DOCA-salt rats. The responses were blocked by iberiotoxin (IBTX) but not by antagonists of other classes of potassium channel. Furthermore, the actions of tempol on large-conductance Ca$^{2+}$-activated potassium (BK) channels were independent of NO availability. IBTX caused larger constrictions in DOCA-salt MA, and Western blot and immunocytochemical data revealed increased expression of the BK channel $\alpha$-subunit protein in DOCA-salt compared with sham MA.

To probe the mechanism of BK channel activation by tempol, whole-cell patch clamp studies were conducted on HEK-293 cells that had been transiently transfected with mslo, the murine BK channel $\alpha$-subunit. These studies showed that tempol increased the amplitude of K$^+$ currents in mslo-transfected cells, and the tempol-activated currents were blocked by IBTX. The data indicated that tempol activates BK channels. It is still unclear whether tempol works directly on the channel or via an indirect pathway involving O$_2^-$. The HEK-293 cells transfected with mslo will express these channels at concentrations higher than those found in myocytes. However, transfected cells may not express the same modulatory proteins as the myocytes. Therefore, it is important to investigate the effects of tempol on BK currents in their native environment (myocytes) rather than in a heterologous expression system (HEK-293 cells). In the present studies, we investigated the mechanisms underlying tempol-induced activation of BK channels in MA myocytes from sham and DOCA-salt hypertensive rats. Effects of tempol on BK channel activity in myocytes were determined using whole-cell and inside-out patch clamp methods. BK channel...
function and expression of α-subunit protein levels were also compared in sham and DOCA-salt MA.

**Methods**

**Animals**

Animal use protocols were approved by the Institutional Animal Use and Care Committee at Michigan State University. Sham operated and DOCA-salt hypertensive rats (male Sprague–Dawley, 170 to 225 g, Charles River Laboratories, Portage, Mich) were prepared as described previously.6,8 Blood pressure was measured in conscious animals by tail-cuff plethysmography 4 weeks after DOCA implantation.

**Whole-Cell and Inside-Out Single Channel Recording in MA Myocytes**

The mesenteric bed was removed from rats after euthanasia. Enzymatic isolation of single MA myocytes was performed according to published methods for dissociation of rat MA (250 to 350 μm, OD).9 Patch-clamp recordings were obtained as reported previously.8 All of the recordings were made using an Axopatch 200A voltage clamp amplifier and a Digidata 1322A (Axon Instruments) A/D converter. pClamp 9.0 software (Axon Instruments) was used to generate voltage-clamp protocols and acquire and analyze whole-cell and single channel currents. Ca2+ (CaCl2) was added to achieve the desired level of [Ca2+]i (http://www.stanford.edu/group/unixmax/clpellmax.html). For whole-cell recordings, the extracellular solution contained (mM): KCl 140, EGTA 1.0, CaCl2 0.018 (300 mmol/L [Ca2+]). The extracellular solution contained (mmol/L): NaCl 140, KCl 5.4, MgCl2 1.0, HEPES 10 (pH 7.4), and amphotericin B (240 μg/mL). Cells were held at −70 mV, and families of macroscopic K+ currents were generated by depolarizing with a series of 200-ms step commands to +80 mV in 10-mV increments at 15-s intervals. Outward current (Io) measured at +80 mV was designated as the maximum current (peak Io). In a single cell, the peak BK current was defined as the difference between the total outward current recorded in drug-free bath solution and the current remaining after treatment with IBTX (0.1 μmol/L). Unitary BK currents in myocytes were obtained in inside-out patches. Myocytes were bathed in a solution containing (mM/L): NaCl 140, 1 EGTA or 1 HEDTA, and 10 HEPES (pH 7.3). Pipettes were filled with the same 140-mmol/L K+ solution without Ca2+ supplementation. Single BK channel currents were recorded from inside-out patches under symmetrical K+ (140 mmol/L) and subjected to membrane test potentials between −60 and +100 mV. Averaged current amplitudes at each potential were obtained for calculation of single-channel conductance. Channel openings were defined as those currents crossing the 50% threshold level of the peak single-channel current. Single channel currents were recorded for 2 minutes after the inside-out patch recording condition had been established. Single channel data were analyzed using Clampfit protocols that are part of the pClamp 9 (Axon Instruments) suite of programs. BK channel number, conductance, and open probability (Po) were determined from all-points amplitude histograms. All of the experiments were performed at room temperature (22 to 25°C).

**Analysis of Vascular BK Channel α-Subunit Expression**

For Western blot analysis, we used a primary antibody targeted to BK channel α-subunits (anti-slo 1, 1:10, Antibodies Inc, Davis, Calif) and a secondary antibody conjugated to horseradish peroxidase. The membranes were developed using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech) and exposed to film (Hyperfilm-ECL, Amersham Pharmacia Biotech). α-Actin was used to normalize protein loading on membranes.

For immunocytochemical analysis, myocytes were isolated and fixed immediately for 20 minutes.8 Myocytes were incubated with anti-slo 1 (1:100; Antibodies Inc) overnight at 4°C. Myocytes were then incubated with sheep anti-mouse IgG conjugated to fluorescein isothiocyanate (1:50, Jackson ImmunoResearch) and 4′,6-diamidino-2-phenylindole (10 μg/mL; Sigma) for 1 hour at room temperature. Images were obtained using a fluorescence microscope (Nikon, TE 2000-U) and a 60× objective. Images were processed using Metamaging Series 6.1 and Adobe Photoshop 8.0 software.

**Drugs**

Tempol, IBTX, 4-aminopyridine (4-AP), and tiron were purchased from Sigma Chemical Co.

**Statistics**

Data are reported as mean±SE from the number of rats. Paired and unpaired t tests were used to make single point comparisons. Current-voltage (I-V) relationships were determined by measuring steady-state current (ie, at the end of the 200-ms test pulse) and compared between the groups. Comparisons of multiple points generated in I-V curves were accomplished using a 2-way ANOVA with repeated measures followed by Student–Newman–Keuls test. Significance was set at P<0.05.

**Results**

Forty-three sham and 44 DOCA-salt rats were used for the studies. In these animals, average systolic blood pressure was higher in DOCA-salt than sham rats (195±4 mm Hg versus 120±5 mm Hg; P<0.05). At the time of the experiments, the body weight of sham rats was 420±8 g and 320±7 g in DOCA-salt rats (P<0.05).

**Tempol, But Not Tiron, Enhanced IBTX-Sensitive Currents in Myocytes From Sham and DOCA-Salt MA; IBTX-Sensitive Currents Were Increased in DOCA-Salt Myocytes**

The effects of tempol on Io were studied in sham and DOCA-salt myocytes (Figure 1A). I-V curves for Io were also obtained (Figure 1B). The activation threshold for Io from sham and DOCA-salt myocytes was ≈0 mV (Figure 1B). Io was recorded before and after the addition of tempol to bath solution. Tempol enhanced peak Io in a concentration-dependent fashion (Figure 1C). In sham myocytes, tempol (3 mmol/L) increased peak Io from 3.1±0.4 nA to 4.4±0.7 nA (≈50%; P<0.05; n=6); this effect was larger in DOCA-salt myocytes (3.4±0.6 nA to 6.8±0.7 nA; ≈90%; P<0.05; n=9; Figure 1A and 1B). Tempol also caused a greater leftward shift in the activation curve for Io in DOCA-salt myocytes (Figure 1B). The increase in Io caused by tempol was completely blocked by IBTX. Finally, IBTX reduced the peak Io below the baseline levels measured before tempol treatment (Figure 1A and 1B), indicating that IBTX blocked both basal and tempol-enhanced Io. The amplitude of BK and other K+ currents in sham and DOCA-salt myocytes were compared before and after treatment with IBTX (0.1 μmol/L; 10 minutes; n=4). Peak Io was similar in sham and DOCA-salt myocytes, but IBTX caused a larger reduction in peak Io in DOCA-salt (3.4±0.5 nA to 1.2±0.2 nA; ≈60%) than in sham myocytes (3.1±0.5 nA to 1.8±0.2 nA, ≈40%; n=4; P<0.05; Figure 1D). Tempol did not increase Io in sham or DOCA-salt myocytes after IBTX treatment (Figure 1D).

The effects of tempol on Io were also studied in DOCA-salt myocytes after treatment with 4-AP (1 mmol/L; voltage-gated K+ channel blocker). Peak Io was reduced by 4-AP treatment, but tempol still increased Io in 4-AP-treated myocytes (Figure 2A and 2B).
To determine whether the effects of tempol on $I_o$ were mimicked by other antioxidants, tiron (3 mmol/L) was applied to myocytes ($n=11005$; Figure 3A and 3B). Tiron did not change $I_o$ in sham and DOCA-salt myocytes.

Tempol Increased $P_o$ of Single BK Channels

These experiments examined the direct effects of tempol on BK channel activity in inside-out patches in myocytes. Unitary BK currents were recorded before and after the addition of tempol (3 mmol/L) to the solution bathing the intracellular surface of the patches. Single-channel conductance of BK channels was similar in sham and DOCA-salt myocytes (Figure 4A through 4C). Tempol did not change single-channel conductance in sham ($210\pm14$ pS versus $220\pm15$ pS; $P>0.05$) or DOCA-salt myocytes ($225\pm10$ pS versus $235\pm11$ pS; $n=6$; $P>0.05$; Figure 4D). Under control conditions, $P_o$ of BK channels was similar in sham and DOCA-salt myocytes (at $+80$ mV). Tempol increased $P_o$ in sham and DOCA-salt myocytes (Figures 4A, 4B, 5A, and 5B), but tempol did not change the mean channel open time in patches from sham or DOCA-salt myocytes (Figure 5C).

Increased BK $\alpha$-Subunit Expression in MA From DOCA-Salt Rats

BK channel $\alpha$-subunit protein levels in MA were analyzed by Western blot. Figure 6A shows that an antibody raised against $slo$ revealed a 100-kDa band in sham and DOCA-salt MA but not in the whole-cell proteins from untransfected HEK-293 cells. This is similar in size to the BK channel $\alpha$-subunit. In 8 separate comparisons using different protein isolations, Western blots revealed that BK channel $\alpha$-subunit expression was increased by $125\pm7\%$ in DOCA-salt MA compared with sham MA (Figure 6B).

Fluorescence images were compared in myocytes obtained from 3 sham and 3 DOCA-salt rats. Under the same exposure setting, immunocytochemical studies in single myocytes showed increased fluorescence intensity related to the BK channel $\alpha$-subunit in DOCA-salt compared with sham myocytes (Figure 6C). The specificity of staining for the BK channel $\alpha$-subunit was confirmed in HEK-293 cells that had been transiently transfected with $m\alpha$lo, whereas no staining was present in untransfected HEK-293 cells (data not shown).

Discussion

Tempol has been used extensively as a tool to probe the contribution of elevated levels of $O_2^*$ to increased vascular tone in animal models of hypertension. Tempol treatment lowers blood pressure in hypertensive animals, and this effect has been attributed to the SOD mimetic properties of tempol. However, recent evidence indicates that tempol can have depressor effects that may be independent of its SOD mimetic properties. One of
these additional effects is to activate vascular BK channels. An effect of tempol on BK channels is also supported by studies showing that tempol can increase BK currents in HEK-293 cells that had been transfected with the BK channel /H9251-subunit. In the present study, tempol enhanced IBTX-sensitive Io in myocytes from sham and DOCA-salt MA. Enhancement of Io by tempol was larger in DOCA-salt myocytes. IBTX also caused a greater reduction of Io in DOCA-salt myocytes. Western blotting and immunocytochemical studies revealed that expression of BK channel /H9251-subunits was increased in DOCA-salt MA. Tempol caused a 4-fold increase of BK channel Po without changing mean open time or single-channel conductance in sham and DOCA-salt myocytes. Furthermore, the effects of tempol on Io were not mimicked by tiron. These data indicate that tempol may directly activate vascular BK channels.

**Activation of BK Channels in MA Myocytes**

Activation of BK channels causes hyperpolarization of the membrane potential, closure of voltage-gated Ca2+/H11001 channels, and reduction of vascular tone. In our previous studies, tempol caused an IBTX-sensitive I, in myocytes from sham and DOCA-salt MA. Enhancement of I, by tempol was larger in DOCA-salt myocytes. IBTX also caused a greater reduction of I, in DOCA-salt myocytes. Western blotting and immunocytochemical studies revealed that expression of BK channel /H9251-subunits was increased in DOCA-salt MA. Tempol caused a 4-fold increase of BK channel Po without changing mean open time or single-channel conductance in sham and DOCA-salt myocytes. Furthermore, the effects of tempol on I, were not mimicked by tiron. These data indicate that tempol may directly activate vascular BK channels.

**Upregulation of MA BK Channels in DOCA-Salt Rats**

BK channels are composed of pore-forming α- and accessory β1-subunits. Increases in BK channel α-subunit expression and function occur in arteries from SHR and aldosterone-salt hypertensive rats suggesting that there is a dynamic relationship between blood pressure and BK channels. Pressure-induced upregulation of BK channel α-subunit protein levels and channel function in vasculature provides an important counterregulatory mechanism to prevent further increases in arterial tone. We found previously that tempol caused larger IBTX-sensitive vasodilations in DOCA-salt MA. In the present studies, IBTX-sensitive I, was larger in DOCA-salt myocytes, but there was no change in single-channel conductance in sham and DOCA-salt myocytes. These data indicate that BK channel expression might be increased in DOCA-salt MA. These functional data are consistent with Western blot and immunocytochemical data that show increased BK channel α-subunit expression in DOCA-salt MA. Based on the current and previously published work, we conclude that increased BK channel expression and function contributes to increased depressor responses caused by tempol in DOCA-salt hypertension.
Smooth muscle cell depolarization, enhanced Ca\(^{2+}\)/H\(_{11001}\) current, and increased \([\text{Ca}^{2+}]_{i}\) contribute to augmented vascular tone in hypertension. Membrane potential is determined, in part, by K\(_{H11001}\) channel activity. Changes in the activity and/or expression of vascular K\(_{H11001}\) channels in hypertension depend on the specific subtype of K\(_{H11001}\) channel, the specific artery, and hypertension model. For example, decreased voltage-gated K\(_{H11001}\) currents occur in MA from SHR, DOCA-salt, and N\(_{H9275}\)-nitro-L-arginine–induced hypertensive rats. However, BK channel currents in arterial myocytes are known to increase as a compensatory response to increased vascular pressure. In the present study, we did not assess the role of voltage-gated K\(_{H11001}\) channel function in DOCA-salt myocytes, but we did find that tempol increased \(I_o\) even in the presence of 4-AP. Therefore, the actions of tempol on \(I_o\) are independent of voltage-gated K\(_{H11001}\) channels.

It is important to note that we measured an increase in IBTX-sensitive currents in DOCA-salt myocytes without a change in peak \(I_o\). The unchanged peak \(I_o\) in DOCA-salt myocytes would be accounted for by the reduction of the amplitude of IBTX-insensitive, voltage-dependent K\(_{H11001}\) currents that we also showed here. The increase in BK currents would be offset by the reduction of voltage-dependent K\(_{H11001}\) currents, and there would be no net increase in total \(I_o\). In addition, BK channel function, particularly in hypertension, is regulated by multiple intracellular signaling pathways and extracellular signaling molecules (\(O_2\), eg, see below). Changes in intracellular or extracellular signaling in hypertension may regulate BK channel function independent of the level of channel expression. It is also possible that upregulated BK channels are not fully functional in DOCA-salt hypertension. Single channel \(P_o\) measured in the inside-out patch configuration was similar in sham and DOCA-salt myocytes. As the intracellular pathways that might modify BK channel function are eliminated under these recording conditions, signaling mechanisms modifying BK channel activity in DOCA-salt myocytes would be eliminated, and the role of additional mechanisms that might modify BK channel activity would not be assessed. It is also unclear whether the expression of BK channels in the cell membrane is increased in DOCA-salt myocytes. Western blot measurements only assess total BK channel protein and not its subcellular distribution.

Figure 4. Effects of tempol on single BK channels in inside-out patches from sham and DOCA-salt myocytes. Unitary BK channel currents in sham (A) and in DOCA-salt myocytes (B) before and after tempol (3 mmol/L). Data show that tempol increased the frequency of channel opening without changing single channel current amplitude. C, Unitary BK channel currents in DOCA-salt myocytes at positive and negative potentials ([\([\text{Ca}^{2+}]_{i}\) 500 nmol/L]. D, Current-voltage relationship for BK channels in sham and DOCA-salt myocytes before and after tempol treatment. Tempol did not change the slope of the current voltage relationship indicating that tempol does not affect single channel conductance.
tion in BK current secondary to diminished expression of the regulatory β1-subunit in SHR and angiotensin II-induced hypertension without a change in expression of α-subunits. Other studies in N-nitro-L-arginine–induced hypertensive rats demonstrated that there was a reduced BK channel α-subunit expression and BK current but no difference in the Ca2+/voltage sensitivity of BK channels.30,31 These data suggest that the effect of hypertension on BK channel expression may be model dependent.

**Tempol Directly Activates BK Channels by Increasing Channel Open Probability**

O2 is elevated in the arteries from DOCA-salt rats,2,34 and O2 directly inhibits BK channel activity,35,36 making the channel...
less sensitive to Ca\(^{2+}\). Tempol is a spin trap reagent and an SOD mimic. In the present study, effects of tempol on whole-cell BK currents were dose dependent, which is consistent with our previous studies in vivo and in vitro, which showed that the tempol causes dose-dependent depressor responses in vasculature.\(^{2,4,6,8}\) However, intracellular pathways that might modify BK channel function were eliminated by using the inside-out patch clamp configuration to study single BK channels. Therefore, the effects of tempol on BK channels would not be affected by intracellular signaling molecules, such as O\(_2\). We found that tempol increased BK channel P\(_o\), in inside-out patches obtained from sham and DOCA-salt myocytes. These data indicate that tempol directly activates vascular BK channels. Although tempol induced larger increases in whole-cell BK currents in DOCA-salt myocytes, there was no difference in the increase in P\(_o\) in patches from sham and DOCA-salt myocytes. Furthermore, tempol did not change single channel conductance or density O\(_2\). Nevertheless, the effects of tempol on BK currents were mimicked by another antioxidant, tiron, and tiron does not affect blood pressure, nor does it cause vasodilatation.\(^9\) The data indicate that the effects of tempol on BK channel function were O\(_2\) independent. However, there is a substantial volume of data indicating that tempol does scavenge O\(_2\), and this effect contributes to the depressor effects of tempol in hypertension. It should also be made clear that tempol is likely to lower blood pressure through multiple mechanisms and that direct BK channel activation is only 1 of these mechanisms.

Conclusions

The SOD mimic drug tempol can act as a direct vascular BK channel activator. Tempol acts to increase BK channel P\(_o\), and the greater effects of tempol on whole-cell currents in myocytes from DOCA-salt MA are because of an increase BK channel \(\alpha\)-subunit expression in these cells. The actions of tempol on BK channels are independent of O\(_2\), but they are likely to contribute to the depressor effects of tempol studied in vitro and in vivo.

Perspectives

BK channels are negative-feedback regulators of vascular tone.\(^{12–15}\) 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 the 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 the vascular 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.

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Disclosures

None.

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