Therapeutic Targeting of Mitochondrial Superoxide in Hypertension

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Rationale: Superoxide ($O_2^-$) has been implicated in the pathogenesis of many human diseases including hypertension; however, commonly used antioxidants have proven ineffective in clinical trials. It is possible that these agents are not adequately delivered to the subcellular sites of superoxide production.

Objectives: Because the mitochondria are important sources of reactive oxygen species, we postulated that mitochondrial targeting of superoxide scavenging would have therapeutic benefit.

Methods and Results: In this study, we found that the hormone angiotensin (Ang II) increased endothelial mitochondrial superoxide production. Treatment with the mitochondria-targeted antioxidant mitoTEMPO decreased mitochondrial $O_2^-$, inhibited the total cellular $O_2^-$, reduced cellular NADPH oxidase activity, and restored the level of bioavailable NO. These effects were mimicked by overexpressing the mitochondrial MnSOD (SOD2), whereas SOD2 depletion with small interfering RNA increased both basal and Ang II–stimulated cellular $O_2^-$. Treatment of mice in vivo with mitoTEMPO attenuated hypertension when given at the onset of Ang II infusion and decreased blood pressure by 30 mm Hg following establishment of both Ang II–induced and DOCA salt hypertension, whereas a similar dose of nontargeted TEMPOL was not effective. In vivo, mitoTEMPO decreased vascular $O_2^-$, increased vascular NO production and improved endothelial-dependent relaxation. Interestingly, transgenic mice overexpressing mitochondrial SOD2 demonstrated attenuated Ang II–induced hypertension and vascular oxidative stress similar to mice treated with mitoTEMPO.

Conclusions: These studies show that mitochondrial $O_2^-$ is important for the development of hypertension and that antioxidant strategies specifically targeting this organelle could have therapeutic benefit in this and possibly other diseases. (Circ Res. 2010;107:106-116.)

Key Words: hypertension ■ mitochondria ■ superoxide ■ mitochondrial targeted antioxidant

During normal mitochondrial function, a small percentage of electrons from the electron transport chain reduce oxygen to form superoxide ($O_2^-$). In several common conditions, such as atherosclerosis, ischemia/reperfusion injury and aging, the mitochondria become dysfunctional and this leak of electrons is increased. The mitochondria contain a unique form of superoxide dismutase (SOD), the manganese-containing mitochondrial SOD (SOD2), which is critical in protecting against excessive production of $O_2^-$. Mice lacking this enzyme die of a cardiomyopathy within 10 days of birth and mice lacking one allele of SOD2 (SOD2$^{+/−}$ mice) develop hypertension with aging and in response to a high salt diet.

The development of hypertension in SOD2$^{+/−}$ mice is in keeping with a role of reactive oxygen species (ROS) in the pathogenesis of this and many other vascular diseases. Hypertension has been associated with increased ROS production in the vasculature, the kidney and in portions of the central nervous system that control blood pressure. The hormone angiotensin II (Ang II), commonly implicated in hypertension, increases ROS production in the sites. Moreover, ROS overproduction leads to decreased bioavailability of NO, impairs endothelium-dependent vasodilation, and promotes vasoconstriction. These alterations occur early in the development of vascular disease.

There is substantial interest in the enzymatic source of ROS in hypertension. Ang II stimulates the NADPH oxidase in many mammalian cells via pathways involving protein kinase C and the tyrosine kinase c-Src. Ang II also activates the NADPH oxidase in vivo and mice lacking components of this enzyme are resistant to both Ang II and salt-dependent hypertension. Specific inhibitors of the NADPH oxidase have antihypertensive effects. Another potential source of ROS in hypertension is the...
mitochondria. We have previously found that Ang II increases production of mitochondrial ROS, decreases mitochondrial membrane potential, and reduces the respiratory control ratio. These deleterious effects of Ang II on mitochondrial function were associated with increased cellular $O_2^-$ production and decreased endothelial NO bioavailability. These studies further indicated that Ang II activation of the NADPH oxidase led to oxidant disruption of mitochondrial function, supporting an important interplay between these 2 sources of ROS, and suggest that mitochondria-derived ROS could contribute to endothelial dysfunction and hypertension. In keeping with this concept, Widder et al recently showed that mice transgenic for the mitochondrial antioxidant enzyme thioredoxin 2, are resistant to Ang II–induced hypertension and endothelial dysfunction. Taken together, these studies suggest that mitochondrial-produced ROS could play an important role in hypertension.

We therefore performed the present study to test the hypothesis that mitochondrial-targeted antioxidant therapy would be effective in both preventing and treating hypertension. To gain further insight into the role of mitochondrial $O_2^-$ in endothelial dysfunction and hypertension, we examined the effects of depleting or overexpressing mitochondrial superoxide dismutase (SOD2) in cultured endothelial cells and transgenic mice with Ang II–induced hypertension. Our data strongly indicate that mitochondrial $O_2^-$ is an important, previously largely ignored, therapeutic target to treat endothelial dysfunction and high blood pressure.

**Methods**

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Reagents**

MitoTEMPO, mitoTEMPO-H, 1-hydroxy-3-carboxy-pyrrolidine (CPH), and nitroxide 3-carboxy-proxyl (CP) were purchased from Alexis Corporation (San Diego, Calif). Xanthine oxidase was purchased from Roche Molecular Biochemicals (Indianapolis, Ind). All other reagents were obtained from Sigma (St Louis, Mo).

**Cell Culture**

Bovine aortic endothelial cells (BAECs) (passage 4 to 8) were cultured on 100-mm plates in media 199 containing 10% FCS supplemented with 2 mmol/L L-glutamine and 1% vitamins. Confluent cells were used for the experiments. Human aortic endothelial cells (HAECs) purchased from Lonza (Chicago, Ill) and cultured in EGM-2 medium supplemented with 2% FBS but without antibiotics. On the day before the study, the FBS concentration was reduced to 1%. In preliminary experiments, we examined the effect of varying doses of Ang II on cellular $O_2^-$ production. We found that 4 hours of Ang II increased cellular $O_2^-$ in a dose-dependent manner with maximum stimulation at 200 nmol/L. This concentration was therefore used in the remainder of the experiments. It should be noted that because degradation in culture, the steady-state concentration of Ang II is substantially lower than that initially added.

**Mitochondrial Isolation and Study**

Mitochondria were isolated as previously described. Complex I–dependent (glutamate plus malate as substrate) or complex II–dependent (succinate as the substrate) mitochondrial respiration was studied using intact mitochondria and fluorescence oxygen monitoring system (Instech Laboratories Inc, Plymouth Meeting, Pa). Mitochondrial $H_2O_2$ was measured by mixing 20 μg of mitochondrial protein with horseradish peroxidase (2 U/mL), peroxidase substrate acetamidophenol (1 mmol/L), SOD (50 U/mL), and spin probe CAT1H (1 mmol/L). Production of mitochondrial $O_2^-$ was visualized in intact cultured HAECs using the fluorescent probe MitoSOX (excitation/emission: 510/580 nm). HAECs were incubated with 2 μmol/L MitoSOX in KHB for 20 minutes at 37°C in CO2 incubator. The mitochondrial subcellular location of MitoSOX was confirmed by colabeling with 50 nmol/L MitoTracker Green FM (excitation/emission: 490/516 nm).

**Measurement of Cellular $O_2^-$, NADPH Oxidase Activity, and NO Levels**

Superoxide was measured using dihydroethidium (DHE) and a high-performance liquid chromatography (HPLC)-based assay with minor modification as described previously. NADPH oxidase activity was measured in membrane preparations prepared as described previously using electron spin resonance (ESR) and the spin probe CPH, and was quantified as NADPH dependent $O_2^-$ production. NO levels in endothelial cells and vessels were quantified by ESR and colloidal Fe(DETC)$_2$ as described previously.

**Modulation of SOD2 Expression**

To manipulate mitochondrial $O_2^-$, we inhibited the expression of mitochondrial SOD2 using small interfering (si)RNA from Qiagen or overexpressed SOD2 using transfection-ready expression plasmid for human SOD2 (Addgene Inc). As a control we used nonsilencing siRNA (Qiagen AllStars negative control) and a green fluorescent protein (GFP) empty plasmid (Lonza). The plasmids were grown in bacteria using standard techniques and purified with a kit (Sigma).

**Animal Experiments**

Hypertension was induced by Ang II (490 ng/kg per minute) as described previously using either C57Bl/6 or mice transgenic for human SOD2 (tgSOD2 mice). In addition, mice received a separate minipump for coinfusion of either TEMPOL, mitoTEMPO or vehi-
cle as described in the figure legends. In other animals, mitoTEMPO treatment was started seven days after saline or Ang II minipump placement. Blood pressure was monitored using either the tail cuff method or telemetry as previously described. Following 14 days of Ang II infusion, the animals were euthanized by CO2 inhalation and aortas were extracted for the analysis of NO and O2 production, and endothelial functions. DOCA salt–induced hypertension was induced as described previously using C57Bl/6 mice. Ten days after surgery, the mice were implanted with osmotic pumps containing saline or mitoTEMPO (0.7 mg/kg per day). Seventeen days after surgery, the animals were euthanized by CO2 inhalation and segments of mouse aorta were used for analysis of vascular NO and O2 production. Endothelium-dependent vasodilatation was analyzed in isolated 3-mm aortic segments in organ chambers as we have previously described.

Statistics
Experiments were analyzed using the Student–Neuman–Keuls post hoc test and ANOVA. P levels of <0.05 were considered significant.

Results
Mitochondrial Accumulation of mitoTEMPO
It has been previously suggested that conjugation to a lipophilic triphenylphosphonium cation allows targeting of an antioxidant to the mitochondria and can prevent mitochondrial oxidative damage and mitochondrial dysfunction. We have developed a new mitochondria-targeted superoxide dismutase mimetic mitoTEMPO (Figure 1A). To confirm mitochondrial accumulation of mitoTEMPO we used ESR to examine the relative intensities of the nitroxide signal in the cell media, mitochondria and cytoplasm following one hour incubation with mitoTEMPO. As evidenced in Figure 1A, the mitoTEMPO accumulation in the cytoplasm fraction was 3-fold greater than that present in the extracellular media, whereas analysis of the mitochondria revealed substantial accumulation of mitoTEMPO up to 15 μmol/L.

Scavenging of O2•− by MitoTEMPO
In additional experiments, we examined the capacity of mitoTEMPO or its reduced form, mitoTEMPO-H, to scavenge O2•−. We used the spin trap EMPO and generated O2•− using xanthine and xanthine oxidase. As evident in Figure 1B, exposure of EMPO to xanthine/xanthine oxidase generated an ESR spectrum typical of the EMPO-OOH radical adduct. Addition of either mitoTEMPO or mitoTEMPO-H inhibited formation of EMPO-OOH radical adduct in dose dependent manner (Figure 1B and 1C). Control experiments showed that neither mitoTEMPO nor mitoTEMPO-H had a direct effect on xanthine oxidase activity (Online Figure II). The estimated IC50’s for

Figure 1. Accumulation of mitoTEMPO in mitochondria and scavenging of O2•− by mitoTEMPO. A, ESR spectra of various compartments from BAECs following incubation with mitoTEMPO (1 μmol/L) for 1 hour. Cells were lysed, cytoplasmic and mitochondrial fractions were isolated by centrifugation. Spectra of the extracellular media, cellular cytoplasm, or mitochondria (10 mg weight for each sample) are shown. B, Spin trapping of O2•− using the spin trap EMPO and xanthine/xanthine oxidase O2•−-generating system in the absence or presence of mitoTEMPO and mitoTEMPO-H. The efficacy of O2•− scavenging was derived from dose-dependent decrease of ESR amplitude of EMPO-OOH in the presence of mitoTEMPO (C) or mitoTEMPO-H (D).
mitoTEMP0 and mitoTEMPO-H were 10 μmol/L and 123 μmol/L, respectively. Based on competition with 50 mmol/L EMPO, which has a rate constant of 74 mol/L⁻¹s⁻¹ for reaction with O₂⁻, the rate constants of reactions of mito-TEMPO and mitoTEMPO-H with O₂⁻ were estimated to be 3.7×10⁵ mol/L⁻¹s⁻¹ and 3.0×10⁴ mol/L⁻¹s⁻¹, respectively. These data are similar to the previously reported rate constants for TEMPOL (6.5×10⁵ mol/L⁻¹s⁻¹) and TEMPONE-H (1.2×10⁴ mol/L⁻¹s⁻¹).³⁰,³¹

The above data indicate that mitoTEMPO would be an effective O₂⁻ scavenger in intact cells. We therefore investigated O₂⁻ dismutation in the cytoplasm and mitochondria of BAECs treated with mitoTEMPO using a commercially available kit (Cayman). Incubation of cells with 25 nmol/L mitoTEMPO increased mitochondrial O₂⁻ dismutation by 3-fold while not affecting cytoplasmic dismutation (Table 1). These data are consistent with mitochondrial accumulation of mitoTEMPO shown in Figure 1A and demonstrate specificity of this agent for mitochondrial protection against O₂⁻.

**Effect of MitoTEMPO on Production of Mitochondrial ROS and Respiration**

We previously reported that Ang II increases mitochondrial ROS production and impairs mitochondrial respiration in endothelial cells.¹⁴ We therefore sought to determine whether mitoTEMPO would ameliorate these effects. To monitor mitochondrial O₂⁻ levels in intact cells, we used the mitochondria-specific fluorescent probe MitoSOX. As expected, stimulation of HAECs with Ang II (200 nmol/L, 4 hours) significantly increased mitochondrial O₂⁻, as reflected by MitoSOX fluorescence (Figure 2A). MitoSOX

![Figure 2. Effect of mitoTEMPO on mitochondrial O₂⁻, endothelial O₂⁻, NO and NADPH oxidase activity. A, Mitochondrial O₂⁻ was measured in control or Ang II–stimulated HAECs using fluorescent probe MitoSOX. Mitochondrial localization of MitoSOX signal was confirmed by colocalization with MitoTracker. B, Activity of NADPH oxidase measured in membrane fractions isolated from unstimulated or Ang II–stimulated BAECs (4 hours, 200 nmol/L) and supplemented for 15 minutes with saline, the mitochondria-impermeable SOD mimetic 3-carboxyproxyl (CP), or the mitochondria-targeted SOD mimetic mitoTEMPO (25 nmol/L). C, Cellular O₂⁻ was measured in intact BAECs using DHE and HPLC. D, Nitric oxide was measured in intact cells after treatment with saline, CP, or mitoTEMPO using ESR and the NO spin trap Fe(DETC)₂.²³ Results are means±SEM (n=5 to 8 each). *P<0.05 vs control; **P<0.05 vs Ang II.](http://circres.ahajournals.org/fig/10.1161.01.CIR.0000310651.17958.62)
fluorescence colocalized with the mitochondria as detected using the probe mitoTracker (Figure 2A). HPLC analysis of the O$_2^-$ specific product of MitoSOX (2-OH-Mito-E$^-$) confirmed the specificity of O$_2^-$ measurements with MitoSOX (Online Figure III). Experiments with PMA and antimycin A confirmed site-specific detection of cellular and mitochondrial O$_2^-$ by DHE and MitoSOX (Online Figure IV). Interestingly, supplementation of HAECs with 25 nmol/L mitoTEMPO for 15-minute after Ang II stimulation abolished the MitoSOX signal indicating that mitoTEMPO decreases mitochondrial O$_2^-$ in intact cells (Figure 2A and Online Figure III).

Scavenging mitochondrial O$_2^-$ by mitoTEMPO could improve mitochondrial function. To determine whether this is correct, we measured respiration of isolated mitochondria in the presence of complex I substrates malate/glutamate or the complex II substrate succinate. Coupling of mitochondrial respiration was estimated by measurements of State 3 and State 4 oxygen consumption in the presence or absence of ADP. In unstimulated cells, treatment with mitoTEMPO did not affect mitochondrial respiration (Table 2). Ang II increased state 4 respiration (without ADP) and reduced state 3 respiration (in the presence of ADP). These changes were reflected in marked reduction of respiratory control ratio (RCR), indicating uncoupling of mitochondrial respiration. Supplementation with mitoTEMPO markedly improved these parameters for both complex I and complex II substrates, indicating that the mitochondrial impairment caused by Ang II could be reversed by mitoTEMPO.

Improvement of respiratory coupling could reduce electron leakage in mitochondria resulting in diminished total mitochondrial ROS production. To address this, we measured total H$_2$O$_2$ production by mitochondria isolated from either control or Ang II–stimulated cells. Ang II increased H$_2$O$_2$ production in response to complex I and complex II substrates. Treatment of cells with mitoTEMPO completely reversed this effect of Ang II (Table 2). MitoTEMPO had no effect on H$_2$O$_2$ production in mitochondria in cells not treated with Ang II. These data indicate that in addition to O$_2^-$ scavenging, mitoTEMPO has the capacity to reduce mitochondrial ROS production by normalizing respiration. Thus, treatment of Ang II–stimulated cells with mitoTEMPO inhibits mitochondrial oxidative stress, improves respiration and inhibits production of mitochondrial H$_2$O$_2$.

### Table 2. Mitochondrial H$_2$O$_2$ and Respiration in Ang II–Stimulated BAECs Treated With MitoTEMPO

<table>
<thead>
<tr>
<th>H$_2$O$_2$ (pmol/mg per minute)</th>
<th>Malate + Glutamate</th>
<th>Succinate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>67±4</td>
<td>234±20</td>
</tr>
<tr>
<td>MitoTEMPO</td>
<td>72±5</td>
<td>235±19</td>
</tr>
<tr>
<td>Ang II*</td>
<td>133±12</td>
<td>377±30</td>
</tr>
<tr>
<td>Ang II + mitoTEMPO†</td>
<td>79±4</td>
<td>227±24</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Malate + Glutamate Respiration (nmol/mg per minute)</th>
<th>State 3</th>
<th>State 4</th>
<th>RCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.6±0.7</td>
<td>3.3±0.4</td>
<td>3.5±0.1</td>
</tr>
<tr>
<td>MitoTEMPO</td>
<td>11.5±0.6</td>
<td>3.2±0.5</td>
<td>3.6±0.4</td>
</tr>
<tr>
<td>Ang II*</td>
<td>8.2±0.5</td>
<td>5.3±1.0</td>
<td>1.5±0.6</td>
</tr>
<tr>
<td>Ang II + mitoTEMPO†</td>
<td>9.8±0.7</td>
<td>3.7±0.5</td>
<td>2.7±0.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Succinate Respiration (nmol/mg per minute)</th>
<th>State 3</th>
<th>State 4</th>
<th>RCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.5±0.3</td>
<td>4.7±0.6</td>
<td>2.0±0.4</td>
</tr>
<tr>
<td>MitoTEMPO</td>
<td>9.3±0.4</td>
<td>4.5±0.7</td>
<td>2.1±0.4</td>
</tr>
<tr>
<td>Ang II*</td>
<td>7.9±0.2</td>
<td>6.9±0.5</td>
<td>1.1±0.3</td>
</tr>
<tr>
<td>Ang II + mitoTEMPO†</td>
<td>9.1±0.3</td>
<td>4.8±0.6</td>
<td>1.9±0.3</td>
</tr>
</tbody>
</table>

Results are means±SEM. *P<0.05 vs control; †P<0.05 vs Ang II.

**Effect of MitoTEMPO on Endothelial O$_2^-$, NO, and NADPH Oxidase Activity**

The studies described above indicate that Ang II increases mitochondrial H$_2$O$_2$ and mitoTEMPO prevents this. H$_2$O$_2$ is freely diffusible and can stimulate the extramitochondrial NADPH oxidase via c-Src-mediated mechanisms.$^{33}$ We therefore tested the hypothesis that inhibition of mitochondrial H$_2$O$_2$ by mitoTEMPO would decrease activity of the NADPH oxidase, reducing cellular O$_2^-$ and improving NO production.

To perform these studies, we stimulated BAECs with Ang II (200 nmol/L for 4 hours) and then exposed the cells to mitoTEMPO (25 nmol/L) for 15 minutes. Membrane fractions were then produced by centrifugation and NADPH oxidase activity measured using ESR. It was found that Ang II significantly increased nonmitochondrial NADPH oxidase activity (Figure 2B). Treatment of cells with mitoTEMPO completely blocked the increase in NADPH oxidase activity caused by Ang II but did not affect basal NADPH oxidase activity in unstimulated cells (Figure 2B). Importantly, supplementation with a mitochondria impermeable SOD-mimetic CP did not affect NADPH oxidase activity. Direct addition of mitoTEMPO (0.5 μmol/L) to membrane fractions did not affect NADPH oxidase activity (Online Figure V).

This decrease of NADPH oxidase activity in mitoTEMPO treated cells was accompanied by reduced production of cellular O$_2^-$ measured in intact cells using dihydroethidium and HPLC (Figure 2C) and an increase in endothelial NO production as detected by ESR and the spin trap Fe[DETC]$_2$ (Figure 2D). Treatment of Ang II–stimulated cells with the mitochondria impermeable analog 3-carboxyproxyl$^{29}$ had no effect on these parameters (Figure 2C and 2D). These data indicate that scavenging of mitochondrial O$_2^-$ with mitoTEMPO results in decrease of cellular O$_2^-$ and recovery of endothelial NO.

**SOD2 Modulates Ang II–Stimulated Cellular O$_2^-$ and NADPH Oxidase Activity**

It is conceivable that the effects of mitoTEMPO are not mediated by O$_2^-$ scavenging but are attributable to nonspecific effects. We therefore performed additional experiments to manipulate the levels of mitochondrial superoxide dismutase (SOD2) and measured NADPH oxidase activity and cellular O$_2^-$ production. Transfection of HAECs with an SOD2 plasmid increased mitochondrial SOD2 activity by 2.4-fold, whereas cytoplasmic SOD1 activity was not
Fluorescent microscopy with MitoSOX showed that SOD2 depletion increased both basal and Ang II–stimulated mitochondrial superoxide production, and that this could be inhibited by mitoTEMPO (Online Figure VI). It is important to note that mitoTEMPO treatment inhibited cellular O$_2^-$ and mimicked SOD2 overexpression (Figure 3B) in SOD2-depleted cells, further validating mitoTEMPO as an SOD2 mimic.

### Antihypertensive Effect of MitoTEMPO

Increased vascular O$_2^-$ production has been implicated in the pathogenesis of endothelial dysfunction and hypertension.$^{9,34}$ Because mitoTEMPO diminished mitochondrial ROS, inhibited Ang II–stimulated endothelial O$_2^-$ and prevented inactivation of NO caused by Ang II in cultured endothelial cells (Figures 2 and 3), we hypothesized that it could improve endothelial function and decrease hypertension in vivo. Co-infusion of mitoTEMPO (Figure 4A) significantly attenuated the Ang II–induced hypertension in a dose-dependent manner (50, 150 and 500 μg/kg per day), while not affecting blood pressure in normal mice. Telemetric measurements of blood pressure confirmed that co-infusion of mitoTEMPO (150 μg/kg per day) with Ang II markedly attenuated hypertension (Figure 4B). In addition, mitoTEMPO inhibited the increase in vascular O$_2^-$ (Figure 4C) and prevented the decrease of vascular NO (Figure 4D) caused by Ang II infusion. Importantly, infusion of the same dose of the nontargeted SOD mimetic TEMPOL (286 nmol/kg per day) did not affect Ang II–induced hypertension (Figure 4A). These data demonstrate that the mitochondrial-targeted SOD2 mimetic mitoTEMPO attenuates Ang II–induced hypertension at an extremely low dose.

The above studies showing that mitoTEMPO can prevent hypertension do not provide insight into whether it could lower blood pressure after hypertension is established. We therefore performed additional studies in which mitoTEMPO was administered after the onset of Ang II–induced hypertension. Following seven days of Ang II infusion (0.7 mg/kg per day) systolic blood pressure reached 160 mm Hg (Figure 4A). These data demonstrate that the mitochondrial-targeted SOD2 mimetic mitoTEMPO attenuates Ang II–induced hypertension at an extremely low dose.

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Because the above findings were made in mice treated with Ang II, there was a question as to whether the effect of mitoTEMPO is limited to the Ang II model. We therefore performed additional experiments in mice with DOCA salt hypertension.$^{35}$ This model of hypertension differs from Ang II–induced hypertension because it is largely volume dependent and is associated with suppressed plasma renin activity. In these experiments, mitoTEMPO...
was administered 10 days after the DOCA salt surgery. As in the case of Ang II–induced hypertension, mitoTEMPO reduced blood pressure in DOCA salt mice but did not affect blood pressure in sham-operated mice (Figure 5C). ESR studies demonstrated that DOCA salt hypertension decreased endothelial NO production and that mitoTEMPO treatment restored this, while not affecting vascular NO in sham mice (Figure 5D).

Overexpression of SOD2 Attenuates Ang II–Induced Endothelial Dysfunction and Hypertension

The above experiments with mitoTEMPO suggest that reducing mitochondrial O$_2^-$ improves endothelial function and hypertension. To confirm this using nonpharmacological means, we investigated production of vascular O$_2^-$ and the development of hypertension in tg$^{SOD2}$ mice. These animals have a 2-fold increase in SOD2 activity and protein levels. Although basal blood pressures in tg$^{SOD2}$ and C57Bl/6 mice were similar, the hypertensive response to Ang II was attenuated and delayed in tg$^{SOD2}$ mice (Figure 6A). The production of vascular O$_2^-$ in Ang II–infused tg$^{SOD2}$ mice was significantly lower compared with C57Blk/6 mice, whereas basal O$_2^-$ level was not different (Figure 6B). These data are in keeping with our findings with mitoTEMPO treatment and confirmed the role of mitochondrial O$_2^-$ in vivo.

Discussion

The present study provides the first evidence that scavenging of mitochondrial O$_2^-$ improves endothelial function and reduces hypertension. In this work we found that treatment with either the mitochondria-targeted SOD mimetic mitoTEMPO or overexpression of SOD2 inhibited oxidative stress and prevented the loss of endothelial NO caused by Ang II both in cultured endothelial cells and intact mice. Furthermore, treatment of hypertensive mice with mitoTEMPO after the onset of either Ang II– or DOCA salt-induced hypertension significantly reduced blood pressure and substantially improved endothelium-dependent vasodilatation. Analysis of SOD2 overexpressing transgenic mice confirmed an important role of mitochondrial O$_2^-$ in endothelial function and hypertension.

We have previously shown that Ang II stimulates production of mitochondrial O$_2^-$. This was dependent on NADPH oxidase activity because siRNA-induced deple-
tion of the NADPH oxidase subunit p22^phox^ or inhibition of NADPH oxidase activity by apocynin prevented mitochondrial impairment and attenuated mitochondrial O_2^\* production,\(^{14}\) demonstrating an upstream role of the NADPH oxidase in modulation of mitochondrial O_2^\* in the present study. We have additionally found that mitochondrial O_2^\* stimulates extramitochondrial NADPH oxidase activity in a feed-forward fashion. Taken together, these studies indicate that the interplay between mitochondrial and NADPH oxidase–derived O_2^\* constitutes a vicious cycle (Figure 6C) in which the NADPH oxidase increases mitochondrial ROS, which further activates the cytosolic NADPH oxidase and increases cellular O_2^\* production, diminishing NO bioavailability and uncoupling endothelial NO synthase.\(^{37}\) The effect of mitochondrial ROS on NADPH oxidase activity is quite likely mediated by c-Src\(^^{38}\) which can be stimulated by H_2O_2.\(^{33}\) Indeed, activation of NADPH oxidase has been reported to be a biphasic process in which the first phase requires direct activation by Ang II followed by a second phase of sustained activation that is H_2O_2 dependent.\(^{39}\) This could explain why inhibition of mitochondrial H_2O_2 by mito-TEMPO (Table 2) results in decrease of NADPH oxidase activity (Figure 2C). Our present findings also indicate that scavenging of mitochondrial O_2^\* using mitochondria-targeted antioxidants can interrupt this vicious cycle.

Our work is in keeping with prior findings that SOD2^\textsuperscript{+/–}\) mice are prone to age-associated and salt-induced hypertension\(^^{8}\) and that treatment with the mitochondria-targeted antioxidant mQ10 attenuates hypertension in spontaneously hypertensive rats.\(^^{40}\) Our findings also provide additional insight into the role of SOD2 in development of these pathological conditions. We suggest that SOD2 depletion increases mitochondrial O_2^\* levels which upregulates NADPH oxidase activity. Indeed, the key role of NADPH oxidase in hypertension and atherosclerosis has been well documented.\(^{34\) The synergism between mitochondrial and cellular O_2^\* production reported in our work may explain these pathological effects in SOD2^+/–\) mice.

Previous studies have shown that the nontargeted SOD mimetic TEMPOL prevents hypertension, renal and vascular dysfunction in several models of hypertension.\(^^{24,41}\) Importantly, we found that targeting of SOD mimetic to mitochondria provided beneficial effects at a dose 1000-fold lower than previously reported for TEMPOL. This finding is important in two respects. First, it confirms a critical role of the mitochondria in hypertension and endothelial dysfunction. Second, our data demonstrate the feasibility of using

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**Figure 5.** Effects of mitoTEMPO treatment after the onset of hypertension on blood pressure, vascular function and NO production. **A,** Blood pressure of mice treated with mitoTEMPO (0.7 mg/kg per day) after onset of Ang II–induced hypertension. **B,** Endothelial-dependent relaxation in aortic vessels isolated from mice infused with saline (sham), mitoTEMPO, or Ang II or Ang II–infused mice treated with mitoTEMPO. Vessels were preconstricted with PGF\_2\alpha, and relaxations to cumulative concentrations of acetylcholine were examined. **C,** Blood pressure of mice treated with mitoTEMPO (0.7 mg/kg per day) after onset of DOCA salt–induced hypertension. **D,** Production of aortic NO measured in isolated aorta by ESR and Fe(DETC)\_2. Results represent means ± SEM for 6 to 8 animals per group. *P < 0.001 vs sham; **P < 0.05 vs Ang II/DOCA.
very low doses of mitochondria-targeted antioxidants for therapeutic purposes.

Importantly, mitoTEMPO not only attenuated development of hypertension but was also effective in treating hypertension after it was established. This is clinically important because treatment is commonly started in humans after hypertension has developed. Our findings indicate that targeting mitochondrial ROS with agents like mitoTEMPO might therefore be effective in treating human hypertension. This is potentially important because many patients’ blood pressure remains poorly controlled despite treatment with multiple drugs. MitoTEMPO and other mitochondria-targeted agents might therefore represent a new class of antihypertensive agent that could add to the currently available therapeutic armamentarium.

MitoTEMPO had no effect on blood pressure in normotensive animals. This is in keeping with the concept that O$_2^-$ does not affect hemodynamics under normal physiological conditions but begins to play a role in pathophysiological states. Indeed, SOD2 overexpression and mitoTEMPO supplementation did not affect basal activity of NADPH oxidase, production of vascular O$_2^-$ or endothelium-dependent relaxation. This is potentially important, because unlike some currently used antihypertensive agents, mitoTEMPO would unlikely cause hypotension in normotensive subjects.

In addition to hypertension, there are many other common conditions including aging, atherosclerosis, diabetes and degenerative neurological disorders in which mitochondrial oxidative stress seems to play a role. Of note, large clinical trials have failed to show a benefit of often-used antioxidants such as vitamin E and vitamin C in many of these conditions, and have paradoxically shown deleterious effects in some trials. There are many potential explanations why these antioxidants have proven ineffective in these studies, but one relates to the fact that agents such as vitamin E and vitamin C are not targeted to sites of ROS generation that are most important in pathological conditions. It is conceivable that the use of SOD mimetics such as mitoTEMPO, targeted to compartments where ROS is generated such as the mitochondria, would be more effective in these conditions. The ability to achieve these effects in relatively low doses might also limit potential untoward effects of antioxidant therapy observed with other agents.

Acknowledgments
We thank Dr Kathy K. Griendling for fruitful discussion and Bernard Lassègue, Lula L. Hilenski, Alexander V. Panov, and Vladimir I. Mayorov for technical assistance.

Figure 6. Analysis of blood pressure and vascular O$_2^-$ in SOD2 overexpressing transgenic mice infused with Ang II. A, Systolic blood pressure in TgSOD2 and C57Blk/6 mice infused with saline or Ang II (0.7 mg/kg per day). B, Production of aortic O$_2^-$ measured with DHE and HPLC. Results represent means±SEM for 4 to 8 animals per group. *P<0.05 vs Ang II. C, Proposed role of mitochondrial O$_2^-$ in endothelial dysfunction and hypertension.
Sources of Funding

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Disclosures

None.

References


**Novelty and Significance**

**What Is Known?**

- Oxidative stress is strongly implicated in the pathogenesis of hypertension.
- Angiotensin II increases superoxide production by NADPH oxidases.
- Angiotensin II causes mitochondrial dysfunction.

**What New Information Does This Article Contribute?**

- Scavenging of mitochondrial superoxide significantly improves endothelial function.
- Inhibition of mitochondrial superoxide reduces the activity of NADPH oxidases.
- Mitochondria-targeted antioxidants can be used as antihypertensive agents.

Despite the fact that the mitochondria are an important source of superoxide in vascular cells, the role of mitochondrial superoxide in endothelial dysfunction remains unclear. We have found that stimulation of endothelial cells with angiotensin II increases the production of mitochondrial superoxide. Overexpression of SOD2 or treatment with mitochondria-targeted SOD mimic mitoTEMPO attenuates activation of vascular NADPH oxidases, inhibits production of cellular superoxide, restores NO production, improves endothelium-dependent vasodilatation and reduces blood pressure in angiotensin II–infused mice. This work demonstrates that angiotensin II–induced superoxide production by NADPH oxidase stimulates mitochondrial superoxide that in turn provides redox-dependent feed-forward stimulation of NADPH oxidase. This vicious cycle can be interrupted at the mitochondrial site by mitochondria targeted antioxidants. For the first time we have found that angiotensin II–induced hypertension was attenuated in SOD2 overexpressing transgenic mice. Furthermore, mitoTEMPO treatment after the onset of DOCA salt or angiotensin II–induced hypertension significantly decreased blood pressure. These studies show that mitochondrial superoxide is important for the development of hypertension and that antioxidant strategies specifically targeting this organelle could have therapeutic benefit in this and possibly other diseases.
Therapeutic Targeting of Mitochondrial Superoxide in Hypertension
Anna E. Dikalova, Aliya T. Bikineyeva, Klaudia Budzyn, Rafał R. Nazarewicz, Louise McCann, William Lewis, David G. Harrison and Sergey I. Dikalov

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**Online Supplement for Dikalova et al.**

**HPLC measurement of cellular and aortic $O_2^\cdot$:** Superoxide was measured using dihydroethidium (DHE) and an HPLC-based assay. Cultured cells were washed with Krebs/HEPES buffer and incubated with 10 μM DHE for 20 min at 37°C. Aortic sections were incubated with 50 μM DHE for 30 min at 37°C. Media was removed and cells or tissue were transferred to methanol for extraction of superoxide-specific product 2-hydroxyethidium (2-OH-E') and kept at -80°C. Separation of ethidium, 2-OH-E' and dihydroethidium was performed using a Beckman HPLC System Gold model with a C-18 reverse phase column (Nucleosil 250, 4.5 mm; Sigma-Aldrich).

**Dose-dependent angiotensin II stimulation of endothelial $O_2^\cdot$:** Cellular $O_2^\cdot$ production was measured in HAEC following 4 hours of treatment with 0, 40, 100 or 200 nM angiotensin II using HPLC and DHE (Online Figure I). Angiotensin II increased $O_2^\cdot$ production in a dose-dependent manner with maximum stimulation at 200 nM concentration.

**Effect of mitoTEMPO and mitoTEMPO-H on xanthine oxidase:** Superoxide was generated using xanthine oxidase and xanthine to measure SOD activity and to investigate SOD-scavenging properties of mitoTEMPO and mitoTEMPO-H. Additional experiments were performed to exclude a potential effect of these agents on xanthine oxidase activity. Neither mitoTEMPO nor mitoTEMPO-H affected xanthine oxidase activity as reflected by the accumulation of urate (Online Figure II).

**HPLC analysis of superoxide specific product of mitoSOX:** MitoSOX can produce both superoxide specific (2-OH-Mito-E') and non-specific (Mito-E') fluorescent products. To provide validation of $O_2^\cdot$ detection with mitoSOX we used HPLC to quantify 2-OH-Mito-E' and Mito-E' as was previously described. Incubation of mitoSOX with xanthine oxidase and xanthine resulted in the specific accumulation of 2-OH-Mito-E' which was inhibited by superoxide dismutase (Online Figure IIIA). Control or angiotensin II stimulated HAEC were supplemented with 4 μM mitoSOX for 20 minutes and then washed with Krebs/HEPES buffer. The cells were then harvest in methanol for extraction of 2-OH-Mito-E' and Mito-E'. Analysis of cellular extracts showed accumulation of both 2-OH-Mito-E' and Mito-E'. Stimulation of cells with angiotensin II (200 nM for 4 hours) increased levels of 2-OH-Mito-E' but did not change Mito-E' (Online Figure IIIB). Supplementation with mitoTEMPO (25 nM for 15 minutes) before addition of mitoSOX inhibited accumulation of 2-OH-Mito-E' (Online Figure IIIC). These data support the specificity of $O_2^\cdot$ measurements with mitoSOX.

**Site-specific detection of cellular and mitochondrial $O_2^\cdot$ by DHE and mitoSOX:** In order to validate $O_2^\cdot$ detection by DHE and mitoSOX we have performed HPLC measurements of $O_2^\cdot$ specific
products 2-OH-E⁺ and 2-OH-Mito-E⁺ in HAEC treated with NADPH oxidase activator phorbol myristate acetate (PMA, 10 µM) or mitochondrial complex III inhibitor antimycin A (AA, 1 µM). HPLC analysis showed that stimulation of \( \mathbf{O}_2^- \) production in cytoplasm with PMA was reflected in accumulation of 2-OH-E⁺ in DHE supplemented cells but did not result in significant accumulation of 2-OH-Mito-E⁺ in mitoSOX supplemented cells (Online Figure IV). Antimycin A induced increase in mitochondrial \( \mathbf{O}_2^- \) was reflected in accumulation of 2-OH-Mito-E⁺ in mitoSOX supplemented cells but did not raised the level of 2-OH-E⁺ in DHE supplemented cells (Online Figure IV). These data demonstrate site-specific detection of cellular and mitochondrial \( \mathbf{O}_2^- \) by DHE and mitoSOX.

Investigation of direct effect of mitoTEMPO on NADPH oxidase activity: To perform these studies, we stimulated BAEC with angiotensin II (200 nM for 4 hours) and measured NADPH oxidase activity in membrane fractions using ESR as described in the Methods section. Angiotensin II significantly increased NADPH oxidase activity (Online Figure V). Supplementation of membrane fraction isolated from unstimulated (Control) or Ang II - stimulated BAEC with 0.5 µM mitoTEMPO did not affect NADPH oxidase activity (Online Figure V).

Effect of mitoTEMPO on \( \mathbf{O}_2^- \) in intact endothelial cells: In this work we have reported that supplementation of BAEC with mitoTEMPO increases \( \mathbf{O}_2^- \) dismutation in mitochondria (Table 1) and reduces the mitoSOX fluorescence (Figure 2, Online Figure III). To further demonstrate that mitoTEMPO scavenges mitochondrial \( \mathbf{O}_2^- \) we performed additional experiments in cells in which SOD2 was downregulated by siRNA.

Analysis of HAEC transfected with non-silencing siRNA (NS siRNA) showed that angiotensin II increased the \( \mathbf{O}_2^- \) similar to non-transfected cells (Figure 2, Online Figure VI). SOD2 depletion increased both basal and angiotensin II – stimulated mitoSOX fluorescence (Online Figure VI). MitoTEMPO significantly inhibited fluorescence of mitoSOX. These data indicate that mitoTEMPO significantly reduces mitochondrial \( \mathbf{O}_2^- \).

Investigation of endothelial-independent relaxation in aortic vessels: Endothelial-independent relaxation was measured by vessels relaxations to cumulative concentrations of sodium nitroprusside (SNP). Endothelium-independent relaxation was similar in vessels isolated from mice infused with saline (Sham), mitoTEMPO, angiotensin II (Ang II) or angiotensin II-infused mice treated with mitoTEMPO (Online Figure VII).

Investigation of heart rate of mice treated with angiotensin II and mitoTEMPO: The potential effect of mitoTEMPO on heart rate was evaluated using telemetry (Online Figure VIII). MitoTEMPO did not affect heart rate in normal mice, however, infusion of angiotensin II for one week increased heart rate by 10% and mitoTEMPO treatment prevented this change in the heart rate (Online Figure IVB).
References


Online Figure Legends

**Online Figure I.** Dose-dependent angiotensin II stimulation of endothelial $O_2^\cdot$. Cellular $O_2^\cdot$ production was measured in HAEC following 4 hours of treatment with angiotensin II using HPLC and DHE as described in Materials and Methods. It was found that angiotensin II increased $O_2^\cdot$ production in a dose-dependent manner with maximum stimulation at 200 nM concentration. Results represent mean ± SEM. $^*P < 0.05$ vs 0 nM, $^{**}P < 0.05$ vs 100 nM Ang II.

**Online Figure II.** Measurements of xanthine oxidase activity in the presence of mitoTEMPO (A) or mitoTEMPO-H (B). The activity of xanthine oxidase was monitored by accumulation of urate (295 nm, $\varepsilon=11.000$) in the sample containing xanthine oxidase (20 mU/ml) and xanthine (200 µM). Data represent the mean values from three separate experiments.

**Online Figure III.** HPLC measurements of superoxide specific (2-OH-Mito-E*) and non-specific (Mito-E*) fluorescent products of mitoSOX as was previously described. (A) Superoxide was generated using xanthine and xanthine oxidase and 2-OH-Mito-E* and Mito-E* were separated using HPLC. Experiments were performed without (upper chromatogram) and with (middle trace) SOD (100 U/ml). The lower panel shows the chromatogram of unreacted mitoSOX. (B) Typical HPLC chromatograms of cellular extracts obtained after incubation of HAEC with 2 µM mitoSOX for 20 minutes. (C) Bar graph showing levels of 2-OH-Mito-E* in unstimulated HAEC or HAEC stimulated with angiotensin II (Ang II, 4 hours, 200nM) and supplemented for 15 minutes with saline or the mitochondria-targeted SOD mimetic mitoTEMPO (25 nM). Results are mean±SEM, n=4 each. $^*P < 0.05$ vs control, $^{**}P < 0.05$ vs Ang II.

**Online Figure IV.** Site-specific detection of cellular and mitochondrial $O_2^\cdot$ by DHE and mitoSOX using HPLC. Superoxide production was induced by NADPH oxidase activator
phorbol myristate acetate (PMA, 10 µM) or mitochondrial complex III inhibitor antimycin A (AA, 1 µM). Cellular and mitochondrial \(O_2^{•−}\) were measured by DHE (A) or mitoSOX (B) using HPLC as was previously described \(^1\,^2\).

**Online Figure V.** NADPH oxidase activity in membrane fractions supplemented with mitoTEMPO (0.5 µM). Activity of NADPH oxidase was measured as NADPH-dependent \(O_2^{•−}\) production in membrane fractions using ESR as described in Materials and Methods \(^2\). NADPH oxidase activity was analyzed in membrane fractions of control unstimulated BAEC or BAEC stimulated with 200 nM angiotensin II for 4-hours. MitoTEMPO (0.5 µM) was applied to the membrane fractions after isolation for 30 minutes prior to measurements of NADPH oxidase activity. Direct supplementation of mitoTEMPO to membrane fractions isolated from control or angiotensin II stimulated BAEC did not affect NADPH oxidase activity. Data are average from three to six separate experiments ± SEM. *P<0.01 vs Control.

**Online Figure VI.** Effect of mitoTEMPO on mitochondrial \(O_2^{•−}\) measured with MitoSOX. HAEC were treated with siSOD2 for 72 hours and then stimulated with 200nM angiotensin II (Ang II) for 4-hours. MitoTEMPO (25 nM, mT) was added for 15 minutes prior to supplementation of mitoSOX. Mitochondrial localization of MitoSOX signal was confirmed by colocalization with MitoTracker.

**Online Figure VII.** Effects of mitoTEMPO treatment on endothelial-independent relaxation in aortic vessels isolated from mice infused with saline (Sham), mitoTEMPO, angiotensin II (Ang II) or angiotensin II-infused mice treated with mitoTEMPO. Results represent mean ± SEM for 6-8 animals per group.

**Online Figure VIII.** Heart rate in C57Blk/6 mice after one week infusion with saline (Control), mitoTEMPO, angiotensin II (Ang II) or mice treated with both angiotensin II and mitoTEMPO (Ang II + mitoTEMPO). Treatment with mitoTEMPO did not affect the heart rate in the control mice. Co-infusion of mitoTEMPO and angiotensin II (0.7 mg/kg/day) attenuated angiotensin II induced increase in heart rate which was associated with antihypertensive effect of mitoTEMPO. Results represent mean ± SEM for 5 animals per group.
Online Figure I

Graph showing cellular superoxide production in response to different concentrations of angiotensin II (0, 40 nM, 100 nM, 200 nM). The y-axis represents cellular superoxide production in nmol/mg protein, while the x-axis represents the concentration of angiotensin II. The graph includes error bars indicating variability. Statistical significance is indicated by asterisks (*) and double asterisks (**) for different concentrations compared to control (0 nM).
Online Figure II

A: Xanthine oxidase activity, %
- mitoTEMPO

B: Xanthine oxidase activity, %
- mitoTEMPO-H

mitoTEMPO concentration, µM

mitoTEMPO-H concentration, µM
Online Figure IV

Cytoplasm

PMA → PKC → NADPHox → \( \text{O}_2^\cdot \) → DHE

AA → Complex III → \( \text{O}_2^\cdot \) → mitoSOX

Mitochondria

A: DHE

B: mitoSOX

Cellular \( \text{O}_2^\cdot \), nmol/mg

Control | PMA | AA

Mitochondrial \( \text{O}_2^\cdot \), nmol/mg

Control | PMA | AA

* Significant difference
Online Figure V

![Graph showing NADPH oxidase activity, pmol/mg/min](image)

- Control
- Control mem. fr. + 0.5 µM mitoTEMPO
- Ang II
- Control mem. fr. + 0.5 µM mitoTEMPO

* Significant difference compared to control
### Online Figure VI

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<th>siSOD2 + AngII</th>
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Online Figure VII

The figure shows a graph plotting relaxation (as % precontraction) against [SNP] log M. Different treatments are represented by distinct line types and markers:
- **SHAM**: Solid square (■)
- **mitoTEMPO**: Open square (◆)
- **Ang II**: Solid triangle (▲)
- **Ang II + mitoTEMPO**: Open triangle (△)

The x-axis represents [SNP] log M values ranging from -9 to -5, while the y-axis represents the percentage relaxation ranging from 0% to 100%. Error bars indicate variability in the data.
Online Figure VIII

Heart Rate per minute

<table>
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<th>Condition</th>
<th>Heart Rate</th>
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<tr>
<td>mitoTEMPO</td>
<td>510</td>
</tr>
<tr>
<td>Ang II</td>
<td>550</td>
</tr>
<tr>
<td>Ang II + mitoTEMPO</td>
<td>490</td>
</tr>
</tbody>
</table>

Significance:
- *: p < 0.05
- **: p < 0.01