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Mechanism by Which Superoxide Potentiates Tubuloglomerular Feedback

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Abstract—The macula densa detects changes in NaCl concentration in tubular fluid and transmits a feedback signal, known as tubuloglomerular feedback (TGF), which helps to control glomerular afferent arteriole resistance. We and other investigators have reported that synthesis of NO in the macula densa inhibits TGF. NO can be scavenged by superoxide (O^-_2) to form peroxynitrite, effectively reducing the bioavailability of NO; there is growing evidence that O^-_2 regulates vascular tone in the kidney. We hypothesized that O^-_2 produced in the macula densa enhances TGF and this effect acts only in an autocrine manner within the cells of the macula densa. Afferent arterioles and attached macula densas from Sprague-Dawley rats were simultaneously microperfused in vitro and TGF response examined before and after perfusing the tubular lumen, bath, or vascular lumen with a superoxide scavenger. The macula densa was perfused with solutions containing either 5 mmol/L Na^+ and 3 mmol/L Cl^- (low NaCl) or 80 mmol/L Na^+ and 77 mmol/L Cl^- (high NaCl) while keeping pressure in the afferent arteriole constant at 60 mm Hg. When 10^{-4} M Tempol, a stable membrane-permeant superoxide dismutase (SOD) mimetic, was added to the tubular lumen, it inhibited TGF by 56% (before Tempol: TGF, 3.2±0.3 μm; after Tempol: TGF, 1.4±0.2 μm; n=6; $P<0.05$, control versus Tempol). Adding Tempol to the bath inhibited TGF by 48% (before Tempol: TGF, 2.5±0.25 μm; after Tempol: TGF, 1.3±0.18 μm; n=6; $P<0.05$). However, adding Tempol to the vessel lumen did not change TGF response significantly (before Tempol: TGF, 2.7±0.37 μm; after Tempol: TGF, 3.2±0.25 μm; n=7; $P=0.25$). When 300 U/mL of the enzyme SOD, which is not membrane-permeant, was added to either the tubular lumen or bath, it had no effect on TGF response. Finally, to determine whether the effect of O^-_2 in the macula densa is mediated by its scavenging of NO, 7-nitroindazole (7-NI) was added to the macula densa to inhibit neuronal nitric oxide synthase (nNOS). In the presence of 7-NI, Tempol had no effect (7-NI only: TGF, 3.0±0.4 μm; 7-NI plus Tempol: TGF, 2.8±0.5 μm; n=6; $P=0.343$). Our findings suggest that (1) reducing O^-_2 increases the bioavailability of NO, which inhibits TGF, (2) both O^-_2 and NO act within the macula densa, and (3) O^-_2 appears to have no effect on its own. **(*Hypertension*. 2002;39[part 2]:624-628.)**

Key Words: arterioles ■ nitric oxide ■ nitric oxide synthase

Tubuloglomerular feedback (TGF) is generally thought to be mediated by the macula densa, which detects changes in NaCl concentration in the distal tubule and transmits a feedback signal to the glomerular vessels.[1] Mundel et al[2] have shown that immunoreactivity, enzymatic activity, and expression of mRNA for neuronal nitric oxide synthase (nNOS) are higher in the cytoplasm of macula densa cells than other cortical cells, and this has stimulated many investigators to study the role of NO produced by macula densa nNOS in the regulation of TGF. These studies show that NO produced within the macula densa decreases the TGF response induced by high concentrations of NaCl at the macula densa.[3–5]

More recently, the role of the superoxide anion (O^-_2) has been examined in relation to endothelial dysfunction. Increased O^-_2 production may decrease NO bioavailability in aortas of stroke-prone spontaneously hypertensive rats (SHR),[6,7] mesenteric arteries of SHR,[8] and afferent arterioles of diabetic rats.[9] SHR reportedly have a diminished TGF response to local inhibition of NOS,[10] apparently due to excessive generation of O^-_2.[11] However, it is not clear whether regulation of O^-_2 plays any role in TGF under normal conditions. We hypothesized that O^-_2 produced in the macula densa enhances TGF and this effect acts only in an autocrine manner within the macula densa cells.

To determine how macula densa nNOS modulates TGF, we decreased reactive oxygen species by adding superoxide dismutase (SOD), which is membrane-impermeant, or Tempol, a stable membrane-permeant SOD mimetic, to the tubular lumen, vascular lumen, or bath. To see whether scavenging O^-_2 increases the bioavailability of NO, we obtained a TGF response after adding the nNOS inhibitor 7-nitroindazole (7-NI) to the macula densa perfusate.

Methods

Afferent arterioles with macula densa attached were isolated and microperfused as described previously.[3,12] Young male Sprague-

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Dawley rats (SD) were anesthetized with ketamine (50 mg/kg, IM) and xylazine (50 mg/kg, IM). The kidneys were sliced along the corticomedullary axis, and slices were placed in ice-cold minimum essential medium (MEM; Gibco) containing 5% bovine serum albumin (BSA; Intergen). A single superficial afferent arteriole and its intact glomerulus from each rat were microdissected together with adherent tubular segments consisting of portions of the thick ascending limb, macula densa, and early distal tubule. Samples were transferred to a temperature-regulated chamber mounted on an inverted microscope (Olympus IMT-2) with Hoffmann modulation. Both the afferent arteriole and the end of either the distal tubule or thick ascending limb were cannulated with an array of glass pipettes as described previously.[12] Intraluminal pressure of the afferent arteriole was measured by Landis' technique, using a fine pipette introduced into the lumen through the perfusion pipette. The afferent arteriole was perfused with oxygenated MEM supplemented with 5% BSA and (in mmol/L) 5 $NaHCO_3$, 10 NaCl, 10 HEPES, and 10 NaOH. Intraluminal pressure was maintained at 60 mm Hg throughout the experiment. The macula densa was perfused with physiological saline consisting of (in mmol/L) 10 HEPES, 3 KCl, 1.2 $MgSO_4$, 2.0 KPO_4, 5 $NaHCO_3$, 5.5 glucose, 1.0 Ca lactate$_2$, and either 74 (high NaCl) or 0 NaCl (low NaCl). MEM was gassed with air and physiological saline was oxygenated with 100% O_2. The pH of each solution was 7.4. The bath was similar to the arteriolar perfusate (except that it contained 0.15% BSA) and was exchanged continuously at a rate of 1 mL/min. Microdissection and cannulation were completed within 90 minutes at 8°C, after which the bath was gradually warmed to 37°C for the rest of the experiment. Once the temperature was stable, a 30-minute equilibration period was allowed before taking any measurements. Images were displayed at magnifications up to ×1980 and recorded with a video system. Afferent arteriole diameter was measured with a MetaMorph image analysis system (Universal Imaging).

Tempol, a membrane-permeant SOD mimetic, and the enzyme SOD were obtained from Sigma Chemical Co; 7-NI was obtained from Cayman Chemical Co.

Statistics

Values are expressed as mean±SEM. A paired *t* test was used to examine whether the diameter at a given concentration was different from control. ANCOVA was used to examine whether dose-response curves differed between groups, and a 2-sample *t* test was used to examine whether the changes in diameter at a given concentration differed between groups. $P<0.05$ was considered significant using Bonferroni's correction for multiple comparisons.

Results

To see if removing O^-_2 would affect TGF, we obtained a control TGF response, added Tempol (100 μmol/L) to the macula densa perfusate, bath, or afferent arteriole perfusate, and then repeated the TGF response (Figure 1A). During the control period, TGF decreased afferent arteriole diameter by 3.2±0.3 μm (from 13.5±0.7 to 10.3±0.7 μm) when the macula densa perfusate was increased from 5 mmol/L Na/3 mmol/L Cl to 80 mmol/L Na/77 mmol/L Cl. After Tempol was added to the macula densa perfusate, the TGF response diminished to 1.4±0.2 μm, with diameter going from 13.5±0.7 to 12.1±0.7 μm. In a separate group, we added Tempol to the bath. During the control period, TGF decreased afferent arteriole diameter by 2.4±0.3 μm (from 14.7±0.7 to 12.3±0.6 μm). After adding Tempol to the bath, the decrease in diameter was significantly less than control and TGF response fell to 1.3±1.3 μm (from 15.1±0.8 to 13.8±0.8 μm) when NaCl concentration was increased. To test whether O^-_2 produced by the afferent arteriole endothelium influences TGF, we added Tempol to the vessel lumen. During the

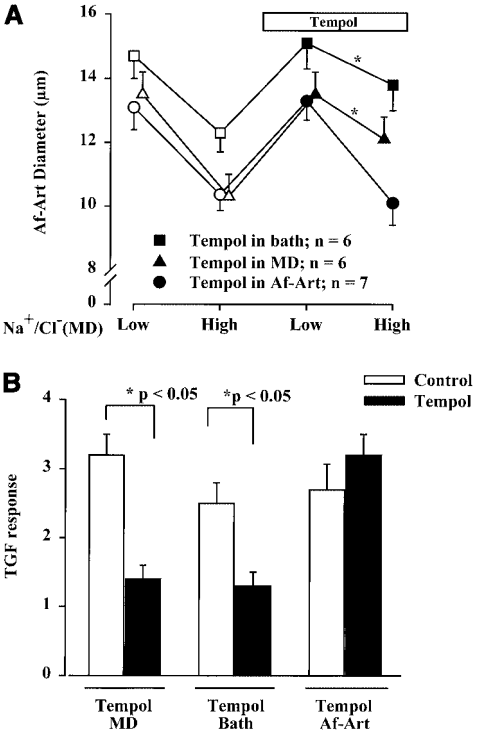

Figure 1. A, Effect of the membrane-permeant SOD mimetic Tempol on TGF. Tempol was added to the macula densa lumen (▲), bath (■), or afferent arteriole lumen (●). B, Paired differences between control TGF and responses to Tempol. Tempol blunted TGF when perfused into the macula densa or interstitium, but not the afferent arteriole, suggesting that its inhibitory effect is compartmentalized.

control period, TGF decreased diameter by 2.7±0.4 μm (from 13.1±0.7 to 10.4±0.5 μm). Adding Tempol to the afferent arteriole perfusate did not alter the TGF response induced by high NaCl at the macula densa; diameter decreased from 13.3±0.6 to 10.1±0.7 μm. Paired differences between control TGF response and Tempol treatment are shown in Figure 1B. Time controls showed no significant change in TGF response. Diameter decreased from 14.3±0.6 to 11.8±0.4 μm ($P<0.05$) when the solution perfusing the macula densa was changed from low to high NaCl. When we repeated the process, diameter decreased from 14.6±0.6 to 12.3±0.3 μm (n=3; $P<0.05$).

To localize the site of O^-_2 generation, we studied the effects of the membrane-impermeant O^-_2 scavenger SOD on TGF. Since Tempol only affected TGF when it was added to the macula densa lumen or bath, but not the Af-Art lumen, we added SOD to the macula densa lumen or bath (Figure 2A). When SOD was added to the macula densa lumen, control TGF was 2.6±0.4 μm (from 13.7±0.5 to 11.1±0.5 μm); after adding SOD, it was 2.4±0.1 μm (from 13.7±0.3 to 11.2±0.4 μm; n=5; $P=0.32$). Similar results were seen when SOD was added to the bath; control TGF was 3.0±0.5 μm (from 13.1±1.5 to 10.1±1.1 μm), while after SOD treatment TGF was 2.7±0.2 μm (from 13.0±1.4 to 10.3±1.2 μm; n=4; $P=0.3$). Paired differences between control TGF response and SOD treatment are shown in Figure 2B.

To test whether if the effect of O^-_2 on TGF involves scavenging of NO produced by the macula densa, we studied

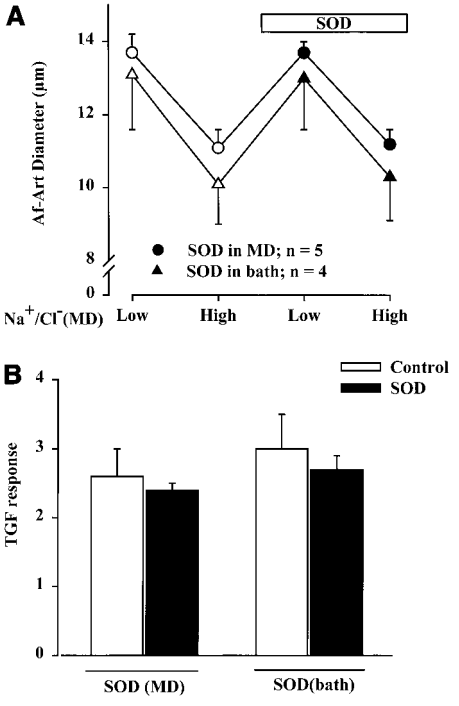

Figure 2. A, Effect of the membrane-impermeable enzyme SOD on TGF. SOD was added to the macula densa lumen (●) or bath (▲). B, Paired differences between control TGF and responses to SOD. SOD had no effect on TGF when it was added to the macula densa or bath, suggesting that O^{-}_{2} may act within cells of the macula densa.

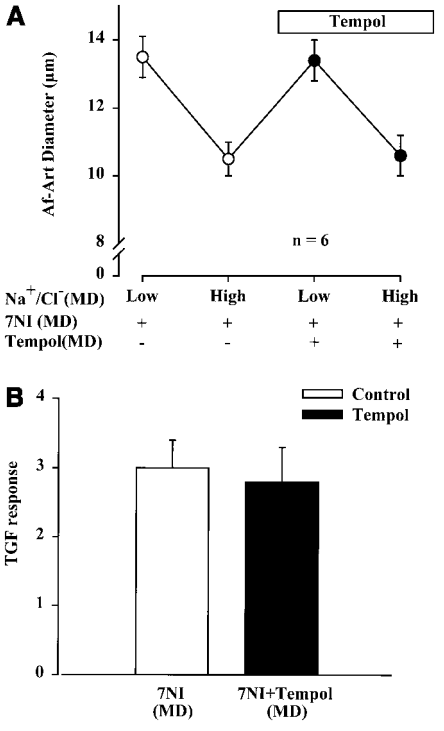

Figure 3. A, Effect of Tempol on TGF when nNOS was inhibited by simultaneous perfusion of 7-NI. B, Paired differences between TGF responses to 7-NI alone and 7-NI plus Tempol. 7-NI blocked the inhibitory effect of Tempol on TGF, suggesting that the effect of O^{-}_{2} on TGF may be mediated by NO.

the effect of Tempol on TGF after adding the nNOS inhibitor 7-NI to the macula densa perfusate. Figure 3A shows changes in afferent arteriole diameter induced by high NaCl at the macula densa in the presence of 7-NI. When 10^{-5} M 7-NI was added to the macula densa perfusate, TGF response was 3.0±0.4, decreasing diameter from 13.5±0.6 to 10.5±0.5 μm. When Tempol was added to the macula densa perfusate, TGF response was unchanged compared with 7-NI alone (2.8±0.5), and diameter decreased from 13.4±0.6 to 10.6±0.6 μm (n=6; P=0.343). Paired differences between control TGF response and Tempol treatment are shown in Figure 3B.

Discussion

We found that Tempol, a membrane-permeant SOD mimetic, diminished TGF when added to the interstitial space (bath) or macula densa perfusate, but not when added to the afferent arteriole perfusate. SOD, which lacks membrane permeability, had no effect on TGF when added to either the macula densa perfusate or bath. In addition, the inhibitory effect of Tempol on TGF was blocked by pretreating the macula densa with 7-NI. Thus, under physiological conditions, constitutive NO generated by macula densa nNOS is scavenged by O^{-}_{2}, and reducing O^{-}_{2} increases the bioavailability of NO, which further inhibits TGF. Both NO and O^{-}_{2} act within the macula densa.

NO is a highly diffusible gas that moves freely through tissues. Unlike NO, O^{-}_{2} is not membrane-permeant and is therefore restricted to the compartment where it is generated. Both O^{-}_{2} and NO are highly reactive and unstable radicals. Thus it is not surprising that they react very rapidly at a rate estimated to be 6.7×10^{9} mol s^{-1} to form the major product $OONO^{-}$,[13] whose site of action is only a few microns.[14] This reaction is approximately 3× faster than dismutation of O^{-}_{2} by SOD, implying that increased generation of O^{-}_{2} in the macula densa may very well inhibit the physiological functions of NO. Previously we showed that NO generated in the macula densa blunts TGF by acting on soluble guanylate cyclase within the macula densa.[4] In this study we found that when the membrane-permeant SOD mimetic Tempol was added directly to the macula densa or adjacent interstitial spaces that may reach the macula densa, it inhibited TGF whereas the membrane-impermeant SOD did not, suggesting that O^{-}_{2} quenches NO within the macula densa.

It is not surprising that adding Tempol to the afferent arteriole lumen did not affect the TGF response. We previously reported that adding the soluble guanylate cyclase inhibitor LY83583 to the afferent arteriole lumen did not affect high NaCl-induced afferent arteriole constriction.[4] Damaging the afferent arteriole endothelium with an antibody against factor VIII-related antigen and complement did not alter TGF response.[15] Thus, the present study combined with the previous studies supports our hypothesis that intracellular O^{-}_{2} in the macula densa enhances TGF and this effect acts only in an autocrine manner within the cells of the macula densa. Although Tempol is relatively cell-permeant, the fact that the bath and luminal solutions of the macula densa and afferent arteriole in our preparation are constantly exchanged

likely limits its effects to the compartment in which it is placed.

On finding that the TGF response was diminished by scavenging O^-_2, we repeated the experiment while inhibiting NO. Removing O^-_2 did not inhibit TGF response in the absence of NO, suggesting that O^-_2 acts by scavenging NO. Several studies have demonstrated that O^-_2 interacts with NO and thus limits its bioavailability. Rubanyi and Vanhoutte[16] showed that O^-_2 inactivates endothelium-derived relaxing factor (EDRF) in coronary artery rings. Alterations in the interaction of O^-_2 with NO signaling are now emerging as an important process in the expression of many vascular diseases, including atherosclerosis, hypertension, and diabetes. Kerr et al[7] confirmed that NO production is greater in SHR compared with normotensive WKY; even so, NO bioavailability was reduced in SHR, suggesting that NO may be scavenged by O^-_2. Recently Welch et al[11] reported that overactive TGF in SHR is partly due to the diminished role of nNOS-derived NO caused by enhanced O^-_2 formation. Interestingly, Ichihara et al[17] found that O^-_2 may predominantly inhibit the influence of nNOS on afferent arteriole diameter in SHR by scavenging NO generated in the macula densa. Whereas most studies have investigated the contribution of O^-_2 under pathophysiological conditions such as hypertension and diabetes, we believe our data are the first to demonstrate that under physiological conditions O^-_2 also plays an important role in the regulation of NO activity in the juxtaglomerular apparatus (JGA).

In vivo studies have shown that O^-_2 inactivates EDRF in many vessels, including the renal microvasculature.[8,9] Tempol reduces mean arteriole pressure and renal vascular resistance more in SHR than in WKY,[18] suggesting that O^-_2 contributes to increased systemic vascular tone in SHR. We did not see any vasodilator effect when Tempol was added to the vessel lumen in normal rats; however, we cannot rule out the possibility that O^-_2 may play a role in the regulation of renal vascular resistance. Our results can be explained by the fact that in our in vitro preparation, isolated arterioles have little tone. Thus, without preconstriction by norepinephrine or some other vasoconstrictor, vasodilatation is difficult to see. This is consistent with Ichihara's finding that Tempol elicits a vasodilator response in afferent arterioles of SHR but not WKY.[17]

Although the source of O^-_2 generated in the JGA is uncertain, nitrotyrosine immunoreactivity in renovascular hypertension is expressed strongly in the interstitium and extraglomerular mesangial cells[19] as well as adventitial fibroblasts[20] and mesangial cells.[21] Therefore, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase could be the predominant source of O^-_2 generation in the JGA. O^-_2 is produced by mitochondrial electron transport chains,[22] and macula densa cells are richly endowed with mitochondria distributed along their basal and lateral aspects.[23,24] NOS,[25,26] cyclooxygenase (COX),[27] xanthine oxidase,[6] and NADPH oxidase[28] are also sources of O^-_2. All of these enzymes are expressed in the kidney, and nNOS and COX are expressed in the macula densa.[2,29] However, the importance of these enzymes for stimulation of O^-_2 in the macula densa remains to be determined.

Our data suggest that Tempol blunts TGF when perfused into the macula densa or interstitium, but not the afferent arteriole. However, these experiments were performed in solutions gassed with air (P_{O2} 152 mm Hg) whereas the P_{O2} of the renal cortex is 40 to 50 mm Hg.[30] The relatively high P_{O2} of our solutions would be expected to enhance O^-_2 production. Although these results were not significantly different from our previous studies in which solutions were gassed with 95% oxygen, it would be interesting to investigate the role of O^-_2 in regulation of TGF under physiological conditions.

Although Tempol has been evaluated extensively as a scavenger of O^-_2 in vitro and in vivo,[31,32] the mechanism by which it inhibits TGF remains unclear. Our data suggest that one pathway may involve an interaction between O^-_2 and NO; however, other mechanisms cannot be excluded. O^-_2 can also stimulate inositol 1,4,5-trisphosphate (IP_3) formation and thus increase intracellular calcium in vascular smooth muscle cells.[33] Previous investigations have demonstrated that luminal perfusion of the Ca^{++} ionophore in the presence of Ca^{++} increased TGF responses, whereas an inhibitor of intracellular Ca^{++} release reduced stop-flow pressure responses.[34,35] We found that when nNOS was blocked by 7-NI, the inhibitory effect of Tempol was completely abolished, suggesting that the action of O^-_2 on TGF mainly involves NO in the macula densa.

In conclusion, our data suggest that intracellular O^-_2 in the macula densa enhances TGF. This effect is due to O^-_2 scavenging NO, which effectively decreases its bioavailability. Thus O^-_2 may exert an enhanced influence on TGF under normal conditions. It may also act as a strong factor under pathological conditions associated with high levels of O^-_2 and impaired endothelial function.

Acknowledgments

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