Superoxide Inhibits Neuronal Nitric Oxide Synthase Influences on Afferent Arterioles in Spontaneously Hypertensive Rats

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Abstract—This study was designed to determine the influence of increased superoxide anion in neuronal nitric oxide synthase (nNOS)-dependent regulation of afferent arterioles in spontaneously hypertensive rats (SHR). Afferent arteriolar diameters of male Wistar-Kyoto rats (WKY) and SHR were assessed in vitro with the blood-perfused juxtamedullary nephron technique and averaged 21.6 ± 1.6 (n = 6) and 18.8 ± 1.2 (n = 7) μm, respectively. The superoxide dismutase mimetic Tempol (1, 10, and 100 μmol/L) did not influence afferent arterioles of WKY but significantly increased afferent arteriolar diameters of SHR by 20.6 ± 5.5%, 25.2 ± 5.4%, and 23.3 ± 4.9%, respectively. In WKY (n = 6), the nNOS inhibitor S-methyl-L-thiocitrulline (L-SMTC; 10 μmol/L) and the NOS inhibitor Nω-nitro-L-arginine (L-NNA; 100 μmol/L) significantly decreased afferent arteriolar diameters (19.6 ± 1.6 μm) by 11.9 ± 3.1% and 21.0 ± 3.9%, respectively. In SHR (n = 7), L-SMTC did not influence afferent arteriolar diameters (21.0 ± 1.5 μm), but L-NNA exerted an afferent arteriolar constriction (14.8 ± 3.2%) that was similar to the response observed in WKY. Experiments were also performed in the presence of 100 μmol/L Tempol. In afferent arterioles of WKY (n = 6), Tempol treatment did not modulate the basal diameters (21.5 ± 1.2 μm) or the constrictor response to L-SMTC (10.6 ± 2.1%) or L-NNA (19.3 ± 3.3%). In SHR (n = 8), Tempol significantly increased afferent arteriolar diameters by 22.5 ± 4.3% and enhanced afferent arteriolar constrictor responses to L-SMTC (18.4 ± 2.7%) and L-NNA (31.9 ± 2.6%). However, the nitric oxide donor S-nitroso-N-acetylpenicillamine (10 μmol/L), which similarly increased afferent arteriolar diameters (17.2 ± 2.3%, n = 6), did not affect afferent arteriolar responses to L-SMTC (1.5 ± 2.7%) or L-NNA (18.6 ± 2.3%). These suggest that superoxide anion inhibits the control of afferent arteriolar diameters by nNOS in SHR. (Hypertension. 2001;37[part 2]:630-634.)

Key Words: Tempol • nitric oxide synthase • arterioles • rats, spontaneously hypertensive • kidney

Nitric oxide (NO) is recognized as a major paracrine regulator of renal microvascular tone. Inhibition of NO synthase (NOS) causes renal microvascular constriction with consequent decreases in renal blood flow and glomerular filtration rate. Therefore, renal NO deficiency may theoretically lead to the development of hypertension. However, constitutive NOS activity has been reported to be maintained in the kidneys of various models of hypertension. In kidneys of spontaneously hypertensive rats (SHR), constitutive NOS activity is also maintained or elevated, suggesting that renal NO production is not reduced. However, the endothelium-dependent relaxation of renal vasculatures has been reported to be impaired in this model of hypertension. This finding can be explained by the reduced production of NO-independent vasodilators, such as endothelium-derived hyperpolarizing factor, or a decrease in NO bioavailability. Recent studies have demonstrated that superoxide anion (O2−) production is increased in the aorta of stroke-prone SHR and in the renal cortex of SHR. Because O2− scavenges NO to form peroxynitrite (ONOO−), a shorter-lived and less potent vasorelaxant than NO, the increase in O2− production may cause the decrease in NO bioavailability that has been observed in the aorta of stroke-prone SHR and the mesenteric arteries of SHR and the afferent arterioles of streptozotocin-treated diabetic rats.

The present study was performed to evaluate the influence of constitutive NOS activity, that is, neuronal NOS (nNOS) and endothelial NOS (eNOS), on the afferent arteriolar diameters in SHR and the normotensive control rats, Wistar-Kyoto rats (WKY). We hypothesized that the influence of NOS on afferent arterioles in SHR would be altered by the decrease in NO bioavailability resulting from an increase in O2− activity. To test this hypothesis, we used the in vitro blood-perfused juxtamedullary nephron technique combined with videomicroscopy.
Methods
Assessment of Afferent Arteriolar Diameter
The experiments were performed in accordance with the guidelines and practices established by the Keio University Animal Care and Use Committee. Afferent arteriolar diameter was assessed in vitro with the blood-perfused juxtamedullary nephron technique combined with videomicroscopy, as previously described.4,18–22 Briefly, each experiment used 2 male WKY or SHR (Charles River Labs), weighing 300 to 350 g, with 1 rat serving as the blood donor and the second rat serving as the kidney donor. The rats were allowed free access to water and standard rat chow (Clea Japan) before the experiments. The rats were anesthetized with sodium pentobarbital (50 mg/kg IP). Donor blood was collected into a heparinized (500 U) syringe and centrifuged to separate the plasma and cellular fractions. Plasma oncotic pressure was adjusted to 18 mm Hg by the addition of BSA (Sigma Chemical Co). After processing of the plasma through filters, erythrocytes were added to achieve a hematocrit of 33%. This reconstituted blood was stirred continuously in a closed reservoir pressurized with a 95% O2, 5% CO2 gas mixture.

The right renal artery of the kidney donor was cannulated and perfused with Tyrode’s solution (pH 7.4), containing 5.1% BSA and a mixture of L-amino acids (Sigma Chemical Co).19 The kidney was excised and prepared as previously described4,18–22 so that the vasculature of the juxtamedullary nephrons was directly visualized and the papilla remained intact. The arterial supply of the exposed artery with fine suture (nylon black monofilament, 10-0; Surgical Specialties Co). After the dissection was complete, the Tyrode’s perfusate was replaced with the reconstituted blood. The perfusion pressure was monitored with a pressure cannula centered in the tip of the perfusion cannula and maintained at 100 mm Hg throughout all experimental protocols. The perfusion pressure was regulated by adjusting the rate of gas inflow into the blood reservoir. The perfusion chamber was warmed, and the inner cortical surface of the kidney was continuously superfused with a warmed (37°C) Tyrode’s solution containing 1% BSA.

The tissue was transilluminated on the fixed stage of a Nikon Eclipse microscope (model E600FN). Video images of the microvessels were transferred via a CCD camera (model C2400-75i; Hamamatsu Photonics) through an image processor (model C5510; Hamamatsu Photonics) and recorded on videotape for later analysis (Videocassette Recorder SVO-260; SONY). The inside diameters of the afferent arterioles were measured at 0.25-second intervals with a width analyzer (model C3161; Hamamatsu Photonics) combined with a computer system (MacLab/8s; AD Instruments Pty Ltd) that yielded diameter measurements reproducible to within 0.5 μm. The afferent arteriolar diameters were measured at sites that were within 100 μm upstream of the glomerulus. A minimum 10-minute equilibration period was allowed before the initiation of each experimental procedure. The average diameter during the final minute of each 5-minute treatment period was used for the statistical analysis of steady-state responses.

Afferent Arteriolar Diameters During Tempol Treatment
The effects of the superoxide dismutase mimetic Tempol on afferent arteriolar diameters were determined in kidneys harvested from WKY and SHR. In WKY, the control diameter of the afferent arterioles was measured before and during exposure to increasing concentrations (0.1, 1, 10, and 100 μmol/L) of Tempol (Sigma Chemical Co).

Effects of Tempol on Afferent Arteriolar Responses to nNOS and NOS Inhibition
To study the effects of O2·− on the regulation of afferent arteriolar diameters by nNOS and eNOS, afferent arteriolar constrictor responses to the selective nNOS inhibitor L-arginine (L-NNA; Sigma Chemical Co) were assessed in kidneys harvested from WKY and SHR. The afferent arteriolar diameters were measured before and during exposure to 10 μmol/L L-SMTC and an additional exposure to 100 μmol/L L-NNA. The same assessment was also performed in the presence of 100 μmol/L Tempol with another group of rats.

Statistical Analysis
The changes in afferent arteriolar diameters resulting from treatment with Tempol and the NOS inhibitors were analyzed with 1-way ANOVA for repeated measures combined with the Newman-Keuls post hoc test. Differences in afferent arteriolar responses to NOS inhibitors in the absence and presence of Tempol treatments were determined with 2-way ANOVA for repeated measures combined with the Newman-Keuls post hoc test. A value of P<0.05 was considered significant. Data are presented as mean±SEM.

Results
Systolic arterial pressure was measured with tail-cuff plethysmography immediately before the experiment. In the present study, the systolic arterial pressure of SHR (183±2 mm Hg, n=28) was significantly higher than that of WKY (120±1 mm Hg, n=18).

Afferent Arteriolar Diameters during Tempol Treatment
Figure 1 shows the dose-response effects of Tempol on afferent arteriolar diameters in WKY and SHR. In WKY, the control diameter of the afferent arterioles averaged 21.6±1.6 μm (n=6). Superfusion with 0.1, 1, 10, and 100 μmol/L tempol did not influence the afferent arteriolar diameters. In SHR, however, the control diameter of the afferent arterioles averaged 18.8±1.2 μm (n=7) and was significantly smaller than that observed in WKY. The afferent arteriolar diameters increased significantly by 6.6±3.7%, 20.6±5.5%, 25.2±5.4%, and 23.3±4.9% in response to 0.1, 1, 10, and 100 μmol/L Tempol, respectively.

Effects of Tempol on Afferent Arteriolar Responses to nNOS and NOS Inhibition
Figure 2 illustrates the afferent arteriolar responses to the nNOS inhibitor L-SMTC and the NOS inhibitor L-NNA in WKY. The control diameter of the afferent arterioles averaged 19.6±1.6 μm (n=6). The afferent arteriole diameter decreased significantly by 11.9±3.1% in response to 10 μmol/L L-SMTC and further decreased by 21.0±5.9% in response to 100 μmol/L L-NNA. In the presence of 100 μmol/L Tempol, the control diameter of the afferent arterioles averaged 21.5±1.2 μm (n=6) and was similar to the control diameter of the afferent arterioles in the absence of Tempol. In the presence of Tempol, the L-SMTC signifi-
cantly decreased the afferent arteriolar diameter by 10.6±2.1%. The L-NNA further decreased the afferent arteriolar diameter by 19.3±3.3%. The afferent arteriolar responses to L-SMTC and L-NNA were similar to those observed in the absence of Tempol.

Figure 3 demonstrates the afferent arteriolar responses to L-SMTC and L-NNA in SHR. The control diameter of the afferent arterioles averaged 21.0±1.5 μm (n=7). The afferent arteriolar diameter was not influenced by 10 μmol/L L-SMTC but decreased significantly by 14.8±3.2% in response to 100 μmol/L L-NNA. In our preliminary study (n=3), 10 μmol/L L-SMTC also did not influence afferent arteriolar diameters in SHR when the perfusion pressure was maintained at 150 mm Hg. In addition, the effects of 100 μmol/L Tempol on the afferent arteriolar responses to L-SMTC and L-NNA were studied with an additional group of rats. Superfusion with 100 μmol/L Tempol significantly increased the afferent arteriolar diameter from 18.6±1.0 to 22.6±0.8 μm (n=8). In the presence of Tempol, L-SMTC significantly decreased the afferent arteriolar diameter by 18.4±2.7%, and L-NNA decreased the afferent arteriolar diameter by 31.9±2.6%. The afferent arteriolar responses to L-SMTC and L-NNA were significantly greater than those obtained in the absence of Tempol. Thus, 100 μmol/L Tempol significantly enhanced the afferent arteriolar responses to L-SMTC and L-NNA.

To test whether the vasodilatation observed during the Tempol treatment enhanced the vasoconstrictor responses to L-SMTC and L-NNA in SHR, the afferent arteriolar responses to L-SMTC and L-NNA were also assessed in a group of kidneys treated with the NO donor S-nitroso-N-acetylpenicillamine (SNAP; Sigma Chemical Co). As shown in Figure 3, the control diameter of the afferent arterioles in this group averaged 19.3±1.2 μm (n=6), and the diameter increased to 22.6±1.5 μm after continuous exposure to 10 μmol/L SNAP. The afferent arteriolar diameter increased by an average of 17.2±2.3% and was similar to the increase that was observed during the treatment with 100 μmol/L Tempol (22.5±4.3%). In the SNAP-treated kidneys, L-SMTC did not influence the afferent arteriolar diameters, whereas L-NNA significantly decreased the afferent arteriolar diameters by 18.6±2.3%. The responses to L-SMTC and L-NNA were similar to those obtained in untreated kidneys.

Figure 2. Afferent arteriolar responses to 10 μmol/L L-SMTC and 100 μmol/L L-NNA in untreated kidneys (open circles and open columns, n=6) and kidneys treated with a superfusion of 100 μmol/L Tempol (filled circles and filled columns, n=6) in WKY. Data are expressed in micrometers (A) and percent changes (B). *P<0.05 vs control diameter.

Figure 3. Afferent arteriolar responses to 10 μmol/L L-SMTC and 100 μmol/L L-NNA in untreated kidneys (open circles and open columns, n=7) and kidneys treated with a superfusion of 100 μmol/L Tempol (filled circles and filled columns, n=8) or 10 μmol/L SNAP (filled triangles and shaded columns, n=6) in SHR. Data are expressed in micrometers (A) and percent changes (B). *P<0.05 vs control diameter. †P<0.05 vs response in untreated kidneys.

Discussion
A recent study demonstrated that the renal cortex of SHR contains increased protein levels of nitrotyrosine, which is formed by the interaction of tyrosine residues and ONOO−.14 Because O2− interacts with NO to yield ONOO−, the data suggested that the renal cortex of SHR has an increased level of O2−. The present study demonstrated that the removal of O2− by the superoxide dismutase mimetic Tempol elicits a vasodilator response in the afferent arterioles of SHR but not in the afferent arterioles of WKY. This result suggests at least that O2− promotes vasoconstriction in the afferent arterioles of SHR, although further biochemical studies will be required to confirm that increased levels of O2− radicals prevail at the juxtaglomerular apparatus in SHR but not in WKY. Thus, increased renal levels of O2− may contribute significantly to the regulation of afferent arteriolar diameters in SHR.

In the kidney of SHR, nNOS inhibition by L-SMTC did not influence the afferent arteriolar diameters, although the same concentration of L-SMTC significantly constricted the afferent arterioles in WKY. This result indicates that the influence of nNOS on afferent arterioles is reduced in SHR compared with WKY. However, NOS inhibition by L-NNA produced a similar decrease in afferent arteriolar diameters in both SHR and WKY, suggesting that the influence of NOS on afferent arterioles is maintained in SHR. Thus, the influence of nNOS on afferent arterioles must be drastically reduced in SHR. During the Tempol treatment, L-SMTC exerted a vasoconstrictor response in the afferent arterioles of SHR, but the tempol treatment did not influence the responses to L-SMTC or L-NNA in the afferent arterioles of WKY. These results suggest that Tempol treatment restores the reduced influence
of nNOS on the afferent arterioles of SHR. In addition, L-SMTC did not influence the afferent arteriolar diameters of SHR in kidneys treated with 10 μmol/L SNAP, causing the afferent arterioles to dilate to the same extent as that observed for the treatment with 100 μmol/L Tempol. Consequently, the vasodilation observed during the Tempol treatment probably does not enhance the vasoconstrictor response to L-SMTC. Instead, the removal of O2− by Tempol probably restores the impaired regulation of nNOS-dependent afferent arteriolar diameters. The inactivation of NO by O2− may account for the impaired nNOS-dependent control of afferent arteriolar diameters in SHR.

Although the afferent arteriolar responses to L-NNA were also enhanced during the Tempol treatment, the percentage by which the afferent arteriolar response to L-NNA increased with Tempol (16.0%) was similar to the percentage by which the afferent arteriolar response to L-SMTC increased with Tempol (17.1%). These data suggest that O2− mainly scavenges the NO derived from nNOS but does not modulate eNOS-derived NO to a great extent in SHR. Bosse and Bachmann demonstrated that nitrotyrosine immunoreactivity is prominently expressed in the interstitium and the extraglomerular mesangial cells between the macula densa and the afferent arteriole in the 2-kidney, 1-clip Goldblatt hypertensive rat. This finding suggests that O2− is mainly produced in the interstitium and the extraglomerular mesangial cells between the macula densa and the afferent arteriole and exerts a similar vasodilation effect in normotensive rats and SHR. Therefore, the NO-vasodilation capacity of the afferent arterioles in SHR is similar to that of normotensive rats.

Continuous exposure to the NO donor SNAP (10 μmol/L) significantly increased the afferent arteriolar diameters in SHR. The increase in afferent arteriolar diameter produced with the SNAP treatment (17±2%) was similar to that observed in normotensive Sprague-Dawley rats (19±2%). These results suggest that a large amount of exogenous NO overwhelms the inactivation by O2− and exerts a similar vasodilation effect in normotensive rats and SHR. Therefore, the NO-vasodilation capacity of the afferent arterioles in SHR is similar to that of normotensive rats.

NO derived from nNOS contributes to the counteraction of tubuloglomerular feedback (TGF) responses through buffering of TGF-mediated afferent arteriolar constriction and inhibition of sodium chloride reabsorption by the macula densa cells. During chronic nNOS inhibition, the enhanced TGF response causes the arterial pressure to increase in rats. Because studies have demonstrated an enhanced TGF response and a decreased role of NO in the TGF response in SHR, the reduced influence of nNOS on the TGF response may play a role in the development of hypertension in SHR. Welch et al recently reported that the overproduction of O2− inactivates NO around the juxtaglomerular apparatus, thus contributing to the reduced role of NO in the TGF response. The present study provides direct evidence that the interaction between O2− and nNOS-derived NO plays an important role in the regulation of afferent arteriolar diameters in SHR. Therefore, an increased level of O2− in the kidneys of SHR may contribute to the enhanced TGF response by diminishing the influence of nNOS on the afferent arterioles.

Because the afferent arterioles of juxtamedullary nephrons supply the renal medullary blood flow, the present findings in juxtamedullary nephrons may also indicate an important role in the medullary hemodynamics of SHR. Previous studies have demonstrated that alterations in medullary blood flow in response to changes in renal arterial pressure are blunted in SHR. This blunted response reportedly resembles that observed in rats treated with the NOS inhibitor N^O-nitro-L-arginine-methyl ester. In addition, the administration of L-arginine, a substrate for NO, was shown to restore the blunted relationship between medullary blood flow and renal arterial pressure. These findings suggest that an impaired NO system may contribute to the blunted blood flow response to changes in pressure in the renal medulla of SHR. Because nNOS inhibition with L-SMTC enhanced the pressure-induced vasoconstriction in afferent arterioles of the juxtamedullary nephrons, the inactivation of NO by O2− may contribute to the blunted relationship between medullary blood flow and renal arterial pressure. These findings suggest that an impaired NO system may contribute to the blunted blood flow response to changes in pressure in the renal medulla of SHR.

In conclusion, Tempol significantly decreased the afferent arteriolar diameters in SHR but did not modulate the afferent arteriolar diameters in WKY, suggesting that O2− significantly contributes to the control of afferent arteriolar diameters in SHR. In addition, the afferent arterioles of SHR constricted in response to the NO inhibition but did not respond to the selective inhibition of nNOS. Because the removal of O2− restored the impaired response to nNOS inhibition, O2− probably selectively inactivates nNOS-generated NO. In the kidney of SHR, O2− may contribute to the regulation of afferent arteriolar diameters by scavenging the NO generated by nNOS.

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