Superoxide Anion Production Is Increased in a Model of Genetic Hypertension
Role of the Endothelium

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Abstract—The hypothesis that the decreased nitric oxide (NO) availability observed in spontaneously hypertensive stroke-prone rats (SHRSP) is due to excess superoxide (O$_2^-$) was examined. O$_2^-$ generation, measured by lucigenin chemiluminescence, was studied in 12- to 16-week male and female Wistar-Kyoto rats (WKY) and SHRSP. In addition, expression of the gene encoding endothelial NO synthase, the enzyme involved in NO generation, was investigated. O$_2^-$ generation was increased in male and female SHRSP (4.11±0.24 and 3.84±0.28 nmol O$_2^-$ · min$^{-1}$ · mg$^{-1}$ respectively) compared with their WKY counterparts and was significantly higher in male than female WKY (1.22±0.08 in males and 0.8±0.08 nmol O$_2^-$ · min$^{-1}$ · mg$^{-1}$ respectively) (SHRSP versus WKY $P<0.0001$, 95% CI $-3.39$, $-2.51$; male versus female WKY $P=0.0029$, 95% CI $-0.67$, $-0.17$). Removal of the endothelium by rubbing or addition of NO synthase inhibitors attenuated O$_2^-$ generation in SHRSP but not WKY. In males, removal of the endothelium reduced O$_2^-$ generation from 3.86±0.12 to 1.35±0.08 nmol · min$^{-1}$ · mg$^{-1}$ ($P<0.0001$, 95% CI 2.29, 2.81), whereas addition of L-NAME caused a reduction from 4.13±0.17 to 1.32±0.16 nmol · min$^{-1}$ · mg$^{-1}$ ($P<0.0001$, 95% CI 2.36, 2.83). Similar reductions were observed in females. L-arginine had no significant effect, but tetrahydrobiopterin significantly decreased O$_2^-$ generation in SHRSP from 4.04±0.11 to 2.36±0.40 nmol · min$^{-1}$ · mg$^{-1}$ ($P=0.0026$, 95% CI 0.89, 2.44). Endothelial NO synthase mRNA expression was significantly greater in SHRSP than in WKY and in WKY males than in WKY females. These results show that O$_2^-$ generation is increased in SHRSP and that the tissue and enzymatic sources of this excess O$_2^-$ appear to be the endothelium and eNOS, respectively. The increase in O$_2^-$ generation could explain the decreased availability of basal NO observed in this model of genetic hypertension. (Hypertension. 1999;33:1353-1358.)

Key Words: superoxide ■ endothelium ■ nitric oxide ■ rats, inbred SHR ■ nitric oxide synthase

Endothelial dysfunction and a relative deficiency in nitric oxide (NO) may be associated with hypertension in humans$^{1,2}$ and in some models of experimental hypertension.$^{3,4}$ In the spontaneously hypertensive stroke-prone rat (SHRSP), a model of genetic hypertension, we have shown an attenuation of functional basal NO despite increased eNOS enzymatic activity.$^5$ Although endothelial NO synthase (eNOS) enzymatic activity was greater in SHRSP than in Wistar-Kyoto rats (WKY) when examined in vitro the possibility that the actual amount of eNOS was reduced in SHRSP in vivo could not be excluded from these results. Alternatively, eNOS levels could be similar or elevated but NO availability decreased because of more rapid removal after synthesis. Superoxide anion (O$_2^-$) is produced in the vasculature and can scavenge NO forming peroxynitrite. Increased scavenging of NO by O$_2^-$ could lead to a decrease in NO availability despite increased synthesis. Raised O$_2^-$ levels have been reported recently in a number of models of endothelial dysfunction including hypertension, induced by either angiotensin infusion$^6$ or aortic banding.$^7$ In the majority of cases the source of excess O$_2^-$ is uncertain, although involvement of NADH/NADPH oxidases$^8$ and xanthine oxidase$^9$ have been suggested.

The aim of this study was to examine the hypothesis that the decreased NO availability observed in SHRSP is due to excess O$_2^-$. To identify the source of this O$_2^-$, to examine other molecular mechanisms involved such as the expression of the gene-encoding enzyme involved in NO generation in the endothelium (eNOS).

Methods

Animals
Three- to 4-month-old male and female WKY and SHRSP were obtained from the colonies established in Glasgow by brother and sister mating as previously described.$^9$ Blood pressure was measured 1 week before study by tail plethysmography according to our published protocol.$^{10}$

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Superoxide Measurement

Animals were killed with barbiturate overdose. The abdominal aorta was removed and placed in chilled buffer. Periadventitial tissue was carefully removed and O$_2^-$ quantified in 5-mm aortic segments with lucigenin chemiluminescence as originally described by O’Hara et al.\textsuperscript{11} and recently reassessed by Li and colleagues.\textsuperscript{12}

In some experiments the endothelium was removed by rubbing. In others either N\textsuperscript{6}-nitro L-arginine methyl ester (L-NNAME, 100 \textmu M/L), N\textsuperscript{6}-monomethyl L-arginine (L-NMMA, 1 mmol/L), or L-arginine (1 mmol/L and 1 mmol/L) was added 30 minutes before determining O$_2^-$ generation. Additionally, tetrahydrobiopterin (100 \textmu M/L), oxyurinol (10 \textmu M/L), or diphenyleneiodonium (DPI, 10 and 100 \textmu M/L) were added 30 to 60 minutes before determining O$_2^-$ generation in some rings. Control rings from the same animal were assayed in parallel to each treatment. O$_2^-$ generation was quantified against a standard curve of O$_2^-$ generation by xanthine/hypoxanthine. Tissue O$_2^-$ generation was expressed as nmol/mg wet wt per minute. Preliminary studies showed no difference in O$_2^-$ generation between thoracic and abdominal aortas from either WKY or SHRSP.\textsuperscript{13} In this study abdominal aortas were taken for O$_2^-$ measurement and thoracic aortas used to confirm the attenuation of basal NO$^+$ in the same animals and for quantification of the mRNA of eNOS.

Reverse Transcription–Polymerase Chain Reaction for eNOS

Total RNA was extracted from homogenized thoracic aortas with the use of RNAzol B. Messenger RNA for eNOS was quantified by reverse transcription–polymerase chain reaction (RT-PCR) as described.\textsuperscript{14} Briefly, competitor RNA was transcribed in vitro from the plasmid pReNIS5, which was kindly donated by F. Soubrier (Paris, France). This plasmid contains a 64 bp fragment of polylinker inserted in the Sac II site of a rat eNOS cDNA.

Total aortic RNA (100 ng) and competitor RNA (0 to 400 fg) were reverse transcribed in the same reaction at 42°C for 45 minutes with oligo(dT)$_{15}$ primer (0.5 \textmu M) and reverse transcriptase (10 U/mL) (AMV) in the presence of RNase inhibitor (40 \textmu U/mL). PCR was carried out on a 3-\textmu L aliquot of each RT reaction with the use of 2 primers, forward (5’-TTC CGG CTG CCA CCT GAT CCT AA-3’) and reverse (5’-AAC ATG TGT CCT TGC TCG AGG CA-3’) surrounding the 64 bp fragment insertion site. Each reaction contained Taq DNA polymerase (5 \textmu U/mL), primers (10 \textmu M/L each), and dNTPs (1.25 mmol/L) and was subjected to an initial denaturation at 94°C for 5 minutes, then 28 cycles of 30 seconds at 94°C, 30 seconds annealing at 62°C, and 1-minute elongation at 72°C; then completion of ongoing reactions at 62°C for 1 second, 72°C for 1 minute, and 72°C for 10 minutes.

The products of each reaction were run on a 2% agarose gel and bands visualized by Southern blotting. The density of each band was quantified with a densitometer and a plot of long competitor density (x-axis) versus log ratio (target density/competitor density) (y-axis) constructed. When the log ratio = 0, then the concentration target and competitor are equal, and this value can be read from the x-axis (Figure 1). For each sample, 10 RT-PCR reactions were run with a range of amounts of competitor mRNA (known).

Analysis of Data

Analysis was by unpaired t test or ANOVA as appropriate. Bonferroni correction was used when multiple comparisons were made. Results are expressed as mean±SE.

Results

Blood Pressure

Blood pressure was elevated in SHRSP compared with WKY and in addition was significantly higher in males than females. Systolic blood pressure (mm Hg) was as follows: male WKY 131±3 (n = 21); female WKY 123±2 (n = 17); male SHRSP 171±6 (n = 23); female SHRSP 149±3 (n = 30).

Superoxide Levels

Superoxide levels were increased in SHRSP males and females compared with their WKY counterparts, as shown in Figure 2. In addition, a gender difference was observed in WKY rats, with males having higher levels than females. However, although overall O$_2^-$ generation was greater in male than female SHRSP (4.11±0.24 versus 3.84±0.28 nmol
O$_2^-$ (min$^{-1}$ mg$^{-1}$), this did not reach significance. Levels of O$_2^-$ in another rat strain, the Sprague-Dawley, were similar to those observed in WKY 1.27±0.08 nmol min$^{-1}$ mg$^{-1}$ (males only).

Removal of the endothelium had no effect on O$_2^-$ generation in either male or female WKY (Figure 3a). In contrast, in male SHRSP O$_2^-$ was reduced from 3.86±0.12 to 1.35±0.08 and in female SHRSP from 3.58±0.15 to 1.41±0.10 nmol O$_2^-$ min$^{-1}$ mg$^{-1}$. These reductions in O$_2^-$ levels were highly significant: P<0.0001, 95% CI 2.29, 2.81, and P=0.0001, 95% CI 1.82, 2.52 for males and females, respectively.

Treatment with L-NAME had effects similar to removing the endothelium. In WKY, O$_2^-$ generation was unaffected by treatment, whereas in SHRSP, levels were significantly reduced, as shown in Figure 3b. In male SHRSP, levels were 4.13±0.17 and 3.2±0.16 nmol O$_2^-$ min$^{-1}$ mg$^{-1}$ for control and L-NAME–treated rings, respectively: P<0.0001, 95% CI 2.36, 2.83 and in females 3.98±0.08 and 1.38±0.06 nmol/L O$_2^-$ min$^{-1}$ mg$^{-1}$, P=0.0001, 95% CI 2.36, 2.83. L-NMMA caused a similar reduction in O$_2^-$ levels in SHRSP values, being 4.17±0.17 and 1.84±0.15 nmol/L O$_2^-$ min$^{-1}$ mg$^{-1}$ in control and L-NMMA–treated rings (n=6; P=0.0003, 95% CI 1.68, 3.0).

Treatment with L-arginine had no significant effect on O$_2^-$ generation in any group. In contrast, although 5 minutes of incubation with tetrahydrobiopterin only caused small, non-significant attenuation of O$_2^-$ generation, 30 minutes of incubation with tetrahydrobiopterin significantly reduced O$_2^-$ generation in SHRSP from 4.04±0.11 to 2.36±0.40 nmol min$^{-1}$ mg$^{-1}$ (P=0.0026, 95% CI 0.90, 2.45, males and females combined). No significant change in O$_2^-$ generation was observed in WKY rats (Figure 4a). The NADH/NADPH oxidase inhibitor DPI caused a dose- and time-dependent reduction in O$_2^-$ generation. Thirty-minute incubation with 100 μmol/L DPI resulted in a decrease in O$_2^-$ from 4.39±0.04 to 2.68±0.12 and from 1.11±0.13 to 0.69±0.06 nmol min$^{-1}$ mg$^{-1}$ in SHRSP and WKY, respectively (males and females combined). When the incubation time was extended to 1 hour, O$_2^-$ generation was reduced from 0.10 nmol/L O$_2^-$ to 0.06 nmol/L O$_2^-$ (Figure 4b).

Expression of eNOS in Thoracic Aorta
As shown in Figure 5a, eNOS mRNA expression (fg/100 ng ± SEM) was significantly greater in SHRSP (308±49) compared with WKY (84.9±14) (95% CI: 107, 339; P=0.002). Within the SHRSP, eNOS mRNA expression was significantly greater in males (417±43) compared with females (170±12) (95% CI: 123, 371; P=0.005), and within the WKY there was a tendency for greater eNOS expression in males (113±17) compared with females (49.2±3.8) 95% CI: 16, 112; (P=0.02), but this failed to achieve statistical significance when corrected for triple comparisons.

Western Blotting for eNOS
Comparison of the amount of NO synthase protein between WKY and SHRSP males is shown in Figure 5b. The aortas
from SHRSP were found to have significantly higher levels than the aortas from the WKY. The ratios of eNOS to α-actin in SHRSP and WKY were 5.24 ± 0.43 and 3.06 ± 0.22, respectively (P < 0.02, 95% CI 0.638, 3.722; n = 4).

**Discussion**

In this study we have shown both increased eNOS mRNA expression and increased O$_2^-$ production in SHRSP compared with WKY. Moreover, in this model of genetic hypertension we have identified the cellular and enzyme sources of the O$_2^-$ excess as the endothelial cells and eNOS, respectively. Despite the increase in expression of mRNA for eNOS and the increased enzymatic activity of eNOS previously demonstrated by ourselves,5 NO availability has been shown to be reduced. This was manifest in a decrease in basal but not agonist-stimulated NO-mediated responses.5 Thus it appears that the excess O$_2^-$ generation more than balances the increase in NO production leading to a net decrease in functional NO availability. Enhanced eNOS expression together with increased O$_2^-$ generation has also been reported in Sprague-Dawley rats made hypertensive by aortic banding.7 It is tempting to speculate that the enhanced eNOS expression is a compensatory mechanism related to the increase in O$_2^-$ generation. However, an inverse relation between eNOS expression and O$_2^-$ generation is not always observed. In studies in mature 16-month animals, Bauersachs et al16 found no increase in eNOS expression in SHR thoracic aorta despite an increased O$_2^-$ generation.

The studies described here using L-NNAME and L-NNMMA suggest that in the SHRSP O$_2^-$ is generated by eNOS. There are other reports of NOS producing O$_2^-$.

**Figure 4.** a, Effect of treatment with tetrahydrobiopterin (BH$_4$, 100 μmol/L) on superoxide generation in rings of abdominal aorta from WKY and SHRSP (males and females combined). Rings were treated with BH$_4$ for 5 or 30 minutes before measuring O$_2^-$ generation by lucigenin chemiluminescence. Groups were compared with the use of ANOVA. P < 0.02 was taken as significant. Results are shown as mean±SEM. n=5 for WKY and 6 for SHRSP. b, Effect of treatment with oxypurinol or DPI on superoxide generation in rings of abdominal aorta from WKY and SHRSP (males and females combined). Rings were treated with oxypurinol (10 μmol/L) for 30 minutes or DPI (100 μmol/L) for 60 minutes before measuring O$_2^-$ generation by lucigenin chemiluminescence. Groups were compared with the use of ANOVA. P < 0.02 was taken as significant. Results are shown as mean±SEM. n=5 WKY; n=6 SHRSP.

The exact mechanism whereby eNOS generates O$_2^-$ is uncertain. However, molecular cloning of NO synthase revealed close amino acid sequence homology between NO synthase and cytochrome P$_{450}$ reductase, a known cellular source of O$_2^-$.
In hypertension caused by aortic banding, increased O$_2^-$ production is reported to be an early event that reached a maximum within 2 weeks of surgery.\textsuperscript{7} In that study O$_2^-$ production was not inhibited by L-NAME, and the source of the excess O$_2^-$ was not identified. Increased O$_2^-$ production has also been observed in angiotensin II–mediated hypertension. In this model of hypertension L-NMMA had no effect on O$_2^-$ generation and the source of the excess O$_2^-$ appeared to be membrane bound vascular NADH/NADPH oxidases.\textsuperscript{8}

In our animals the major source of vascular smooth muscle O$_2^-$ appeared to be NADH/NADPH oxidases, as illustrated by the attenuation of O$_2^-$ generation in the presence of DPI. NADPH is a cofactor for eNOS. Thus the proportionally greater reduction in O$_2^-$ generation in SHRSP compared with WKY is likely to be due to inhibition of O$_2^-$ production by eNOS, in addition to inhibition of NADH/NADPH oxidases in vascular smooth muscle in SHRSP. Oxyurinol had no effect on O$_2^-$ generation in either SHRSP or WKY, suggesting that the xanthine oxidase pathway did not contribute to O$_2^-$ in these animals. O$_2^-$ generation was not completely abolished in the tissues incubated with 100 µmol/L DPI for 1 hour. The effects of DPI were dose and time dependent. It is possible that complete inhibition of NADH/NADPH oxidase was not achieved. Alternatively, there are a number of other potential sources of O$_2^-$ including aldehyde oxidase, dihydro-oroic dehydrogenases, flavin dehydrogenases, peroxidases, and auto-oxidation compounds such as catecholamines.\textsuperscript{24}

In WKY animals we observed a significant gender effect on O$_2^-$ levels, O$_2^-$ being greater in males than females. This would be consistent with the higher blood pressure in males. Brandes and Mugge\textsuperscript{25} also found that levels of O$_2^-$ were higher in male than female Wistar rats. In the SHRSP O$_2^-$ levels tended to be higher in the males than the females, but this difference was not significant. It is probable that any gender effect was overwhelmed by the much larger hypertensive effect. One explanation for the gender effect would be that the higher levels of estrogen in the females resulted in increased scavenging of O$_2^-$ . Arnal et al\textsuperscript{26} have shown the synthetic estrogen ethynyl estradiol to increase release of bioactive NO by inhibiting superoxide anion production in cultured bovine endothelial cells, whereas Kleiner et al\textsuperscript{27} have shown increased transcription of human eNOS gene on treatment with estrogens in culture.

In summary, we have shown O$_2^-$ generation to be increased in SHRSP. The tissue and enzymatic sources of this excess O$_2^-$ appear to be the endothelium and eNOS, respectively. The increase in O$_2^-$ generation in SHRSP could contribute to the decreased availability of basal NO observed in this model of genetic hypertension.

Our findings reconcile previous controversies that surrounded molecular and functional analysis of endothelial function in the SHRSP and related models of genetic hypertension. Despite an excessive production of the eNOS mRNA combined with the increased eNOS protein levels, there is NO-dependent endothelial dysfunction that is best explained by an excess of O$_2^-$ generated by the eNOS enzyme within the endothelial cells.

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