Superoxide Excess in Hypertension and Aging
A Common Cause of Endothelial Dysfunction

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Abstract—There is evidence in humans that hypertension and aging similarly impair endothelial function, although the mechanism remains unclear. Superoxide anion (O$_2^-$) is a major determinant of nitric oxide (NO) bioavailability and thus endothelial function. We sought to determine the relationship between endothelial function, O$_2^-$, and age in normotensive Wistar-Kyoto (WKY) and stroke-prone spontaneously hypertensive rats (SHRSP). Aortic rings were removed from female WKY and SHRSP at 3 to 4 months (young) and 9 to 12 months (old). O$_2^-$ generation by aortic rings was measured before and after removal of the endothelium or incubation with N$^G$ nitro-L-arginine methyl ester, diphenyleneiodonium, or apocynin. Levels of p22phox were studied with immunohistochemistry and used as a marker of NAD(P)H oxidase expression. NO bioavailability was significantly lower in old WKY compared with young WKY ($P=0.0009$) and in old SHRSP compared with young SHRSP ($P=0.005$). O$_2^-$ generation was significantly greater in old WKY compared with young WKY ($P=0.0001$). Removal of the endothelium and N$^G$ nitro-L-arginine methyl ester treatment resulted in a significant reduction in O$_2^-$ generation in old SHRSP ($P=0.009$ and 0.001, respectively). Diphenyleneiodonium significantly reduced O$_2^-$ generation in 12-month WKY ($P=0.008$) and 12-month SHRSP ($P=0.009$). Apocynin attenuated O$_2^-$ generation by older WKY ($P=0.038$) and SHRSP ($P=0.028$), p22phox was increased in older animals compared with young. We conclude that NO bioavailability decreases with age in female WKY and SHRSP. O$_2^-$ generation increases with age in WKY and is higher in SHRSP and may contribute to the reduced NO by scavenging. NAD(P)H oxidase may contribute to the age-related increase in O$_2^-$. (Hypertension. 2001;37[part 2]:529-534.)

Key Words: endothelium  ■ nitric oxide  ■ hypertension, experimental  ■ aging

There is evidence that in animal models and in humans, impaired endothelial function and a decrease in nitric oxide (NO) bioavailability may occur in hypercholesterolemia, hypertension, and decreased NO bioavailability may also occur with aging. A decrease in NO bioavailability may occur with aging. However, the effects of aging on O$_2^-$ production are less well defined. Huraux and colleagues observed a negative correlation between O$_2^-$ levels and age in human internal mammary arteries. In contrast, Berry et al found basal O$_2^-$ production in human internal mammary arteries to be weakly but positively associated with age.

Potential vascular sources of O$_2^-$ are endothelial NO synthase (eNOS), xanthine oxidase, and NAD(P)H oxidase. eNOS and NAD(P)H oxidase have been proposed to be involved in O$_2^-$ production in different models of hypertension, whereas xanthine oxidase may be involved in O$_2^-$ production in hypercholesterolemia. eNOS can be inhibited by arginine analogues such as N$^G$ nitro-L-arginine methyl ester (L-NAME). NAD(P)H oxidase is composed of at least 5 subunits, and apocynin can inhibit enzymatic activity by preventing association of the subunits. Diphenyleneiodonium (DPI) is a less specific inhibitor of flavin-containing oxidases, including NAD(P)H oxidase. In this study, the hypothesis that both hypertension and aging result in increased levels of O$_2^-$ and decreased NO bioavailability in blood vessels from Wistar-Kyoto rats (WKY) and stroke-prone spontaneously hypertensive rats (SHRSP) has been examined. The likely source(s) of O$_2^-$ was also investigated.
Methods

Animals
Four groups of female rats were studied: 3- to 4-month-old WKY (n=28), 3- to 4-month-old SHRSP (n=28), 9- to 12-month-old WKY (n=48), and 9- to 12-month-old (n=46) SHRSP. Fewer young animals were used because studies comparing O$_2^-$ production in young WKY and SHRSP animals had already been undertaken. The animals were obtained from the colonies established in Glasgow by brother-and-sister mating, as previously described. Blood pressure was measured by tail-cuff plethysmography 1 week before study, according to our published protocol. All experiments were approved by the Home Office according to regulations regarding experiments in animals in the United Kingdom.

O$_2^-$ Measurement
The animals were given an overdose of barbiturate. The thoracic aorta and carotid arteries were removed, and periadventitial tissue was cleaned from the vessels. O$_2^-$ was quantified in 4- to 5-mm segments by lucigenin chemiluminescence, as originally described by O’Har et al and previously used by our group. In some experiments, the endothelium was removed by rubbing. In others, either L-NAME (0.1 mmol/L), DPI (0.1 mmol/L), or apocynin (3 mmol/L) was added 60 minutes before determining O$_2^-$ generation. Control rings from the same animal were always assayed in parallel to each treatment. O$_2^-$ generation was quantified against a standard curve of O$_2^-$ generation by xanthine/xanthine oxidase. Tissue O$_2^-$ was expressed as nanomoles per minute per milligram of wet weight.

Liochev and colleagues have reported that high concentrations of lucigenin may produce redox cycling leading to artificial increases in O$_2^-$ concentrations of lucigenin used for our initial studies (250 mmol/L) was relatively high; however, we wanted to be able to compare our results with these previously obtained in young animals. Studies in which a range of concentrations of lucigenin have been examined report no change or lower levels of O$_2^-$ with lower concentrations of lucigenin but with any differences between experimental groups retained.

NAD(P)H Oxidase Activity
Aortas and carotids were cleaned of any adhering connective tissue, rinsed, minced finely with scissors, and homogenized for 30 seconds with an Ultraturrax T8. The homogenate was centrifuged for 5 minutes at 1000g and the pellet discarded. Two milliliters of supernatant was taken for measurement of NAD(P)H oxidase activity by lucigenin chemiluminescence in the presence of 500 μmol/L NADH or NADPH and 25 μmol/L lucigenin. Protein concentrations were measured by the method of Bradford, and O$_2^-$ generation was expressed as nanomoles per minute per milligram of protein.

Organ Bath Studies
Arteries were prepared as for measurement of O$_2^-$, except that they were cut into 2- to 3-mm rings. The rings were suspended under 1 g tension in individual 10-mL muscle baths containing physiological saline solution of the following composition (mmol/L): NaCl 130, KCl 4.7, NaHCO$_3$ 14.9, KHPO$_4$ 1.18, MgSO$_4$ 0.7, H$_2$O 1.17, CaCl$_2$ 0.2, H$_2$O 1.6, glucose 5.5, and CaNa$_2$ EDTA 0.03. The physiological saline solution was aerated with 95% O$_2$/5% CO$_2$ and indomethacin was added to a final bath concentration of 0.1 mmol/L to inhibit any prostaglandin-mediated responses. Isometric tension was measured with Grass force transducers and displayed on a MacLab.

NO bioavailability was determined as previously described. Rings were constricted to their individual EC$_{50}$ values to phenylephrine (PE). The NOS inhibitor L-NAME was added at a final concentration of 0.1 mmol/L. The increase in the contractile response was taken as a measure of NO bioavailability and expressed as a percentage of the PE EC$_{20}$.

Immunohistochemistry
Small blocks of thoracic aortas from young and old rats were embedded in OCT and frozen at −70°C. Sections of 5 μm were cut, and immunohistochemistry was performed with standard techniques. Briefly, sections were blocked in 20% horse serum and then incubated overnight (in a humidified box) at 4°C with a monoclonal antibody against p22phox kindly supplied by Dr. Mark Quinn. For negative control, the primary antibody was replaced with mouse IgG. Biotinylated anti-mouse (Vector Labs) at a dilution of 1:200 in 2% horse serum was incubated for 60 minutes followed by streptavidin conjugated to horseradish peroxidase. Color was developed by the addition of DAB (Sigma). The sections were lightly stained in hematoxylin and then dehydrated through alcohol and xylene. Sections were viewed and scored by an independent observer unaware of the age or genotype of the rats. Sections were scored as endothelial or medial, with 1 representing no staining, 2 representing faint brown, 3 moderate brown, and 4 intense brown.

Statistics
Vessels from different animals were compared by 2-tailed unpaired t test, whereas vessels from the same animal with and without treatment were compared by a paired t test. Statistical significance was taken as P<0.05. Results are shown as mean±SEM, with 95% confidence intervals (CI) where significance was achieved. Bonferroni correction was applied for analysis of NADH/NADPH-driven O$_2^-$ generation to allow for multiple comparisons.

Results
Blood Pressure
The blood pressures (mm Hg±SEM) of the 4 groups of female rats studied were as follows: 3- to 4-month WKY, 117±1; 3- to 4-month SHRSP, 137±4; 9- to 12-month WKY, 115±2; and 9- to 12-month SHRSP, 141±3. Blood pressure was significantly higher in SHRSP than WKY at both ages (P<0.0001): 95% CI for 9- to 12-month WKY versus 9- to 12-month SHRSP, −33.6, −18.7, and for 3- to 4-month WKY versus 3- to 4-month SHRSP, −29.5, −11.8. No age-related effect was noted in either strain.

Basal NO Bioavailability
Addition of L-NAME caused an increase in the contractile response to PE in all groups studied. However, as shown in Figure 1a, this increase (% of PE±SEM) was significantly lower in vessels for 9- to 12-month WKY (296±30, n=11) than for 3- to 4-month WKY (523±51, n=15, P=0.0009, 95% CI 144, 349) and in vessels from 9- to 12-month SHRSP.
(220±28, n=8) than for 3- to 4-month SHRSP (341±26, n=16, P=0.005; 95% CI, 41, 201).

**O$_2^-$ Levels**

O$_2^-$ generation in aortas (nmol · min$^{-1} ·$ mg$^{-1}$SEM) was significantly higher in 9- to 12-month WKY (2.83±0.30, n=21) compared with 3- to 4-month WKY (1.06±0.2, n=7, $P=0.001$; 95% CI, 1.07, 2.54), but the difference between 9- to 12-month SHRSP (3.44±0.31 n=23) and 3- to 4-month SHRSP (2.98±0.49 n=9) did not reach statistical significance (Figure 1b).

Similar increases in O$_2^-$ levels with age and hypertension were observed in carotid arteries. O$_2^-$ values of 0.88±0.18 (n=8) and 3.88±0.50 (n=12) were obtained in vessels from 3- to 4- and 9- to 12-month WKY, respectively ($P=0.002$; 95% CI, 1.39, 3.55), and 3.35±0.46 (n=12) and 4.89±0.88 (n=12) in 3- to 4- and 9- to 12-month SHRSP ($P=0.19$). These results are expressed per milligram of wet weight tissue. There was considerable hypertrophy of both carotid arteries and aortas from the older SHRSP, and it is possible that this resulted in an underestimation of O$_2^-$ levels in these animals.

**Sources of O$_2^-$ in Aorta From 9- to 12-Month Animals**

In older animals, incubation of the aortas with the NAD(P)H oxidase inhibitor DPI caused a significant decrease in O$_2^-$ levels (nmol · min$^{-1} ·$ mg$^{-1}$SEM) from 2.13±0.30 to 0.89±0.18 (n=6, $P=0.008$) in WKY and from 3.04±0.43 to 1.19±0.14 (n=10, $P=0.009$; 95% CI, 0.39, 0.09) in SHRSP (Figure 2a).

As shown in Figure 2b, inhibition of NAD(P)H oxidase activity with apocynin also decreased O$_2^-$ generation (nmol · min$^{-1} ·$ mg$^{-1}$SEM) in older animals, with levels being 1.86±0.25 and 1.06±0.36, respectively, in control and treated vessels from older WKY (n=7, $P=0.038$; 95% CI, 0.07, 1.78) and 2.29±0.53 and 1.44±0.43, respectively, in control and treated vessels from older SHRSP (n=7, $P=0.028$; 95% CI, 0.13, 1.57). In addition, apocynin had no significant effect in young WKY, with levels being 1.65±0.41 and 1.65±0.31 in control and treated vessels, respectively, but reduced O$_2^-$ generation in aortas from young SHRSP from 2.36±0.47 to 1.48±0.27 (n=6, $P=0.037$; 95% CI, 0.08, 1.77).

The NOS inhibitor L-NAME had no significant effect on O$_2^-$ generation in 9- to 12-month-old WKY, being 2.58±0.39 and 2.08±0.23 (n=9, $P=0.08$) in control and treated segments, respectively, but significantly reduced levels in 9- to 12-month-old SHRSP from 2.04±0.44 to 1.55±0.34 (n=6, $P=0.02$; 95% CI, 0.14, 0.79). Similarly, in WKY, the difference between (3.42±0.34) and endothelium-denuded vessels (3.01±0.29, n=10) was not significant. In contrast, removal of the endothelium by rubbing decreased O$_2^-$ levels in SHRSP from 3.63±0.38 to 2.79±0.18 (n=13, $P=0.006$; 95% CI, 0.30, 1.54).

**NADH/NADPH-Driven O$_2^-$ Production**

In aortas and carotid arteries, NADH-driven O$_2^-$ generation was greater than NADPH-driven O$_2^-$ generation in all groups. Mean NADH- and NADPH-driven O$_2^-$ generation was higher in older animals (Table). This difference was significant for NADH-driven O$_2^-$ generation in carotid arteries from 3- to 4-month versus 9- to 12-month WKY ($P=0.038$; 95% CI, 91, 5033) but failed to reach statistical significance in carotid arteries from SHRSP or in aorta from either WKY or SHRSP.

**Immunohistochemistry**

Representative sections from young and old WKY and SHRSP are shown in Figure 3. Moderate brown staining was evident in the endothelium of the young vessels in both strains as 1±1 (Figure 3, B and D), whereas the media was scored as 0±1 for both. In the older WKY rats (Figure 3C), the endothelium scored 2±1, whereas that of the SHRSP (Figure 3E) consistently scored 3. Moderate staining, 1±1, was present in the media of both old WKY and old SHRSP. Because much of the periadventitial tissue is routinely removed from these vessels, it was not possible to comment, reliably, on the staining patterns.
Discussion

In these studies, we showed that both hypertension and aging result in a decrease in basal NO bioavailability and a corresponding increase in the generation of vascular $\text{O}_2^-$ in female rats. We then went on to investigate the tissue and enzymatic sources of this excess $\text{O}_2^-$. In the older SHRSP but not WKY, both removal of the endothelium by rubbing and L-NAME treatment caused a significant reduction in $\text{O}_2^-$ levels. Previously, we have made similar observations in young SHRSP, and the present results would substantiate our conclusion that eNOS is an important source of $\text{O}_2^-$ in SHRSP.

However, eNOS is not the only source of $\text{O}_2^-$. Both DPI and apocynin attenuated $\text{O}_2^-$ production in vessels from SHRSP and older WKY. DPI is frequently used as an inhibitor of NAD(P)H pathways, although it has other actions, including inhibition of NOS. The vascular NAD(P)H oxidase consists of at least 5 subunits, with those that make up the membrane-bound cytochrome $b_{558}$, p22phox, and gp91phox being important for the electron transport and the reduction of molecular oxygen to $\text{O}_2^-$. Apocynin acts by interfering with NAD(P)H subunit assembly in the membrane and is therefore a more specific inhibitor than DPI. Taken together, the inhibition of $\text{O}_2^-$ production by these compounds would be consistent with a role for NAD(P)H oxidase as a source of $\text{O}_2^-$, particularly in older animals.

Further support for this hypothesis comes from the immunohistochemical studies that showed staining for p22phox. In both WKY and SHRSP, staining was lowest in young WKY and highest in old SHRSP. However, both endothelial and vascular smooth muscle cell expression was upregulated in all the older rats.

As expected for vascular tissue, NADH generated tissue was greater than that generated by NADPH in both aortas and carotid arteries from all groups of animals studied. However, although NADH-generated $\text{O}_2^-$ levels tended to be higher in the older animals, this only reached statistical significance for NADH-driven $\text{O}_2^-$ generation in WKY carotid arteries. The
immunohistochemical data suggested that p22phox levels were highest in the endothelium and lowest in vascular smooth muscle. The proportion of vascular smooth muscle was greater in blood vessels from older animals, which is likely to lead to an underestimation of the $O_2^-$ generation per milligram of protein in the older animals. It is also possible that not all subunits of the NAD(P)H oxidase complex were upregulated to the same extent as p22phox in the older animals.

Although these studies indicate that NAD(P)H oxidase activity increases with age in female rats, these studies do not exclude an additional increase in $O_2^-$ from other sources in the older animals. For example, although $O_2^-$ generation from xanthine oxidase is negligible in young WKY and SHRSP, its contribution to $O_2^-$ generation was not examined in older animals.\(^{12}\)

In the studies reported here, a range of techniques was used to substantiate and extend our original findings. Taken together, these studies point to both eNOS and NAD(P)H oxidase as sources of $O_2^-$ in SHRSP and suggest that the endothelium is an important source of $O_2^-$ in both young and old SHRSP. In contrast, in young WKY, there is less endothelial involvement in $O_2^-$ production. $O_2^-$ generation by NAD(P)H oxidase appears to increase with age, and its primary source appears to be endothelium and adventitia.

All of the studies reported here were carried out in female rats. In contrast to female rats, we have previously observed no decrease in basal nitric oxide bioavailability with age in male WKY or SHRSP.\(^{10}\) Zalba et al.\(^{22}\) found no difference in NAD(P)H-driven $O_2^-$ production in aortas from 16- and 30-week-old male WKY, although an increase was observed in male SHR at 30 weeks. This could suggest that some of the age-related changes reported here are gender-specific. Decreased estrogen levels with age would provide a potential explanation because estrogen has been reported to act as an antioxidant decreasing LDL oxidation and uptake,\(^{30}\) to up-regulate eNOS,\(^{31}\) and to decrease vascular $O_2^-$ production.\(^{32}\) However, decreased estrogen levels are unlikely to be the cause of any of the age-related changes reported here. Most of the older animals used in our study were ex-breeders whose last litter had been weaned <1 month previously. Moreover, plasma estrogen levels do not differ significantly between 3- and 9-month-old animals (unpublished observations).

Conclusions
As with hypertension, the endothelial dysfunction with aging is due to reduced NO bioavailability as a result of scavenging by excess vascular $O_2^-$ production. Endothelial NOS contributes significantly to $O_2^-$ production in hypertensive animals, whereas NAD(P)H oxidase appears to be an important contributor to age-related increases in $O_2^-$.

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