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,[1,2] diabetes,[3] and hypertension[4–6] despite normal or increased NO production by the endothelium.[6] A decrease in NO bioavailability may also occur with aging.[7–10]

In a number of animal models of disease, including hypertension[11,12] and hypercholesterolemia,[13] an increase in superoxide (O_2^-) occurs concurrent to the decrease in NO bioavailability. O_2^- rapidly reacts with NO, forming peroxynitrite and decreasing NO bioavailability.[14] Thus, it has been proposed that elevations in O_2^- levels contribute to the impaired endothelial function associated with atherosclerotic disease.[13,15]

Taddei et al[9] proposed that the endothelial dysfunction that occurs in hypertension represents an accelerated form of the dysfunction that occurs with aging. However, the effects of aging on O_2^- production are less well defined. Huraux and colleagues[16] observed a negative correlation between O_2^- levels and age in human internal mammary arteries. In contrast, Berry et al[17] 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),[18] xanthine oxidase,[19] and NAD(P)H oxidase.[20,21] eNOS[18] and NAD(P)H oxidase[22,23] 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.[13] 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.

Received October 25, 2000; first decision November 27, 2000; revision accepted December 11, 2000.

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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.[12] The animals were obtained from the colonies established in Glasgow by brother-and-sister mating, as previously described.[24] Blood pressure was measured by tail-cuff plethysmography 1 week before study, according to our published protocol.[25] 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'Hara et al[13] and previously used by our group.[12,17] 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[26] have reported that high concentrations of lucigenin may produce redox cycling leading to artificial increases in O_2^-. The concentration 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.[12] Studies in which a range of concentrations of lucigenin have been examined report no change[17] or lower levels of O_2^- with lower concentrations of lucigenin but with any differences between experimental groups retained.[27]

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 1000*g* 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,[28] 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, KH_2PO_4 1.18, $MgSO_4$ 0.7, H_2O 1.17, $CaCl_2$ 0.2, H_2O 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 prostanoid-mediated responses. Isometric tension was measured with Grass force transducers and displayed on a MacLab.

NO bioavailability was determined as previously described.[6] Rings were constricted to their individual EC_{20} 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}.

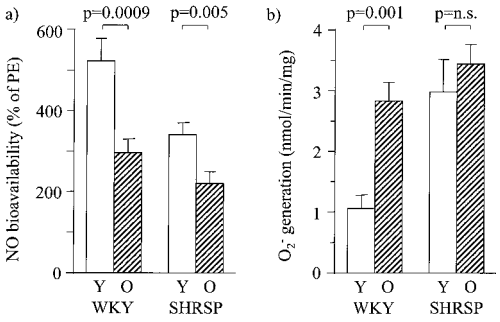

Figure 1. a, NO bioavailablity as measured by contraction in response to L-NAME (% of PE±SEM) for young (Y) and old (O) WKY and SHRSP. b, O_2^- generation as measured by lucigenin chemiluminescence in young and old WKY and SHRSP.

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

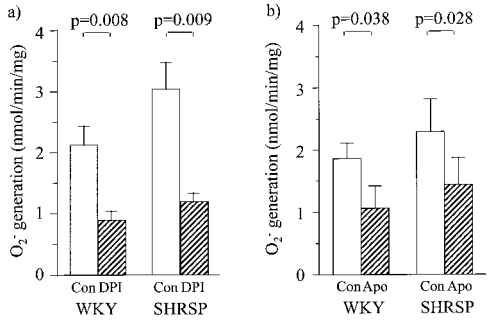

Figure 2. a, O_2^- generation as determined by lucigenin chemiluminescence in control (Con) and DPI-treated (0.1 mmol/L) vessels from WKY and SHRSP. b, O_2^- generation as determined by lucigenin chemiluminescence in control (Con) and apocynin-treated (Apo, 3 mmol/L) vessels from WKY and SHRSP.

(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, 3.09) 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 control (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- and NADPH-Driven O_2^- Production in Aortas and Carotid Arteries From Young and Old WKY and SHRSP

		O_2^- Production, nmol · min^{-1} · mg^{-1}	
Tissue	Rat Strain	NADH	NADPH
Aorta	3- to 4-mo WKY	2490±261*	1157±151
	9- to 12-mo WKY	2962±407*	1251±98
	3- to 4-mo SHRSP	2354±351*	1212±302
	9- to 12-mo SHRSP	3334±540*	1757±340
Carotid arteries	3- to 4-mo WKY	2877±787*	1519±158
	9- to 12-mo WKY	5524±912*†	2918±554
	3- to 4-mo SHRSP	3207±286*	2812±473
	9- to 12-mo SHRSP	4821±760*	2114±334

*NADH significantly higher than NADPH O_2^- generation (P<0.05).

†NADH O_2^- generation significantly greater (P<0.05) in carotid arteries from 9- to 12-month than 3- to 4-month WKY.

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.

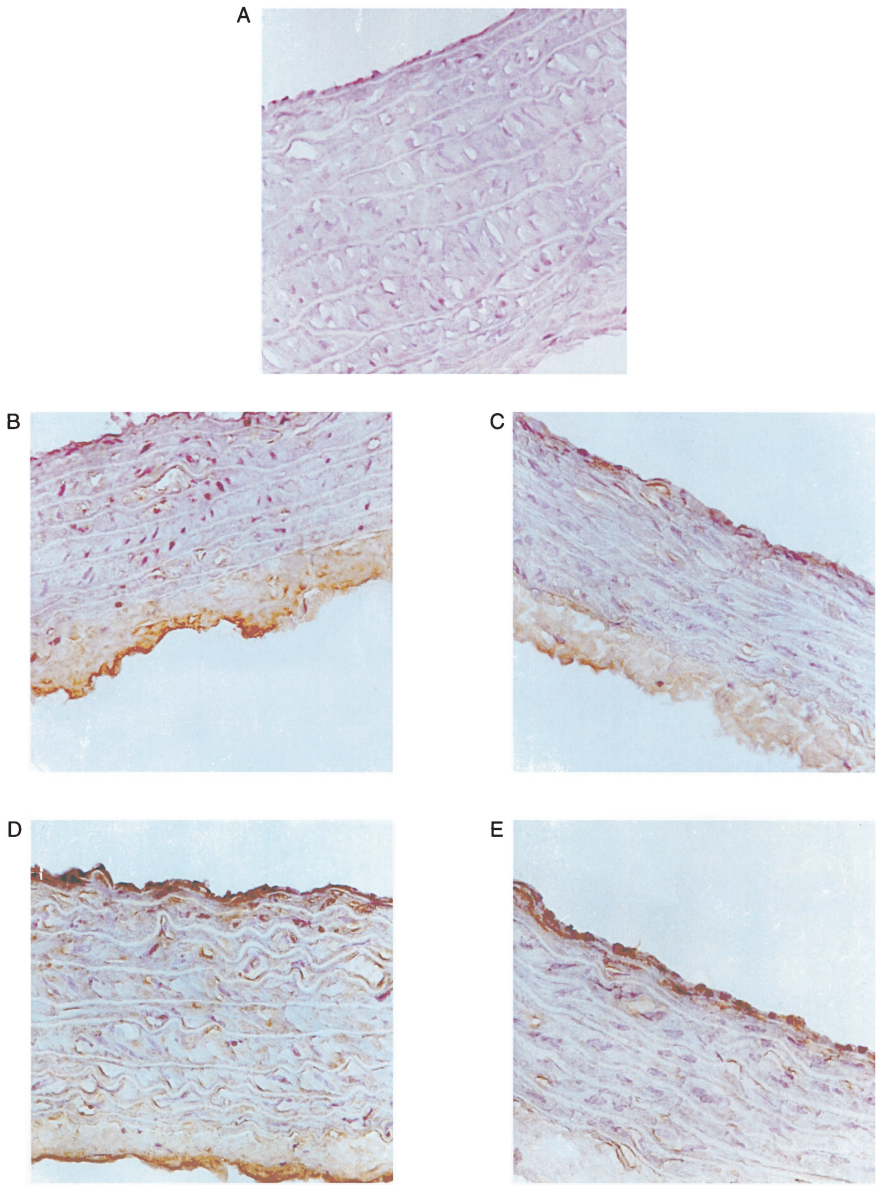

Figure 3. Expression of p22phox. Immunohistochemistry of aorta from young (B and D) and old (C and E) normotensive and hypertensive rats. Photographs are shown at ×400 magnification. A, Negative central vessel, where primary antibody was replaced with mouse IgG.

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 O_2^- in female rats. We then went on to investigate the tissue and enzymatic sources of this excess 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 O_2^- levels. Previously, we have made similar observations in young SHRSP,[12] and the present results would substantiate our conclusion that eNOS is an important source of O_2^- in SHRSP.

However, eNOS is not the only the source of O_2^-. Both DPI and apocynin attenuated 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.[29] 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 O_2^-. Apocynin acts by interfering with NAD(P)H subunit assembly in the membrane and is therefore a more specific inhibitor than DPI.[20] Taken together, the inhibition of O_2^- production by these compounds would be consistent with a role for NAD(P)H oxidase as a source of 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. Semiquantitatively, this 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 O_2^- levels tended to be higher in the older animals, this only reached statistical significance for NADH-driven 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 upregulate 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^-.

Acknowledgments

This work was supported by the British Heart Foundation Program and Project Grants RG-97009 and PG-200023. The authors thank Emma Jardine for expert technical assistance.

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