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Increased Levels of Superoxide in Brains from Old Female Rats

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Hypertension, aging and a range of neurodegenerative diseases are associated with increased oxidative damage. The present study examined whether superoxide ($O_2^{\cdot-}$) levels in brain are increased during aging in female rats, and the role of superoxide dismutase (SOD) and oestrogen in regulating $O_2^{\cdot-}$ levels.

Young adult (3 month) and old (11 month) female spontaneously hypertensive stroke prone rats (SHRSP) and normotensive Wistar-Kyoto rats (WKY) were studied. $O_2^{\cdot-}$ levels were measured in brain homogenates by lucigenin chemiluminescence and SOD expression by Western blotting. Ageing significantly increased brain $O_2^{\cdot-}$ levels in WKY (cortex +216%, hippocampus +320%, striatum +225%) and to a greater extent in SHRSP (cortex +540%, hippocampus +580%, striatum +533%). Older SHRSP showed a decline in cortical Cu/Zn SOD expression compared to young adult SHRSP. Oestrogen did not attenuate $O_2^{\cdot-}$ levels.

The results show a significant age-dependent increase in brain $O_2^{\cdot-}$ levels which is exaggerated in SHRSP. The excess cortical $O_2^{\cdot-}$ levels in the SHRSP may be associated with a down-regulation of Cu/Zn SOD but are not related to a decrease in oestrogen.

Keywords: Rat; Age; Hypertension; Superoxide; Oestrogen; Female

INTRODUCTION

It has been suggested that accrual of macromolecular damage induced by oxidative stress is a central causal factor in the aging process.[1,2] Oxidative stress has also been implicated in a number of neurodegenerative disorders associated with aging.[3] The pathological role of reactive oxygen species (ROS) has been intensively studied in Alzheimer's disease and ischaemic reperfusion injury amongst others.[3,4]

Superoxide ($O_2^{\cdot-}$) is a major source of ROS. It can cause oxidative damage directly and, via metabolism, form a number of toxic species including hydrogen peroxide, hydroxyl radicals, peroxynitite and nitrotyrosine.[5] Elevated levels of $O_2^{\cdot-}$ could be due to increased production, decreased removal or a combination of both. Removal of $O_2^{\cdot-}$ occurs by enzymatic pathways, in particular dismutation to hydrogen peroxide by a family of superoxide dismutases (SODs). Over-expression of SOD increases life span in some but not all studies.[6] SOD mimetics have been reported to be effective in attenuating oxidative stress associated disease processes.[7] Decreased levels of SOD have been suggested to be one of the causative factors for vulnerability of myocardium and brain against $O_2^{\cdot-}$ radicals in spontaneously hypertensive stroke prone rats (SHRSP).[8] As well as dismutation to hydrogen peroxide $O_2^{\cdot-}$ levels may also be reduced by direct scavenging by antioxidants. Low molecular weight antioxidants such as α-tocopherol, ascorbate and selenium have been shown to be effective in many animal studies.[9] Epidemiological studies also indicate an inverse association between vitamin intake and heart disease and stroke.[10] However, more recent prospective clinical trials have been

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disappointing with none showing a reduction in stroke.[11,12] 17β oestradiol (E_2) can also act as an antioxidant, it may have a role to play in transcriptional regulation of diverse genes and has been suggested to be neuroprotective. Premenopausal women exhibit a lower susceptibility to stroke related brain damage and other forms of cardiovascular disease than males or postmenopausal women.[10] E_2 related neuroprotection has been reported in rodent models of stroke.[13,14] Epidemiological evidence and clinical reports indicate a positive role for E_2 both in Alzheimer's and Parkinson's disease.[15]

Despite evidence that $O_2^{\cdot-}$ may play a pivotal role in various neurological disorders many questions remain to be answered. Indeed few attempts have been made to measure $O_2^{\cdot-}$ levels directly in the brain in relation to neurological disease or aging. One problem is that there are few suitable animal models for human neurological pathologies. An exception is the SHRSP. Older animals stroke spontaneously with a greater preponderance of stroke in males than females. The SHRSP also shows increased sensitivity to cerebral ischaemia compared to its normotensive counterpart, the Wistar-Kyoto rat (WKY).[14,16] Brains from SHRSP have been reported to exhibit enhanced lipid peroxidation[17] and lower SOD levels.[8]

The aim of the present study was therefore to examine levels of $O_2^{\cdot-}$ in brains of young and old WKY and SHRSP and to determine the role of SOD's and E_2 in regulating $O_2^{\cdot-}$ levels.

MATERIALS AND METHODS

Animals

All experiments were carried out under a Home Office project license and were subject to the Animals (Scientific Procedures) Act, 1986. Experiments were conducted on 3 month (young) and 11 month (old) female SHRSP and WKY rats obtained from colonies established in Glasgow as previously described.[18] Some studies were also carried out in 3 month male WKY. At 3 months of age rats have reached sexual maturity. By 11 months of age fertility is greatly reduced in female rats and they start to experience irregular oestrous cycles. Few male SHRSP survive to 11 months, most having suffered strokes by this age.

Blood Pressure

Systolic blood pressure was measured in conscious young adult and old SHRSP and WKY ($n = 6$ each group) by tail plethysmography.[18]

Measurement of Superoxide by Lucigenin-mediated Chemiluminescence

SHRSP and WKY rats were anaesthetised with barbiturate (sodium pentobarbitone, Euthetal®), decapitated and brains removed. Cortex, hippocampus and striatum were dissected, weighed, homogenised and diluted in 0.1 mol/l Krebs buffer (pH 7.4). $O_2^{\cdot-}$ measurement was by lucigen chemiluminescence using 15 μmol/l lucigenin.[19] Brain samples from SHRSP and WKY rats were analysed in parallel. Results were expressed in nmol/min/mg of wet weight. Lucigenin has been widely used to monitor $O_2^{\cdot-}$ formation. It is sensitive with high temporal resolution, but may undergo redox-cycling leading to artificially elevated estimation of $O_2^{\cdot-}$ generation when high concentrations of lucigenin are used. From pilot studies we selected 15 μmol/l lucigenin as the optimum concentration balancing reproducibility against minimising the concentration of lucigenin.[20–22] Studies were carried out in freshly prepared and boiled brain homogenates to confirm the specificity of $O_2^{\cdot-}$ generation. Diphenyleneiodonium inhibition of the reaction in fresh but not boiled homogenates indicates a flavin containing enzyme, presumably NAD(P)H oxidase is an important enzymatic source of $O_2^{\cdot-}$. In contrast N^G-nitro L-arginine methyl ester and allopurinol had no effect on $O_2^{\cdot-}$ levels suggesting that neither nitric oxide synthase nor xanthine oxidase contributed to $O_2^{\cdot-}$ generation in these brain extracts. In addition to examining $O_2^{\cdot-}$ generation the role of endogenous scavenging of $O_2^{\cdot-}$ by Cu/Zn SOD was studied. Cortical samples were incubated with the selective Cu/Zn SOD inhibitor, sodium diethyldithio-carbamate (DETC) (10 mmol/l) for 45 min before measurement of superoxide.

Western Blotting

In a separate series of experiments, brains were rapidly removed from SHRSP and WKY and the cortex, hippocampus and striatum were snap frozen in liquid nitrogen. The proteins were extracted by homogenisation in boiling 250 mmol/l Tris–HCl pH 6.8, 4% SDS, 10% Glycerol, 0.006% bromophenol blue and 2% β-mercaptoethanol. Protein (10 μg) was electrophoresed on an SDS polyacrylamide gel and then transferred onto a PVDF (polyvinyl difluoride) membrane overnight. Prestained rainbow markers (Amersham) were used as molecular weight standards. The membranes were blocked in 5% skimmed dried milk in 10 mmol/l Tris pH 7.5, 100 mmol/l NaCl. 0.1% Tween 20 for 1 h at room temperature. Thereafter they were sequentially incubated with antibodies directed against Cu/Zn SOD, Mn SOD and GAPDH

(glyceraldehyde-3-phosphate dehydrogenase). Bands were detected using an enhanced chemiluminescence test (Amersham) and autoradiography by exposure to X-ray film (Kodak X-OMAT). The resultant bands were quantified with the use of a Phospho-Imager (Bio-Rad) (using GAPDH as an internal control). Results were expressed as a percentage of the mean value for young WKY in each region. Anti-Mn SOD was a gift from Professor Taniguchi (University of Osaka, Japan) and anti-Cu/Zn SOD was purchased from Calbiochem. These antibodies have been used previously by us and others to detect Cu/Zn SOD and Mn SOD.[19,23]

Overiectomy and E_2 Replacement

Female SHRSP and WKY underwent a bilateral ovariectomy under halothane anesthesia. E_2 pellets (21 day controlled release) were implanted subcutaneously in the dorsal neck of 6 rats from each group, and the remaining animals implanted with a placebo pellet. Two weeks later the animals were killed, blood removed for plasma E_2 assay and cortex, stratum and hippocampus dissected out for $O_2^{\cdot-}$ measurement. At the same time brain samples were obtained from age matched WKY males. Samples from male animals were divided into 2, one half being treated with E_2 1 μmol/l for 30 min and the other being treated with vehicle before measuring $O_2^{\cdot-}$ levels.

17β-oestradiol Assay

Samples were analysed for E_2 by radioimmunoassay (Coat-a-Count Radioimmunoassay, Diagnostic Products Corporation).

Statistical Analysis

Data are expressed as mean ± S.E. Data were analysed by ANOVA followed by *t*-test correcting for multiple comparisons using Bonferani or Tukeys test.

RESULTS

Systolic Blood Pressure

There was no difference in systolic blood pressure between young adult (107 ± 4 mmHg, $n = 6$) and old (110 ± 5 mmHg, $n = 6$) WKY female rats. Likewise, there was no difference in systolic blood pressure between young adult (140 ± 9 mmHg, $n = 6$) and old (146 ± 8 mmHg, $n = 6$) female SHRSP. In both age groups SHRSP displayed significantly higher systolic blood pressure compared to normotensive WKY rats ($p < 0.01$).

$O_2^{\cdot-}$ Levels

Steady-state $O_2^{\cdot-}$ levels in brain homogenates from young adult and old SHRSP and WKY are shown in Fig. 1. These *in vitro* steady-state $O_2^{\cdot-}$ levels were significantly higher in old animals compared to young adult animals in all brain regions examined, increases of 540, 580 and 535% being observed in cortex, hippocampus and striatum of SHRSP and of 216, 322 and 225% in the corresonding brain regions of WKY. The higher steady state $O_2^{\cdot-}$ levels in old SHRSP compared to WKY reached significance in cortex ($p < 0.05$ 95% CI −2.386, −0.214) but not in other brain regions. There was no difference in levels between SHRSP and WKY in any of the brain regions analysed in the young adult animals.

Incubation of the cortical tissue with DETC had no effect in boiled samples but significantly increased the level of $O_2^{\cdot-}$ in freshly prepared cortical homogenates from young adult SHRSP from 0.5 ± 0.1 to 6.2 ± 0.2 nmol/mg/min ($n = 3$ $p < 0.001$ 95% CI −7.020, −4.360). In the old SHRSP rats the increase was much smaller from 3.2 ± 0.5 to 5.1 ± 0.3 nmol/mg/min ($n = 3$ $p < 0.01$ 95% CI −3.230, −0.5701). In WKY DETC caused a highly significant increase in cortical $O_2^{\cdot-}$ levels in both young adult and old animals. In young WKY $O_2^{\cdot-}$ levels were increased from 0.6 ± 0.1 to 7.42 ± 0.4 nmol/mg/min ($n = 3$ $p < 0.001$ 95% CI −8.140, −5.480) and in old WKY from 1.9 ± 0.2 to 6.0 ± 0.2 nmol/mg/min ($n = 3$ $p < 0.001$ 95% CI −8.140, −2.700).

In ovariectomised female rats E_2 replacement had no effect on $O_2^{\cdot-}$ levels in any of the brain regions examined (Table I). Values did not differ significantly to those found in intact females. No gender difference in brain $O_2^{\cdot-}$ levels was observed between age matched male and female WKY, nor did acute, 30 min *in vitro*, exposure to relatively high levels of E_2 (1 μmol/l) attenuate $O_2^{\cdot-}$ levels in brains from male rats (Table I).

SOD Levels

Levels of Cu/Zn SOD and Mn SOD expression are shown in Table II. Cu/Zn SOD expression was significantly lower in old SHRSP compared to young adult SHRSP in the cortex ($p < 0.05$) as illustrated in Fig. 2. A similar trend was observed in WKY but the difference did not reach significance. There was no significant difference in Mn SOD expression between young adult and old animals in any of the regions examined (Table II).

Plasma 17β-Oestradiol Levels

E_2 levels were similar in the two age groups studied being 22.7 ± 4.7 pg/ml in young and 26 ± 5.5 pg/ml

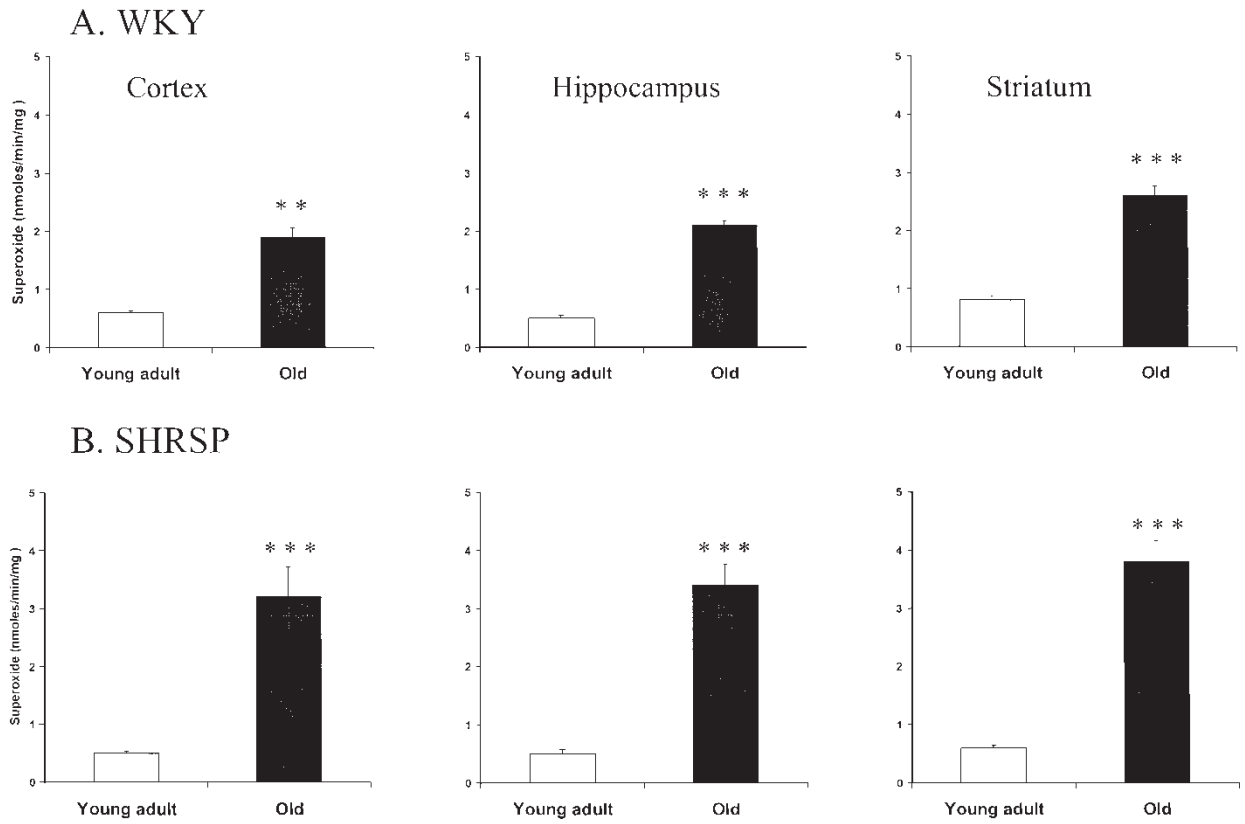

FIGURE 1 Levels of superoxide ($O_2^{\cdot-}$) in the cortex, hippocampus and striatum of young adult and old (A) Wistar-Kyoto rats (WKY) and (B) spontaneously hypertensive stroke prone rats (SHRSP) ($n = 6$ per group, $**p < 0.005$, $***p < 0.001$).

TABLE I Effect of oestrogen supplementation on brain $O_2^{\cdot-}$ levels

Brain area	E_2 supplementation	Male WKY $O_2^{\cdot-}$ nmol/mg/min	Female WKY $O_2^{\cdot-}$ nmol/mg/min	Female SHRSP $O_2^{\cdot-}$ nmol/mg/min
Cortex	−	0.83 ± 0.29	1.14 ± 0.24	1.30 ± 0.30
	+	0.84 ± 0.30	0.93 ± 0.92	1.26 ± 0.33
Hippocampus	−	0.69 ± 0.28	0.89 ± 0.21	1.14 ± 0.25
	+	0.79 ± 0.30	0.70 ± 0.10	1.00 ± 0.14
Striatum	−	0.82 ± 0.11	0.80 ± 0.12	1.04 ± 0.24
	+	−	0.91 ± 0.16	1.15 ± 0.23

Male rats brains were treated with 1 μmol/l E_2 or vehicle for 30 min. Female rats underwent ovariectomy and implanted with placebo or E_2 pellets for 2 weeks. $n = 7$ for male WKY $n = 6$ for female WKY and SHRSP. Results are shown as mean ± SEM.

TABLE II Levels of Cu/Zn and MnSOD in young adult and old WKY and SHRSP

Brain region	SOD	Young WKY	Old WKY	Young SHRSP	Old SHRSP
Cortex	Cu/Zn	100 ± 21	58 ± 9	132 ± 25	60 ± 12*
Hippocampus	Cu/Zn	100 ± 6	83 ± 2	93 ± 12	82 ± 5
Striatum	Cu/Zn	100 ± 15	62 ± 21	67 ± 7	81 ± 5
Cortex	Mn	100 ± 19	121 ± 21	116 ± 16	169 ± 16
Hippocampus	Mn	100 ± 11	102 ± 25	80 ± 2	98 ± 16
Striatum	Mn	100 ± 30	90 ± 25	54 ± 5	62 ± 3

Results are expressed as a percentage of the mean absorbance in young adult WKY and are shown as mean ± SEM $n = 4–5$ per group. $*P < 0.05$ when levels in young animals compared to those in older animals of the same strain.

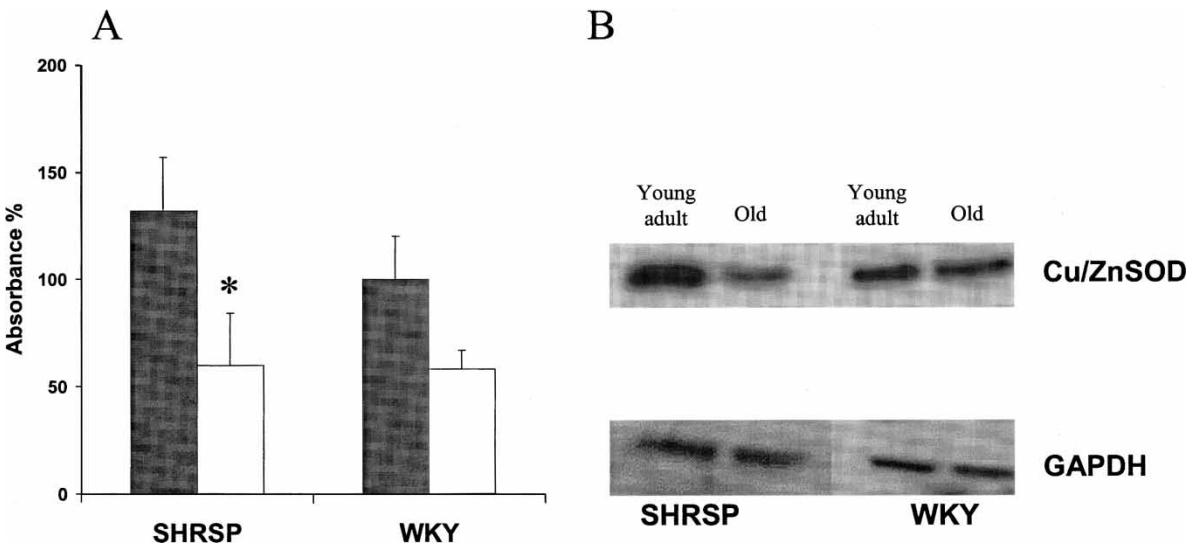

FIGURE 2 (A) Levels of superoxide dismutase expression in the cortex, of young adult and old Wistar-Kyoto rats (WKY) and spontaneously hypertensive stroke prone rats (SHRSP) ($n = 4$ per group, $*p < 0.05$). ■ young adults □ old. (B) Representative Western blot showing copper–zinc superoxide dismutase (Cu/Zn SOD) expression in the cortex illustrating the decline in old SHRSP compared with young adult SHRSP. Below are the blots for the internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (Results are expressed as a percentage of the mean absorbance in young adult WKY).

in older animals. Levels of E_2 were reduced in placebo treated ovariectomised animals, but in E_2 treated ovariectomised rats levels were similar to those found in intact females (WKY ovariectomised placebo treated $E_2 = 7.3 \pm 1.8$ oestrogen treated $E_2 = 22.9 \pm 4.1$ pg/ml).

DISCUSSION

A key finding in the present study is the significant age-dependent increase in $O_2^{\cdot-}$ steady state levels in brain homogenates from both WKY and SHRSP. The largest increase was of 580% in hippocampus from SHRSP and the smallest 216% in WKY cortex. These results are consistent with the hypothesis that $O_2^{\cdot-}$ is involved in both normal ageing and pathological processes associated with ageing.[1,2] The increase in $O_2^{\cdot-}$ levels in the cortex, in SHRSP, might be partially attributed to the decrease in Cu/Zn SOD. $O_2^{\cdot-}$ levels were significantly higher in cortical homogenates from old SHRSP compared to WKY and it was only in SHRSP that the decrease in SOD protein and activity reached significance. Neither the effect of age nor hypertension on SOD levels have been investigated previously in female animals. However, Kimoto *et al.*[8] found that both MnSOD and Cu/Zn SOD levels tended to be lower in male SHRSP than WKY cerebral cortex and hippocampus and to decrease significantly between 15 and 31 weeks of age in SHRSP. Although in our study neither Mn SOD nor Cu/Zn SOD levels were decreased in hippocampus or stratum of the old animals the importance of SOD in regulating $O_2^{\cdot-}$ levels was demonstrated by the large increase in $O_2^{\cdot-}$ levels observed in the presence of the Cu/Zn SOD inhibitor DETC in cortical tissue from young WKY and SHRSP.

It must be noted that these *in vitro* observations in brain homogenates may not relate directly to the *in vivo* situation. The $O_2^{\cdot-}$ levels measured in this study are dependent on both the rate of production and removal. Using selective enzyme inhibitors we have provided evidence that NAD(P)H oxidase is involved in $O_2^{\cdot-}$ generation and Cu/Zn SOD in $O_2^{\cdot-}$ removal. However, the activities of the enzyme systems involved may not be identical *in vivo* although a qualitative relationship between experimental *in vitro* studies and the *in vivo* situation is assumed.

Although levels of Cu/Zn SOD expression were reduced in cortical tissue from older animals no significant decreases were observed in hippocampus or striatum. Thus changes in Cu/Zn SOD levels would not be sufficient to account for the observed increases in $O_2^{\cdot-}$ levels which were observed in all brain regions examined. There in much indirect evidence to suggest that decreased levels of E_2 in older women could contribute to an increase in oxidative stress and neurological disease.[15] However, we found no evidence that E_2 attenuated ROS production in the brains of either WKY or SHRSP. Despite the increase in $O_2^{\cdot-}$ levels E_2 levels did not differ significantly between young or old WKY or SHRSP. In addition, $O_2^{\cdot-}$ levels were similar in the brain regions studied in male and female WKY, neither did $O_2^{\cdot-}$ levels differ in brains of ovariectomised females receiving E_2 replacement nor placebo treatment. In light of these observation it is interesting to note that in recent well-controlled

studies E_2 replacement has not been associated with a reduced incidence of stroke.[24,25,28] In most cases in which E_2 has been reported to act as an antioxidant in the brain supraphysiological concentrations were required.[27,28] In our studies even acute exposure of brain homogenates from male WKY to high levels of E_2 (1 μmol/l) had no effect on $O_2^{\cdot-}$ levels. However, E_2 has a plethora of cellular effects including increased expression of anti-apoptotic proteins, interactions with second messenger cascades, alterations in glutaminergic activation and maintenance of intracellular calcium homeostasis activation.[15,29] E_2 may well be neuroprotective in some situations despite the lack of antioxidant effect observed in this study.

In conclusion, increased ROS production can lead to cell death or an acceleration in ageing and age-related disease.[2] Neurodegenerative processes of ageing and cerebrovascular injury have been suggested to be associated with $O_2^{\cdot-}$ generation.[30] In this *in vitro* study measuring $O_2^{\cdot-}$ in brain hemogenates we substantiate these hypothesis and provide further insights into the relationship between ROS and aging. The increase in $O_2^{\cdot-}$ observed in the older animals were large, (200–300% in WKY) and over 500% in all brain regions examined in SHRSP. Thus, in addition to showing an age related effect these results suggest an interaction between hypertension and ageing in ROS production.

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