Anti-oxidant defences and peroxidation in liver and brain of aged rats

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Old rats (28 months), when compared with young adults (9 months), did not show differences in activities of superoxide dismutase (SOD) or selenium-dependent and -independent glutathione peroxidases (GPx), or in levels of GSH, GSSG, GSSG/GSH and endogenous peroxidation in liver and brain. Rates of stimulated peroxidation *in vitro* were decreased in the livers of old rats. Old animals showed decreased levels of hepatic catalase and glutathione reductase. Nevertheless, when enzyme activities were referred to cytochrome oxidase activity these decreases disappeared, and GPx and SOD (brain) were even increased in old rats.

INTRODUCTION

Many reports dealing with the free radical theory of aging [1] have focused on the possibility that aging may be accelerated in old individuals due to lowering of the anti-oxidant defences. Thus levels of discrete anti-oxidants or tissue peroxidation have been measured in tissues of old and young rats. Nevertheless, many of these studies have given contradictory results concerning hepatic superoxide dismutase (SOD) [2–6] and catalase (CAT) activities [5,7,8], and production of thiobarbituric acid-reactive substances (TBARS) by stimulated peroxidation *in vivo* or *in vitro* [12,13]. Similar observations have been made in the brain for SOD [4,5,14–17], CAT [5,18,19], selenium (Se)-dependent glutathione peroxidase (GPx) [15,16,18–21], glutathione reductase (GR) [15,16,20], GSH [22,23] and TBARS *in vivo* [5,10,16]. These contradictory results may be due to the fact that the majority of these studies were not comprehensive. The measurement of a limited number of free radical-related parameters in a tissue can lead to inconclusive results due to the known compensatory homeostasis which occurs among the different cellular anti-oxidants.

A further complication can arise due to the reference usually used for expressing the activities of anti-oxidant enzymes. Even if a decrease in these activities occurs in old individuals when expressed per mg of protein, this does not necessarily mean that a higher susceptibility to oxidative stress is present in the tissue. In order for this increased susceptibility to occur, the total cellular generation of oxygen radicals per unit time must not similarly decrease as a function of age. Such a decrease may occur due to a decrease in mitochondrial activities in old tissues, since mitochondria play a principal role in oxygen radical generation. Thus analogous decreases in both oxygen radical generation and detoxification in old age would leave tissue oxidative stress unchanged. In that case, a decrease in the anti-oxidant defence should be regarded as a down-regulation phenomenon rather than a cause of additional free radical damage.

In an attempt to resolve these questions, we have performed a comprehensive study in the liver and cerebral cortex of the male Wistar rat. We measured the five principal anti-oxidant enzymes, as well as GSH, GSSG and TBARS levels *in vivo*, and we performed a kinetic study on the susceptibility of the tissues to peroxidation *in vitro* in both young and old rats. The total oxygen consumption ($\dot{V}_{O_2}$) of the animals was measured as an

index of basal aerobic metabolism, and cytochrome *c* oxidase (COX) activity was assayed in order to correct for changes in the maximal aerobic mitochondrial capacity. All of these parameters were measured in the same two groups of young mature (9 months) and old (28 months) rats.

MATERIALS AND METHODS

Animals and preparation of samples

Male 8- and 27-month-old Wistar rats were obtained from the Centre d'Élevage Raymond Janvier (CERJ, Le Genest-Laval, France). They were maintained for 1 month in the laboratory at $23 \pm 2^\circ\text{C}$ and $50 \pm 10\%$ relative humidity, and they were fed on normal laboratory diet (as pellets) and water *ad libitum*. Animals of 9 months were selected as young adults, since previous publications have shown that anti-oxidants such as SOD [2], GPx [24] or GSH [22] can be still increasing in younger animals which are not fully mature. Rats aged 28 months were selected as 'old' in order to avoid the presence of changes which are secondary to pathological states of the tissues typical in very old animals.

Enzymes

Animals were killed by decapitation. Samples from liver and cerebral cortex were quickly rinsed and homogenized in 20 vol. of cold 50 mM-potassium phosphate buffer, pH 7.4, using an automatic Wheaton homogenizer with a Teflon pestle (three strokes at 1000 rev./min). The homogenates were sonicated at 38 W for 30 s and centrifuged at 5°C and 3200 *g* for 20 min. CAT and both total and Se-dependent GPx were measured by spectrophotometric methods as previously described [25]. GPx was assayed using cumene hydroperoxide or H_2O_2 as substrate. The assay with H_2O_2 measures the Se-dependent enzyme, whereas the assay with cumene hydroperoxide (total GPx) measures both Se-dependent and Se-independent GPx activity. COX was measured in samples of the supernatants, after addition of 1% Lubrol, by the method of Smith [26]. Commercial cytochrome *c* was reduced with 0.5% ascorbate. The ascorbate was eliminated from the cytochrome *c* solution (0.6 ml) by overnight dialysis against two changes of phosphate buffer (300 ml). Before the measurements, in order to check the percentage reduction of cytochrome *c*, the ratio of absorbances at 550 nm/565 nm was measured, and values greater than 9 were always obtained (values greater than 6 indicate 90% cytochrome *c* reduction).

Abbreviations used: SOD, superoxide dismutase (EC 1.15.1.1); CAT, catalase (EC 1.11.1.6); GPx, glutathione peroxidase (EC 1.11.1.9); GR, glutathione reductase (EC 1.6.4.2); COX, cytochrome *c* oxidase (EC 1.9.3.1); TBARS, thiobarbituric acid-reactive substances; $\dot{V}_{O_2}$, total oxygen consumption.

The rate of oxidation of reduced cytochrome *c* (56 μM) was monitored at 550 nm. Cytochrome *c* oxidized by 0.9 mM-potassium ferricyanide was used as a blank.

A portion of the supernatants was dialysed overnight against the 50 mM-phosphate buffer, pH 7.4, and was used to measure SOD and GR activities. GR was assayed by measuring NADPH oxidation at 340 nm in the presence of 4 mM-GSSG and 0.3 mM-NADPH in 50 mM-phosphate buffer, pH 7.4. GR and both GPx activities were corrected for spontaneous reaction in the absence of enzyme. After dialysis, the haemoglobin present in the samples used to measure SOD was precipitated with ethanol/chloroform (supernatant/ethanol/chloroform, 1:0.33:0.18, by vol.), and Triton X-100 was added to a final concentration of 1%. SOD activity was measured in the presence and absence of supernatant by measuring the rate of NADH oxidation at 340 nm in the presence of 5 mM-EDTA, 2.5 mM-MnCl₂, 0.27 mM-NADH and 0.56 mM-mercaptoethanol [27] in 50 mM-phosphate buffer. One unit of activity is defined as the amount of enzyme that inhibits the rate of NADH oxidation by 50%. All of the enzymic activities were measured at 25 °C in a 1201 Milton-Roy spectrophotometer. Protein concentrations were measured by the method of Lowry *et al.* [28].

Glutathione system

Tissue samples were homogenized in 20 vol. of cold 5% trichloroacetic acid containing 0.01 M-HCl. This solution was deoxygenated by bubbling with N₂. The homogenates were centrifuged at 3200 *g* for 5 min under an N₂ atmosphere. The supernatants were divided into two portions. One was used for the measurement of total glutathione; and to the second sample, 4 μl of 2-vinylpyridine was added per 0.1 ml of supernatant. This procedure led to GSH elimination and at the same time neutralized the samples [29]. After 1 h in the presence of 2-vinylpyridine, GSSG was measured. Total glutathione and GSSG were assayed by the method of Tietze [30] by monitoring the change in absorbance at 412 nm in the presence of 0.6 mM-5,5'-dithiobis-(2-nitrobenzoic acid), 0.21 mM-NADPH and 0.5 units of GR/ml of assay mixture in 50 mM-phosphate buffer, pH 7.4. GSH and GSSG values were corrected for spontaneous reaction in the absence of sample. The assay was performed at 25 °C.

Peroxidation

Tissue peroxidation in supernatants from the phosphate buffer homogenates was measured by the thiobarbituric acid (TBA) test as previously described [25]. Sensitivity to peroxidation *in vitro* was estimated by incubating supernatant samples with 0.4 mM-ascorbate and 0.05 mM-FeSO₄ for 0, 5, 15 or 30 min (brain) or 0, 30, 60 or 90 min (liver) at 25 °C before performing the TBA assay.

Oxygen consumption

The $\dot{V}_{\text{O}_2}$ of individual animals at rest was measured in 18-litre closed system respirometers at 25 ± 0.5 °C. The changes in chamber $p\text{O}_2$ were monitored throughout the experiment with an ESD O₂ air analyser. Mean and minimal $p\text{O}_2$ inside the chamber were 17.5 and 16.0 kPa (131 and 120 mmHg) respectively. Soda lime (50 g) was used in every experiment as a CO₂ absorber. An air pump recirculated the chamber air at 3 litres/min throughout the experiment. Measurements were started after 15 min of equilibration.

Statistical analysis

After testing for normality and variance homogeneity, the data were subjected to Student's *t* test. The *P* < 0.05 level was selected as the point of minimal statistical significance.

RESULTS

When liver enzyme activities were expressed per mg of protein, significant decreases were observed in old rats for CAT and GR, whereas SOD and GPx (Se-dependent and total) did not change (Table 1). The decrease that was observed for COX when expressed per mg of tissue (results not shown) was not significant when expressed per mg of protein (Table 1). In the brain all of the anti-oxidant enzyme activities remained constant in old rats, whereas COX activity was acutely decreased (Table 1). When the activities of the anti-oxidant enzymes of both tissues were expressed in relation to COX activity (Table 2), no decreases were observed in tissues from old animals in any case. Furthermore, brain SOD and hepatic and cerebral total and Se-dependent GPx showed clear increases in old animals.

The concentrations of GSH and GSSG and the GSSG/GSH ratio did not differ between young and old animals in any tissue (Table 3). The endogenous levels of TBARS did not change in liver and brain as a function of age. The rate of accumulation of TBARS during stimulated peroxidation *in vitro* did not show age-related differences in the brain, whereas it was significantly lower in the livers of old compared with young animals at all time points studied (results not shown). The basal oxygen consumption of the whole animals was similar in young (0.79 ± 0.03 ml of O₂/h per g; mean $\pm$ S.D.) and old (0.86 ± 0.05 ml of O₂/h per g) rats.

DISCUSSION

Previous studies performed in the rat have shown an absence during aging of changes in GSH [23,24] and GSH transferases [24,31] in the liver, and in total GPx [5] and peroxidation *in vitro* [11,18] in the brain. These results are fully in agreement with

Table 1. Activities of anti-oxidant enzymes and COX in relation to protein content

Young rats were 9 months old and old rats were 28 months old. Values are means $\pm$ S.E.M. from eight animals of the same age. Activities were expressed as units (SOD), μmol (of H₂O₂ for CAT; of cytochrome *c* for COX), or nmol of NADPH (both GPx and GR) oxidized/min per mg of protein. Significant differences in old compared with young animals in the same tissue are indicated by **P* < 0.05; ***P* < 0.01.

Tissue	Group	Activity					
		SOD	CAT	GPx (total)	GPx (Se-dependent)	CR	COX
Liver	Young	82 $\pm$ 8	365 $\pm$ 27	1029 $\pm$ 100	665 $\pm$ 60	30 $\pm$ 2	0.17 $\pm$ 0.01
	Old	77 $\pm$ 12	259 $\pm$ 27**	1343 $\pm$ 187	873 $\pm$ 87	24 $\pm$ 1*	0.14 $\pm$ 0.02
Brain	Young	39 $\pm$ 6	4.3 $\pm$ 0.6	63 $\pm$ 5	48 $\pm$ 5	8.4 $\pm$ 0.8	0.16 $\pm$ 0.04
	Old	47 $\pm$ 6	4.6 $\pm$ 0.8	71 $\pm$ 4	45 $\pm$ 4	7.3 $\pm$ 1.6	0.08 $\pm$ 0.01*

Table 2. Activities of anti-oxidant enzymes in relation to COX activity

Values are means $\pm$ S.E.M. from eight animals of the same age. Activities were expressed as units (SOD), μ mol of H_2O_2 (CAT) or nmol of NADPH (GPx and GR) oxidized in relation to the μ mol of COX oxidized per unit time. Significant differences in old compared with young animals in the same tissue are indicated by * $P < 0.05$; ** $P < 0.01$.

Tissue	Group	SOD/COX	CAT/COX	GPx (total)/COX	GPx (Se-dependent)/COX	GR/COX
Liver	Young	490 $\pm$ 58	2175 $\pm$ 128	6105 $\pm$ 506	3910 $\pm$ 313	179 $\pm$ 13
	Old	575 $\pm$ 95	1995 $\pm$ 222	10597 $\pm$ 1636*	7080 $\pm$ 1116*	193 $\pm$ 24
Brain	Young	360 $\pm$ 99	40 $\pm$ 10	572 $\pm$ 111	399 $\pm$ 68	73 $\pm$ 15
	Old	624 $\pm$ 97*	56 $\pm$ 6	980 $\pm$ 160*	601 $\pm$ 60*	90 $\pm$ 16

Table 3. Peroxidation *in vivo* (TBARS) and glutathione system

Values are means $\pm$ S.E.M. from six (GSH and GSSG) or eight (TBARS) animals of the same age. TBARS are measured in nmol of malonaldehyde equivalents/g.

Tissue	Group	TBARS <i>in vivo</i> (nmol/g)	GSH (μ mol/g)	GSSG (μ mol/g)	GSSG/GSH
Liver	Young	138 $\pm$ 23	6.5 $\pm$ 1.3	0.50 $\pm$ 0.10	0.091 $\pm$ 0.026
	Old	100 $\pm$ 23	5.5 $\pm$ 0.7	0.47 $\pm$ 0.08	0.091 $\pm$ 0.020
Brain	Young	516 $\pm$ 65	0.99 $\pm$ 0.16	0.07 $\pm$ 0.01	0.076 $\pm$ 0.011
	Old	450 $\pm$ 66	0.97 $\pm$ 0.16	0.05 $\pm$ 0.01	0.066 $\pm$ 0.015

those obtained in the present work, since the difference in activity between total and Se-dependent GPx is due to the activity of some GSH transferases. On the other hand, the livers of old rats have been reported to show similar [2] or decreased [3–6] SOD activities compared with young rats. In the liver, CAT has been reported to be decreased [5–7] or unchanged [8], production of TBARS *in vivo* to be increased [9–10] or decreased [5,12], and production of TBARS *in vitro* to be increased [13] or decreased [9,12] in old rats. In the old compared with the young rat brain, SOD activity has been reported to be decreased [14,15], increased [17,19] or unchanged [4,5,16,18]; CAT activity to be decreased [5] or unchanged [18,19], Se-dependent GPx activity to be decreased [20], unchanged [16] or increased [15,19,21]; GR activity to be decreased [15], unchanged [16] or even increased [19,20]; GSH to be decreased [22] or unchanged [23], and TBARS production *in vivo* to be increased [10,16] or decreased [5]. A limitation of the majority of these studies was that they assayed only a few free radical-related parameters simultaneously.

In our study, no age-dependent variations were found for the glutathione system in either tissue or for any anti-oxidant enzyme in the brain. In the liver, only CAT and GR were decreased in old animals. Nevertheless, no decreases were observed in any organ when the enzymic activities were referred to COX activity. The basal oxygen consumption of the whole animals did not change with age, whereas our COX results suggest that the maximal aerobic capacity of the tissues was decreased in old animals. An absence of variation of basal $\dot{V}_{O_2}$, together with a decrease of maximal $\dot{V}_{O_2}$, is typical in old mammals, including humans [32]. In addition, it has been shown that situations that increase O_2 consumption also increase the rate of O_2 radical generation [33], and that the rate of superoxide radical production by brain mitochondria is decreased in old rats [11]. These last results were obtained *in vitro* after supplementing mitochondria with high levels of substrates. Thus previous results, together with our $\dot{V}_{O_2}$ and COX data, suggest that the maximal

(but not basal) capacity for oxygen consumption, and thus for oxygen radical generation, is smaller in the tissues of old rats. The decreases observed for some anti-oxidant enzymes may then represent a down-regulation which is secondary to this decrease, rather than a higher susceptibility to free radical-induced damage. The absence of a change in the GSSG/GSH ratio, a potent indicator of tissue oxidative stress, is also in accordance with the suggestion that free radical damage is not increased in tissues of old animals. This ratio is maintained at a low value provided that GR is not rate-limiting.

Endogenous levels of TBARS did not change as a function of age, showing that accumulation of peroxidation products does not occur in old tissues. When the sensitivity of the tissues to additional peroxidative damage was kinetically studied *in vitro*, no increases were observed in the livers or brains of old animals. In the brain no changes were detected, confirming previous single point data [11,18]. In the liver, in agreement with a previous report using rat hepatic microsomes [9], the TBARS production rate *in vitro* was clearly decreased. Interpretation of the cause of these TBARS changes is difficult, because data obtained in the TBA test can be influenced by factors such as vitamin E or polyunsaturated fatty acid content of membranes. Nevertheless, from TBARS data *in vitro* it can be concluded that these rat tissues do not show an increase in their susceptibility to free radical-induced peroxidation in old age, and that this sensitivity may even be decreased in the liver.

In summary, the available data suggest that the livers and brains of old rats are not more susceptible to oxidative stress than those of their young counterparts. Thus the rate of aging will not be accelerated in old age due to an increased sensitivity to free radical damage. This does not preclude the possibility that a constant presence of free radicals in the tissues throughout the life span may be an important determinant of aging.

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