J Neural Transm (2001) 108: 955–967

Journal of Neural Transmission

ROS generation, lipid peroxidation and antioxidant enzyme activities in the aging brain

S. Leutner, A. Eckert, and **W. E. Müller**

Department of Pharmacology, Biocenter, University of Frankfurt, Federal Republic of Germany

Received December 18, 2000; accepted March 12, 2001

Summary. The objective of this study was to determine the specific relationship between brain aging and changes in the level of oxidative stress, lipid peroxidation (LPO) and in the activities of antioxidant enzymes. We used four different age groups (2–3 months, 10–11 months, 16–17 months and 20–21 months) which represented young adults, adults, beginning senescence and senescence, respectively.

Basal levels of LPO products measured as malondialdehyde increased gradually with age in mouse brain homogenate. The extent of stimulated LPO products, however, was clearly decreased in the brain of adult mice compared to young mice but increased again in the brain of senescent mice. We could not detect any appreciable age-related changes in the basal as well as in stimulated levels of ROS measured with the fluorescent dyes dichlorofluorescein and dihydrorhodamine123. Nevertheless, there was a significant delay in the time course of ROS-generation in brain cells from old mice. The activities of the antioxidant enzymes CuZn-superoxide dismutase and glutathione reductase increased with age whereas glutathione peroxidase remained unchanged.

On the basis of our present findings, we envisage a potential model that integrates several divergent findings described in the literature about the role of oxidative stress in brain aging.

Keywords: Brain aging, ROS, antioxidant enzymes, lipid peroxidation, free radicals, oxidative stress.

Introduction

Oxidative stress as well as gene expression profile have been identified as causal factors in the aging process (Harman, 1981; Ames et al., 1993; Sohal et al., 2000). The brain is especially vulnerable to oxidative damage as a result of its high oxygen consumption rate, its abundant lipid content, and the relative paucity of antioxidant enzymes compared to other tissues. Endogenous reac-

tive oxygen species (ROS) that are generated from aerobic metabolism are probably the most important causes of age-related neuronal damage.

Reactive oxygen species are generated by different mechanisms. They are constantly produced within the body from normal oxidative metabolism. Sources of ROS are mainly the mitochondrial electron transport chain, but also the arachidonic acid cascade or nitric oxide (Miquel, 1992). During evolution, living organisms have been faced with the necessity to inactivate these free radicals, and they have developed several ways to protect themselves from oxidative attacks. These defense mechanisms include a variety of antioxidant enzymes like superoxide dismutase or glutathione reductase, which catalyzes the NADPH-dependent reduction of glutathione disulfide (GSSG) to glutathione (GSH). This reaction is essential for the maintenance of glutathione levels. Glutathione itself plays a major role as a reductant in oxidant-reduction processes, and also serves in detoxification (Carlberg and Mannervik, 1985).

There have been many conflicting reports on age-associated alterations in oxidative damage and antioxidant enzyme activity in brain. These inconsistent results may arise from differences in strains or ages of the animals investigated or from differences in animal maintenance conditions and the variety of experimental procedures used (Benzi and Moretti, 1995). More importantly, most studies measured only one or two parameters and nearly none tried to determine the equilibrium between free radical generation, detoxification, and the induced tissue damage. This point is especially important, since all of the above mentioned parameters might change differently over life span. Moreover, most studies just compared young and old or adult and old animals, leaving out any information about the changes that may be specific for adult life.

In the context of the above mentioned problems, one major purpose of this study was to determine whether many different biochemical parameters pertaining to oxidative stress follow a continuous or fluctuated pattern over the whole life span. Therefore, we examined mice at four different ages (2–3 months, 10–11 months, 16–17 months and 20–21 months) to determine aging as well as development during life time.

Another objective of this study was to obtain a comprehensive pattern of various parameters associated with oxidative stress, e.g., rates of ROS generation, the activities of antioxidant enzymes, namely SOD, GPx and GR, and cellular damage as indicated by lipid peroxidation.

Materials and methods

Animals

In our study, we used female NMRI mice, which were purchased from Harlan Winkelmann (Wiesbaden, Germany) and were aged at the animal facilities of the Biocenter at the University of Frankfurt. All mice had access to food and water ad libitum. Mice of 2–3, 10–11, 16–17 and 20–21 months of age were used in this study.

In total, 90 mice were included in the study. 43 animals were used for the determination of ROS and 47 for the determination of LPO and the enzyme activities. In each group we had between nine and thirteen animals.

Preparation of brain tissue

Mice were sacrificed by decapitation and brains were quickly dissected on ice. After removing the cerebellum, we used two different methods of tissue preparation. For the determination of dynamic processes like ROS measurement we needed live cells. Therefore, we mechanically dissociated brain cells, which were prepared basically following the method of Stoll et al. (1992). Vitality of the brain cell aggregates was previously tested by Trypan blue exclusion test and was >90% in the mouse brain (Hartmann et al., 1996).

On the other hand, we prepared brain homogenates for the determination of lipid peroxidation and enzyme activities. Tissue samples without cerebellum were homogenized in a Potter-Elvehjem homogenizer in 5 mM or 20 mM Tris-HCl buffer (Merck, Darmstadt, Germany), pH 7.4 to produce a 1/10 or 1/5 homogenate (wt/vol) depending on the assay performed.

Determination of reactive oxygen species (ROS)

Determination of ROS was based on several methods (LeBel et al., 1992; Oyama et al., 1994; Rothe et al., 1988) with slight modifications.

Levels of cellular oxidative stress were measured by using the fluorescent probes 2′,7′-dichlorofluorescein diacetate (DCFDA) and dihydrorhodamine 123 (DHR123). Brain cells were loaded for 30 minutes in the presence of 10 μM DCFDA or for 15 minutes in the presence of 10 μM DHR123 at 37°C in a shaking water bath (Driver et al., 2000). After washing twice with HBSS, the formation of the fluorescent product DCF was monitored by a SLM-Aminco fluorescence spectrometer with excitation wavelength of 488 nm and emission wavelength of 510 nm. DCF is trapped mainly in the cytoplasm and is oxidized by several ROS, most notably hydrogen peroxide (Keller et al., 1998).

DHR localizes to mitochondria and fluoresces when oxidized by ROS, particularly peroxynitrite, to the positively charged rhodamine 123 derivate (Rothe et al., 1988). The fluorescence was measured at excitation and emission wavelengths of 500 nm and 536 nm, respectively.

For in vitro induction of oxidative stress, $FeCl_3$ (20 μM) was added after the initial fluorescence reading (Hempel et al., 1999).

Production of ROS was expressed as fluorescence unit/mg protein.

CuZn-SOD, glutathione peroxidase and glutathione reductase activity assays

Brain homogenate was prepared in 20 mM Tris-HCl buffer (20% wt/vol) and centrifuged at 8,500×g for 10 minutes at 4°C. The clear supernatant was used for determining the activity of SOD, glutathione peroxidase (GPx) and glutathione reductase (GR).

SOD activity was measured as previously described (Leutner et al., 2000). The assay is based on the method described by Nebot et al. (1993) utilizing a specific reagent (R1) that undergoes alkaline autoxidation, which is accelerated by superoxide dismutase. Autoxidation of R1 (V) yields a chromophore, which absorbs maximally at 525 nm.

Determination of glutathione reductase activity was performed as previously described by Leutner et al. (2000). Briefly, this assay measures the rate of NADPH oxidation to $NADP^+$, which is accompanied by a decrease in absorbance at 340 nm, so GR activity can be monitored spectrophotometrically. Thus, one GR unit is defined as the reduction of one μM of GSSG per minute at 25°C and pH 7.6.

Glutathione peroxidase activity was assayed spectrophotometrically by using the Cellular Glutathione Peroxidase Assay Kit by Calbiochem© (Bad Soden, Germany) which measures GPx activity indirectly by determing the rate of NADPH oxidation to $NADP^+$, which is accompanied by a decrease in absorbance at 340 nm. One GPx unit is directly proportional to the amount of NADPH consumed in nmol per minute at 23–25°C and pH 7.6.

Measurement of basal and stimulated lipid peroxidation in brain tissue

Brain was homogenized in 10 volumes of ice-cold Tris-HCl (5 mM, pH 7.4). Brain homogenates were incubated with 100 μM $FeCl_3$ (Merck, Darmstadt, Germany), 10 mM H_2O_2 or buffer as control for 30 min at 37°C in a shaking water bath. After the incubation, the homogenates were centrifuged at 3,000× g for 10 min. The supernatants were collected and tested for lipid peroxidation by measuring the concentration of malondialdehyde (MDA). The LPO-586 kit, purchased from Calbiochem©, was used for this purpose. This kit takes advantage of a special chromogenic reagent, which reacts specifically with MDA at a comparatively low temperature of incubation (45°C).

Protein determination

The protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard (BIO-RAD, Munich, Germany).

Statistical analysis

Data are expressed as means +/− S.E.M. of n experiments each representing an individual animal. Statistical analysis was performed by Student's t-test for independent samples and by one way ANOVA followed by a post-hoc test (Bonferroni) using Prism 3.0 originated from Graph Pad Software Inc., San Diego, USA.

Results

Effects of aging on basal levels of lipid peroxidation

For our study, we chose population cohorts representing 4 different ages to give precise information about age-related changes in the murine organism. Therefore, studies were conducted on animals of several ages representing young adults (2–3 months), adults (10–11 months) beginning senescence (16–17 months) and senescence (20–21 months). At the age of 21 months, approximately 25% of the animals were still alive.

Lipid peroxidation is a well-established mechanism of cellular injury. It leads to the production of lipid peroxides and their by-products such as aldehydes and finally to the destruction of membrane lipids. Malonaldehyde (MDA) represents an end product of this process derived from the breakdown of polyunsaturated fatty acids and related esters. Measurement of MDA provides an exact and well established index of oxidative damage. Figure 1 shows that the basal level of lipid peroxidation, measured as formation of MDA, gradually increased in an age-dependent fashion indicating an accumulation of oxidative damage with age. There was a significant correlation between age and basal levels of MDA ($r = 0.992$; $p < 0.01$).

Effects of aging on antioxidant enzyme activity

The activity of SOD significantly increased between the ages of 2–3 and 10–11 months by about 87% and remained still elevated by about 70% in the brains of aged mice (see Fig. 2A). As shown in Fig. 2B, the activity of GR also had a tendency to increase with age. There was a significant correlation between age and the activity of GR ($r = 0.959$; $p < 0.05$).

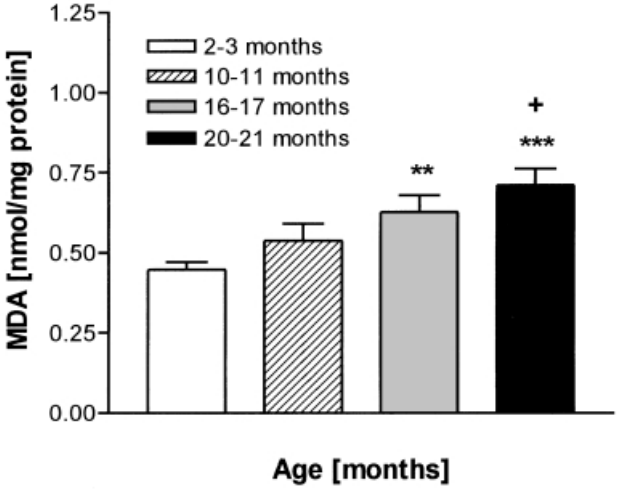

Fig. 1. Effect of age on basal levels of lipid peroxidation. Lipid peroxidation was measured in mouse brain homogenates as concentration of MDA (nmol/mg protein). Values are means ± S.E.M. of 11–12 mice at the age of 2–3 months, 10–11 months, 16–17 months and 20–21 months. Basal levels of the lipid peroxidation product MDA increased with increasing age. ANOVA indicated a significant effect over all groups ($p < 0.01$). Student's t-test: $^{+}p < 0.05$ vs. 10 months; $^{**}p < 0.01$, $^{***}p < 0.001$ vs. 2 months. In addition, there was a significant correlation between age and the lipid peroxidation product MDA ($r = 0.992$; $p < 0.01$)

However, only the 20–21 months old mice showed a statistically significant elevation of the activity of GR in brain (about 22%) over the level detected in young animals. No significant age-related changes in the activity of GPx could be observed (see Fig. 2B). There was only a small increase in the activity by about 17% at the age of 16–17 months.

Effects of aging on ROS generation

Figure 3A shows that the basal levels of ROS in brain homogenates detected with the fluorescent dye DCFDA were not significantly different among mice of different ages. DCF itself is oxidized by several ROS, most notably hydrogen peroxide (Cavazzoni et al., 1999). The mitochondrial electron transport chain is widely viewed as the main locus for the generation of ROS in the cell. Therefore, we used DHR123 as a second fluorescent dye. Inside live cells, the colourless DHR123 is oxidized by ROS, particularly peroxynitrite, to the positively charged rhodamine 123 derivative that stains mitochondria (Keller et al., 1998). When using DHR123, we observed a slight but not significant tendency to decreased levels of ROS in the adult brain compared to young brain. ROS levels did not seem to drop further in the aged brain, but slightly increased again (see Fig. 3B).

Effects of aging on LPO induced by $Fe3^{+}$ or H_2O_2

Brain tissue of both, young and aged mice, was more susceptible to peroxidation of polyunsaturated fatty acids after Fe^{3+}-treatment compared to

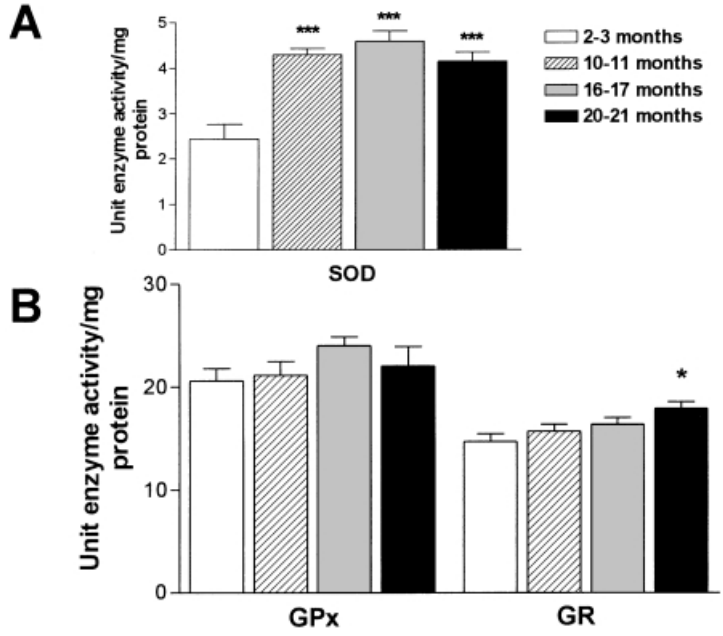

Fig. 2. Effect of age on the antioxidant enzyme activities of superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) in mouse brain. All three enzyme activities were measured in brain homogenates from female NMRI mice. Values are means ± S.E.M. **A** *Activity of SOD*. 11–12 animals were used in each group. The activity of CuZnSOD significantly increased in brains of adult and senescent mice compared to young ones ($p < 0.001$, ANOVA: ***$p < 0.001$ vs. 2–3 months, post-hoc Student's t-test). **B** *Activity of GPx* (left). The activity of GPx did not change with age. 11 animals were used in each group. *Activity of GR* (right). The activity of GR increased gradually with age ($p = 0.01$, ANOVA: *$p < 0.05$ vs. 2–3 months, post-hoc Student's t-test. There was a significant correlation between age and the activity of GR ($r = 0.959$; $p < 0.05$). 9–11 animals were used in each group

adult animals. This was demonstrated by significantly elevated levels of MDA in young and aged mice (Fig. 4A). Very similar results could be observed with hydrogen peroxide treatment (Fig. 4B).

The observed changes in MDA levels as a function of age are independent of the baseline values since an analysis of the stimulation data with subtraction of the baseline values still gives a statistically significant difference (see Fig. 4C+D). Again, the in vitro susceptibility of lipids to peroxidation induced by $FeCl_3$ (100 μM) or H_2O_2 (10 mM) first decreased in brains of adult animals compared to young ones and then increased again in brains of aged animals compared to the adult ones.

Effects of aging on generation of ROS

ROS production was stimulated by Fe^{3+} (20 μM) in dissociated mouse brain cell aggregates. Figure 5A shows the difference between the basal levels of ROS and the maximum iron-stimulated levels measured with DCFDA

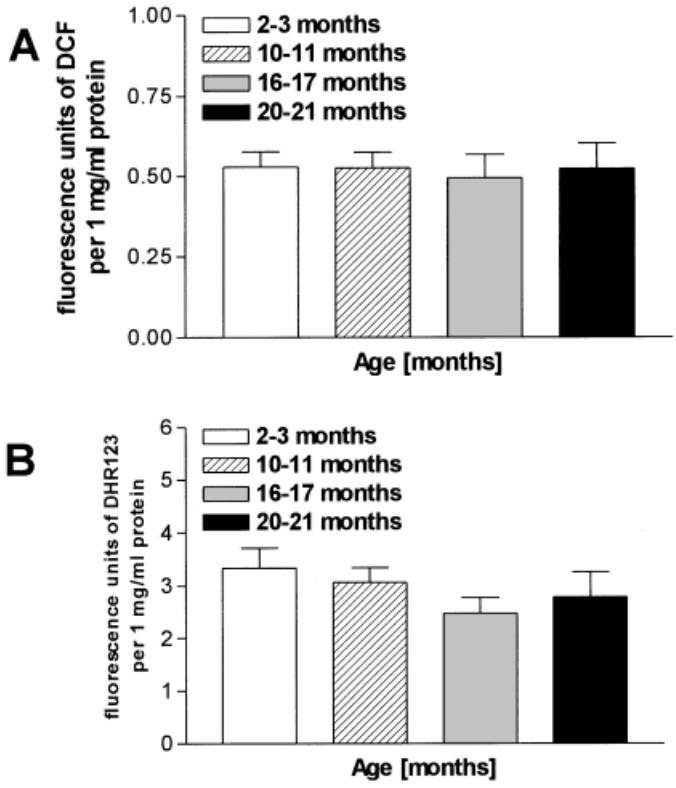

Fig. 3. Effect of age on basal levels of ROS. **A** Basal levels of cytoplasmic ROS were measured in dissociated brain cells with the fluorescent dye DCFDA. ROS levels are expressed as fluorescent units per 1 mg/ml protein. No differences in the basal levels of ROS could be detected between the four groups. **B** Basal levels of ROS were measured in dissociated mouse brain cells with DHR. ROS levels are expressed as fluorescent units per 1 mg/ml protein. There was no significant increase in brains of aged mice

(stimulated level minus basal level). The stimulation of ROS production by Fe^{3+} did not vary significantly with age.

Similarly, no significant differences between young, adult, and aged mice could be detected for ROS levels detected with DHR123 (Fig. 5B). However, there was again a tendency for lower levels of DHR123 fluorescence in adult vs. young and aged mice.

Interestingly, the time to reach the maximum of stimulated ROS-generation was significantly delayed in brains of 16–17-month-old animals as well as in brains of aged 20–21 months old animals as measured with both dyes (Fig. 5C+D). There was a significant correlation between age and the time to reach the maximum of stimulated ROS ($r = 0.997$, $p < 0.01$).

Discussion

Brains are generally liable to peroxidation by oxygen free radicals as already mentioned. Lipid peroxides readily decompose to liberate highly reactive carbonyl fragments, the most prominent being malondialdehyde (MDA). In

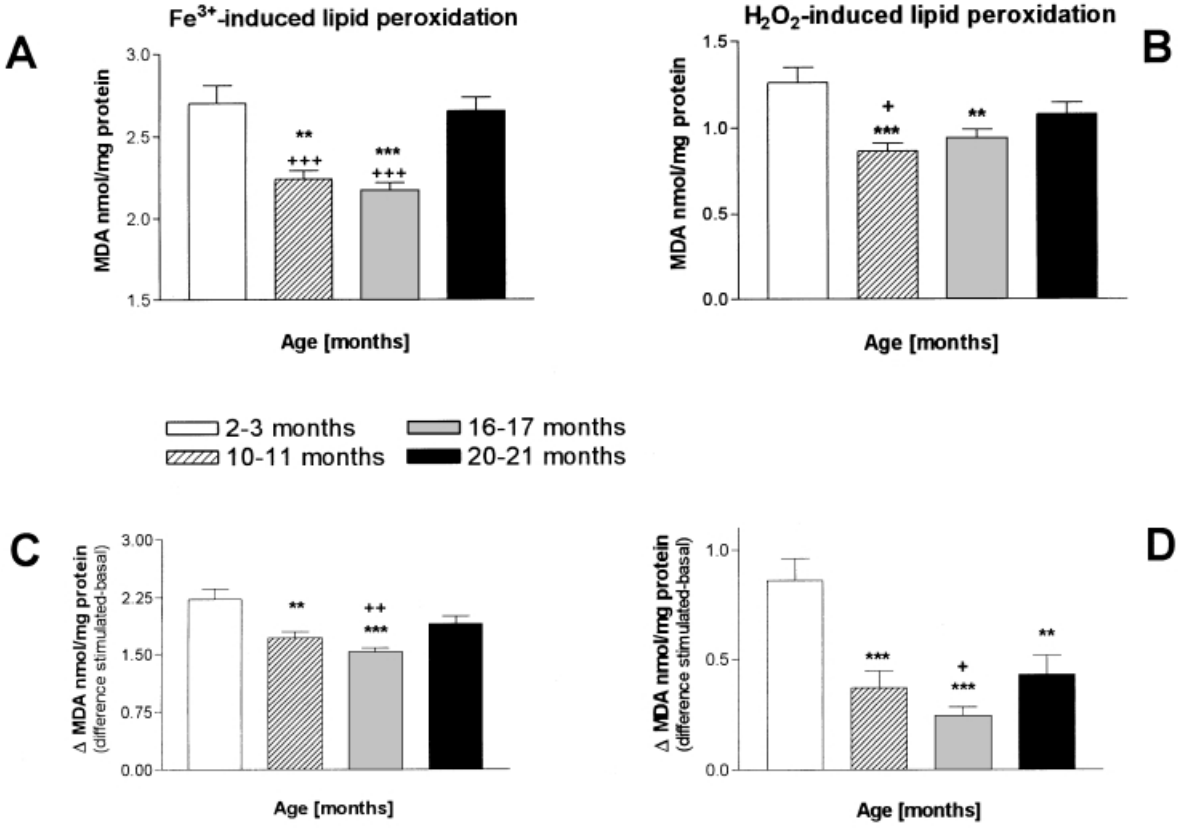

Fig. 4. Effect of age on iron- or hydrogen peroxide-induced levels of the lipid peroxide product MDA. **A** Effect of age on the iron-induced lipid peroxidation. Mouse brain homogenates were incubated for 30 minutes at 37°C in the presence of 100 μM Fe^{3+} followed by determination of MDA. Adult animals (10–11 and 16–17 months) were less sensitive to in vitro lipid peroxidation than young adults (2–3 months) or senescent mice (20–21 months) ($p < 0.001$, ANOVA: $^{**}p < 0.01$, $^{***}p < 0.001$ vs. 2 months; $^{+++}p < 0.001$ vs. 20 months, post-hoc Student's t-test). 9–13 animals were used in each group. **B** Effect of age on the hydrogen peroxide-induced lipid peroxidation. Homogenates were incubated for 30 minutes at 37°C in the presence of 10 mM H_2O_2. Adult animals (10–11 and 16–17 months) were less sensitive to in vitro lipid peroxidation than young adults (2–3 months) or senescent mice (20–21 months) ($p < 0.001$, ANOVA: $^{**}p < 0.01$, $^{***}p < 0.001$ vs. 2–3 months; $^{+}p < 0.05$ vs. 20–21 months, post-hoc Student's t-test). 11–13 animals were used in each group. **C** Effect of age on the susceptibility of lipids to oxidation (ΔMDA). Adult animals (10–11 and 16–17 months) were less sensitive to in vitro lipid peroxidation than young adults (2–3 months) or senescent mice (20–21 months). The bars represent the differences between the levels of MDA obtained after induction with Fe^{3+} and the basal level ($p < 0.001$, ANOVA: $^{**}p < 0.01$, $^{***}p < 0.001$ vs. 2–3 months; $^{++}p < 0.01$ vs. 20–21 months, post-hoc Student's t-test). 11–12 animals were used in each group. **D** Effect of age on the susceptibility of lipids to oxidation (ΔMDA). The bars represent the differences between the levels of MDA obtained after induction with hydrogen peroxide and the basal level. Adult animals (10–11 and 16–17 months) were less sensitive to in vitro lipid peroxidation than young adults (2–3 months) or senescent mice (20–21 months) ($p < 0.001$, ANOVA: $^{**}p < 0.01$, $^{***}p < 0.001$ vs. 2–3 months; $^{+}p < 0.05$ vs. 20–21 months, post-hoc Student's t-test). 11–12 animals were used in each group

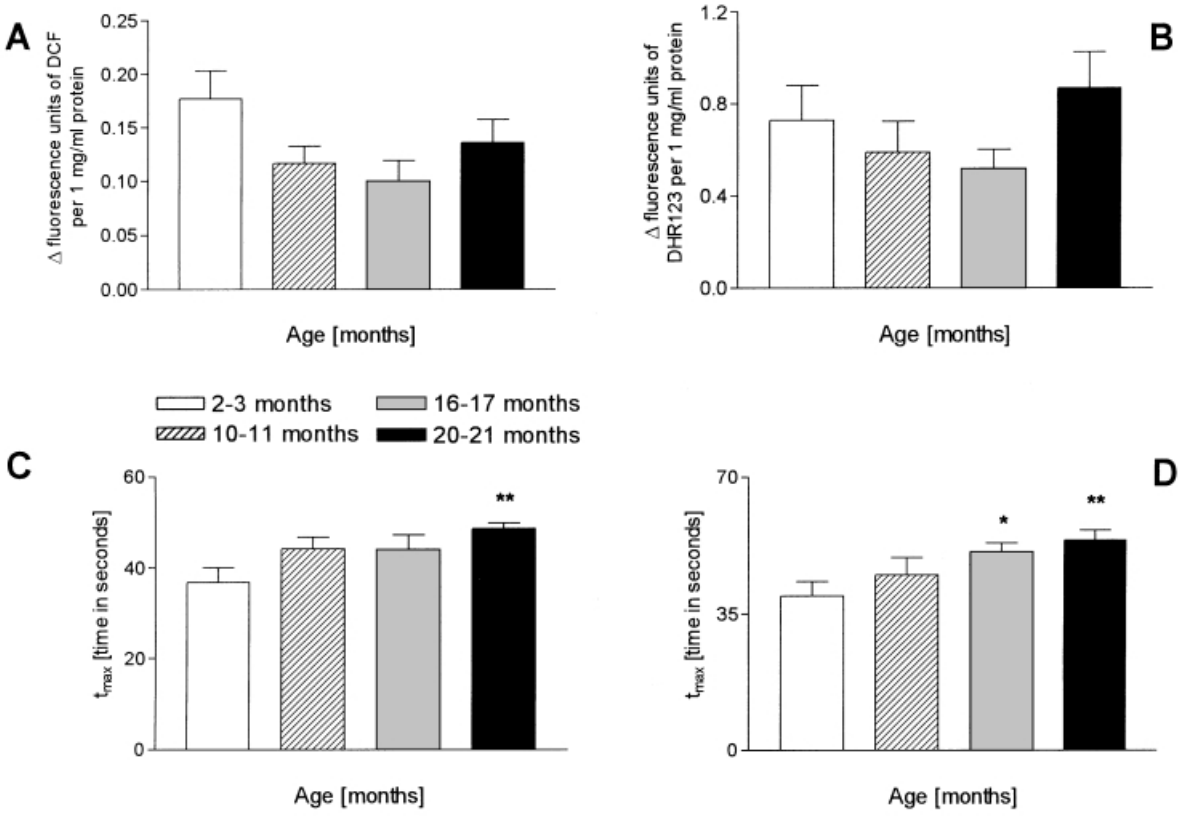

Fig. 5. Effect of age on the generation of ROS. **A** Effect of age on the iron-induced levels of ROS in dissociated mouse brain cells (Δ fluorescence of DCF). The bars represent the difference between the maximum levels of DCF fluorescence obtained after induction by Fe^{3+} (20 μM) and the basal level of ROS. No significant differences in sensitivity could be detected. 11 animals were used in each group. **B** Effect of age on the iron-induced levels of ROS (Δ fluorescence of DHR). The bars represent the difference between the maximum levels of DHR fluorescence obtained after induction by Fe^{3+} (20 μM) and the basal level of ROS. 10–11 animals were used in each group. No significant differences could be detected. **C** Effect of age on the time when maximum stimulation of ROS was achieved (t_{max} DCF). The time to reach the maximum of stimulated ROS-generation was significantly delayed in senescent mice ($p < 0.05$, ANOVA: $^{**}p < 0.01$ vs. 2–3 months, post-hoc Student's t-test). 10–11 animals were used in each group. **D** Effect of age on the time when maximum stimulation of ROS was achieved (t_{max} DHR). The time to reach the maximum of stimulated ROS-generation was significantly delayed in senescent mice ($p < 0.05$, ANOVA: $^{*}p < 0.05$, $^{**}p < 0.01$ vs. 2–3 months, post-hoc Student's t-test). Moreover, there was a significant correlation between age and the time to reach the maximum of stimulated ROS ($r = 0.997$, $p < 0.01$). 10–11 animals were used in each group

the literature, an increase of MDA with age is a consistent finding in most tissues and is paralleled by increased DNA and protein oxidation and mitochondrial oxidative changes in several animals as well as in humans (Ando et al., 1990; Smith et al., 1991; Pansarasa et al., 2000). In the present study, basal MDA levels increased constantly over the whole age range in the mouse brain, suggesting an involvement of oxygen free radicals in brain aging. These

results are in accordance with studies using rat brain or brain from C57BL mice (Mo et al., 1995; Tian et al., 1998).

A different picture emerged when LPO levels after in vitro stimulation by iron and hydrogen peroxide were examined. Comparing young and adult animals, we detected an age-related decrease in the magnitude of the response. These findings are associated with previous studies, which showed changes in fatty acid composition during aging as well as in species with long life spans (Imre et al., 2000; Pamplona et al., 2000). On the other hand, there was a significant increase in MDA-production after iron- or hydrogen peroxide-stimulation comparing adult and aged mice. These results suggest that the brains of adult mice have an elevated capacity to buffer or sequester exogenous stimuli. In contrast, aged mice are no longer able to cope with these stimuli resulting in an enhanced generation of MDA. As it seemed likely that the elevated levels of lipid peroxidation are a result of increased free radical generation during brain aging, we examined the generation of ROS levels in dissociated mouse brain cell aggregates using the probes DHR123 and DCF, the fluorescent product of DCFDA. Rather than analyzing the products of oxidative degradation, these methods directly assay the production of ROS. Under the conditions used in the present study, we could not detect increased levels of ROS measured with both fluorescent dyes in brains of adult or aged mice. These results are in agreement with findings made by Baek et al., who found no significant difference in basal ROS generation in hippocampus of young and aged rats measured with DCFDA (Baek et al., 1999). However, there are conflicting data presented by two previous studies, which observed increased levels of ROS in adult and aged rats (Driver et al., 2000) or even a lower basal oxygen formation in aged rat brain (LeBel and Bondy, 1991).

By acutely generating large amounts of ROS in mouse brain cells by in vitro stimulation, we tested how brain cells are able to cope with this exogenous stimulus. The rate of ROS-generation was not significantly different in the four examined age groups but again a tendency for lower levels was seen in the adult mice. However, brains of aged mice showed a significant delay in reaching the maximum level of ROS-stimulation by iron. Thus, brain cells of aged mice can compensate elevated ROS levels even better and take longer to reach maximum ROS levels.

Antioxidant enzymes are considered to be a primary defense mechanism that protects biological macromolecules from oxidative damage. Thus, elevated levels of antioxidant enzymes could be involved in the increased protection of aged brain tissue against free radical-mediated damage even if several studies report decrease protection against oxidative stress (Mo et al., 1995). Therefore, we determined the changes in enzyme activity during the aging process in mouse brain. We found significantly increased activity of SOD in brains of adult and aged mice in agreement with previous work by de Haan et al. (1992). There was an increase in the activity of SOD in brains of adult mice by about 87%, which slightly declined in the aged animals but was still considerably (about 70%) elevated relative to the young animals.

We also investigated two further important antioxidant enzymes GPx and GR. There was no significant change in GPx activity within the observed life

span, which was already reported by other groups (Dogru-Abbasoglu et al., 1997). Similarly, we also detected only a slight increase of GR activity with age, which only became statistically significant for the aged animals (about 22% over the level of young animals).

Generally, any imbalance between prooxidant and antioxidant factors can lead to a chronic accumulation of damaging effects like lipid peroxidation. A clear picture of the age-related changes taking place in mouse brain can only be given when many different parameters are known. Therefore, we not only investigated mice of four different ages but also several mechanisms related to the generation and detoxification of free radicals. Based on these findings, a theory can be postulated, how the well documented enhanced free radical production in the aging brain mainly due to mitochondrial dysfunction (Benzi and Moretti, 1995; Lenaz, 1998) finally leads to enhanced tissue damage. Our data suggest that initially elevated free radical production in the aging brain can be compensated by an increased activity of the antioxidant enzyme SOD, which finally leads to an elevated production of hydrogen peroxide. Since the activity of GPx is not increased in brains of aged mice to the same extent, hydrogen peroxide-induced damage may accumulate with age. Hydrogen peroxide is rapidly converted into the toxic hydroxyl radical, which represents the main ROS product responsible for lipid peroxidation. Accordingly, our data demonstrate elevated levels of lipid peroxidation with increasing age. The age-related increase of SOD activity might also be responsible for the unchanged basal levels of ROS and the decreased levels of ROS and LPO after stimulation. Since free iron solution alone, without added oxidant or reductant, is sufficient to generate free radicals and induce lipid peroxidation (Braughler et al., 1986), cellular agents such as superoxide radical anion may reduce Fe^{3+}. The resulting ferrous iron (Fe^{2+}) may initiate lipid peroxidation reactions mostly in combination with hydrogen peroxide known as Fenton's reaction. The observed increase in SOD activity may be involved in the decreased levels of LPO after stimulation because of its ability to decompose superoxide radical anion.

Thus, from the present data a rather clear pattern emerges when analyzing the relationship between the aging process and the generation and detoxification of oxygen free radicals. Oxidative stress develops when the well-regulated balance between pro-oxidants and protective antioxidants gets out of control. This seems to happen in the brains of aged NMRI mice even if the activities of the antioxidant enzymes SOD and GR are considerably increased.

Thus, our findings and the suggested model can integrate many divergent findings reported in the literature (Cand and Verdetti, 1989; Hussain et al., 1995; Mo et al., 1995) about the role of oxidative stress for brain aging.

References

Ames BN, Shigenaga MK, Hagen TM (1993) Oxidants, antioxidants, and the degenerative diseases of aging. Proc Natl Acad Sci USA 90: 7915–7922

Ando S, Kon K, Aino K, Totani Y (1990) Increased levels of lipid peroxides in aged rat brain as revealed by direct assay of peroxide values. Neurosci Lett 113: 199–204

Baek BS, Kwon HJ, Lee KH, Yoo MA, Kim KW, Ikeno Y, Yu BP, Chung HY (1999) Regional difference of ROS generation, lipid peroxidation, and antioxidant enzyme activity in rat brain and their dietary modulation. Arch Pharm Res 22: 361–366

Benzi G, Moretti A (1995) Age- and peroxidative stress-related modifications of the cerebral enzymatic activities linked to mitochondria and the glutathione system. Free Radic Biol Med 19: 77–101

Braughler JM, Duncan LA, Chase RL (1986) The involvement of iron in lipid peroxidation. Importance of ferric to ferrous ratios in initiation. J Biol Chem 261: 10282–10289

Cand F, Verdetti J (1989) Superoxide dismutase, glutathione peroxidase, catalase, and lipid peroxidation in the major organs of the aging rats. Free Radic Biol Med 7: 59–63

Carlberg I, Mannervik B (1985) Glutathione reductase. Methods Enzymol 113: 484–490

Cavazzoni M, Barogi S, Baracca A, Castelli GP, Lenaz G (1999) The effect of aging and an oxidative stress on peroxide levels and the mitochondrial membrane potential in isolated rat hepatocytes. FEBS Lett 449: 53–56

de Haan JB, Newman JD, Kola I (1992) Cu/Zn superoxide dismutase mRNA and enzyme activity, and susceptibility to lipid peroxidation, increases with aging in murine brains. Brain Res Mol Brain Res 13: 179–187

Dogru-Abbasoglu S, Tamer-Toptani S, Ugurnal B, Kocak-Toker N, Aykac-Toker G, Uysal M (1997) Lipid peroxidation and antioxidant enzymes in livers and brains of aged rats. Mech Ageing Dev 98: 177–180

Driver AS, Kodavanti PR, Mundy WR (2000) Age-related changes in reactive oxygen species production in rat brain homogenates. Neurotoxicol Teratol 22: 175–181

Harman D (1981) The aging process. Proc Natl Acad Sci USA 78: 7124–7128

Hartmann H, Velbinger K, Eckert A, Muller WE (1996) Region-specific downregulation of free intracellular calcium in the aged rat brain. Neurobiol Aging 17: 557–563

Hempel SL, Buettner GR, O'Malley YQ, Wessels DA, Flaherty DM (1999) Dihydrofluorescein diacetate is superior for detecting intracellular oxidants: comparison with 2′,7′-dichlorodihydrofluorescein diacetate, 5(and 6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, and dihydrorhodamine 123. Free Radic Biol Med 27: 146–159

Hussain S, Slikker WJ, Ali SF (1995) Age-related changes in antioxidant enzymes, superoxide dismutase, catalase, glutathione peroxidase and glutathione in different regions of mouse brain. Int J Dev Neurosci 13: 811–817

Imre S, Firbas JH, Noble RC (2000) Reduced lipid peroxidation capacity and desaturation as biochemical markers of aging. Arch Gerontol Geriatr 31: 5–12

Keller JN, Guo Q, Holtsberg FW, Bruce-Keller AJ, Mattson MP (1998) Increased sensitivity to mitochondrial toxin-induced apoptosis in neural cells expressing mutant presenilin-1 is linked to perturbed calcium homeostasis and enhanced oxyradical production. J Neurosci 18: 4439–4450

Keller JN, Kindy MS, Holtsberg FW, St Clair DK, Yen HC, Germeyer A, Steiner SM, Bruce-Keller AJ, Hutchins JB, Mattson MP (1998) Mitochondrial manganese superoxide dismutase prevents neural apoptosis and reduces ischemic brain injury: suppression of peroxynitrite production, lipid peroxidation, and mitochondrial dysfunction. J Neurosci 18: 687–697

LeBel CP, Bondy SC (1991) Persistent protein damage despite reduced oxygen radical formation in the aging rat brain. Int J Dev Neurosci 9: 139–146

LeBel CP, Ischiropoulos H, Bondy SC (1992) Evaluation of the probe 2′,7′-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chem Res Toxicol 5: 227–231

Lenaz G (1998) Role of mitochondria in oxidative stress and ageing. Biochim Biophys Acta 1366: 53–67

Leutner S, Czech C, Schindowski K, Touchet N, Eckert A, Muller WE (2000) Reduced antioxidant enzyme activity in brains of mice transgenic for human presinilin-1 with single or multiple mutations. Neurosci Lett 292: 87–90

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275

Mo JQ, Hom DG, Andersen JK (1995) Decreases in protective enzymes correlates with increased oxidative damage in the aging mouse brain. Mech Ageing Dev 81: 73–82

Nebot C, Moutet M, Huet P, Xu JZ, Yadan JC, Chaudiere J (1993) Spectrophotometric assay of superoxide dismutase activity based on the activated autoxidation of a tetracyclic catechol. Anal Biochem 214: 442–451

Oyama Y, Hayashi A, Ueha T, Maekawa K (1994) Characterization of 2′,7′-dichlorofluorescin fluorescence in dissociated mammalian brain neurons: estimation on intracellular content of hydrogen peroxide. Brain Res 635: 113–117

Pamplona R, Portero-Otin M, Riba D, Requena JR, Thorpe SR, Lopez-Torres M, Barja G (2000) Low fatty acid unsaturation: a mechanism for lowered lipoperoxidative modification of tissue proteins in mammalian species with long life spans. J Gerontol A Biol Sci Med Sci 55: B286-B291

Pansarasa O, Castagna L, Colombi B, Vecchiet J, Felzani G, Marzatico F (2000) Age and sex differences in human skelatal muscle: role of reactive oxygen species. Free Radic Res 33: 287–293

Rothe G, Oser A, Valet G (1988) Dihydrorhodamine 123: a new flow cytometric indicator for respiratory burst activity in neutrophil granulocytes. Naturwissenschaften 75: 354–355

Smith CD, Carney JM, Starke-Reed PE, Oliver CN, Stadtman ER, Floyd RA, Markesbery WR (1991) Excess brain protein oxidation and enzyme dysfunction in normal aging and in Alzheimer disease. Proc Natl Acad Sci USA 88: 10540–10543

Sohal RS, Mockett RJ, Orr WC (2000) Current issues concerning the role of oxidative stress in aging: a perspective. Results Probl Cell Differ 29: 45–66

Stoll L, Schubert T, Muller WE (1992) Age-related deficits of central muscarinic cholinergic receptor function in the mouse: partial restoration by chronic piracetam treatment. Neurobiol Aging 13: 39–44

Tian L, Cai Q, Wei H (1998) Alterations of antioxidant enzymes and oxidative damage to macromolecules in different organs of rats during aging. Free Radic Biol Med 24: 1477–1484

Tsay H, Wang P, Wang S, Ku H (2000) Age-associated changes of superoxide dismutase and catalase activities in the rat brain. J Biomed Sci 7: 466–474

Authors' address: Dr. W. E. Müller, Biocenter Niederursel, Marie-Curie-Strasse 9, N 260, D-60439 Frankfurt, Federal Republic of Germany, e-mail: W.E.Mueller@em.uni-frankfurt.de