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Sensitivity to *in vitro* Lipid Peroxidation in Liver and Brain of Aged Rats

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Lipid peroxidation in rat liver and brain has been studied to see if it increases with old age. No significant differences in the level of endogenous, nonstimulated lipid peroxidation (TBA-RS) is found between 9 month-old (mature adults) and 28 month-old animals in liver or cerebral cortex. Liver homogenates subjected *in vitro* to an oxidative stress (ascorbate-Fe^{++}), show a clearly slower peroxidation rate in old than in young animals. On the other hand, the *in vitro* peroxidation rate of cerebral homogenates was similar in young and old animals. The *in vitro* peroxidation rate was much higher in brain than in liver tissue. These results do not support the view that old rats liver and brain are more susceptible to free radical oxidative damage than those of young ones.

Key words: Aging, Lipid peroxidation, Oxygen radicals, Free radicals.

Free radicals are currently proposed to be the principal determinants of the aging process (10, 14, 20). The main proposal of the free radical theory of aging (20) affirms that aging is due to oxidative damage caused by the continuous generation of free radicals in biological tissues. Even though direct demonstration that free radicals are at the root of the aging process is still lacking, the present versions of the theory are based on various indirect circumstancial evidences. Among them, particular attention has been paid to the suggestion that old animals are more susceptible to oxidative stress due to a decline in their endogenous antioxidant defenses. Perhaps the only animals in which decreases of antioxidants, together with increases in lipid peroxidation in old age, have been clearly shown (20) in the absence of opposite information, are whole *Musca domestica* flies. Nevertheless, clear

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decreases in lipid peroxidation during aging of *Drosophila melanogaster* flies have been recently reported from the same laboratory (21). In addition, in rodents there are contradictory reports concerning variations of enzymatic and nonenzymatic antioxidants in both brain (2) and liver (13).

Although it is sometimes assumed that lipid peroxidation increases in old animal tissues, nevertheless reports on increases (15, 19), absence of changes (1, 6) or decreases (5, 8) in old age can be found in the literature. The situation is further complicated since many reports do not distinguish or state clearly if their lipid peroxidation values correspond to *in vivo* (endogenous) or *in vitro* stimulated peroxidation. In an attempt to clarify these points, we have studied both *in vivo* and *in vitro* lipid peroxidation in the brain and liver of mature adult rats and old rats. *In vitro* lipid peroxidation was studied kinetically at various time points in order to gain a wider perspective about the sensitivity of the tissues to a peroxidative challenge. Liver and brain were selected as subjects of study due to their character of vital organs which show important declines of function during aging.

Materials and Methods

Animals. — Male Wistar rats of 8 and 27 months of age were obtained from the Centre d'élevage Raymond Janvier (CERJ, France), where they had been maintained throughout their life span. After arriving at the laboratory, they were maintained for one month at 23 ± 2 °C and 50 ± 10 % relative humidity, and were fed a standard pelleted laboratory diet (Panlab, Spain) and water *ad libitum*. Mean composition of this diet was as follows (in %): carbohydrates, 58.7; cellulose, 4.3; protein, 17; fat, 3; mineral-vitamin mix, 5 and water, 12. The age of the animals was specifically selected to detect changes associated with the part of the life span during which a decline in physiological capabilities occurs. Thus, 9 month-old animals were selected as young adults in order to avoid the implication of changes associated with growth and development involved in lower age animals. Twenty-eight-month-old rats were selected as old in order to avoid the presence of changes secondary to pathological states of the tissues, typical in very old animals.

Samples. — The animals were sacrificed by decapitation and liver and cerebral cortex samples were immediately excised. The samples were rinsed and homogenized in 20 volumes of cold 50 mM phosphate buffer (pH 7.4) using an automatic Wheaton homogenizer (teflon-glass) with three strokes at around 1000 rpm. The homogenates were sonicated over ice with a Vibra-Cell ultrasonic processor with a power output of 38 W. Sonication was performed in three bursts of 10 s separated by two time intervals of 20 s. The sonicated homogenates were centrifuged at 3,200 × *g* for 20 min at 5 °C and the supernatants were used for lipid peroxidation assays.

Lipid peroxidation in vivo. — The concentrations of products of tissue peroxidation *in vivo* in liver and brain supernatants from young and old animals were estimated by the TBA assay as described by UCHIYAMA and MIHARA (22) with some modifications. An aliquot of 0.14 ml of the supernatants was mixed with 1 ml of 1 % phosphoric acid, 0.33 ml of 0.6 % TBA and 0.033 ml of ethyl alcohol containing 0.01 % butylated hydroxytoluene (BHT). BHT was added in order to avoid an overestimation of lipid peroxidation. The inclusion of BHT in the TBA mixture has been reported to prevent a further peroxidation of the sample during the heating step of the assay which would lead to artificially high TBA values (17). The addition of 3.3 µg of BHT in the assay mix-

ture is enough to completely protect the samples from extra peroxidation during the assay, and addition of higher BHT amounts does not provide further antioxidant protection. The mixture was heated for 45 min in a boiling water bath and, after cooling, 1.4 ml of n-butanol were added and mixed vigorously. The butanol phase was separated by centrifugation at 1,700 *g* for 15 min. The absorbance was read at 535 nm, and referred to a standard curve obtained by performing the assay at various known concentrations of pure malondialdehyde (MDA). The results are presented as TBA-reacting substances (TBA-RS) and are expressed in nmole equivalents of MDA/g tissue.

Lipid peroxidation in vitro. — Sensitivity to *in vitro* peroxidation was estimated by incubating supernatant samples with 0.4 mM ascorbate and 0.05 mM $FeSO_4$ for 5, 15, and 30 min (brain) or 30, 60, and 90 min (liver) at 25 °C. At the end of these incubation times an aliquot of the sample was separated and subjected to the TBA assay as described in the previous section.

Statistical analyses. — Two-Way Analysis of Variance (ANOVA) were used to perform comparisons among ages and times of incubation in the *in vitro* peroxidation system. When significant effects of age and incubation time were found in the absence of interaction, a one-way ANOVA was performed separately in young and old animals in order to study the incubation time effect. After the ANOVA, Fisher's least significant difference (LSD) test was used when necessary to analyze significance among groups. The 0.05 level was selected as the point of minimal statistical significance in every comparison.

Results

Levels of endogenous lipid peroxidation products were measured in the liver and brain of both young and old Wistar rats. The results are represented in figs. 1 and 2 as points obtained at time 0, without any *in vitro* stimulated peroxidation. No significant differences were found when the *in vivo* lipid peroxidation values were compared between young and old animals in either brain or liver.

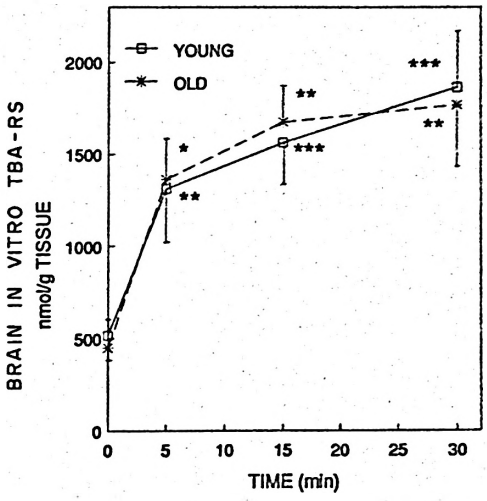

Fig. 1. In vitro *peroxidation kinetics (mean ± S.E.M.) of cerebral cortex supernatants from young (9 months) and old (28 months) male rats.* Supernatants were incubated during 0, 5, 15 and 30 min at 25 °C in the presence of 0.4 mM ascorbate and 0.05 mM $FeSO_4$ in 50 mM phosphate buffer pH 7.4. Values obtained at time 0 represent *in vivo* tissue peroxidation. n = 6 animals. Significant differences from values at time 0: * $P > 0.05$; ** $P < 0.01$; *** $P < 0.001$.

In vitro stimulation of lipid peroxidation was very effective in supernatants from both organs. Brain TBA-reactive substances (TBA-RS) increased very quickly as a function of incubation time and significantly higher values (around a 300 % increase) were obtained at as early as 5 min of incubation (fig. 1). The rate of brain TBA-RS increase was afterwards slowed, and at 30 min of incubation peroxidation was almost saturated as shown

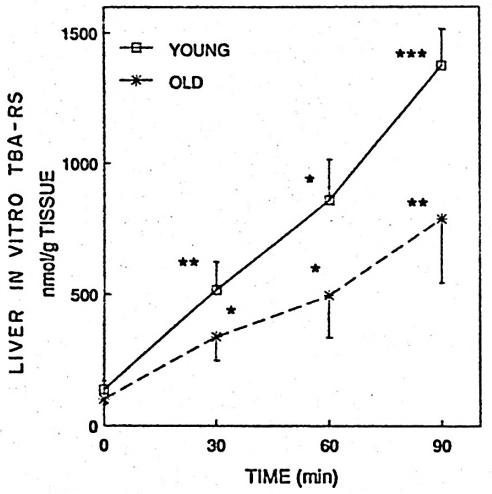

Fig. 2. In vitro *peroxidation kinetics (mean ± S.E.M.) of liver supernatants from young (9 months) and old (28 months) male rats.* Supernatants were incubated during 0, 30, 60 and 90 min at 25 °C as in fig. 1. Values obtained at 0 time represent *in vivo* tissue peroxidation. n = 6 animals. Significant difference from the previous time point: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

by a plateau phase. The one-way ANOVA ($P < 0.001$ in young and $P < 0.01$ in old rats) showed a significant increase from 0 to 5 min in both groups and did not show significant differences between 5 min and longer incubation times. In liver samples the peroxidation process was much slower, but significantly increased TBA-RS values (300 %) were obtained at as early as 30 min of incubation. However, in this case a significant stimulation continued to be present at a similar rate between 30 and 60 and between 60 and 90 min of incubation in animals of both ages (one-way ANOVA: $P < 0.001$ in young and $P < 0.01$ in old rats). The peroxidation process was not saturated even after such a long incubation period. Total stimulation of TBA-RS values (between 0 and 90 min of incubation) was much greater for liver (1,000 % increase in young animals) than for brain tissue (350 % increase).

When the TBA-RS values obtained after *in vitro* peroxidation of brain supernatants were compared between young and old animals, no significant differences between them were obtained at any time point (fig. 1). The two-way ANOVA showed a $P < 0.97$ for the age effect and a $P < 0.001$ for the incubation time effect. A very different situation was observed for liver, where a significantly lower TBA-RS value was obtained in old animals in relation to young ones at as early as 30 min of incubation (fig. 2). This difference in the rate of ascorbate-Fe^{++} peroxidation was again shown at 60 and 90 min of incubation. The two-way ANOVA showed highly significant effects due to age ($P < 0.01$) and time of incubation ($P < 0.001$). Peroxidation proceded at a rather constant rate in the liver of both young and old rats, but this rate was clearly slower in old animals.

Discussion

Increases (7, 19), and decreases (5, 9, 11) of lipid peroxidation *in vivo* during aging have been described for rat liver. Similarly, the *in vivo* lipid peroxidation either increased (15, 19), decreased (5, 8) or did not change in smooth endoplasmic reticulum (7) in old rat brain in relation to young ones.

Althougt the TBA assay is convenient for lipid peroxidation assays because of its simplicity and high sensitivity, the assay nevertheless, is not specific for malondialdehyde and many other substances present in biological samples can contribute to TBA values. In fact, the validity of the *in vivo* lipid peroxidation-TBA-test has recently been questioned as an indicator of changes in peroxidative damage during aging (21). However, if the biological sample is peroxidized *in vitro*, lipid peroxi-

dation produces high amounts of malondialdehyde, hydroperoxides and other substances that give positive results in the TBA test. This greatly minimizes the contribution to TBA values from substances not related to lipid peroxidation. Liver TBA values, for instance, increased from 137 to 1375 malondialdehyde equivalents/g from 0 to 90 min of incubation in our *in vitro* lipid peroxidation system. Even assuming an initial endogenous interference as high as 50 % for *in vivo* peroxidation (at 0 time), this would involve only a 5 % interference for *in vitro* peroxidation at 90 min, since the difference between TBA values at both time points is exclusively due to the generation of lipid peroxidation products during the *in vitro* assay. Thus, it is suggested that the TBA assay can accurately estimate the concentration of lipid peroxidation products in complex biological samples only after *in vitro* stimulation, the *in vivo* values being highly inaccurate.

Fortunately, there have been previous reports on *in vitro* lipid peroxidation in young and old rats, where the previous contradictions are almost totally resolved. Thus, clear decreases of *in vitro* TBA values in old rat liver (7, 9, 12) and brain tissue (8) have been described. No significant changes in rat brain in *in vitro* TBA values have been described (1). Our results are consistent with these reports, since *in vitro* peroxidation clearly decreased in the liver and did not change in the brain with old age. The present results and those of DEVASAGAYAM (7), are the only ones in which the *in vitro* peroxidation process was followed kinetically as a function of time, whereas in the rest of the reports it was studied at a single time point. The total agreement between DEVASAGAYAM's (7) results and our concerning the kinetics of *in vitro* liver peroxidation in young and old rats, gives further support to this kind of result. Our data also extend the decrease of *in vitro* lipid peroxidation to the whole liver, since DEVASAGAYAM's data were obtained using rough and smooth liver microsomes. It should be noted that an absence of changes in *in vitro* lipid peroxidation as a function of age has been found in liver and brain of various animal species and humans ranging from 15 to 80 years of age (6).

The TBA values obtained after *in vitro* peroxidation are subject to many influences, including endogenous levels of antioxidants, free radical generation systems, and polyunsaturated fatty acid (PUFA) content. This last factor can be important since PUFA are the most susceptible lipids to peroxidation, while important decreases in the unsaturated/saturated fatty acid ratio (3) and corresponding increases in the microviscosity of liver membranes (16) have been shown to occur in old rat liver. In agreement with this, a decrease in 6-desaturase activity has been recently shown to take place in the liver but not in the brain of old rodents (4). Furthermore, a high vitamin E content in membranes strongly decreases their sensitivity to peroxidation. It should be noted that aging rats progressively accumulate diet-derived vitamin E in the liver (200 % increase) but not in the brain when they age from 4 to 29 months (18, 23). This could also explain why liver but not brain *in vitro* TBA-RS values decreased in old animals. Thus, the changes observed can be a reflection of the different chemical composition of the tissues of old versus young rats. In any case, even though the basic causes of the decrease in liver peroxidation cannot be ascertained at present, the available data allow us to conclude that the liver and brain of old rats are not more susceptible to peroxidative damage than the homologous organs of young animals. This conclusion should not be considered as contradictory with the free radical theory of aging since free radical damage can still continuously occur from the mature adult to the aged animal.

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Resumen

Se estudia el posible incremento de peroxidación lipídica en función de la edad en hígado y en cerebro de rata. Los niveles de peroxidación lipídica endógena no estimulada no varían significativamente entre animales adultos maduros (9 meses) y viejos (28 meses) en el hígado y la corteza cerebral. Los homogeneizados de hígado, sometidos a un estrés oxidativo *in vitro* (ascorbato-Fe^{++}), muestran una tasa de peroxidación lipídica claramente menor en los animales viejos que en los jóvenes, no encontrándose diferencias en homogeneizados cerebrales. La tasa de peroxidación lipídica *in vitro* es mucho mayor en el cerebro que en el hígado. Estos resultados no apoyan que el hígado y el cerebro de las ratas viejas sean más sensibles que los de las jóvenes al daño oxidativo inducido por radicales libres.

Palabras clave: Envejecimiento, Peroxidación lipídica, Radicales de oxígeno, Radicales libres.

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