ELSEVIER

Mechanisms of Ageing and Development
102 (1998) 1–13

mechanisms of ageing and development

Iron accumulation in aging: modulation by dietary restriction

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Received 18 August 1997; received in revised form 6 December 1997; accepted 16 December 1997

Abstract

Male Fischer 344 rats fed ad libitum or dietary restricted (maintained on 60% of ad libitum food intake) were sacrificed at 6, 12 and 24 months of age. Portions of kidney, liver and brain were removed for total iron content analysis and oxidative stress assessment. Total iron content was measured directly and lipid peroxidation (LPO) was assayed as an index of oxidative stress. Tissue total iron content was shown to increase significantly with age in animals fed ad libitum (AL). At 24 months, these animals showed comparable iron content increases in the liver and kidney, but were significantly greater than measurements found in brain. This age-related iron accumulation, however, was found to be markedly suppressed by dietry restriction (DR) in all tissues. Similarly, LPO measurements increased in an age-related, tissue-specific fashion. At 24 months of age, measurements of LPO in AL rats brain and liver exceeded measurements in kidney. Again, we found DR to markedly suppress age-related LPO in all tissues. Reported here are our findings on the ability of DR to modulate iron status at the tissue level. Consistent with the proposed anti-oxidative mechanism of DR, these findings further suggest that the modulation of tissue total iron content is an important component of that mechanism.

Keywords: Tissue iron content; Lipid peroxidation; Oxidative stress; Dietary restriction; Aging

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0047-6374/98/$19.00
PII S0047-6374(98)00005-0

1. Introduction

Aging may be defined as gradual, progressive changes in an organism that increase the probability of its death. These alterations compromise an organism's ability to meet both internal and external challenges (Baker and Martin, 1994; Yu, 1996). A prime example of such alterations is the age-related accumulation of damage due to endogenous generation of free radicals. The condition is worsened by an age-related decline in the organism's ability to counteract these changes. These phenomena result in what is referred to as oxidative stress (Baker and Martin, 1994; Yu, 1996).

The oxidative stress theory of aging asserts that, with time, the balance between free radical-mediated oxidative damage and anti-oxidative protection shifts towards greater oxidative damage. A primary source of damage brought about by oxidative stress is lipid peroxidation (LPO), which is attributed to its highly propagative nature and cytotoxicity from its metabolic byproducts. LPO is therefore an established index of age-related oxidative stress (Koizumi et al., 1987; Draper and Hadley, 1990; Janero, 1990; Yu and Yang, 1996).

Another hallmark of biological aging is the accumulation of iron in tissue as indicated by age-related increases in total iron content. Iron is recognized as a potent pro-oxidant and a necessary catalyst for in vivo LPO (Dunford, 1987; Minotti and Aust, 1992; Floyd and Carney, 1993; Fonteclave and Pierre, 1993; Olanow, 1993; Baker and Martin, 1994). These characteristics imply a causal relation between age-related iron accumulation and LPO. Indeed, as proposed by Massie et al. (1985), the rate of age-related iron accumulation correlates inversely with the life span in some species.

Our laboratory recently reported on the ability of dietary restriction (DR), an established anti-aging intervention, to attenuate age-related LPO and iron accumulation in serum (Choi and Yu, 1994). This finding is consistent with the proposed anti-oxidative mechanism of DR (Fishbein, 1991; Davis et al., 1993; Djuric and Kritscheusky, 1993; Feuers et al., 1993; Yu, 1995). To further explore the relation between age-related oxidative stress and iron accumulation, our current research established the following two objectives: (1) Determine whether or not total iron content and LPO increase in tissues in a correlated, age-dependent fashion; and (2) ascertain whether the proposed anti-oxidative mechanism of DR implicates an ability to modulate total iron content and LPO in tissues.

2. Materials and methods

2.1. Animals

Specific-pathogen-free (SPF) Fischer 344 male rats purchased from Charles River Laboratory were used for this research. Rats were maintained in a barrier facility, housed singly in plastic cages with wire mesh floors and fed a semi-synthetic diet (Ralston-Purina). SPF status of each shipment of rats was verified and maintained

as described by Yu et al. (1985, 1982). Dietary restriction (60% of ad libitum Fed) began at 6 weeks of age, as previously described (Yu et al., 1985), and continued throughout life. Rats were sacrificed via decapitation at 6, 12 and 24 months of age and the required tissues were removed.

2.2. Preparation of tissues

Approximately 0.8–1.3 g of tissue from the kidney, liver and brain (forebrain) were obtained from rats and prepared at 4°C. Tissues were minced and rinsed several times with 5 mM sodium-potassium phosphate, buffer at pH 7.4. Prior to homogenization, care was taken to remove the blood from the tissues. Tissues were homogenized using a Polytron homogenizer and samples were divided into aliquots for the measurement of protein, hemoglobin, total iron content, and malondialdehyde (MDA) or were stored at −78°C.

2.3. Determination of protein

Protein concentration was measured in the homogenate by the method of Bradford (1976) using bovine serum albumin as a standard.

2.4. Determination of tissue preparation hemoglobin content

Hemoglobin (Hb) content of tissue homogenate preparations from ad libitum fed rats was measured by a method that employs Drabkin's reagent (Wendell, 1962). The reagent was prepared by dissolving 200 mg $K_3Fe(CN)_6$, 50 mg KCN and 140 mg KH_2PO_2 in 900 ml distilled water. The Fe^{2+} ion of heme in hemoglobin, oxyhemoglobin and carboxyhemoglobin is oxidized to Fe^{3+} by ferricyanide to form methemoglobin. Methemoglobin combines with ionized cyanide to produce the stable, red cyanmethemoglobin, which was measured spectrophotometrically at 540 nm. Methemoglobin (30 g/dl), obtained from Sigma (St. Louis, MI) was used as a standard. Hb content was analyzed in the kidney, liver and brain samples from 6, 12 and 24-month-old ad libitum (AL) rats.

2.5. Determination of tissue total iron content

The method used for the quantitation of total iron in the experimental samples relied on an initial treatment that releases complexed iron for its subsequent quantitative determination. A standard iron solution was prepared by dissolving 10.25 mg of ferrous ethylenediammonium sulfate (Fe^{2+}-EDS) obtained from Sigma (St. Louis, MI), in 250 ml of 0.01 N HCl (Fish, 1988).

Reagents necessary for this assay (A and B) were prepared as follows: 100 ml of a 1.2-N HCl solution and 100 ml of a 4.5% w/v of (0.285 M) $KMnO_4$ solution were prepared. Equal volumes of each (HCl and $KMnO_4$ solutions) were gently mixed together in a fume hood to yield reagent A. Reagent B was prepared by dissolving 9.7 g of ammonium acetate and 8.8 g of ascorbate in 15 ml of distilled water. To

this mixture, 80 mg of ferrozine and 80 mg of neocuprine were added and brought to 25 ml with distilled water. This gave final concentrations of 5 M ammonium acetate, 2 M ascorbate, 6.5 mM ferrozine and 13.1 mM neocuprine.

The digestive phase of this procedure involves the addition of 500 μl of reagent A to tissue samples (3–5 mg protein) in a 15-ml polystyrene conical tube. Tubes were capped and incubated at 60°C with moderate agitation for 2 h according to prescribed measures for complete tissue digestion and release of iron. The tubes were cooled for 15–20 min to ambient temperature (70–75°F). After cooling, 100 μl of reagent B was added to the digestion mixture. Following a 30-min incubation at room temperature, all tubes were vortexed and 1 ml of the contents was transferred to 1.5 ml eppendorf tubes and centrifuged at 1000 g for 10 min. A clear violet to purple supernatant was obtained for spectrophotometric measurements at 562 nm. The values obtained were proportional to the iron content of the sample. Iron content was expressed as ng/mg protein.

2.6. Determination of lipid peroxidation

Tissue lipid peroxidation was measured using an MDA assay kit (LPO-586) obtained from Oxis International (Portland, OR). The principle of the procedure used in this study was based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA (Janero, 1990). A product with a maximal absorbency of 586 nm was produced; the absorbency values obtained spectrophotometrically were proportional to the MDA content of the sample. An MDA standard solution was obtained from Sigma (St. Louis, MI). MDA is expressed as nanomoles per microgram protein.

2.7. Statistics

One-way analysis of variance (ANOVA) was conducted to analyze significant differences between all possible age and diet pairs. Analysis of relevant pairs revealed the effect of age on Hb, iron, and MDA content. Two-way (ANOVA) was used to detect interactions between variables (age and diet) on tissue total iron and MDA content. P values less than 0.05 were recognized as significant and reported.

3. Results

3.1. Tissue preparation hemoglobin content

Hb content measurements of kidney tissue preparations from AL fed rats at 24 months was significantly lower than measurements at 6 and 12 months ($P < 0.05$). However, the Hb content of liver and brain tissue preparations revealed no significant difference among the three age groups ($P > 0.05$). Furthermore, measurements of Hb content in tissue preparations revealed that iron content due to Hb contamination of samples was less than 0.02%. The contribution of Hb iron to

tissue total iron measurements was therefore negligible. In addition there was no significant age-related effect on Hb content in any of the three tissues analyzed, therefore data is not shown.

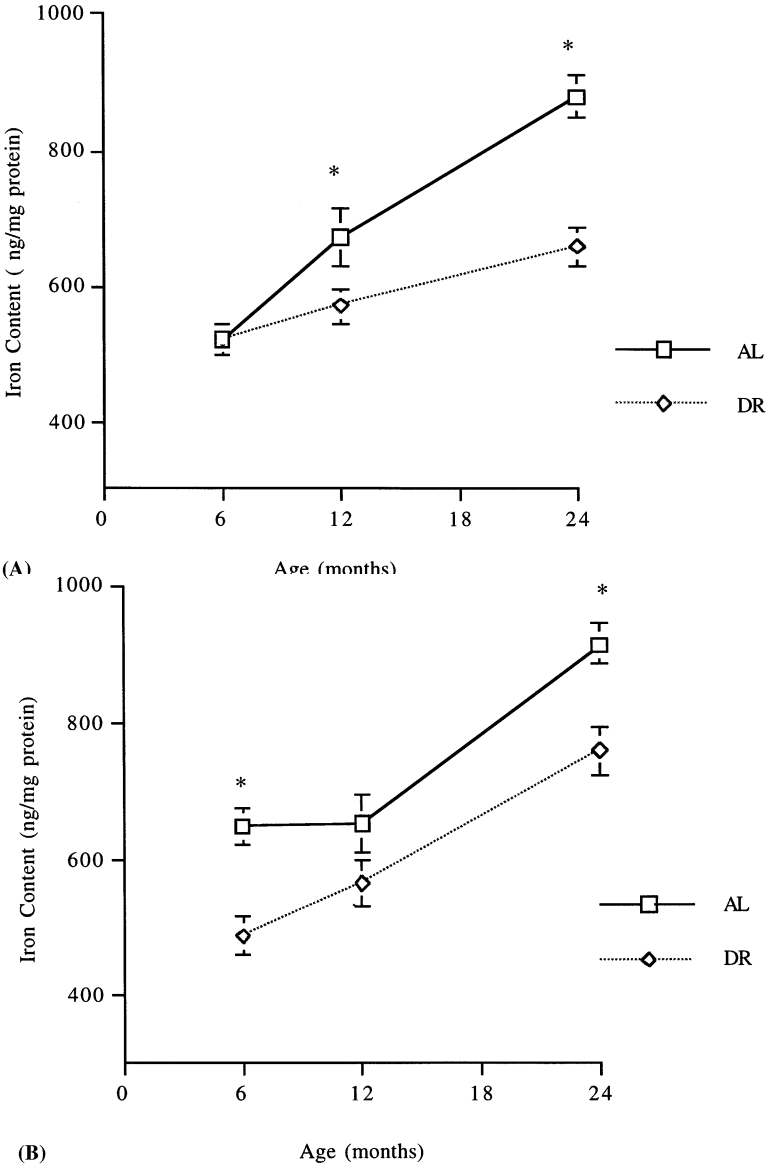
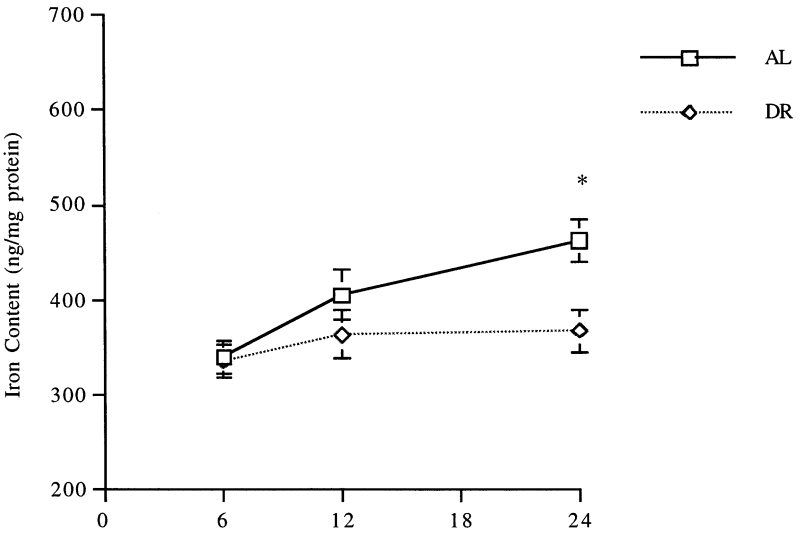


Fig. 1.



(C)

Fig. 1. (A) Tissue total iron content in kidney. The effects of age and diet on total iron content in kidney are shown. Total iron content is expressed in nanograms per milligram protein. Each datum is the mean ± S.E.M. of six samples. Iron measurements obtained from the AL rats are represented by squares and DR by diamonds. Asterisks represent significant ($P < 0.05$) diet-dependent differences between iron measurements. (B) Tissue total iron content in liver. The effects of age and diet on total iron content in liver are shown. Total iron content is expressed in nanograms per milligram protein. Each datum is the mean ± S.E.M. of six samples. Iron measurements obtained from the AL rats are represented by squares and DR by diamonds. Asterisks represent significant ($P < 0.05$) diet-dependent differences between iron measurements. (C) Tissue total iron content in brain. The effects of age and diet on total iron content in brain are shown. Total iron content is expressed in nanograms per milligram protein. Each datum is the mean ± S.E.M. of six samples. Iron measurements obtained from the AL rats are represented by squares and DR by diamonds. Asterisks represent significant ($P < 0.05$) diet-dependent differences between iron measurements.

3.2. Tissue total-iron content

Fig. 1A–C show significant ($P = 0.0001$) age-related iron accumulation in kidney and liver of both AL and DR rats. In brain, however, iron accumulation was significant ($P < 0.015$) only in tissue obtained from AL animals. Kidney iron content at 12 and 24 months was significantly ($P < 0.0173$) suppressed by DR. In addition, there was a significant ($P = 0.0025$) interaction between age and diet on kidney total iron content. Similarly, iron content in liver at 6 and 24 months, and brain at 24 months was significantly ($P < 0.0018$) suppressed by DR, although the age–diet interaction was not observed in either tissue.

3.3. Tissue lipid peroxidation

Fig. 2A–C show significant ($P < 0.05$) age-related increases in LPO in all tissues obtained from both AL and DR rats. In kidney obtained from 24-month-old rats, LPO was significantly ($P = 0.0001$) suppressed by DR. In addition, a significant

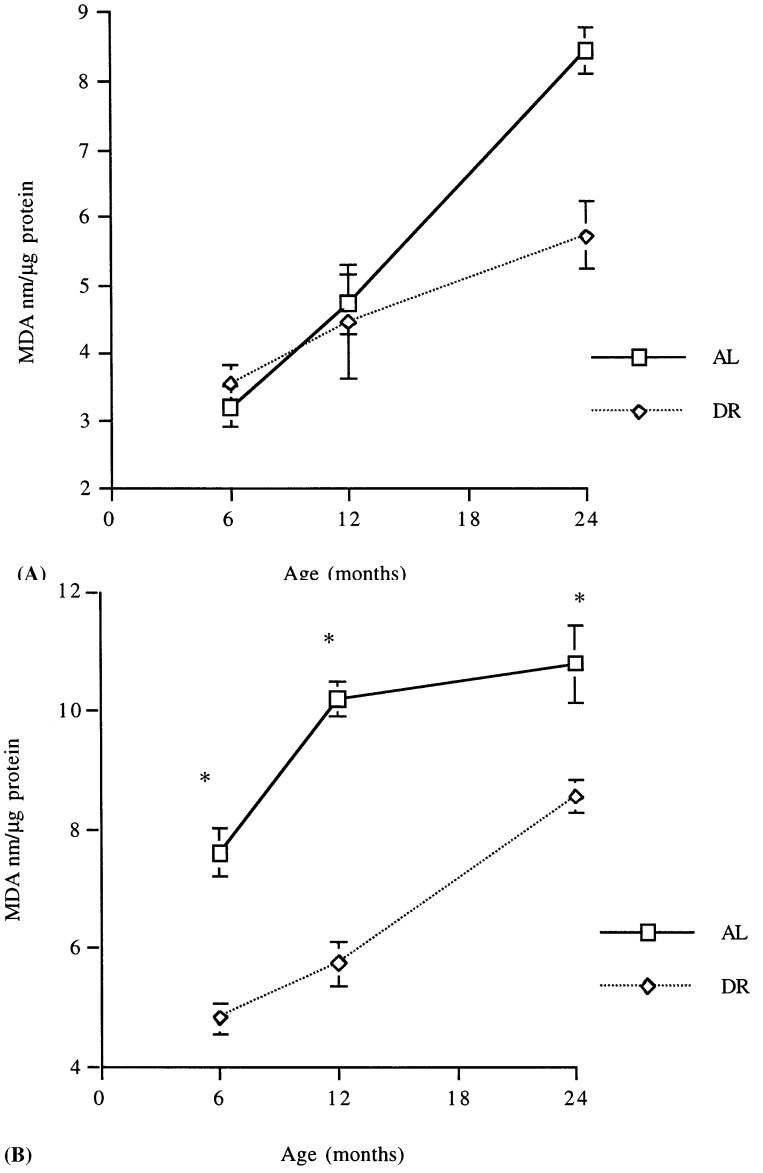


Fig. 2.

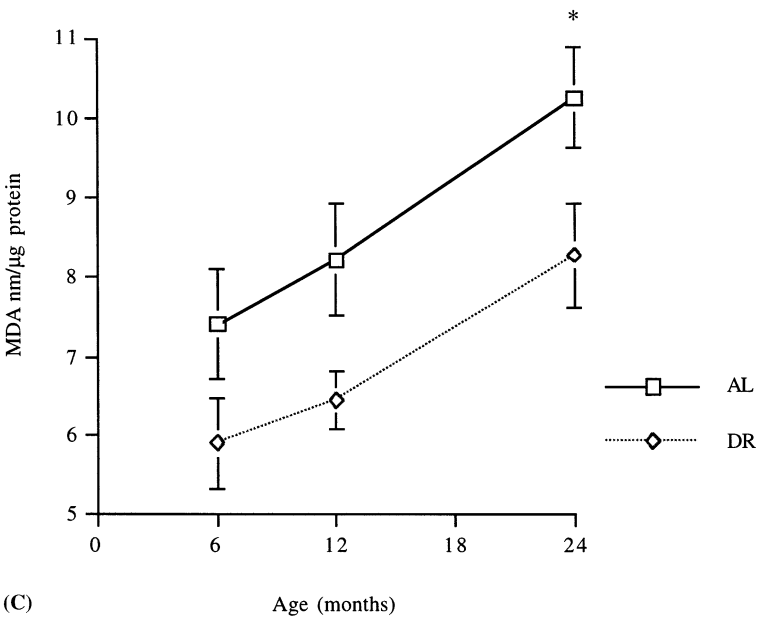


Fig. 2.(A) Tissue lipid peroxidation in kidney. The effects of age and diet on MDA content in are shown. MDA is expressed in nanomoles per microgram protein. Each datum is the mean ± S.E.M. of six samples. MDA measurements obtained from the AL rats are represented by squares and DR by diamonds. Asterisks represent significant ($P < 0.05$) diet-dependent differences between MDA measurements. (B) Tissue lipid peroxidation in liver. The effects of age and diet on MDA content in liver are shown. MDA is expressed in nanomoles per microgram protein. Each datum is the mean ± S.E.M. of six samples. MDA measurements obtained from the AL rats are represented by squares and DR by diamonds. Asterisks represent significant ($P < 0.05$) diet-dependent differences between MDA measurements. (C) Tissue lipid peroxidation in brain. The effects of age and diet on MDA content in brain are shown. MDA is expressed in nanomoles per microgram protein. Each datum is the mean ± S.E.M. of six samples. MDA measurements obtained from the AL rats are represented by squares and DR by diamonds. Asterisks represent significant ($P < 0.05$) diet-dependent differences between MDA measurements.

($P = 0.0005$) interaction between age and diet on LPO was observed. Similarly, LPO in liver samples was suppressed by DR in all age groups ($P < 0.0005$); and a significant ($P = 0.0261$) interaction between age and diet on liver LPO was observed. Furthermore, LPO in brain was also significantly suppressed by DR in tissue obtained from 24-month-old rats ($P = 0.0343$) but no age–diet interaction was observed.

3.4. Correlation analysis of LPO and iron content

Correlation analysis of LPO and iron content within each diet group (AL and DR) in kidney, liver and brain is illustrated in Table 1. Significant ($P < 0.0001$) correlated increases in iron content and LPO were observed in kidney and liver tissue obtained from AL and DR animals. However, a positive correlation between

iron content and LPO was not observed in brain tissue obtained from either AL or DR animals.

4. Discussion

Aging may be defined as gradual changes in cellular homeostasis that compromise an organism's ability to meet both internal and external challenges (Baker and Martin, 1994; Yu, 1996). Altered iron homeostasis is a recognized age-related phenomenon of micronutrient accumulation. Our work examines the relation between iron content and aging based on observations that iron accumulates with senescence, suggesting age-related changes in iron homeostasis (Baker and Martin, 1994). In 1985, Massie et al. (1985) reported that the rate of iron accumulation inversely correlates with the life span in some species; this was recognized as strong evidence that iron accumulation has an adverse effect on aging. Our laboratory further demonstrated that serum ferritin and total iron content, two indices of total-body iron status, increase in an age-related fashion in male Fischer 344 rat (Choi and Yu, 1994).

In our current research, prior to measuring tissue iron content, it was necessary to ascertain the possible blood contamination in tissue by analyzing hemoglobin (Hb) content of kidney, liver and brain preparations. It was observed that kidney preparations contained the least and liver the most ng Hb/mg protein, with no significant age-related trend observed in any of the three tissues analyzed. This data indicated that the contribution of Hb iron was less than 0.02% of the total iron measured.

The total iron content of kidney, liver and brain (forebrain) was found to significantly increase in an age-related fashion (Fig. 1), in accordance with previous reports on tissue iron in aging. Sharma (1977) reported total iron accumulation increases in liver and brain of rats with respect to age (0–240 days), which appeared to plateau in liver between 60 and 240 days of age, but with no marked brain iron content accumulations. The primary limitation of Sharma's study was that the age range analyzed failed to delineate between developmental events and aging. Massie et al. (1983), however, when analyzing organs from C57BL/6J male mice ranging from 45 to 900 days of age, observed an accumulation of iron in liver, kidney and brain. Although liver iron content increased by 216%, kidney by 54%, and brain by

Table 1
Correlations between iron content and MDA in tissues from AL and DR rats

Kidney		Liver		Brain	
AL	DR	AL	DR	AL	DR
0.8229	0.7491	0.3836	0.6990	0.2790	0.940

All values expressed are correlation coefficients.

27%, neither kidney nor liver showed a significant increase until after 355 days, once growth had been completed. After that time, liver iron increased by 140% and kidney iron by 44%, suggesting true age-related events. Evidence in support of age-related iron accumulation was recently reported by Ghio et al. (1997), who observed that non-heme (Fe^{3+}) increased in the lung of both humans and rats.

One of our current findings is the first analysis of age-related changes in tissue iron content in SPF Fischer 344 rats. It was determined that iron content increased in an age-related fashion by 69% in kidney, 41% in liver, and 37% in brain. Liver and brain showed the largest increase in iron content at 24 months, well after development.

The data from our iron analysis experiments is in agreement with others and indicates that iron accumulates with age in a tissue-specific fashion, which suggests age-related alterations in tissue iron homeostasis. These findings are important in relation to aging because oxidative stress is recognized as a principal component of the aging process and iron is a potent pro-oxidant and essential catalyst for free radical reactions.

LPO is commonly measured as an index of oxidative stress through the quantitation of malondialdehyde (MDA), one of the major byproducts of LPO. Although numerous measurements of LPO with respect to age have been reported, not all of these have been consistent. For example, Yoshikawa and Hirai (1967) observed an age-related increase in MDA in the brains of Wistar rats, yet Grinna and Barber (1973) found an age-related decrease in MDA in livers and kidneys of Sprague-Dawley rats. One of the major problems has been the non-specific nature of thiobarbituric acid (TBA) reaction with MDA. These problems have been reduced by recent developments of more appropriate and sensitive spectrophotometric techniques. Choi and Yu (1994) measured lipid peroxide content in serum via the modified TBA method of Yagi (1987) and reported an age-related increase in MDA. Our current research reports on the concurrent measurements of LPO and iron content in tissues with respect to age. In these experiments, a more specific technique based on the reaction of N-methyl-2-phenylindole with MDA was used (Janero, 1990). Significant age-related increases in MDA content were observed in kidney, liver and brain tissue preparations (Fig. 2), indicating that oxidative stress increases in these tissues with age.

Our findings demonstrate that both iron and LPO increase in tissues as a function of age. The potential pro-oxidative role of iron and the fact that no regulated means of iron excretion from the body has been identified (Lash and Saleem, 1995), suggest that age-related increases in iron accumulation and LPO may be more than coincidental. However, two questions remain to be addressed before suggesting a significant pro-oxidative role for iron in relation to the oxidative stress theory of aging. One is whether the anti-aging paradigm, DR, which has been hypothesized to function via an anti-oxidative mechanism, attenuates age-related iron accumulation. The other is whether there is a significant positive correlation between oxidative stress and iron in tissues. Choi and Yu (1994) explored these questions and reported that DR simultaneously attenuates both LPO and iron accumulation in serum suggesting that iron is an important factor in the aging process.

Our current research shows that DR suppresses LPO and iron content at 24 months of age in all three tissues studied (Figs. 1 and 2). The effect of diet on age-related iron and MDA content were more apparent in kidney and liver than in brain preparations (Figs. 1 and 2). Two-way analysis of variance (ANOVA) indicated a significant interaction between age and diet on both iron and MDA content in the kidney ($P < 0.005$; data not shown). Although this interaction was observed only in kidney, the results indicate that DR significantly slows the rate of MDA and iron accumulation in this tissue with advancing age. Generally, the greatest effect of DR on iron accumulation and LPO in all tissues studied was observed at 24 months of age (Figs. 1 and 2).

The mechanism by which DR suppresses oxidative stress is known to involve: (a) increased upregulation of free radical scavenger enzymes and antioxidants (i.e. catalase, superoxide-dismutase, glutathione, glutathione-reductase, glutathione-peroxidase and ascorbic acid); and (b) suppression of oxidant generation (i.e. $O_2^{\cdot -}$, $OH^{\cdot}$ and H_2O_2) (Yu et al., 1982, 1985; Fishbein, 1991; Davis et al., 1993; Djuric and Kritscheusky, 1993; Feuers et al., 1993; Choi and Yu, 1994; Yu, 1995). Iron is a potent pro-oxidant that accumulates with age; therefore, the modulation of iron content appears to be a significant part of the anti-oxidative mechanism of DR.

To further investigate the relation between age-related oxidative stress and iron content, correlation analysis of the dependent variable MDA versus iron was conducted for each tissue (Table 1). A relatively large positive correlation was observed between LPO and iron content in AL kidney, DR kidney and liver, but was absent in AL liver and brain and DR brain. Furthermore, multiple regression analysis revealed that iron accumulation and LPO increase independently of one another in the kidney and liver as a consequence of age. Tissue specificity probably accounts for the marked discrepancy between kidney, liver and brain correlation analysis.

This research demonstrates the ability of DR to attenuate both age-related iron accumulation and MDA content at the tissue level. Furthermore, these data are consistent with the proposal that DR functions via an anti-oxidative mechanism (Olanow, 1993; Baker and Martin, 1994). Future investigation of the relation between iron and oxidative stress in aging should include an analysis of free iron content and ferritin as a potential source of redox-active iron in tissues. Such analysis will further define the role of iron in age-related oxidative stress and the importance of iron modulation in the anti-oxidative mechanism of DR.

Acknowledgements

The authors would like to thank Drs Helen A. Bertrand, Jeremiah T. Herlihy, John Nishimura and Yuji Ikeno for their encouragement and assistance throughout the progress of this work. Part of this work was supported by the National Institute on Aging (Grant AG-0118).

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