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MODULATION OF AGE-RELATED ALTERATIONS OF IRON, FERRITIN, AND LIPID PEROXIDATION IN RAT SERUM

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ABSTRACT

It is generally accepted that oxidative stress increases with age, as indicated by age-related increases in lipid peroxidation. Also found in the aged organism is the accumulation of iron in various tissues. The increase in lipid peroxidation and tissue iron accumulation may not have been coincidental, but rather may be a causally related phenomenon, since iron involvement in lipid peroxidation is a well-accepted phenomenon in free radical metabolism. Our present study examined serum iron and ferritin as means to compare age-related changes in lipid peroxides and peroxidizability in aged rats. In addition, the anti-aging effect of dietary restriction on the modulation of iron status and lipid peroxidation was assessed. The results showed that the age-related increase in serum iron concurs with increased lipid peroxidation, and that these adverse changes were effectively attenuated by dietary restriction. Our finding is the first report on the modulation of iron status by long-term calorie restriction, and is consistent with the previous findings on the anti-peroxidative action of dietary restriction.

INTRODUCTION

Increased lipid peroxidation with age is well accepted as an index of age-related increases in oxidative stress, and gerontological research confirms that such age-related increases occur widely (1). Elevated peroxidation has been linked to various physiological abnormalities and to the pathogenesis of many age-related diseases (2-4). Increased lipid peroxidation is usually attributed to a) the vulnerability of lipid molecules to oxidative reactions, due to their unstable double bondings, and b) a wide distribution of lipid molecules throughout biological systems as plasma components and cell membrane constituents. In the aged organism, this increased oxidative stress coupled with weakened antioxidant defenses further exacerbates oxidative insults (5).

An additional factor responsible for acceleration of lipid peroxidation is the involvement of transition metals (2). Metal catalysis is known to enhance free radical reactions. Iron (Fe^{++} or Fe^{+++}), for instance, is essential for the conversion of H_2O_2 to more reactive hydroxyl radicals, as illustrated by the well known Haber-Weiss reaction. For lipid peroxidation, Fe catalyzes activation of oxygen molecules to form lipid hydroperoxides (2,6). Therefore, Fe is involved in the initiation and propagation of lipid peroxidation (7-9). Iron requirement in lipid peroxidation is also confirmed by in vivo evidence that in the absence of iron, little lipid peroxidation is detected (10).

A careful review of gerontological literature reveals a substantial disruption of iron homeostasis during aging, as indicated by accumulation of iron in aged organisms. Massie, who first reported iron homeostasis to be altered with aging, provided strong evidence that life spans of some species are inversely correlated with iron accumulation (11,12). These and other findings (13,14) underscore the importance of iron homeostasis in relation to lipid peroxidation in aging.

Our study examines iron status in relation to lipid peroxidation in aging rats. Furthermore, this study scrutinizes the anti-oxidative, free radical inhibitory effects of dietary restriction on the modulation of iron status in aging. This interesting finding could be another important mechanism by which dietary restriction may suppress oxidative stress, thereby slowing age-related physiological declines.

DISCUSSION

Iron, essential to cellular functions, becomes cytotoxic when its normal homeostasis is disrupted. Increased iron in blood and tissues due to aging (12) has been consistently observed in many species, including Drosophila, rodents (11), and humans (14,16). At present, the reason for this elevation is not fully understood. It is of interest, then, that our study also found both ferritin and total serum iron, the most reliable indicators of total body iron homeostasis (17), elevated during aging. Additionally, these elevated levels of iron are consistent with findings of increased lipid peroxidation. Our findings that total iron and ferritin concentrations in serum increase progressively with age in ad libitum fed rats are in accord with Massie's conclusions about iron accumulation (12).

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This type of increase in ferritin is a well-known response to increased iron load (18,19). Based on what is known about catalytic action of iron in free radical redox reactions, we propose that age-related increased lipid peroxidation may well be closely related to increased iron accumulation in tissues.

Data on iron modulation by dietary restriction appear to confirm this proposal. Our study marks the first time that age and dietary effects on iron status and lipid peroxidation, examined together, show that the modulation of iron by dietary restriction leads to age-related alterations in the total serum, and ferritin. Our data on rat serum lipid peroxide levels lend further supporting evidence to the susceptibility of lipids to oxidative stress. Our analysis of the peroxidizability index of serum phospholipids, as shown in Figure 3, also provides a biochemical explanation for increased peroxides, which probably involves iron catalysis.

Our data regarding modulation of iron status by dietary restriction are consistent with recently available information on the antioxidant properties of dietary restriction (20). These findings emphasize the importance of the regulation of iron status as part of an overall biological strategy by which to attenuate free radical induced oxidative stress. This possibility has already been suggested as a means by which to reduce oxidative insults of neurodegeneration in Parkinson's disease, in which high regional levels of iron accumulation occur in substantia nigral neurons (21,22).

While this study focuses on iron and iron-storing proteins, other transition metals such as copper and other metal chelating proteins should not be overlooked in the free radical-modulated aging process. For instance, a copper binding protein, ceruloplasmin, has also been suggested as a modulator of iron status (23). Similarly, many known metal-chelating proteins should be considered major antioxidant components and included in a list of essential cellular defense systems (24). To obtain a complete picture of the involvement of transition metals in age-related lipid peroxidation, much more intensified investigations are warranted.

RESULTS

The effects of age and diet on total serum iron are illustrated in Figure 1. In the ad libitum group, serum iron levels increased steadily with age until 18 months; no further increase was noted at 24 months of age. Dietary restriction significantly decreased total serum iron at all ages. Dietary-restricted rats exhibited no age-related increase in serum iron, and their total iron levels were significantly reduced from those of the ad libitum fed group at all ages studied.

Figure 2 shows the impact of age and diet on ferritin levels in serum. Serum ferritin levels of ad libitum fed rats are significantly higher than levels found in restricted rats at all ages. Unlike the profile seen with serum iron, however, the serum ferritin content of restricted rats increases with age.

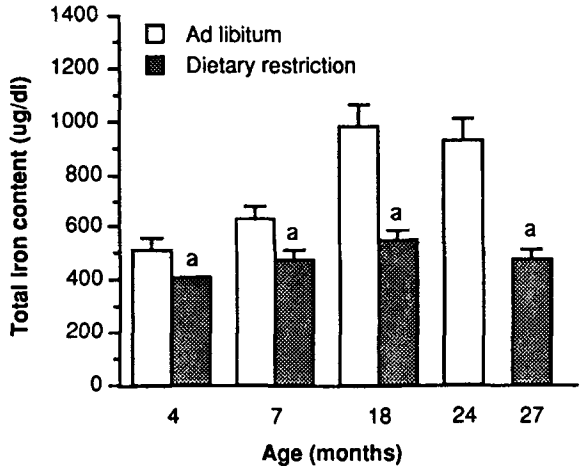

Figure 1. The Effect of Age and Dietary Restriction on the Total Iron Content in Serum.
a indicates significant difference ($p<0.001$) from ad libitum group. *b* $p<0.001$ compared with 4 months group. Maintained group shows no changes compared with 4 months group. $n=6$ in each group.

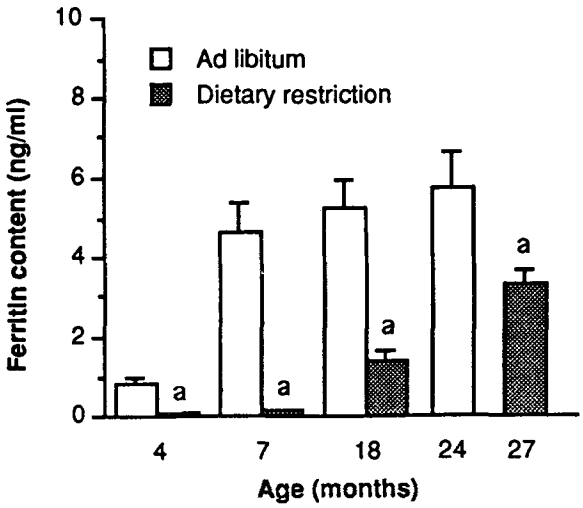

Figure 2. The Ferritin Levels in Serum in Ad Libitum Fed and Dietary Restricted Rats at Various Ages.
a indicates significant difference ($p<0.001$) from ad libitum group. *b* $p<0.001$ compared with 4 month age group and indicates age trend in dietary restricted groups. $n=6$ in each group.

Effects of age and diet on serum phospholipids may be assessed by analyzing the peroxidizability index, as shown in Figure 3, which measures the susceptibility of the serum lipid to peroxidation (15). Previous studies confirmed this correlation of membrane lipid hydroperoxide levels with age. In restricted rats, no changes are evident throughout life span. Inconsistent with this antioxidant effect of dietary restriction is the age-related increase in levels of lipid peroxides in serum, as shown in Figure 4. Dietary restriction suppressed lipid hydroperoxide at much lower levels throughout the life span.

Similarly, amounts of MDA, measured as TBAR substance, are elevated in serum of ad libitum fed rats, but

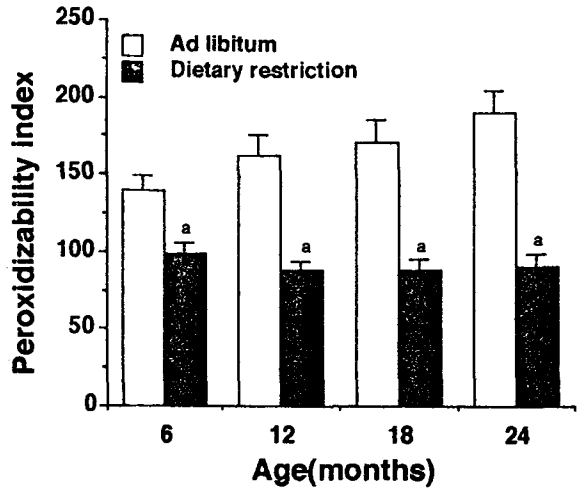

Figure 3. The Effect of Age and Dietary Restriction on Peroxidizability of Serum Phospholipids.
a indicates p<0.001 compared with 6 months group, but no change is indicated in dietary restricted groups. *n*=6 in each group.

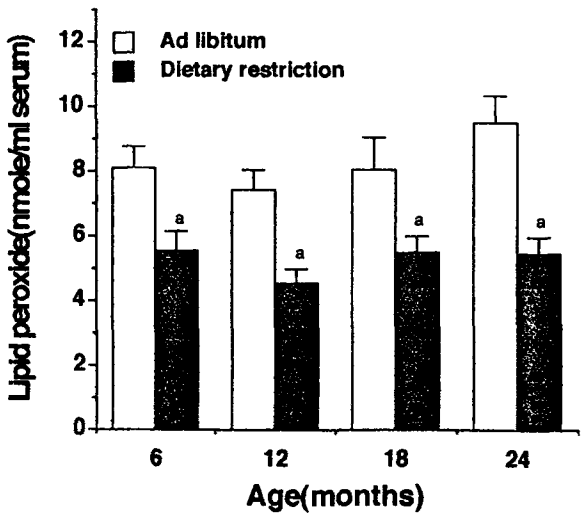

Figure 4. Attenuation of Age-Related Increase in Serum Lipid Peroxide by Dietary Restriction.
a indicates p<0.05 compared with 6 months age group, but increases significantly from 12 months of age. *n*=6 in each group.

were kept low throughout the life span through dietary restriction, as shown in Figure 5.

EXPERIMENTAL PROCEDURES

Animal and Dietary Intervention

Specific-pathogen free Fisher 344 male rats (Charles River Laboratory) were maintained in a barrier facility, housed singly in plastic cages with wire mesh floors, and fed a semi-synthetic diet (Ralston-Purina) (25). Dietary restriction (60% of ad libitum) began at 6 weeks of age, as previously described (25), and continued throughout life. Rats were sacrificed at various ages as shown in figures. Blood samples were collected from heart puncher under anesthetic conditions, with ether.

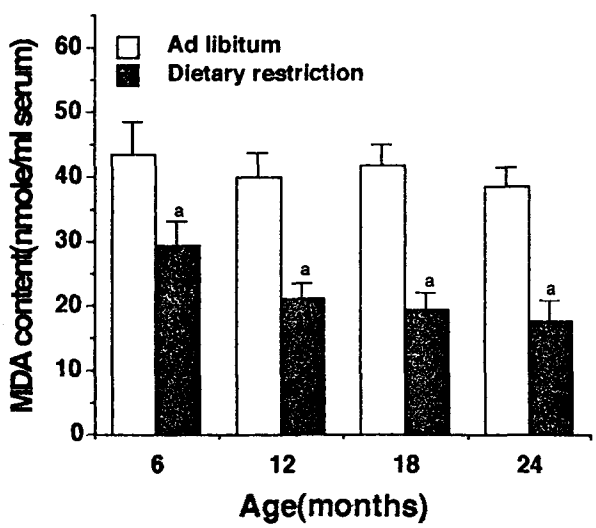

Figure 5. Modulation of Serum Lipid Peroxidation by Dietary Restriction. *a* indicates p<0.05 compared with 6 months group. *n*=6 in each group.

Determination of Serum Iron

Total non-heme iron content was determined by a modified method of Brumby and Massey (26). Briefly, 0.1 ml of serum was ashed with 0.1 ml of 60% perchloric acid in water bath (100°C) for 40 minutes, then 0.3 ml of hydrogen peroxide was added to these tubes dropwise until serum was colorless, and heated an additional 30 minutes to effect complete breakdown of hydrogen peroxide. Excess perchlorate was complexed with 0.10 ml of pyridine, 0.15 ml of distilled water, 0.15 ml of 0.1% 1,10-phenanthroline, 0.05 ml of 1.0% ascorbid acid, 0.02 ml of saturated ammonium acetae, and 0.03 ml of 12.6% ammonia which was added and the tubes centrifuged at 4,500 rpm for 15 minutes. Supernatant was carefully removed and read at 510 nm.

Radioimmunoassay of Ferritin

Ferritin in serum was determined by Ferritin ^{125}I Radioimmunoassay Kit purchased from Medical Products (DuPont Co.).

Fatty Acid Analysis for Determination of Peroxidizability Index

Serum lipids were extracted with chloroform/methanol (2:1, V/V), and transmethylation was done with Instant Methanolic-HCl Kit (Alltech/Applied Science, IL). Fatty acid esters were analyzed using heptadecanoic acid as an internal standard by gas-liquid chromatograph (Model 8420), Perkin-Elmer) with a silica capillary column (DB 225, J & W Scientific, CA), according to the method of Choi and Yu (27). Peroxidizability index of serum lipids (15) was based on the percentage of monoenoic, dienoic, trienoic, tetraenoic, pentaenoic, and hexaenoic acids, multiplied by 0.025, 1, 2, 4, 6, and 8, respectively, as described in Laganiere and Yu (28).

Determination of Lipid Peroxide Content

Lipid peroxide content in serum was determined by the method of Yagi (29). Four microliters of 1/12N H_2SO_4 and 0.5 ml of 10% phosphotungstic acid were added to 20 *ul* of serum, mixed, allowed to stand at room temperature for 10 min., and centrifuged at 800xg for 10 min. The sediment was suspended in 4.0 ml of distilled water, and 1.0 ml of 0.67% Thiobarbituric acid was added. The mixture was heated in a water bath (95°C) for 60 min. After cooling, 5.0 ml of n-butanol was added, shaken vigorously, and then centrifuged at 800xg for 15 min. The n-butanol layer was measured using emission and excitation wavelengths of 553 and 575, emission at 553 and excitation at 515 nm respectively. Lipid peroxide level (L_p) was expressed in tens of serum malondialdehyde as follows: serum L_p = 0.5 x f/F x 1.0/0.02 n mol/ml serum, where f refers to the measured fluorescence intensity, and F refers to the intensity of the standard, respectively (29).

Determination of Protein

Protein contents were determined by the method of Lowry et al. (30) with bovine serum albumin as a standard.

Statistics

Two-way analyses of variance were utilized to determine significant interaction of all age and dietary effects. Pair-wise comparisons were made using student's t-test to evaluate differences between groups.

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