Effect of Dietary Restriction on Age-Related Increase of Liver Susceptibility to Peroxidation in Rats

Tae Il Jeon[a], Beong Ou Lim[a,b], Byung Pal Yu[c], Yoongho Lim[a], Eun Ja Jeon[a], and Dong Ki Park[a,*]

[a]Department of Applied Biology and Chemistry, Konkuk University, Korea, [b]Graduate School of East-West Medical Science, Kyunghee University, Korea, and [c]Department of Physiology, The University of Texas Health Science Center, San Antonio, Texas

ABSTRACT: Dietary restriction (DR) increases life span and decreases age-related diseases in experimental animals. It has received a great deal of attention in connection with the relationship between aging, nutrition, and oxidative stress because oxidative injury in several organ systems is a prominent feature in aging. We investigated the possibility that DR can protect vulnerable liver lipids against age-related increases of peroxidation. Male Fischer 344 rats fed *ad libitum* (AL) or dietarily restricted (maintained on 60% of AL food intake) were killed by decapitation at 4 (young) or 12 mon (adult) of age. Phosphatidylcholine hydroperoxide (PCOOH) concentration of liver was determined using a chemiluminescent high-performance liquid chromatographic method. Liver PCOOH increased with age in adult rats, but less of an increase of PCOOH was seen in DR rats, which is consistent with results on production of thiobarbituric acid-reactive substances and oxygen-derived free radicals. No significant differences were found in liver superoxide dismutase and catalase activity between AL and DR groups of young and adult rats. Liver triglyceride and cholesterol contents were lower in DR than AL rats at 12 mon. Fatty acid compositions of phosphatidylcholine and phosphatidylethanolamine indicated that the ratio of (20:3n-6 + 20:4n-6)/18:2n-6, an index of linoleic acid (18:2n-6) desaturation, was lower in DR than in AL rats. We concluded that DR suppresses age-related oxidative damage in liver by modulating the amount of lipid as well as fatty acid composition.

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Aging can be characterized as an accumulation of deleterious changes that increase the risk of death. These changes can be attributed to both genetic and environmental factors (1), and they inevitably compromise an organism's ability to meet both internal and external challenges. Oxidative stress is causally related to irreversible damage due to endogenously generated free radicals (2,3). Under normal conditions, cells of aerobic organisms utilize reactive oxygen species (ROS) as physiological messengers. The redox balance is altered by the age-related decline in an organism's ability to counteract oxidative damage (4,5).

Reducing the caloric intake of laboratory animals while maintaining nutrition can increase their lifespans (6–8). Dietary restriction (DR) delays age-associated pathological and physiological changes and extends longevity. DR postpones the accumulation of damaging effects that accompany *ad libitum* (AL) food intake. One major mechanism by which DR retards the aging process is its remarkable ability to reduce oxidative damage (5,9). For example, Kim *et al*. (10) showed that DR decreases the malondialdehyde content of cardiac mitochondria, indicating a decrease in lipid peroxidation. DR feeding regimens enhanced the organism's ability to attenuate levels of harmful reactive free radicals in various organs (11,12). Oxidative stress-mediated injury is causally related to aging since ROS-caused damage accumulates with age (13,14). Although several previous studies reported a relationship between aging and lipid peroxidation, most examined thiobarbituric acid-reactive substance (TBARS) levels to indicate peroxidative status.

In the present study, we determined phospholipid hydroperoxide (PLOOH), a sensitive key indicator for oxidative injury, because phospholipids (PL) are important structural and functional components of the biological system (15,16), and are commonly recognized as a major target of lipid peroxidation. The technique we used to determine PLOOH was a chemiluminescence–high-performance liquid chromatography (CL-HPLC) method. Previous reports from this laboratory described a simple and continuous one-step flow injection system based on cytochrome c-amplified chemiluminescence for the assay of radical scavenging activity (17,18). The present study attempts to quantify age-associated changes of the lipid profile and the degree of attenuation on oxidative damage in DR.

The liver is an important metabolic organ, and is susceptible to a wide variety of disorders, possibly because it is constantly exposed to potentially harmful agents. It is generally recognized that oxidative end-products accumulate with age and therefore free radical-mediated damage to liver cells occurs. Thus, the liver was selected as a model organ for this study in recognition of the significant health benefits of DR.

*To whom correspondence should be addressed at Department of Applied Biology and Chemistry, Konkuk University, 1 Hwayang-dong, Kwangjin-gu, Seoul, 143-701, Korea. E-mail: parks@konkuk.ac.kr

Abbreviations: AL, *ad libitum*; CL-HPLC, chemiluminescence-high-performance liquid chromatography; DR, dietary restriction; FI-CL, flow injection-chemiluminescence; FID, flame-ionization detector; PC, phosphatidylcholine; PCOOH, phosphatidylcholine hydroperoxide; PE, phosphatidylethanolamine; PL, phospholipid; PLOOH, phospholipid hydroperoxide; PUFA, polyunsaturated fatty acids; ROS, reactive oxygen species; SOD, superoxide dismutase; SPF, specific-pathogen-free; TBARS, thiobarbituric acid-reactive substances; TC, total cholesterol; TG, triglyceride.

EXPERIMENTAL PROCEDURES

Animals. Specific-pathogen-free (SPF) male Fisher 344 rats were housed in plastic cages at the University of Texas Health Science Center with approval of the Institutional Animal Care and Utilization Committee. The SPF status of shipments of rats was verified and maintained as described by Yu *et al*. (19). Dietary restriction (60% of *ad libitum* fed) was begun at 6 wk of age as described by Yu *et al*. (20), and continued throughout life. Rats were weighed biweekly, and were killed by decapitation at 4 or 12 mon of age. The required tissues were then removed.

Antioxidant activities measurement by the flow injection (FI)-CL system. To determine changes in antioxidant activity in liver CL intensity was measured with a filter-equipped photon counting-type spectrophotometer (CLD-110; Tohoku Electronic Industry, Miyagi, Japan) connected to a pump (model 303; Gilson Medical Electronics S.A., Villiers-le-Bel, France) and a sample injection valve (model 7125; Rheodyne, Cotati, CA). The mobile phase was 50 mM phosphate buffer (pH 7.4) containing 50% methanol (for solvent-soluble samples), cytochrome c (10 mg/L), and luminol (2 mg/L). The flow rate was maintained at 1.0 mL/min with the pump. For the purpose of measuring the abilities of radical scavengers, a mixture of 0.06% H_2O_2 (5 μL) and sample solution (5 μL) was injected. The reduced CL intensity of the mixture compared to the CL intensity of 0.06% H_2O_2 (5 μL) enables analysis of radical-scavenging activity (17).

Phosphatidylcholine hydroperoxides (PCOOH) measurement. Total lipids were extracted from the liver by the method of Folch *et al*. (21). The CL-HPLC procedure for quantification of PCOOH concentration followed the method of Miyazawa *et al*. (15). Standard PCOOH was prepared by oxidation of PC using a method of Terao *et al*. (22).

TBARS measurements. TBARS were quantified as described by Buege and Aust (23) using 1,1,3,3,-tetraethoxypropane as a standard material (Sigma, St. Louis, MO).

Antioxidant enzyme activities measurements. Liver catalase activity was determined spectrophotometrically by observing the decomposition of hydrogen peroxide at 240 nm. One catalase unit is defined as the amount of enzyme required to decompose 1.0 μmol H_2O_2/min at pH 7.0 at 25°C (24,25). Total superoxide dismutase (SOD) activity was determined by monitoring the inhibition of reduction cytochrome c at 550 nm using xanthine and xanthine oxidase system. One SOD unit was defined as the amount of enzyme that inhibited reduction of cytochrome c by 50% (26).

Analysis of lipids. Liver total lipids were extracted and purified by the method of Folch *et al*. (21). PL, total cholesterol (TC), and triglyceride (TG) were measured as described by Gu *et al*. (27). PC and phosphatidylethanolamine (PE) were separated by thin-layer chromatography with chloroform/ethanol/water/triethylamine (30:35:6:35, by vol) as the developing solvent (28). The fatty acid composition of PC and PE were analyzed by gas–liquid chromatography (Varian, Palo Alto, CA) in an Omegawax 320 capillary column (30 m × 0.32 mm i.d., 0.25 μm film, Supelco, Bellefonte, PA) equipped with a flame-ionization detector (FID) after direct transmethylation with 14% BF_3 in methanol at 70°C (28). Chromatographic conditions were as follows: column temperature 200°C; carrier gas N_2 30 mL/min; FID temperature 260°C; injector temperature 250°C. Fatty acid methyl esters were identified by comparing their retention times with those of standard methyl esters (Supelco).

Statistics. Differences between the means of the individual groups were assessed by one-way analysis of variance with Duncan's multiple range test (SPSS version 7.5, SPSS Institute, Chicago, IL). Differences of $P < 0.05$ were considered to be significant (29).

RESULTS

DR decreased body weight of each group. DR rats had body weights lower than those of AL rats by 59.7 and 55.7% at 4 and 12 mon, respectively.

To determine the effect of DR on lipid peroxidation, PCOOH (as a representative PLOOH), TBARS production, and active oxygen-derived radical levels were examined in AL or DR rats at 4 and 12 mon. The liver peroxidation indices increased with age in AL rats. PCOOH and TBARS concentrations in livers of AL rats were 3 and 1.4 times higher in the 12-mon group than in the 4-mon group (top and middle panels, Fig. 1). Consistent with these results, the radical activity evaluated by FI-CL assay was significantly higher in the 12-mon group than in the 4-mon group ($P < 0.05$; bottom panel, Fig. 1). However, DR was associated with much less peroxidation in the 12-mon rats. Concentrations of PCOOH or TBARS in adult rat livers were 50 and 76% less, respectively, in the DR rats as compared to those of AL rats (top and middle panels, Fig. 1).

No significant differences were found in catalase and SOD activity between AL and DR groups (Fig. 2). These results demonstrate that lipid peroxidation products increase with age, while DR attenuates the extent of free radical damage.

We further analyzed lipid contents in each group as shown in Figure 3. The TC and TG concentrations of AL rats increased with age of rats (top and middle panels, Fig. 3). The TC and TG concentrations were significantly lower in the DR group than the AL group at 12 mon ($P < 0.05$). No significant difference was found in the PL concentration among all groups (bottom panels, Fig. 3).

The constituent fatty acids in PC and PE are indicated in Table 1. At 4 mon, PC showed an increasing trend of 18:2n-6 (linoleic acid). At 12 mon, a decreasing trend of 20:4n-6 (arachidonic acid) in DR rats was evident when compared to that of AL rats. Consequently, the ratio of (20:3n-6 + 20:4n-6)/18:2n-6, an index of linoleic acid desaturation, was lower in the DR group, and the proportion of 22:5n-6 in the DR group at 12 mon also decreased. A similar, but lesser degree of difference was observed in PE and there was a nonsignificant trend toward decreasing 22:6n-3 in the DR rats.

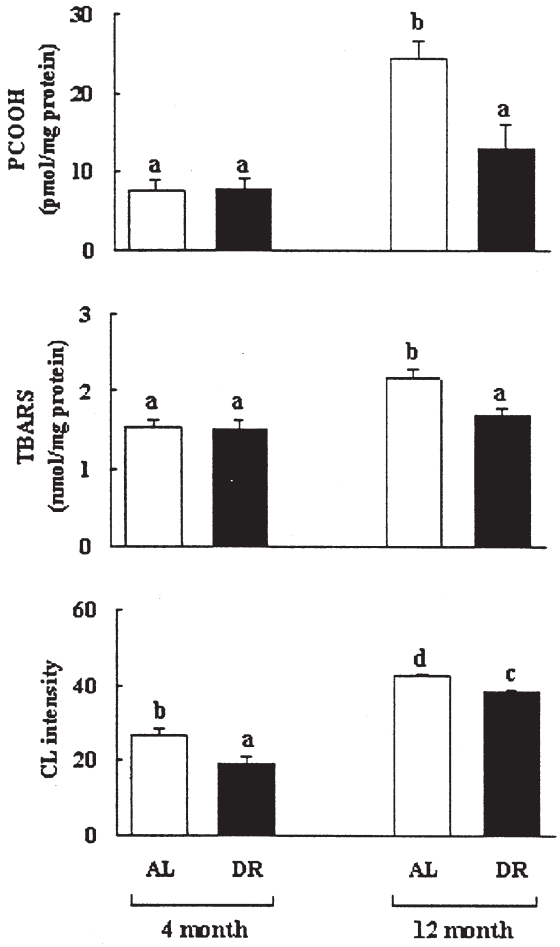

FIG. 1. Effects of dietary restriction (DR) on the amount of phosphatidylcholine hydroperoxide (PCOOH; top panel), thiobarbituric acid-reactive substances (TBARS; middle panel), and oxygen-derived radical activity (bottom panel) in the liver of rats between 4 and 12 mon. Each bar represents the mean ± SEM of five rats. Mean values with different superscripts are significantly different ($P < 0.05$). CL, chemiluminescent; AL, *ad libitum*-fed rats.

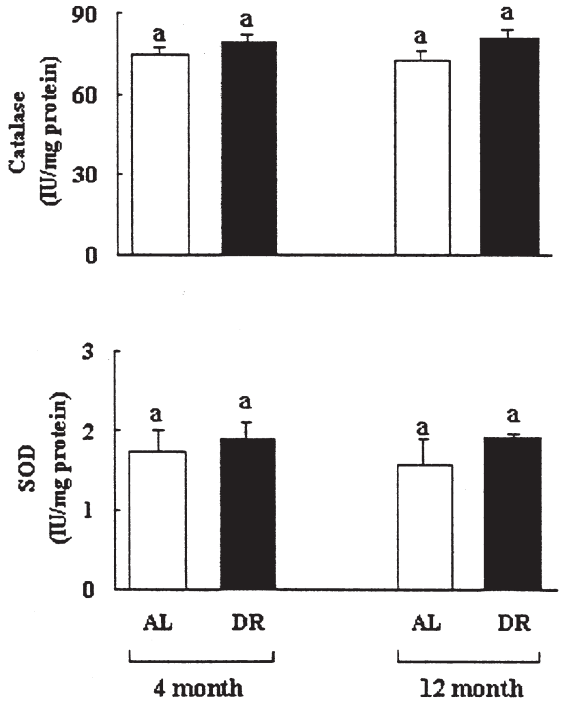

FIG. 2. Effects of DR on the cytosolic catalase activity (top panel) and superoxide dismutase (SOD) activity (bottom panel) in the liver of rats between 4 and 12 mon. Each bar represents the mean ± SEM of five rats. Mean values with different superscripts are significantly different ($P < 0.05$). For other abbreviations see Figure 1.

DISCUSSION

In this study we demonstrated that indices of hepatic oxidative stress increased with age and were less prominent in DR rats. The index of linoleic acid desaturation and unsaturation degree of fatty acids in PC and PE was significantly reduced by DR. A number of investigators have previously examined the effect of DR with age on lipid peroxide concentrations, mostly using TBARS (13,30). They suggested that fatty acid unsaturation is a main factor in determining the sensitivity to lipid peroxidation. To evaluate the effect of DR on oxidative stress, we measured PCOOH as a sensitive marker of oxidative liver injury because of PC's vulnerability to peroxidation (31).

PLOOH was shown to increase significantly with age in AL rats (top panel, Fig. 1). Moreover, polyunsaturated fatty acid (PUFA) contents of 20:4n-6, 22:5n-6, and 22:6n-3 increased in 12-mon AL rats (Table 1) over those present at 4 mon (Table 2), implying an age-related increase of PUFA susceptibility to peroxidation. However, major PUFA amounts were markedly lowered by DR in adult rats, with lower TBARS and PCOOH levels than in AL rats.

The present study found that catalase and SOD activities were not significantly different between DR and AL rats (Fig. 2). Catalase and SOD activities at basal levels were not statistically different between the young and the adult groups. Interestingly, DR led to significant changes in cholesterol and TG levels, indicating that DR primarily modulates the reduction of neutral lipids, not PL (Fig. 3).

To further explore the notion that tissue lipid vulnerability is a crucial factor contributing to cellular oxidative status, constituent fatty acids in PC and PE were analyzed. The trends in fatty acid composition to increase toward peroxidizable PUFA with age are evident (Table 1). Among the most obvious modifications by DR have been the compositional changes related to membrane lipid composition, specifically the age-related membrane fatty acid composition (32). It has been proposed that the modulation of the fatty acid profile by DR results in lower age-related oxidative stress as a possible adaptive strategy (9,11,14). In our study, 18:2n-6 and 18:3n-3 increased in PE of DR rats, whereas the content of PUFA derivatives (20:4n-6, 22:5n-6, and 22:6n-3) is decreased (Table 2).

We conclude that DR protects liver against age-related increases in oxidative stress *in vivo* by modifying lipids and their composition to reduce peroxidizable substrates such as TG and PUFA. Our study strongly supports earlier data

TABLE 1
Effect of Dietary Restriction (DR) Compared with *ad Libitum* (AL) Feeding on the Fatty Acid Composition of Phosphatidylcholine (PC) and Phosphatidylethanolamine (PE) in the Liver of Rats Between 4 and 12 mon[a]

Fatty acids	PC				PE			
	4 mon		12 mon		4 mon		12 mon	
(weight %)	AL	DR	AL	DR	AL	DR	AL	DR
14:0	0.2 ± 0.0[a]	0.2 ± 0.0[a]	0.1 ± 0.0[a]	0.1 ± 0.0[a]	0.2 ± 0.0[a]	0.3 ± 0.1[a]	0.6 ± 0.4[b]	0.2 ± 0.1[a]
16:0	15.4 ± 0.4[b]	16.3 ± 0.9[b]	13.2 ± 0.4[a]	13.2 ± 1.1[a]	14.7 ± 0.6[a]	17.6 ± 1.4[b]	12.3 ± 1.6[a]	13.2 ± 0.9[a]
16:1	0.9 ± 0.0[c]	1.1 ± 0.1[c]	0.5 ± 0.0[b]	0.3 ± 0.0[a]	0.9 ± 0.1[b]	0.9 ± 0.2[b]	0.5 ± 0.2[a]	0.4 ± 0.1[a]
18:0	25.1 ± 0.8[a]	24.4 ± 1.5[a]	27.6 ± 5.1[b]	24.2 ± 2.1[a]	22.7 ± 0.5[a]	23.0 ± 1.0[a]	25.1 ± 1.4[b]	22.7 ± 0.7[a]
18:1n-9	2.7 ± 0.1[b]	3.6 ± 0.3[c]	2.2 ± 0.1[a]	2.6 ± 0.2[b]	3.9 ± 0.3[b]	5.1 ± 1.0[c]	2.3 ± 0.8[a]	3.9 ± 0.6[b]
18:2n-6	11.3 ± 0.9[b]	15.2 ± 0.7[c]	8.8 ± 0.3[a]	11.1 ± 0.9[b]	8.7 ± 0.9[b]	9.1 ± 0.9[b]	5.1 ± 0.7[a]	14.3 ± 0.8[c]
18:3n-3	0.3 ± 0.0[a]	0.4 ± 0.0[a]	0.2 ± 0.0[a]	0.3 ± 0.0[a]	0.2 ± 0.0[a]	0.3 ± 0.1[a]	0.2 ± 0.0[a]	0.2 ± 0.0[a]
20:3n-6	0.7 ± 0.1[a]	1.2 ± 0.1[b]	0.5 ± 0.0[a]	0.4 ± 0.1[a]	0.5 ± 0.1[b]	0.6 ± 0.1[b]	0.3 ± 0.1[a]	0.5 ± 0.0[b]
20:4n-6	31.8 ± 0.6[b]	28.1 ± 1.0[a]	34.0 ± 0.3[c]	30.7 ± 2.7[b]	29.2 ± 0.9[b]	29.2 ± 1.6[b]	25.2 ± 2.8[a]	27.1 ± 1.1[a,b]
22:4n-6	0.9 ± 0.0[a,b]	0.8 ± 0.1[a]	1.0 ± 0.1[b]	1.0 ± 0.1[b]	2.0 ± 0.1[b]	1.5 ± 0.1[a]	2.4 ± 0.1[c]	2.3 ± 0.1[c]
22:5n-6	2.3 ± 0.3[b]	1.4 ± 0.1[a]	4.3 ± 0.3[c]	2.3 ± 0.4[b]	4.7 ± 0.5[b]	2.5 ± 0.2[a]	9.1 ± 0.9[c]	4.2 ± 0.6[b]
22:5n-3	0.5 ± 0.0[a]	0.7 ± 0.1[b]	0.4 ± 0.0[a]	0.6 ± 0.1[a,b]	1.1 ± 0.1[b]	1.1 ± 0.1[b]	0.7 ± 0.1[a]	1.2 ± 0.1[b]
22:6n-3	3.0 ± 0.1[a]	2.9 ± 0.3[a]	3.3 ± 0.1[a]	2.6 ± 0.3[a]	7.2 ± 0.4[b]	5.8 ± 0.5[a]	7.9 ± 1.0[b]	5.4 ± 0.3[a]
20:3+20:4								
18:2	3.0 ± 0.3[c]	1.9 ± 0.1[a]	3.9 ± 0.1[d]	2.8 ± 0.1[b]	3.7 ± 0.5[b]	3.5 ± 0.5[b]	5.1 ± 0.8[c]	2.0 ± 0.2[a]

[a]Values represent the mean ± SEM of five rats. Values in row with a different roman superscript are significantly different ($P < 0.01$).

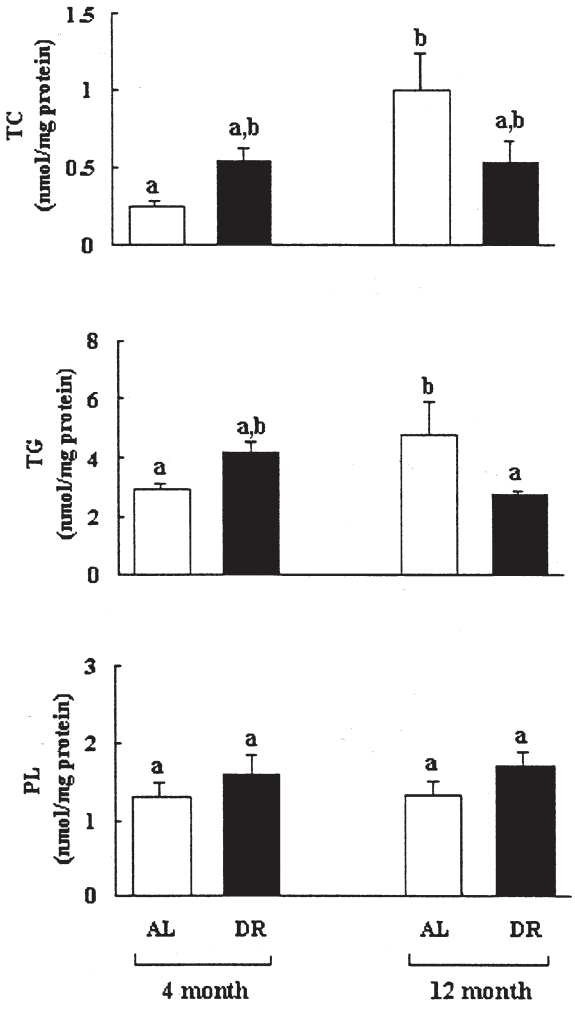

FIG. 3. Effects of DR on the concentrations of total cholesterol (TC; top panel), triglyceride (TG; middle panel), and phospholipid (PL; bottom panel) in the liver of rats between 4 and 12 mon. Each bar represents the mean ± SEM of five rats. Mean values with different superscript are significantly different ($P < 0.05$).

(7,11,14,33) that DR is a potent for antioxidative strategy to alleviate liver oxidative injury, thereby slowing down the progression of aging.

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