High-fat Diet Increases Phospholipid Peroxidation in the Liver of Mature Fischer 344 Rats

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Abstract: Phospholipid peroxidation is considered to be involved in the pathophysiology of various diseases. While dietary antioxidants are believed to help prevent these diseases via inhibition of phospholipid peroxidation, further evaluation is needed to prove this hypothesis. For this, it is crucial to establish an animal model with accelerated phospholipid peroxidation. In this study, we hypothesized that a combination of aging and high-fat diet feeding may accelerate phospholipid peroxidation *in vivo*. High-fat diets were fed to mature and juvenile Fischer 344 rats for 12 weeks. The mature rats in particular accumulated body fat and liver phosphatidylcholine hydroperoxide (PCOOH). Interestingly, the increase in PCOOH levels was abrogated by the co-administration of antioxidants to mature rats. This may be attributed to factors including the decrease in body fat, functions of vitamin E, and/or the involvement of antioxidant-related genes, each caused by antioxidant administration. These results indicate that the high-fat diet-fed aging animal model may be suitable for investigation of the relationship between phospholipid peroxidation, oxidative stress-related diseases, and dietary antioxidants.

Key words: antioxidant, liver, oxidative stress, phosphatidylcholine hydroperoxide, rat

1 INTRODUCTION

Phospholipid peroxidation is considered to be involved in the pathophysiology of human diseases such as arteriosclerosis, diabetes, and dementia¹⁻⁵. While dietary antioxidants (e.g., vitamin E, carotenoids, vitamin C and polyphenols) are believed to help prevent these diseases via inhibition of phospholipid peroxidation⁶⁻⁸, further evaluation is needed to prove this oxidation and antioxidation hypothesis. To this end, the establishment of an animal model with accelerated phospholipid peroxidation will be highly valuable.

With the exception of administering certain chemicals such as carbon tetrachloride^{7,9}, aging and high-fat diet feeding are the most plausible ways to accelerate phospholipid peroxidation *in vivo*. Studies indicate that phospholipid peroxidation [e.g., phosphatidylcholine hydroperoxide (PCOOH) formation] increases in the liver of mature rats¹⁰, and in rats receiving high-fat diet⁸. However, other studies also suggest that the individual effects of aging or high fat

diet feeding are limited and/or occasionally vary among animals, and that it takes a significant amount of time (e.g., over 7-18 month¹⁰) to amplify oxidative stress. We therefore hypothesized that the combination of aging and high-fat diet feeding may provide a promising way to elevate and accelerate phospholipid peroxidation *in vivo*. To the best of our knowledge, very few studies have examined the effect of the combination of aging and high-fat diet feeding on PCOOH formation.

In this study, to evaluate this hypothesis, we fed high-fat diets to mature (20 weeks old) and juvenile (control; 6 weeks old) Fischer 344 rats. As expected, liver PCOOH levels increased significantly after 12 weeks of high-fat diet feeding. Since we employed Fischer 344 rats (known to rapidly accumulate body fat vis-à-vis other rat strains¹¹) and high-linoleic safflower oil [enhances the levels of easily oxidizable fatty acids (polyunsaturated fatty acids) in tissues¹²], therefore, we speculated that these factors, along with aging and high-fat diet feeding, may further ac-

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celerate PCOOH formation. Furthermore, the increase in PCOOH levels was abrogated by the co-administration of dietary antioxidants. Therefore, such an animal model with high tissue PCOOH levels will become valuable to study the relationship between phospholipid peroxidation, oxidative stress-related diseases, and dietary antioxidants.

2 EXPERIMENTAL

2.1 Animals and diets

Male 6 weeks old Fisher 344/Jcl rats, and 20 weeks old Fisher 344, retired male breeders were obtained from CLEA Japan, Inc. (Tokyo, Japan). In this study, we defined 6 weeks old rats as juvenile (control) and 20 weeks old rats as mature. Rats were housed individually in stainless steel wire mesh cages at a constant room temperature of $24 \pm 1^\circ\text{C}$ with a 12 h light-dark cycle (light period: 8:00-20:00) and acclimated to the animal housing with ad libitum commercial laboratory chow (CE-2; CLEA Japan Inc.).

After 1 week of acclimation, mature ($n=5$) and juvenile ($n=8$) rats were fed with 20 wt% high-fat diet (modified AIN-93G diet supplemented with high-linoleic safflower oil, Table 1) for 12 weeks. Mature ($n=5$) and juvenile ($n=8$) rats were also fed with 20 wt% high-fat diet supplemented with 1 wt% commercial dietary antioxidant mix (0.14 wt% vitamin E, 0.67 wt% fish oil, 0.018 wt% tomato polyphenol, 0.018 wt% green tea polyphenol, 0.018 wt% grape polyphenol, 0.018 wt% cacao polyphenol, 0.018 wt% blueberry polyphenol, 0.037 wt% black soybean polyphenol; Yubera zeitaku polyphenol Kiwami from Eisai Co., Tokyo,

Japan). The major vitamin E components were 90.7 wt% α -tocopherol (Toc), 4.4 wt% γ -tocotrienol (T3), and 3.4 wt% α -T3. Fish oil included 28 wt% eicosapentaenoic acid (EPA) and 14 wt% docosahexaenoic acid (DHA). During the experimental period, rats had free access to the experimental diet and distilled water. Body weights and food consumption were measured every other day. The experiments were conducted according to Regulations for Animal Experiments and Related Activities at Tohoku University and the protocol was approved by the institutional Animal Care and Use Committee of Tohoku University (2013-AgA-009).

2.2 Sampling of liver, plasma, and other tissues

After feeding experimental diets, the rats were fasted for 12 h and euthanized by decapitation under isoflurane inhalation anesthesia. Tissues including liver and fat were collected and weighed. A small part of the liver (~ 100 mg) was immediately immersed in RNAlater[®] solution (Life Technologies, Carlsbad, CA, USA) overnight, and was subsequently stored at -30°C until PCR analysis. The remaining liver for biochemical experiments was perfused with cold saline, snap frozen in liquid N_2 , and stored at -80°C until analysis. Blood was collected in heparinized tubes, and plasma was obtained from the blood by centrifugation (4°C , 1000 g, 20 min) and stored at -80°C .

2.3 Liver PCOOH analysis

Liver PCOOH was measured by chemiluminescence detection-high-performance liquid chromatography (CL-HPLC)¹³. Briefly, liver total lipids (TL) were extracted from liver homogenate by the Folch method¹⁴, with 0.002% butylated hydroxytoluene (BHT). Liver TL were applied on a Sep-Pak Vac, 100 mg 1 cc NH_2 cartridge (Waters Corporation, MA, USA)¹⁵. The phospholipid (PL) fraction was eluted, and then subjected to CL-HPLC equipped with a Finepak SIL- NH_2 -5 column (4.6×250 mm; JASCO Corporation, Tokyo, Japan) and a chemiluminescence detector (CLA-FL2; Tohoku Electronic Industries; Miyagi, Japan). The mobile phase was 2-propanol/methanol/water (135:45:20) at a flow rate of 1.0 mL/min. The post column chemiluminescence reagent for the specific detection of lipid hydroperoxides was a mixture of luminol and cytochrome *c* in 50 mM borate buffer (pH 10.0) at a flow rate of 1.5 mL/min. The concentration of PCOOH was calculated using an external standard prepared by a method previously reported by us^{15, 16}.

2.4 Measurement of liver TBARS, vitamin E, and other parameters

Liver thiobarbituric acid reactive substances (TBARS) were obtained from TL via reaction with thiobarbituric acid in the presence of BHT and sodium dodecyl sulfate. The TBARS contents were determined by HPLC-fluorescence

Table 1 Dietary compositions of the experimental diets.

	HFD	HFD+Antiox
Casein	20.00	20.00
L-Cystine	0.30	0.30
Cornstarch	26.75	25.75
α -Cornstarch	13.20	13.20
Sucrose	10.00	10.00
High-linoleic safflower oil	20.00	20.00
Cellulose	5.00	5.00
AIN-93G mineral mix	3.50	3.50
AIN-93 vitamin mix	1.00	1.00
Choline bitartrate	0.25	0.25
t-Butylhydroquinone	0.0014	0.0014
Antioxidant mix*	—	1.00
	(g/100 g diet)	

* Compositional details of the commercial dietary antioxidant mix are described in the main article.

HFD, high fat diet; Antiox, antioxidant mix.

detection¹⁷⁾. Liver vitamin E homologues, namely, α -, β -, γ -, and δ -Toc and α -, β -, γ -, and δ -T3, were determined by HPLC-MS/MS¹⁸⁾. Liver PL was determined as phosphorus in the TL by the modified Bartlett method¹⁹⁾. Triglyceride (TG) and total cholesterol (T-Cho) were measured using commercially available kits (Wako, Osaka, Japan). Plasma TL, TG, T-Cho, PL and nonesterified fatty acid (NEFA) were measured by the LSI Medience Corporation (Tokyo, Japan).

2.5 Analysis of mRNA levels

The mRNA levels of genes involved in the antioxidant system [*Glutathione peroxidase1 (Gpx1)*, *Glutathione peroxidase2 (Gpx2)*, *catalase (Cat)*, *Mn-Superoxide dismutase (Mn-Sod)* and *Cu/Zn-Superoxide dismutase (Cu/Zn-Sod)*] were analyzed by real-time quantitative RT-PCR. Total mRNA was extracted from liver with a commercially available kit (Qiagen, Hilden, Germany) and converted to cDNA by Ready-To-Go™ T-Primed First-Standard Kit (GE Healthcare, Piscataway, NJ, USA). Real-time PCR was performed on the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, CA, USA) and SYBR Premix Ex Taq II (Takara, Shiga, Japan). All primer sequences used in this study have previously been reported^{8, 20–23)}.

2.6 Statistical analysis

Results are expressed as mean $\pm$ standard deviation (SD). All statistical processing of the data was carried out by two-way factorial (diet $\times$ age) analysis of variance (two-way ANOVA). For factors where interaction was confirmed by two-way ANOVA, the Bonferroni test was further performed for multiple comparison. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp. in Armonk, NY).

3 RESULTS AND DISCUSSION

3.1 Food consumption, body weight, and tissue profile

As described in the Introduction, aging and high-fat diet feeding are two individual factors known to accelerate phospholipid peroxidation *in vivo*^{8, 10)}. However, their effect may be limited and takes a long time for oxidative stress to accumulate^{8, 10)}. In this study, we fed high-fat diets to mature (20 weeks old) and juvenile (control; 6 weeks old) Fischer 344 rats to evaluate whether the combination of aging and high-fat diet feeding will elevate as well as accelerate phospholipid peroxidation *in vivo*.

After 12 weeks of high-fat diet feeding to mature and juvenile rats, no difference was found in total food consumption between the two groups (Table 2), thus food efficiency was lower in mature rats than in juvenile rats. Mature rats that received high-fat diet showed greater body, liver, and mesenteric fat weights than juvenile rats. Mature rats also demonstrated higher concentrations of lipids (TL, TG and T-Cho) in the liver but not in plasma (Table 3).

Fischer 344 rats, compared with SD rats, are known to have lower food consumption and body weights but higher body fat percentage, and this tendency can be increased by feeding high-fat diets¹¹⁾. Previous studies indicate that high-fat diet feeding increases body weight^{24, 25)}, body fat weight^{8, 25, 26)}, and liver lipids levels^{8, 27)} compared to control diet feeding in Fischer 344 rats. McDonald *et al.* fed high-fat diets to 5 months old and 2 years old Fischer 344 rats, and reported higher fat accumulation in older rats²⁵⁾. These previous findings^{8, 11, 24–27)}, along with our results in the present study (Tables 2 and 3), indicate that the combination of aging and high-fat diet feeding can indeed enhance obesity of Fischer 344 rats in a rather short time.

Table 2 Body weights, total caloric food consumptions, and tissue weights in rats fed with high fat diets (HFD) or HFD supplemented with an antioxidant mix (HFD+Antiox) for 12 weeks.

	Juvenile		Mature		age	diet	age $\times$ diet
	HFD	HFD+Antiox	HFD	HFD+Antiox			
Initial body weight (g)	140 $\pm$ 5	141 $\pm$ 4	323 $\pm$ 3	328 $\pm$ 25	***	ns	ns
Final body weight (g)	358 $\pm$ 20	349 $\pm$ 26	394 $\pm$ 10	401 $\pm$ 19	***	ns	ns
Body weight increase (g)	218 $\pm$ 19	207 $\pm$ 25	70.6 $\pm$ 12.2	73.0 $\pm$ 8.4	***	ns	ns
Total food consumption (g)	1096 $\pm$ 48	1037 $\pm$ 72	1082 $\pm$ 27	1090 $\pm$ 241	ns	ns	ns
Feed efficiency (g/100 kcal)	4.31 $\pm$ 0.20	4.32 $\pm$ 0.24	1.41 $\pm$ 0.22	1.45 $\pm$ 0.17	***	ns	ns
Liver (mg/g body weight)	23.7 $\pm$ 0.9	24.4 $\pm$ 1.4	23.9 $\pm$ 0.6	26.0 $\pm$ 0.6	*	**	ns
Epididymal fat (mg/g body weight)	37.3 $\pm$ 3.1	35.2 $\pm$ 3.5	33.9 $\pm$ 3.3	34.3 $\pm$ 2.2	ns	***	ns
Perirenal-retroperitoneal fat (mg/g body weight)	38.6 $\pm$ 1.7	34.5 $\pm$ 1.4	37.7 $\pm$ 2.5	33.4 $\pm$ 2.7	ns	***	ns
Mesenteric fat (mg/g body weight)	26.2 $\pm$ 2.5	23.6 $\pm$ 3.2	30.9 $\pm$ 4.7	28.8 $\pm$ 3.2	***	ns	ns

Values are expressed as mean $\pm$ SD (juvenile; n=8, mature; n=5), * : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$

Table 3 Plasma and liver lipid contents of rats fed with high fat diets (HFD) or HFD supplemented with an antioxidant mix (HFD+Antiox) for 12 weeks.

	Juvenile		Mature		age	diet	age × diet
	HFD	HFD+Antiox	HFD	HFD+Antiox			
Plasma							
TL (mg/dL)	488 ± 111	366 ± 37	467 ± 38	410 ± 41	ns	***	ns
TG (mg/dL)	185 ± 65	120 ± 19	183 ± 23	154 ± 24	ns	**	ns
T-Cho (mg/dL)	96 ± 15	80 ± 7	92 ± 8	84 ± 5	ns	**	ns
PL (mg/dL)	159 ± 26	126 ± 13	147 ± 10	130 ± 10	ns	**	ns
NEFA (mEq/L)	1.13 ± 0.32	1.03 ± 0.18	1.14 ± 0.10	0.85 ± 0.13	ns	*	ns
Liver							
TL (mg/g)	63.9 ± 8.2	91.8 ± 22.3	88.7 ± 13.4	127.4 ± 15.4	***	***	ns
TG (mg/g)	22.6 ± 2.7	52.8 ± 19.6	49.6 ± 16.1	85.5 ± 17.4	***	***	ns
TC (mg/g)	2.12 ± 0.35	4.23 ± 2.02	4.44 ± 2.05	7.22 ± 1.27	***	***	ns
PL (mg/g)	29.1 ± 6.3	30.7 ± 6.1	34.7 ± 10.6	32.7 ± 7.2	ns	ns	ns

Values are expressed as mean ± SD (juvenile; n = 8, mature; n = 5), * : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$

Fig. 1 Liver PCOOH (A) and TBARS (B) of mature or juvenile rats fed with experimental diets for 12 weeks. TBARS was expressed as MDA contents. Closed square; high fat diet (HFD), Open square; high fat diet supplemented with antioxidant mix (HFD + Antiox). Data are shown as means ± SD, Juvenile (n = 8), Mature (n = 5).

3.2 Liver phospholipid peroxidation and antioxidant parameters

As it was confirmed that obesity was enhanced in a short time with the combination of aging and high-fat diet feeding (Tables 2 and 3), we next determined whether peroxidation of phospholipids was accelerated in the Fischer 344 rats. To this end, PCOOH was measured as a reliable phospholipid peroxidation marker^{28, 29)}. We found that after 12 weeks of high-fat diet feeding, mature rats demonstrated higher PCOOH levels in the liver (Fig. 1), and thereby, it was confirmed that phospholipid peroxida-

tion can be accelerated under the conditions used in this study, even without the administration of chemicals such as carbon tetrachloride. The PCOOH level in mature rats was approximately one and a half times higher than that in the juvenile group; this increase in PCOOH was almost equivalent to that found in the plasma PCOOH of hyperlipidemic patients²⁾ and diabetic patients⁵⁾.

Additionally, to identify why phospholipid peroxidation was accelerated in the liver, we examined the correlation between PCOOH and various parameters. We found a significant correlation between mesenteric fat weight and

Fig. 2 The correlation between liver PCOOH and mesenteric fat. Data are shown as means $\pm$ SD, Juvenile ($n=8$), Mature ($n=5$).

PCOOH ($R=0.517$, $p=0.007$) (Fig. 2). Compared with other fat tissues, mesenteric fat is more likely to develop inflammation³⁰. Therefore, the accumulation of mesenteric fat that was observed in this study may have induced oxidative stress from inflammation (e.g., generation of inflammatory reactive oxygen species such as singlet oxygen), leading to the increase in liver PCOOH levels. It should also be noted that high-linoleic safflower oil, which is known to increase the levels of polyunsaturated fatty acids in tissues¹², was used as the dietary fat source in this study. Further study is necessary to evaluate the effect of dietary fat source (high-linoleic safflower oil) on PCOOH accumulation *in vivo*.

In contrast to that of PCOOH, levels of TBARS (a classi-

cal lipid peroxidation marker³¹) did not significantly differ between mature and juvenile rat groups (Fig. 1). This contradiction may be attributed to the difference in the substances that were measured. The TBARS method is based on the measurement of malondialdehyde (MDA), which forms as a result of the degradation of lipid hydroperoxides. TBA reacts with MDA along with other various carbonyl compounds, resulting in a rather low specificity³². On the other hand, as the CL-HPLC method directly detects PCOOH^{28, 29}, the method is more sensitive and suitable to determine the levels of liver phospholipid peroxidation. With regard to liver antioxidant (vitamin E) levels (Fig. 3) and mRNA levels of crucial antioxidant-related genes (*Gpx1*, *Gpx2*, *Cat*, *Mn-Sod* and *Cu/Zn-Sod*), these also did not differ between mature and juvenile rat groups (Table 4).

Based on these results, it was concluded that the combination of aging and high-fat diet feeding certainly increased PCOOH levels in the liver of mature Fischer 344 rats in a short period of time. The increase in liver PCOOH levels is presumably due to the mesenteric fat-induced oxidative stress from inflammation, rather than changes in the antioxidative factors (vitamin E and antioxidant-related genes) in the liver.

3.3 Co-administration of dietary antioxidants

The results mentioned above confirm, at least in part, that we were able to establish an animal model with accelerated phospholipid peroxidation. To validate its usability, we investigated the effect of antioxidant administration in the model. Co-administration of dietary antioxidants ameliorated PCOOH levels, but not those of TBARS in the liver

Fig. 3 Total Toc (A) and T3 (B) of liver of mature or juvenile rats fed with experimental diets for 12 weeks. Closed square; high fat diet (HFD), Open square; high fat diet supplemented with antioxidant mix (HFD+Antiox). Data are shown as means $\pm$ SD, Juvenile ($n=8$), Mature ($n=5$). Different letters denote statistical difference ($p<0.05$).

Table 4 Liver mRNA expression levels of antioxidant-related genes in rats fed with high fat diets (HFD) or HFD supplemented with an antioxidant mix (HFD+Antiox) for 12 weeks.

	Juvenile		Mature		age	diet	age × diet
	HFD	HFD+Antiox	HFD	HFD+Antiox			
<i>Gpx1</i>	1.00 ± 0.85	0.562 ± 0.261	0.690 ± 0.330	1.33 ± 0.91	ns	ns	ns
<i>Gpx2</i>	1.00 ± 0.79 ^a	0.805 ± 0.473 ^a	0.888 ± 0.692 ^a	2.63 ± 2.10 ^b	*	*	*
<i>Cat</i>	1.00 ± 0.47	0.548 ± 0.286	0.898 ± 0.700	1.12 ± 1.05	ns	ns	ns
<i>Mn-Sod</i>	1.00 ± 0.65	0.793 ± 0.369	0.906 ± 0.441	1.62 ± 0.90	ns	ns	ns
<i>Cu/Zn-Sod</i>	1.00 ± 0.86 ^a	1.00 ± 0.53 ^a	1.06 ± 0.38 ^a	3.41 ± 2.18 ^b	*	*	*

Values are expressed as mean ± SD (juvenile; n=8, mature; n=5), * : $p < 0.05$. Different letters denote statistical difference ($p < 0.05$).

of the mature rats. Similar response was noted in juvenile rats, although to a lesser extent (Fig. 1). The commercial dietary antioxidants used in this study contained several polyphenols and n-3 unsaturated fatty acids, which are known to suppress fat accumulation^{33, 34}. Since antioxidant supplementation decreased epididymal and perirenal-retroperitoneal fat weight as well as plasma TL, TG, T-Chol, PL and NEFA levels (Tables 2 and 3), it was speculated that the intake of antioxidants ameliorated the high-fat diet-induced body fat accumulation, thereby preventing obesity-induced phospholipid peroxidation. As an exception, liver TL, TG and T-Chol levels significantly increased upon antioxidant supplementation. The elevation of liver lipid levels has previously been reported in rats fed with high-linoleic acid diets along with vitamin E to an extent similar to that in this experiment^{35, 36}. This suggests that excess intake of vitamin E, high-fat diet, and linoleic acid may lead to the accumulation of liver lipids. Although the mechanism is still unclear, further evaluation is necessary.

Another possible factor in the reduction in PCOOH levels after antioxidant administration is liver vitamin E, a well-known membrane antioxidant against phospholipid peroxidation. It was observed that liver Toc content decreased in the antioxidant treated mature rats in comparison with the antioxidant treated juvenile rats (Fig. 3), this suggests that mature rats utilized Toc to reduce oxidative stress in the liver, thus leading to the decrease in PCOOH levels.

Additionally, high mRNA levels of crucial antioxidant-related genes were observed in the antioxidant treated mature rats (Table 4), which may have also contributed to the decrease in PCOOH. This finding corroborates that of a previous study where plant-derived polyphenols present in dietary antioxidants were shown to induce elevation of the gene levels of antioxidative enzymes³⁷. However, unvarying levels of antioxidative enzymes in the juvenile group post antioxidant treatment was contradictory. It is speculated that oxidative stress level (PCOOH levels) in juvenile group might be insufficient to elicit the upregulation of antioxidant-related genes.

Notably, we focused on PCOOH as the main lipid peroxi-

dation marker in this study. On the basis of the above results, aging and high-fat diet feeding may also affect levels of other phospholipid hydroperoxides, including phosphatidylethanolamine hydroperoxide, as well as cholesterol hydroperoxide and triglyceride hydroperoxide. Since such other lipid peroxides are of interest, we plan to investigate them in the future.

To conclude, it was found that the increased liver PCOOH levels induced by the combination of aging and high-fat diet feeding were suppressed by the co-administration of a commercial antioxidant mix. Mechanistically, this antioxidant-mediated suppression may be attributed to factors such as decrease in body fat, vitamin E function, and/or upregulation of antioxidant-related genes. Moreover, an animal model with high tissue PCOOH levels can be derived from the rearing conditions described in this study. This may be a suitable model to study the relationship between phospholipid peroxidation, oxidative stress-related diseases, and dietary antioxidants. While we focused on the effect of antioxidants on liver PCOOH levels in this study, further research is needed to validate the effectiveness of this model. Ongoing detailed analysis in our group is focused on phospholipid peroxidation in other tissues and plasma, as well as on the influence of antioxidant dosage on the levels of phospholipid peroxidation.

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Conflict of interest

None of the authors have any conflicts of interest.

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