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Fish Oil Enriched Diet-Induced *in vivo* Lipid Peroxidation and Increased Excretion of Urinary Lipophilic Lipid Metabolites *in Rats*

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ABSTRACT

Peroxidative stimuli mediated by high polyunsaturated fatty acid administration in rats induced *in vivo* lipid peroxidation and resulted in increased urinary excretion of a number of lipophilic aldehydes and related carbonyl compounds. These secondary lipid peroxidation products, measured as 2,4-dinitrophenylhydrazine derivatives, were detected and identified by the newly developed HPLC method. The identified urinary lipophilic nonpolar aldehydes and related carbonyl compounds were butanal, butan-2-one, pentan-2-one, hexanal, hex-2-enal, hepta-2,4-dienal, hept-2-enal, octanal, and oct-2-enal. Lipophilic polar aldehydes such as 4-hydroxyhex-2-enal and 4-hydroxyoct-2-enal were also identified. A polyunsaturated fatty acid diet containing n-3 fatty acids generally caused high levels of urinary excretion of lipophilic aldehydes and related carbonyl compounds in rats than a normal diet. Significantly increased secondary lipid peroxidation products were hexanal, hepta-2,4-dienal, octanal, 4-hydroxyhex-2-enal, 4-hydroxyoct-2-enal, and a number of unidentified compounds.

KEY WORDS lipid peroxidation, urinary lipophilic aldehydes, urinary carbonyl compounds, fish oil diet.

INTRODUCTION

Major constituents of all biological membranes are phospholipids with unsaturated fatty acid side chains, making them potential targets for oxygen radical attack.[1] Polyunsaturated fatty acids (PUFA) rich in cell membranes are readily attacked by oxidizing radicals. Oxidation of even a small portion of PUFA in the phospholipid bilayer can induce changes in membrane permeability, transmembrane ionic gradients, lipid fluidity, and lipid-protein interactions.[2] Therefore, free radical-induced peroxidative damage to membrane lipids has been regarded as a critical step leading to oxidative cell injury.[3]

It is known that increasing the content of PUFA in cells and tissues by dietary manipulation readily can modify the extent of cellular oxidative damage, and enhance the amount of endogeneous peroxidized lipids.[4] Thus, dietary lipids were suggested to induce extensive modification in the fatty acid composition of cell membranes, which in turn affects various cellular functions.[5] It has been reported so far that diets rich in ω-3 PUFA (eicosapentaenoic acid: $C_{20:5}$ and docosahexaenoic acid: $C_{22:6}$), present in fish oil, are recommended for treatment and prevention of atherosclerosis and heart disease.[6,7] However, the substitution of membrane fatty acids with unstable ω-3 PUFA generally potentiated the susceptibility of cellular membranes and tissues to oxidative stress and lipid peroxidation.[8,9] The oxidative destruction of these labile PUFA, known as lipid peroxidation, is particularly damaging because it proceeds as autocatalytic chain reaction and then leads to the formation of lipid hydroperoxides, lipid radical intermediates, and other secondary peroxidation products.

The decomposition of lipid hydroperoxides in biological systems is an inevitable event associated with the production of a great variety of carbonyl compounds. In addition to the hydrophilic malondialdehydes (MDA), lipid peroxidation also leads to the formation of a wide range of other hydrophilic and lipophilic aldehydic compounds. The lipophilic aldehydes such as alkanals, alkenals, alkadienals, and hydroxyalkenals have mainly been studied *in vitro* production.[10,11] Very few *in vivo* studies have been done on lipophilic aldehydes, and these mostly focus on studies of 4-hydroxynon-2-enal (HNE).[12,13]

The low molecular weight lipophilic aldehydes have been proposed to be preferentially accumulated near or within peroxidizing biomembranes, therefore can attack essential cell components.[14] On the other hand, the hydrophilic MDA does not remain in the membrane and only exhibits toxic effects at a relatively high concentration.[15] Several studies have presented evidence that the

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lipophilic aldehydes are capable of damaging functional proteins, inhibiting enzyme activities, and causing cell lysis and loss of cellular reproductive integrity.[16][17] Therefore, the diffusible aldehydes have been hypothesized to act as "secondary toxic messengers"[16] for free radical events. Pathological conditions such as atherosclerosis, liver injury, diabetes, cancer, ischemia, inflammation, aging of cells and tissues as well as other conditions have been suggested as outcomes associated with lipid peroxidation and the cytotoxic action of lipophilic aldehydes.[18]

In the previous study using the newly developed non-invasive method,[19] analysis of normal rat and human urine samples revealed the presence of several lipophilic aldehydes and related carbonyl compounds of lipid peroxidation. Our finding indicates that lipid peroxidation is a continuous physiological process and that a permanent low state of oxidative stress exists in normal rats and humans. Therefore, the detection of high levels of lipophilic aldehydes and related carbonyl compounds in urine of pro-oxidant-intoxicated animals could represent clear evidence for the occurrence of lipid peroxidation *in vivo*. The objective of this study was to evaluate the effects of the dietary pro-oxidant stimuli on *in vivo* lipid peroxidation in rats by measuring urinary lipophilic aldehydes and related carbonyl compounds. Lipid peroxidation was induced by the high PUFA diet supplement. Urinary lipophilic aldehydes and related carbonyl compounds were measured as 2,4-dinitrophenylhydrazine derivatives by the HPLC method developed by Kim *et al.*.[19]

MATERIALS AND METHODS

1. Animals and diets

Sprague-Dawley female weanling rats were divided at random into two groups of eight to nine animals: the normal group and the high PUFA group. The normal group fed a normal diet containing 8% corn oil and the PUFA group fed a PUFA diet containing 3% cod liver oil and 5% corn oil for nineteen weeks. The composition of the diets is shown briefly in Table 1. The food was changed with fresh food every day to be prevented from being oxidized.

2. Urine collection

At the end of dietary treatments, the animals were individually held in stainless steel metabolic cages and were fasted 48 hours. The urine samples were collected during the second 24 hours of fasting. Urine were stored at −70 ℃ until analysis.

Table 1. Composition of normal and PUFA diets

Ingredients	% Composition	
	Normal	PUFA
Anhydrous d (+)-dextrose[1]	65 8	65.8
Vitamin-free casein[2]	20.0	20.0
Salt mixture[2]	4.0	4.0
Vitamin mixture[2][3]	2.2	2.2
Vacuum distilled corn oil[4]	8.0	5.0
Cod liver oil[4]	–	3.0
RRR-tocopheryl acetate[5]	0.003	0.003

1) United States Biochemical Corp., Cleveland, OH
2) Salt mixture 4179 and vitamin mixture ICN Biochemicals, Aurora, OH
3) Composition of special vitamin diet fortification mix without vitamin E (g/kg mixture) Vatamin A acetate (500,000 I.U./g), 1.80, Vitamin D cleiferol (850,000 I.U./g); Inositol 5 0, Choline chloride, 75.0, Menadione, 2.250, Biotin, 0.020, p-Aminobenzoic acid, 5.0, Ascorbic acid; 45.0, Niacin; 4.250, Riboflavin; 1 0, Pyridoxine hydrochloride, 1.0, Thiamin hydrochloride, 1.0, Calcium pantothenate; 3 0, Folic acid, 0. 090, Vitamin B; 0.00135, Dextrose; 855.46365
4) United States Biochemical Corp., Cleveland, OH.
5) ICN Biochemicals, Aurora, OH.

3. Measurement of urinary lipophilic aldehydes and related carbonyl compounds

The new method developed by Kim *et al.*[19] was used for urine analysis and a brief description of the method is as follows. First, ultrafiltration of urine was performed by an Amicon cell equipped with a YC05 Diaflo Ultrafilter (Amicon Corp., Beverly, MA) to remove compounds with molecular masses larger than 500 daltons. Urine was derivatized with 2,4-dinitrophenylhydrazine (DNPH), extracted with dichloromethane, and applied to a silica gel thin layer chromatographic plate (Silica gel 60, Aluminum Backed 20 cm × 20 cm, 0.2mm thickness, Alltech Ass. Inc., Deerfield, IL). Nonpolar (NPC) and polar (PC) carbonyl compounds separated from the plates using dichloromethane were eluted with methanol. Further separation of lipophilic aldehydes and carbonyl compounds were achieved by high performance liquid chromatography (HPLC) on a Ultrasphere ODS C_{18} reverse phase column (25 cm × 4.6 mm i.d., 5 μm particle size, Altex, Berkeley, CA) using two different solvent systems.[28] NPC and PC were eluted by 75% methanol in water and 50% methanol in water, respectively. Identification of DNPH derivatives of urinary lipophilic aldehydes and carbonyl compounds was accomplished by cochromatography with pure standards in three different solvent systems.[28]

4. Analysis of urinary creatinine

To determine urinary lipophilic aldehydes and related

carbonyl compounds based on the urinary creatinine concentrations, urinary creatinine was analyzed by the modified Gradwhol's method.[20] Duplicates of 0.02 ml of 15 urine samples were added to the screw top test tubes. 0. 38 ml of HPLC grade water, 3.2 ml of 0.42 N H_2SO_4, and 0.4 ml of 5% sodium tungstate were added to series of tubes. After the mixture was vortexted and centrifugated for 10 min. at 2000 rpm, 1.6 ml of alkaline picrate reagent was added to the mixture, with 30 sec. interval between each sample. Alkaline picrate reagent was prepared by combining 5 vol. of 1% picric acid with 1 vol. of 10% NaOH. After exactly 16 min., the absorbance of alkaline picrate solution was measured at 520 nm on a Spectronic 20 spectrophotometer. The absorbance was used to calculate the amount of urinary creatinine. Various concentrations of creatinine standard solution were subjected to the same creatinine test procedure, and a standard curve was prepared. The amount of urinary creatinine(mg/ml urine) was calculated from the standard curve.

5. Statistical analysis

Data was presented as means ± SEM. Student's t-test was conducted for comparison of mean values between the normal group and the PUFA group. Statistical significance was considered at $p < 0.05$ for all analysis.

RESULTS

A number of lipophilic nonpolar and polar aldehydes and related carbonyl compounds were separated and identified by HPLC in the urine of rats fed a normal diet or a PUFA diet. The presence of the urinary lipophilic lipid peroxidation metabolites were detected as 2,4-DNPH derivatives. Typical HPLC separations of urinary lipophilic nonpolar and polar aldehydes and related carbonyl compounds from rats in the PUFA group are presented in Fig. 1 and 2, respectively. Nine compounds out of fourteen NPC were identified by using cochromatography in three different solvent systems. These are butanal, butan-2-one, pentan-2-one, hexanal, hex-2-enal, hept-2-enal, hepta-2,4-dienal, octanal, and oct-2-enal. Two lipophilic polar aldehydes out of thirteen PC were also identified. These are 4-hydroxyhex-2-enal (HHE) and 4-hydroxyoct-2-enal (HOE). Some of NPC and PC were not identified in this study because of lack of standards.

In order to increase the peroxidative stress *in vivo* and to detect the resulting increases of urinary NPC and PC,

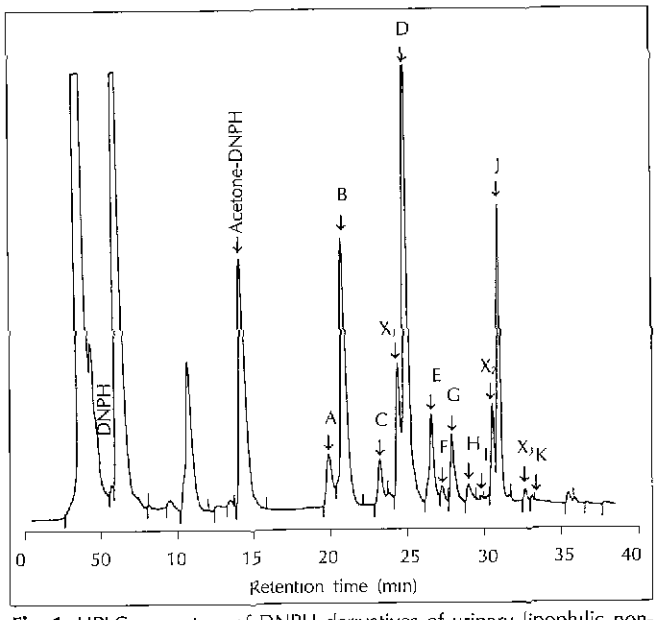

Fig. 1. HPLC separation of DNPH derivatives of urinary lipophilic nonpolar aldehydes and carbonyl compounds from rats fed a high PUFA diet. A butanal, B butan-2-one, D pentan-2-one, F: hex-2-enal, G: hexanal, H hepta-2,4-dienal, I: hept-2-enal, K. octanal, X_3: oct-2-enal, C, E, J, X_1 and X_2: unidentified

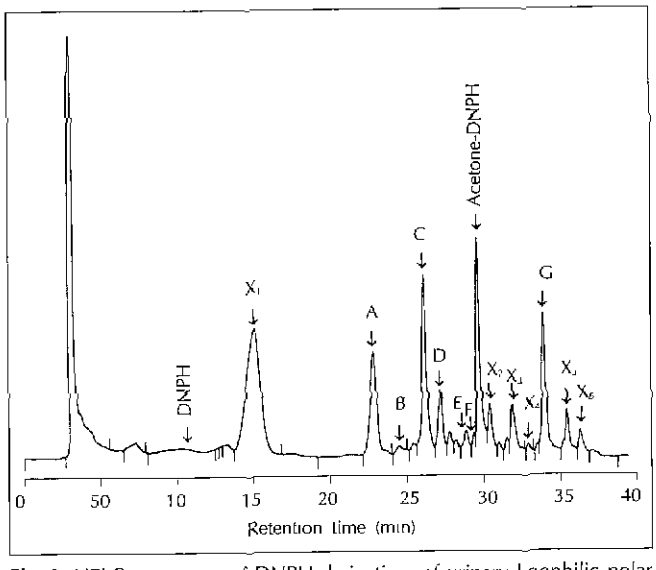

Fig. 2. HPLC separation of DNPH derivatives of urinary lipophilic polar aldehydes and carbonyl compounds from rats fed a high PUFA diet. E 4-hydroxyhex-2-enal. X_4: 4-hydroxyoct-2-enal, A, B, C, D, F, G, X_1, X_2, X_3, X_5 AND X_6: unidentified.

the rats of the PUFA group were fed a PUFA diet containing 3% cod liver oil and 5% corn oil for 19 weeks. The cod liver oil in the PUFA diet is rich in ω-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). A normal diet containing only 8% corn oil was fed to rats in the normal group. The effects of feeding a diet high in PUFA on the urinary excretion of

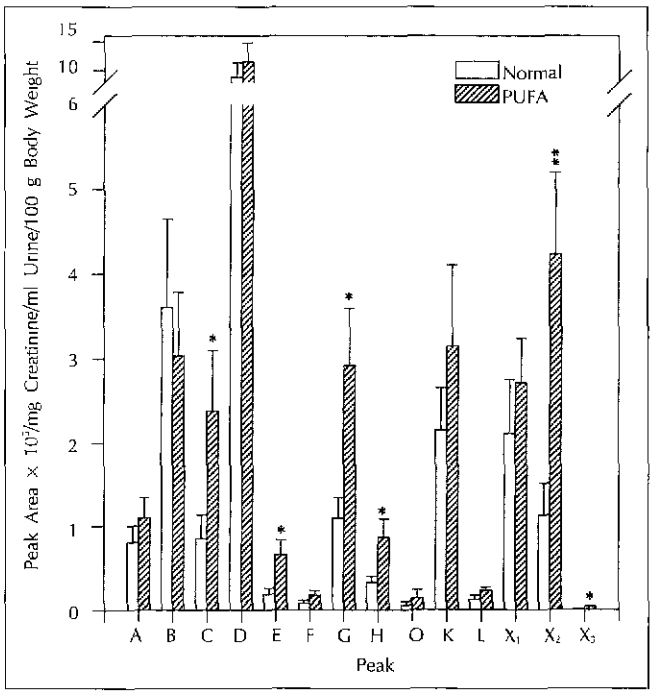

Fig. 3. Urinary excretion of nonpolar aldehydes and carbonyl compounds measured as DNPH derivatives from rats fed either a normal diet containing 8% corn oil or a PUFA diet containing 3% cod liver oil and 5% corn oil. A: butanal, B butan-2-one, D pentan-2-one, F: hex-2-enal, G: hexanal, H: hepta-2,4-dienal, I hept-2-enal, K: octnal, X_1 oct-2-enal, C, E, J, X_1, and X_2: unidentified. Values represent mean ± SEM of 9 and 6 animals in Normal and PUFA groups, respectively. statistical significance of difference is determined by Student's t-test. *$p < 0.05$ and **$p < 0.01$.

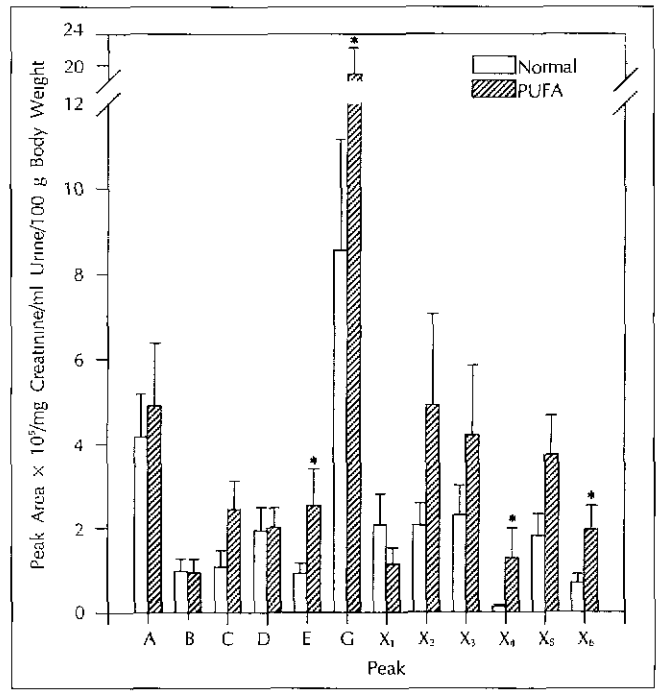

Fig. 4. Urinary excretion of polar aldehydes and carbonyl compounds measured as DNPH derivatives from rats fed either a normal diet containing 8% corn oil or a PUFA diet containing 3% cod liver oil and 5% corn oil. A, B, C, D, G, X_1, X_2, X_3, X_5 and X_6: unidetified, E 4-hydroxyhex-2-enal, X_4: 4-hydroxyoct-2-enal. Values represent mean ± SEM or 9 and 6 animals in Normal and PUFA groups, respectively. statistical significance of difference is determined by Student's t-test; *$p < 0.05$.

lipophilic NPC and PC are illustrated in Fig. 3 and 4, respectively. The urinary excretion of the compounds was expressed as mean area/mg creatinine/ml urine/100 g body weight. The urinary levels of all thirteen NPC except butan-2-one were increased in the PUFA group as compared to the normal group. In particular, hexanal, hepta-2,4-dienal, oct-2-enal, and unidentified compounds C, E, and χ_2 were significantly increased in the PUFA group. Ten PC except unidentified compounds B and χ_1 were increased in the urine of rats in the PUFA group as compared to the normal group. HHE, HOE, and unidentified compounds G and χ_6 of PC were significantly increased in the PUFA group. Neither total NPC nor total PC showed any statistical differences between normal and PUFA groups, as indicated in Fig. 5. However, rats in the PUFA group tended to excrete a higher amount of total NPC and total PC than in the normal group.

DISCUSSION

In the current study, the increased urinary lipophilic aldehydes and related carbonyl compounds seem to reflect *in vivo* peroxidizing abilities of a PUFA diet containing ω-3 fatty acids. Several investigators have reported an increased incorporation of ω-3 PUFA in plasma, tissues, and cell membranes of human subjects and animals due to dietary cod liver oil or other fish oil administration.[21,23] It has been shown in rats that dietary substitution with unstable ω-3 PUFA causes increased lipid peroxidation in liver microsomal membranes.[24] Incorporation of ω-3 PUFA like EPA into the lipids of cultured cells enhanced the cellular susceptibility to oxidants, especially H_2O_2.[25] Other *in vivo*[26] and *in vitro*[27] studies have demonstrated that cellular membranes and tissues containing relatively high amounts of ω-3 PUFA-constraining phospholipids became more vulnerable to peroxidative damage. Recent studies have also shown that a fish oil-enriched diet induced vitamin E deficiency in animals[28] and increased lipid peroxides in human plasma.[29]

A number of the lipophilic aldehydes found in the present study are suggested to be formed from the primary hydroperoxides of PUFA by the β-cleavage reaction.[16] Among different groups of lipophilic aldehydes, most saturated aldehydes show a low reactivity towards biological molecules such as amino acids, proteins, nucleic acids,

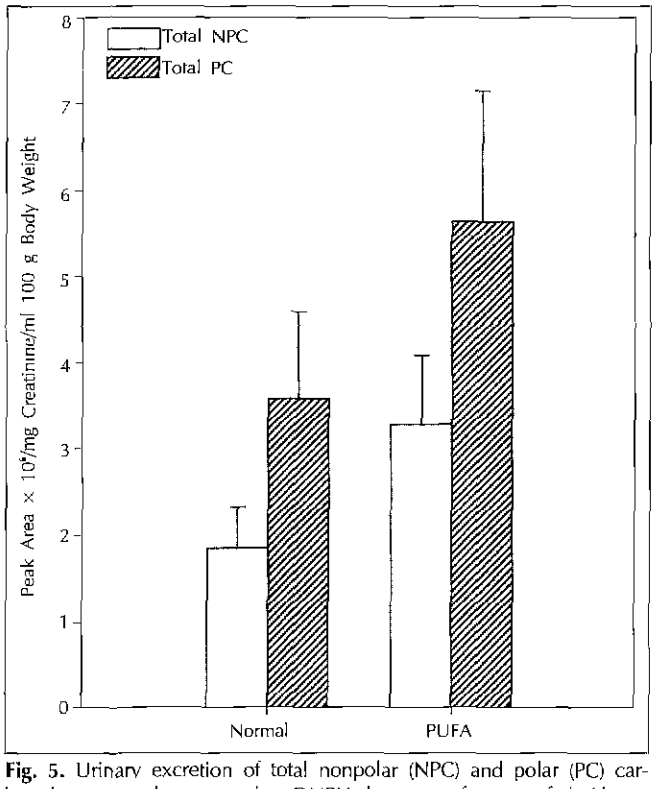

Fig. 5. Urinary excretion of total nonpolar (NPC) and polar (PC) carbonyl compounds measured as DNPH derivatives from rats fed either a normal diet containing 8% corn oil or a PUFA diet containing 3% cod liver oil and 5% corn oil. Values represent mean ± SEM of 9 and 6 animals in Normal and PUFA groups, respectively. The differences in means of either total NPC or total PC between two groups were not significant by Student's t-test at $p < 0.05$.

etc.[30] In contrast, α, β-unsaturated aldehydes (alk-2-enals, alk-2,4-dienals, and 4-hydroxyalk-2-enals) highly react on them even at a neutral pH and at low concentrations with biomolecules.[16] The reactive unsaturated aldehydes appear to inhibit several biological functions such as DNA and RNA synthesis, respiration, and glycolysis because of their high reactivity with thiols.[31] Thus, increases of *in vivo* production of these lipophilic aldehydes might be critical since these are highly reactive to biomolecules. It was reported that dietary peroxidation products, mostly medium chain-length lipophilic aldehydes, caused hepatic dysfunction due to decreases in the activities of hepatic enzymes and to the depletion of coenzymes.[32] The process of the LDL oxidation has been suggested to be involved by the lipophilic lipid metabolites.[33] Studies of lipophilic aldehydes with cultured human endothelial cells[34] or rat hepatocytes[35] indicated the influence of various structures of aldehydes on their cyto- and genotoxic potential. In particular, α, β-unsaturated aldehydes with a hydroxyl group or additional double bonds were highly toxic and caused rapid cell lysis.[34] As indicated from the above studies, lipophilic secondary lipid peroxidation products found in the present study seem to be relevant factors in the occurrence of cellular damage and in the consequent induction of some pathological conditions related to lipid peroxidation.

Besides saturated and unsaturated aldehydes, 4-hydroxyalkenals were assumed to be generated from ω-6 or ω-3 PUFA during the course of peroxidation of fatty acids. HHE found in the present study was a degradation product of DHA,[36] whereas HNE was derived from ω-6 PUFA.[52] A number of studies have shown that 4-hydroxyalkenals possess hepatotoxic, mutagenic, and genotoxic properties.[37,38] In particular, detection of cyclic adducts of deoxyguanosine with HNE or HHE *in vitro*[39] provides the possibility that these 4-hydroxyalkenals may be capable of adduct formation with DNA in physiological conditions. The hydroxyaldehydes have been reported to be metabolized by isolated rat hepatocytes, and are then partially excreted in urine as mercapturic acid conjugates.[40] In the present experiment, the findings of significant urinary excretion of HHE and HOE due to the oxidizing action of PUFA diet indicate that these aldehydes might be generated *in vivo* to such high levels that some of the DNPH reactive aldehydic metabolites are excreted in the urine. In addition, the urinary excretion of various PC including HHE and HOE are of special significance since they can react readily with biomolecules. Besides these well known 4-hydroxyaldehydes, other urinary PC were found in the present experiments. These unidentified PC may include 2- or 4-hydroxyaldehydes detected in peroxidizing systems *in vitro*.[41,42]

In conclusion, *in vivo* lipid peroxidation stimulated by the highly oxidizable PUFA diet in rats led to increased levels of urinary lipophilic nonpolar and polar aldehydes and related carbonyl compounds. The secondary lipid peroxidation products generated as part of the lipid peroxidation process are partially excreted in the urine through detoxification process. However, it should be mentioned that other aldehydes and related carbonyl compounds, not yet isolated or identified in tissues, could possibly be formed during lipid peroxidation, and that these compounds may also exert cytotoxic effects on cell components.

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