Production of Aliphatic Aldehydes on Peroxidation of Various Types of Lipids

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***In vitro* peroxidation by air, or xanthine–xanthine oxidase (xanthine–XOD) was performed to estimate the production of aliphatic aldehydes from free polyunsaturated fatty acids (PUFA), triglycerides, phospholipids and rat liver microsomes and mitochondria. The aldehyde contents in peroxidized lipids were determined by liquid chromatography and fluorescence detection. In both peroxidation, pentanal, (*E*)-4-hydroxy-2-nonenal (4-HN), and hexanal were produced from ω-6 PUFA rich lipids and propanal was mainly produced from ω-3 PUFA rich lipids. The aldehyde production was markedly enhanced by increasing the degree of fatty acid unsaturation. The ratios of 4-HN to hexanal production in xanthine–XOD peroxidation of the ω-6 PUFA rich lipids, and rat liver microsomes and mitochondria were much higher than those in air peroxidation. The ratios (4-HN/hexanal) obtained in microsomes and mitochondria by xanthine–XOD were similar to those in rat liver observed in vitamin E deficient studies. The determination of these aldehydes may be useful to estimate the kinds of fatty acids peroxidized and investigate *in vivo* lipid peroxidation mechanism.**

Keywords lipid peroxidation; aliphatic aldehyde; polyunsaturated fatty acid; rat liver microsome; mitochondria; 4-hydroxy nonenal; hexanal

Several investigators have reported that some aldehydes were produced *in vitro* or *in vivo* lipid peroxidation. Esterbauer[2)] demonstrated that hexanal and (*E*)-4-hydroxy-2-nonenal (4-HN) were mainly produced in the peroxidation of linoleic acid or arachidonic acid from the study of air oxidation in acetate buffer. The production of hexanal and 4-HN were also observed in rat liver microsomes by adenosine diphosphate (ADP)/$Fe^{2,3)}$ or reduced nicotinamide adenine dinucleotide phosphate (NADPH)/$Fe^{4)}$ dependent *in vitro* lipid peroxidation and in mouse liver by the oral administration of bromobenzene.[5)] However, these reports did not include quantitative analysis of the aldehydes. We have shown recently that high levels of propanal, pentanal, hexanal, and 4-HN were observed in plasma and liver from vitamin E deficient rats compared with vitamin E supplemented rats by the high performance liquid chromatography (HPLC)-fluorometric method.[6)] The ratios of 4-HN to hexanal production in plasma and liver were 69% and 88%, respectively. While, in the report of Esterbauer,[3)] the ratios in air oxidation of free polyunsaturated fatty acids (PUFA) were lower than 20%. Most PUFA in biological tissues are in esterified forms,[7)] and the PUFA in triglyceride (TG) and phosphatidylcholine (PC) were peroxidized *in vivo* by the superoxide anion radical, lipoxygenase (EC 1.13.11.12), *etc.*[8–11)] The process of aldehyde production *in vivo* would be different from the *in vitro* production by air.

In this study, we compared the production of aliphatic aldehydes from various types of lipids and biological samples by atmospheric oxygen and xanthine–xanthine oxidase (EC 1.2.3.2) (xanthine–XOD) lipid peroxidation.

Experimental

Chemicals Linoleic acid and linolenic acid were purchased from Tokyo Kasei Kogyo Co., Ltd. Arachidonic acid was from Serdary Research Laboratories, Inc. and docosahexaenoic acid was from Nu-Chek-Prep, Inc. Safflower oil was obtained from Yuro Yakuhin Co. and linseed oil was from Wako Pure Chemical Industries, Ltd. L-α-Phosphatidylcholine (type VII-E, from frozen egg yolk) and XOD (grade IV, from milk) were purchased from Sigma Chemical Co. Other chemicals used were of reagent grade quality.

Preparation of Phosphatidylcholine Liposomes Vitelline PC liposomes were prepared by the method of Ursini *et al.*[12)]

Preparation of Rat Liver Microsomes and Mitochondria A male Wistar rat of 3 weeks old obtained from Clea Japan Inc. was sacrificed and the liver was removed after perfusion with ice-cold physiological saline. The liver was homogenized in 0.25 M sucrose solution including 5 mM Tris–HCl buffer (pH 7.2) and the microsomal and mitochondrial fraction were prepared by the method of Sato.[13)]

Peroxidation of Lipids and Rat Liver Fractions by Air Seven micro mol of each PUFA (linoleic acid, linolenic acid, arachidonic acid, and docosahexaenoic acid) or TG (safflower oil and linseed oil; *ca.* 98.5% of the oil was composed of TG) was put in the flasks and spread into the films by a rotary evaporator. These flasks were rotated at 37 °C for 24 h and for 48 h in the case of PUFA and TG peroxidation, respectively. After peroxidation, the lipids were diluted with ethanol–acetone (3 : 2) or distilled water. The suspensions of rat liver microsomes and mitochondria were added into 0.1 M phosphate buffer (pH 7.4) and incubated at 37 °C for 2 h. These samples were used for the determination of thiobarbituric acid reactive substances (TBARS) and aliphatic aldehydes.

Peroxidation of Lipids and Rat Liver Fractions by Xanthine–XOD PUFA and TG were diluted with ethanol–acetone (3 : 2). PC liposomes were suspended in 0.1 M Tris–HCl buffer (pH 7.5) and rat liver microsomes and mitochondria were in 0.01 M Tris–0.15 M KCl buffer (pH 7.4). These samples were used as the substrate for the peroxidation. Each substrate was added into 0.01 M Tris–0.15 M KCl buffer (pH 7.4) containing 10 μM ethylenediaminetetraacetic acid (EDTA), 0.15 M ADP, 0.01 M ferric chloride, 1 mM xanthine, and 0.032 units of XOD. Each substrate, 3.5 μM PUFA, 3.5 μM TG, 1 mg/ml PC liposomes, 4.8 mg protein/ml microsomes, or 6.4 mg protein/ml mitochondria, was added. The mixture was incubated at 25 °C for 60 min. TBARS and aliphatic aldehydes in the reaction mixture were assayed as follows.

Assay of TBARS and Aliphatic Aldehydes TBARS was determined by the method of Masugi *et al.*[14)] and was expressed as malondialdehyde equivalents. The determination of aliphatic aldehydes was performed by the method reported in a previous paper.[15)] The results shown in this study were obtained from the mean of the three experiments. The protein content was determined by the method of Lowry *et al.*[16)] with bovine serum albumin as the standard.

Results

Production of Aliphatic Aldehydes in Peroxidation of Free Polyunsaturated Fatty Acids Authentic free PUFA, *i.e.* linoleic acid, linolenic acid, arachidonic acid, and docosahexaenoic acid were peroxidized by two different peroxidations. Table I shows the production of aliphatic aldehydes from these lipids. The production of pentanal, 4-HN and hexanal were observed in the peroxidation of ω-6 PUFA such as linoleic acid and arachidonic acid by both lipid peroxidations. In air peroxidation, the ratios of

Table I. Formation of Aliphatic Aldehydes and TBARS in the Peroxidation of Free Polyunsaturated Fatty Acids

Aldehydes	Linoleic acid	Linolenic acid	Arachidonic acid	Docosahexaenoic acid
Non treatment (control)				
Propanal	Trace	0.20	Trace	1.80
Butanal	0.07	0.38	0.16	0.38
Pentanal	0.11	0.17	1.50	0.18
4-HN	0.10	Trace	0.17	0.10
Hexanal	0.27	0.15	1.18	Trace
Nonanal	Trace	Trace	Trace	Trace
Decanal	Trace	Trace	0.04	0.05
TBARS	0.04	0.11	0.07	1.85
Peroxidation by air				
Propanal	Trace	2.55	Trace	237.60
Butanal	1.84	3.70	12.94	9.35
Pentanal	3.13	1.06	124.60	9.68
4-HN	1.90	Trace	137.00	7.20
Hexanal	6.06	3.15	260.70	3.58
Nonanal	Trace	0.11	13.13	1.31
Decanal	Trace	Trace	4.55	0.43
TBARS	0.32	1.76	18.91	33.92
Peroxidation by xanthine–XOD				
Propanal	1.59	55.11	19.69	207.46
Butanal	2.71	9.66	30.09	20.63
Pentanal	86.72	10.34	150.60	16.85
4-HN	50.75	4.81	146.22	3.59
Hexanal	59.19	1.52	183.40	7.21
Nonanal	Trace	0.01	0.08	0.66
Decanal	0.01	Trace	0.01	0.67
TBARS	0.30	1.65	14.38	22.28

Aliphatic aldehydes, μmol/mmol free fatty acid. TBARS, μmol as malondialdehyde/mmol free fatty acid.

Table II. Formation of Aliphatic Aldehydes and TBARS in the Peroxidation of Safflower Oil and Linseed Oil

Aldehydes	Safflower oil	Linseed oil
Non treatment (control)		
Propanal	Trace	2.11
Butanal	Trace	Trace
Pentanal	1.18	0.01
4-HN	Trace	Trace
Hexanal	2.03	Trace
Nonanal	Trace	Trace
Decanal	Trace	Trace
TBARS	0.05	1.09
Peroxidation by air		
Propanal	Trace	7.95
Butanal	0.30	0.03
Pentanal	6.00	0.02
4-HN	1.60	Trace
Hexanal	14.91	0.20
Nonanal	0.10	Trace
Decanal	Trace	Trace
TBARS	0.43	10.87
Peroxidation by xanthine–XOD		
Propanal	0.51	66.80
Butanal	0.99	2.98
Pentanal	7.09	2.73
4-HN	13.35	0.71
Hexanal	68.11	4.32
Nonanal	0.01	0.02
Decanal	Trace	Trace
TBARS	3.89	19.16

Aliphatic aldehydes, μmol/g oil. TBARS, μmol as malondialdehyde/g oil.

Table III. Formation of Aliphatic Aldehydes and TBARS in the Peroxidation of Phosphatidylcholine Liposomes

Aldehydes	Non treatment (control)	Peroxidation by xanthine–XOD
Propanal	0.08	1.23
Butanal	0.02	1.69
Pentanal	0.61	5.62
4-HN	0.04	3.71
Hexanal	0.75	9.45
Nonanal	Trace	0.73
Decanal	Trace	0.01
TBARS	0.56	3.42

Aliphatic aldehydes, μmol/g phosphatidylcholine. TBARS, μmol as malondialdehyde/g phosphatidylcholine.

4-HN to hexanal production in the peroxidation of linoleic acid and arachidonic acid were 31% and 53%, respectively. While in xanthine–XOD peroxidation, the ratios were 86% and 80%, respectively. A large amount of propanal, and a small amount of butanal and pentanal were produced from ω-3 PUFA by xanthine–XOD, though the formation of propanal from linolenic acid by air was not significant. The amount of aldehydes and TBARS were associated with the number of double bonds in ω-6 and ω-3 PUFA.

Production of Aliphatic Aldehydes in Peroxidation of Esterified Fatty Acids Most fatty acids in biological tissues exist in TG or phospholipids as esterified form. Safflower oil and linseed oil, 98.5% of the oils were composed of TG, were peroxidized by air or xanthine–XOD. And PC liposomes were peroxidized by xanthine–XOD. Tables II and III show the production of aldehydes from the oils and PC liposomes, respectively. The species of aldehydes produced by air or xanthine–XOD peroxidation would be decided by the species of PUFA in the oil. Pentanal, 4-HN, and hexanal were produced by the peroxidation of safflower oil in which *ca.* 70% of the constituent PUFA is linoleic acid.[17] The ratios of 4-HN to hexanal production in air and xanthine–XOD peroxidation were 11% and 20%, respectively. While propanal was produced in the peroxidation of linseed oil in which *ca.* 60% of constituent PUFA is linolenic acid.[18] The 4-HN to hexanal ratios in xanthine–XOD peroxidation was higher than that in air peroxidation. In the peroxidation of PC liposomes in which *ca.* 15% of constituent PUFA is linoleic acid and 5% of constituent PUFA is arachidonic acid, the productions of pentanal, 4-HN, and hexanal were observed, and the ratio of 4-HN to hexanal in the xanthine–XOD peroxidation was 39%.

Production of Aliphatic Aldehydes in Lipid Peroxidation of Rat Liver Microsomes and Mitochondria Rat liver microsomes and mitochondria containing a large amount of PUFA as phospholipid and TG forms[19] were frequently used for *in vitro* lipid peroxidation study. The productions of propanal, pentanal, 4-HN, and hexanal were observed in the peroxidation of these biological samples by xanthine–XOD as shown in Table IV. The ratios of 4-HN to hexanal production were 75% and 61% in microsomes

TABLE IV. Formation of Aliphatic Aldehydes and TBARS in the Peroxidation of Rat Liver Microsomes and Mitochondria

Aldehydes	Microsomes	Mitochondria
Non treatment (control)		
Propanal	0.86	1.10
Butanal	0.15	0.07
Pentanal	0.16	0.10
4-HN	0.16	0.06
Hexanal	0.20	0.12
Nonanal	0.04	0.05
Decanal	0.05	0.02
TBARS	0.86	0.85
Peroxidation by air		
Propanal	1.46	1.44
Butanal	0.26	0.13
Pentanal	0.20	0.17
4-HN	0.18	0.08
Hexanal	0.56	0.21
Nonanal	0.09	0.10
Decanal	0.06	0.03
TBARS	1.12	1.21
Peroxidation by xanthine–XOD		
Propanal	2.42	2.19
Butanal	1.09	0.05
Pentanal	2.95	1.83
4-HN	2.56	1.54
Hexanal	3.43	2.53
Nonanal	0.10	0.01
Decanal	0.01	Trace
TBARS	7.29	5.22

Aliphatic aldehydes, nmol/mg protein. TBARS, nmol as malondialdehyde/mg protein.

and mitochondria, respectively. The production of these aldehydes from microsomes and mitochondria by air were markedly low compared with production by xanthine–XOD. TBARS and aldehyde levels in the microsomes were slightly higher than those in the mitochondrial fraction.

Discussion

Some studies have been reported on the formation of aliphatic aldehydes from the breakdown of monohydroperoxides, hydroperoxy cyclicperoxides, or cyclic dioxides.[20,21]

Frankel *et al.*[20] have reported that propanal was formed by the thermal decomposition of hydroperoxy cyclicperoxide of methyl linolenate. We showed that a large amount of propanal was produced in the peroxidation of linolenic acid and docosahexaenoic acid, and linseed oil containing ω-3 fatty acids by air or xanthine–XOD.

Esterbauer[2] has shown that hexanal and 4-HN were produced from ω-6 free PUFA in the study of air oxidation of free PUFA. Our study in the peroxidation of various lipids by air and xanthine–XOD confirmed his findings. A marked difference of the ratio of 4-HN to hexanal production between air and xanthine–XOD peroxidation was observed. The ratios in the peroxidation of linoleic acid and arachidonic acid by air were 31% and 53%, respectively, while the ratios in the peroxidation by xanthine–XOD were 86% and 80%, respectively. The ratio observed in xanthine–XOD peroxidation was higher than that in air peroxidation. The high ratios of 4-HN to hexanal production were also observed in the peroxidation of rat liver microsomes and mitochondria by xanthine–XOD. Since 4-HN has several harmful effects on biological membranes compared with the other aliphatic aldehydes,[2,4] an increase of the ratio in the peroxidation by xanthine–XOD would be inconvenient in biological tissues. Our previous study with a rat fed vitamin E deficient diet showed increased levels of 4-HN and hexanal in plasma and liver and high ratios of 4-HN to hexanal production in their samples (69% and 88%, respectively).[6] The ratios in the peroxidation of these rat liver fractions by other peroxidation systems such as NADPH/Fe or lipoxygenase were lower (19—49%) than those by xanthine–XOD (data not shown). It is known that the superoxide anion radical is produced from the reaction of xanthine–XOD.[22] Lipid peroxidation observed in vitamin E deficient rats may be caused partly by the superoxide anion radical. Significant productions of 4-HN and hexanal were observed in the peroxidation of safflower oil and PC liposomes by xanthine–XOD, however, the ratios of 4-HN to hexanal were low (19.6%, 39.0%, respectively) compared with free PUFA or rat liver samples. Tappel[23] and Thayer[8] suggested that PUFA peroxidized in phospholipids were hydrolyzed first by phospholipase A_2 (EC 3.1.1.4) bound to the membrane and were treated with glutathione peroxidase (EC 1.11.1.9). We found the 4-HN to hexanal ratio in the peroxidation of free PUFA, mitochondria, and microsomes. Therefore, it was thought that lipid peroxides in microsomes and mitochondria were hydrolyzed to free PUFA peroxides by phospholipase A_2 before they were decomposed directly and then these aldehydes were produced. Rat liver microsomes and mitochondria contained a few ω-3 fatty acids, however, the ratios of propanal to hexanal production were very high. In our previous paper, the levels of propanal in plasma liver from vitamin E deficient rats were slightly higher than those of hexanal. These results might be explained by the evidence that ω-3 fatty acid could be peroxidized easily as compared with ω-6 fatty acids,[24] or that the specific activities of metabolic enzymes could be different between these aldehydes.[25,26]

References and Notes

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