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Lipid peroxide levels and accumulation of hydroxy lipids in live fish with oxidative stress

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Lipid peroxides (LPO) are helpful in studying peroxidation levels in biological materials, although the levels might reflect only the physiological conditions just now. In the living body, it is considered that LPOs and related compounds are decomposed to more inactive compounds shortly after their formation and/or reduced to more stable hydroxy compounds by enzymes such as glutathione peroxidase (GPx). Thus, the levels of hydroxy lipids (L-OH) rather than LPOs may provide more important information concerning oxidative condition of the living body. We studied the levels of L-OH of liver in live fish. The levels of hydroxy lipids were noted to be higher in the order of diseased fish, cultured fish and wild fish, reflecting the strength of oxidative stress. Additionally, in order to investigate the relationships between levels of LPO, L-OH and of antioxidative enzymes in live fish, cultured cells from tilapia liver were treated with oxidative stressors. These results suggest that the elevation of the L-OH level is closely associated with the elevation of GPx activity. Consequently, oxidative status of live fish was shown clearly for the first time using new indices of L-OH. L-OH levels may be a good index for the integrated level of oxidative stress in live fish.

Key words : hydroxy lipids, lipid peroxides, glutathione peroxidase, diseased fish, Hepa-T1

Introduction

Reactive oxygen species (ROS) result in either positive responses, such as proliferation and activation, or negative responses, such as lipid peroxidation, DNA damage, cell growth inhibition and cell death to living body. Injury to cells by oxidative stress occurs only when ROS overwhelm the biochemical defenses of the cell.[1-3] ROS, in particular, hydroxy radicals can react with all biological molecules, i.e. lipids, proteins, nucleic acids and carbohydrates. Among them the more susceptible targets are polyunsaturated fatty acids (PUFA) of lipids.[4] Lipid radicals generated during an early encounter with an oxidant add molecular oxygen to produce lipid radicals. This prooxidant abstracts allylic hydrogen from another unsaturated side chain, producing lipid peroxide (LPO) and thus propagating the chain.[1,2] LPOs are an important biological consequence of oxidative cellular damage and aging.[5] Therefore, LPOs have attracted the attentions of many researchers for their important roles in a variety of disorders and pathological condition. These studies are not confined to mammals but also applicable to aquatic organisms. Considering from an aquacultural standpoint, disturbances under conditions of intensive fish feeding are usually of a prolonged nature and the accompanying chronic stress will lead to a continued loss of homeostasis, to which adaptation is not possible.[6] Fish contain more PUFA than land organisms and aquaculture feeds contain a large amount of PUFA which are easily oxidized. Oxidized PUFA might lead to oxidative stress in fish.[7] These phenomenons suggest that LPOs are helpful in studying peroxidation levels in biological materials; although the levels might reflect only the physiological conditions just now. Since LPOs are hazardous in living bodies, in live organisms, LPOs are reduced more preferentially by antioxidative enzymes such as glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD) than by low molecular weight antioxidants such as vitamain A, C, and E. GPx has been noted

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for reducing LPOs directly, and therefore many researchers have studied LPOs and GPx detoxification system. GPx contain an active site selenocysteine, which participates in two-electron reduction of peroxides to hydroxy lipids (L-OH). Therefore, estimation of the levels of L-OH *in vivo* will provide a more meaningful measure of oxidative stress during the recent past. However, there have been some problems in measuring L-OH in biopsy materials due to their small quantities and the coexistence of a large amount of normal lipids. Therefore, very few reports are available concerning L-OH levels in living bodies, not only in fish but also in land animals.

In this study, in addition to LPO levels, the author determined L-OH levels in several antioxidative statuses of many kinds of fish using fluorometric high performance liquid chromatography (HPLC). Hydroxy lipids from lipids in live fish were reacted with 1-anthroyl nitrile. This reagent is not only extremely fluorescent but it is also reactive with hydroxy groups under very mild conditions. The analytical method of the resulting anthroyl hydroxy lipids was developed and the levels in fish were determined by HPLC. Additionally, in order to investigate the relationships between LPO and L-OH, and antioxidative enzymes such as GPx in live fish, the author used cultured cells from tilapia liver (Hepa-T1). Hepa-T1 cells were treated with 2,2'-azobios(2-amidino-propane) dihydrochloride (AAPH) and H_2O_2 as a model of oxidative stress. AAPH is a water-soluble radical initiator, which undergoes spontaneous thermal decomposition to form carbon-centered radicals. These radicals can initiate a chain reaction to produce LPOs in the presence of oxygen and PUFA.[8,9] H_2O_2 is an active oxygen species which is produced during normal oxygen metabolism in all aerobic cells, and can react with major constituents of cells such as membrane lipids, proteins, and DNA.[10-12] The purpose of this study is to estimate the LPO and L-OH levels in normal and diseased fishes and to find an index for the internal peroxidation in live fish.

Materials and Methods

Fish

Cultured live fish; yellowtail *Seriola quinqueradiata* (average length, 67.2 cm; weight, 5,390 g) with streptococcal infection (*Lactococcus garvieae*) or jaundice disease, sweetfish *Plecoglossus altivelis* (18.5 cm, 79 g) with ulcer disease, globefish *Fugu rubripes rubripes* (17.2 cm, 152 g) with *Heterobothrium okamotoi* and its complication (emaciation disease), red sea bream *Chysophrys major* (26.3 cm, 283 g) with *Vibrio anguillarum* and its complication (*Edwardsiella tarda*), carp *Cyprinus carpio* (24.5 cm, 240 g) with cold water disease, and striped jack *Caranx delicatissimus* (34.8 cm, 746 g) with Irido viruses, as well as their respective normal fish fed in the same cage under identical cultural conditions were obtained from private fish farms via the Fisheries Research Center of Kumamoto and the Fukuoka Fisheries & Marine Technology Research Center in Japan. Wild live fish; yellowtail (average length, 37.5 cm; weight, 723 g), sweet fish; (17.0 cm, 79.2 g) and the other wild fish for analysis of LPOs and hydroxy lipids in the liver; flatfish *Pleuronichthys cornutus* (28.5 cm; weight, 310 g), flathead *Platycerhalus indicus* (length, 35.8 cm; weight, 272 g), girella *Girella punctata* (length, 32.0 cm; weight, 498 g), gray mullet *Mugil cephalus* (length, 40.8 cm; weight, 632 g), hairy stingfish *Sebastiscus marmoratus* (length, 27.2 cm; weight, 342 g), horse mackerel *Trachurus japonicus* (length, 17.5 cm; weight, 55 g), parrot fish *Oplegnathus fasciatus* (length, 23.8 cm; weight, 277 g), red spotted grouper *Epinephelus akara* (length, 16.5 cm; weight, 78 g), and stone wall perch *Oplegnathus fasciatus* (length, 23.4 cm; weight, 275 g) were purchased from local fishermen. Diseased and normal fish judged by external appearance were diagnosed by expert scientists of the Research Center.

In vivo Peroxidation with hepatotoxic substances

Following an abdominal injection of carbon tetrachloride (2 ml / kg body weight) to a carp, 1 ml each of serial blood samples was withdrawn *via* a cannula.[13] Tissues of yellowtail obtained after an abdominal injection of a culture of jaundice-bacterium were donated by Prof. H. Murata (Faculty of Agriculture, Miyazaki University, Japan). Cultured fishes were used for both experiments.

Culture of Hepa-T1 cells

Hepa-T1 cell line, epithelial-like hepatocytes from the

tilapia *Oreochromis niloticus*, was used in this study. This cell line, established by Dr. Kong Zwe-Ling, was purchased from The Institute of Physical and Chemical Research (RIKEN) in Japan. The cells were cultured in 90 mm petri dishes (Nalge Nunc Int. Co., Ltd., Rochester, NY) with E-RDF (Kyokuto Pha. Ind. Co., Ltd., Japan) medium containing 10% heat inactivated fetal bovine serum (FBS) (Invitrogen Corp., Carlsbad, CA) at 56℃ for 30 min. The morphology of the cells was observed using a hemocytometer under a phase-contrast microscope (Nikon Corporation, Japan). The cells were then harvested in a logarithmic growth phase of 80% confluence (cell number; $2x10^6$ - $3x10^6$/dish) using 0.2% trypsin (Invitrogen Corp., Carlsbad, CA) fortified with 0.02 mM ethylenediaminetetraacetic acid (EDTA).

Oxidative treatments of Hepa-T1 cells with AAPH and H_2O_2

At 80% confluent, Hepa-T1 cells were exposed to the oxidizing agents by replacing E-RDF without FBS containing AAPH (5 mM) or H_2O_2 (5 mM). After exposure of the cells to the oxidative stressors at 28℃ for 1 or 2 hours, floating dead cells were removed by decantation. The surviving cells attached to the petri dish were then rinsed twice with phosphate-buffered saline (PBS), and transferred to a 15 mL centrifugal tube (Nalge Nunc Int. Co., Ltd., Rochester, NY) using a cell scraper (Nalge Nunc Int. Co., Ltd., Rochester). The cells harvested were again washed with PBS by centrifugation at 150 g for 5 min. The washed living cells were dispersed in 1 ml of 0.05 M Tris-HCl buffer (pH 7.6) and then disrupted by sonication for 60 s in an ice bath. This cell suspension was used for the following assays.

Determination of protein in Hepa-T1 cells

The protein concentration of the cell suspension was measured with a BCA protein assay kit (PIERCE, Co. Ltd., Rockford, IL), using bovine serum albumin as a standard.

Determination of antioxidative enzymic activity of Hepa-T1 Cells

GPx activity was determined by the method of Ursini et al[14] using *t*-butyl hydroperoxide as a substrate. Briefly, NADPH reagent consisting of 24 μmol glutathione, 12 U glutathione reductase, and 4.8 μmol NADPH in 0.05 M Tris-HCl (pH 7.6) containing 5 mM EDTA was used. Then 20 μl of the cell suspension was placed in a quartz cuvette followed by 350 μl of 0.05 M Tris-HCl (pH 7.6) containing 5 mM EDTA and 175 μl of NADPH reagent. The quartz cuvette was placed in a spectrophotometer (Ultrospec 3000, Amraham Pharmacia Bioteck Biotech UK Ltd., England). The reaction was started by the addition of 175 μL of 776 μmol/l t-butyl hydroperoxide in a water solution. Oxidation of NADPH was monitored by recording the absorbance at 340 nm every 30 sec for 180 sec. One unit of enzymic activity is defined as the oxidation of 1 μmol of NADPH per min. The specific activity of GPx was expressed as units per mg protein.

CAT activity was determined using a modification of the method of Aebi[15] and Garcia et al.[16] Briefly, 20 μl of the supernatant was placed in a quartz cuvette followed by 960 μl of 25 mM H_2O_2 diluted with 0.05 mM Tris-HCl buffer (pH 7.6) and 20 μl of water. The quartz cuvette was placed in the spectrophotometer (Ultrospec 3000, Amraham Pharmacia Bioteck Biotech). H_2O_2 degradation was monitored by recording the absorbance at 240 nm every 10 sec for 60 sec. The specific activity of CAT was expressed as μmol of H_2O_2 degraded per minute per mg protein.

SOD activity was determined by the modified method of Oberley et al.[14] Briefly, 20 μl of the cell suspension were placed in a quartz cuvette followed by 20 μl of 0.066 U/mL xanthine oxidase solution by dissolving 0.05 M ammonium sulfate (pH 10.2) containing 5 mM EDTA. The quartz cuvette was placed in the spectrophotometer (Ultrospec 3000, Amraham Pharmacia Bioteck Biotech). The reaction was started by the addition of 560 μl of 0.1 mM xanthine and 0.025 mM nitro blue tetrazolium (NBT) by dissolving 50 mM Na_2CO_3 (pH 10.2) containing 0.1 mM EDTA. NBT reduction was monitored by recording the absorbance at 560 nm every 10 sec for 80 sec. One unit of enzymic activity is defined as the amount of protein required for 50% inhibition under the assay conditions given above. The specific SOD activity was expressed as units per mg protein.

Extraction of Lipids

In case of live fishes, immediately after decapitation and

exsanguination, tissues were excised and lipids were extracted by homogenizing the tissue with chloroform/methanol (2/1, by vol) containing 0.005% 2,6-di-*tert*-butyl-*p*-cresol (BHT) under an ice-cold conditions, and recovered in a manner similar to that described by Folch et al.[17] In case of Hepa-T1, to the cell suspension (0.9 mL), 1.8 ml of chloroform and 1.2 ml of methanol were added. The mixture was stirred and then separated into two layers by centrifugation at 2,000 g for 10 min. The lipid extracts in the lower layer were used for the following assays.

Analysis of LPO Levels

To an aliquot (1 mg) of the lipids in a screw-capped tube, 100 μl of cyclohexane and 100 μl of triphenylphosphine reagent (11 mg triphenylphosphine in 10 ml cyclohexane) were added and the mixture was shaken gently at 30℃ in the dark for 30 min. Triphenylphosphine oxide (TPO) generated stoichiometrically was determined by HPLC on a column of Nova-pack Silica (3.9 i.d. x 150 mm, Waters ltd., Milford, MA) using *n*-hexane/ 2 -propanol (98/3, by vol) as eluent (TP-method).[18]

Analysis of L-OH Levels

In case of live fishes, L-OH was determined as anthroyl esters. To a lipid sample used for LPO analysis (*ca.*1 mg) in a screw-capped test tube, 100 μl of 1 -hexadecanol (internal standard; 24 μg/ml acetonitrile), 100 μl of 1 -anthroyl cyanide reagent (8 mg/ml acetonitrile) and 50 μl quinuculidine reagent (2.4 mg/ml acetonitrile) were added. The mixture was stirred for 40 min at 60℃. After cooling, the reaction was terminated by adding 1 ml methanol. Five μl of the resulting mixture was injected into the following HPLC system. Anthroyl derivatives of hydroxy lipids were detected fluorometrically with a HITACHI F-1050 detector (Ex 370 nm and Em 470 nm). The HPLC column used in the experiment was an Inertsil ODS- 2 (4.6 mm x 250 mm, GL Sciences Inc.). The elution gradient was programmed linearly from A: acetonitrile/water (80/20, by vol) to A/acetone (20/80, by vol) for 30 min; this condition was then maintained for 20 min. The flow rate was 1.5 ml/min. Hydroxy lipids that eluted between 1-hexadecanol (internal standard) and cholesterol, corresponding to a retention time of *ca.* 32-41 min, were tentatively defined as L-OH depending on the agreement of their retention time with that of authentic hydroxy triglycerides (castor oil and reduced-autoxidized tuna triglycerides). L-OH levels were expressed as nmol per g tissue.

In case of Hepa-T1, to the TP-reduced lipids, 20 μL of 1 -hexadecanol (internal standard, 2.4 mg/ml acetonitrile), 50 μl of 1-anthroyl cyanide reagent (8 mg/mL acetonitrile) and 25 μl of quinuculidine reagent (2.4 mg/ml acetonitrile) were added. The mixture was stirred gently for 40 min at 60℃. After cooling, the reaction was stopped by the addition of 100 μl of methanol followed by the addition of 800 μl of *n*-hexane. The mixture was stirred and centrifuged at 2,000 g for 10 min. The upper layer was transferred to another 2 ml safe-lock tube and evaporated to dryness using a centrifugal concentrator (Taitex, Japan). The anthroyl derivatives of L-OH were dissolved in 50 μl of benzene/methanol (50/50, by vol). The anthroyl derivatives of L-OH were detected aboved HPLC system. L-OH levels were expressed as nmol per mg protein.

Statistical analyses

Data presented in Fig. 6 is statistically treated using one-way ANOVA and Scheffe's test. Data presented in Fig. 4 , 9 , and 10 are statistically treated using Student's *t*-test. A *P*-value of less than 0.05 denoted a statistically significant difference.

Results

LPO levels in muscles and liver of live fish

LPO levels in muscles and liver of wild fishes are shown in Fig. 1 . They showed a tissue-characteristic steady state. The LPO levels of muscle and liver, 10-50 and 100-200 nmol/g tissue, respectively, seemed to reflect physiological activity of the tissue.

Elevation of LPO levels by oxidative stress

Effects of exogenous hepatotoxins, carbon tetrachloride and culture of a jaundice-bacterium, on carp and yellowtail are shown in Fig. 2 . LPO levels in muscle and liver of the carp increased even 22 hours after abdominal injection of carbon tetrachloride. Likewise, LPO levels in liver and

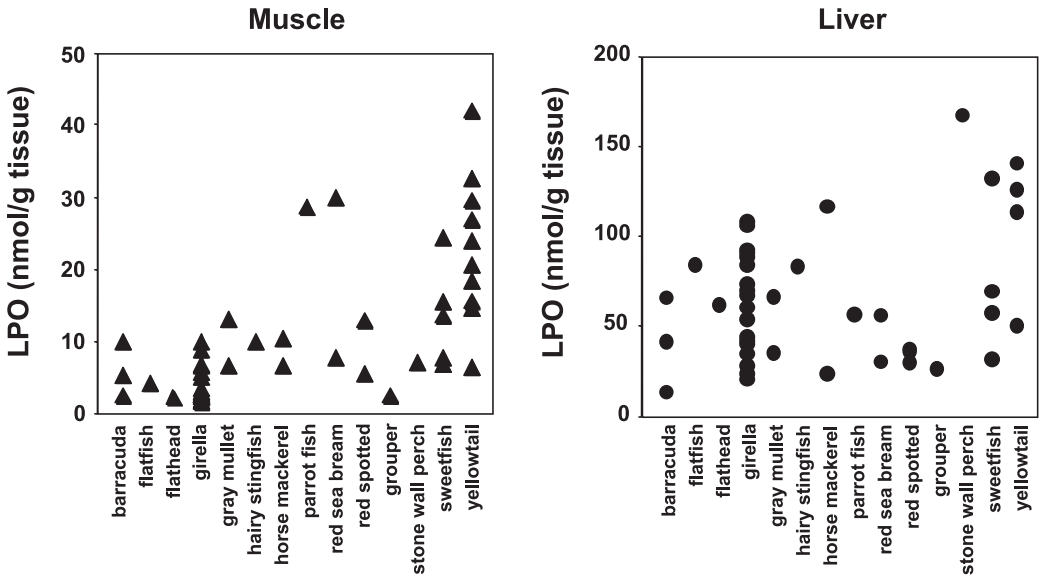

Fig. 1. LPO levels in mascle and liver of wild normal fish analyzed by using TP-method ▲, muscle; ●,liver.

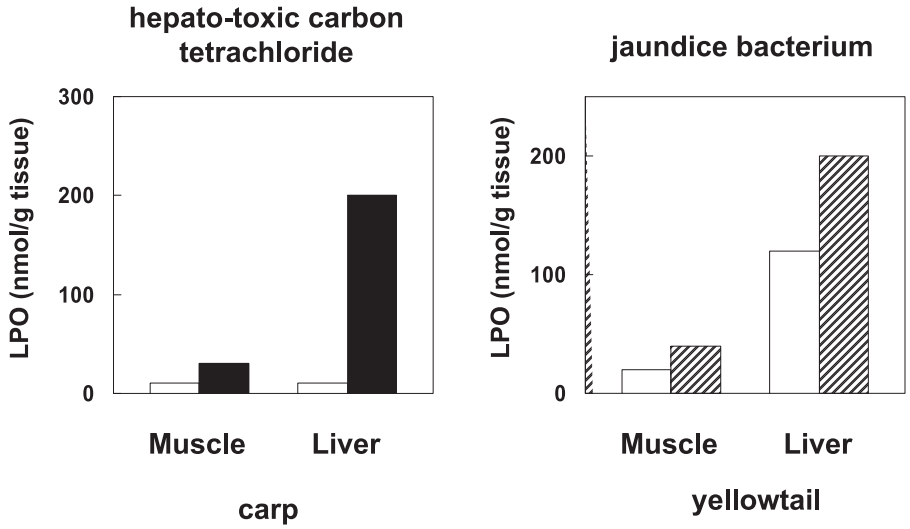

Fig. 2. Effects of hepatotoxins, carbon tetrachloride on carp and jaundice-bacterium on yellowtail. □, control; ■, carbon tetrachloride injected carp; ▨, jaundice-bacterium injected yellowtail

lateral muscle of the yellowtails increased after the abdominal injection of the bacterial culture.

Separation of anthroyl L-OH by reversed phase HPLC

The fluorescence derivatives of L-OH in liver of yellowtail were separated by reversed phase HPLC and typical chromatograms are shown in Fig. 3. The peak-area from the 31-min peak of the 1-hexadecanol derivative (internal standard) to the 41-min peak of the intact cholesterol derivative; tentatively defined as L-OH, was integrated.

LPO and L-OH Level in tissues of red sea bream with administration of hepatotoxic carbon tetrachloride

Effects of carbon tetrachloride, an exogenous hepatotoxin, on red sea bream are shown in Fig. 4. Although LPO levels in the liver were not increased significantly ($P<0.05$ by Student-t test) after carbon tetrachloride injection, the L-OH level in the liver remarkably increased from 343±7 to 223 x 10±120 (mean±standard deviation, nmol/g tissue). In the muscle, LPO and L-OH levels in the inflamed portion (carbon tetrachloride injected-portion) were the

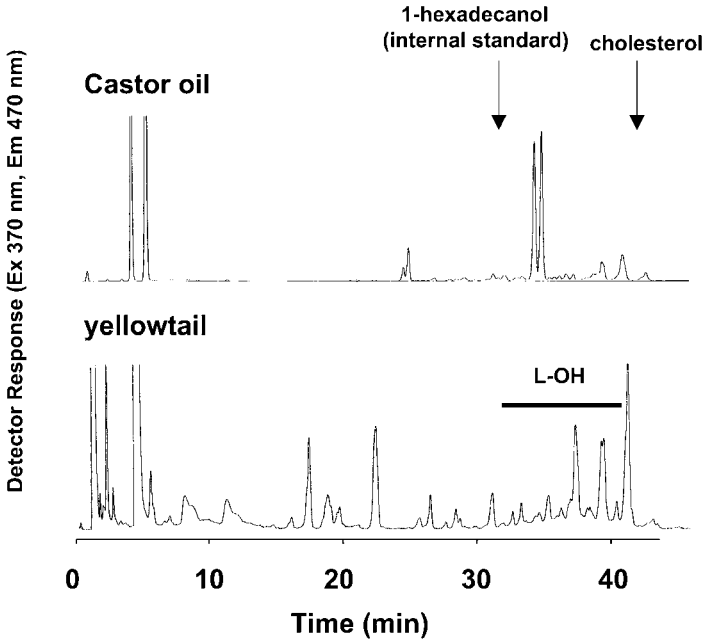

Fig. 3. HPLC chromatogram of anthroyl derivatives of L-OH from liver of cultured yellowtail and castor oil. Anthroyl derivatives of hydroxy lipids were detected fluorometrically with reversed-HPLC method. L-OH eluted between 1-hexadecanol (internal standard) and cholesterol, corresponding to a retention time of *ca.* 31-40 min depending on the agreement of their retention time with that of authentic hydroxy triglycerides such as castor oil that contained ricinolic acid.

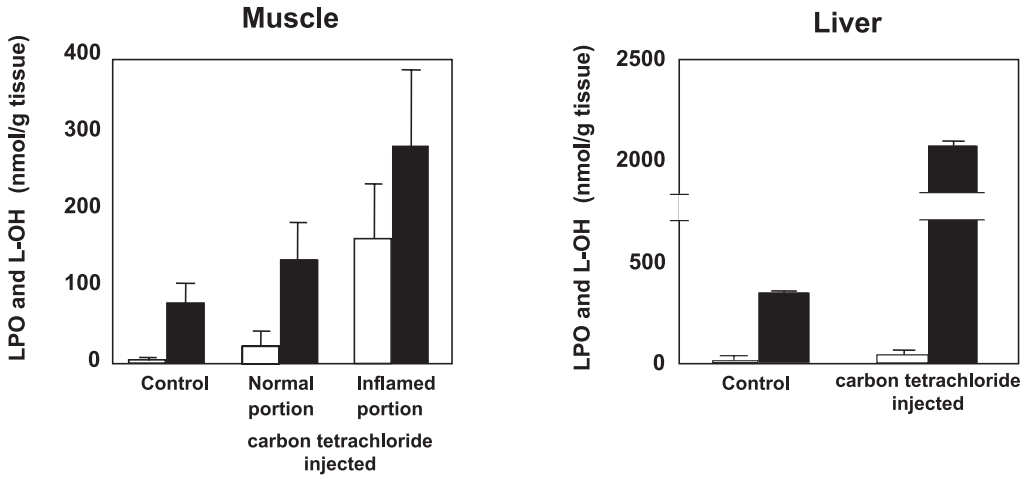

Fig. 4. Elevation of LPO and L-OH levels in tissues of red sea bream after injection of carbon tetrachloride. □, LPO (nmol/g tissue) ; ■, L-OH (nmol/g tissue). Sample number : n=3 for control and n=4 for carbon tetrachloride injected fish. Muscles of carbon tetrachloride -injected fish were divided into normal and inflamed portions, and lipids were extracted. Columns and bars represent the means± SD.

highest. The L-OH level was increased from 79±28 in control fish to 275±119 nmol/g tissue in fish that had been inflamed by the carbon tetrachloride injection.

LPO and L-OH Levels in tissues of cultured and wild yellowtails

HPLC chromatograms of L-OH and L-OH and LPO levels in tissues of the yellowtails are shown in Fig. 5 and Fig. 6, respectively. The highest levels of L-OH were in the liver and seem to reflect the oxidative environment of this tissue. The levels of L-OH in the liver were much higher than those of LPO, and indicated an accumulation of L-OH in this tissue after reduction of LPO to L-OH. L-OH levels in each tissue of the cultured yellowtails were higher than those in that of wild fish. This tendency was also observed in other fish, although the levels corresponding to

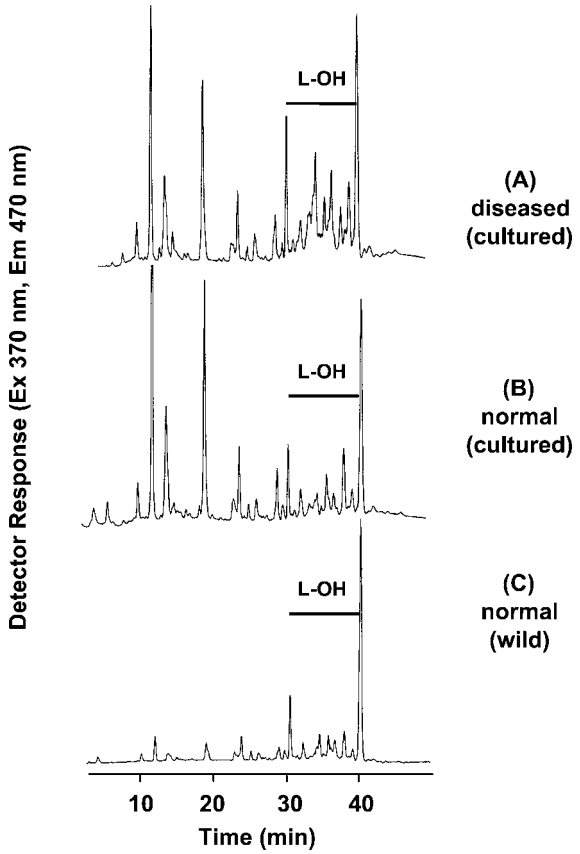

Fig. 5. Separation of anthroyl derivatives of L-OH in the liver of normal and diseased yellowtails. (A) Streptococcal-infected yellowtail; (B) normal yellowtail cultured with the same feeds as (A); (C) wild normal yellowtail. Strong peaks at 31 and 37 min in the chromatograms represent 1-hexadecanol as internal standard and cholesterol, respectively. Hydroxy lipid peaks eluted between the two peaks were defined as L-OH based on the simultaneous analysis of standard hydroxy triglycerides.

each tissue were much lower than those of yellowtail were.

Elevation of L-OH level in liver of diseased fish

The increases in L-OH levels in the liver of other diseased fish mentioned above were investigated and the results are illustrated in Fig. 7. In all diseased fish; carp infected with cold water disease, globefish infected with *H. okamotoi* and its complication (emaciation disease), red sea bream infected with *Edwardsiella* and its complication (*Vibrio*), yellowtail infected with jaundiced disease, and wild sweetfish with ulcer disease the level of L-OH was significantly higher in comparison with that in normal fish cultured with the same cultural conditions. Generally, in all fish tested, L-OH and LPO levels in wild fish whose diets could not be specified were distinctly lower than those of cultured fish were.

Elevation of LPO and L-OH levels of Hepa-T1 cells induced by AAPH and H_2O_2

The fluorescence derivatives of L-OH in Hepa-T1 cells were separated by reversed phase HPLC and a typical chromatogram is shown in Fig. 8. The peak-area between the 12-min peak of the 1-hexadecanol derivative (internal standard) and the 30-min peak of the intact cholesterol derivative, defined as L-OH, was integrated.

The effects of oxidative stressors on Hepa-T1 cells are

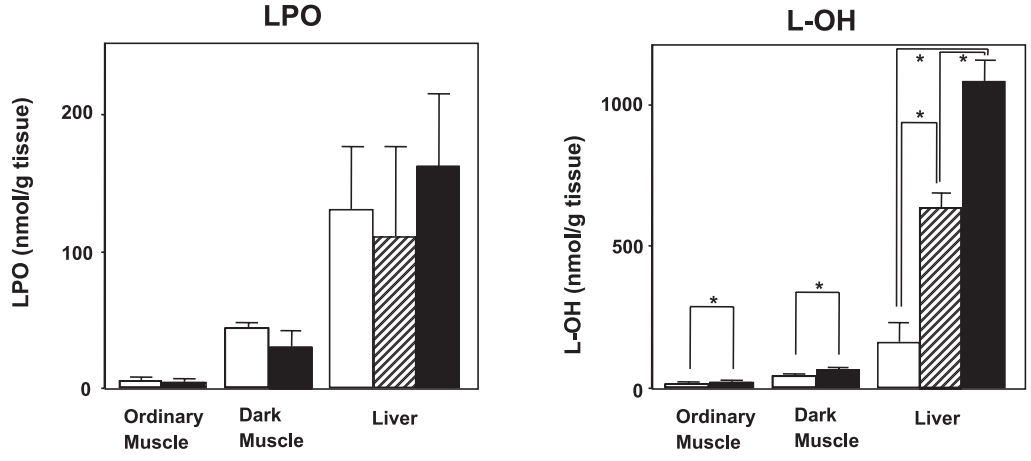

Fig. 6. LPO and L-OH levels in muscle and liver of cultured normal and diseased, and wild normal yellowtails. □, wild normal yellowtail (n = 4); ▨, normal cultured fish (n = 2); ■, streptococcal infected yellowtail culturedwith the same feed as the normal fish (n = 3). Columns and bars represent meanFstandard deviation, nmol/g tissue. An asterisk means a significant difference from each category at $P<0.05$ by using one-way ANOVA and Scheffe's test.

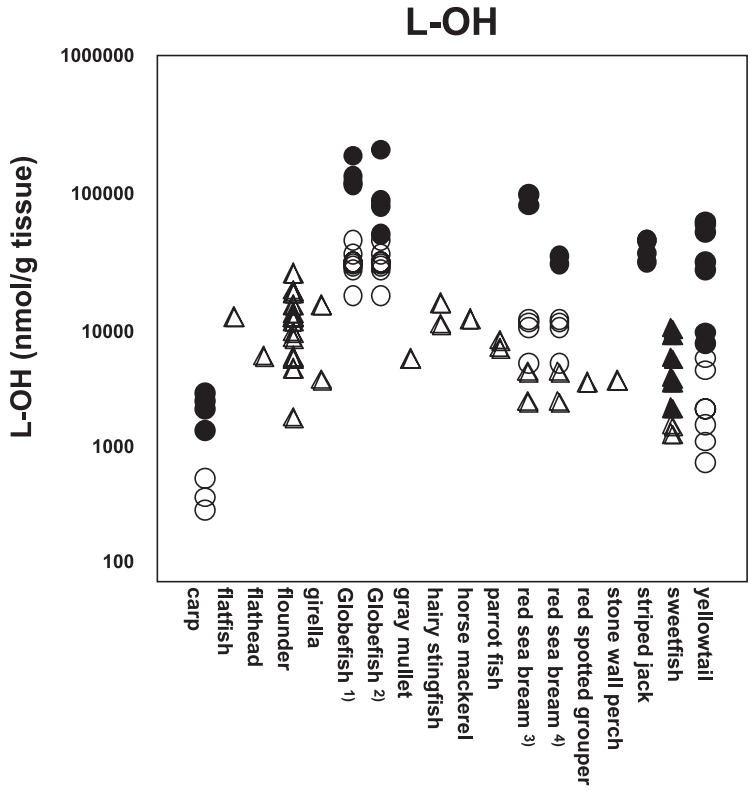

Fig. 7. L-OH levels in liver of normal and diseased fish. □, wild normal fish; ▲, wild diseased fish caught at the same place as the normal wild fish; ○, cultured normal fish; ●, cultured diseased fish reared at the same cage as the normal fish.[1]) Globefish infected with *H. okamotoi*;[2]) globefish infected with complication of *H. okamotoi* and emaciation disease;[3]) red sea bream infected with Edwardsiella;[4]) red sea bream infected with complication of Edwardsiella and *Vibrio*.

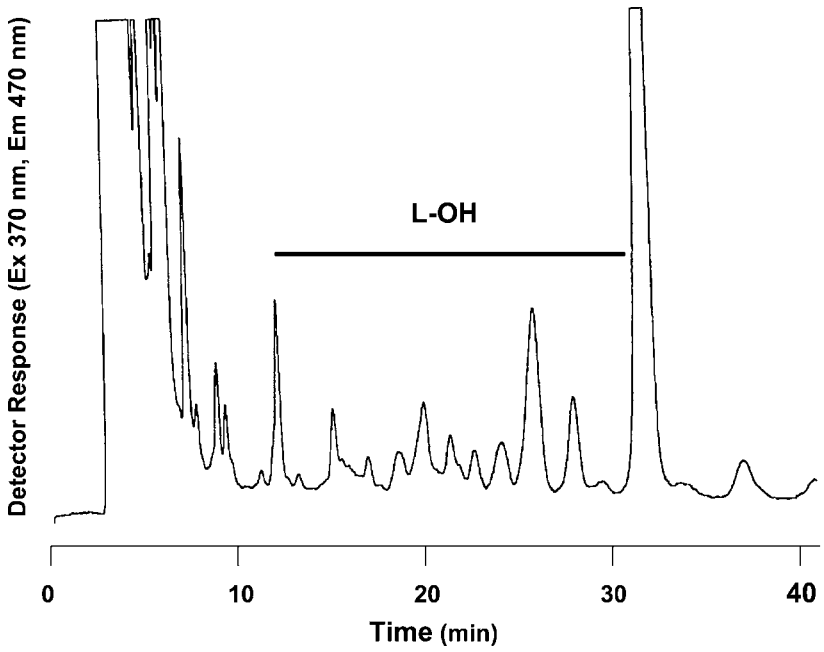

Fig. 8. HPLC chromatogram of anthroyl derivatives of L-OH from liver of Hepa-T1 cells treated with AAPH. Anthroyl derivatives of hydroxy lipids were detected fluorometrically with reversed-HPLC method. L-OH eluted between 1-hexadecanol (internal standard) and cholesterol, corresponding to a retention time of *ca.* 12-30 min depending on the agreement of their retention time with that of authentic hydroxy triglycerides such as castor oil that contained ricinolic acid.

shown in Fig. 9. After the administration of various concentration of AAPH or H_2O_2 for 2 hours to Hepa-T1 cells, the rate of survival were 80% at 5 mM of AAPH or H_2O_2 respectability and suddenly decreased at administration of 5 mM over. This concentration induced highly oxidative stress to Hepa-T1 cells in this experiment. Therefore, to investigate the relationship between lipid oxidation and the activity of antioxidative enzymes, 5 mM of AAPH or H_2O_2 were used for the following assays. LPO and L-OH levels increased time-dependently and significantly after the addition of 5 mM AAPH: the L-OH level increased remarkably from 7.6±0.7 to 23.6±1.8 (mean±standard deviation, nmol/mg protein) after 2 hours exposing. In the case of H_2O_2 treatment, although the LPO levels were increased slightly after 2 hours exposing, no significant change was observed in the L-OH levels through the experimental period.

Antioxidative enzyme activity of Hepa-T1 cells induced by AAPH and H_2O_2

After the administration of AAPH, GPx activity increased time-dependently and significantly from 259±106 to 738±125 mU/mg protein (1 hour) and 1,970±135 mU/mg protein (2 hours) whereas SOD activity did not change and CAT activity was remarkably decreased after 2 hours (Fig.10). By treatment with H_2O_2, CAT activity increased time-dependently and significantly from 217±21 to 647±106 mU/mg protein (1 hour) and 2,506±120 mU/mg protein (2 hours), while the SOD activity increased twice after 1 and

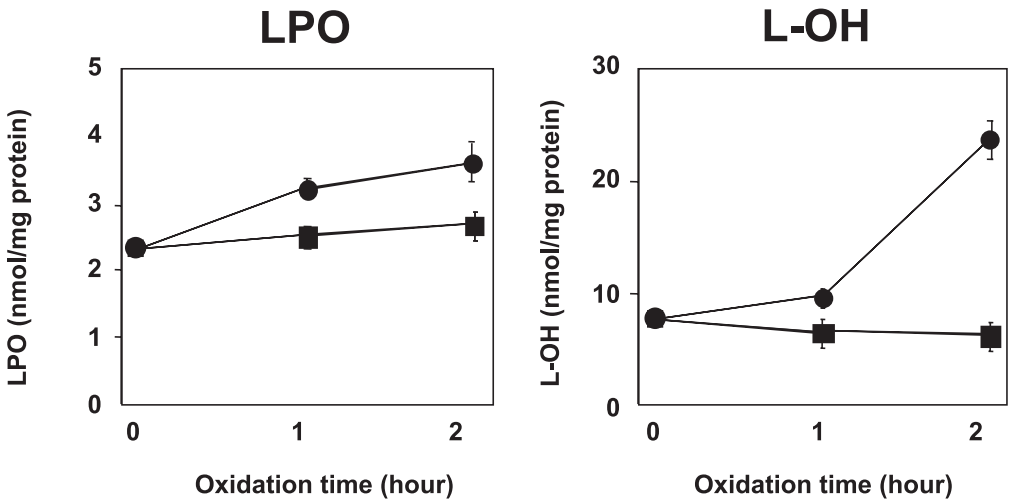

Fig. 9. Changes in LPO and L-OH levels of Hepa-T1 induced by AAPH and H_2O_2. Hepa-T1 treated with 5 mM AAPH ($n=3$) ●, and Hepa-T1 treated with 5 mM H_2O_2 ($n=3$) ■. Points and bars represent the mean ± standard deviation. Superscripts a and b means a significant difference from the pre-administration at $P<0.05$ by Student's t-test.

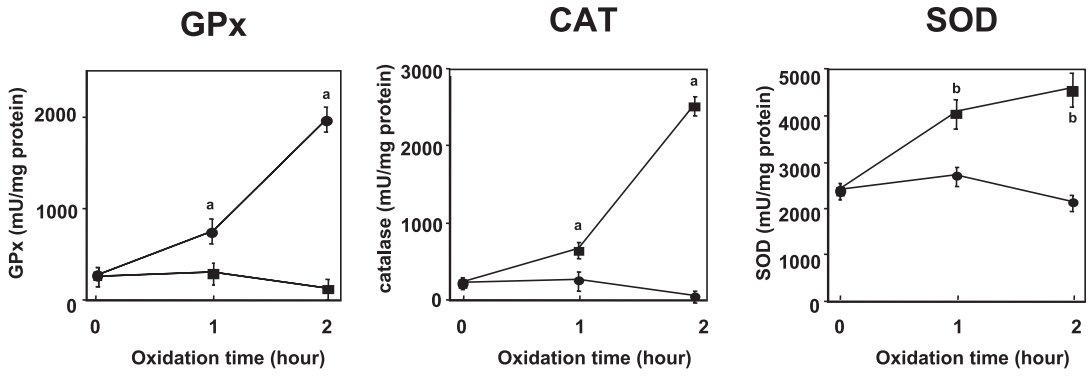

Fig. 10. Changes in antioxidative enzymatic activity of Hepa-T1 induced by AAPH and H_2O_2. Hepa-T1 treated with 5 mM AAPH ($n=3$) ●, and Hepa-T1 treated with 5 mM H_2O_2 ($n=3$) ■. Points and bars represent the mean ± standard deviation. Superscripts a and b indicate a significant difference from the pre administration at $P<0.05$ by Student's t-test. CAT, catalase; GPx, glutathione peroxidases; SOD, superoxide dismutase.

2 hours. However, GPx activities were decreased after 2 hours.

Discussion

LPO levels *in vivo* may increase reflecting the physiological conditions induced by oxidative stress such as pathological conditions and/or tissue injuries.[1, 19-23] Even under the normal physiological conditions, reactive oxygen and free radicals are generated in mitochondria, capillaly endothelium, peroxisomes in almost all tissues, microsomes in liver, and so on.[24, 25] Normally, the reactive oxygens produced are scavenged by in vivo antioxidant systems, and lipid levels are kept at a steady-state. However, when production exceeds the scavenging capacities of cellular defense systems, lipid peroxide levels in living individuals may increase up to certain levels. As shown in Fig. 1, usually, the levels in lateral ordinary muscle of normal fish were distributed between 10 to 50 nmol/g tissue. Much higher levels, over 100 nmol/g tissue which reflect higher physiological activity, were present in liver and levels increased following injection of carbon tetrachloride or the bacterial culture.

In this study, in addition to LPO levels, the author determined L-OH levels in several antioxidative statuses of many kinds of fish using fluorometric HPLC. We verified the accumulation of L-OH in diseased, normal cultured, and wild live fish by use of a fluorometric HPLC. As shown in Fig. 1, many peaks of anthroyl derivatives of hydroxy lipids were detected in both normal and diseased fish. L-OH that eluted between 1-octadecanol (internal standard) and intact cholesterol, which were tentatively identified as L-OH, were our main focus. L-OH levels in liver of all diseased fish tested in this study were significantly higher in comparison with those of normal fish cultured with the same feed at the same cage. A similar trend was observed in the levels of LPO; however, the elevation was not always significant. Some fish diseases, such as muscular dystrophy, hemolysis and jaundice, are known to be closely related to lipid peroxidation and thought to be catalyzed by oxidative damage to tissue. Ploch et al.[26] induced oxidative stress in fish by treating them with tert-butyl hydroperoxide and they confirmed the total depletion of glutathione and an increase of LPO in the liver of channel catfish *Ictalurus punctatus* and brown bullhead *Amerurus nebulosus*. However, no previous reports have mentioned an elevation in the level of hydroxy lipid in vivo in response to oxidative stress. Generally, when the production of reactive oxygen exceeds the scavenging capacities of the cellular defense system, LPO levels in living individuals increase up to some maximum level. However, since LPOs are unstable and hazardous, they are reduced to more stable and nontoxic products like hydroxy lipids shortly after their formation. In other words, the LPO level may reflect current or differential peroxidative conditions and the L-OH level may reflect historical or cumulate peroxidative conditions during the recent past in the living body. For these reasons, the hydroxy lipid level is estimated to be superior to the LPO level as an index of peroxidative conditions in the living body of not only fish but also humans.

Additionally, in order to investigate the relationships between LPO and L-OH, and antioxidative enzymes in live fish, the author used Hepa-T1. Antioxidative enzymes such as GPx, CAT and SOD are the primary defenses against oxidative cellular damage. AAPH, which is capable of initiating lipid peroxidation, elevated GPx but not CAT or SOD activity, and L-OH levels increased significantly (Fig.10). This indicates that GPx served to protect against oxidative cell damage of LPO by converting them to more inactive L-OH. In the evaluation of *in vivo* lipid peroxidation, oxidation products from phospholipids in the cellular membrane, which include large amounts of PUFA, should be considered. Phospholipid hydroperoxide (P-OOH) was estimated as a part of the LPO in this study, while its reduced products such as hydroxy phospholipid (P-OH) were not determined in this study because an analytical method has yet to be established. However, levels of LPO including P-OOH were not so high as those of L-OH as shown in Fig. 9. P-OOH, if produced, most of them in the cells were converted to P-OH by phospholipid hydroperoxide GPx (PHGPx), which is believed to be present mostly in cytosolic and partly membrane-bound forms in animal tissues.[14] The elevation of GPx activity in Fig.10 may depend partly on PHGPx activity, since the assay used in this experiment can not differentiate PHGPx and GPx activities and generally PHGPx activity is much lower than that of GPx in cultured cells and in tissues.[27] Imai et al re-

ported that over expression of PHGPx in rat basophile leukemia cells suppress the peroxidation of membrane lipids such as phosphatidylcholine induced by AAPH administration and concluded that PHGPx can prevent cell death in response to extracellular by a lipid peroxide.[28] Further, in live fish, Sakai et al[29] reported that when yellowtails were fed on a diet containing LPO, GPx activity in liver was significantly elevated. Murata et al[24] reported that GPx activity in liver of yellowtails were significantly higher in cultured fish than in wild fish. These reports also support a close correlation of elevation of GPx activity in Hepa-T1 with the elevation of that in liver induced by oxidative stress. Either way, GPx played an important role in the conversion of LPO to L-OH like hydroxy triglycerides, and thereby resulted in the accumulation of L-OH in Hepa-T1 cells exposed to AAPH. On the other hand, exposure of the cells to H_2O_2 stimulated CAT and SOD remarkably but not GPx, and no elevation of L-OH level were observed. These results also support a close correlation of GPx with the accumulation of L-OH. Usually, cytotoxic H_2O_2 itself can not initiate lipid peroxidation, but reactive oxygen species (ROS) induced by H_2O_2 are known to cause critical cell damage including lipid peroxidation.[30] Therefore, SOD, which does not catalyze H_2O_2 decomposition, may be stimulated as a defensive enzyme to eliminate ROS like a superoxide anion. In addition H_2O_2 did not elevate GPx activity, which is capable of reducing H_2O_2 as well as CAT under normal concentrations of H_2O_2 in living cells.[31] This is probably because CAT activity is predominantly stimulated in the cultured cells surrounded by a high concentration of H_2O_2.[31] Under the our experimental conditions, H_2O_2 should be reduced by CAT preferentially rather than GPx. CAT and SOD play roles cooperatively in protecting living Hepa-T1 cells from cytotoxic H_2O_2, but there is no evidence that they are involved in converting LPO to L-OH. In this report, Hepa-T1 cells treated with AAPH or H_2O_2 as oxidative stressor. AAPH are excellent free radical initiators for the quantitative study of lipid peroxidation, because they are able to generate peroxyl radicals at a known and constant rate and at a specific site.[32]

In conclusion, oxidative statuses of live fish in healthy and diseased conditions were shown clearly for the first time using new indices of LPO and L-OH. Especially, the findings of cumulative accumulation of the hydroxy lipids in liver with oxidative stress are noteworthy. Additionaly, L-OH level is closely associated with the increase of GPx activity. These results obtained cleared the first step elucidating the detoxification pathway of peroxidized lipids *in vivo*.

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