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# In the presence of ferritin, visible light induces lipid peroxidation of the porcine photoreceptor outer segment

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**Abstract**

We studied the synergistic effect of visible light and ferritin on the lipid peroxidation on a fraction of porcine photoreceptor outer segment (POS). Reaction mixtures containing the POS fraction and horse spleen ferritin were irradiated under white fluorescent light mainly at 17,000 *lx* or incubated under dark conditions at 37°C. The lipid peroxidation was evaluated by both the thiobarbituric acid method and the ferrous oxidation/xylenol orange method. The irradiation-induced lipid peroxidation was affected by some experimental factors such as the irradiation dose and acidity of the material. When the irradiation was stopped, the lipid peroxidation was also stopped; thereafter, the re-irradiation induced lipid peroxidation. Moreover, this lipid peroxidation was inhibited by desferrioxamine, an iron chelator, or by dimethylthiourea, a hydroxyl radical scavenger, suggesting that the lipid peroxidation involves hydroxyl radicals generated via the Fenton reaction by iron ion released from ferritin. The lipid peroxidation did not take place under dark conditions or in the absence of ferritin. This study suggested the possibility that the visible light-induced lipid peroxidation of the POS fraction in the presence of ferritin may participate in the etiology of human retinal degenerative diseases as the human retina is exposed to light for life.

**Keywords:** *Ferritin, iron release, lipid peroxidation, photoreceptor outer segment, retina, visible light*

## Introduction

Living tissue is in the presence of light for more than half of its lifetime. In particular, the retina [1–3], the lens [4], and the skin [5] may suffer from the photic injuries from such long exposures. It is well known that the retinas of Primates as well as rodents are at the risk of photic injury by visible light irradiation. The main retinal photic injury is composed of the massive destruction of the outer retina (photoreceptor cell nuclei and photoreceptor segments) through photoreceptor cell apoptosis [6–8]. Though, there are several mechanisms proposed for retinal photic injury, the lipid peroxidation explanation has attracted much attention because the retina, especially the photoreceptor outer segment (POS), contains a large amount of polyunsaturated fatty acids, especially docosahexaenoic acid (22:6, $n-3$; DHA) [9], which are very susceptible to lipid peroxidation [9,10]. Indeed, intervention of lipid peroxidation was proven in the POS of rats after constant light irradiation [11].

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It is clinically well known that the iron-containing foreign bodies retained in the eye may result in retinal degeneration associated with the constricted visual fields and the reduction of electro-retinogram amplitudes (ERG amplitudes) (*ocular siderosis*). Hiramitsu et al. found that following surgical implantation of an iron nail in rabbit vitreous, retinal lipid peroxides increased concomitant with ERG amplitude reduction [12]. The increase of lipid peroxides can account for the mechanism of the severe retinal degeneration clinically caused by iron injury.

Recently, Yefimova et al. demonstrated that ferritin and iron showed the same distribution pattern in photoreceptor cells of rat eyes [13]. There are several studies to illustrate the relationship between retinal photic injury and iron. For example, Li et al. [1] reported that albino rats given intraperitoneally desferrioxamine before light exposure, the intense fluorescent light-induced disappearance of photoreceptor cells was suppressed. Further, Organisciak et al. [3] reported the protective effect of dimethylthiourea, a hydroxyl radical scavenger, on the retinal photic injuries in albino rats. Intravitreal implantation of iron particles in rat eyes without light exposure also induces apoptosis limited to photoreceptor cells like photic injury [14]. Thus, they suggested that free iron and hydroxyl radicals ($^{\cdot}OH$) are important mediators in retinal photic injuries.

Iron is distributed as one of essential vital elements in cells and tissues and plays an important role in the $^{\cdot}OH$ generation via the Fenton reaction. The free radicals lead to lipid peroxidation in membranes and injury to cells and tissues. At the same time, a large proportion of iron in tissues is stored in the form of ferritin and haemosiderin preventing $^{\cdot}OH$ generation via the Fenton reaction [15]. However, ferritin also acts as an iron source for the lipid peroxidation by the released iron from ferritin [16]. It was shown that iron could be released from ferritin by biological molecules such as urate, ascorbate, citrate, bicarbonate, lactate and apo-transferrin [3]. Since superoxide radical anion ($O_2^{\cdot-}$) causes the iron release from ferritin, the harmful inflammatory effects on tissues are not only attributed to the toxicity of $O_2^{\cdot-}$ but also to the $^{\cdot}OH$ produced by the secondary reaction induced by the released iron [17]. In our recent study, we found that the irradiation of ferritin in the presence of ADP with white fluorescent light can induce iron release from ferritin [18]. The iron released from ferritin by the white light irradiation might induce the free radical generation and lipid peroxidation *in vitro* and *in vivo*. In this study, we clarified that visible light can induce lipid peroxidation of the porcine POS fraction in the presence of ferritin.

(Part of this work was published in the proceedings of the 1st Asian Conference on Photobiology [19]).

## Materials and methods

### *Materials*

Horse spleen ferritin (lot no. 90K7039) was obtained from Sigma Chemical Co. (St Louis, MO, USA), containing *ca.* 1060 iron atoms per molecule [18]. Desferrioxamine mesylate was purchased from Ciba Giegy (Takarazuka, Japan). 5,5′-dimethyl-1-pyrrolline *N*-oxide (DMPO) was purchased from Nacalai tesque, Inc. (Kyoto, Japan). All other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). To minimize contamination by the transition metal ions, all reagents were prepared in purified water with specific resistance of 18.0 MΩ-cm Milli-Q SP reagent water system, Millipore Co., (Bedford, MA, USA) [20].

Fresh porcine eyes were obtained from a local slaughterhouse. They were placed on ice under dark conditions, and transported to the laboratory for subsequent preparation and analysis. In this study, we used Tris–maleate buffer solution, because our previous study showed that the Tris–maleate buffer solution had low inhibitory effects on iron-induced lipid peroxidation due to the weak $Fe^{2+}$-binding activity and the low $Fe^{2+}$-autoxidation-accelerating ability [21].

### *Preparation of porcine photoreceptor outer segment (POS) fraction*

The porcine POS fraction was prepared by a modification of Anderson's procedure [22]. Retinas were removed from the porcine eyes, and ten fresh retinas were homogenized in 15 ml of 10 mM tris buffer solution (pH 7.35) containing 1.17 g/ml sucrose, 0.42% NaCl and 2 mM $MgCl_2$ by five strokes of a Teflon pestle in a glass homogenization tube. The homogenate was transferred to a 94 ml cellulose nitrate centrifuge tube, leading to overlaying 15 ml buffer solutions containing 1.13 and 1.11 g/ml sucrose, respectively. Membranes were separated by the centrifugation in an RP42 rotor (himac CP56G, Hitachi Koki Co., Ltd., Tokyo, Japan) at 32,000 rpm for 60 min. The fluffy pink layer at the 1.11/1.13 interface was removed and diluted with 50 ml of the Tris–acetate buffer solution (pH 7.4) containing 5 mM $MgCl_2$. A pellet of the membranes was prepared by centrifuging the solution at 12,000 rpm for 20 min. The pellet was twice washed with the buffer solution and centrifuged again at 12,000 rpm for 20 min. The pellet was resuspended in 10 mM Tris–maleate (pH 7.0). The protein concentration of the pellet was determined by the Bradford method [23] using the Bio-Rad protein assay kit (Bio-Rad laboratories, Hercules, CA, USA). The POS solution was adjusted to 5.0 mg protein/ml. All procedures were carried out at 4°C under low light conditions.

*Light irradiation and incubation*

Irradiation was carried out by a white fluorescent light (27 W; FML27EX-N; National Co., Osaka, Japan). The irradiance of the light was adjusted by altering the distance (17,000 *lx* at the light-sample distance of 10 cm), and was estimated with an irradiance meter (IM-3, Topcon Co., Tokyo, Japan). Dark controls (0 *lx*) were obtained by covering the samples with an absorbing black plastic sheet.

The reaction mixture (100 μM) contained horse spleen ferritin (0.5 mg/ml) and the POS fraction (0.5 mg protein/ml) in 20 mM Tris–maleate buffer solution at an indicated pH value, adjusted with 1 M NaOH at the incubation temperature. The reaction mixture was placed into a well in a 96-well microplate. Irradiation of the incubation mixtures was performed at 37°C in a water bath.

*Lipid peroxidation assay*

The lipid peroxidation was evaluated by two assay methods, i.e. thiobarbituric acid (TBA) method [24] and ferrous oxidation/xylenol orange (FOX) method [25]. The TBA method is one of the most widely used tests for measuring the aldehydic lipid peroxidation products, i.e. malondialdehyde (MDA), as TBA reactive substance. The FOX method is one of lipid hydroperoxide (LOOH)-specific tests used increasingly in the last decade.

*Thiobarbituric acid method*. The reaction mixtures were mixed with 1.7 ml of 4.4% acetate–NaOH buffer solution (pH 3.5) containing 0.35% TBA, 0.5% SDS, 0.1% butylated hydroxytoluene and 27.5 μM EDTA, and boiled for 60 min. After the reaction, the solution was immediately cooled on ice. The reaction products were extracted with 2 ml of pyridine–*n*-butanol solution (1:15, v/v) by the application of the centrifugation at 3000 rpm for 10 min. The upper organic layer was carefully removed, and the absorbances at 535 nm corresponding to the TBA reactive substance peak formation were measured. In this study, 1,1,3,3-tetraethoxypropane was used as an external standard and lipid peroxide level was expressed as nmol/mg protein of MDA.

*Extraction of lipid hydroperoxide and ferrous oxidation/xylenol orange (FOX) method*. Lipid hydroperoxides (LOOH) in the incubated samples were extracted with ethyl acetate–methanol extraction [25]. Water containing 3.3 mM EDTA (300 μl) and methanol (250 μl) was added into the incubated samples (100 μl). After 10 s of vortex mixing, ethyl acetate (500 μl) was added and the mixture was re-vortexed. The suspension was centrifuged at 3000 rpm for 5 min. The 200 μl of the upper organic layer was then transferred to the glass test tube, and completely dried under nitrogen gas. LOOH contents in the incubated samples were estimated by the FOX method [25]. FOX reagent was prepared by dissolving xylenol orange and ammonium ferrous sulfate in 250 mM $H_2SO_4$ to the final concentration of 1 and 2.5 mM, respectively. The extracted LOOH fractions were dissolved with 450 μl methanol containing 4.4 mM butylated hydroxytoluene. The solution was added to 50 μl of FOX reagent and incubated at room temperature for 30 min. The absorbance of the solution was monitored spectrophotometrically at 560 nm. Cumene hydroperoxide was used for the standard curve of LOOH.

*Spin trapping technique using an electron spin resonance (ESR) spectrometer*

The spin trapping technique, a general method to convert transient free radicals to stable free radicals, was used to measure hydroxyl radical ($^{\cdot}OH$) formation. The spin trap used in this study was DMPO, which was purified by treatment with charcoal under the dim light. The sample solution containing 50 μM $Fe^{2+}$ without DMPO and dimethylthiourea was pre-incubated at 37°C under dark or irradiated conditions for 10 min. The defined concentration of DMPO and antioxidants was added into the sample solution. The sample solutions were incubated for 30 min. Eight microlitre of the incubating reaction mixture was removed, which was further transferred to a glass capillary tube (inner and outer diameters of 1.1 and 1.5 mm, respectively) at the middle of the capillary tube. After the capillary tube containing the sample was moreover put into a quartz sample tube, it was inserted into the cavity of an ESR spectrometer (FE2XG, JEOL Ltd., Tokyo, Japan). ESR measurement conditions were as follows: X-band frequency, 9.2 GHz; modulation width, 3.2 G; amplitude, 5000 times; response time, 1 s; sweep time, 1.6 min; magnetic field, 3280 ± 500 G; scan width, 100 G; temperature, at room temperature.

*Statistics*

All data are reported as arithmetic means ± S.D. Two sample comparisons were analyzed by the unpaired Student's *t*-test. The comparisons among three groups were analyzed by multiple comparison methods. One-way analysis of variance (ANOVA) was carried out to determine the statistical significance of the data. To establish the statistical significance of the differences between pairs of means, an *a posteriori* comparison was calculated using the Tukey's or Dunnett's *post hoc* test. A correlation within in each group was assessed with Pearson's correlation coefficient.

## Results

*Irradiation and ferritin-induced lipid peroxidation*

Before incubation, the formation of LOOH in sample solution (basic value of LOOH) containing porcine POS fraction was observed at 10.2 nmol/mg protein. With irradiation, the formation of LOOH was enhanced in the time-dependent fashion. For example, in the case of 4000 *lx*, the formation of LOOH was 19.3 nmol/mg protein for 20 min, 43.8 nmol/mg protein for 40 min and 57.4 nmol/mg protein for 60 min. This time-dependent increase of LOOH was also observed at 8500 and 17,000 *lx* irradiation (Figure 1A).

Without irradiation, only small amounts of MDA were observed in the sample (less than 3 nmol/mg protein) by keeping the sample in the dark. However, the irradiation markedly enhanced the formation of MDA time-dependently. For example, the irradiation of the sample at 4000 *lx* for 20 min produced the formation of MDA at 6.5 nmol/mg protein, following 11.0 and 15.4 nmol/mg protein for 40 and 60 min irradiation, respectively. Similar results were observed of 8500 and 17,000 *lx* irradiation (Figure 1B).

Next, we examined the light effect on the ferritin structure. If the irradiation induced the decomposition of ferritin structure, the iron storing activity of ferritin would be lost, leading to iron release (leakage) from ferritin even under dark conditions, resulting in the increase of the LOOH formation. When the sample solutions were irradiated for 10 min, a marked increase of LOOH and MDA was observed corresponding to more than five times compared to the baseline. After irradiation, the sample solutions were kept in the dark for 30 min and the LOOH and MDA levels were subsequently measured. As a result, no increase of LOOH and MDA were observed. Further irradiation of the sample solutions increased the LOOH level up to 84.2 nmol/mg protein and the MDA level up to 23.2 nmol/mg protein, respectively (Figure 2).

The pH conditions significantly influences MDA formation under irradiated conditions (Figure 3). Namely, an acidic condition corresponding to a pH 5.0, the irradiation produced 26.6 nmol/mg protein MDA. However at the basic level of a pH 8.0, the formation of MDA was the about half of that at a pH 5.0. On the other hand, the lipid peroxidation under dark conditions was not observed at the tested pH values (1.3–1.8 nmol/mg protein).

Irradiation with an optical filter (cut-off ≤ 390 nm) produced an MDA at 6.1 nmol/mg protein, while the irradiation without optical filter produced MDA at 13.1 nmol/mg protein (Figure 4). The formation of MDA with an optical filter is attributed to visible light irradiation. The difference of the values with and without an optical filter indicates the value induced by UV-A irradiation.

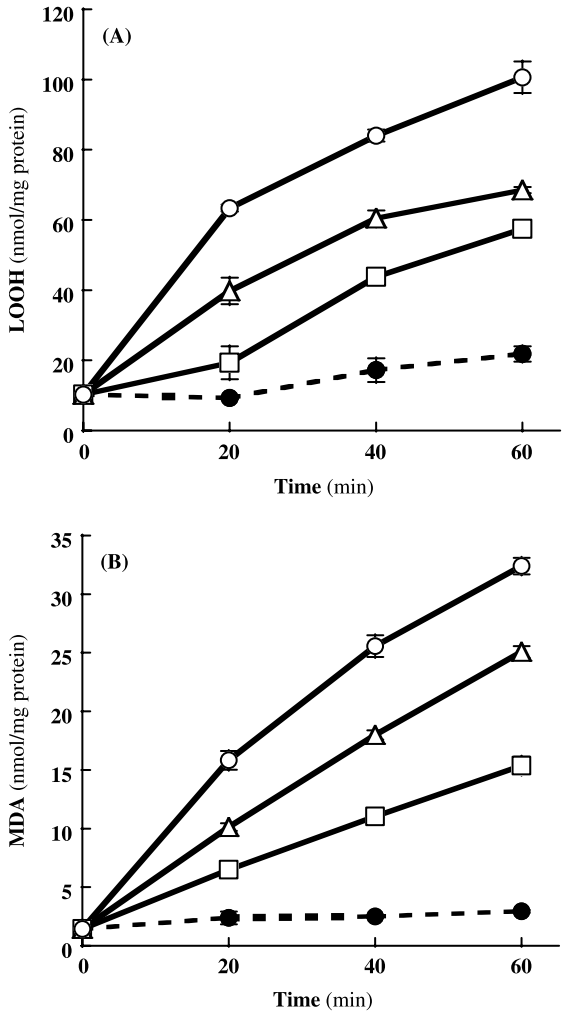


Figure 1. Time dependence of irradiation-induced LOOH (A) and MDA (B) formations in the POS solution in the presence of ferritin. The POS solution (0.5 mg protein/ml) containing ferritin (0.5 mg/ml) was incubated at pH 7.0 and 37°C. Solid lines show the values under irradiated conditions (open circle, 17,000 *lx*; open triangle, 8500 *lx*; open square, 4000 *lx*). Dashed lines show the values under dark conditions (closed circle). The incubated samples were subjected to the FOX method (A) and the TBA method (B). Values presented are arithmetic means ± S.D. of triplicates for LOOH and MDA concentrations.

*Effects of antioxidants*

To clarify the mechanism of irradiation-induced lipid peroxidation in the presence of ferritin, we examined the effects of antioxidants on the lipid peroxide formation. The results shown in Table I demonstrate the antioxidant effect on irradiation-induced lipid peroxidation in the presence of ferritin. An addition of 500 μM desferrioxamine completely inhibited the formations of LOOH (100%) and MDA (93%) in the reaction medium. Furthermore, 50 mM dimethylthiourea completely inhibited the LOOH formation (100%). Although, the inhibition by 50 mM dimethylthiourea of the MDA formation was only 16%, dimethylthiourea diminished the MDA

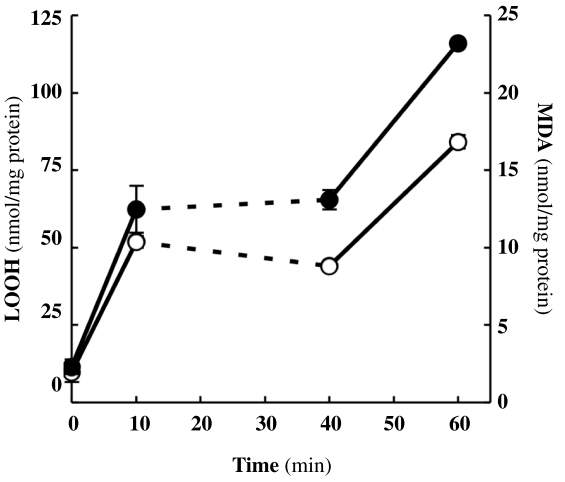


Figure 2. Effects of irradiation on time dependence of ferritin-mediated lipid peroxidation of the POS fraction. The POS solution (0.5 mg protein/ml) containing ferritin (0.5 mg/ml) buffered by 20 mM Tris–maleate was incubated at pH 7.0 and 37°C. Solid lines illustrate the values under irradiated conditions at 17,000 *lx*. Dashed lines show the values under dark conditions. Open circle corresponds to LOOH products; closed circle, MDA products. Values presented are arithmetic means ± S.D. of triplicates for LOOH and MDA concentrations.

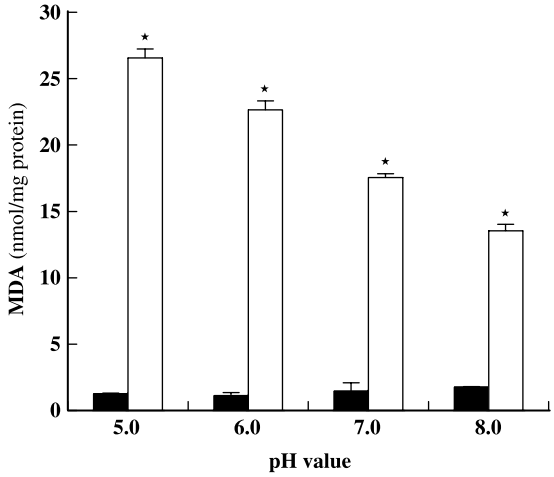


Figure 3. Effects of pH values on ferritin-mediated MDA formation of the POS fraction under irradiated conditions. The POS solution (0.5 mg protein/ml) with ferritin (0.5 mg/ml) buffered by 20 mM Tris–maleate was incubated at 37°C for 20 min. Open columns correspond to irradiated conditions at 17,000 *lx*; shaded columns, under dark conditions. Values presented are arithmetic means ± S.D. of triplicates for MDA concentrations. ⋆, $p < 0.05$ vs. each dark condition.

formation in a concentration-dependent manner ($IC_{50\%} = 202$ mM; Figure 5). Therefore, $^{\cdot}OH$ is involved in the LOOH and MDA formations. A water-soluble free radical scavenger, DMPO also diminished it in the concentration-dependent manner ($IC_{50\%} = 56$ mM; Figure 5). On the other hand, 1000 U/ml SOD, 1000 U/ml catalase, a mixture of 1000 U/ml SOD and 1100 U/ml catalase, and 50 mM sodium azide had no effect on the LOOH and MDA formations (Table I). These results indicate that $O_2^{\cdot -}$, $H_2O_2$, and $^1O_2$ may not be involved in the formation of LOOH and MDA.

### *Hydroxyl radical generation*

We examined the possibility of the $^{\cdot}OH$ generation using the ESR-spin trapping technique using DMPO. As an oxidant, 50 μM $FeCl_2$ was used, because the ferritin-mediated lipid peroxidation under irradiated conditions is attributable to $Fe^{2+}$ released from ferritin (refer to "Discussion" section). The ESR signal was detected in the $Fe^{2+}$-induced lipid peroxidation of POS in the presence of 100 mM DMPO (Figure 6A). The ESR signal shows only the 1:2:2:1 quartet signal due to DMPO–OH adduct with the coupling constants ($a^H = 1.49$ mT, $a^N = 1.49$ mT). This signal was not obtained from the decomposition of DMPO–OOH [26] or the iron-dependent nucleophilic addition of water to DMPO [27], because the signal was inhibited to 30% of control by 200 mM dimethylthiourea (Figure 6B).

Although, in the presence of ferritin, the irradiation did not induce ESR signal of DMPO–OH, which is explained by a small amount of free iron released from ferritin under irradiated conditions.

## Discussion

This work is the first evidence that visible light induces lipid peroxidation in the presence of ferritin. In order

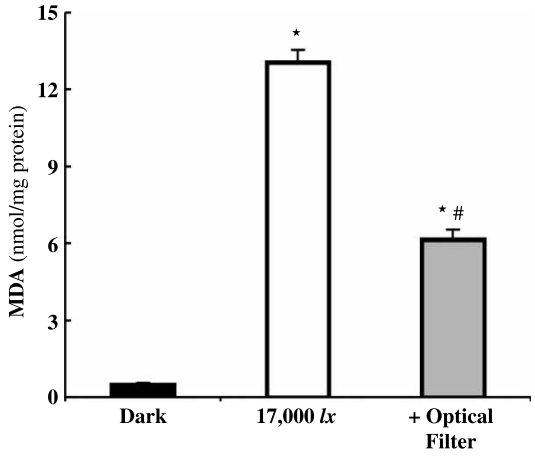


Figure 4. Effects of an optical filter on ferritin-mediated lipid peroxidation of POS fractions under irradiated conditions. The POS solution (0.5 mg protein/ml) with ferritin (0.5 mg/ml) buffered by 20 mM Tris–maleate was irradiated at 17,000 *lx* for 20 min at 37°C and pH 7.0 with and without an optical filter, which can truncate the wavelength range of light to less than 390 nm. Values presented are arithmetic means ± S.D. of triplicates for MDA concentrations. ⋆, $p < 0.05$ vs. dark condition; #, $p < 0.05$ vs. irradiated condition (17,000 *lx*).

Table I. Ferritin-mediated lipid peroxidation of POS under irradiated conditions.

| | LOOH (nmol/mg protein) | MDA (nmol/mg protein) |
|---|---|---|
| Complete system | 64.6 ± 5.3 (-) | 19.4 ± 0.7 (-) |
| −Light | 15.2 ± 1.6 (100%)** | 3.2 ± 1.7 (100%)** |
| −Ferritin | 10.2 ± 0.7 (≥100%)** | 0.6 ± 0.9 (≥100%)** |
| −POS | 13.1 ± 3.7 (≥100%)** | 0.0 ± 0.2 (≥100%)** |
| +SOD† | 60.4 ± 4.7 (8%) | 19.6 ± 0.9 (≤0%) |
| +Catalase† | 63.2 ± 6.2 (2%) | 21.7 ± 1.2 (≤0%) |
| +SOD/Catalase‡ | 75.1 ± 4.3 (≤0%) | 22.4 ± 0.2 (≤0%) |
| +Desferrioxamine¶ | 15.3 ± 1.3 (100%)** | 4.4 ± 0.3 (93%)** |
| +Dimethylthiourea§ | 8.4 ± 5.1 (≥100%)** | 16.8 ± 1.5 (16%)* |
| +Sodium azide§ | 63.0 ± 7.1 (3%) | 21.4 ± 0.5 (≤0%) |

*Note*: As complete system, samples contained 0.5 mg/ml ferritin and 0.5 mg protein/ml POS fraction in 20 mM Tris–maleate, pH 7.0. Light irradiation of 17,000 *lx* was carried out at 37°C for 20 min.
† 1000 U/ml.
‡ 1000/1100 U/ml.
¶ 500 μM.
§ 50 mM.
Values presented are arithmetic means ± S.D. of triplicates for LOOH and MDA concentrations and the suppressing rates in parentheses. 2mu*, $p < 0.05$; **, $p < 0.01$ vs. the complete system.

to analyze the lipid peroxidation by the synergistic effect of visible light and ferritin, we used the biological sample as a substrate, i.e. the POS isolated from porcine retinas. It is important to investigate the light-mediated oxidation of the POS fraction, because the exposure of the eyes to sunlight is one of risk factors of age-related macular degeneration (AMD) [28]. We have already reported in our recent study [18] that a white fluorescent light can induce iron release from ferritin in the presence of ADP. We found that the iron release from ferritin induced by visible light can proportionally induce lipid peroxidation of POS. Here, we discuss the synergistic effect of white light and ferritin on the lipid peroxidation of the POS fraction.

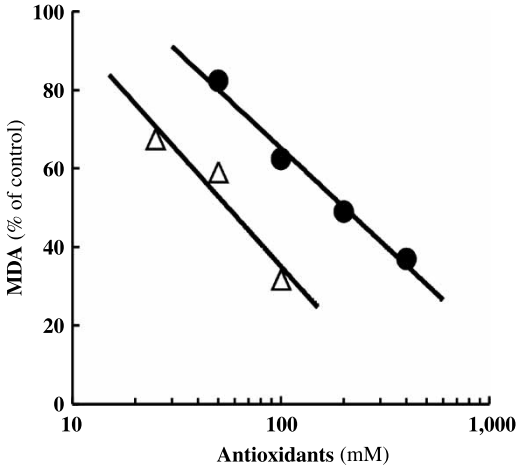


Figure 5. Effects of antioxidants on ferritin-mediated lipid peroxidation of the POS solution under irradiated conditions. The POS solution (0.5 mg protein/ml) with ferritin (0.5 mg/ml) buffered by 20 mM Tris–maleate was irradiated at 17,000 *lx* with dimethylthiourea (50, 100, 200 and 400 mM) or DMPO (25, 50 and 100 mM) at 37°C and pH 7.0 for 20 min. Closed circle correspond to dimethylthiourea; open triangle, DMPO. All results are subtracted by the amount of MDA formed under dark conditions, and expressed as percentages of the amount of MDA formed in control samples without dimethylthiourea.

Two independent assay methods, FOX and TBA methods, indicated that the lipid peroxidation was enhanced only under irradiated conditions. In addition, there was a proportional correlation between iron amount released from ferritin under irradiated conditions [18] and the amounts of LOOH and MDA formations ($r = 0.992$ and 0.999, respectively) (Figure 7). In on/off light switching effects, LOOH and MDA levels were suppressed by turning off the light, and the restart of the irradiation increased the LOOH and MDA levels (Figure 2). Moreover, we showed that the white fluorescent light using $a \leq 390$ nm cut-off filter could also induce the iron release [18] and the lipid peroxidation (Figure 4) (corresponding to 60.1% for the iron release [18] and 55.0% for the MDA formation of each control without an additional optical filter, respectively). Taken all these factors together, it is quite reasonable to consider that visible light participates in the iron release from ferritin and the subsequent formations of LOOH and MDA can be attributed to iron release from ferritin under irradiated conditions.

Aubailly et al. have reported the UV-A and ferritin-induced lipid peroxidation of human high-density lipoprotein (HDL) fraction [29]. In their study, the irradiance of UV-A is *ca.* 60 mW/cm$^2$ at 365 nm, this value is over 600 times higher than the irradiance at 365 nm in our study (*ca.* 0.09 mW/cm$^2$). Despite the much lower irradiance at 365 nm, our results showed that irradiation of 17,000 *lx* is enough to induce ferritin-mediated lipid peroxidation. As for this difference of irradiance needed for the lipid peroxidation, we believe that one of the most important factors might be the difference of fatty acid sample compositions between the two studies (i.e. porcine

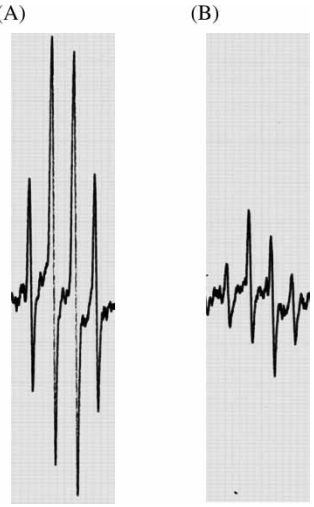


Figure 6. Effect of dimethylthiourea on the ESR signal of DMPO–OH. The 70 μl of the POS solution with $FeCl_2$ buffered by Tris–maleate was pre-incubated for 10 min at pH 7.0 and 37°C in the dark. Thereafter, DMPO (10 μl, 1 M) and/or dimethylthiourea (20 μl, 1 M) were added and further post-incubated for 30 min. Final concentrations in post-incubation were as follows: POS fraction, 0.5 mg protein/ml; ferrous ions, 50 μM; buffer ingredient, 20 mM; DMPO, 100 mM. Spectrum (A) corresponds to control, and spectrum (B), 200 mM dimethylthiourea addition. Under irradiated conditions, the similar ESR signal was detected as spectrum (A).

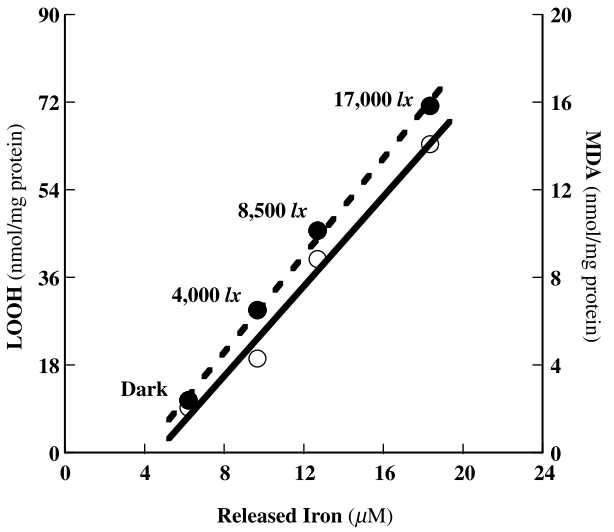


Figure 7. Correlation between released iron concentration and the amounts of LOOH and MDA products. The values of iron released from ferritin (the amounts of iron released from ferritin in the presence of 10 mM ADP at pH 7.0 and 37°C for 20 min: 6.2 μM in the dark, 9.7 μM at 4000 *lx*, 12.7 μM at 8500 *lx*, 18.3 μM at 17,000 *lx*) [18] and the values of LOOH and MDA formations of the POS solution after 20 min incubation under the various irradiated conditions were plotted. Solid line corresponds to the correlation between the released iron and LOOH products (open circle); Dashed line corresponding to the correlation between the released iron and MDA products (closed circle).

POS vs. human HDL). The retina, especially the POS is quite a special sample very susceptible to lipid peroxidation as it contains extremely high concentrations of DHA residues [9,10]. It is quite difficult to determine the precise reaction mechanism of the iron release from ferritin, however, it is worthy to describe not only UV-A light but also visible light can induce the iron release from ferritin and subsequent lipid peroxidation. This is the first evidence to clarify the participation of visible light in the induction of the ferritin-mediated lipid peroxidation.

In our previous study, the visible light-induced iron release from ferritin at neutral pH values needed the presence of ADP in the media as an iron chelator [18]. However, this visible light-induced lipid peroxidation did not require it. This suggests the possibility that acidic phospholipids such as phosphatidic acid and phosphatidylserine, as well as ADP, may chelate iron released from ferritin under irradiated conditions. Kuross et al. demonstrated the participation of non-haem iron with polar head groups of phosphatidylserine (but not phosphatidylcholine) in the lipid peroxidation [30]. Besides, Tadolini et al. showed that $Fe^{2+}$-induced lipid peroxidation occurred in liposomes containing phosphatidylcholine and phosphatidic acid (but not in single phosphatidylcholine liposome) [31]. This observation suggests the possibility that the presence of acidic phospholipids on the membrane would favor $Fe^{2+}$ binding and acidic-phospholipid/iron complexes leading to lipid peroxidation. Therefore, all these papers suggest that acidic phospholipids can chelate iron released from ferritin under irradiated conditions as observed in the case of ADP.

Irradiation and ferritin-induced formations of LOOH and MDA were completely inhibited in the presence of 500 μM desferrioxamine (Table I), indicating that the lipid peroxide formation was catalyzed by free iron released from ferritin, but not by the light-excited ferritin molecule (protein). The irradiation and ferritin-induced MDA formation was suppressed by dimethylthiourea as function of concentration (Figure 5), whereas the LOOH formation was completely inhibited by 50 mM dimethylthiourea (Table I). These results suggest that ·OH and iron are involved in this irradiation-induced lipid peroxidation of POS in the presence of ferritin.

However, the inhibiting effect of dimethylthiourea was very weak with a high $IC_{50}$ value of 202 mM (Figure 5). When DMPO, as a water-soluble free radical scavenger similar to dimethylthiourea, was applied to the irradiation and ferritin-induced lipid peroxidation, DMPO inhibited the MDA formation with an $IC_{50}$ value of 56 mM (Figure 5), and the value is nothing but one-forth of that of dimethylthiourea in spite of its broad reactivity against various free radicals [32]. These results suggest that water-soluble free radical scavengers such as dimethylthiourea and DMPO are not effective to inhibit the formation of MDA. If one can only suppress the ·OH produced in

aqueous media, the escaped ROS such as lipid peroxyl radical (or alkoxyl radical ($LO^{\cdot}$)) will have a chance to induce the formation of MDA under these experimental conditions. In support for this, we observed the formation of $^{\cdot}OH$ by ESR-spin trapping technique using DMPO, however, we could not detect the DMPO spin adduct of $O_2^{\cdot-}$ or $LO^{\cdot}$ under the ESR conditions (Figure 6). So, it is reasonable to consider that the formation of $^{\cdot}OH$ amount is bigger than the other radicals. However, we could not exclude the possibility of the formation of other radicals under the reaction conditions. Especially, the high $IC_{50}$ value of dimethylthiourea suggests the possibility that the $LO^{\cdot}$ or other radicals also contribute the formation of MDA. The high $IC_{50}$ value of dimethylthiourea is somewhat contributed to be vulnerability of highly unsaturated fatty acid residues in POS. Namely, once free radicals in POS are produced, a very rapid radical chain reaction is anticipated to take place. The inhibitory effect of dimethylthiourea is quite restricted because the participation coefficient of dimethylthiourea is quite low. In this meaning also, dimethylthiourea is not effective toward radicals produced in the lipid phase.

Hydroxyl radicals are produced by ultraviolet- or metal-catalyzed decomposition of $H_2O_2$ formed via superoxide dismutation reaction. However, the involvement of $O_2^{\cdot-}$ and $H_2O_2$ seems not to be mainly involved because SOD and catalase did not inhibit the reactions (Table I). Generally, iron-catalyzed LOOH degradation leads to $LO^{\cdot}$ formation (Reaction 1), not to $^{\cdot}OH$ formation (Reaction 2).

$$LOOH + Fe^{2+} \rightarrow LO^{\cdot} + {}^{-}OH + Fe^{3+} \quad (1)$$

$$LOOH + Fe^{2+} \rightarrow LO^{-} + {}^{\cdot}OH + Fe^{3+} \quad (2)$$

However, there are a few reports on iron-catalyzed $^{\cdot}OH$ formation from LOOH. Yagi et al. reported by the spin-trapping technique with ESR spectrometry that $^{\cdot}OH$ could be generated upon reduction of the LOOH [33] or oxidatively modified low-density lipoprotein [34] with $Fe^{2+}$ chelated by epinephrine. Besides, Chamulitrat et al. reported that, by the HPLC–ESR method, DMPO–OH spin adduct was detected by the methyl linoleic acid hydroperoxide reduction with iron, however, they explain that ~80–90% of the total DMPO–OH derived from water by an iron-dependent nucleophilic addition reaction [35]. We think that the ESR signal in Figure 6 with the 1:2:2:1 quartet signal is the DMPO–OH signal, and that it did not derive from the spontaneous decomposition of a $O_2^{\cdot-}$ reaction product, DMPO–OOH, [26] or the iron-dependent nucleophilic addition of water to DMPO [27], because the signal was inhibited by dimethylthiourea (Figure 6B), which does not react with $O_2^{\cdot-}$ [36]. Then, it suggests that the free radicals scavenged by dimethylthiourea are $^{\cdot}OH$, and that $^{\cdot}OH$ is generated via the LOOH reduction reaction by iron released from ferritin under irradiated conditions. However, we consider from the high $IC_{50}$ value of dimethylthiourea that the $LO^{\cdot}$ formation (Reaction 1) is a major reaction in this lipid peroxidation system instead of this $^{\cdot}OH$ generation via the LOOH reduction (Reaction 2).

It is difficult to determine whether or not ferritin is involved in the retinal photic injury *in vivo* at present. However, as mentioned in the introduction, it is suggested that iron and $^{\cdot}OH$ participate in the rat retinal photic injury. In human cases, due to direct sunlight exposure, people enjoying the outdoors during the daytime for long periods of time are quite susceptible in affecting retinal degeneration such as AMD [28]. This disease is the most popular cause of irreversible blindness in the USA in individuals older than 65 years [37]. This disease is known to increase the iron concentration in the macula as evidenced in postmortem AMD-affected retina [38]. The abnormality of the retinal iron transport mechanism is suggested to be related to the pathogenesis of AMD. Thus, the light exposure and the oxidative stress via iron are the common factors for human AMD and animal's acute photic injury.

Our studies (this study and [18]) demonstrated that visible light and ferritin act synergistically to induce the lipid peroxidation of the POS fraction through the Fenton reaction by iron released from the irradiated ferritin. This finding proves the possibility that retinal photic injury can mediate the oxidative stress stimulated by iron released from the irradiated ferritin in photoreceptor cells. Therefore, it is plausible that visible light and iron synergistically play an important role in the etiology of human retinal degenerative diseases.

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