Lipid Peroxidation in Linoleic Acid Micelles Caused by H$_2$O$_2$ in the Presence of Myoglobin

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We investigated the lipid peroxidation in linoleic acid micelles caused by H$_2$O$_2$, in the presence of metmyoglobin by monitoring the oxygen consumption. O$_2$ consumption usually consisted of two phases. In the first phase, it occurred slowly and linearly until the concentration of linoleic acid hydroperoxide reached a certain value, rapid consumption, presumably by a chain reaction, then followed in the second phase. No effects of diethylenetriaminepentaacetic acid (DTPA) on the induction period (the period during the first phase) and the maximum oxygen consumption rate (MOCR) in the second phase indicate that free ferric ions liberated from myoglobin had no role in any phases during the lipid peroxidation. The differing dose effects of ascorbic acid, z-tocopherol, and sodium nitrite on the induction period and MOCR reflect their respective antioxidative mechanisms during lipid peroxidation.

Key words: myoglobin; metmyoglobin; hydrogen peroxide; lipid peroxidation; sodium nitrite

Lipid peroxidation initiated by active oxygen species such as hydrogen peroxide (H$_2$O$_2$) has been regarded as a trigger that causes deterioration in foods and various diseases in the human body. Although H$_2$O$_2$ is normally present in some foods and in aerobic cells, it cannot cause lipid peroxidation in the absence of transition metals such as Fe and Cu. Among the biological substances consisting of transition metals, hemoproteins such as myoglobin and cytochrome c have attracted strong attention as the catalysts of lipid peroxidation caused by H$_2$O$_2$, in red meat, LDL, and mitochondria.

It has been reported that H$_2$O$_2$ oxidized Fe ions in the heme of myoglobin, and that the activated myoglobin (ferrylmyoglobin) abstracted a hydrogen atom from polyunsaturated fatty acid, resulting in lipid peroxidation. One problem in the studies on lipid peroxidation caused by H$_2$O$_2$ in a meat model, it is necessary to establish the experimental conditions under which we can compare the time-dependent effects of various factors on lipid peroxidation in the presence of myoglobin with those in the presence of free ferric ions. In previous studies, the O$_2$ consumption has been used to investigate the time-course of lipid peroxidation caused by H$_2$O$_2$ in the presence of myoglobin. However, there are few reports on the time-dependent effects of various factors on lipid peroxidation based on a quantitative index. In the present study, we tried to modify this method in order to clarify the role of myoglobin and free ferric ions on the lipid peroxidation caused by H$_2$O$_2$ by a quantitative index. Since the dose-dependent effect of free ferric ions has been rarely reported, it is necessary to establish a model system which would enable us to investigate the effects of free ferrous or ferric ions on the lipid peroxidation caused by H$_2$O$_2$ in a dose-dependent manner. Fukuzawa et al. have reported that free ferrous ions dose-dependently caused lipid peroxidation in the presence of H$_2$O$_2$ in tetradecyltrimethylammonium bromide (TTAB) micelles. According to their method for preparing the linoleic acid micelles with TTAB, we compared the effects of ferric ions on the H$_2$O$_2$-induced peroxidation of linoleic acid with those of metmyoglobin and studied the effects of various factors on the lipid peroxidation occurring in the latter case.

Materials and Methods

Materials. We purchased H$_2$O$_2$ from Mitsubishi Gas Chemical Co. (Tokyo, Japan), obtained metmyoglobin from horse skeletal muscle, and purchased z-tocopherol and linoleic acid from Sigma Chemical Co. (St. Louis, MO, U.S.A.). We purchased tetradecyltrimethylammonium bromide (TTAB), triphenylphosphine, and ascorbic acid from Wako Pure Chemical Industries (Osaka, Japan), sodium nitrite (NaNO$_2$) from Kanto Chemical Co. (Tokyo, Japan), and diethylenetriaminepentaacetic acid (DTPA) from E. Merck Japan (Tokyo, Japan). Linoleic acid hydroperoxide (a mixture of isomers) was prepared by allowing linoleic acid to stand at room temperature for several days. It was purified by high-performance liquid chromatography with a Develosil packed column (8.0 i.d. x 250 mm; Nomura Chemical Co., Aichi, Japan). The mobile phase consisted of hexane, 2-propanol, and acetic acid (97.3 : 2.5 : 0.2).

All other chemicals were of reagent grade and were used without further purification. We prepared all aqueous solutions with distilled water treated with Milli-Q Lab from Japan Millipore (Tokyo, Japan).

Preparation of linoleic acid micelles. We added 400 pmol of triphenylphosphine to 50 μmol of linoleic acid in chloroform in order to quench any trace amount of linoleic acid hydroperoxide present in the received preparation, which would be a cause of inconsistent results for the time-course of lipid peroxidation. After evaporating the solution to dryness with nitrogen gas, we added 10 ml of a TTAB aqueous solution (50 mm) to the dried linoleic acid, and then vortexed and sonicated the mixture under a nitrogen gas atmosphere to prepare the linoleic acid micelles according to the method of Fukuzawa et al. Unless otherwise indicated, we added DTPA (50 μmol of final concentration) to the prepared linoleic acid micelles (5 mm of final concentration) in order to prevent the Fenton reaction (H$_2$O$_2$ + Fe$^{2+}$ → OH$^-$ + OH$^-$ + Fe$^{2+}$), because ferric DTPA is not readily reduced and consequently not effective in the Fenton reaction, whereas ferric EDTA is reduced and effective in the Fenton reaction.

Abbreviations: TTAB, tetradecyltrimethylammonium bromide; DTPA, diethylenetriaminepentaacetic acid; MOCR, maximum oxygen consumption rate.

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reaction.

Analysis of lipid peroxidation by O2 consumption. We measured the O2 consumed in the process of lipid peroxidation of the linoleic acid micelles in a glass chamber at 37°C with a Clark-type YSI 5300 oxygen electrode (Yellow Spring Inst.). After confirming that the addition of metmyoglobin to the micelles had not caused any O2 consumption for several minutes, we added H2O2 to the micelles. We carried out 4 independent experiments at each measuring point with the same linoleic acid micelles and the results are expressed as means with standard deviations.

Analysis of conjugated dienes from the reaction mixture. At indicated times during the incubation, we sampled 250 µl of the reaction mixture in the glass chamber through a fine tube with a syringe in order to avoid exposing the reaction mixture to atmospheric oxygen. After the successive addition of 10 µl of a NaBH4 aqueous solution (0.8 M), one drop of 1 N HCl and 1.75 ml of methanol to the sampled reaction mixture, we measured its absorbance at 234 nm.

Results

Definition of the induction period and the maximum oxygen consumption rate (MOCR)

Figure 1 illustrates the typical pattern for O2 consumption and conjugated diene formation during linoleic acid oxidation in the presence of H2O2 and metmyoglobin. O2 consumption usually consisted of two phases. It started slowly and linearly, before rapid O2 consumption, assumed to have been caused by the chain reaction of lipid peroxidation, proceeded. We obtained the induction period (min) and MOCR (µM/s) by drawing two straight lines on each pattern as depicted in Fig. 1 and by assuming that the initial reaction mixture contained 217 µM of molecular oxygen at 37°C. The arrow in Fig. 1 designates the starting point for the rapid O2 consumption. Although it was difficult to maintain a constant induction period and MOCR by incubating linoleic acid micelles prepared on different days, the effect of various factors on the two parameters was always reproducible. The pattern of the conjugated diene formation in Fig. 1 also shows that the oxidation occurred in two phases and suggests that the O2 consumption reflected the formation of linoleic acid hydroperoxides.

Dose-dependent effects of metmyoglobin, H2O2, and linoleic acid hydroperoxide

Metmyoglobin dose-dependently decreased the induction period and increased MOCR (Fig. 2). On the other hand, H2O2 dose-dependently decreased the induction period and had no effect on MOCR (Fig. 3a). These results suggest that metmyoglobin was involved in the reaction in both phases, while H2O2 was only involved in the first phase of the reaction. When we added the prepared linoleic acid hydroperoxide instead of H2O2 to the linoleic acid micelles, it also dose-dependently decreased the induction period and had no effect on MOCR (Fig. 3b). A significant difference between the dose effects of H2O2 and linoleic acid hydroperoxide was observed. More than 500 µM of H2O2 was necessary to make the induction period less than 10 min. On the other hand, rapid O2 consumption started immediately after the addition of 15 µM of linoleic acid hydroperoxide. Even at a concentration higher than 15 µM, e.g., 200 µM, MOCR was not significantly changed (data not shown), suggesting that, at such a concentration of linoleic acid hydroperoxide, the catalytic sites of metmyoglobin would be filled and the reaction rate would then reach a maximum.

Effects of DTPA

The addition of ferric sulfate instead of metmyoglobin to the linoleic acid micelles in the absence of DTPA also resulted in O2 consumption. Although the starting point for the rapid O2 consumption was not as clear as it was for metmyoglobin (data not shown), we observed a dose-dependent decrease in the induction period and a dose-dependent increase in MOCR, which was much lower than the effect of metmyoglobin (Fig. 4). This indicates that the catalytic effect of metmyoglobin on the chain reaction was much higher than that of ferric ions. Since Fe3+ might be released from metmyoglobin during the peroxidation of linoleic acid caused by H2O2, we investigated the effect of DTPA, an Fe3+ chelator, to elucidate the role of free ferric ions or of the heme moiety during H2O2-induced lipid peroxidation under conditions giving a similar induction period. DTPA (10 µM) completely inhibited the O2 consumption in the lipid peroxidation system with 200 µM of H2O2 and 5 µM of ferric sulfate. This indicates that Fe3+ chelated by DTPA in the reaction mixture lost its catalytic activity during the lipid peroxidation process. In the lipid

Fig. 1. O2 Consumption (---) and Conjugated Diene Formation (-----) during Linoleic Acid Oxidation in the Presence of H2O2 and Metmyoglobin.

The oxidation was started by adding 200 µM of H2O2 to linoleic acid micelles containing 5 mM of linoleic acid and 5 µM of metmyoglobin. The arrow indicates the starting point for rapid O2 consumption.
assess amount in and that the hemepiety of myoglobin was directly involved. Thus, we concluded that Fe^{3+} was not significantly released from metmyoglobin during the reaction process and that the heme moiety of myoglobin was directly involved in the lipid peroxidation caused by H_{2}O_{2}. By excluding any effects of free ferric ions by the addition of a sufficient amount of DTPA, the described method was applied to assess various factors affecting the two parameters for lipid peroxidation in the presence of metmyoglobin.

**Effects of antioxidants and sodium nitrite**

We investigated the effects of ascorbic acid and α-tocopherol on H_{2}O_{2}-induced lipid peroxidation in the presence of metmyoglobin (Fig. 5). Ascorbic acid dose-dependently increased the induction period and had no effect on MOCR. α-Tocopherol also increased the induction period, but only slightly. Furthermore, it dose-dependently decreased MOCR.

Figure 6 shows that NaNO_{2} dose-dependently increased the induction period and decreased MOCR. The addition...
of sodium nitrite to a reaction mixture consisting of 5 mM linoleic acid micelles, 200 μM hydrogen peroxide and 10 μM ferric sulfate in the absence of DTPA made the starting point for rapid O₂ consumption unclear. Thus, it was difficult to quantify the two parameters. We only observed the slow and constant O₂ consumption phase, whose rate decreased with increasing concentration of sodium nitrite; e.g., MOCR at 15 μM of sodium nitrite was 0.01. These results show that sodium nitrite also acted as an antioxidant against the oxidation of linoleic acid caused by H₂O₂ in the presence of free ferric ions instead of myoglobin.

Discussion

To the best of our knowledge, this is the first report describing that the lipid peroxidation caused by H₂O₂ in the presence of myoglobin comprised the two phases. The clear separation of these two phases in the present study would have resulted from the treatment of linoleic acid with triphenylphosphine to quench any trace of linoleic acid hydroperoxide that may have been present in the received preparation. Otherwise, the induction period would have been shorter and less consistent.

We postulate the mechanism for lipid peroxidation comprising the two phases based on the following facts: (1) Theoretically, 217 μM of lipid hydroperoxide should be produced as a result of the complete consumption of 217 μM of O₂ in the reaction mixture. At the starting point for rapid O₂ consumption shown by the arrow in Fig. 1, 8% of the O₂ had been consumed, meaning that 17 μM of linoleic acid hydroperoxide was produced. (2) The calculated amount of conjugated diene, using an extinction coefficient of 25,000 M⁻¹ cm⁻¹, was 12 μM at the starting point. (3) Figure 3b shows that 15 μM of linoleic acid hydroperoxide caused rapid O₂ consumption without passing through the first phase.

These results suggest that the linoleic acid hydroperoxide accumulated at the starting point for rapid O₂ consumption was sufficient to start this rapid consumption. Since the production and consumption of linoleic acid hydroperoxide occur at the same time, the real kinetics for lipid peroxidation would be more complicated. From these results, we speculate that, in the first phase, the rate of lipid peroxidation depended on the concentration of H₂O₂, and this phase remained until the concentration of linoleic acid hydroperoxide had reached at a certain value, when rapid lipid peroxidation in the chain reaction occurred. Since linoleic acid hydroperoxide was also a product of the chain reaction in the second phase, the net amount of linoleic acid hydroperoxide would have been enough to keep the O₂ consumption rate in the second phase constant (Fig. 3b). Consequently, MOCR would be independent of the concentration of H₂O₂ (Fig. 3a).

Ascorbic acid and α-tocopherol showed different effects on the induction period and MOCR (Fig. 5). An increase in the length of the induction period and no effect on MOCR by ascorbic acid suggest that it exerted its antioxidant effect by reducing ferrylmyoglobin in the first phase. No effect on MOCR corresponds well with the assumed mechanism, as the chain reaction in the second phase caused by linoleic acid hydroperoxide would occur in the micelles, where ascorbic acid could not be present. On the other hand, α-tocopherol effectively worked as a free radical scavenger in the chain reaction in the second phase. The different results of these typical two antioxidants on the two parameters imply that our method is suitable to elucidate the mechanisms of various antioxidants such as plant polyphenols.

Sodium nitrite (NaNO₂) used for curing and preserving meat reacts with the heme of myoglobin to form nitrosomyoglobin. Igene et al. have suggested that nitrite functions as an antioxidant in cured meat products in three possible ways: (1) by the formation of a strong complex with heme pigments; (2) by directly interacting with the liberated non-heme iron; and (3) by stabilization of the unsaturated lipids. We tried to elucidate the mechanism for the antioxidative activity of NaNO₂ with our model and found that NaNO₂ exerted its antioxidative effect in both the first and second phase (Fig. 6). This effect, which is opposite to the observed concentration effect of metmyoglobin in Fig. 2, implies that NaNO₂ dose-dependently formed nitrosomyoglobin, which would not have been involved in the peroxidation of the linoleic acid micelles during any phase. If nitrosomyoglobin is resistant to H₂O₂-induced activation, we can expect that the addition of NaNO₂ to a mixture of linoleic acid micelles prior to the addition of H₂O₂ should prevent the lipid peroxidation caused by H₂O₂. Since NaNO₂ also acted as an antioxidant in the presence of free ferric ions instead of myoglobin, the addition of NaNO₂ to meat could prevent the lipid peroxidation initiated by H₂O₂, possibly both by the formation of nitrosomyoglobin and interaction with the liberated non-heme iron.

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References