Oxymyoglobin Oxidation and Membrane Lipid Peroxidation Initiated by Iron Redox Cycle: Prevention of Oxidation by Enzymic and Nonenzymic Antioxidants

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The red color of muscle is principally due to the presence of oxymyoglobin. Oxidation of heme iron from the ferrous to the ferric state produces a brownish color, which consumers find undesirable. The aim of this study was to use enzymic and nonenzymic antioxidants to simulate in situ muscle antioxidation reactions in order to understand better the mechanism by which the iron redox cycle catalyzes membrane lipid peroxidation and oxymyoglobin oxidation. The inclusion of superoxide dismutase (SOD) in the model system decreased oxymyoglobin oxidation by 10% without affecting lipid peroxidation. Addition of catalase decreased oxymyoglobin oxidation by ~40% but not lipid peroxidation. Increasing the ceruloplasmin concentration inhibited lipid peroxidation but increased oxymyoglobin oxidation, which was inhibited by SOD and catalase. Conalbumin (50 μM), a specific iron chelator, inhibited peroxidation and oxymyoglobin oxidation by almost 50%. The addition of the antioxidant catechin (500 μM) decreased lipid peroxidation by 90% but oxymyoglobin oxidation by only 50%. Feeding turkeys with vitamin E at several levels significantly increased the α-tocopherol level of membranes, thus preventing oxymyoglobin and lipid oxidation. In conclusion, oxymyoglobin stability in the model system was affected by two pathways: (a) oxygen active species, such as $O_2^{\bullet -}$, H_2O_2, $HO^{\bullet}$, and ferryl, generated during autoxidation of myoglobin and oxidation of ferrous ions and ascorbic acid; and (b) lipid radicals, such as $ROO^{\bullet}$, $RO^{\bullet}$, and hydroperoxides, generated during lipid peroxidation. Maximum inhibition could be achieved only by introducing inhibitors of both pathways into the system.

Keywords: *Oxidation; oxymyoglobin; lipids; microsomes; enzymic; nonenzymic antioxidants*

INTRODUCTION

Myoglobin, an oxygen-binding heme protein found in muscle fibers, is responsible for intracellular oxygen storage and oxygen transport from the plasma membrane to the cell mitochondria (*1*), although some skepticism about its function has been reported (*2*).

The red color of muscle is principally due to the presence of oxymyoglobin. The redox state of the heme iron and the presence or nature of a ligand bound to myoglobin account for the color. Oxidation of heme iron from the ferrous state in oxymyoglobin or deoxymyoglobin to the ferric state, in metmyoglobin, produces the brownish color on fresh meat, which consumers find undesirable (*3*).

Heme proteins such as oxymyoglobin and oxyhemoglobin are particularly prone to oxidation and autoxidation and are affected by a wide spectrum of active oxygen species and metal ions, such as $O_2^{\bullet -}$, H_2O_2, $HO^{\bullet}$, HOCl, $NO_2^{\bullet}$, and lipid oxy radicals (*4*−*10*).

Our data have shown that the free iron redox cycle, provided by ascorbic acid, is the main initiator of lipid peroxidation in fresh muscle foods (*11*−*13*) and that such a system could greatly affect the oxidation of oxymyoglobin.

Several authors have postulated that oxymyoglobin and lipid peroxidation in muscle tissue are interrelated (*14*−*16*). Most recently, we have demonstrated that membrane lipid peroxidation by an iron redox cycle, provided by ascorbic acid, greatly accelerates oxymyoglobin oxidation. It was found that oxymyoglobin is a weak catalyzer of membrane lipid peroxidation. In the presence of an iron redox cycle system, oxymyoglobin-affected muscle membrane undergoes lipid peroxidation by a mechanism that depends on oxymyoglobin concentration. A low oxymyoglobin concentration slightly affected peroxidation, but at a high concentration the process is inhibited (*17*). The involvement of heme proteins as catalyzers of lipid peroxidation was previously described by us (*18*, *19*) and others (*20*−*24*).

Most recently, measurement of the accumulation of oxidized heme proteins has been applied as a method of evaluating the protective effects of antioxidants against oxidative stress (*25*, *26*).

The aim of the present study was to arrive at a better understanding of the mechanism by which iron redox cycle membrane lipid peroxidation and myoglobin oxidation are interrelated reactions and how they can be prevented by enzymic and nonenzymic antioxidants.

MATERIALS AND METHODS

Materials. Hydrogen peroxide (30% for synthesis), ascorbic acid, D-α-tocopherol, and trichloroacetic acid were purchased from Merck (Darmstadt, Germany). Myoglobin type I from bovines, bovine serum albumin, sodium dithionite, 2-thiobarbituric acid, and L-histidine free-base were obtained from Sigma Chemical Co. (St. Louis, MO). Sephadex G-15 medium was obtained from Pharmacia Biotech (Uppsala, Sweden). Ferric chloride was obtained from Mallinckrodt Chemical Works (St. Louis, MO).

Methods. *Oxymyoglobin Preparation.* Metmyoglobin, treated with Chelex-100 and neutralized to pH 7.0, was separated from low molecular weight compounds on a column of Sephadex

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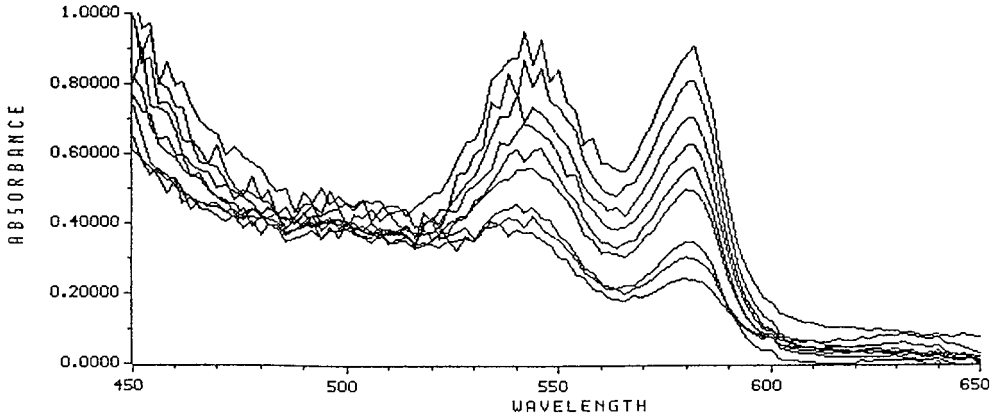

Figure 1. Oxymyoglobin (20 μM) oxidation by the microsomal (1 mg of protein/mL) model system containing ascorbic acid (200 μM) and ferric chloride (50 μM). Each line denotes 15 min of incubation.

G-15. Oxymyoglobin was prepared by reducing 1 mL of metmyoglobin (1 mM) with dithionite (3 mg) under aerobic conditions and purifying by gel filtration on a column of Sephadex G-15 using 0.01 M phosphate buffer, pH 8.0, for elution (*11*, *27*). Oxymyoglobin concentration was calculated using an extinction coefficient, $E_{582} = 15.1\ M^{-1}\ cm^{-1}$.

Oxidation of oxymyoglobin was determined by measuring the decrease in absorption by the extract solution, by means of a method previously developed in our laboratory (*17*). To reduce the effect of turbidity, an appropriate number of layers of Parafilm (usually one) was used as a background, to subtract some of the absorbency caused by the turbidity of the samples. The samples were scanned from 450 to 650 nm, and the change in absorption at 582 nm was calculated (Figure 1).

Lipid Peroxidation. Isolation of the microsomal fraction from muscle tissues was done according to a procedure described previously (*18*). Thiobarbituric acid reactive substances (TBARS) were determined according to the method of Bidlack et al. (*28*). The results are reported as nanomoles of malondialdehyde (MDA) per milligram of protein, calculated on the basis of a molar extinction coefficient of $E_{532} = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$. The accumulation of conjugated dienes produced during lipid peroxidation was monitored by the increase in A_{233} (*29*). Protein determination was conducted according to the modified Lowry procedure (*30*), with BSA as a standard.

Turkey bird diet was supplemented with DL-α-tocopherol acetate at 0, 29, or 150 mg/kg of feed. Microsomes from turkey muscles were prepared, containing α-tocopherol at various concentrations. Day-old males of a large strain of Broad Breasted White turkeys (British United Turkeys, T6) were raised in electrically heated battery brooders placed in a temperature-controlled room at 25 ± 2 °C to 28 days of age and then in individual cages with wire floors in a temperature-controlled (22 ± 2 °C) building.

Vitamin E (Rovimix E-50 SD; Hoffmann-La Roche, Basel, Switzerland) was fed ad libitum in mash form.

At slaughtering, either during the course of the experiments or at their termination at 22−23 weeks of age, meat from the thigh and the drumstick (the muscle around the femur and the tibia, respectively) was removed and stored at −18 and 4 °C, respectively, for various periods to determine its stability.α-*Tocopherol determination* was performed on a 5-g sample of muscle tissue or on 1 g of microsomes, which were frozen at −70 °C. The samples were freeze-dried and ground to a powder. α-Tocopherol from the powdered sample (100−500 mg) was extracted with 4 mL of ethanol containing 1% BHT. The homogenate was centrifuged for 15 min at 5000*g* and the supernatant filtered through a 0.2 μm membrane. A 20-μL aliquot was injected into an HPLC (LKB Brome). The antioxidant was detected by a spectrofluorometer detector (JESCO FP 210), excited at 290 nm and emitting at 329 nm. D-α-Tocopherol was used to prepare a standard calibration curve. In all of the experiments the results are means of triplicates; in the figures, each bar (I) denotes the standard deviation.

Table 1. Composition of the Basal Diets (Turkeys)

ingredient	age period (weeks) 20−24 (%)
yellow corn	25.00
sorghum grains	49.15
soybean meal (44.5% protein)	16.60
acidulated soybean soapstock	5.50
DL-methionine	0.05
L-lysine	0.05
dicalcium phosphate	2.00
limestone	1.00
mineral mix[a]	0.40
vitamin mix[b]	0.25
calculated values	
AME (kcal/kg)	3330
crude protein	14.20
crude fat	7.40
calcium	0.90
phosphorus	0.66

[a] Supplying (in mg/kg of feed): sodium chloride, 2500; sodium sulfate, 1000; Mn, 80; Zn, 75; Fe, 20; Cu, 5; I, 1.2; Co, 0.2; Se, 0.3. [b] Supplying (per kg of food): retinyl acetate, 3.44 mg; cholecalciferol, 72.5 μg; DL-α-tocopherol acetate, 0, 28, or 150 mg, menadione sodium bisulfite, 2 mg; riboflavin, 7.2 mg; calcium pantothenate, 12.8 mg; niacin, 64 mg; cyanocobalamine, 0.012 mg; folacin, 1.2 mg; pridoxine hydrochloide, 3.6 mg; thiamin hydrochloride, 1.2 mg; biotin, 0.12 mg; choline chloride, 250 mg; ethoxyquin, 62.5 mg; BHT, 62.5 mg.

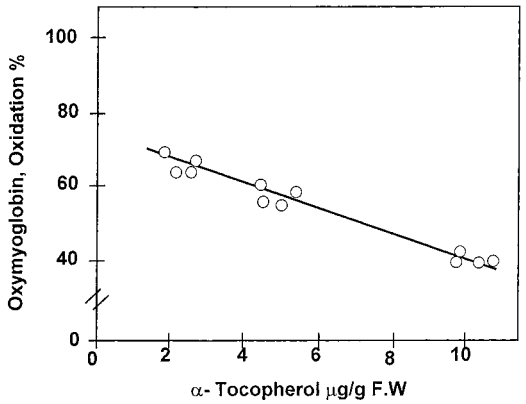

Figure 2. Effect of membrane α-tocopherol concentration on oxymyoglobin oxidation catalyzed by iron redox cycle. The system contained microsomes (1 mg of protein/mL), oxymyoglobin (20 μM), $FeCl_3$ (50 μM), and ascorbic acid (200 μM) in buffer acetate, pH 6.5, incubated at 37 °C for 30 min.

RESULTS

α-Tocopherol. Feeding turkeys with α-tocopherol at several levels significantly increased the amount of vitamin E in the membranes of the muscle cells. Figure 2 demonstrates the interrelationship between the concentration of α-tocopherol in membranes and oxymyo-

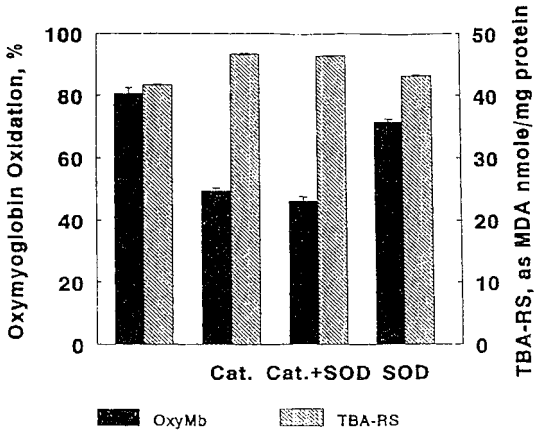

Figure 3. Effect of superoxide dismutase and catalase on oxymyoglobin oxidation and membrane lipid peroxidation catalyzed by iron redox cycle. The system contained the same concentrations of microsomes, oxymyoglobin, $FeCl_3$, and ascorbic acid as listed in Figure 2, SOD (400 units/mL) and catalase (400 units/mL), incubated for 60 min.

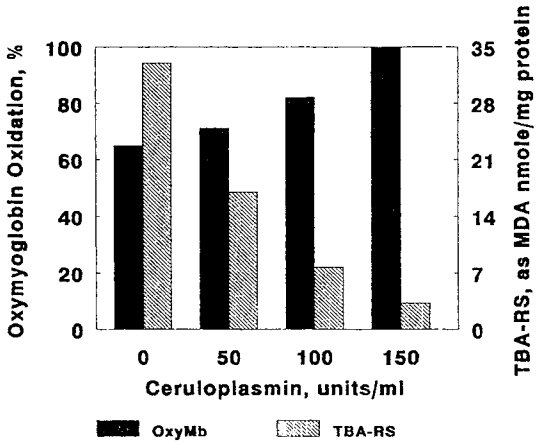

Figure 4. Effect of ceruloplasmin concentration on oxymyoglobin oxidation and membrane lipid peroxidation catalyzed by iron redox cycle. The system contained microsomes (1 mg of protein/mL), oxymyoglobin (20 μM), $FeCl_3$ (50 μM), and ascorbic acid (200 μM) in buffer acetate, pH 6.5, incubated at 37 °C for 30 min.

globin oxidation, as affected by iron redox cycle catalysis. In controls without added α-tocopherol, its concentration in the membranes was <2 μg/g of fresh weight and the oxidation of oxymyoglobin was at a maximum. Increasing the level of α-tocopherol in the feed to 150 mg/kg increased the concentration of α-tocopherol in the membrane to >8 μg/g of fresh weight. Concurrently with this 4-fold increase in α-tocopherol concentration in the membrane, there was a decrease in oxymyoglobin oxidation of almost 50% (Figure 2).

Effects of Superoxide Dismutase (SOD), Catalase, and Ceruloplasmin. The inclusion of SOD in the reaction mixture, at a level of 400 units/mL, decreased oxymyoglobin oxidation by 10% without affecting lipid peroxidation. Addition of catalase to the mixture, at a level of 400 units/mL, decreased oxymyoglobin oxidation by ~ 40%, but not lipid peroxidation. Addition of catalase plus SOD had no greater effect on oxymyoglobin oxidation than catalase alone (Figure 3).

The effect of ceruloplasmin on the system is presented in Figure 4. Increasing the ceruloplasmin concentration inhibited lipid peroxidation but enhanced oxymyoglobin oxidation. This effect was found to be dependent on the oxidation of ascorbic acid and increased production of

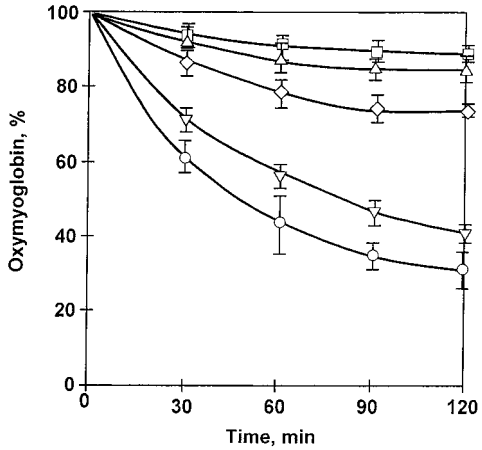

Figure 5. Effects of SOD and catalase on ceruloplasmin (100 units/mL) affected oxymyoglobin oxidatiion catalyzed by iron redox cycle. The system contained microsomes (1 mg of protein/mL), oxymyoglobin (20 μM), $FeCl_3$ (50 μM), and ascorbic acid (200 μM) in buffer acetate, pH 6.5, incubated at 37 °C for 30 min: (◇) control without ceruloplasmin; (○) control with ceruloplasmin; (▽) addition of SOD at 200 units/mL; (△) addition of catalase at 200 units/mL; (□) addition of catalase and SOD (200 units/mL each).

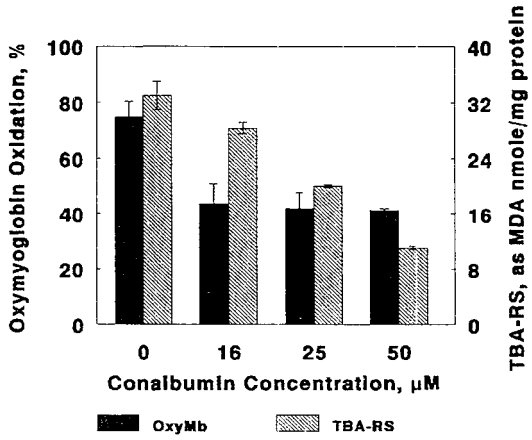

Figure 6. Oxymyoglobin oxidation and membrane lipid peroxidation catalyzed by iron redox cycle as affected by conalbumin concentration. The system contained microsomes (1 mg of protein/mL), oxymyoglobin (20 μM), $FeCl_3$ (50 μM), and ascorbic acid (200 μM) in buffer acetate, pH 6.5, incubated at 37 °C for 30 min.

$O_2^{\bullet -}$ and H_2O_2 in the system. It was possible to prevent the oxidation of oxymyoglobin in the presence of ceruloplasmin partially by including SOD and totally by including catalase (Figure 5).

Effect of Conalbumin and Catechin. Iron ions are very important catalysts, which greatly affect the oxidation of the system. Conalbumin significantly affected oxymyoglobin oxidation and lipid peroxidation. The effect was concentration dependent, and at 50 μM, conalbumin inhibited both reactions by ~50% (Figure 6).

The addition of catechin, a well-known antioxidant and scavenger of free radicals, partially inhibited lipid peroxidation and oxymyoglobin oxidation (Figure 7). An increase in catechin concentration in the model system from 50 to 500 μM decreased lipid peroxidation by almost 90% but inhibited oxymyoglobin oxidation by only 50% (Figure 8).

"Cocktail" Effect. Figure 9 demonstrates the potential to inhibit oxymyoglobin oxidation and lipid peroxidation by the addition of a reagent such as catalase or SOD, which should decrease the effects of

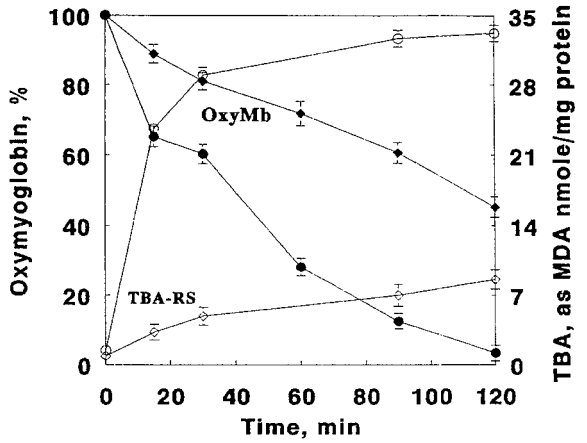

Figure 7. Oxymyoglobin oxidation and membrane lipid peroxidation catalyzed by iron redox cycle as affected by catechin (100 μM). The system contained microsomes (1 mg of protein/mL), oxymyoglobin (20 μM), $FeCl_3$ (50 μM), and ascorbic acid (200 μM) in buffer acetate, pH 6.5, incubated at 37 °C for 30 min: (○, ●) control; (◇, ◆) catechin (100 μM).

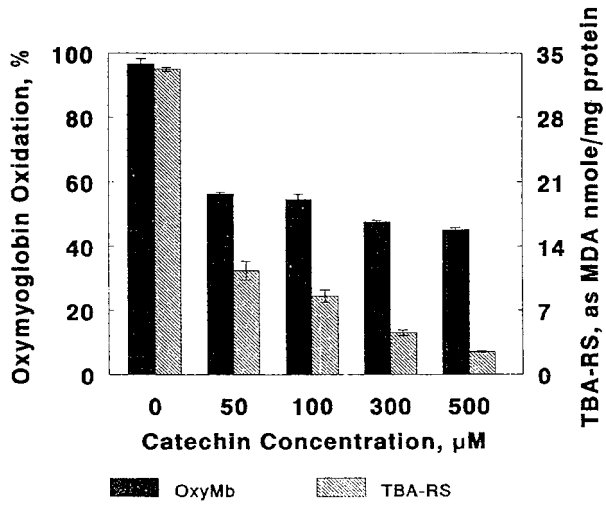

Figure 8. Effect of catechin concentration on oxymyoglobin oxidation and membrane lipid peroxidation catalyzed by iron redox cycle. The system contained microsomes (1 mg of protein/mL), oxymyoglobin (20 μM), $FeCl_3$ (50 μM), and ascorbic acid (200 μM) in buffer acetate, pH 6.5, incubated at 37 °C for 30 min.

$O_2^{\bullet-}$ and H_2O_2 and the effects of a free radical scavenger such as catechin in the same system. The results showed a significant reduction of oxymyoglobin oxidation by catalase plus SOD without any effect on lipid peroxidation. The addition of SOD, catalase, and catechin prevented oxymyoglobin oxidation by >70% and lipid oxidation by 95%. The addition of conalbumin (50 μM) to the system containing SOD, catalase, and catechin decreased myoglobin oxidation and lipid peroxidation by 80 and 95%, respectively (Figure 10).

DISCUSSION

Autoxidation of oxymyoglobin is affected by several ions and compounds such as Cl^-, SCN, and CN^- and other conditions, which decrease oxygen binding to the heme (*9*, *19*). This effect could oxidize the ferrous form to ferric, with the production of superoxide anion and H_2O_2 by dismutation (*6*, *9*, *10*, *19*).

Either $O_2^{\bullet-}$ or H_2O_2 could further oxidize oxymyoglobin, and thus reactions increase the autoxidation of

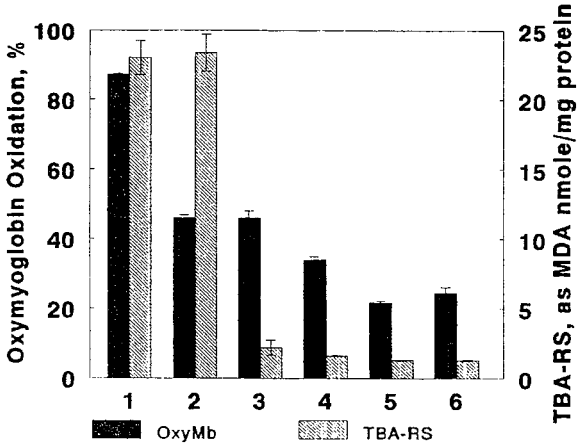

Figure 9. Effects of SOD and catalase on catechin (500 μM) inhibition of oxymyoglobin oxidation and membrane lipid peroxidation catalyzed by iron redox cycle. The system contained microsomes (1 mg of protein/mL), oxymyoglobin (20 μM), $FeCl_3$ (50 μM), ascorbic acid (200 μM), catechin (500 μM), and SOD and catalase (different concentrations) in buffer acetate, pH 6.5, incubated at 37 °C for 120 min: (1) control without catechin and enzymes; (2) without catechin, catalase, and SOD (1000 units/mL each); (3) catechin, without catalase and SOD; (4) catechin, catalase, and SOD (100 units/mL each); (5) catechin, catalase, and SOD (400 units/mL each); (6) catechin, catalase, and SOD (1000 units/mL each).

oxymyoglobin as shown by the following reactions:

$$\text{Mb-Fe}^{2+}\text{-O}_2 \leftrightarrows \text{Mb-Fe}^{2+} + \text{O}_2 \quad (1)$$

$$\text{Mb-Fe}^{2+} + \text{O}_2 \xrightarrow{\text{Cl}^-} \text{Mb-Fe}^{3+} + \text{O}_2^{\bullet-} \quad (2)$$

$$\text{O}_2^{\bullet-} + \text{O}_2^{\bullet-} \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad (3)$$

$$\text{Mb-Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Mb-Fe}^{4+}\text{=O} + \text{H}_2\text{O} \quad (4)$$

$$\text{Mb-Fe}^{2+} + \text{Mb-Fe}^{4+}\text{=O} \xrightarrow{\text{H}^+} 2\text{Mb-Fe}^{3+} + \text{HO}^- \quad (5)$$

$$\text{Mb-Fe}^{2+} + \text{O}_2^{\bullet-} \xrightarrow{2\text{H}^+} \text{Mb-Fe}^{3+} + \text{H}_2\text{O}_2 \quad (6)$$

The iron redox cycle generates $O_2^{\bullet-}$, H_2O_2, $HO^{\bullet}$, and also alkoxyl and peroxyl radicals in the presence of hydroperoxides (*13*, *31*−*33*). All of these species oxidize oxymyoglobin. The significant inhibition of oxymyoglobin oxidation by catalase demonstrated that H_2O_2 was generated in the system and was involved in the oxidation of oxymyoglobin, but the finding that lipid peroxidation was not affected by catalase showed that this process, in our model system, was not dependent significantly on hydrogen peroxide, formed in the external microsome area.

Ceruloplasmin, which oxidizes ferrous to ferric ions, was found to inhibit lipid peroxidation very significantly, but it enhanced oxymyoglobin oxidation. These results indicate that lipid peroxidation in our model system depended greatly on Fe^{2+} ions, which most probably enhanced the reduction of hydroperoxides to alkoxyl free radicals.

$$\text{Fe}^{2+} + \text{LOOH} \rightarrow \text{Fe}^{3+} + \text{LO}^{\bullet} + \text{HO-} \quad (7)$$

$$\text{LH} + \text{LO}^{\bullet} \rightarrow \text{L}^{\bullet} + \text{LOH} \quad (8)$$

It is possible to explain the acceleration of oxymyoglobin oxidation by ceruloplasmin as a reaction of ferric

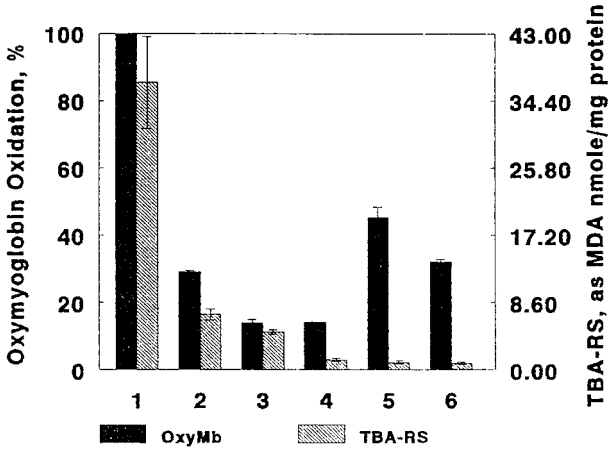

Figure 10. Cocktail effect. Addition of SOD, catalase, catechin, and conalbumin affected oxymyoglobin oxidation and membrane lipid peroxidation catalyzed by iron redox cycle. The system contained microsomes (1 mg of protein/mL), oxymyoglobin (20 μM), $FeCl_3$ (50 μM), and ascorbic acid (200 μM) in buffer acetate, pH 6.5, incubated at 37 °C: (1) control; (2) conalbumin (50 μM); (3) conalbumin (50 μM) and catalase and SOD (400 units/mL each); (4) as for (3) plus catechin (500 μM); (5) catechin (500 μM); (6) conalbumin (500 μM) and catechin (500 μM), incubation for 120 min.

iron with oxymyoglobin or an enhancement of ascorbic acid oxidation and a greater generation of $O_2^{\bullet-}$ and H_2O_2, which could oxidize oxymyoglobin to metmyoglobin very significantly. Inhibition of ceruloplasmin-dependent oxymyoglobin oxidation by catalase in the presence of iron−ascorbic acid demonstrated the involvement of H_2O_2 in this reaction.

Conalbumin, a well-known protein extract from eggs, works very similarly to transferrin by binding 2 mol of "free" iron to each mole of the protein (*34*). Conalbumin inhibited both MbO_2 oxidation and lipid peroxidation. This demonstrates the importance of free iron in the general oxidation of the system. Catechin was found to inhibit lipid peroxidation very effectively, but MbO_2 oxidation only partially. The results indicate that MbO_2 oxidation is only partially dependent on lipid peroxidation.

In conclusion, MbO_2 oxidation is a process that is affected mainly by two pathways, as shown by our model system: the first pathway generates active oxygen species such as $O_2^{\bullet-}$ and H_2O_2; the second generates lipid peroxides and lipid free radicals. Maximum inhibition could be achieved only by introducing inhibitors of both pathways, such as SOD, catalase, conalbumin, catechin, and α-tocopherol, into the system. Lipid peroxidation was affected mainly by the iron redox cycle. The best stabilization of oxymyoglobin and the arresting of lipid peroxidation were achieved when both oxymyoglobin and ascorbic acid were present in the system at high concentrations of 120 and 2000 μM, respectively (*17*).

Oxymyoglobin did not affect lipid peroxidation significantly at a concentration of 20 μM; however, at a higher concentration it did inhibit it, because of the decomposition of hydroperoxides and autoreduction (*17*, *31*, *35*, *36*).

In fresh muscle foods that contain active enzymes such as SOD, catalase, and glutathione peroxidase, lipid peroxidation and MbO_2 oxidation seem to be strongly affected by supplementation of free radical scavengers such as α-tocopherol. Indeed, such results have been obtained by many researchers including ourselves (*3*, *14*, *16*, *37*−*39*).

ABBREVIATIONS USED

MbO_2, $MbFe^{2+}$-O_2, oxymyglobin; $MbFe^{2+}$, deoxymyoglobin; $MbFe^{3+}$, methmyoglobin; Mb-Fe^{4+}=O, myoglobin ferryl; SOD, superoxide dismutase.

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