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Muscle Lipid Peroxidation Dependent on Oxygen and Free Metal Ions

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In studies to determine lipid peroxidation by the accumulation of thiobarbituric acid reactive compounds and conjugated dienes, muscle lipid peroxidation was dependent on the presence of oxygen and free metal ions. Lipid peroxidation in raw and heated samples was prevented by vacuum and ethylenediaminetetraacetic acid (EDTA). In membrane model systems, a low concentration of EDTA inhibited enzymatic and nonenzymatic free metal ion catalysis, and only a very high concentration of EDTA inhibited activated metmyoglobin catalysis. Lipid peroxidation in minced turkey muscle is affected especially by "free" metal ions.

Lipid peroxidation is one of the primary mechanisms of quality deterioration in stored foods, especially in muscle tissues. The changes in quality can be manifested by deterioration in flavor, color, texture, and nutritive value and the production of toxic compounds (Simic and Karel, 1980; Pearson et al., 1983; Benedetti et al., 1984). The problems of lipid peroxidation in meats have become even more important with modern food formulation of restructured and precooked products and the utilization of deboned muscle tissues (Pearson et al., 1983). The oxidation of muscle lipids involves peroxidation of the unsaturated fatty acids, in particular the polyunsaturated fatty acids (PUFA) (Allen and Foegeding, 1981). The PUFA are associated with phospholipids, which are critical to the development of the off-flavor in muscle products (Keller and Kinsella, 1973; Moerck and Ball, 1974; Igene et al., 1979). Most of these highly unsaturated fatty acids are located in the membranes of muscle foods.

The mechanism of lipid peroxidation in muscle foods was studied by several researchers utilizing model systems of linoleate emulsion (Lin, 1970a,b; Lee et al., 1975; Koizumi et al., 1976; Fisher and Deng, 1977; Sklan et al., 1983; Tay et al., 1983). These model systems could be conducted for studying general problems of lipid peroxidation but not for simulating lipid peroxidation in muscle foods (Kanner et al., 1986).

Recently, we adopted the utilization of muscle microsome membranes (sarcosomes) to study lipid peroxidation in muscle foods (Harel and Kanner, 1985; Kanner and Harel, 1985; Kanner et al., 1986). During these studies we evaluated membrane lipid peroxidation by three possible pathways: (i) microsome enzymic lipid peroxidation dependent on NADPH or NADH and iron ions; (ii) nonenzymatic microsome lipid peroxidation stimulated by H_2O_2-activated metmyoglobin; (iii) nonenzymic lipid peroxidation catalyzed by the iron redox cycle system. Membrane lipid peroxidation initiated by these three pathways was inhibited by several reducing agents, hydroxyl radical scavengers, chelators, enzymes, and food antioxidants. From the inhibitors employed in our research, it was found that EDTA could be used for evaluating the role of free iron or heme proteins in in situ muscle lipid peroxidation.

Ethylenediaminetetraacetic acid, a good sequestering agent, has been studied extensively in recent years in many model systems of oxygen radical research. Numerous studies with EDTA have demonstrated the complexity it imparts upon the reactivity of iron. EDTA stimulates ferrous ion oxidation by oxygen or H_2O_2-producing superoxide and hydroxyl radicals (Udenfried et al., 1954; Halliwell, 1978; McCord and Day, 1978; Cohen and Sinet, 1980; Kanner et al., 1986). EDTA also stimulates ferrous ion oxidation by hydroperoxides, but only at a concentration less than that of iron (Grinstead, 1960;, Barber, 1966; Gutteridge et al., 1979; Tien et al., 1983; Gutteridge, 1984) and only when the fatty acids are emulsified (Tien et al., 1982, 1983; Girotti and Thomas, 1984). EDTA inhibits lipid peroxidation at a concentration higher than that of the iron ion (Wills, 1965; Tien et al., 1982; Gutteridge, 1984) or even at a low concentration if the PUFA are associated in liposomes (Tien et al., 1982) or in mem-

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branes (Girotti and Thomas, 1984; Kanner et al., 1986).

In this research we studied the effect of oxygen and EDTA on the initiation of lipid peroxidation in several muscle membrane model systems and in situ tissues.

MATERIALS AND METHODS

Materials. Hydrogen peroxide (30% for synthesis), ascorbic acid, trichloroacetic acid, and chloroform were purchased from Merck (Darmstadt, FGR). Myoglobin type I from equine skeletal muscle, potassium chloride, L-histidine-free base, thiobarbituric acid (TBA), bovine serum albumin (BSA), and β-nicotinamide adenine dinucleotide disodium salt reduced from type X (NADPH) were obtained from Sigma Chemical Co. (St. Louis, MO). Ethylenediaminetetraacetic acid disodium salt (EDTA) and ferric chloride were purchased from BDH Chemicals Ltd. (Poole, England). Adenosine 5′-diphosphate salt (ADP) was from Boehringer-Mannheim GmbH (Mannheim, FGR).

Methods. Isolation of the microsome fraction from fresh turkey muscle tissues was done by a procedure described previously (Apgar and Hultin, 1982; Kanner and Harel, 1985). The enzymatic microsome model system contained KCl (0.12 M), NADPH (0.4 mM), ADP (0.4 mM), $FeCl_3$ (0.01 mM), histidine (5 mM), and microsomes (0.5 mg of protein/mL) at pH 7.3. Microsome nonenzymic lipid peroxidation initiated by the iron redox cycle system contained ascorbic acid (0.2 mM), $FeCl_3$ (0.01 mM), acetate buffer (50 mM at pH 7.0), and microsomes (1.0 mg/mL of protein). Microsome nonenzymic lipid peroxidation initiated by activated metmyoglobin contained MetMb (0.03 mM) and H_2O_2 (0.03 mM), preincubated for 3 min, microsomes (1 mg of protein/mL), and acetate buffer (50 mM at pH 7.0). All model systems were incubated in air in a shaking water bath at 25 °C.

Fresh dark turkey muscle was ground in a Moulinex food processor with a plastic work bowl and stainless steel blades for 60 s. One part of the minced tissues was incubated in polyethylene bags before or after heating to 68.5 °C. Portions (approximately 120 g) of the minced muscle tissues were also placed in 86 × 29 nm drawn round cans, closed under vacuum, and cooked in a 77 °C water to an internal temperature of 68.5 °C within 20–25 min. The polyethylene bags and canned products were chilled in iced water at 5 °C within less than 30 min. The samples were stored at 4 °C and tested periodically for lipid peroxidation. Thiobarbituric acid reactive substances were determined in model systems by a procedure of Bidlack et al. (1973) and in muscle tissues by a method developed by Witte et al. (1970) and calculated as malonodialdehyde (MDA) with an extinction coefficient of $\epsilon_{532} = 1.55 \times 10^5$ M^{-1} cm^{-1} (Buege and Aust, 1978). The accumulation of conjugated diene was performed by the method of Parr and Swoboda (1976) following lipid extraction (Bligh and Dyer, 1959). The samples (250 mg of fat) were dissolved in 5 mL of isooctane–ethanol (1:1, v/v). One-milliliter aliquots of this stock solution were transferred to 25-mL stoppered volumetric flasks, and the volume was made up with ethanol. Lipid peroxidation was monitored by the increase in absorbance at 233 nm, with a molar extinction coefficient of $\epsilon_{233} = 2.52 \times 10^4$ M^{-1} cm^{-1} (Buege and Aust, 1978).

The results are means of triplicates of one microsome preparation; in the figures, each bar (I) denotes the standard deviation.

RESULTS AND DISCUSSION

Lipid peroxidation in muscle tissues could be propagated by breakdown of preformed hydroperoxides. Our results show that a small amount of TBA-RC is produced even

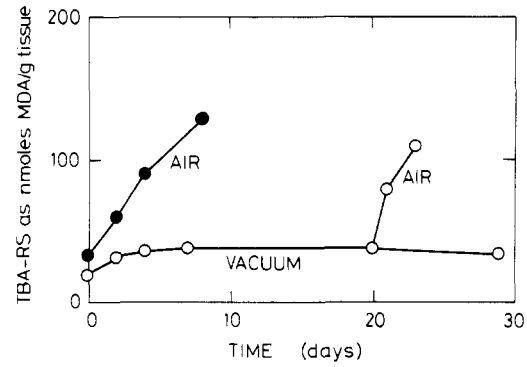

Figure 1. Effect of the presence of oxygen on lipid peroxidation of minced turkey dark muscle stored at 4 °C, as determined by TBA-RS accumulation.

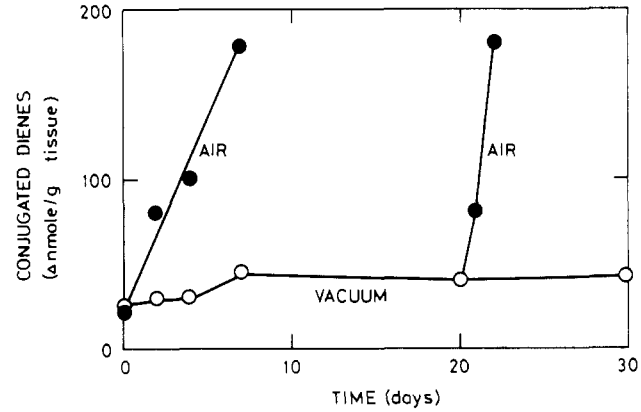

Figure 2. Effect of the presence of oxygen on lipid peroxidation of minced turkey dark muscle stored at 4 °C, as determined by conjugated diene accumulation.

if the minced turkey muscle was canned and heated under vacuum. During storage under vacuum, the amount of TBA-RC does not change significantly (Figure 1). The same results were obtained if lipid peroxidation was determined by the accumulation of conjugated dienes (Figure 2). If, however, the cans have been opened to air and stored at 4 °C for several days, the minced muscle starts to peroxidize at a very rapid rate. The same results were obtained after 20 days of storage. Our results demonstrate the importance of oxygen in the propagation of lipid peroxidation in muscle tissues, as determined by two different methods.

The second part of our results shows that lipid peroxidation in minced turkey muscle tissues depends on catalysts, in particular free metal ions. Membrane lipid peroxidation in a model system was adopted for studying the effect of metal ion chelating agents, especially EDTA, on the oxidative process. Enzymatic and iron redox cycle membrane lipid peroxidation were inhibited by EDTA at the relatively low concentration of 20–25 μM; H_2O_2-activated MetMb membrane lipid peroxidation was also inhibited, but at a concentration of 1 mM and above.

Many researchers utilize a high concentration of EDTA in raw and heated muscle tissues to inhibit in situ lipid peroxidation and evaluate the role of non-heme iron or heme proteins in the catalysis of this reaction (Lin and Watts, 1970; Sato and Hegarty, 1971; Igene et al., 1979; Tay et al., 1983; Shahidi et al., 1986). Igene et al. (1979) and Tay et al. (1983) used EDTA at a concentration of 2%, which is equivalent to 50 mM. This concentration, as shown in Figure 3, could inhibit not only free iron catalysis but also activated hemeproteins.

Studying in situ raw and heated minced muscle lipid peroxidation showed that EDTA at a concentration lower

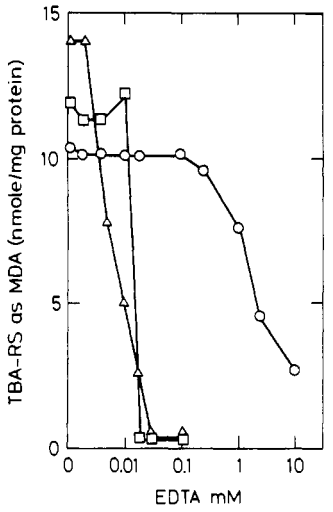

Figure 3. Effect of EDTA concentration on membrane lipid peroxidation: □, enzymic microsome system; △, nonenzymic microsome iron redox cycle system; ○, nonenzymic microsome H_2O_2-activated metmyoglobin system.

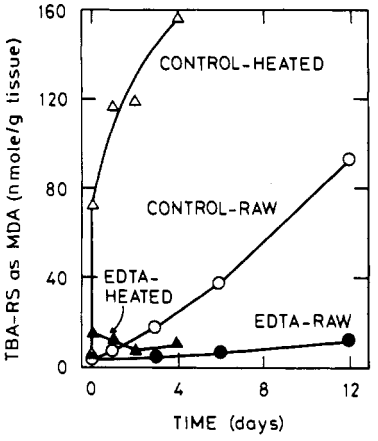

Figure 4. Effect of EDTA (400 μM) on the inhibition of minced turkey dark muscle lipid peroxidation before and after heat treatment.

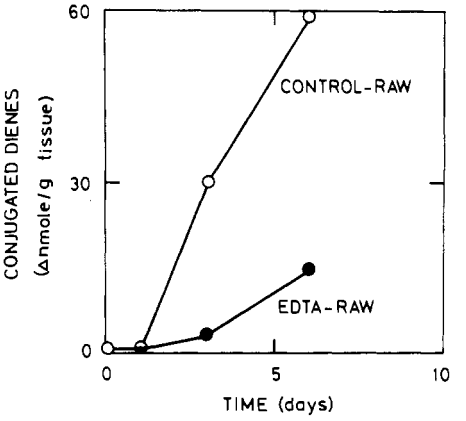

Figure 5. Effect of EDTA (400 μM) on the inhibition of minced turkey dark muscle lipid peroxidation as determined by conjugated dienes accumulation.

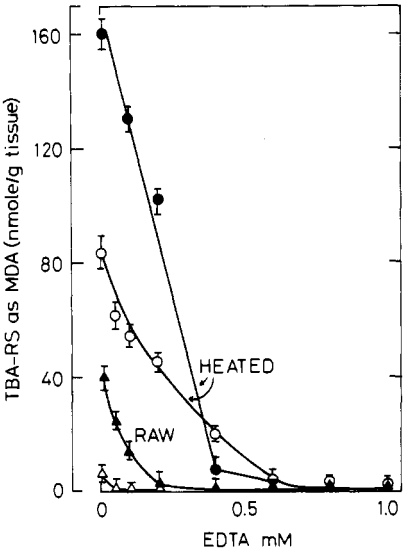

Figure 6. Effect of EDTA concentration on the inhibition of lipid peroxidation of minced turkey dark muscle stored at 4 °C: △, raw, zero time; ▲, raw, 5 days; ○, heated, zero time; ●, heated, 5 days.

than that which could affect activated MetMb inhibits lipid peroxidation (Figures 4 and 5). We assume that in turkey minced muscles the most important catalysts for lipid peroxidation are free metal ions (easily chelated, which could participate in redox cycle reactions) and most probably iron ions (Kanner et al., 1988). In model systems, EDTA at a concentration of ~20 μM inhibits lipid peroxidation almost completely; in situ in muscle tissues this was inactivated at a concentration of ~400 μM (Figure 6). As the amount of free iron ions in turkey muscle tissues is ~2.5 μg/g (Kanner et al., 1988), this is equivalent to ~40 μM free iron, which is fourfold higher than the amount of free iron in our model systems. It seems that the higher amounts of EDTA needed to inhibit in situ muscle lipid peroxidation derived from the compartmentation of iron in muscle tissues.

Most PUFA in muscle tissues are associated with the cell membrane, plasma, and organelles. The special arrangement of the membrane constituents provides it with a structure that prevents spontaneous lipid peroxidation by exogenous species (Kanner et al., 1986). Ferrous ions, which seem to be produced by reducing compounds in muscle tissues (Kanner et al., 1986), can stimulate PUFA peroxidation by generating hydroxyl radicals from hydrogen peroxide but also by decomposing preformed lipid peroxides to form the alkoxyl radical (Gutteridge, 1984). Hydroxyl and alkoxyl radicals are substances with a high reactivity, and for this reason the radicals will not move far away from their site of formation Ferrous ion, in order to affect unsaturated fatty acids, which are in the endogenous part of the membranes, should be bound at or near the target molecule.

It seems that EDTA inhibits muscle lipid peroxidation by two different mechanisms: It pulls metals off membranes' endogenous binding sites. At a concentration twice that of iron, it deactivates the metal by surrounding it with tightly bound ligands that cannot be replaced by reagents such as hydroperoxides (Waters, 1971).

In membrane model systems and raw minced muscle, lipid peroxidation seems to be inhibited especially by the first mechanism. In heated minced muscle, however, because of the distruction of the special arrangement of the membranes, the inhibitory effect of EDTA seems to derive also from the second mechanism. This might explain in part the inhibition of heated minced muscle only by higher concentrations of EDTA.

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Catalytic "Free" Iron Ions in Muscle Foods

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Catalytic "free" iron was determined in muscle foods by the bleomycin method. The results demonstrated that turkey and chicken muscle contain significant amounts of free catalytic iron. Base propenal derived from DNA during the incubation of bleomycin with muscle water extracts was inhibited by the iron chelator desferrioxamine. The amounts of free iron in turkey and chicken dark muscles were found to be 2.5 and 0.5 $\mu g/g$, and those of light muscles, 0.9 and 0.2 $\mu g/g$, respectively. The amount of free iron in turkey and chicken dark muscle correlates well with the tendency of this muscle tissue to undergo lipid peroxidation. Turkey and chicken dark and white muscle stored at 4 °C for 1 week releases free iron ions, the concentration of which increases by 2–3-fold over fresh samples. The implication of free iron in the quality of muscle foods is discussed.

Iron is an important catalyst in biological systems. Transition metals, e.g. iron and copper, with their labile d-electron system, are well suited to catalyze redox reactions. The stable oxidation states of iron in aqueous systems are exclusively the ferric (Fe^{3+}) and ferrous (Fe^{2+}) forms (Spiro and Saltman, 1969). Free iron, especially ferric ions, is insoluble in water above pH 3.0, and therefore in order to participate in aqueous-phase reactions they must be chelated in some form of soluble ligand complex, such as EDTA, ascorbate, or ADP. The redox chemistry of iron is itself directly affected by the coordinating ligands (Richter and Waddel, 1983).

The two oxidation states of iron allow it to act as an electron donor and acceptor, which give it important catalytic properties. In the presence of oxygen, ferrous ions produce the superoxide anion radical which dismutates to H_2O_2. Ferrous ions further reduce H_2O_2 to hydroxyl radicals (Cohen and Sinet, 1980). The hydroxyl radical ($HO^{\bullet}$) can attack and oxidize any bioorganic compounds present in live cells. Recently, we demonstrated that hydroxyl radicals are generated during the iron redox cycle by ascorbic acid; however, it seems that most of the membrane lipid peroxidation was stimulated by breakdown of hydroperoxides during interaction with the reduced metal (Kanner et al., 1986).

Ferrous ion can stimulate polyunsaturated fatty acid (LH) peroxidation by decomposing preformed lipid peroxides (LOOH) to the alkoxyl radical ($LO^{\bullet}$), which will attack other lipid molecules.

In muscle foods, e.g. meat, poultry and fish, iron accumulates in myoglobin, hemoglobin, ferritin, and transferrin. A small pool of non-protein-bound iron moving among transferrin, cell cytoplasma, and ferritin could provide

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