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Lipid Oxidation in a Chicken Muscle Model System: Oxidative Response of Lipid Classes to Iron Ascorbate or Methemoglobin Catalysis

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Catalysis by iron ascorbate and activated methemoglobin generated different oxidative responses in chicken muscle model systems. In iron ascorbate systems, large increases in hydroperoxides and thiobarbituric acid-reactive substances (TBARS) occurred during the initial stage of incubation. Thereafter, iron ascorbate catalysis led to a slow increase in the oxidation of triacylglcyerol (TG) and sarcoplasmic reticulum (SR) membrane lipids. By the end of incubation, 24, 36, and 32% of the initial content of *n*−3 fatty acids in free fatty acids, TG, and SR single-lipid model systems catalyzed by iron ascorbate had been lost. Reduced losses of *n*−3 fatty acids were observed in the SR and TG fractions (0 and 24%, respectively) when iron ascorbate model systems contained all three lipid fractions (mix). Hydroperoxides and TBARS in model systems catalyzed by activated methemoglobin were characterized by a lag phase during most of the incubation. Consistent with their role as antioxidants, losses of α-tocopherol (42−49%), γ-tocopherol (36−42%), and protein sulfhydryls (41−52%) were observed in model systems catalyzed by activated methemoglobin. SR and mix model systems were 30−50% slower to oxidize than TG model systems when activated methemoglobin served as the catalytic agent.

Keywords: *Membrane; triacylglycerol; free fatty acid; sulfhydryl; tocopherol; hydroperoxide*

INTRODUCTION

Lipid oxidation is implicated not only in clinical conditions such as cancer and rheumatoid arthritis (Halliwell and Gutteridge, 1984) but also in quality deterioration of foods. Although model systems are often used to understand complex biological systems, they are also used to comprehend and explain chemical and biochemical reactions in food systems. In a previous study, a model system was assembled to simulate chicken muscle tissue for subsequent lipid oxidation studies (Sista and Erickson, 1999). The model system contained chicken muscle fibers, lipid, and water, in proportions typically found in chicken tissue.

Responses to oxidation by the lipid classes, free fatty acids (FFA), triacylglycerols (TG), and phospholipids (PL), have been previously investigated. Labuza et al. (1969) demonstrated that oxidation of FFA was faster than if the fatty acids were esterified in TG. Membrane PL was also found to be more susceptible to oxidation than emulsified TG (Slabyj and Hultin, 1984). The greater oxidative susceptibility of PL was attributed to the arrangement of fatty acids in the membrane bilayer, thereby making it more accessible to the catalytic sites and facilitating propagation. Additionally, the degree of unsaturation in PL is higher compared to that in TG (Igene et al., 1980; Lokesh et al., 1981). This composi-

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tional difference may be responsible for the rapid oxidation of PL in cooked meats that leads to the presence of warmed-over flavors (Igene, 1980; Keller and Kinsella, 1973; Tichivangana and Morrisey, 1982).

The environment of each lipid class has a major impact on the susceptibility of a lipid class to oxidize. For example, higher oxidation rates were seen in the fatty fish Pacific mackerel and sardines compared to the lean fish plaice and carp (Toyomizu and Hanaoka, 1980). Whereas the predominant lipid class in dark muscle is TG, higher concentrations of myoglobin and lipase play a major role in the higher oxidative rates seen in fatty fish (Foegeding et al., 1996; Tichivangana and Morrissey, 1982). Similarly, the hydrolytic enzyme, phospholipase, will affect the participation of membrane PL, but the outcome is dependent on the state of the released fatty acids. If oxidized, the FFA will accelerate oxidation, whereas if the FFA have not been oxidized, they will inhibit subsequent lipid oxidation (Mazeaud and Bilinski, 1976; Shewfelt et al., 1981; Borowitz and Montgomery, 1989). In the latter case, it is theorized that unoxidized FFA cause physical changes in the membrane that limit attack by initiating radicals (Hultin, 1979).

In a few studies, lipid susceptibility to oxidation has also been found to be dependent on the source of oxidant stress. Addition of hemoglobin accelerated the oxidation of phospholipids but had little effect on neutral lipids (Hsieh and Kinsella, 1989). Alternatively, cobalt and copper ions were effective catalysts when emulsions of fatty acids were examined and ineffective catalysts when phospholipid liposomes served as the oxidative substrate (Yoshida and Niki, 1992). More recently, Mei et al. (1998) found that the rate of iron-catalyzed oxidation of emulsified oils was dependent on the type of surfactant and surface charge of the lipid. Proximity to the dominating oxidative catalysts may therefore play a primary role in dictating the degree to which each lipid class oxidizes.

To identify the oxidative susceptibility of lipids in the face of changing catalytic conditions, a chicken model system incorporating FFA, TG, or membrane PL, individually or in combination, was monitored for its oxidative response. Several oxidative measures were used in this study to determine if the relative contribution of the lipid classes to the oxidation of the system varied with time.

MATERIALS AND METHODS

Muscle Fiber Isolation. Fresh, skinless, boneless chicken breast from Gold Kist Farms (Ellijay, GA) was shipped on ice to the laboratory. Immediately upon arrival, the meat was cut up and ground for isolation of muscle fibers according to the procedure of Sista and Erickson (1999).

Lipid Fraction Preparation. Skinless, boneless chicken breast (Tyson Foods, Inc.) was obtained from a local grocery store, and its lipid was extracted with chloroform/methanol (2:1) following the procedure described in Erickson (1993). Separation and isolation of lipid fractions (TG and PL) were conducted according to the procedures described in Sista and Erickson (1999). A FFA fraction was prepared by hydrolysis of TG using the procedure described in Kates (1986). Isolation and reconstitution of sarcoplasmic reticulum (SR) membrane fractions were accomplished using the modified procedure of Decker et al. (1988). Isolated lipid fractions and SR membrane vesicles were stored at −20 and −85 °C, respectively, until used.

Model System Preparation. Model systems containing chicken muscle fibers (0.15 g), buffer (0.45 mL, 0.12 M KCl, 30 mM histidine, pH 6.8) and lipid were prepared as described in Sista and Erickson (1999). Lipid in these systems was furnished as either TG (3 mg) emulsions, SR (2 mg of PL) membrane vesicles, FFA (0.3 mg), or a mix consisting of TG, SR, and FFA (3 mg, 2 mg of PL, and 0.3 mg, respectively). Two freshly prepared catalysts, iron−ADP−ascorbate (15 μM:150 μM:60 μM) or activated methemoglobin, 30 μM (Decker and Faraji, 1990), challenged the systems. The systems were incubated for 0, 10, 20, and 30 h at 15 °C, after which time they were analyzed for hydroperoxides, thiobarbituric acid reactive substances (TBARS), protein sulfhydryls, fatty acids, and tocopherols.

Controls that contained everything except the catalyst were run simultaneously. All reactions were carried out in duplicate, and each experiment was repeated twice.

Hydroperoxides. Chloroform/methanol (2:1) was used for the extraction of lipid from model systems. Hydroperoxides in the extracted lipids were analyzed and calculated using the procedure outlined by Buege and Aust (1978).

TBARS. The method described by Sista and Erickson (1999) was used for the analysis of TBARS in oxidized model systems. The absorbance values were compared to a standard curve prepared with 1,1,3,3-tetraethoxypropane for the calculation of TBARS concentrations.

Sulfhydryls. The modified procedure of Sista and Erickson (1999) was used to quantify protein sulfhydryls in model systems. A molar extinction coefficient of 14150 M^{-1} cm^{-1} was used in the calculation of concentrations.

Fatty Acids. Lipid from oxidized model systems was extracted using 2:1 chloroform/methanol. For model systems containing only one lipid class, a portion of the lipid extract was evaporated and dissolved in benzene (0.8 mL). For model systems containing the three lipid classes, the lipid extract was first evaporated, reconstituted, and then separated on a thin-layer chromatography plate into lipid bands using conditions described in the lipid fractions preparation section. Thebands were scraped into separate tubes before benzene (0.8 mL) was added. Following the addition of benzene, 4% sulfuric acid in methanol was added to the lipid fractions for esterification of fatty acids according to the procedure of Erickson and Selivonchick (1988). Fatty acid methyl esters (FAME) were separated on a Hewlett-Packard 5790 series gas chromatograph (GC) that was installed with an Omegawax 250 (30 m × 0.25 mm × 0.25 μm film, Supelco Inc., Bellefonte, PA) column and equipped with a flame ionization detector. The initial temperature of the column was set at 180 °C, increased to 220 °C at a rate of 4 °C/min, and then held at 220 °C for an additional 20 min. The detector temperature was set at 250 °C while the temperature at the injection port was maintained at 200 °C. Helium was used as the carrier gas at a flow rate of 30 cm/s. Relative retention times of FAME standards (Supelco Inc., Bellefonte, PA), subjected to the same conditions as the samples, were used to identify chromatographic peaks.

Tocopherol. Model systems containing SR or the mix (mixture of SR, TG, and FFA) were saponified in the presence of ascorbic acid and ethanol followed by extraction of tocopherols with 10% ethyl acetate in hexane (Erickson, 1992). The extract was evaporated to dryness under nitrogen, reconstituted in 100 μL of methanol/water (98:2), and filtered through a 0.2 μm filter. Tocopherol separation of the extract was carried out by reverse phase high-performance liquid chromatography as described by Vatassery and Smith (1987). The ESA Coulochem II (model 5200A, Chelmsford, MA) installed with an analytical cell (model 5011) and detection limit set at 10 μA served as the instrument for the detection of eluting substances. Tocopherol standards (Sigma, St. Louis, MO) were used to identify and quantify α- and γ-tocopherol.

Statistical Analyses. All statistical analyses were carried out using SAS (Cary, NC). The level of significance was set at $\alpha = 0.05$ for all tests.

RESULTS

Hydroperoxides. Hydroperoxide levels in model systems challenged with iron ascorbate over time are

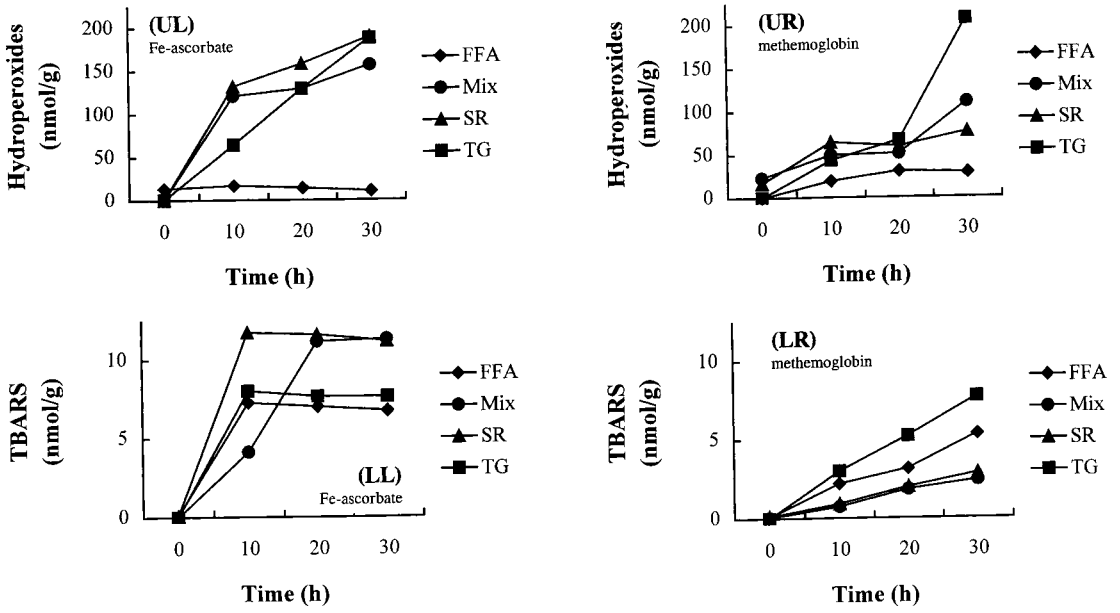

Figure 1. Hydroperoxide (U-) and TBARS (L-) concentrations in model systems over time when challenged with iron ascorbate (-L) or activated methemoglobin (-R). Lipid in the model system was added as SR (2 mg of PL), FFA (0.3 mg), TG (3 mg), or a mix consisting of SR, FFA, and TG (2, 0.3, and 3 mg, respectively). Other constituents of the model system were chicken muscle fibers (0.15 g) and buffer (0.45 mL, 0.12 M KCl, 30 mM histidine, pH 6.2). The systems were challenged with either an iron/ADP/ascorbate catalyst (15 μM:150 μM:60 μM) or an activated methemoglobin catalyst (30 μM). All systems were incubated at 15 °C for 0, 10, 20, and 30 h.

shown in Figure 1-UL. In model systems containing FFA, hydroperoxide concentrations did not increase from initial values. In contrast, significant differences were noted over time for SR, TG, and mix model systems. At 10 and 20 h, model systems containing TG had lower hydroperoxide concentrations than SR model systems.

Using activated methemoglobin as the catalyst, hydroperoxide concentrations increased significantly over time for SR, mix, and TG model systems (Figure 1-UR). The periods of time at which these increases were noted, however, varied with the system under study. In the case of SR model systems, significant increases in hydroperoxide concentrations were already noted by 10 h, whereas in TG and mix model systems, these increases were not observed until 20 and 30 h, respectively. The largest accumulation of hydroperoxides over the entire treatment period occurred in model systems containing TG.

TBARS. Concentrations of TBARS increased significantly over time for all model systems when challenged by either iron ascorbate or activated methemoglobin (Figure 1-LL and -LR). Different patterns of accumulation, however, were noted among the catalyst treatments. For model systems challenged with activated methemoglobin, TBARS increased linearly over time. In contrast, TBARS concentrations in FFA, TG, or SR model systems challenged with an iron ascorbate catalyst were maximal at 10 h. TBARS concentrations in the mix model systems containing the iron ascorbate catalyst continued to increase until 20 h; thereafter, levels were similar to those for SR.

Protein Sulfhydryls. No significant sulfhydryl losses were noted over time for any of the model systems when iron ascorbate served as the catalytic agent (Table 1). In contrast, losses of protein sulfhydryls were noted in the model systems when activated methemoglobin served as the catalyst. In these catalyzed systems, ~50% of the protein sulfhydryls had been lost by 30 h. The systems did vary in the time period at which significant losses could first be detected. For both FFA and SR model systems, losses were detected by 10 h, whereas significant losses were not detected until 20 h for TG and mix model systems.

Table 1. Protein Sulfhydryl Concentrations (Micromoles of Sulfhydryl per Gram of Model System) over Time in Model Systems Challenged with either Iron Ascorbate or Activated Methemoglobin[a]

catalyst	lipid system	time 0 h	10 h	20 h	30 h
iron ascorbate	FFA	10.1a	6.4a	8.4a	10.8a
	mix	16.9a	18.3a	17.9a	17.7a
	SR	15.7ab	13.4b	15.2b	16.0a
	TG	7.6a	6.7a	10.2a	9.6a
activated methemoglobin	FFA	27.3a	22.5b	16.3c	13.1c
	mix	39.9a	39.7a	29.7b	23.5c
	SR	37.2a	29.9b	25.0c	17.9d
	TG	24.4a	21.0a	16.8b	12.5c

[a] Means followed by the same letter within a row are not significantly different.

Fatty Acids. The greatest degree of unsaturation in the lipid fraction was found in the $n-3$ fatty acids. As susceptibility to oxidation increases with increased level of unsaturation (Cosgrove et al., 1987), it was anticipated that this group of fatty acids would serve as a sensitive indicator of lipid oxidative activity. Table 2 illustrates the response of the $n-3$ fatty acids in the model systems to the two different catalyzing agents. All lipid classes when present by themselves in model systems were susceptible to iron ascorbate catalyzed lipid oxidation as evidenced by the loss of $n-3$ fatty acids over time. If present in the mix, only the SR and FFA fractions from treated samples displayed losses of $n-3$ fatty acids in iron ascorbate systems. In contrast, no measurable losses of $n-3$ fatty acids were detected in SR or mix model systems when activated methemoglobin served as the catalyst.

Table 2. *n*−3 Fatty Acid Composition (Weight Percent) of Model Systems Challenged by Iron Ascorbate or Activated Methemoglobin[a]

			wt % at time			
catalyst	system	lipid	0 h	10 h	20 h	30 h
iron ascorbate	single lipid	FFA	7.1ab	7.5a	6.0bc	5.4c
		TG	3.6 a	3.5a	2.6b	2.3b
		SR	13.4a	11.4b	9.7c	9.1d
	mixed lipid	FFA	3.3a	2.0b	2.5b	1.9b
		TG	1.1a	1.2a	1.1a	1.1a
		SR	13.6a	10.7b	10.0b	10.4b
activated met-hemoglobin	single lipid	FFA	7.6a	6.1b	5.6b	5.4b
		TG	6.0a	3.3b	3.4b	2.1c
		SR	12.6a	12.8a	12.4a	12.0a
	mixed lipid	FFA	2.0a	2.4a	2.6a	2.7a
		TG	1.1a	1.1a	0.9a	0.9a
		SR	10.6a	11.8a	11.5a	11.8a

[a] Means followed by the same letter within a row are not significantly different.

Tocopherol. Iron ascorbate challenged mix model systems led to significant losses of α- and γ-tocopherol after 10 h of incubation (Figure 2-UL and -LL). At 20 and 30 h, no further losses were recorded in mix model systems. In the case of SR model systems, mean α-tocopherol levels were not found to have statistically changed over time due to the large variability in the data. A significant loss of γ-tocopherol was observed in SR model systems over the first 10 h of treatment. In SR and mix model systems challenged with activated methemoglobin, both α- and γ-tocopherol concentrations decreased significantly over time (Figure 2-UR and -LR). When catalyst was absent, no significant loss of either α- or γ-tocopherol was recorded over the trial period in SR or mix model systems (data not shown).

DISCUSSION

The model systems catalyzed by iron ascorbate and activated methemoglobin had distinctive responses to the oxidative treatments. Different profiles over time were found in levels of hydroperoxides, TBARS, protein sulfhydryls, fatty acids, and tocopherols for the two types of catalyzed systems. Most evident were the large increases in hydroperoxides and TBARS by 10 h of treatment in systems catalyzed by iron ascorbate (Figure 1-UL and -LL). This type of response is contrary to the typical response displayed during the initiation of lipid oxidation, when a lag phase is present in initial phases and then an exponential phase follows. During the first 10 h of treatment in this study, homolytic degradation of pre-existing hydroperoxides by the low molecular weight iron (Tappell, 1953) added to the system could have accounted for such a large accumulation of hydroperoxides and TBARS. Over this same period of time, large losses were also observed for both α- and γ-tocopherol in SR and mix model systems. The concurrent generation of hydroperoxides and consumption of tocopherol support the interpretation that alkoxyl radicals had been generated as tocopherol does not preferentially scavenge these types of radicals in the presence of an excess of lipid substrate (Fukuzawa and Fujii, 1992).

During the incubation period from 10 to 30 h in iron ascorbate systems, hydroperoxide concentrations increased linearly in mix, SR, and TG model systems (Figure 1-UL) as would be typical of a lag phase. The lack of further accumulation of TBARS during this period for SR and TG systems is also typical of a lag phase, when initiation reactions predominate over propagating reactions. Over the same period of time, however, no losses of tocopherol were recorded in model systems containing membranes (Figure 2-UL and -LL). The absence of tocopherol degradation would imply either that tocopherol had no antioxidant capabilities in the iron ascorbate catalyzed system or that tocopherol had been depleted in the first 10 h to levels that were ineffective against peroxyl radicals.

On the basis of the hydroperoxide and TBARS data, no oxidation of the FFA lipid fraction occurred after 10 h in iron ascorbate catalyzed systems (Figure 1-UL and -LL). In contrast, the fatty acid data did show losses of *n*−3 fatty acids from the FFA model systems throughout incubation (Table 2). In mix model systems containing FFA, TG, and SR lipid fractions, levels of TBARS were similar to those in SR model systems after 30 h of incubation (Figure 1-LL). At the same time, levels of hydroperoxides in mix model systems were significantly lower than model systems containing either SR or TG lipid fractions (Figure 1-UL). The lower hydroperoxide levels may be attributed to the lack of oxidation of TG *n*−3 fatty acids in mix model systems (Table 2). It is conjectured that FFA in the mix model systems inhibited oxidation of TG by ionically complexing with low molecular weight iron in the system and thus inhibiting the interaction of Fe with the nonpolar TG fraction.

Similar to low molecular weight iron, heme compounds have also previously been demonstrated to decompose pre-existing fatty acid hydroperoxides (Tappell, 1953). In this case, however, model systems catalyzed by activated methemoglobin were characterized by a lag phase in hydroperoxide accumulation over the first 20 h of incubation (Figure 1-UR). Reduced access to pre-existing hydroperoxides in these muscle fiber systems by the bulky heme proteins may have occurred. TBARS, on the other hand, were generated at a slow steady rate in these systems (Figure 1-LR). Consequently, it is conceivable that activated methemoglobin not only contributed to the generation of hydroperoxides but aided in the breakdown of these same hydroperoxides to secondary products. This scenario would explain the accumulation of TBARS in FFA model systems when hydroperoxides were not seen to increase.

Loss of protein sulfhydryls occurred in all model systems catalyzed by activated methemoglobin (Table 1). In fact, when the sulfhydryl data were compared to the TBARS data, a strong negative correlation (−0.86) was found, suggesting that sulfhydryls were utilized to stabilize primary reaction products. In these systems, protein sulfhydryls were furnished by both protein fibers and methemoglobin; consequently, losses could conceivably have been primarily from the methemoglobin fraction. Alternatively, the sulfhydryl losses seen in systems catalyzed by activated methemoglobin may be due to the absence of a reducing agent, such as ascorbic acid, that would be capable of scavenging and regenerating sulfhydryl radicals (Scheschonka et al., 1990).

No significant differences in SR *n*−3 fatty acid levels were found following incubation of SR or mix model systems catalyzed by activated methemoglobin (Table 2). The absence of oxidation of SR *n*−3 fatty acids may account for the lower levels of hydroperoxides and TBARS found in systems containing SR compared to systems containing TG (Figure 1-UR and -LR). Limited interaction between the catalyst and membrane lipids may be responsible for the slow oxidation of these

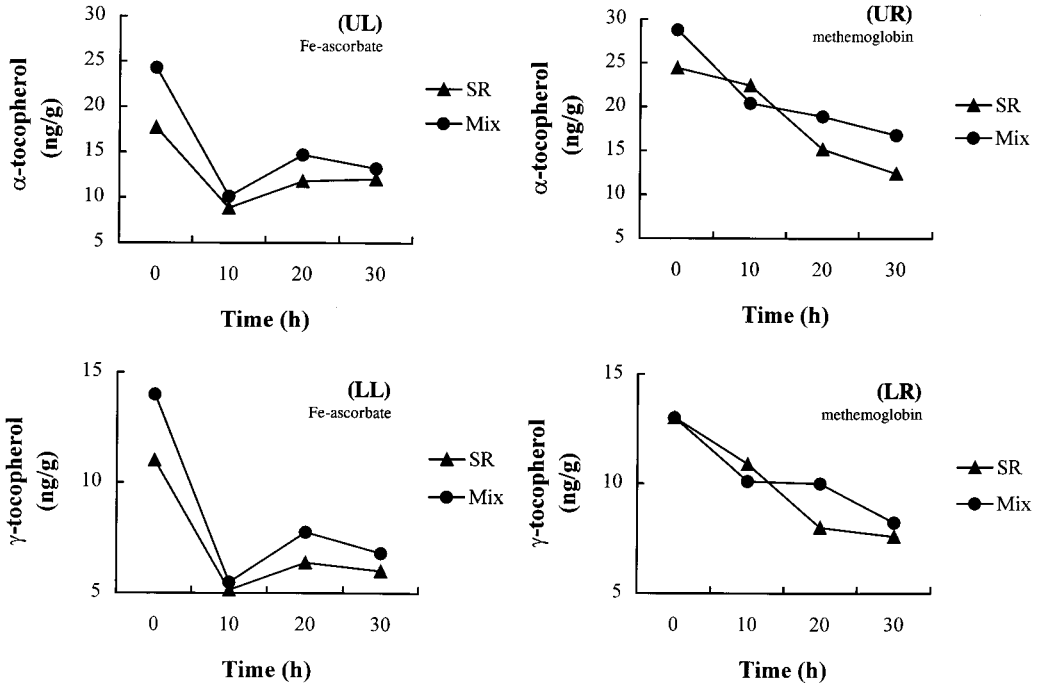

Figure 2. Levels of α-tocopherol (U-) and γ-tocopherol (L-) in model systems over time when challenged with iron ascorbate (-L) or activated methemoglobin (-R). Lipid in the model system was added as SR (2 mg of PL), FFA (0.3 mg), TG (3 mg), or a mix consisting of SR, FFA, and TG (2, 0.3, and 3 mg, respectively). Other constituents of the model system were chicken muscle fibers (0.15 g) and buffer (0.45 mL, 0.12 M KCl, 30 mM histidine, pH 6.2). The systems were challenged with either an iron/ADP/ascorbate catalyst (15 μM:150 μM:60 μM) or an activated methemoglobin catalyst (30 μM). All systems were incubated at 15 °C for 0, 10, 20, and 30 h.

systems. Alternatively, preferential scavenging of oxidants by tocopherol may have occurred as a progressive loss of α- and γ-tocopherol (Figure 2-UR and -LR) was also observed in these systems. Tocopherol or another component of SR may also serve to protect other lipids in the mix model system. In mix model systems, no losses of *n*−3 fatty acids occurred for the TG and FFA fractions, whereas losses had been observed in single-lipid model systems.

In conclusion, different patterns of oxidative responses were observed in model systems when different catalysts were used. Lipid classes also responded differently when present in single-lipid model systems compared to multiple-lipid systems. Attention should therefore be given to compositional variations in the system under study when one is attempting to identify the most susceptible site for lipid oxidation. In addition, this study also demonstrated the importance of multiple assays to interpret the complicated mechanism of lipid oxidation. The pattern that emerged from the varied responses of individual and mixed-lipid systems emphasized that the intricacies of lipid oxidative reactions could not be explained by one assay alone. Therefore, to understand the influence of lipid on oxidation and its effect on other components of the system, a combination of assays is necessary.

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