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Mechanism of Oxymyoglobin Oxidation in the Presence of Oxidizing Lipids in Bovine Muscle

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Lipid oxidation and oxymyoglobin oxidation were measured in bovine muscle homogenates. *M. longissimus dorsi* homogenates (25% w/w, pH 5.7) were prepared, held at 4 °C, and subjected to one of the following treatments: (i) stirred and bubbled with oxygen, (ii) stirred with no oxygen, or (iii) neither stirred nor bubbled with oxygen (control). Lipid oxidation was initiated with 45 μM ferric chloride/sodium ascorbate. Lipid oxidation was highest and oxymyoglobin oxidation lowest in the homogenate bubbled with oxygen while lipid oxidation was lowest and oxymyoglobin oxidation highest in the control homogenate. Dissolved oxygen became depleted over time in the control homogenate, remained high in the homogenate bubbled with oxygen, but decreased and then increased in the homogenate stirred with no oxygen. Free radical formation was lower in the control homogenate than in the stirred homogenates as determined by spin trapping and electron spin resonance detection. The data indicated that lipid oxidation-induced oxygen depletion, as opposed to primary or secondary lipid oxidation products, is a likely cause of oxymyoglobin oxidation in muscle systems.

KEYWORDS: Lipid; oxymyoglobin; oxidation; oxygen concentration; free radicals

INTRODUCTION

The relationship between lipid oxidation and oxymyoglobin oxidation has been the subject of numerous research papers over the years. Experiments using muscle (*1*−*3*), muscle homogenates (*4*), and oxymyoglobin in the presence of oxidizing liposomes (*5*, *6*) or microsomal lipid (*7*, *8*) have demonstrated that oxymyoglobin oxidizes to metmyoglobin in the presence of oxidizing lipids. However, the mechanism by which oxidizing lipids, primarily those associated with the membranal lipid fraction of muscle tissue (*9*), can bring about oxidation of oxymyoglobin, which is associated with the muscle aqueous phase, remains unclear.

In red meats, the relationship is of particular interest because both flavor and color are adversely affected by oxidation, flavor because lipid oxidation products give rise to off-flavors, particularly during storage, and color because oxidation of myoglobin leads to meat discoloration (*9*, *10*). It has been suggested that free radicals or peroxides derived from lipid oxidation could react directly with myoglobin causing it to oxidize (*4*, *10*). Chan et al. (*11*) demonstrated that secondary products of lipid oxidation, mainly aldehydes, could accelerate oxymyoglobin oxidation. Lynch et al. (*3*) showed that aldehyde−protein adducts were formed in ground beef at 4 °C and implied that oxymyoglobin could oxidize following adduct formation. Furthermore, Alderton et al. (*12*) found that the redox stability of bovine myoglobin decreased following adduct formation with 4-hydroxy-2-nonenal. However, the ratios of aldehyde lipid oxidation products to myoglobin used in these recent studies (*11*, *12*) appear to be greatly in excess of those likely to occur in oxidized meat (*4*). O'Grady et al. (*4*) suggested that depletion of oxygen during lipid oxidation in meats could cause the oxygen levels to fall to levels conducive to myoglobin oxidation (*13*), a suggestion that is investigated in the present study. On the other hand, free radicals generated during the redox activity of myoglobin derivatives have been suggested to attack lipids in interphases or membranes, in effect initiating lipid oxidation chain reactions (*14*, *15*).

The action of antioxidants in delaying both lipid oxidation and myoglobin oxidation has provided some insight into the relationship between the two reactions. Thus, the addition of vitamin E to muscle model systems (*6*) or increasing muscle vitamin E levels through preslaughter dietary supplementation (*16*) has been shown to inhibit both lipid oxidation and oxymyoglobin oxidation. While the mechanism by which vitamin E inhibits lipid oxidation is well-established, the mechanism by which it inhibits oxymyoglobin oxidation remains unclear. A number of proposals have been made, mainly focusing on the inhibition of lipid oxidation by the antioxidant as a prerequisite for inhibition of oxymyoglobin oxidation (*17*, *18*). In their work with vitamin E supplementation of beef cattle, Faustman et al. (*16*) supported the suggestion of Greene (*10*) that antioxidants inhibit oxymyoglobin oxidation by protecting the reducing systems in muscle, which are largely responsible

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for maintaining myoglobin in its reduced form, from attack by lipid oxidation intermediates such as free radicals. Alternatively, vitamin E could, by inhibiting lipid oxidation, prevent a reaction between lipid-derived free radicals (*10*) or α,β-unsaturated aldehydes (*19*) and oxymyoglobin or the depletion of dissolved oxygen to levels that promote oxymyoglobin oxidation (*4*).

The objective of the research was to study the relationship between lipid oxidation and oxymyoglobin oxidation in an oxidizing muscle system and to investigate the hypothesis that lipid oxidation-induced oxygen depletion may be responsible for oxymyoglobin oxidation.

MATERIALS AND METHODS

Reagents. [N-Morpholino]ethanesulfonic acid (MES), ferric chloride hexahydrate, α-(4-pyridyl-1-oxide)-N-*tert*-butylnitrone (POBN), trichloroacetic acid, and 2-thiobarbituric acid were obtained from Sigma Aldrich (Steinheim, Germany). L-Ascorbic acid (sodium salt) was from Fluka Biochemica (Buchs, Germany).

Preparation of Oxidizing Muscle Homogenates. Bovine *M. longissimus dorsi* (LD) was obtained from a local butcher, cut into 2.5 cm steaks, and stored vacuum packaged at −20 °C until used (less than 2 weeks). Muscle homogenates (25%, w/w) were prepared by homogenizing 25 g of LD in 75 g of 50 mM MES buffer, pH 5.7, using an Ultra Turrax T25 (Janke and Kunkel, IKA-Labortechnik, GmbH & Co., Germany) at 8000 rpm for 3 min. The muscle tissue and buffer were held on ice during homogenization.

LD homogenate samples (100 g) were held in 250 mL beakers at 4 °C in a refrigerated room and subjected to one of three treatments. One sample was stirred continuously at 375 rpm on a Heidolph MR3000 magnetic stirrer (Heidolph, Schwabach, Germany) and simultaneously bubbled with oxygen at ∼400 mL/min. The second sample was stirred but not bubbled with oxygen, and the third sample (control) was neither stirred nor bubbled with oxygen. The water soluble spin trap POBN (40 mM) was added to each homogenate 20 min prior to the initiation of lipid oxidation. Lipid oxidation was initiated in each sample by the addition of ferric chloride/sodium ascorbate (1:1), at 45 μM, using freshly prepared stock solutions (45 mM) of ferric chloride and sodium ascorbate (*20*). Lipid oxidation, oxymyoglobin oxidation, and free radical formation were measured immediately prior to the addition of the prooxidants and at 30 (lipid oxidation and oxymyoglobin oxidation measurement) and 60 (free radical measurement) min intervals thereafter for up to 4 h.

Lipid Oxidation. Lipid oxidation in homogenates was measured following a modification of the 2-thiobarbituric acid procedure of Siu and Draper (*21*). In screw cap test tubes, 3 mL of muscle homogenate was added to 3 mL of 10% trichloroacetic acid, the tube contents were mixed using a vortex mixer, and the precipitate formed was removed by centrifugation (MSE Mistral 2000) at 1000*g* for 10 min. The clear supernatant (4 mL) was added to 0.75 mL of 0.06 M 2-thiobarbituric acid in a screw-cap test tube. The tube contents were incubated at 100 °C for 40 min, and the absorbance at 532 nm was measured against a blank containing all reagents except the LD homogenate. 2-Thiobarbituric acid reactive substances (TBARS) were expressed in units of absorbance at 532 nm.

Oxymyoglobin Oxidation. Muscle homogenate (1.5 mL) was added to 1.5 mL of MES buffer and centrifuged (Sorval RMC 14 microfuge, Dupont) at 6500 rpm for 10 min at 4 °C. The supernatant obtained was filtered through Advantec 5C filter paper, and the absorbance spectrum of the filtrate was obtained on a double-beam spectrophotometer (Varian Cary 3 UV−visible). The proportion of oxymyoglobin (% of total myoglobin) was calculated using absorbance measurements at 525, 545, 565, and 572 as described by Krzywicki (*22*). Myoglobin was assumed to be the main heme pigment present, and residual hemoglobin was treated as a tetramer of myoglobin with similar absorbing properties (*22*).

Free Radical Formation. Muscle homogenate (3 mL) was filtered through Advantec 5C filter paper, and the supernatant obtained was transferred to a flat aqueous cell (Wilmad Glass, Buena, NJ). A ECS 106 electron spin resonance (ESR) spectrophotometer (Bruker, Rheinstetten, Germany) equipped with and ER4103 TM cavity was used for the ESR measurements. The settings were as follows: microwave power, 20 mW; modulation amplitude, 1.0 G; modulation frequency, 100 kHz; conversion time, 163 ms; and time constant, 328 ms. The trapping of radicals in the homogenate gave rise to adducts with six-line ESR spectra (triplets of doublets). The signal height of the second line of the center field doublet was used as a measure of the relative concentration of radical adducts formed in the homogenate at a given time. The response of the ESR instrument was checked daily by recording the spectra of a 2.0 μM aqueous solution of the spin probe TEMPO, and its signal intensity was used to standardize signal intensities in the emulsions in order to compare experiments from different days. The ESR spectra of POBN spin adducts were fitted to simulated spectra by using the Winsim program (*23*).

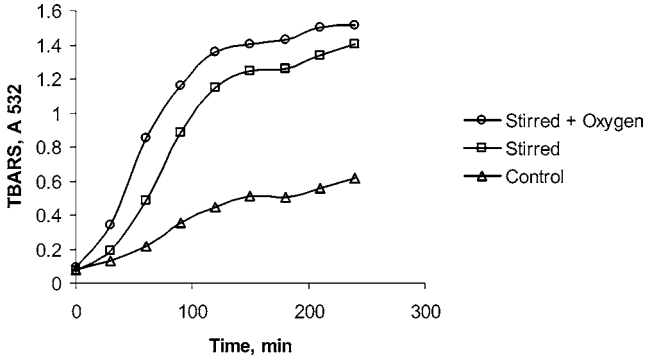

Figure 1. Lipid oxidation (TBARS) in 25% muscle homogenates, held at 4 °C, following the addition of 45 μM ferric chloride/sodium ascorbate. Homogenates were either stirred and bubbled with oxygen (stirred + oxygen) or stirred with no oxygen (stirred) or neither stirred nor bubbled with oxygen (control).

Oxygen Consumption. LD homogenates were prepared as described above and transferred to a cell (90 mL, thermostated at 4 °C) equipped with a microcathode Clark electrode (Sable Systems, Henderson, NV). The homogenates were subjected to the same stirring and oxygen saturation treatments, and lipid oxidation was initiated as described above. The cells were left open to the atmosphere during measurement. Samples were removed for lipid oxidation and oxymyoglobin oxidation measurement as described above, and measurement of oxygen concentration was made every 20 s using a ReadOX-4 oxygen analyzer (Sable Systems). A two point calibration using air-saturated water at 4 °C and N_2-saturated water (4 °C, with added sodium dithionite to remove residual oxygen) was used to calibrate the electrode and oxygen analyzer before use.

Statistics. The experiment was carried out three times on three separate LD steaks. The data were subjected to one way analysis of variance with repeated measures in SPSS (*24*). Differences between treatment means were determined using the least significant difference test.

RESULTS AND DISCUSSION

The progression of lipid oxidation following the addition of the initiators to the homogenates is shown in **Figure 1**. As compared to the control sample, higher levels ($p < 0.05$) of lipid oxidation occurred in homogenates, which were stirred with or without supplementary oxygen, and this may be attributed to the availability of oxygen to support the lipid oxidation reaction (*2*, *25*, *26*). In the control sample, lipid oxidation once initiated proceeded at a lower initial rate probably because available dissolved oxygen became limiting. The dissolved oxygen measurements (**Figure 2**) clearly showed that the oxygen concentration was higher in the stirred homogenate than in the control homogentate and that oxygen concentrations fell in both homogenates following initiation of lipid oxidation; the rate of decrease was greater in the stirred homogenate probably because the rate of lipid oxidation was greater.

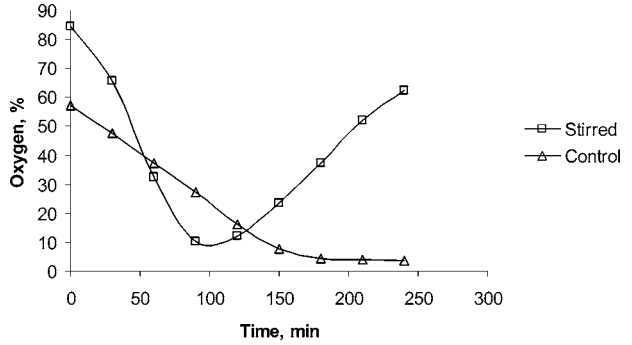

Figure 2. Dissolved oxygen (%) in 25% muscle homogenates, held at 4 °C, following the addition of 45 μM ferric chloride/sodium ascorbate. Homogenates were either stirred with no oxygen (stirred) or neither stirred nor bubbled with oxygen (control).

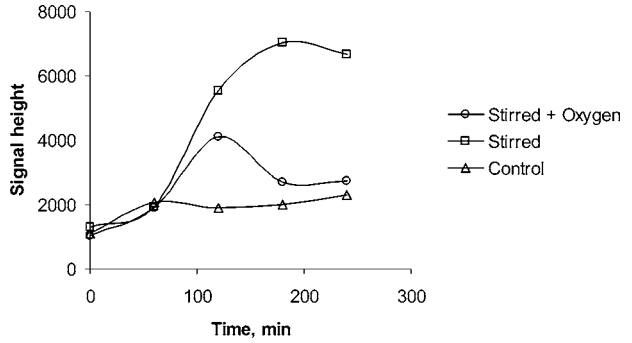

Figure 3. Signal height (at the second peak in the second signal) in 25% muscle homogenates containing 40 mM POBN, held at 4 °C, following the addition of 45 μM ferric chloride/sodium ascorbate. Homogenates were either stirred and bubbled with oxygen (stirred + oxygen) or stirred with no oxygen (stirred) or neither stirred nor bubbled with oxygen (control).

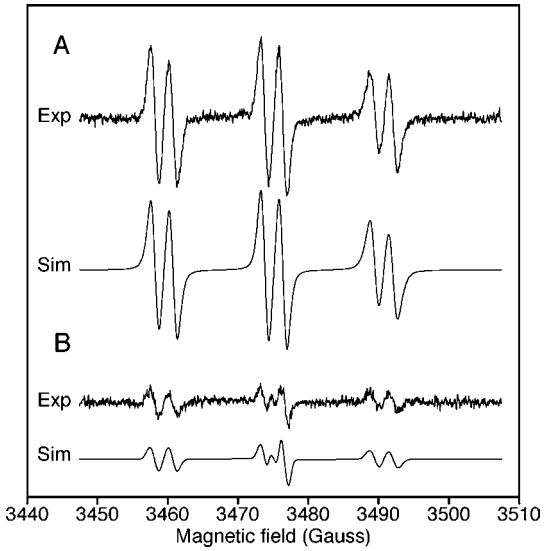

Figure 4. ESR spectra of spin adducts formed in 25% muscle homogenates containing 40 mM POBN, held at 4 °C, following the addition of 45 μM ferric chloride/sodium ascorbate. Homogenates were either stirred with no oxygen (**A**) or neither stirred nor bubbled with oxygen (**B**). The spectra (Exp) were recorded after 240 min. Simulated spectra (Sim) were fitted to the experimental spectra.

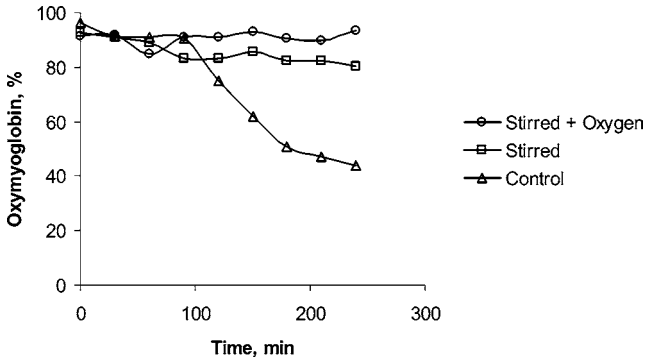

Figure 5. Oxymyoglobin (% of total myoglobin) in 25% muscle homogenates, held at 4 °C, following the addition of 45 μM ferric chloride/sodium ascorbate. Homogenates were either stirred and bubbled with oxygen (stirred + oxygen) or stirred with no oxygen (stirred) or neither stirred nor bubbled with oxygen (control).

However, the oxygen concentration in the stirred sample began to increase after 100 min, when ~10% dissolved oxygen was present, suggesting that oxygen incorporated in the homogenate as a result of the stirring was then in excess of that needed to support lipid oxidation. At the end of the experiment, the dissolved oxygen level in the stirred homogenate was close to that present at the beginning. In the control sample, the oxygen concentration continued to decrease for the duration of the experiment indicating that little oxygen was re-entering this static system. As expected, in the LD homogenate stirred with supplementary oxygen, the level of dissolved oxygen remained constant and high (~500%, based on calibration of the oxygen electrode to 100% with oxygen in air) throughout the experiment (data not shown in **Figure 2**).

In agreement with the lipid oxidation data, free radical adduct formation was lower ($p < 0.05$) in the control homogenate as compared to the homogenate stirred without oxygen (**Figure 3**). Unexpectedly, fewer free radical adducts were detected in the oxygen-saturated homogenate as compared to the homogenate stirred without oxygen. Because TBARS levels were not significantly different between the two homogenates, this suggests that the type of free radical formed may have differed in the two systems or that the free radical adducts formed may have been less stable in an oxygen-saturated environment. However, the ESR spectra obtained from the homogenates stirred with and without oxygen could be fitted to a simulated ESR spectrum of a single radical having hyperfine coupling constants $a_N = 15.6$ G and $a_H = 2.7$ G but with varying intensity and line widths (**Figure 4**). The values of the hyperfine coupling constants suggest that a single POBN spin adduct is formed by trapping a carbon-centered radical (•R) (*27*). It is therefore likely that identical radicals are formed in the homogenates stirred with and without oxygen but that the stability of the spin adducts depends on the level of oxygen in the system.

The ESR spectra of the spin adducts formed in the nonstirred control homogenate had very low intensities. None of the spectra could be satisfactorily fitted to the POBN/•R spin adduct that was observed in the two stirred experiments. However, including a second radical, with a doublet splitting and a coupling constant $a = 1.7$ G, together with the POBN/•R adduct, improved the fitting significantly. The ESR spectrum of the second radical is tentatively assigned to the ascorbyl radical based on the value of the hyperfine coupling constant (*28*). The fitting also revealed that the mixture of observed stable radicals in the nonstirred control experiment consisted of 30−40% of the ascorbyl radical spectrum and 60−70% of the POBN/•R adduct.

Oxymyoglobin oxidation, indicated by the decrease in percentage oxymyoglobin in homogenates, occurred to the greatest extent in the control sample (**Figure 5**) although significant differences ($p < 0.05$) in percentage oxymyoglobin

existed between all treatments. Because the control sample was also the one with the least amount of lipid oxidation (**Figure 1**), the results suggest the peroxides or lipid oxidation products alone are not the critical determinants of whether oxymyoglobin oxidation occurs and, therefore, that lipid oxidation and oxymyoglobin oxidation may not always be positively correlated, as often reported. The data indicate that the level of dissolved oxygen in the oxidizing system is a critical factor in determining whether oxymyoglobin oxidizes. It is well-established that oxymyoglobin oxidizes to metmyoglobin as oxygen partial pressures fall below 80 mmHg (equivalent to ~10% oxygen) while remaining above 0 mmHg (*29*, *30*). In the control homogenate, the dissolved oxygen concentration fell to ~8% after 180 min and to ~4% by the end of the experiment. In the homogenate stirred with supplementary oxygen, the dissolved oxygen concentration remained high (~five times that in air), as expected, throughout the experiment and oxymyoglobin levels were maintained. However, in the homogenate stirred without supplementary oxygen, the oxygen concentration did in fact decrease after 90 min to ~10%, a level at which oxymyoglobin oxidation would be expected to occur, but increased thereafter. This may explain why oxymyoglobin decreased, although not significantly, relative to the homogenate stirred with oxygen, to ~83% of total myoglobin after 90 min but was maintained at this level thereafter as the oxygen concentration increased to a level no longer conducive to oxymyoglobin oxidation (**Figures 2** and **5**).

From the results, we conclude that in an oxidizing muscle system the depletion of dissolved oxygen as a result of lipid oxidation is the primary cause of oxymyoglobin oxidation. While the results do not support the earlier contention (*4*, *10*, *16*) that free radical or peroxide intermediates of lipid oxidation could directly oxidize oxymyoglobin, they are not at odds with some previous findings. For example, O'Grady et al. (*4*) observed that discoloration of oxidizing beef homogenates stored in open sample vessels occurred at the base of the vessel first and gradually moved upward and suggested that closer to the surface the rate of oxygen depletion was lower because atmospheric oxygen could diffuse into solution to replace oxygen utilized in the lipid oxidation reaction. With regard to other proposed mechanisms, O'Grady et al. (*4*) found that secondary lipid oxidation products incubated with oxymyoglobin at levels appropriate to those found in oxidized beef did not cause oxymyoglobin oxidation. Furthermore, Mooney et al. (*31*) showed that metmyoglobin reductase activity was not significantly lowered in the presence of oxidizing lipids. Ordonez and Ledward (*32*) demonstrated that the rate of metmyoglobin formation in pork decreased with increasing oxygen concentration although the rate of lipid oxidation was not significantly affected by oxygen.

If the results of the present work are extrapolated to a practical situation, they suggest that red meats stored in high oxygen atmospheres could undergo lipid oxidation, with associated flavor deterioration, yet maintain an acceptable red color. There is some evidence that this may indeed occur (*33*). Furthermore, it may be concluded that the extension of color shelf life achieved by the elevation of muscle vitamin E (*1*, *16*, *26*) is due to the inhibition of lipid oxidation-induced depletion of dissolved oxygen in muscle during storage and that it is essential that native antioxidants, such as vitamin E, are present at sufficient levels in muscle tissue to retard lipid oxidation particularly when high oxygen packaging is used.

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