Peroxynitrite-Induced Oxidation of Lipids: Implications for Muscle Foods

Robert G. Brannan and Eric A. Decker*

Department of Food Science, University of Massachusetts, Amherst, Massachusetts 01002

Peroxynitrite ($ONOO^-$), formed from the nearly diffusion limited reaction between nitric oxide and superoxide, could be an important prooxidant in muscle foods. The objective of this study was to determine whether peroxynitrite caused oxidation of pyrogallol red, liposomes, muscle microsomes, and skeletal muscle homogenate. Oxidation of pyrogallol red, liposomes, and microsomes initiated by peroxynitrite continuously produced by 3-morpholinosydnonimine (SIN-1, 2 mM) was time-dependent and enhanced by CO_2 (1 mM). Reagent peroxynitrite (2 mM) caused concentration-dependent oxidation of pyrogallol red, liposomes, and muscle microsomes that was very rapid with no change after 5 min. Peroxynitrite-induced oxidation was suppressed by CO_2 and low pH. Skeletal muscle homogenate oxidized by reagent peroxynitrite (0.5 mM) exhibited gradual oxidation with time and was suppressed by CO_2, low pH, and metal chelators. These data suggest that peroxynitrite could be an important prooxidant in muscle foods.

Keywords: *Peroxynitrite; nitric oxide; superoxide; lipid oxidation; muscle foods; prooxidants*

INTRODUCTION

Lipid oxidation in muscle food causes many undesirable changes, including development of rancid off-flavor, changes in texture, color, and water-holding capacity, and loss of nutrients (*1*). Oxidation of cellular membrane lipids in muscle food is one reaction by which this process is thought to be initiated (*2*). Recent research has described reactive nitrogen species such as peroxynitrite as an initiator of lipid oxidation. Studies determining whether peroxynitrite and its precursor, nitric oxide (NO), can initiate lipid oxidation in muscle food have not been undertaken.

NO is produced by nitric oxide synthase (NOS). NOS catalyzes a complicated five-electron oxidation of L-arginine to form L-citrulline and NO, needing several cofactors including oxygen, NADPH, and flavin-containing molecules (*3*). A number of NOS isoenzymes have been identified, which vary in molecular size and tissue distribution. The constitutive form is nNOS, so named because its activity was demonstrated first in neurons. nNOS has been reported in several mammalian species, including humans, subhuman primates, and rodents. Not surprisingly, nNOS is found in skeletal muscle, located near the sarcolemma of skeletal muscle fibers (*4*).

NO is a low-energy radical that resists dimerization to N_2O_2 due to its resonance-stabilized unpaired electron (*5*). NO has a surprisingly long half-life (up to 3 min) and thus can diffuse through membranes to reach biological targets (*6*). Although considered a long-lived molecule, NO can react with other free radicals at nearly diffusion-controlled rates. The focus of this paper is the product of such a reaction, the termination reaction of NO with superoxide ($O_2^{\bullet -}$) to form the peroxynitrite anion ($ONOO^-$; reaction 1).

$$NO^\bullet + O_2^{\bullet -} \rightarrow ONOO^- \quad (1)$$

Numerous biomedical studies have documented the ability of peroxynitrite to oxidize biological molecules such as lipids, proteins, and DNA. Of particular relevance are peroxynitrite-induced oxidations of membrane lipids (*7*), low-density lipoproteins (*8*−*10*), myelin lipids from brain (*11*), and methyl linoleate model systems (*12*). Peroxynitrite can also contribute to the weakening of oxidative protection by oxidizing antioxidants (*13*). It has been shown that peroxynitrite oxidations are enhanced in the presence of carbon dioxide (*14*, *15*). Peroxynitrite reacts with carbon dioxide to form $ONOOCO_2^-$ (1-carboxylato-2-nitrosodioxidane) (*16*), which undergoes homolysis to form $CO_3^\bullet$ (carbonate radical) and $NO_2^\bullet$ (nitrogen dioxide radical) (*17*) according to reaction 2. The reaction of CO_2 with peroxynitrite can modulate both nitrations of aromatic residues on proteins (*15*) and lipid oxidation (*18*, *19*).

$$ONOO^- + CO_2 \rightarrow ONOOCO_2^- \rightarrow CO_3^\bullet + NO_2^\bullet \quad (2)$$

Characterizing the role of peroxynitrite as a lipid oxidation initiator could provide new insights into the oxidative stability of muscle foods. Skeletal muscle contains NOS, and superoxide ($O_2^{\bullet -}$) is known to be produced in muscle foods, suggesting that peroxynitrite could be generated. Unregulated concentrations of superoxide, nitric oxide, and carbon dioxide in postmortem tissue may lead to increased oxidation of unsaturated membrane lipids by peroxynitrite-mediated mechanisms.

The objective of this research was to determine if exogenous peroxynitrite could promote lipid oxidation in model systems and skeletal muscle homogenate. Characterization of the effect of CO_2, pH, and metal chelators on peroxynitrite-promoted oxidation was determined.

* Corresponding author [telephone (413) 545-1026; fax (413) 5451262; e-mail edecker@foodsci.umass.edu].

10.1021/jf001407q CCC: $20.00
Published on Web 06/01/2001

MATERIALS AND METHODS

All solvents were acquired from Fisher Scientific Co. (Pittsburgh, PA), and other chemicals were obtained from Sigma (St. Louis, MO). Natipide II liposome suspension (20% phospholipid) was obtained from American Lecithin Co. (Oxford, CT). Rainbow trout (*Salmo gairdneri*) were obtained from Mohawk Trout Hatchery (Sunderland, MA), filleted within 1 h of harvest, and immediately processed into muscle microsomes or minced and stored frozen (−80 °C) for later use in muscle homogenates.

Muscle microsome preparations (*20*) were isolated as follows: Fresh trout muscle was homogenized in 4 volumes of 0.12 M KCl/25 mM phosphate buffer, pH 7.2, in a Tissuemizer (20000 rpm; Tekmar, Cincinnati, OH) for 1 min followed by centrifugation (30 min at 10000*g*, 25 °C; Sorvall Superspeed RC2-B, Newtown, CT). The resulting supernatant was ultracentrifuged for 1 h at 100000*g* (Sorvall Ultra 80, DuPont, Wilmington, DE) to pellet insoluble muscle components. Myofibrillar proteins were solubilized from the pellet in 0.6 M KCl/25 mM phosphate buffer, pH 7.2 and the microsomes were pelleted by ultracentrifugation for 1 h at 100000*g*. Isolated microsomes were standardized to 100 mg of protein/mL with 0.12 M KCl/25 mM phosphate buffer, pH 7.2, and stored at −80 °C until analyzed. Protein analysis was performed using a protein determination kit (Sigma) according to the modified micro-Lowry method. Liposomes were prepared immediately before use by vortexing (2 min) 1 part of the liposome preparation (American Lecithin Co.) with 1.2 parts of 0.12 M KCl/200 mM phosphate buffer, pH 7.2, to give a final phospholipid concentration of 10%. Skeletal muscle homogenates (1 g of tissue/5 mL of buffer) were prepared from minced trout muscle and 0.12 KCl/200 mM phosphate buffer using a Tissuemizer (Tekmar) at 20000 rpm for 30 s.

Reagent peroxynitrite was synthesized via the reaction of reagent isoamyl nitrite with 9 M hydrogen peroxide at pH 13 (*21*). Residual hydrogen peroxide was removed from peroxynitrite with manganese dioxide. Peroxynitrite was stored at −80 °C for up to 1 week and standardized spectrophotometrically ($\epsilon = 1670\ M^{-1}\ cm^{-1}$) on each sampling day. 3-Morpholinosydnonimine (SIN-1) was used in some experiments to generate peroxynitrite.

Oxidation of 50 mM pyrogallol red in 0.12 M KCl/200 mM phosphate buffer, pH 7.2, by peroxynitrite was measured over time as the loss of absorbance at 540 nm ($\epsilon = 2.04 \times 10^4\ M^{-1}\ cm^{-1}$). Microsome and liposome suspensions were diluted to 10 mg of protein/mL assay and 1% concentrations, respectively, with 0.12 M KCl/200 mM phosphate buffer, pH 7.2 Oxidations were initiated by the addition of SIN-1 or reagent peroxynitrite to pyrogallol red, liposomes, microsomes, or muscle homogenate. Diethylenetriaminepentaacetic acid (DTPA; 100 mM), bathophenanthrolinedisulfonic acid (BPS; 100 μM), and $NaHCO_3$ (25 mM) in 0.12 M KCl/200 mM phosphate buffer, pH 7.2, were added immediately prior to addition of the peroxynitrite source. $NaHCO_3$ (25 mM) equilibrates in solution with ∼1.0−1.3 mM aqueous CO_2 (*19*).

Lipid oxidation was monitored using conjugated dienes (CD), thiobarbituric acid substances (TBARS), and/or lipid hydroperoxides (LOOH). CD were extracted with hexane, read spectrophotmetrically at 234 nm, and quantified on the basis of a standard curve prepared from conjugated linoleic acid in hexane. TBARS were determined in muscle microsome and liposome suspensions using a modified method of McDonald and Hultin (*22*) by mixing 0.5 mL of the sample with 1 mL of 15% TCA/0.375% TBA/0.25 M HCl solution. TBARS in skeletal muscle homogenate were determined using a modified method of Srinivasan and Xiong (*23*) by mixing homogenate (1 g) with 2.0 mL of 7.5% TCA/0.1% propyl gallate/100 μM DTPA solution followed by centrifugation at 2000*g* for 5 min. A 1 mL aliquot of the supernatant was then mixed with 1 mL of 0.02 M TBA. The TBA sample solutions were incubated for 15 min in a boiling water bath, centrifuged (5 min at 2000*g*), read spectrophotometrically at 532 nm, and quantified on the basis of the molar extinction coefficient of malondialdehyde ($1.56 \times 10^5\ M^{-1}\ cm^{-1}$). LOOH were extracted from an 8 mL muscle microsome sample with 4 mL of chloroform/methanol (2:1) or a 3 g sample of skeletal muscle homogenate with 10 mL of cholorform/methanol (2:1). After centrifugation (5 min at 2000*g*), an aliquot (2 mL) of the lower chloroform layer was reacted with 16.7 μL each of 3.94 M ammonium thiocynate and 0.072 M ferrous chloride after the addition of 1.3 mL of chloroform/methanol (2:1) (*24*). Absorbance was measured at 500 nm after 20 min of incubation, and LOOH were quantified on the basis of a standard curve prepared from cumene hydroperoxide.

All measurements were made in triplicate and reported as means ± standard deviation. SAS (Cary, NC) was used to analyze data using the general linear model (PROC GLM), regression (PROC REG), and simple statistics (PROC MEANS). The level of significance for all tests was set at $\alpha = 0.05$. Means separations were achieved according to Duncan's multiple-range test.

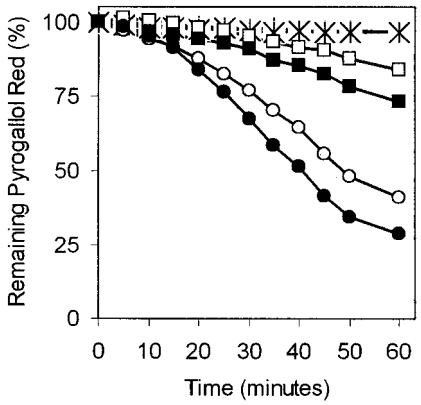

Figure 1. Oxidation kinetics of 50 μM pyrogallol red by SIN-1 (open symbols) and SIN-1 plus 1 mM CO_2 (solid symbols): controls (×, +); 1 mM SIN-1 (squares); 10 mM SIN-1 (circles).

RESULTS

To characterize the kinetics of peroxynitrite oxidation by SIN-1 (1 and 10 mM), a dye, pyrogallol red (50 μM), was used to monitor oxidation in the presence and absence of CO_2. SIN-1 is a peroxynitrite generator because it simultaneously produces superoxide and NO, which quickly react to form $ONOO^-$ (reaction 1). At pH 7.4 and 37 °C, SIN-1 (1 mM) provides continuous production of peroxynitrite on the order of ∼10 μM/min (*25*). Pyrogallol red was chosen because it has been used to quantify relative oxidative activities of pro- and antioxidants in aqueous solutions (*26*), and CO_2 was used because it was shown to have modulating activity on peroxynitrite oxidations (*19*). As shown in Figure 1, 1 and 10 mM SIN-1 decreased pyrogallol red concentrations by 13 and 58%, respectively, compared to a no-SIN-1 control after 60 min of incubation. The presence of CO_2 (1 mM) accelerated oxidation of pyrogallol red by 13% (1 mM SIN-1) and 30% (10 mM SIN-1) after 60 min. Bolus addition of different concentrations of reagent peroxynitrite resulted in nearly instantaneous (<1 min) oxidation of 50 mM pyrogallol red (data not shown).

To examine the effect of SIN-1 oxidations on lipids, liposomes and muscle microsomes were oxidized and the formation of CD and TBARS was monitored over time (Figure 2). SIN-1 was able to promote production of CD and TBARS in both lipid models. In liposomes, CD increased nearly 3.5-fold after 120 min, from 1.8 to 6.2 μM. Accumulation of CD in muscle microsomes increased 6-fold after 120 min, from 1.2 to 7.4 μM. After a short lag phase in both models, TBARS increased

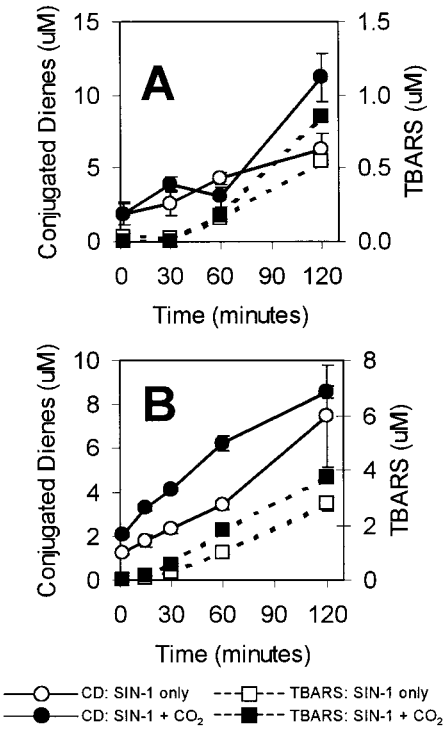

Figure 2. CD and TBARS formation by 2 mM SIN-1 ± 1 mM CO_2 in (A) liposomes (1% phospholipid) and (B) microsomes (10 mg of protein/mL). Data points represent means ($n = 3$) plus standard deviation (some error bars are smaller than the data symbol).

rapidly, reaching concentrations of 2.7 μM in liposomes and 0.5 μM in muscle microsomes after 120 min. The presence of CO_2 (1 mM) increased the formation of CD by SIN-1 1.8-fold in both liposomes and microsomes after 120 min. CO_2 increased SIN-1-induced TBARS formation 1.6-fold in liposomes and 1.9-fold in microsomes after 120 min.

Bolus addition of reagent peroxynitrite induced lipid oxidation in liposomes and muscle microsomes. Kinetics of oxidation induced by bolus addition of reagent peroxynitrite differed from that induced by SIN-1 in that oxidation occurred very rapidly (≤5 min), after which no further change in oxidation was observed (Table 1). Although significant oxidation occurred in systems to which CO_2 (1 mM) was added, TBARS formation by reagent peroxynitrite was suppressed by 26% (liposomes) and 48% (microsomes) compared to systems without CO_2 after 10 min of incubation.

Oxidation kinetics of skeletal muscle homogenate by bolus addition of reagent peroxynitrite (0.5 mM) was monitored (Figure 3). Oxidation of skeletal muscle homogenates increased gradually over time. A nearly 8-fold increase in LOOH and TBARS was observed at 4 h of incubation. CO_2 (1 mM) suppressed reagent peroxynitrite-induced formation of LOOH and TBARS by 70 and 68%, respectively, in the muscle homogenate system after 4 h of oxidation.

To assess the role of metals in oxidation of skeletal muscle by reagent peroxynitrite (0.5 mM), the metal chelator DTPS and the iron-specific chelator BPS were used (Table 2). BPS has been shown to form ferrous iron complexes with reduced activity between pH 5.5 and 8.0 (*27*), whereas DTPA chelates both iron and copper and is used extensively in peroxynitrite-induced oxidation systems (*28*). Compared to oxidation initiated by 0.5 mM peroxynitrite alone, BPS and DTPA inhibited oxidation 45 and 70%, respectively, after 4 h of incubation.

The extent of lipid oxidation in model systems and skeletal muscle homogenate by bolus addition was dependent on peroxynitrite concentration as evidenced by formation of TBARS, CD, and LOOH. Peroxynitrite concentration-dependent oxidation has been reported in phospholipid liposomes (*7*), myelin lipids (*11*), and linoleic acid (*12*). In liposomes oxidized with 0−2.0 mM peroxynitrite, formation of CD increased from 7.2 to 10.7 μM and that of TBARS from 0.7 to 1.8 μM (Figure 4A). In microsomes, CD formation was not different from the control at any concentration (Figure 4B). Over the range of 0−2.0 mM reagent peroxynitrite, muscle microsome LOOH increased from 0.27 to 0.71 μM and TBARS increased from 0.46 to 1.58 μM. Skeletal muscle homogenates oxidized over the range of 0−0.5 mM peroxynitrite (Figure 5) exhibited a 15-fold increase in the formation of LOOH from 4.1 to 63.6 μmol/kg of tissue and a 9-fold increase in TBARS from 1.8 to 15.9 μmol/kg of tissue.

As shown in Figure 6, decreasing the pH from 7.2 to 5.6 decreased the ability of 2 mM reagent peroxynitrite to form TBARS 83 and 42% in liposomes and microsomes, respectively, after 10 min of incubation. In liposomes, lowering the pH from 7.2 to 5.6 resulted in a 58% decrease in CD formation. No significant changes were observed in the formation of LOOH from pH 7.2 to 5.6 in muscle microsomes. In skeletal muscle homogenate oxidized with 0.5 mM peroxynitrite after 4 h of incubation, TBARS concentration was 47% lower at pH 7.2 than at pH 5.6. Formation of LOOH from muscle homogenates increased 1.5-fold from pH 7.2 to 5.6.

DISCUSSION

Oxidation of pyrogallol red, liposomes, microsomes, and skeletal muscle homogenate exhibited different characteristics when challenged with peroxynitrite continuously produced by SIN-1 or by bolus addition of reagent peroxynitrite. Oxidation of pyrogallol red (Figure 1), liposomes, and microsomes (Figure 2) initiated by SIN-1 occurred in a time-dependent manner for up to 2 h, whereas oxidation induced by bolus addition of reagent peroxynitrite was very rapid with no subsequent change after 5 min of incubation (pyrogallol red, data not shown; liposomes and microsomes, Table 1). Similar results have been observed in low-density lipoprotein (LDL), where SIN-1 caused a time-dependent consumption of α-tocopherol and formation of fatty acid hydroperoxides, whereas reagent peroxynitrite caused instantaneous loss of α-tocopherol and formation of fatty acid hydroperoxides (*9*). Because SIN-1 produces peroxynitrite continuously for several hours as a consequence of its decay (*29*), time-dependent oxidation is not unexpected. Conversely, addition of reagent peroxynitrite at high concentration would be expected to create immediate nitrosative stress causing rapid loss of antioxidant protection and subsequent formation of oxidative markers.

The oxidation of skeletal muscle homogenate by bolus reagent peroxynitrite caused a gradual increase in LOOH and TBARS over several hours (Figure 3), unlike the rapid oxidation that was essentially complete within a few minutes in pyrogallol red, liposomes, and microsomes. This observation suggests that the mechanism of reagent peroxynitrite-induced oxidation is different in skeletal muscle homogenate than in purified

Table 1. Formation of TBARS (Micromolar ± Standard Deviation) in Liposomes and Microsomes ± Reagent Peroxynitrite ($ONOO^-$, 2 mM) and CO_2 (1 mM)

	1.5 min	5 min	10 min[a]	20 min	30 min
			Liposomes		
control	0.96 ± 0.01	0.96 ± 0.01	0.96^c ± 0.01	0.99 ± 0.01	1.00 ± 0.03
$ONOO^-$ only	1.53 ± 0.02	1.57 ± 0.01	1.56^a ± 0.04	1.57 ± 0.01	1.56 ± 0.01
$ONOO^-$ + 1 mM CO_2	1.14 ± 0.01	1.12 ± 0.02	1.12^b ± 0.03	1.14 ± 0.02	1.12 ± 0.02
			Microsomes		
control	0.78 ± 0.03	0.75 ± 0.06	0.77^c ± 0.02	0.78 ± 0.03	0.77 ± 0.01
$ONOO^-$ only	1.73 ± 0.10	2.12 ± 0.11	2.09^a ± 0.04	2.10 ± 0.06	2.03 ± 0.16
$ONOO^-$ + 1 mM CO_2	0.95 ± 0.04	0.98 ± 0.04	0.95^b ± 0.03	1.01 ± 0.04	1.00 ± 0.04

[a] Means within model system (liposomes or microsomes) at 10 min of oxidation with different superscripts are significantly different ($p < 0.05$).

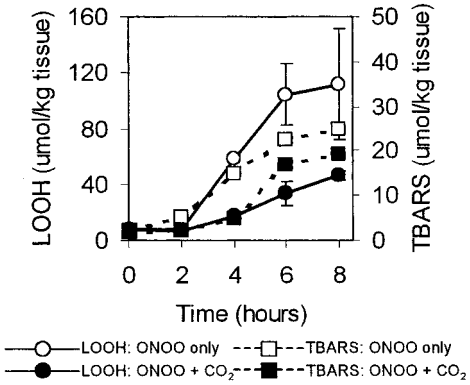

Figure 3. LOOH and TBARS formation by 0.5 mM reagent peroxynitrite (ONOO) ± 1 mM CO_2 in skeletal muscle homogenate. Data points represent means ($n = 3$) plus standard deviation (some error bars are smaller than the data symbol).

Table 2. Effect of BPS and DTPA on Peroxynitrite-Induced Formation of TBARS and LOOH in Skeletal Muscle Homogenate after 4 h of Incubation

	TBARS[a]	LOOH[a]
control	3.2^d ± 0.1	8.9^d ± 3.3
0.5 mM $ONOO^-$	23.9^a ± 0.5	119.7^a ± 12.1
+100 μM BPS	12.7^b ± 0.2	66.7^b ± 15.4
+100 μM DTPA	5.5^c ± 0.1	30.7^c ± 1.7

[a] Micromoles per kilogram of tissue; means within a column with different superscripts are significantly different ($p < 0.05$).

lipid and lipid membranes. Oxidation of peroxynitrite-induced skeletal muscle homogenate was suppressed by the nonspecific metal chelator DTPA and the ferrous iron chelator BPS (Table 2). This suggests that transition metals originating from skeletal muscle could enhance oxidation induced by peroxynitrite. LOOH account for as much as 60% of all linolenic acid emulsion oxidation products formed by bolus peroxynitrite (*30*). LOOH are decomposed by transition metals to form free radicals, which can participate in chain-propagating lipid oxidation reactions (*2*). Therefore, peroxynitrite could be stimulating oxidation by producing LOOH, which in turn are decomposed by endogenous transition metals. However, DTPA and BPS were not able to completely inhibit oxidation of the skeletal muscle homogenate. Heme proteins could represent a class of prooxidants that can be activated by peroxynitrite but not inhibited by metal chelators. When dihydrorhodamine was used as an oxidation substrate, peroxynitrite (in the form of molar equivalents of superoxide and nitric oxide) increased metmyoglobin-induced oxidation 43% (*31*). In addition, preformed LOOH increase the ability of methemoglobin to promote lipid oxidation (*32*), suggesting that hydroperoxides produced by peroxynitrite could further increase the prooxidant activity

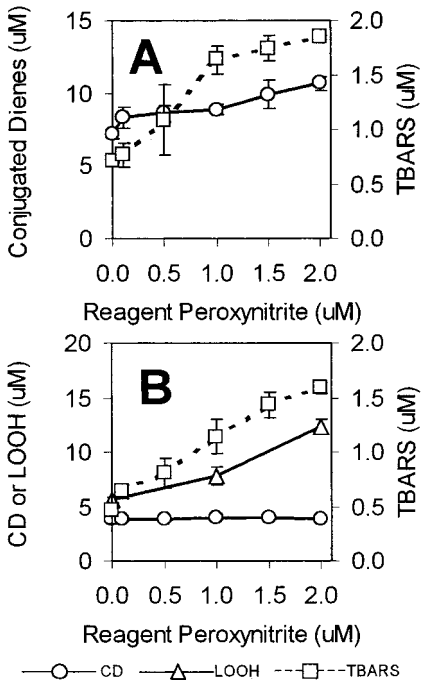

Figure 4. Effect of reagent peroxynitrite concentration on formation of TBARS, CD, and LOOH in (A) liposomes (1% phospholipid) and (B) microsomes (10 mg of protein/mL). Data points represent means ($n = 3$) plus standard deviation (some error bars are smaller than the data symbol).

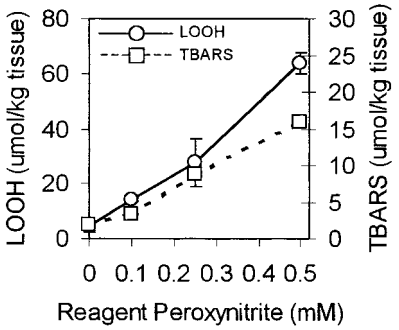

Figure 5. Effect of reagent peroxynitrite concentration on formation of TBARS and LOOH in skeletal muscle homogenate. Data points represent means ($n = 3$) plus standard deviation (some error bars are smaller than the symbol).

of heme proteins. Peroxynitrite-promoted oxidation of skeletal muscle homogenate could also be affected by consumption of endogenous antioxidants. Peroxynitrite has been shown to cause oxidation of carotenoids (*33*) and tocopherols (*7*) in LDL and liposomes, respectively. If peroxynitrite decreased antioxidant concentrations in the skeletal muscle homogenate, this could increase the activity of endogenous prooxidants.

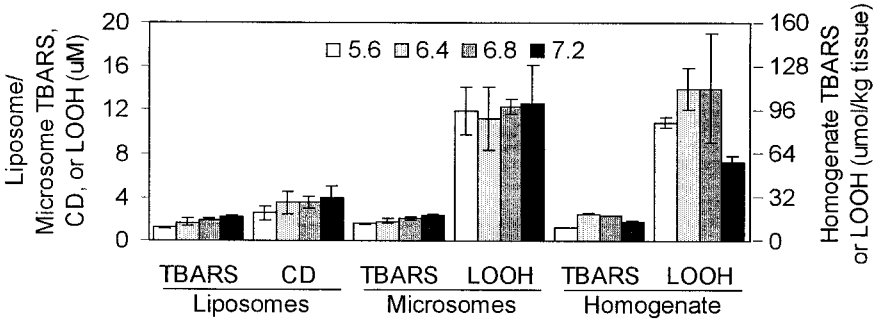

Figure 6. Effect pH on formation of TBARS, CD, and LOOH formation by 2 mM reagent peroxynitrite in liposomes (1% phospholipid) and microsomes (10 mg of protein/mL) after 10 min of incubation and 0.5 mM reagent peroxynitrite in skeletal muscle homogenate after 4 h of incubation. Data points represent means ($n = 3$) plus standard deviation.

The modulating activity of CO_2 on liposomes, microsomes, and skeletal muscle homogenate was dependent on the peroxynitrite-delivery system. Oxidation in the presence of CO_2 (~1 mM) was enhanced in pyrogallol red (Figure 1), liposomes, and microsomes (Figure 2) oxidized by SIN-1, but CO_2 suppressed the prooxidant effect of reagent peroxynitrite in liposomes and microsomes (Table 1) as well as in the skeletal muscle homogenate (Figure 3). It has been shown that CO_2 inhibits LDL and plasma oxidation induced by bolus reagent peroxynitrite while enhancing LDL and plasma oxidation induced by SIN-1 (*9*). Previous research has reported that peroxynitrite rapidly reacts with CO_2 to generate oxidizing species such as carbonate ($CO_3^{\bullet}$) and nitrogen dioxide ($NO_2^{\bullet}$) radicals (*17*). The radicals formed via the reaction of peroxynitrite with CO_2 are speculated to be highly reactive and short-lived, thus unable to diffuse from the aqueous to the lipophilic environment before being consumed or decomposed (*34*). Bolus addition of reagent peroxynitrite could produce high numbers of reactive radicals where radical−radical termination type reactions may predominate, decreasing the number of radicals available to promote lipid oxidation. In addition, bolus addition of reagent peroxynitrite may result in sacrificial decomposition of peroxynitrite by CO_2, thus decreasing peroxynitrite concentrations. In the SIN-1/CO_2 system, the slower production of peroxynitrite may cause lower numbers of radicals, decreasing termination reactions, thereby allowing the radicals to diffuse into the lipid to promote oxidation.

Muscle foods exist at a variety of pH values, generally in the range of 5.6−7.2. The equilibrium of peroxynitrite and its conjugate acid, peroxynitrous acid ($pK_a = 6.8$), is affected by pH. If differences exist in the reactivity and/or stability of the anion (peroxynitrite) versus the protonated acid (peroxynitrous acid), the prooxidant activity of peroxynitrite may vary with the pH of the muscle food product. It has been reported that peroxynitrite decomposes rapidly with decreasing pH as it becomes protonated (*35*), even in the presence of oxidants (*36*). The oxidative influence of peroxynitrite should therefore decrease with decreasing pH because the greater amount of peroxynitrous acid would result in more decomposition, thus removing peroxynitrite from the system. Suppression of oxidation in liposomes by decreasing pH has been shown (*7*). Results of our study partially support this reasoning as a significant decrease ($p < 0.05$) in TBARS formation in both liposomes and microsomes was observed at pH 5.6 compared to pH 7.2 (Figure 6). However, no significant differences in the formation of either CD in liposomes or LOOH in microsomes were observed over this pH range.

The effect of pH on peroxynitrite-induced oxidation in the skeletal muscle homogenate was less predictable. Formation of TBARS was reduced at pH 5.6 compared to that at pH 7.2, whereas the opposite was observed for LOOH concentrations. Differences of the impact of pH on peroxynitrite-induced oxidation in skeletal muscle homogenate could be due to endogenous pro- and antioxidants in the skeletal muscle, the activity of which is also influenced by pH. Therefore, it is unknown if the influence of pH on oxidation in the skeletal muscle homogenate was due to its effect on peroxynitrite or other oxidative factors in the muscle. In any event, substantial oxidation in skeletal muscle homogenate was observed at pH values common to muscle foods.

Results of this study provide evidence to support two findings. First, reagent peroxynitrite and SIN-1 were able to initiate oxidation in dye and lipid model systems and skeletal muscle homogenate. Oxidation was peroxynitrite concentration-dependent and was modulated by CO_2. Even at low pH, where peroxynitrite has lower prooxidative activity, significant oxidation was observed. Second, the activity of reagent peroxynitrite exhibited important differences in pyrogallol red, liposomes, and microsomes versus skeletal muscle homogenate, with pyrogallol red, liposomes, and microsomes showing nearly instantaneous oxidation and skeletal muscle homogenate showing gradual oxidation with time. The skeletal muscle homogenate data suggest that peroxynitrite could enhance oxidation rates by inactivation of endogenous antioxidants or activation of endogenous prooxidants. Because peroxynitrite can potentially increase lipid oxidation in skeletal muscle, control of peroxynitrite during the early stages of processing may be crucial to delay the onset of oxidation. Further research using biochemical markers specific for peroxynitrite oxidation, such as nitrated proteins, antioxidants, or lipids, as well as determining if the conditions that impact peroxynitrite production, that is, the activity of nitric oxide synthase, will be important before a conclusion as to the relative role of peroxynitrite-induced oxidation in muscle food can be reached. In any event, we believe that the results of this study provide enough initial evidence to warrant expansion of research into the oxidative chemistry of peroxynitrite in muscle food systems.

LITERATURE CITED

(1) Ladikos, D.; Lougovois, V. Lipid oxidation in muscle foods: A review. *Food Chem.* **1990**, *35*, 295−314.

(2) Kanner, J.; German, J. B.; Kinsella, J. E. Initiation of lipid peroxidation in biological systems. *CRC Crit. Rev. Food Sci. Nutr.* **1987**, *25*, 317−364.
(3) Gaston, B.; Stamler, J. S. Biochemistry of nitric oxide. In *Nitric Oxide and Infection*; Fang, F. C., Ed.; Kluwer Academic/Plenum Publishers: New York, 1999; pp 37−49.
(4) Grozdanovic, Z.; Baumgarten, H. G. Nitric oxide synthase in skeletal muscle fibers: a signaling component of the dystrophin-glycoprotein complex. *Histol. Histopathol.* **1999**, *14*, 243−256.
(5) Fontecave, M.; Pierre, J.-L. The basic chemistry of nitric oxide and its possible biologic reactions. *Bull. Soc. Chim. Fr.* **1995**, *131*, 620−631.
(6) Rosen, G. M.; Britigan, B. E.; Halpern, H. J.; Pou, S. *Free Radicals. Biology and Detection by Spin Trapping*; Oxford University Press: New York, 1999; pp 83−139.
(7) Radi, R.; Beckman, J. S.; Bush, K. M.; Freeman, B. A. Peroxynitrite-induced membrane lipid peroxidation: The cytotoxic potential of superoxide and nitric oxide. *Arch. Biochem. Biophys.* **1991**, *288*, 481−487.
(8) Hogg, N.; Kalyanaraman, B. Nitric oxide and low-density lipoprotein oxidation. *Free Radical Res.* **1998**, *28*, 593−600.
(9) Thomas, S. R.; Davies, M. J.; Stocker, R. Oxidation and antioxidation of human low-density lipoprotein and plasma exposed to 3-morpholinosydnonimine and reagent peroxynitrite. *Chem. Res. Toxicol.* **1998**, *11*, 484−494.
(10) Stanbro, W. D. Modeling the interaction of peroxynitrite with low-density lipoproteins. I. Plasma levels of peroxynitrite. *J. Theor. Biol.* **2000**, *205*, 457−464.
(11) van der Veen, R. C.; Roberts, L. J. Contrasting roles for nitric oxide and peroxynitrite in the peroxidation of myelin lipids. *J. Neuroimmunol.* **1999**, *95*, 1−7.
(12) O'Donnell, V. B.; Eiserich, J. P.; Chumley, P. H.; Jablonsky, M. J.; Krishna, N. R.; Kirk, M.; Barnes, S.; Darley-Usmar, V. M.; Freeman, B. A. Nitration of unsaturated fatty acids by nitric oxide-derived reactive nitrogen species peroxynitrite, nitrous acid, nitrogen dioxide, and nitronium ion. *Chem. Res. Toxicol.* **1999**, *12*, 83−92.
(13) Hogg, N.; Kalyanaraman, B. Nitric oxide and lipid peroxidation. *Biochim. Biophys. Acta* **1999**, *1411*, 378−384.
(14) Radi, R.; Cosgrove, T. P.; Beckman, J. S.; Freeman, B. A. Peroxynitrite-induced luminol chemiluminescence. *Biochem. J.* **1993**, *290*, 51−57.
(15) Santos, X. C.; Bonini, M. G.; Augusto, O. Role of the carbonate radical anion in tyrosine nitration and hydroxylation by peroxynitrite. *Arch. Biochem. Biophys.* **2000**, *377*, 146−152.
(16) Lymar, S. V.; Jiang, Q.; Hurst, J. K. Mechanism of carbon dioxide-catalyzed oxidation of tyrosine by peroxynitrite. *Biochemistry* **1996**, *35*, 7855−7861.
(17) Merenyi, G.; Lind, J. Thermodynamics of peroxynitrite and its CO_2 adduct. *Chem. Res. Toxicol.* **1997**, *10*, 1216−1220.
(18) Bartberger, M. D.; Olson, L. P.; Houk, K. N. Mechanisms of peroxynitrite oxidations and rearrangements: the theoretical perspective. *Chem. Res. Toxicol.* **1998**, *11*, 710−711.
(19) Radi, R.; Denicola, A.; Freeman, B. A. Peroxynitrite reactions with carbon dioxide-bicarbonate. *Methods Enzymol.* **1999**, *301*, 353−367.
(20) Player, T. J.; Hultin, H. O. Some characteristics of the NAD(P)H-dependent lipid peroxidation system in the microsomal fraction of chicken breast muscle. *J. Food Biochem.* **1977**, *1*, 153−171.
(21) Uppu, R. M.; Pryor, W. A. Synthesis of peroxynitrite in a two-phase system using isoamyl nitrite and hydrogen peroxide. *Anal. Biochem.* **1996**, *236*, 242−249.
(22) McDonald, R. E.; Hultin, H. O. Some characteristics of the enzymic lipid peroxidation system in the microsomal fraction of flounder skeletal muscle. *J. Food Sci.* **1987**, *52*, 15−21, 27.
(23) Srinivasan, S.; Xiong, Y. L. Sodium chloride-mediated lipid oxidation in beef heart surimi-like material. *J. Agric. Food Chem.* **1996**, *44*, 1697−1703.
(24) Shantha, N. C.; Decker, E. A. Rapid, sensitive, iron-based spectrophotometric methods for determination of peroxide values of food lipids. *J. AOAC Int.* **1994**, *77*, 421−424.
(25) Brunelli, L.; Crow, J. P.; Beckman, J. S. The comparative toxicity of nitric oxide and peroxynitrite to *Escherichia coli*. *Arch. Biochem. Biophys.* **1995**, *316*, 327−334.
(26) Balavoine, G. G.; Geletii, Y. V. Peroxynitrite scavenging by different antioxidants. Part I: convenient assay. *Nitric Oxide* **1999**, *3*, 40−54.
(27) Cowart, R. E.; Singleton, F. L.; Hind, J. S. A comparison of bathophenanthrolinedisulfonic acid and ferrozine as chelators of iron(II) in reduction reactions. *Anal. Biochem.* **1993**, *211*, 151−155.
(28) Beckman, J. S.; Chen, J.; Ischiropoulos, H.; Crow, J. P. Oxidative chemistry of peroxynitrite. *Methods Enzymol.* **1994**, *233*, 229−240.
(29) Hoglen, N. C.; Liebler, D. C. Products from reaction of peroxynitrite with gamma-tocopherol. *Methods Enzymol.* **1999**, *301*, 483−490.
(30) Rubbo, H.; Radi, R.; Trujillo, M.; Telleri, R.; Kalyanaraman, B.; Barnes, S.; Kirk, M.; Freeman, B. A. Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation. Formation of novel nitrogen-containing oxidized lipid derivatives. *J. Biol. Chem.* **1994**, *269*, 26066−26075.
(31) Jourd'heuil, D.; Mills, L.; Miles, A. M.; Grisham, M. B. Effect of nitric oxide on hemoprotein-catalyzed oxidative reactions. *Nitric Oxide* **1998**, *2*, 37−44.
(32) Richards, M. P.; Hultin, H. O. Effect of pH on lipid oxidation using trout hemolysate as a catalyst: a possible role for deoxyhemoglobin. *J. Agric. Food Chem.* **2000**, *48*, 3141−3147.
(33) Pannala, A. S.; Singh, S.; Rice-Evans, C. Interaction of carotenoids and tocopherols with peroxynitrite. *Methods Enzymol.* **1999**, *301*, 319−32.
(34) Lymar, S. V.; Hurst, J. K. Carbon dioxide: physiological catalyst for peroxynitrite-mediated cellular damage or cellular protectant? *Chem. Res. Toxicol.* **1996**, *9*, 845−850.
(35) Pfeiffer, S.; Gorren, A. C. F.; Schmidt, K.; Werner, E. R.; Hansert, B.; Bohle, D. S.; Mayer, B. Metabolic fate of peroxynitrite in aqueous solution. Reaction with nitric oxide and pH-dependent decomposition to nitrite and oxygen in a 2:1 stoichiometry. *J. Biol. Chem.* **1997**, *272*, 3465−3470.
(36) Koppenol, W. H. Chemistry of peroxynitrite and its relevance to biological systems. *Met. Ions Biol. Syst.* **1999**, *36*, 597−619.

Received for review November 22, 2000. Revised manuscript received April 3, 2001. Accepted April 10, 2001.

JF001407Q