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Reactive Sulfur Species Act as Prooxidants in Liposomal and Skeletal Muscle Model Systems

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Reactive sulfur species (RSS) are redox-active sulfur compounds formed under conditions of oxidative stress that may be capable of initiating oxidation reactions. The objective of this research was to determine if two RSS, sulfite radicals and disulfide *S*-oxides (DSSO), induce oxidation in n-3 liposomes and muscle homogenates. Sulfite radicals and DSSO caused an increase in secondary oxidation products compared to the unoxidized control within 5 min of addition to liposomes. In muscle homogenates, DSSO caused immediate oxidation of sulfhydryls, whereas sulfite radicals caused a slow and linear oxidation of sulfhydryls and lipid. These results suggest that if conditions exist in muscle foods that promote the formation of RSS, they could be a factor in the course of lipid oxidation by either weakening antioxidant defenses or directly oxidizing lipid molecules.

KEYWORDS: Reactive sulfur species; sulfite radicals; disulfide *S*-oxides; lipid oxidation

INTRODUCTION

The literature is replete with examples of sulfur-containing compounds such as low-molecular-weight thiols and protein sulfhydryls that act as antioxidants in a variety of systems. However, this paradigm—that all sulfur-containing molecules in muscle and meat primarily occur in nonoxidizing redox states—may overlook naturally occurring redox-active sulfur compounds that are capable of initiating cellular oxidation reactions. The oxidative chemistry of reactive sulfur is an emerging area.

As oxidative stress commences in food, a number of ROS such as superoxide and various peroxides are generated chemically or enzymatically. Once these ROS are formed, other reactive species can be chemically produced via the reaction of certain molecules with ROS. An example of this is peroxynitrite, formed from the reaction between superoxide and nitric oxide (*1*). In protein-rich systems that contain high concentrations of thiols and disulfides, sulfur is a likely target for oxidation. Under these conditions, the oxidation of thiols and disulfides can lead to sulfur species, including sulfur-centered radicals, with oxidation states different from those of the mildly reducing sulfur species that are commonly known. The formation of reactive sulfur species is not a theoretical assumption, as the presence of sulfur-centered radicals has been observed in several systems (*2*−*4*).

Reactive sulfur is either a byproduct of the reactions of major biologically important thiol-containing molecules such as glutathione, cysteine, and dithiols (i.e., lipoate) or a result of the oxidation of sulfite or sulfate molecules. Some of these sulfur molecules and their oxidation states are shown in **Figure 1**. In general, the more positive the oxidation state, the higher the oxidation potential of the compound. Thiols occur in oxidation states that have been shown to be capable of producing free radical mediated reactions with molecules such as proteins (*5*), fatty acids (*6*), and cholesterol (*7*). Sulfite radicals can be produced via autoxidation initiated by divalent metals or from the one-electron reduction of *S*-sulfocysteine, although the first path is considered more likely (*8*). The first evidence of the sulfite radical in food was likely when it was shown to be directly responsible for the oxidative pathway leading to the formation of methanethiol in soy protein isolate (*4*). The best known example of another class of reactive sulfur molecule, disulfide *S*-oxides (DSSO), is the well-studied allicin molecule (diallyldisulfide *S*-monoxide) from garlic, about which several reviews have been published (*9*−*11*), and pathways in skeletal muscle that are similar to allicin synthesis in plants could account for its presence in muscle foods (*12*).

A series of papers by Giles and co-workers has shed light on reactive sulfur, especially DSSO. First, they speculated that reactive sulfur species are "second-generation" reactive species in that they likely are formed by a chemical reaction involving reactive oxygen or reactive nitrogen, they are formed in vivo under conditions of oxidative stress, and they are likely to include disulfide *S*-oxides, sulfenic acids, and thiyl radicals (*3*, *13*). They then showed that disulfide *S*-monoxides (thiosulfinates) and disulfide *S*-dioxides (thiosulfonates) are highly reactive oxidizing species that preferentially and rapidly oxidize thiols (*14*). They also characterized the DSSO oxidation pathway as being independent of inhibition by high concentrations of antioxidants such as ascorbate, NADH, trolox, and melatonin (*15*). These important findings have ushered in a growing amount of research on reactive sulfur species in the biochemical literature.

In spite of this, little research on reactive sulfur exists in the food science literature. A recent paper has shown that a plant extract was capable of reducing the amount of oxidation biomarkers in a model system that did not contain thiols (*16*), but no research was uncovered that examined reactive sulfur species in more complex food systems. The objective of this research was to

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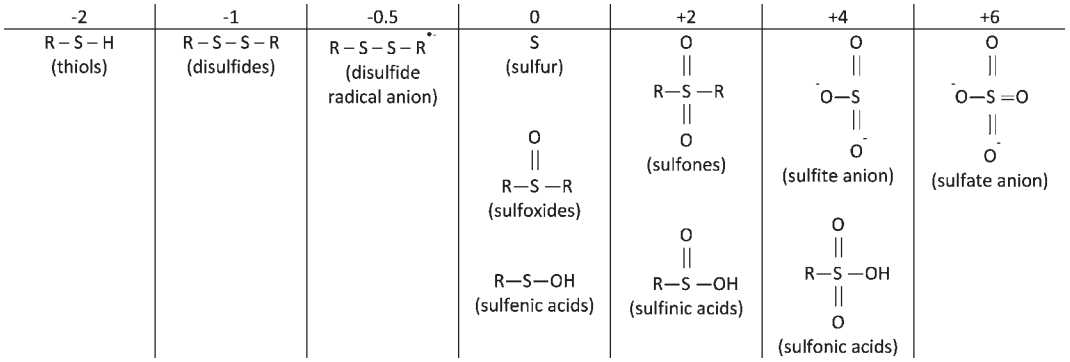

Figure 1. Some reactive sulfur species categorized by oxidation state.

determine if sulfite radicals and disulfide *S*-oxides (DSSO) induce oxidation in n-3 liposomes and muscle homogenates.

MATERIALS AND METHODS

All chemicals and reagents were obtained from Fisher Scientific (Waltham, MA) or Sigma−Aldrich (St. Louis, MO), except 1,2-didocosahexaenoyl-sn-glycero-3-phosphocholine (22:6 PC), which was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Fresh turkey breast was purchased from a local retailer.

Suspensions of 22:6 PC liposomes were prepared from 22:6 PC in chloroform by first evaporating to dryness under nitrogen and then homogenizing in 25 mM histidine buffer (pH 7.25) by 10 passes with a Potter homogenizer. The resulting suspensions were packed in ice and sonicated for 15 min using a Braun-Sonic 2000 U ultrasonic generator (Braun Biotech, Allentown, PA) equipped with a 5 T standard probe at a power setting of +250 and a 0.3 s repeating cycle. The sonicated suspensions were collected, and the resulting liposome suspensions were stored for less than 24 h before being subjected to the conditions of the experiment. The phospholipid concentration of the liposome suspensions was estimated by measuring phosphorus content (*17*) and was standardized at 0.25 mg of 22:6 PC/mL via the addition of buffer.

Skeletal muscle homogenates (1 g of tissue/5 mL of buffer) were prepared from fresh turkey breast muscle purchased from a local retailer previously that day. The muscle was trimmed of visible fat, cut into approximately 20 mm square cubes, weighed, and then mixed with 0.12 M KCl/200 mM phosphate buffer (pH 7.2) using a Tissuemizer (Tekmar, Cincinnati, OH) at 20 000 rpm for 30 s. Homogenates were used immediately after preparation.

Oxidation of 22:6 PC liposome suspensions and tissue homogenates was induced by the addition of aqueous solutions of sulfite radicals and disulfide *S*-oxides (DSSO). Sulfite radicals were derived from a freshly prepared mixture of $MnCl_2$, methionine, and sodium sulfite (*4*) to achieve a final concentration in the liposomes and homogenates of 3.6 M $MnCl_2$, 3.2 M methionine, and 3.2 M sodium sulfite. DSSO were generated from a freshly prepared mixture of glutathione, H_2O_2, and methyltrioxorhenium (VII) that was incubated for 30 min prior to use (*18*) to achieve a final concentration of 50 mM glutathione, 100 mM H_2O_2, and 50 mM methyltrioxorhenium (VII) in the liposomes and homogenates. Control samples did not contain oxidants, but an appropriate amount of deionized water was added to account for the volume. In addition to the two reactive sulfur oxidants and the control samples, H_2O_2 alone was added to certain homogenate samples to achieve a final concentration of 4 mM, to account for the effects in DSSO systems that may have been due to unreacted H_2O_2. Once the oxidants were added, the liposome suspensions were vortexed for 1 min and then incubated at room temperature (22 °C) for up to 120 min, while the homogenates were thoroughly mixed and then incubated for up to 6 h.

Lipid hydroperoxides (LOOH) were extracted from a 3 g sample of skeletal muscle homogenate with 10 mL of chloroform/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). 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 (*19*).

Thiobarbituric acid reactive substances (TBARS) were determined in liposome suspensions by mixing 0.5 mL of the liposome sample with 1 mL of a 15% tricholoracetic acid (TCA)/0.375% thiobarbituric acid (TBA)/0.25 M HCl solution (*20*). TBARS in skeletal muscle homogenate were determined by mixing the skeletal muscle 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 20 min in a boiling water bath, centrifuged (5 min at 2000*g*), read spectrophotometrically at 532 nm, and quantified on the basis of a standard curve prepared from 1,1,3,3-tetramethoxypropane (*1*).

Protein and nonprotein sulfhydryl (SH) content of skeletal muscle homogenates were quantified using Ellman's reagent according to published methods (*21*). For total SH, homogenate (1 g) was diluted with 5 mL of 0.1 M phosphate buffer/1 mM EDTA, pH 8.0, and then reacted directly with Ellman's reagent (50 uL). After 15 min of centrifugation at 2000*g*, the absorbance of the solution was determined at 412 nm. For nonprotein SH, homogenate (3 g) was precipitated with 1 mL of 5% TCA and then centrifuged at 2000*g* for 15 min. A 250 μL aliquot was reacted with Ellman's reagent (50 μL), and after 15 min the absorbance of the solution was determined at 412 nm. Protein SH values were calculated as the difference between total SH and nonprotein SH.

For each measurement, mean values were generated from six trials ($n = 6$). SPSS (Chicago, IL) was used to analyze data using the general linear model procedure. The main effects of oxidant and incubation time and the two-way interaction effect were included in the analysis. The level of significance for all tests was set at 0.05. Mean separations were achieved according to Duncan's multiple-range test.

RESULTS AND DISCUSSION

Initiation of Oxidation in 22:6 PC Liposomes. A simple experiment was undertaken to determine if exogenous sulfite radicals or DSSO can initiate oxidation under controlled conditions during 2 h of incubation. The model liposomal system was generated using phosphatidylcholine produced with only 22:6 fatty acid chains; therefore, it was very susceptible to oxidation. The results showed that TBARS formation was significantly affected by the main effects of incubation time ($p < 0.001$) and the addition of oxidant ($p < 0.001$). The two-way interactions, shown in **Figure 2**, indicate that sulfite radicals and DSSO caused a significant increase in TBARS formation over time, although different patterns of TBARS formation were observed. In the sulfite radical system, an initial burst of oxidation occurred within 5 min, with further increases not observed until after 60 min of

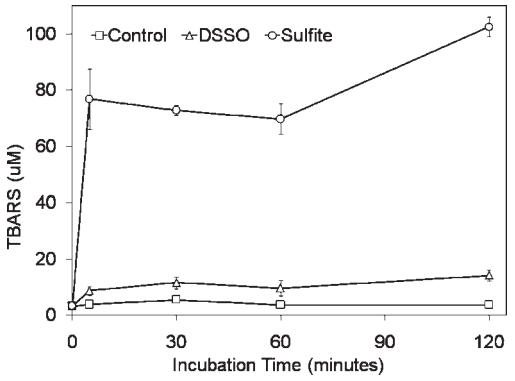

Figure 2. Formation of thiobarbituric acid reactive substances (TBARS) as affected by the addition of reactive sulfur species disulfide *S*-oxides (DSSO) and sulfite radicals (Sulfite) in 22:6 phosphatidylcholine liposomes.

incubation. In the DSSO system, increased TBARS formation compared to the untreated control were not observed until after 60 min of incubation. The magnitude of TBARS formation was nearly an order of magnitude greater in the sulfite radical system compared to the DSSO system; however, the relevance of this comparison is questionable, since the amount of oxidants added to each system was not well characterized. However, one explanation could be that sulfite radicals are known to react very quickly with O_2 to form peroxyl radicals (*8*), which is not the case for DSSO.

Oxidation of Sulfhydryls in Muscle Homogenate. The main effects and two-way interactions for protein SH and nonprotein SH as affected by the addition of oxidant (sulfite radicals or DSSO) and incubation time in turkey muscle homogenates are shown in **Table 1**. These main effects and two-way interactions were significant except for the main effect of incubation time with respect to nonprotein SH.

Protein and nonprotein SH content of the turkey muscle homogenates as affected by incubation time and oxidant are shown in **Table 2**. Non-protein SH accounted for just less than 1 order of magnitude fewer SH than protein SH, which is in agreement with the case for other species (*22*). In the control samples to which no oxidant was added, no decrease in protein SH was observed between 0 and 6 h of incubation, while a slight but significant increase in nonprotein SH content was observed in the untreated controls over 6 h of incubation. By 6 h of incubation, the addition of sulfite radicals had caused a significant decrease in protein and nonprotein SH levels of 14% and 23%, respectively, compared to the control. The addition of DSSO caused immediate and nearly complete oxidation of protein and nonprotein SH of 98% and 88%, respectively. The technique used to generate aqueous solutions of DSSO involved the use of H_2O_2 which, if unreacted, could account for some or all of the SH oxidation observed. The addition of this strong oxidant did cause a loss in nonprotein SH content of about 30%; however, it cannot account for the decline in nonprotein SH content observed in the DSSO-treated homogenates. These findings are consistent with research that has shown that DSSO selectively targets protein and non-protein sulfhydryl groups (*14*).

Non-protein sulfhydryls are likely to be small-molecular-weight thiols such as glutathione or free, sulfur-containing amino acids. If conditions in meat favored the formation of sulfur radicals or disulfides such as DSSO with higher oxidation states, a loss of nonprotein SH such as the 12% and 88% exhibited in this study (**Table 2**) could alter the antioxidant defenses of the system. The redox properties of proteins can be affected due to oxidation, producing side-chain modifications, cross-linking, or fragmentation. Conditions commonly encountered in food—harsh processing conditions, irradiation, exposure to divalent metals, light, or existing peroxides—could shift the balance between mildly reducing species such as thiols to mildly or strongly oxidizing species such as DSSO, which in turn could reduce the antioxidant defenses of the system.

Table 1. Main Effects (Oxidant, Incubation Time) and Two-Way Interactions for the Formation of Lipid Hydroperoxides (LOOH, μmol/kg of tissue), Thiobarbituric Acid Reactive Substances (TBARS, μmol/kg of tissue), and Non-Protein and Protein Sulfhydryls (SH, μmol/g of tissue) in Turkey Muscle Homogenates

	main effects		
measurement	oxidant	incubation time	two-way interaction (oxidant × incubation time)
LOOH[a]	0.370	<0.001	<0.001
TBARS[a]	<0.001	<0.001	<0.001
non-protein SH[b]	<0.001	0.289	<0.001
protein SH[b]	<0.001	<0.001	<0.001

[a] Two levels of oxidant (control, sulfite radicals), four levels of incubation time (0, 2, 4, 6 h). [b] Three levels of oxidant (control, sulfite radicals, disulfide *S*-oxides), four levels of incubation time (0, 2, 4, 6 h).

Lipid Oxidation in Muscle Homogenate. Main effects and two-way interactions for the dependent variables LOOH and TBARS as affected by the addition of sulfite radicals and incubation time in turkey muscle homogenates are shown in **Table 1**. All main effects and two-way interactions for LOOH and TBARS were significant, except for the main effect of oxidant with respect to LOOH. Unfortunately, interferences with the redox LOOH assay and the colorimetric TBARS assay precluded elucidation of these values in the DSSO system. H_2O_2 probably was not responsible for the interference in the LOOH assay, since H_2O_2 is not chloroform-soluble as would be required to interfere with the LOOH assay and no interference was observed for TBARS in homogenates to which H_2O_2 was added (data not shown).

Two-way interactions for the formation of LOOH and TBARS in turkey muscle homogenates as affected by incubation time and sulfite radicals are shown in **Table 3**. LOOH represents a primary oxidation product of lipid oxidation, and often a lag time is observed before an increase in LOOH is detected as the first few radicals are formed. Slight but significant accumulation of LOOH was observed during 6 h of incubation for the samples to which sulfite radicals were added in comparison to the untreated control, suggesting that sulfite radicals are a class of reactive sulfur species capable of oxidizing lipids. The addition of sulfite radicals to the homogenates produced a lag phase of TBARS formation, followed by an increase in TBARS formation after 2 h of incubation.

Implications. The results of this study show that reactive sulfur species were able to initiate sulfhydryl and lipid oxidation in lipid model systems and skeletal muscle homogenate. Sulfite radicals and DSSO caused an initial increase in secondary oxidation products in comparison to the unoxidized control within 5 min of addition to liposomes, with no gradual accumulation occurring over the incubation period. However, the magnitude of formation of oxidation products was nearly 1 order of magnitude higher in the sulfite radical system compared to the DSSO system. In skeletal muscle homogenates, DSSO caused immediate oxidation of sulfhydryls, whereas sulfite radicals caused a slow and linear oxidation of sulfhydryls and lipid.

There were major differences observed between the loss of protein and non-protein SH in the two oxidizing systems. In the sulfite radical system, a lag phase was observed in the muscle

Table 2. Protein and Non-Protein Sulfhydryl Content (SH, μmol/g of tissue) of the Turkey Muscle Homogenates As Affected by Incubation Time and Oxidant[a]

	oxidant			
time (h)	none (control)	sulfite radical	disulfide *S*-oxide	hydrogen peroxide
		Non-Protein SH		
0	0.035 ± 0.001 BC	0.033 ± 0.001 CD	0.004 ± 0.000 H	0.024 ± 0.001 G
3	0.036 ± 0.001 B	0.032 ± 0.000 D	0.004 ± 0.001 H	0.026 ± 0.003 F
6	0.038 ± 0.003 A	0.029 ± 0.000 E	0.005 ± 0.000 H	0.027 ± 0.000 F
		Protein SH		
0	0.276 ± 0.003 AB	0.286 ± 0.013 A	0.007 ± 0.001 F	0.253 ± 0.009 E
3	0.253 ± 0.007 D	0.277 ± 0.002 AB	0.008 ± 0.001 F	0.284 ± 0.004 AB
6	0.264 ± 0.007 BC	0.246 ± 0.005 E	0.005 ± 0.000 F	0.269 ± 0.004 CD

[a] Different letters within each sulfhydryl type are significantly different at $p < 0.05$.

Table 3. Effect of Sulfite Radicals on the Formation of Lipid Hydroperoxides and Thiobarbituric Acid Reactive Substances (TBARS) in Turkey Muscle Homogenates during 4 h Incubation[a]

	lipid hydroperoxide (μmol/kg)		TBARS (μmol/kg)	
time (h)	control (no oxidant)	sulfite radical	control (no oxidant)	sulfite radical
0	109.9 ± 25.3 B	65.5 ± 5.9 C	4.61 ± 0.44 BC	3.59 ± 2.31 C
2	106.7 ± 7.5 B	67.6 ± 19.4 C	5.25 ± 0.51 BC	5.94 ± 0.51 B
4	102.7 ± 19.2 B	119.8 ± 20.7 B	5.47 ± 0.48 BC	9.31 ± 1.04 A
6	121.7 ± 10.8 B	162.8 ± 15.7 A	5.76 ± 0.71 B	10.13 ± 0.88 A
p value	<0.001		<0.001	

[a] Different letters within lipid hydroperoxides or TBARS columns are significantly different at $p < 0.05$.

homogenates for the loss of protein and non-protein SH, which corresponded to the increase observed in LOOH and TBARS. However, in the DSSO system, a nearly immediate and complete oxidation of protein and non-protein SH was observed. In the DSSO system, the immediate oxidation of SH groups could lead to either the alteration of the pro-oxidant/antioxidant balance or the activation of radical activity in the homogenates. Methods to measure primary and secondary oxidation products other than those employed in this study will be beneficial to elucidating the action of DSSO's on lipid oxidation in muscle. The liposome data from this study (**Figure 2**) does show that DSSO (in concentration similar to that added to the skeletal muscle homogenates) did not cause accumulation of oxidation products; thus, it seems unlikely that DSSO somehow activated radical activity. On the other hand, the sulfite system acted as a more "traditional" oxidant, with the decline in SH accompanied by the formation of primary and secondary lipid oxidation products. Sulfite radicals are known to react with oxygen to produce peroxyl radicals (*8*), which could account for its behavior in both the liposome and homogenate systems. In any event, the data presented in this study suggest that if conditions exist that promote the formation of sulfite radicals, they could be a major factor in the propagation step of lipid oxidation.

However, whether conditions exist to promote the formation of reactive sulfur species of higher oxidation states or if these compounds are even redox active under physiological conditions remains an open question in the biochemical literature (*23*). Complicating the issue is the fact that many of the compounds shown in **Figure 1** can be oxidized to form species that exhibit both oxidant and reductant ability. Such is the case with sulfite radicals (*8*), which under the conditions of the present study were clearly oxidative. A review of reactive sulfur in the literature has accumulated data to show that similar conditions that produce other oxidants, such as peroxynitrite, can also produce reactive sulfur species and that a variety of reactive oxygen species also can participate in reactions that produce reactive sulfur (*13*). Although endogenous formation of peroxynitrite has not been directly observed in meat (*24*), the enzyme that produces a precursor to peroxynitrite (nitric oxide synthase) is active in postmortem muscle (*1*), suggesting that conditions may also exist for the formation of reactive sulfur. Because sulfite radicals and DSSO can potentially increase lipid oxidation in skeletal muscle, control of conditions that promote formation of these compounds during processing may be beneficial to maintaining the proper pro-oxidant/antioxidant balance in muscle food. However, more work on reactive sulfur that includes examining an in vivo source and its reactivity under conditions commonly encountered in muscle food should be performed before a conclusion as to the relative role of reactive sulfur in oxidative reactions in muscle food can be reached.

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