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Heme-Mediated Production of Free Radicals via Preformed Lipid Hydroperoxide Fragmentation

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Electron spin resonance (ESR) spectroscopy and the spin-trapping technique were used to investigate the capacity of several hemoglobin (Hb) forms of rainbow trout (oxyHb and metHb), free hemin (oxidized form of heme group), and hemin complexed with bovine serum albumin (BSA) to promote formation of free radicals via fragmentation of preformed lipid hydroperoxides. Cumene hydroperoxide (CumOOH) was used as a model for lipid hydroperoxide, and free radicals were monitored by stabilizing with the spin traps α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN) and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO). Two different types of free radicals, hydroxyl and carbon-centered radicals, were identified as a result of the interaction of the heme-containing systems and CumOOH. Carbon-centered radicals were found to be mainly heme-mediated because the addition of the iron-chelating agent EDTA did not affect the formation of POBN/carbon-centered adducts. Hemin alone was the best promoter for the production of POBN/carbon-centered radicals in the presence of low hydroperoxide concentrations (below equimolar condition over heme group), whereas hemin/BSA and oxyHb were more active in generating radicals at high hydroperoxide concentrations or after successive interactions with hydroperoxides. This finding can be explained by the coexistence of two different facts: (i) the interaction between hemin and lipid hydroperoxides seems to be more efficient in the case of free hemin compared to heme−protein complexes and (ii) a faster degradation of hemin is produced without the presence of a protein fraction, globin or albumin. The comparison of oxyHb and metHb also suggested that both Hb redox states have similar capacities to generate oxidative stress via cleavage of preformed lipid hydroperoxides.

KEYWORDS: Heme group; pro-oxidant; free radicals; hydroperoxides; ESR; spin trapping; lipid oxidation

INTRODUCTION

Lipid peroxidation is one of the major mechanisms in the deterioration of food quality during processing and storage (*1*), as well as in the development of a wide variety of diseases ranging from atherosclerosis, cancer, and neurodegenerative disorders to even the normal aging process (*2*−*4*). Polyunsaturated fatty acids (PUFAs) are the main components of tissue membranes and one of the major targets of free radicals, resulting in peroxidation and production of lipid hydroperoxides under aerobic conditions (*5*). Lipid hydroperoxides can be formed in situ in the body by peroxidation of PUFAs contained in cell membranes (*6*). However, dietary lipids can also be a source of lipid hydroperoxides either by direct consumption of peroxidized lipids (*7*) or by oxidation of dietary lipids in the gastric compartment, where the combination of oxidizable lipids with dietary iron and heme compounds in an acid environment creates conditions for a relatively fast peroxidation (*8*). In fact, lipid hydroperoxides were found to increase in plasma in the postprandial state after the consumption of a fatty meal (*9*).

Hemoglobin (Hb) and myoglobin (Mb) are known to trigger lipid peroxidation in muscle-based foods (*10*−*12*) and play also an oxidative role in the pathology of certain disease states, such as malaria, thalassemia, and rhabdomyolysis (*13*). Two main pathways are suggested to explain the activation of oxidative stress by Hb and Mb: (i) hypervalent ferryl Hb/Mb radical ($^{\bullet}$HbFe(IV)═O/($^{\bullet}$MbFe(IV)═O) formed by reaction of metHb/metMb (HbFe(III)/MbFe(III)) and hydrogen peroxide can abstract an H atom from a polyunsaturated fatty acid (*1*) and (ii) heme proteins stimulate the generation of free radicals via cleavage of lipid hydroperoxides (*14*). Several investigations indicated that the presence of lipid hydroperoxides is a crucial factor in the heme protein-initiated lipid peroxidation (*15*), but there is not a clear understanding of the individual contribution of each mechanism on the pro-oxidative role of Hb. The reaction of Hb/Mb with hydroperoxides leads to Hb/Mb modifications, being described as the formation of oxidized Hb/Mb forms,

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dissociation of the heme group, and oxidative modifications of the heme group (*13, 16*).

The present study aimed to investigate the efficiency of the heme group and representative Hb forms in the generation of free radicals in the presence of lipid hydroperoxides. With that purpose, the organic hydroperoxide, cumene hydroperoxide (CumOOH), was used as a model for lipid hydroperoxide and incubated with several trout Hb species (OxyHb and metHb), free hemin (oxidized form of heme group), and hemin coupled to bovine serum albumin (BSA). CumOOH-derived free radicals formed were monitored using electron spin resonance (ESR) spectroscopy and the spin-trapping technique. The radical-generating activity under continuous episodes of oxidative stress was also evaluated by monitoring the capacity of heme-containing systems after successive interactions with hydroperoxides.

MATERIALS AND METHODS

Materials. Rainbow trout (*Oncorhynchus mykiss*) was provided alive by local fish suppliers in Denmark. α-(4-Pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN), CumOOH, hemin chloride, BSA, ammonium persulfate, sodium heparin, tris[hydroxymethyl]aminomethane (Tris), bovine hemoglobin, sodium dithionate, dibasic sodium phosphate ($Na_2HPO_4 \cdot 2H_2O$), and monobasic sodium phosphate ($NaH_2PO_4 \cdot H_2O$) were purchased from Sigma (Steinheim, Germany). 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) was obtained from Fluka (Buchs, Swizerland). Sephadex G-25 columns were obtained from Pharmacia Biotech (Uppsala, Sweden). All chemicals were of analytical grade, and water was previously distilled and further deionized using a Milli-Q system (Millipore, Billerica, MA).

Isolation of Hemoglobin. Blood was taken from the caudal vein after the tail of individual rainbow trouts in the rigor state had been cut off. Blood was collected with a transfer glass pipet rinsed with 150 mM NaCl and sodium heparin solution (30 units/mL), and it was immediately mixed with approximately 1 volume of the saline sodium heparin solution. Hemolysate was then prepared according to the modifications by Richards and Hultin (*17*) of the procedure of Fyhn et al. (*18*). The visible spectrum at pH 8.6 showed that Hb was mostly oxygenated because of the appearance of two absorption peaks at 540 and 570 mn, characteristic of OxyHb species. Hb was stored at −80 °C and was thawed just before use.

Preparation of Methemoglobin (MetHb). Trout OxyHb was fully oxidized by modification of the procedure of Svistunenko et al. (*19*). Briefly, trout Hb was incubated with 4 mM ammonium persulfate for 1 h at room temperature and then passed through a Sephadex G-25 column. MetHb was stored at −80 °C and was thawed just before use.

Quantification of Hemoglobin. The concentration of Hb was quantified according to the method of Brown (*20*). Briefly, Hb diluted in 50 mM Tris, pH 8.6, was mixed with around 1 mg of sodium dithionite and bubbled with carbon monoxide gas for 20 s. The sample was then scanned from 400 to 480 nm against a blank that contained only buffer. The peak absorbance was recorded, and absorbance at the peak (≈ 416 nm) minus absorbance at the valley (465 nm) was related with the concentration of hemoglobin. A standard curve was built with hemoglobin from bovine blood.

Preparation of Hemin and Albumin Solutions. Hemin stock solutions were prepared by dissolving solid hemin in 0.010 N NaOH. BSA stock solutions were prepared by dissolving solid BSA in 1 mM Tris buffer, pH 8.0. Working solutions containing hemin and BSA at 4:1 (hemin/BSA) molar ratio were prepared by diluting the stock solutions in 1 mM Tris buffer, pH 8.0, and were incubated at room temperature for at least 30 min prior to use. The stock and working solutions were protected from light, and used only on the day of preparation.

ESR Spin-Trapping Experiments. Solutions of the spin traps POBN and DMPO were daily prepared in 50 mM phosphate buffer at pH 6.8. The heme-containing systems were incorporated into the above spin trap solution, and reaction was then initiated by the addition of CumOOH in ethanol. Ethanol constituted approximately 2.5% of the reaction mixture. Final concentrations of POBN and DMPO spin traps were, respectively, 60 and 280 mM, and heme-containing systems were added on the basis of heme content up to a concentration of 92 μM. CumOOH was added at low (23 μM), medium (114 μM), and high concentration levels (571 μM). The effect of EDTA on POBN−radical adducts was evaluated using a final concentration of 1.5 mM for this iron-chelating agent. The reaction mixture was immediately subjected to vigorous vortex stirring and transferred to a 50 μL disposable glass capillary micropipette (Blaubrand intraMARK, Brand, Wertheim, Germany). ESR spectra were recorded at room temperature on a Miniscope MS200 spectrometer (Magnettech, Berlin, Germany) using the following parameters for detection of POBN−spin adducts: microwave power, 9.4 GHz; sweep width, 70 G; sweep time, 60 s; modulation amplitude, 2 G; time constant, 0.1 s. DMPO−spin adducts were monitored using the ESR settings indicated above, but with a sweep width of 100 G. At the experiments proposed to evaluate the effect of successive interactions with hydroperoxides on the radical-generating activity of heme-containing systems, heme-containing systems and CumOOH were incubated at equimolar conditions (92 μM) for 10 min prior to the incorporation of POBN spin trap (60 mM) and an additional quantity of CumOOH (114 μM). Subsequently, POBN−radical adducts were detected as described above.

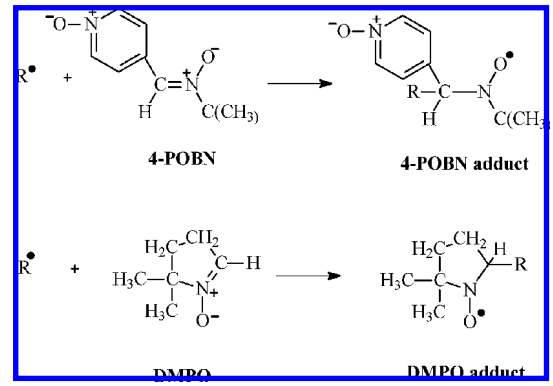

Figure 1. Formation of long-lived nitroxyl radical spin adducts by addition of radicals to the nitrone spin trap, 4-POBN, and pyrroline-*N*-oxide spin trap, DMPO.

Peak-to-peak amplitudes were used for the quantification of the intensity signal of POBN−spin adducts (**Figure 1**). Simulation and fitting of ESR spectra were performed by the WinSIM program (*21*).

Heme Stability in the Presence of Hydroperoxide and Albumin. Hemin alone or associated with albumin in a 4:1 (hemin/BSA) molar ratio was exposed to CumOOH in 50 mM phosphate buffer, pH 6.8. Final concentrations of hemin and CumOOH were 40 μM and 2 mM, respectively. The stability of hemin was determined by measuring the loss of the absorbance at 390 nm after 1, 3, or 6 min of interaction with CumOOH. The initial absorbance at 390 nm (100% of absorbance) was determined by substituting the ethanolic solution of CumOOH by pure ethanol.

Statistical Analysis. The experiments were performed at least twice, and data are reported as mean ± standard deviation of two replicates. The data were analyzed by one-way analysis of variance (ANOVA) and the least-squares difference method. Statistical analyses were performed with the software Statistica 6.0.

RESULTS

Detection of CumOOH-Derived Free Radicals. ESR spectroscopy and the spin-trapping technique were employed to monitor free radicals produced from interaction between the heme-containing systems studied and a model hydroperoxide, CumOOH. POBN or DMPO spin traps were used to stabilize the free radicals by forming long-lived radical spin adducts, as illustrated in **Figure 1**. ESR spectra recorded after 1 min showed the formation of POBN−radical adducts consisting of a triplet of doublets, which were successfully simulated by assuming a

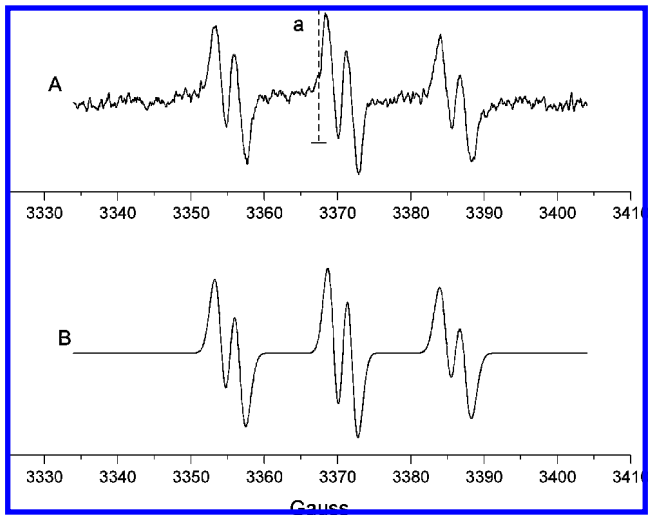

Figure 2. ESR spectrum (**A**) and computer simulation (**B**) of POBN spin adducts formed from the reaction of cumene hydroperoxide (CumOOH) with Hb species or hemin. The reaction mixture contained 92 μM trout oxyHb (on basis of heme group), 114 μM CumOOH, and 60 mM POBN in 50 mM phosphate buffer, pH 6.8, and the ESR spectrum was recorded after 1 min of reaction at room temperature. Peak-to-peak amplitudes (a) were used for the quantification of the POBN−radical adducts as indicated.

Table 1. Structural Assignment for the POBN and DMPO Spin Adducts Detected in the Reaction of Hb Species or Hemin with Cumene Hydroperoxide (CumOOH)

	spin trap	a_N (G)	a_H (G)	assignment
I	POBN	15.4	2.4	carbon-centered radical
II	DMPO	15.7	22.5	carbon-centered radical
III	DMPO	14.8	14.8	hydroxyl radical
IV	DMPO	14.5		DMPO ring broken

single spin adduct **I** with coupling constants $a_N = 15.4$ G and $a_H = 2.4$ G (**Figure 2** and **Table 1**). The formation of spin adduct **I**, previously reported and assigned to a POBN/carbon-centered radical adduct (*22, 23*), was detected in the presence of either trout OxyHb or hemin and quantified on the basis of the peak-to-peak amplitude as shown at **Figure 2**. Carbon-centered radicals may be generated from the iron-mediated decomposition of CumOOH (*24*). However, under the actual experimental conditions the formation of POBN/carbon-centered radicals seems to be mainly heme-mediated. POBN−radical adduct **I** was detected only in the presence of the heme-containing system (data not shown), and the addition of the iron-chelating agent EDTA did not significantly affect the formation of the spin adduct (**Figure 3**).

DMPO spin trap originated complex spectra after 1 min of reaction of CumOOH with OxyHb or hemin, which were successfully simulated assuming the simultaneous presence of three different radical adducts characterized by the following coupling constants: (i) $a_N = 15.7$ G and $a_H = 22.5$ G (**II**), (ii) $a_N = 14.8$ G and $a_H = 14.8$ G (**III**), and (iii) $a_N = 14.5$ G (**IV**) (**Figure 4** and **Table 1**). The coupling constants corresponding to the radical adduct **II** have been previously assigned to a DMPO/carbon-centered radical (*25, 26*), whereas adducts **III** and **IV** were respectively reported as DMPO−hydroxyl radical adducts (*25*) and DMPO-ring broken adducts (*27*). After 12 min of incubation, the triplet signal characteristic of adduct **IV** dominated the ESR signal (**Figure 4**).

Capacity of OxyHb, MetHb, and Hemin To Generate Free Radicals in the Presence of Hydroperoxides. Heme-containing

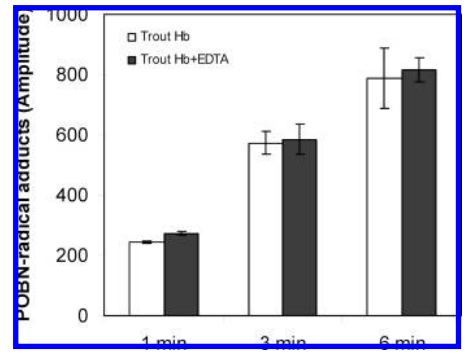

Figure 3. Effect of EDTA on POBN−radical adducts generated by trout oxyHb in the presence of CumOOH. The reaction was performed in the presence or absence of 1.5 mM EDTA, together with 92 μM trout oxyHb (on the basis of the heme group), 114 μM CumOOH, and 60 mM POBN in 50 mM phosphate buffer, pH 6.8. The intensity of POBN−radical adducts was monitored after 1, 3, and 6 min of the reaction initiation.

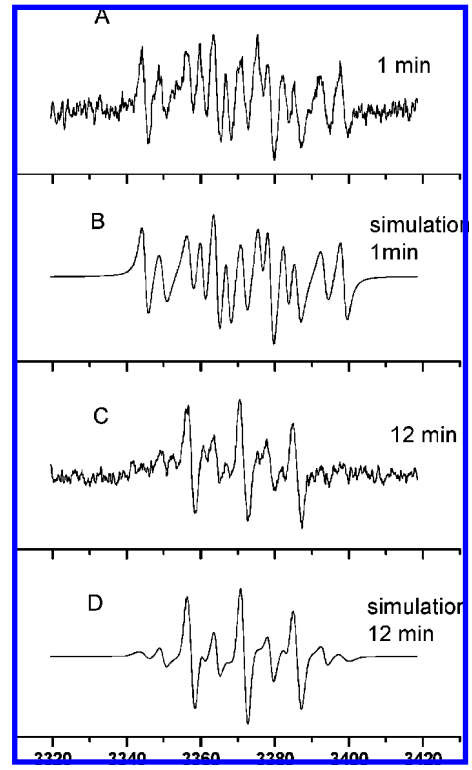

Figure 4. ESR spectra and computer simulation of DMPO−radical adducts formed from the reaction of cumene hydroperoxide (CumOOH) with Hb species or hemin. The reaction mixture contained 92 μM hemin, 114 μM CumOOH, and 280 mM DMPO in 50 mM phosphate buffer, pH 6.8. The ESR spectrum was recorded after 1 and 12 min of reaction at room temperature.

systems such as hemin, hemin/BSA (4:1), and trout oxyHb were evaluated to compare the radical-generating activity of free heme group alone, in the presence of protein, and as a tightly bound complex constituting Hb. All heme-containing systems were studied at the same molar concentration (92 μM on heme basis) under conditions of low (23 μM), medium (114 μM), and high (571 μM) concentration of CumOOH. At the lowest hydroperoxide/heme molar ratio (1:4), hemin gave the highest levels of POBN−radical adducts during the entire monitoring time, whereas hemin/BSA generated more radical adducts than oxyHb at 1 min (**Figure 5a**). After longer incubation times, hemin/BSA and oxyHb exhibited similar activities in generating POBN−radical adducts. Those results also indicated different

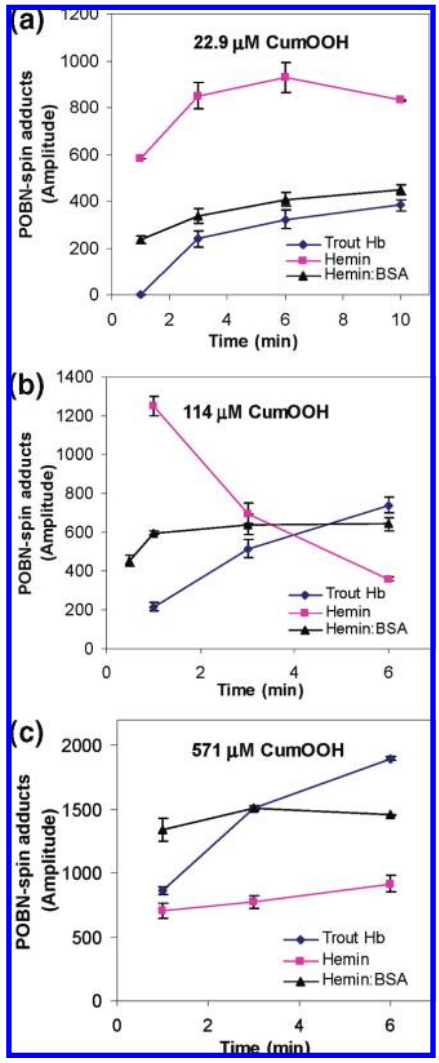

Figure 5. Capacity of oxyHb, hemin, and hemin/BSA (4:1) to generate POBN−radical adducts in the presence of different concentrations of CumOOH (23, 114, and 571 μM). Heme-containing systems and POBN were incubated at concentrations of 92 μM (heme basis) and 60 mM, respectively, in 50 mM phosphate buffer, pH 6.8.

kinetic patterns for the formation of POBN−radical adducts, because the level of spin adducts reached a maximum after 6 min for hemin, whereas hemin/BSA and oxyHb both gave a net formation of radicals during the entire monitoring time of 10 min (**Figure 5a**).

The interaction with a medium concentration of CumOOH (114 μM) for 1 min generated higher spin adduct formation for all heme-containing systems than low CumOOH concentration, although similar radical-promoting behaviors were observed for those obtained with low hydroperoxide concentration: hemin > hemin + BSA > trout oxyHb (**Figure 5**). However, POBN−radical adducts generated by hemin decreased continuously for longer reaction times, and hemin gave the lowest spin adduct concentration after 6 min as a result of increasing levels of spin adducts observed for hemin + BSA and oxyHb.

Under conditions of high hydroperoxide concentration, hemin was found to be the weakest promoter of free radicals during the entire time of monitoring, whereas hemin/BSA gave the highest spin adduct concentrations at 1 min of incubation. However, oxyHb was significantly more active in generating radical adducts after longer incubation periods (**Figure 5c**). In summary, hemin generated the highest levels of free radicals under low−medium hydroperoxide concentration, whereas

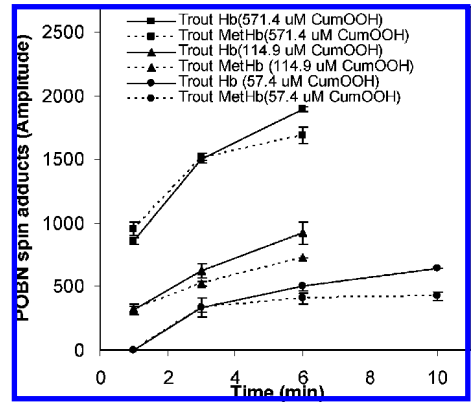

Figure 6. Comparison of oxyHb and metHb as promoters of POBN−radical adducts in the presence of different concentrations of CumOOH (58, 114, and 571 μM). Hb species were tested at 92 μM (heme basis) in 50 mM phosphate buffer, pH 6.8, containing 60 mM of the spin adduct POBN. Heme/hydroperoxide molar ratios reached 1:0.63, 1:1.25, and 1:6.24 in the presence of 58, 114, and 571 μM CumOOH, respectively.

hemin/BSA and oxyHb were more active in generating free radicals at the high hydroperoxide concentrations (**Figure 5**).

The capacity of trout metHb to generate free radicals via fragmentation of preformed hydroperoxides was also evaluated, and the results indicated no significant differences as compared to oxyHb during short-term incubations for up to 3 min (**Figure 6**). MetHb and oxyHb were accordingly concluded to generate similar amounts of CumOOH-derived radicals under different heme/hydroperoxide molar ratios (1:0.63, 1:1.25, and 1:6.24) during the initial 3 min of reaction. After longer incubation times, metHb seemed to lose efficiency to generate hydroperoxide-derived free radicals in comparison to oxyHb ($p < 0.05$).

Effect of Interaction of OxyHb, Hemin, and Hemin/BSA with Hydroperoxides on Subsequent Free Radical Formation. OxyHb, hemin, and hemin/BSA were initially incubated with CumOOH at equimolar conditions (91 μM) for 10 min, and the POBN−radical adducts were then monitored after incorporation of the spin trap POBN and an additional supply of CumOOH (114 μM). Such experiments were designed to explore the remaining activity of the heme-containing systems after an episode of oxidative stress. This earlier exposure of heme-containing systems to hydroperoxides led to a major reduction of hemin capacity to generate POBN−radical adducts (**Figure 7**), in comparison with the direct incubation with CumOOH (114 μM) (**Figure 5**). Under conditions of previous exposure to hydroperoxide, hemin gave an approximately constant level of POBN−radical adducts. Hemin produced the lowest levels of free radicals during the entire incubation time, together with oxyHb that also generated the lowest levels of free radical at 1 min. Hemin/BSA was the best free radical promoter at 1 min ($p < 0.05$), and the high activity was maintained almost constant during the next 6 min of incubation. However, oxyHb gave an increasing level of spin adducts with time, and it was the best free radical promoter after 6 min (**Figure 7**).

Heme Stability in the Presence of Hydroperoxides. Heme stability was evaluated during the incubation of hemin alone and during incubation of hemin/BSA (4:1) with CumOOH by monitoring the absorbance at 390 nm, at which wavelength hemin shows maximal absorption. Hemin showed decomposition either alone or associated with BSA; however, the absence of BSA produced a faster loss of heme integrity (**Figure 8**). Approximately 32% of hemin was lost by incubating hemin/

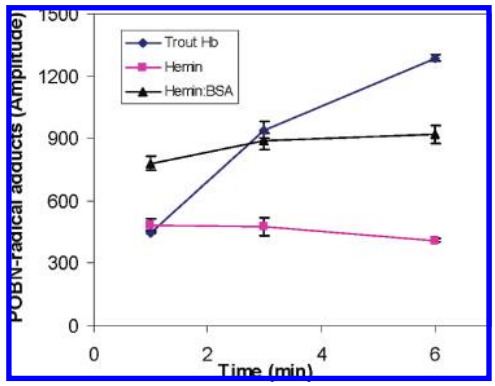

Figure 7. Effect of a previous incubation of OxyHb, hemin, and hemin/BSA (4:1) with CumOOH on their free radical promoter activity. Heme-containing systems (92 μM on heme basis) were incubated with CumOOH (92 μM) during 10 min in 50 mM phosphate buffer, pH 6.8. After this initial incubation, POBN (60 mM) and CumOOH (114 μM) were added, and POBN−radical adducts were detected by ESR.

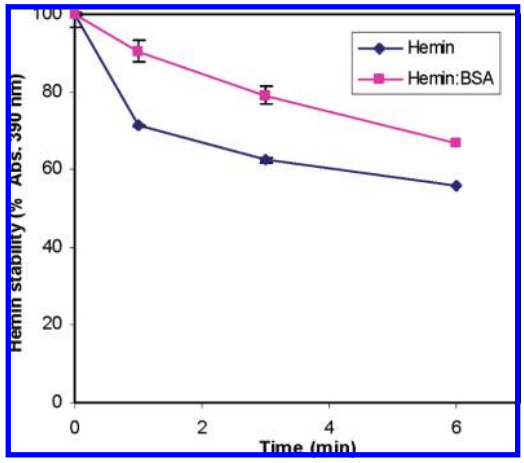

Figure 8. Effect of bovine serum albumin (BSA) on hemin stability in the presence of CumOOH. Hemin (40 μM) and hemin/BSA (at molar ratio 4:1) were incubated in the presence of CumOOH (2000 μM), and hemin stability was monitored as absorbance at hemin absorption maximum at 390 nm.

BSA and CumOOH for 6 min. In contrast, hemin alone gave losses of 30 and 48% of hemin after 1 and 6 min of incubation, respectively.

DISCUSSION

A procedure based on ESR and spin-trapping technique has been used to distinguish the capacity of several heme-containing systems to generate free radicals via fragmentation of preformed hydroperoxides. The present paper demonstrates that the heme group either alone or attached to the hemoglobin structure catalyzes effectively the cleavage of CumOOH, leading to the production of two different types of free radicals, that is, hydroxyl and carbon-centered radicals. The production of carbon-centered radicals was not found to be significantly affected by adding an iron-chelating agent (EDTA), and therefore these carbon-centered radicals seem to be mainly heme-mediated and not generated by free ionic iron. This conclusion is in agreement with the result of a previous investigation that reported a reduced formation of carbon-centered radicals through iron-catalyzed fragmentation of CumOOH in 50 mM phosphate buffer, which was also the phosphate concentration in the present experiments (*24*). Moreover, hemoglobin was found to cleavage hydroperoxides 40 and 430 times more rapidly than free ferrous iron and free ferric iron, respectively (*28*). Several mechanisms have been postulated for the reaction between hemoglobin and organic hydroperoxides, including both heterolytic and hemolytic cleavage of the peroxide bond and a peroxidase-type mechanism (*29*). These mechanistic pathways include the formation of peroxyl or alkoxyl oxygen-centered radicals as initial radical products. The carbon-centered radicals could then arise by subsequent intramolecular β-scission of tertiary alkoxyl radicals (*30*), and the methyl radical has previously been found to be a β-scission product of the corresponding alkoxyl radical of CumOOH, cumyloxyl radical (CumO$^{\bullet}$) (*24, 31*). With regard to the hydroxyl radicals, the formation of this highly reactive radical has previously been detected for either free iron or cytochrome P-450-induced decomposition of linoleic acid hydroperoxide (*29, 32*). Those investigations suggest that hydroxyl radical may be generated through Fenton type decomposition of hydrogen peroxide produced by disproportionation of superoxide radicals ($O_2^{\bullet-}$), which was identified during the iron- and heme-induced decomposition of linoleic acid hydroperoxide under aerobic conditions.

Hb/Mb in the reduced ferrous state undergoes oxidation to metHb/metMb [HbFe(III)/MbFe(III)] and even to ferryl species such as $^{\bullet}$HbFe(IV)═O/$^{\bullet}$MbFe(IV)═O, as a consequence of the reaction with hydroperoxides (*13*). The oxidative modification of Hb/Mb is likely responsible for the loss of redness reported during the propagation of lipid oxidation in muscle foods. The results obtained in the present study show some differences in the behavior of oxyHb, metHb, and hemin in the generation of free radicals through hydroperoxide fragmentation, suggesting that hemin is especially efficient in promoting hydroperoxide-derived free radical at low concentration of hydroperoxides, which is likely at the initiation phase of lipid peroxidation, whereas Hb forms are more active during the propagation step of lipid peroxidation that is characterized by higher concentration of lipid hydroperoxides. Hemin activated more quickly and more extensively the formation of hydroperoxide-derived free radicals at low hydroperoxide/heme molar ratio (1:4), whereas the increment of hydroperoxide/heme ratio up to around 6:1 was found to deactivate moderately its free radical promoter capacity. Accordingly, hemin was also a less active free radical generator when it had been pretreated with hydroperoxides. On the contrary, the radical-generating capacity of oxyHb and hemin/BSA was not diminished by the previous contact with hydroperoxides. These observations could be explained, at least in part, by considering the coexistence of two different facts: (i) a more efficient interaction between hemin and lipid hydroperoxides should occur in the case of free hemin in comparison with that constituting heme−protein complexes and (ii) a faster degradation of hemin in the absence of the protein fraction and the possible stabilizing role of proteins (globin and albumin) on hemin by trapping radicals. Grunwald and Richards (*33*) also showed more rapid lipid oxidation in washed cod muscle treated with hemin in the presence of BSA than with hemin alone. Other investigations have indicated that prevention of heme degradation by inhibition of the heme oxygenase, an enzyme that degrades heme, is associated with an enhancement of oxidative damage in animal models, and overexpression of heme oxygenase is protective (*34*). All of these findings suggest that heme-related systems are more important than free iron in mediating oxidative damage in tissues, and therefore procedures that increase the degradation of heme groups could result in less pronounced lipid oxidation.

OxyHb and metHb exhibited similar capacities to promote the formation of hydroperoxide-derived free radicals during short incubation times,; however, oxyHb seems to be a better free radical promoter during long-term exposures to hydroperoxides. These results are in contrast with the higher activity of met Hb/Mb in promoting lipid peroxidation in washed cod muscle, in comparison with ferrous Mb (*33*). Reeder and Wilson (*35*) demonstrated that metMb reacts with the lipid hydroperoxide hydroperoxyoctadecadienoic acid to render mainly the ferrylMb form, whereas changes for the oxyMb are more complex, with ferrous, ferric, and ferryl species present. Therefore, a possible explanation for the superior activity of metHb to catalyze lipid peroxidation compared with oxyHb could be that the former provides higher production of the ferryl form, which is also able to initiate lipid peroxidation by abstraction of a hydrogen atom from PUFAs (*1*).

The present paper has demonstrated the importance of heme-mediated decomposition of lipid hydroperoxides for the generation of free radicals with the capacity of propagating lipid oxidation in biological tissues and meat-based foods and even to degrade heme catalyst. Hemin alone, without a protein fraction present (globin or albumin), was shown to be significantly less stable, an observation that may explain the poor free radical-promoting activity of hemin under conditions of high hydroperoxide/heme molar ratios. On the contrary, Hb and hemin/BSA showed an increased generation of free radicals for conditions of high hydroperoxide concentrations. A strategy for the design of more effective treatments of muscle-based foods to retard the propagation of heme-mediated lipid peroxidation could accordingly be based on controlled degradation of heme pigments.

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