ARTICLES

Effect of Aldehyde Lipid Oxidation Products on Myoglobin

M. P. Lynch and C. Faustman*

Department of Animal Science, University of Connecticut, Storrs, Connecticut 06269

The effects of aldehyde lipid oxidation products on myoglobin (Mb) were investigated at 37 °C and pH 7.2. Oxymyoglobin (OxyMb) oxidation increased in the presence of 4-hydroxynonenal (4-HNE) compared to controls ($P < 0.05$). Preincubation of metmyoglobin (MetMb) with aldehydes rendered the heme protein a poorer substrate for enzymatic MetMb reduction compared to controls, and the effect was inversely proportional to preincubation time; unsaturated aldehydes were more effective than saturated aldehydes ($P < 0.05$). The order of MetMb reduction as affected by preincubation was control > hexanal > heptanal > octanal > nonanal = decanal = hexenal > heptenal = octenal > nonenal = decenal = 4-HNE ($P < 0.05$). Preincubation of MetMb with 4-HNE enhanced the subsequent ability of the heme protein to act as a prooxidant in both liposomes and microsomes when compared to controls ($P < 0.05$); the effect was reduced in microsomes containing elevated concentrations of α-tocopherol ($P < 0.05$). MetMb preincubation with mono-unsaturated aldehydes enhanced the catalytic activity of MetMb to a greater degree than saturated aldehydes ($P < 0.05$). These results suggest that aldehyde lipid oxidation products can alter Mb stability by increasing OxyMb oxidation, decreasing the ability of MetMb to be enzymatically reduced and enhancing the prooxidant activity of MetMb.

Keywords: *Myoglobin; oxidation and reduction; 4-hydroxynonenal; aldehydes*

INTRODUCTION

Pigmentation in beef is principally due to the oxidation state of myoglobin (Mb). Oxymyoglobin (OxyMb) may be maintained in meat by delaying its oxidation to metmyoglobin (MetMb) or through nonenzymatic reduction of MetMb by reduced cytochrome b_5 (Cyt b_5; Livingston et al., 1985). Lipid oxidation and Mb oxidation can occur concurrently as MetMb formation is positively correlated with lipid oxidation in vitro (Yin et al., 1993) and in meat (Schaefer et al., 1995). OxyMb and MetMb are also known catalysts of lipid oxidation in model systems (Baron et al., 1997) and meat (Johns et al., 1989). The process by which lipid oxidation products alter Mb stability is unknown.

Lipid oxidation results in a wide range of secondary aldehyde products which are predominantly *n*-alkanals, *trans*-2-alkenals, 4-hydroxy-*trans*-2-alkenals, and malondialdehyde (Esterbauer et al., 1991). The aldehyde products are more stable than free radical species and readily diffuse into the cellular media where they may exert toxicological effects by reacting with critical biomolecules in vivo (Esterbauer et al., 1991; Szweda et al., 1993). Protein modification by aldehydes is believed to play a central role in many pathophysiological conditions (Szweda et al., 1993). 4-Hydroxynonenal (4-HNE) is an α,β-unsaturated aldehyde formed by oxidation of ω6 unsaturated fatty acids (Esterbauer and Cheeseman, 1990; Sakai et al., 1995) and is thought to be an important indicator of free radical-stimulated lipid oxidation. Previous work has shown covalent attachment of malondialdehyde to LDL (Haberland et al., 1988) and of 4-HNE to glucose-6-phosphate dehydrogenase, hemoglobin, and LDL (Esterbauer et al., 1991; Szweda et al., 1993; Bruenner et al., 1995; Uchida et al., 1994). It is possible that the presence of these lipid oxidation products may alter Mb through covalent modification (Faustman et al., 1998). The objective of this project was to determine the effect of aldehyde lipid oxidation products on OxyMb oxidation, MetMb reduction, and the catalytic activity of MetMb as a lipid prooxidant in vitro.

MATERIALS AND METHODS

Reagents and Assay Conditions. All assays were incubated at 37 °C (Yin and Faustman, 1993) and pH 7.2 (0.1 M sodium phosphate). Horse heart Mb, dinitrophenylhydrazine (DNPH), methanol, acetonitrile, and tetrahydrofuran were obtained from Sigma Chemical Co. (St. Louis, MO). Hexanal, hexenal, heptanal, heptenal, octanal, octenal, nonanal, nonenal, decanal, and decenal were obtained from Aldrich Chemical Co., (Milwaukee, WI) and 4-HNE from Cayman Chemical Co., (Ann Arbor, MI). Egg yolk phosphatidylcholine was obtained from Avanti Polar Lipids Inc. (Alabaster, AL). All other chemicals were reagent grade or purer.

Mb and Lipid Oxidation Analysis. OxyMb and MetMb concentrations were determined by the method of Krzywicki (1982) using a Shimadzu Inc. (Columbia, MD) UV2101PC spectrophotometer. Lipid oxidation was measured by the thiobarbituric acid (TBARS) test as described by Yin et al. (1993).

Oxidation of OxyMb in the Presence of 4-HNE. A stock solution of OxyMb was prepared from commercial MetMb as

* To whom correspondence should be addressed. Department of Animal Science, U-40, University of Connecticut, Storrs, CT 06269. Telephone: 860-486-2542. Fax: 860-486-4375. E-mail: CFAUSTMA@ansc1.cag.uconn.edu.

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described by Yin and Faustman (1993). OxyMb was combined with 4-HNE (stock 10 mg/mL ethanol), pH 7.2, in a rubber-capped test tube (16 × 100 mm) to obtain final concentrations of 0.15 and 1 mM, respectively; controls were aldehyde-free but contained an equivalent volume of ethanol (15 μL/mL assay used to deliver the aldehyde). Samples (1.5 mL) were withdrawn at 0, 30, 60, and 90 min to determine OxyMb and free 4-HNE concentrations.

4-HNE Concentration Determination. 4-HNE was derivatized using DNPH according to the method of Goldring et al. (1993) with the modification of redissolving the nitrogen-dried sample in methanol rather than chloroform. The concentration of derivatized 4-HNE was then determined using reverse-phase HPLC, (5 μm spherical C_{18} column, Waters, Milford, MA). The mobile phases were A (water/acetonitrile/tetrahydrofuran 60/30/10 v/v/v) and B (water/acetonitrile 20/80 v/v). Elution was as follows: 100% A for 1.5 min, then a linear gradient from 100% A to 100% B in 12 min, and 100% B for 5 min. A flow rate of 1 mL/min was used with an injection volume of 35 μL and detection at 378 nm. Peak identification and standard curves were generated by injecting a 4-HNE standard under the same conditions.

Effect of Aldehyde Preincubation on Enzymatic MetMb Reduction. MetMb was combined in a rubber-capped test tube (16 × 100 mm) with the aldehydes at a molar ratio of 1:7; controls were prepared as previously described. 4-HNE preincubation samples were removed at 60, 90, and 120 min and applied to a PD-10 column (Pharmacia Biotech, Piscataway, NJ) to remove free aldehyde. All other aldehydes were preincubated with MetMb for 90 min prior to chromatography. Enzymatic MetMb reduction was based on the method of Lynch et al. (1998). MetMb reduction assays contained buffer, MetMb (0.15 mM) from the PD-10 eluant, and 45.0 μL bovine liver extract (Faustman et al., 1988); NADH (0.15 μM) was added to initiate the reaction. MetMb reduction assays were incubated for 30 min, and the MetMb concentration was determined.

Liposome and Microsome Preparation. Phosphatidylcholine liposomes were prepared according to Yin and Faustman (1993). Beef liver microsomes from vitamin E-supplemented and -nonsupplemented animals (Chan et al., 1995) were prepared according to Guengerich (1977), and microsomal protein concentration was determined using a BCA protein assay kit (Sigma). The microsomal α-tocopherol (α-Toc) content was determined according to Lang et al. (1992).

Effect of Aldehyde Preincubation on the Catalytic Activity of MetMb as a Prooxidant. Aldehydes were combined with MetMb for 0, 15, 30, 60, 90, and 120 min. Free aldehyde was removed, and controls were prepared as previously described. MetMb eluant (0.15 mM) was incubated with liposomes and microsomes (1 mg of microsomal protein/mL); lipid oxidation was analyzed as previously described.

Statistical Analysis. Analysis of variance (ANOVA) was computed using the SAS general linear model procedure (SAS, 1985). Differences among means at the 5% level were determined by the least significance difference test. Each reaction condition was performed in triplicate on three different days.

RESULTS AND DISCUSSION

The results of 4-HNE incubation with OxyMb are presented in Figure 1. During a 90 min incubation, OxyMb oxidation was greater in the presence of 4-HNE than in controls (Figure 1A; $P < 0.05$), a result consistent with that of Faustman et al. (1999). At the same time, 4-HNE concentration decreased approximately 40% ($P < 0.05$) in the presence of OxyMb, whereas controls decreased only 4% ($P > 0.05$; Figure 1B). This latter observation suggested that there is no significant loss of 4-HNE through volatilization. In the presence of OxyMb, the measured loss of free 4-HNE (Figure 1B) is believed to be due to covalent attachment of 4-HNE to OxyMb, thereby rendering OxyMb more susceptible

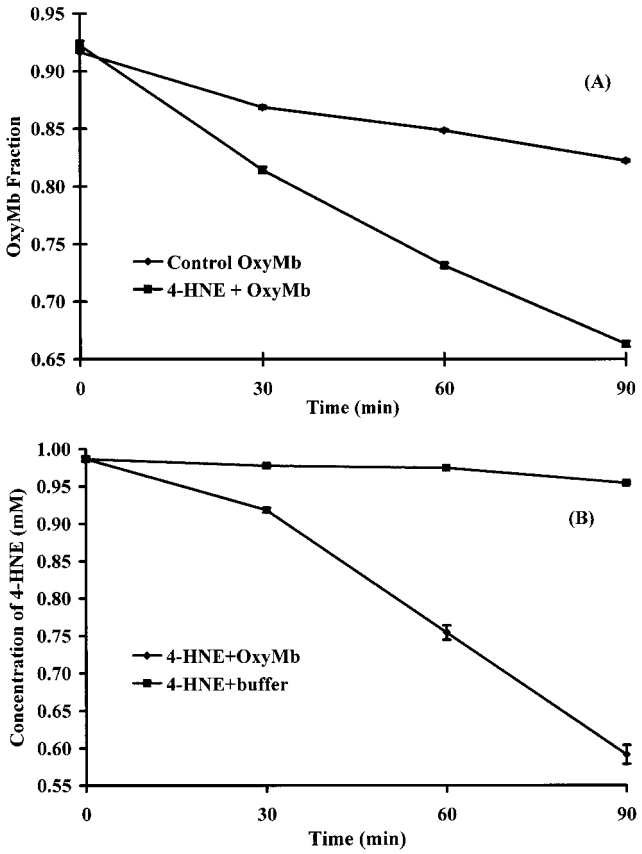

Figure 1. (A) Effect of 4-hydroxynonenal (4-HNE) on oxymyoglobin (OxyMb) oxidation and (B) disappearance of 4-HNE, at 37 °C and pH 7.2. Standard error bars are included.

to oxidation (Faustman et al., 1998). Greater OxyMb oxidation in the presence of 4-HNE was highly correlated with the aldehyde's disappearance from solution ($R^2 = 0.96$). Faustman et al. (1999) showed that 4-HNE adducts of OxyMb were formed under similar experimental conditions using electrospray ionization mass spectrometry. They also showed increased MetMb formation in the presence of several other aldehyde lipid oxidation products. The significant effect of 4-HNE on OxyMb oxidation led us to investigate the possible effects of 4-HNE and other aldehyde lipid oxidation products on both enzymatic MetMb reduction and the ability of MetMb to act as a lipid prooxidant.

The effect of 4-HNE on the ability of MetMb to be reduced enzymatically was investigated. MetMb preincubated with 4-HNE was less desirable as a substrate for enzymatic reduction than controls ($P < 0.05$; Figure 2A). Additionally, increasing the length of 4-HNE preincubation resulted in less total MetMb reduction ($P < 0.05$), indicating that MetMb was rendered a poorer substrate by reaction with the aldehyde. When MetMb was preincubated with other aldehyde products, the fraction of MetMb reduced was lower than that in controls ($P < 0.05$; Figure 2B). Preincubation of MetMb with unsaturated aldehydes was more detrimental than that with saturated aldehydes ($P < 0.05$; Figure 2B). The order of MetMb reduction after 30 min incubation with the enzymatic system was control > hexanal > heptanal > octanal > nonanal = decanal = hexenal > heptenal = octenal > nonenal = decenal = 4-HNE ($P < 0.05$). These results suggested that aldehyde lipid oxidation products react with MetMb and decrease its ability to be reduced. In addition, the apparent differences in the extent of reduction by saturated versus unsaturated

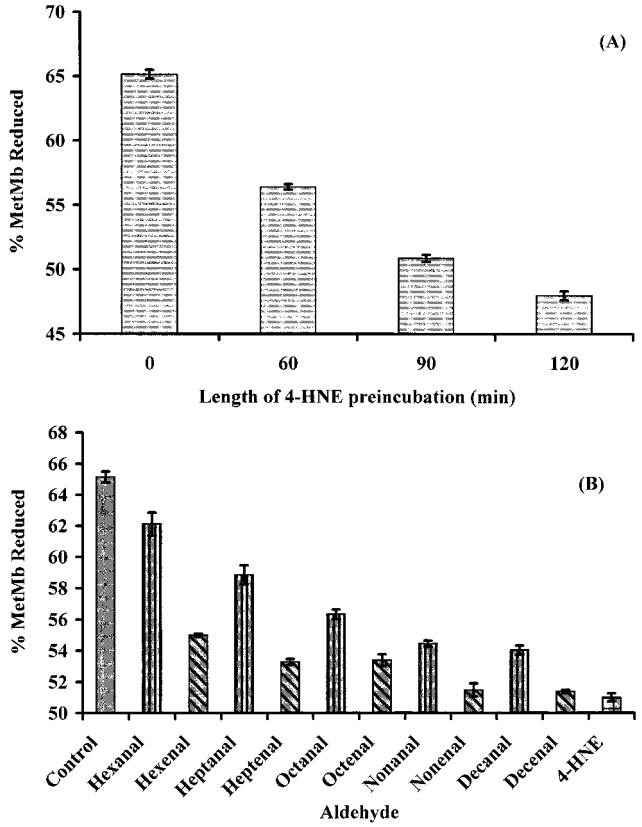

Figure 2. Effect of (A) length of 4-HNE preincubation and (B) preincubation of aldehyde lipid oxidation products with metmyoglobin (MetMb) on the extent of enzymatic MetMb reduction at 30 min, 37 °C and pH 7.2. Symbols for 2B are as follows: shaded bars, control; alternating vertical dark and light bars, saturated aldehydes; alternating slanted dark and light bars, monounsaturated aldehydes; dark grid over shaded bar, 4-HNE. Standard error bars are included.

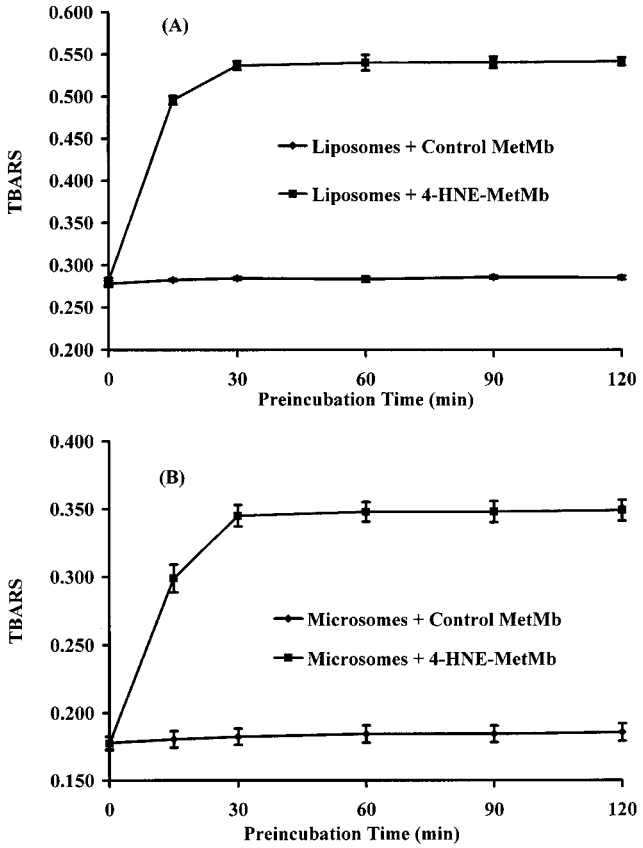

Figure 3. Effect of the length of 4-HNE preincubation on the catalytic activity of metmyoglobin MetMb as a prooxidant (TBARS) in (A) liposomes after 6 h and (B) microsomes after 2 h, incubation at 37 °C and pH 7.2. Standard error bars are included.

aldehydes for a given carbon length suggests potentially different mechanisms of reactivity with MetMb.

The effect of aldehyde preincubation with MetMb on heme protein-catalyzed lipid oxidation was investigated in vitro. MetMb preincubated with 4-HNE for as little as 15 min was enhanced in its ability to catalyze lipid oxidation when compared to controls in both liposomes (Figure 3A) and microsomes (Figure 3B; $P < 0.05$). The greater catalytic effect of 4-HNE-treated MetMb in both lipid systems appeared to reach a maximum at ≥30 min preincubation ($P < 0.05$; Figure 3). Faustman et al. (1999) showed that Mb was monoadducted with 4-HNE after 30 min when reacted under similar conditions, and this covalent modification could be related to the enhanced prooxidative activity of 4-HNE-treated MetMb.

Liver microsomes from cattle supplemented with vitamin E contained 19.9 ± 3.2 μg α-Toc/mg of protein, whereas liver microsomes from nonsupplemented cattle contained 9.0 ± 2.8 μg α-Toc/mg of protein. In microsomes containing a high concentration of α-Toc, the prooxidative effect of MetMb preincubated with 4-HNE was significantly reduced and resulted in lower TBARS compared to control MetMb ($P < 0.05$; Figure 4A). The reduced prooxidative activity of MetMb in the presence of microsomes containing higher concentrations of α-Toc is consistent with previous studies, both in vitro and in meat (Anton et al., 1991; Liu et al., 1995), which show that prooxidative effects in lipid systems can be reduced in the presence of this antioxidant.

TBARS in liposomes were higher following incubation with aldehyde-treated MetMb than with control MetMb (Figure 4B; $P < 0.05$). MetMb preincubated with unsaturated aldehydes demonstrated greater catalytic activity than when it was reacted with saturated aldehydes ($P < 0.05$). The order was control < hexanal < heptanal < octanal < nonanal = decanal < hexenal < heptenal = octenal < nonenal = decenal = 4-HNE ($P < 0.05$). These results suggest that aldehyde lipid oxidation products increase the prooxidant activity of MetMb in lipid systems and that different lipid oxidation products appear to have different inherent reactivity toward Mb. Interestingly, the trend toward unsaturated aldehydes being more effective than saturated aldehydes and the greater effect with increased carbon chain length were similar for MetMb reduction (Figure 2B) and catalytic activity of MetMb as a prooxidant (Figure 4B). Faustman et al. (1999) observed the same trends for the effect of aldehydes on OxyMb oxidation; the basis for the minor but significant effect of saturated aldehydes is not known.

The greater lipid oxidation observed with aldehyde-treated MetMb may be due to structural changes in MetMb induced by covalent attachment of the aldehyde. Although most work has focused on the covalent attachment of 4-HNE and malondialdehyde to protein (Haberland et al., 1988; Uchida et al., 1994), investigators have shown LDL modification by hexanal (Chen et al., 1992) and hemoglobin modification by pentanal, hexanal, hexenal, heptenal, octenal, and nonenal (Kautiainen, 1992). These studies demonstrate that both saturated and unsaturated aldehydes may react with

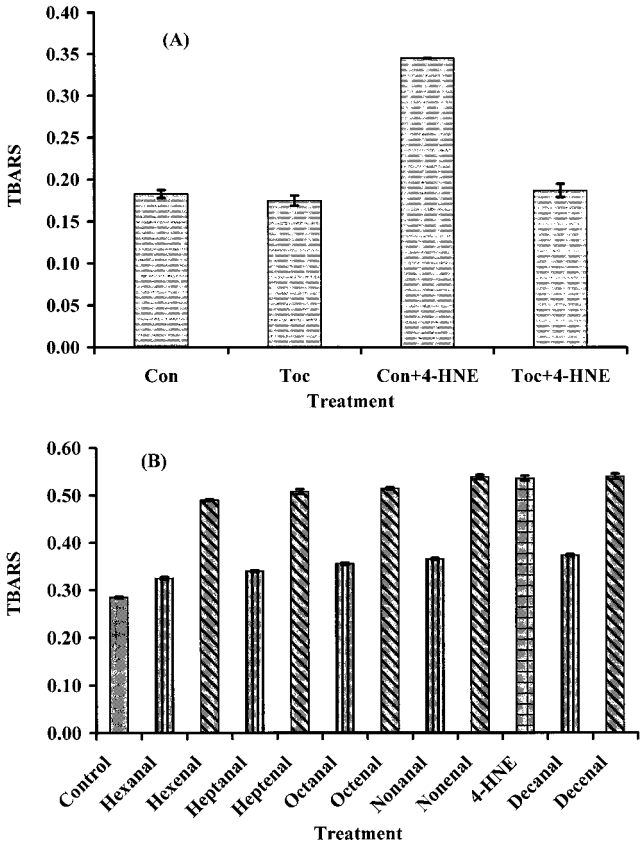

Figure 4. (A) Effect of 30 min of 4-HNE preincubation with MetMb on lipid oxidation (TBARS) in liver microsomes from vitamin E-supplemented (Toc; 19.9 ± 3.2 μg Toc) and -non-supplemented cattle (Con; 9.0 ± 2.8 μg Toc) after 2 h at 37 °C and pH 7.2. (B) Effect of preincubation of different aldehydes with MetMb on TBARS formation in liposomes after 6 h at 37 °C and pH 7.2. Symbols for 4B are the same as those used for 2B. Standard error bars are included.

protein. Our results suggest that MetMb modification by aldehydes may enable the heme group of MetMb to catalyze lipid oxidation more readily. Structural changes in heme proteins induced by heat at temperatures belowdenaturation have resulted in greater catalysis of lipid oxidation (Eriksson et al., 1971). This increased prooxidant activity was attributed to heat-induced structural changes leading to increased exposure of the heme group to the surrounding lipid. More recently, Kristensen and Andersen (1997) showed that temperatures immediately below the thermal denaturation point of MetMb induced structural changes which increased the prooxidative activity of MetMb in linoleic acid emulsions. It may be that reaction with aldehydes causes conformational changes which permit MetMb to be more effective in a similar manner.

In this study, we showed that preincubation of OxyMb with 4-HNE increased heme protein oxidation. Preincubation of MetMb with aldehyde lipid oxidation products decreased its ability to be reduced and increased its catalytic activity as a lipid prooxidant. The aldehydes used in this study are formed during lipid oxidation of both liposomes and microsomes (Esterbauer et al., 1982) and in meat (Sakai et al., 1995). 4-HNE has been shown to be produced in quantities greater than those of other mono-unsaturated aldehydes during oxidation of liposomes in the presence of Mb (Lynch and Faustman, 1999), and Esterbauer et al. (1982) showed similar results during microsomal oxidation. While Faustman et al. (1999) showed covalent attachment of 4-HNE to Mb, further work is needed to ascertain the sites of binding and effects (if any) on protein conformation.

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