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Species-Specific Myoglobin Oxidation

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ABSTRACT: The effect of the lipid oxidation product, 4-hydroxy-2-nonenal (HNE), on oxidation of oxymyoglobin (OxyMb) from seven different meat-producing species was investigated. Relative to controls, HNE increased OxyMb oxidation within all species ($p < 0.05$) at both 25 and 4 °C, pH 5.6. The relative effect of HNE was greater for myoglobins (Mbs) that contained 12 ± 1 histidine (His) residues than for those that contained 9 His residues ($p < 0.05$); HNE efficacy in all species except chicken and turkey decreased with time. Mono-HNE adducts were detected in all species except chicken and turkey. In general, HNE alkylation increased the Mbs' ability to accelerate lipid oxidation in a microsome model. However, neither an HNE nor a Mb species dependent effect was observed. Results suggested that microsome model system associated lipid oxidation overshadowed HNE and species effects on OxyMb oxidation observed in lipid-free systems.

KEYWORDS: myoglobin, lipid oxidation, 4-hydroxy-2-nonenal, mass spectrometry, meat color

INTRODUCTION

Color is an important meat quality parameter which directly influences the consumer's perception of meat quality and purchase decision. The heme protein myoglobin (Mb) is responsible for meat color. Mb consists of a single polypeptide of approximately 153 amino acids and a heme group. The exact number and sequence of amino acids are species-specific (Table 1). The color imparted to meat by Mb is determined by the molecule's redox state and the ligand located at the sixth coordination site of the heme iron. In meat, the oxidation of ferrous oxymyoglobin (OxyMb) to ferric metmyoglobin (MetMb) causes a change from cherry-red to brown coloration, and this is perceived as undesirable.

Meat from different species has been reported to discolor at different rates. Venison and beef color stability was investigated by Farouk et al.[1] They reported that venison demonstrated lower a^* (redness), b^* (yellowness), and L^* (lightness) values than beef, but beef demonstrated greater changes in a^*, b^*, and L^* values during storage. Gutzke and Trout[2] investigated the autoxidation rates of bovine, ovine, porcine, and cervine OxyMbs and showed that porcine OxyMb had a significantly lower oxidation rate than OxyMbs from the other three species.

Many factors influence Mb redox stability including lipid oxidation products; the mechanisms by which they accomplish this have been reviewed recently.[3] It is recognized that reactive aldehydes and ketones generated by lipid oxidation can negatively impact protein stability; α,β-unsaturated aldehydes are particularly reactive.[4] Redox instability of OxyMb has been attributed to modification of the protein's primary structure.[5,6] 4-Hydroxy-2-nonenal (HNE) is an α,β-unsaturated aldehyde formed during linoleic acid oxidation and has been identified in meat.[7−9]

The relationship between HNE and Mb redox stability has previously been reported.[5,10] Mb modified by HNE exhibits decreased redox stability compared to unmodified Mb.[6] HNE−Mb adducts have been formed primarily at histidine (His) residues via Michael addition and resulted in a protein mass increase of 156 Da (MW of HNE) for monoadducts. HNE-induced redox instability of Mbs from horse, cattle, pig, tuna, bison, chicken, and turkey was studied separately for each species by different investigators under different conditions, and in all cases OxyMb oxidation was promoted by HNE.[5,11−14] Suman et al.[10] compared HNE-adducted porcine and bovine Mbs at pH 5.6, 4 °C. Both mono- and diadducts were detected with bovine Mb, while only monoadducts were present in porcine Mb. LC−MS/MS revealed four His adduction sites in bovine Mb while only two were identified in porcine Mb. These results suggested a species-specific effect of HNE on Mb. However, no direct comparison of the susceptibility of Mbs from different meat-producing species under the same conditions has been completed to date.

The extent to which Mb can accelerate lipid oxidation in meat has been the subject of several studies.[15−18] Interestingly, alkylation of Mb by HNE appeared to enhance its ability to function as a prooxidant.[12,19,20] This is noteworthy because it establishes an interaction wherein the process of lipid oxidation leads to formation of products that exacerbate myoglobin oxidation which in turn could further facilitate lipid oxidation. The relative effectiveness of various species' myoglobins for enhancing lipid oxidation in the absence and presence of HNE has not been previously reported.

Therefore, our objectives were (1) to investigate species-specific Mb redox stability in the presence and absence of

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Table 1. Mb Sequences and Histidine Residue Numbers of Seven Meat-Producing Species[a]

His group	Species	Source	Sequence	His number
9	Chicken	Gallus gallus (chicken)	1 mglsdqewqq vltiwgkvea diaghghevl mrlfhdhpet ldrfdkfkgl ktpdqmkgse 61 dlkkhgatvl tqlgkilkqk gnheselkpl aqthatkhki pvkylefise viikviaekh 121 aadfgadsqa amkkalelfr ndmaskykef gfqg	9
9	Turkey	Turkey	1 mglsdqewqq vltiwgkvea diaghghevl mrlfhdhpet ldrfdkfkgl ktpdqmkgse 61 dlkkhgatvl tqlgkilkqk gnheselkpl aqthatkhki pvkylefise viikviaekh 121 aadfgadsqa amkkalelfr ndmaskykef gfqg	9
9	Porcine	Sus scrofa (pig)	1 mglsdgewql vlnvwgkvea dvaghgqevl irlfkghpet lekfdkfkhl ksedemkase 61 dlkkhgntvl talggilkkk ghheaeltpl aqshatkhki pvkylefise aiiqvlqskh 121 pgdfgadaqg amskalelfr ndmaakykel gfqg	9
12±1	Bovine	Bos Taurus (cattle)	1 mglsdgewql vlnawgkvea dvaghgqevl irlftghpet lekfdkfkhl kteaemkase 61 dlkkhgntvl talggilkkk ghheaevkhl aeshankhki pvkylefisd aiihvlhakh 121 psdfgadaqa amskalelfr ndmaaqykvl gfhg	13
12±1	Ovine	Ovis aries (sheep)	1 mglsdgewql vlnawgkvea gvaghgqevl irlftghpet lekfdkfkhl kteaemkase 61 dlkkhgntvl talggilekk ghheaevkhl aeshankhki pvkylefisd aiihvlhakh 121 psdfgadaqg amskalelfr ndmaaqykvl gfqg	12
12±1	Equine	Equus caballus (horse)	1 mglsdgewqq vlnvwgkvea diaghgqevl irlftghpet lekfdkfkhl kteaemkase 61 dlkkhgtvvl talggilkkk ghheaelkpl aqshatkhki pikylefisd aiihvlhskh 121 pgdfgadaqg amtkalelfr ndiaakykel gfqg	11
12±1	Cervine	White tail deer	1 mglsdgewql vlnawgkvea dvaghgqevl irlftghpet lekfdkfkhl kteaemkase 61 dlkkhgntvl talggilkkk ghheaevkhl aeshankhki pvkylefisd aiihvlhakh 121 psnfgadaqg amskalelfr ndmaaqykvl gfqg	12

[a] Histidine, h, residues are shaded.

HNE and (2) to investigate the species-specific relationships between Mbs and lipid oxidation in vitro.

MATERIALS AND METHODS

Materials and Chemicals. Sodium bicarbonate, sodium citrate, sodium chloride, sodium hydrosulfite, ethanol, bicinchoninic acid (BCA) protein assay kit, horse cardiac muscle myoglobin, tris-hydroxymethyl-aminomethane hydrochloride (Tris-HCl), Sephacryl 200-HR, ammonium sulfate, EDTA, sodium hydrosulfite, and Bicinchoninic Acid Protein Assay Kit were purchased from Sigma Chemical Co. (St. Louis, MO). 4-Hydroxynonenal was obtained from Cayman Chemical (Ann Arbor, MI). HiTrap DEAE FF (1 mL) and PD-10 (1.0 to 2.5 mL) columns were obtained from Amersham Biosciences (GE Healthcare; Piscataway, NJ). All chemicals were of reagent grade or greater purity.

Mb Isolation from Different Animal Species. Bovine and porcine Mbs were isolated from cardiac muscle, and ovine and cervine Mbs were prepared from skeletal muscle.[21] The method reported by Maheswarappa et al.[22] was used to purify Mb from chicken skeletal (thigh) muscle and turkey cardiac muscle. For all species, muscle samples devoid of visible fat were cut into small pieces and ground through an 8 mm plate. Bovine, porcine, ovine, and cervine muscles were homogenized with 10 mM Tris-HCl, 1 mM EDTA buffer (pH 8.0, 4 °C) at a ratio of 1:3 and centrifuged (Thermo Fisher Scientific, Waltham, MA) at 5000*g* for 10 min at 4 °C. Chicken and turkey muscle was homogenized with 20 mM ammonium bicarbonate (pH 9.0, 4 °C) at a ratio of 1:1. The supernatants were brought to 70% ammonium sulfate saturation (for chicken and turkey Mbs, homogenates were brought to 50% ammonium sulfate saturation). Homogenates were stirred for 1 h and then centrifuged at 18000*g* for 20 min, 4 °C. The pellet was discarded, and the resulting supernatant was brought to 100% ammonium sulfate saturation, followed by 1 h stirring and centrifugation at 20000*g* for 1 h at 4 °C. The supernatant was discarded, and the pellet was resuspended in 10 mM Tris-HCl, 1 mM EDTA buffer (pH 8.0, 4 °C) for bovine, porcine, ovine, and cervine preparations, or 20 mM ammonium bicarbonate (pH 9.0, 4 °C) for chicken and turkey Mbs. The protein solutions were then dialyzed against 10 vol of 5 mM Tris-HCl, 1 mM EDTA buffer (pH 8.0, 4 °C), or 20 mM ammonium bicarbonate (pH 9.0, 4 °C) for 24 h with 3 buffer changes. Sephacryl 200-HR gel filtration (2.5 × 100 cm) was used to separate Mb from hemoglobin with the dialysis buffer at a flow rate of 60 mL/h. HiTrap DEAE-FF columns were used to further purify chicken and turkey Mbs. The first elution buffer was 10 mM Tris buffer (pH 8.4, 4 °C), and Mb was eluted out in this fraction. The second elution buffer (50 mM Tris and 0.2 M sodium chloride buffer, pH 8.6, 4 °C) was used to wash out nontarget proteins.

Reaction of OxyMb with HNE. Mbs isolated from the different species' muscles was reduced to deoxymyoglobin (DeoxyMb) by sodium dithionite and then oxygenated to form OxyMb.[23] PD-10 columns precalibrated with 50 mM sodium citrate (pH 5.6, 4 °C) were used to remove excess sodium dithionite. OxyMb concentrations were adjusted to 0.075 mM for seven species and incubated with 0.525 mM HNE or with a volume of ethanol (control) equivalent to that used to deliver HNE (82 μL of ethanol in 10 mL of reaction system) at 25 °C for 6 h, and 4 °C for 96 h. The temperatures for all experiments were within ±0.1 range. At specific time points of incubation (0, 30, 60, 120, 180, 240, 300, and 360 min for 25 °C and 0, 24, 48, 72, and 96 h for 4 °C), samples were scanned from 650 to 450 nm using a UV−vis spectrophotometer (Shimadzu UV-2101PC spectrophotometer, Kyoto, Japan). The percentage of MetMb was calculated according to wavelength maxima at 503, 557, and 582 nm.[24] Following Mb redox status measurement at the last time point, HNE-treated samples were passed through a PD-10 desalting column precalibrated with deionized water to remove unreacted HNE and stored at −80 °C for subsequent ESI-Q-TOF MS analysis.

ESI-Q-TOF MS. One-hundred microliters of HNE-treated Mb samples (0.035 mM) from all species were prepared in 100 μL of methanol:distilled water (1:1) with addition of 0.1% acetic acid to enhance protonation. Samples (100−200 μL) were applied to an electrospray ionization-Q-TOF mass spectrometer (model: QSTAR Elite, Applied Biosystems/MDS SCIEX, Ontario, Canada). The ESI-MS raw data was

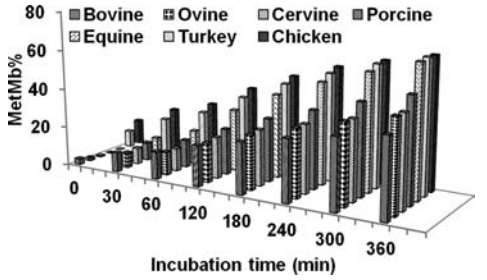

Figure 1. OxyMb autoxidation of Mbs isolated from seven Mb species at 25 °C and pH 5.6 (SE M = 2.00).

deconvoluted into a true mass scale in the mass range between 10,000 and 60,000 Da (Da) to determine the molecular mass of the Mb by the instrumentation software.

Microsome Preparation. Ovine livers ($n = 3$) were obtained from MacGlaflin's study.[25] Microsomes were isolated according to Guengerich's protocol.[26] The microsomal protein concentration was determined using a BCA protein assay.

Incubation of OxyMb-HNE with Microsomes. MetMb proteins from bovine, porcine, ovine, cervine, equine, and turkey were preincubated with HNE (1:7) at 37 °C for 2 h. Preincubated MetMb was reduced to DeoxyMb by sodium dithionite and passed through PD-10 columns precalibrated with 50 mM sodium citrate (pH 5.6, 4 °C) to remove excess of sodium dithionite and unreacted HNE. The treated OxyMbs (0.075 mM) were incubated with microsomes (1 mg of protein/mL) at 25 °C for 6 h. MetMb was determined as previously described with a Shimadzu UV-2101PC spectrophotometer with integrating sphere assembly. Following color measurement, lipid oxidation in microsome−myoglobin mixture was determined by measuring thiobarbituric acid reactive substances (TBARS) according to Yin et al.[27]

Statistical Analysis. Each experiment was replicated thrice ($n = 3$). A completely randomized design was used to determine the effects of HNE on species-specific Mb redox stability at pH 5.6, 4, and 25 °C. The data for myoglobin redox stability at 4 and 25 °C were analyzed separately. A randomized block design was used to assess the species-specific relationships between Mbs and lipid oxidation in vitro, where liver used for microsome isolation from each lamb served as a block. For both experiments, repeated TBARS values and % MetMb were determined at specific time points during incubation. Fixed effects include species, HNE, incubation time, and their interactions. The MIXED procedure with repeated option of SAS (Version 9.1, SAS Institute Inc. Cary, NC, USA) was used for the type-3 test of fixed effects and interactions. Least-squares means were generated for significant *F*-tests ($P < 0.05$), and the diff option was used to separate least-squares means.

RESULTS AND DISCUSSION

Reaction of OxyMb with HNE. MetMb accumulation in the Mbs from all seven species in the absence of HNE at 25 °C, pH 5.6, is presented in Figure 1. The relative redox stability of OxyMbs followed the order chicken = turkey > equine > bovine = ovine = porcine = cervine ($p < 0.05$). Since the MetMb% at time 0 min was not the same among species, the difference in MetMb accumulation during incubation was calculated (MMb $\%_{endofincubation}$ − MMb$\%_{time0}$) and followed the order equine > turkey = chicken = porcine = venison = ovine > bovine ($p < 0.05$; data not shown). Within all species, the percentages of MetMb increased with time at both 25 and 4 °C, pH 5.6.

HNE enhanced MetMb formation in all species ($p < 0.05$) relative to their respective controls at both 25 and 4 °C, pH 5.6 (4 °C results not shown). The effect of HNE on cervine Mb has

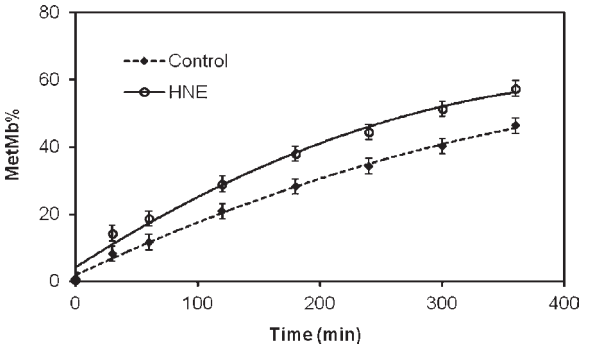

Figure 2. Effect of HNE on cervine OxyMb at 25 °C and pH 5.6.

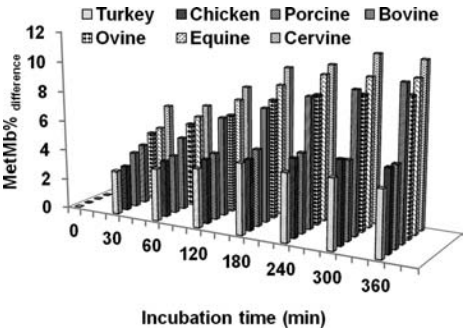

Figure 3. MetMb% difference ($\%Met_{difference} = \%MetMb_{HNE} - \%MetMb_{Control}$) between HNE-induced oxidation and autoxidation of Mbs isolated from seven species at 25 °C and pH 5.6 (SE M = 1.06).

not been previously reported and is presented in Figure 2. This permits visualization of the difference between control and HNE-treated Mb. Previous studies concluded that HNE altered OxyMb's redox stability by covalently binding to His residues of Mb.[5,12] Suman et al.[10] reported that effect of HNE on OxyMb redox stability was species-specific.

The primary sequences of Mbs in different meat-producing species and the number of His residues within them are presented in Table 1. For the species evaluated in this study, the number of His residues ranged from 9 (chicken, turkey and porcine) to 13 (bovine). In the current study, based on His number, species were divided into either 12 ± 1 His residue group (i.e., bovine, equine, ovine and cervine Mbs) or those with 9 His residues (i.e., porcine, chicken and turkey Mbs). As expected, the effect of HNE on OxyMb led to differences in MetMb percentage between control and HNE-treated groups ($\%Met_{difference}$) that increased with time. Differences in MetMb formation between HNE-treated samples and controls were calculated as $\%Met_{difference} = \%MetMb_{HNE} - \%MetMb_{Control}$ for the different time points of incubation (Figure 3). Interestingly, the relative degree of $\%Met_{difference}$ appeared to differ between Mbs of the 12 ± 1 His group and those of the 9 His group at 25 °C such that $\%Met_{difference}$ of the 12 ± 1 His residue group was greater ($p < 0.05$). It is important to note that, within either of these groups, there were no differences between species for HNE or control treatments ($p > 0.05$).

In the current study, the effect of HNE was most significant at the initiation of incubation. In an attempt to better characterize this, we calculated HNE efficacy, the $\%Met_{difference}$ expressed relative to the control MetMb formation (HNE efficacy = $[\%MetMb_{HNE} - \%MetMb_{Control}]/\%MetMb_{Control}$) (Figure 4).

For bovine, equine, ovine, cervine, and porcine Mbs, HNE efficacy decreased with time ($p < 0.05$) while HNE efficacy toward chicken and turkey Mbs remained constant ($p > 0.05$).

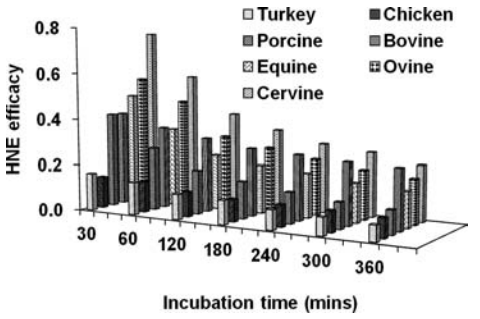

Figure 4. HNE efficacy (HNE efficacy = $[\%MetMb_{HNE} - \%MetMb_{Control}]/\%MetMb_{Control}$) for redox stability of Mbs isolated from seven species at 25 °C and pH 5.6 (SE M = 0.04).

This suggested that HNE alkylated Mbs of all species relatively quickly, except chicken and turkey, at the beginning of incubation to produce the observed effects. With increased time, HNE efficacy decreased because fewer new HNE−Mb adducts formed. In addition, the HNE efficacy of Mbs in the 12 ± 1 His residue group was greater at all time points compared to chicken and turkey Mbs in the 9 His residue group. These results were supported by mass spectral analysis which revealed mono-HNE adducts with Mb in all species, except chicken and turkey, following incubation at 25 °C (6 h) or 4 °C (96 h; results not shown). Mass-transformed spectra for cervine (12 ± 1 His) and turkey (9 His) Mbs at 4 °C and 96 h are shown in Figure 5. Mb with mass of 17078 is clearly evident in spectra of cervine Mb incubated with HNE and corresponds to a monoadduct of HNE, which is consistent with previous studies (Figure 5a).[5,11−14] Under the conditions of the present investigation, no HNE adducts were observed in chicken and turkey Mbs (Figure 5b). The relative lack of an HNE effect on MetMb formation in turkey

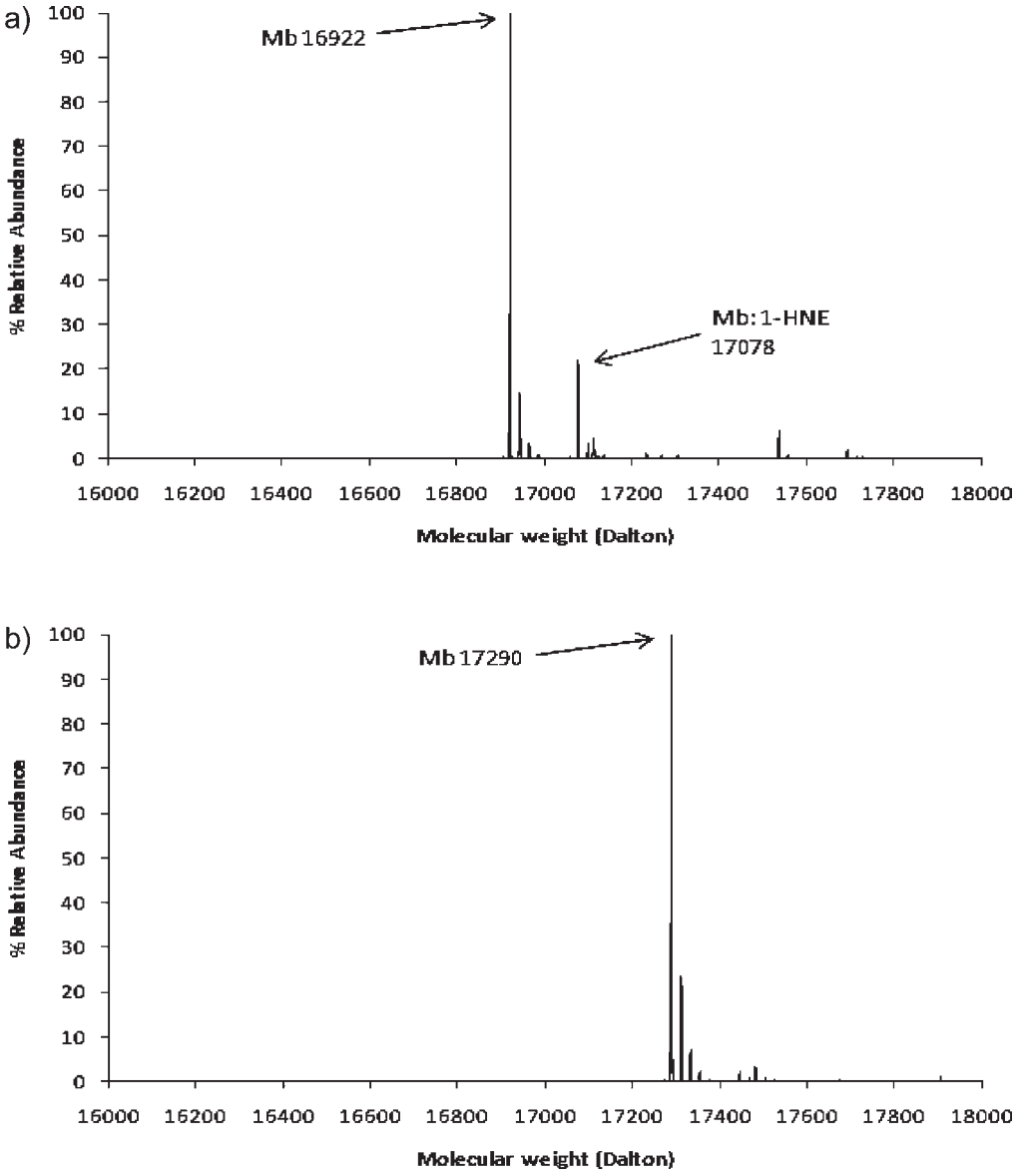

Figure 5. (a) Deconvoluted mass spectra of cervine Mb incubated with HNE for 96 h at 4 °C and pH 5.6. (b) Deconvolued mass spectra of turkey Mb incubated with HNE for 96 h at 4 °C and pH 5.6.

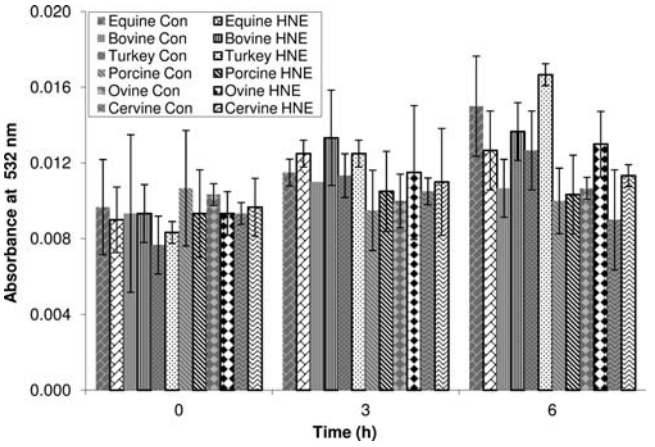

Figure 6. Effect of HNE preincubation with Mb on lipid oxidation in an ovine liver microsome model system at 25 °C and pH 5.6.

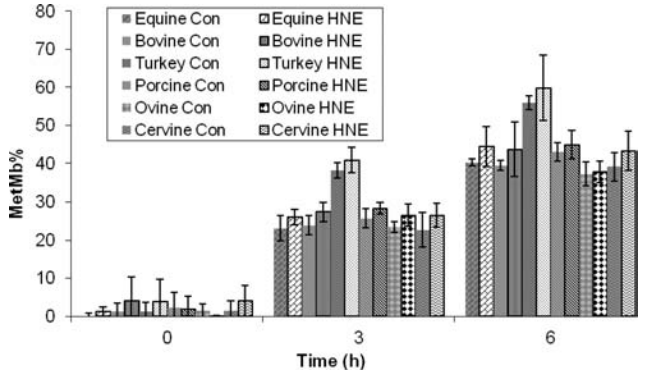

Figure 7. Effect of preincubation of Mb with HNE on OxyMb oxidation in an ovine liver microsome system at 25 °C and pH 5.6.

and chicken Mbs is attributed to this observation. However, this did not agree with previous chicken and turkey Mb studies where monoadducts of HNE were detected at pH 5.8 and 25 °C and both mono- and diadducts of HNE were detected at pH 7.4 and 25 °C.[11,22] One possible explanation is that the relatively lower pH 5.6 used in the present study made the His residues (p I = 5.8) partially positively charged relative to pH 5.8 and 7.4 and reduced their ability to serve as nucleophiles.

Incubation of OxyMb-HNE with Microsomes. While the adduction of HNE to OxyMb accelerates its oxidation, alkylation also made modified MetMb a more reactive prooxidant toward lipids.[20] Yin and Faustman[28] studied the relationship between Mb and lipid oxidation in a microsome model but did not compare the species-specific effect of different Mbs on lipid oxidation. The effect of Mb from bovine, porcine, ovine, cervine, equine, and turkey, with or without HNE adduction, on lipid oxidation in microsomes at 25 °C and pH 5.6 is presented in Figure 6. TBARS values increased with incubation time (p < 0.05). However, no effect of HNE relative to controls was observed (p > 0.05), nor did any single species' Mb appear to be more prooxidative than another toward lipid. Nevertheless, prooxidant effect of equine Mb preincubated with HNE at pH 7.4 was observed in microsomal model by Lynch and Faustman.[20]

Mb oxidation within microsomes was concurrently monitored with the assessment of lipid oxidation. MetMb accumulation with or without HNE in microsomes is presented for bovine, porcine, ovine, cervine, equine, and turkey Mbs (Figure 7). MetMb percentage increased with time for these species (p < 0.05). However, MetMb accumulation in microsomes was not affected by inclusion of HNE unlike the observations for Mbs in the absence of the lipid system (p > 0.05). There are a couple of possible explanations for this unanticipated observation. First, the turbulent nature of microsomal models necessitates the use of a diffuse integrating sphere for analysis of changes in Mb redox stability, and some sensitivity is lost with this approach. In addition, it is possible that lipid oxidation products generated by the microsomes themselves could have overshadowed the effect of HNE. Turkey Mb demonstrated greater MetMb accumulation relative to the other species, and this was consistent with the autoxidation results obtained for the isolated proteins.

In conclusion, differences in His residue number among different species' Mbs resulted in a difference of Mb redox instability induced by HNE. The adduction of HNE to Mb was positively correlated with HNE efficacy for OxyMb oxidation. The greater HNE efficacy observed early in incubation suggested that OxyMb reacted with HNE immediately after mixing and that the addition of HNE to OxyMb decreased its redox stability.

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ABBREVIATIONS USED

Mb, myoglobin; OxyMb, oxymyoglobin; MetMb, metmyoglobin; DeoxyMb, deoxymyoglobin; MS, mass spectrometry; ESI-MS, electrospray ionization mass spectrometry; ESI-Q-TOF, electrospray ionization-triple quadrupole; LC−MS/MS, liquid chromatography−tandem mass spectrometry; BCA, bicinchoninic acid; DEAE, diethylaminoethyl; His, histidine; HNE, 4-hydroxy-2-nonenal; L^*, lightness; a^*, redness; b^*, yellowness

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