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Short communication

Species-specific effects on non-enzymatic metmyoglobin reduction in vitro

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ABSTRACT

Our objectives were to determine the non-enzymatic metmyoglobin reduction properties of bovine, porcine, and equine myoglobins and to characterize the effects of pre-incubation of 4-hydroxy-2-nonenal (HNE) with myoglobins on non-enzymatic metmyoglobin reduction in vitro. Purified bovine, porcine, and equine metmyoglobins (0.05 mM) were reduced at pH 5.6 and 7.4 in the presence or absence of HNE. Rates of metmyoglobin reduction were monitored by spectrophotometry, and myoglobin adducts were characterized by high-resolution mass-spectrometry. Results showed that the species origins of individual myoglobins determined rates of non-enzymatic reduction (beef > equine > pork; $P < 0.05$). Irrespective of species, pre-incubation of HNE myoglobin decreased non-enzymatic metmyoglobin reduction compared with control at both pH 5.6 and 7.4 ($P < 0.05$). Mass spectrometric analysis revealed adducts of HNE with bovine, porcine, and equine myoglobins. The results indicate that the amino acid composition and the covalent binding of HNE with myoglobin can significantly decrease the ability of heme to accept electrons.

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1. Introduction

Myoglobin is the sarcoplasmic protein that is primarily responsible for meat color. Myoglobin can exist in three different forms, namely deoxy-, oxy-, or metmyoglobin, with the oxymyoglobin form yielding the desirable bright cherry-red color to beef. Yin et al. (2011) compared the oxidation of oxymyoglobin from seven different species, and concluded that myoglobin sequences cause differences in myoglobin oxidation rates.

Metmyoglobin reducing activity (MRA) is the ability of the postmortem muscle to regenerate ferrous oxy- or deoxymyoglobin by enzymatic and/or non-enzymatic reactions. This reducing activity of meat is critical to limiting myoglobin oxidation (Ledward, 1985; Seyfert et al., 2006), and meat contains endogenous metmyoglobin reducing systems (Arihara, Cassens, Greaser, Luchansky, & Mozdziak, 1995; Bekhit, Illian, Morton, Sedcole, & Bickerstaffe, 2003; Faustman, Cassens, & Greaser, 1988; Hagler, Coppes, & Herman, 1979; Mohan, Hunt, Muthukrishnan, Barstow, & Houser, 2010; Watts, Kendrick, Zipser, Hutchins, & Saleh, 1966). Although there are several metmyoglobin reducing systems present in meat, the NADH-dependent metmyoglobin reducing enzyme system has received much attention. This system involves an electron donor (NADH), electron carrier (NADH-dependent cytochrome b5 reductase), and cytochrome b5 (electron acceptor). However, enzyme-independent electron carriers can also contribute to metmyoglobin reduction. Brown and Snyder (1969) were the first to report non-enzymatic reduction of metmyoglobin by NADH in the presence of EDTA and methylene blue in vitro. These authors also noted that the non-enzymatic metmyoglobin reduction is as efficient as the enzymatic reduction.

Numerous oxidative processes in meat can generate primary and secondary reactive lipid oxidation products. One of these secondary lipid oxidation products, 4-hydroxy-2-nonenal (HNE) results in myoglobin being a poor substrate for enzymatic metmyoglobin reduction (Lynch & Faustman, 2000). HNE binds to metmyoglobin, and this binding is influenced by species-specific variations in the number of histidine residues present (Suman, Faustman, Stamer, & Liebler, 2007; Yin et al., 2011), likely reflecting the absence of cysteine residues in livestock myoglobin sequences (Alderton, Faustman, Liebler, & Hill, 2003).

Although studies have characterized species-specific effects on oxymyoglobin oxidation, limited information is available determining the species-specific effects on non-enzymatic metmyoglobin reduction at postmortem muscle pH. Similarly, although it is appreciated that HNE can increase oxymyoglobin oxidation and limit enzymatic metmyoglobin reduction, the effects of HNE on non-enzymatic MRA are

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largely unknown. Therefore, we compared the in-vitro non-enzymatic reduction of bovine, porcine, and equine metmyoglobin at pH 5.6 and 7.4, and also determined the effects of HNE on these processes in vitro.

2. Materials and method

2.1. Materials and chemicals

Equine skeletal myoglobin, methylene blue, Tris hydroxymethyl aminomethane hydrochloride (Tris–HCl), potassium phosphate monobasic (KH_2PO_4), potassium phosphate dibasic (K_2HPO_4), NADH, Sephacryl 200-HR, ammonium bicarbonate, and EDTA tetra sodium hydrate were purchased from Sigma Chemical Co. (St. Louis, MO). Ammonium sulfate was purchased from Fisher Scientific (Fair Lawn, NJ). HNE was obtained from Cayman Chemical Co. (Ann Arbor, MI). PD-10 columns were obtained from GE Healthcare (Piscataway, NJ). All chemicals were of reagent grade or greater purity.

2.2. Myoglobin isolation

The ammonium sulfate precipitation and gel-filtration techniques were used to isolate myoglobin from porcine and bovine hearts (Faustman & Phillips, 2001). Briefly, beef and porcine cardiac muscles devoid of fat and connective tissue were homogenized in buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0, 4 °C) and centrifuged at 5000 ×*g* for 10 min. The supernatant was brought to 70% ammonium sulfate saturation and the resulting solution was stirred for 1 h at 4 °C and then centrifuged at 18,000 ×*g* for 20 min. The supernatant was saturated with ammonium sulfate (100%) and centrifuged at 20,000 ×*g* for 1 h. The precipitate was re-suspended in homogenization buffer and dialyzed (3 volumes) against 10 mM Tris–HCl, 1 mM EDTA, at pH 8.0, 4 °C for 24 h. Myoglobin was separated from hemoglobin using a Sephacryl 200-HR gel filtration column (2.5 × 100 cm) using 5 mM Tris–HCl, 1 mM EDTA at pH 8.0 as the mobile phase at 1 mL/min.

Equine skeletal myoglobin was commercially purchased. Myoglobins from all species were converted to metmyoglobin and the pH of the myoglobin solutions were adjusted to either 5.6 or 7.4 by passing through PD-10 chromatographic columns equilibrated with 50 mM phosphate buffer at either pH 5.6 or 7.4 (Ramanathan, Mancini, Suman, & Beach, 2014). The myoglobin concentrations of all species were adjusted to 0.05 mM and were confirmed using absorbance at 525 nm (assuming an extinction coefficient of 7.6 mM^{-1} cm^{-1}; Broumand, Ball, & Stier, 1958).

2.3. Non-enzymatic metmyoglobin reduction

The methodology used by Brown and Snyder (1969) was modified to determine the species-specific effects on non-enzymatic metmyoglobin reduction. The assay mixture contained metmyoglobin from either beef, pork, or equine at pH 5.6 or 7.4 (0.05 mM), EDTA (0.5 mM), methylene blue (0.1 mM), and NADH (0.014 mM). The reaction was initiated by the addition of NADH. The absorbance at 582 nm was used to measure metmyoglobin reduction with the time course option in a Shimadzu UV–Vis 2600 spectrophotometer (Shimadzu Inc., Columbia, MD) combined with a temperature-regulated (25 °C) 2-cell holder (TCC-240A, thermoelectrically controlled, Shimadzu, Kyoto, Japan) for 100 s. Non-enzymatic metmyoglobin reducing activity was calculated as nanomoles of metmyoglobin reduced per minute during the initial linear phase of the reaction (slope of the progress curve), using a difference in molar absorptivity of 12,000 mol^{-1} cm^{-1} at 582 nm for 100 s.

2.4. Reaction of HNE with myoglobin

Previous research has shown that pre-incubation of HNE with myoglobin and lactic dehydrogenase (LDH) can decrease enzymatic metmyoglobin reduction and LDH activity, respectively (Lynch & Faustman, 2000; Ramanathan et al., 2014). Myoglobins from different species at either pH 5.6 or 7.4 were pre-incubated with HNE (1:7, ratio of myoglobin:HNE) for 2 h at 37 °C to promote binding (Yin et al., 2011). Control samples received a volume of ethanol vehicle similar to that used to deliver HNE. Following incubation, both control and HNE incubated myoglobin samples were passed through PD-10 columns to remove unbound HNE. The resultant myoglobin solutions were used to determine non-enzymatic metmyoglobin reduction as described in the previous section, and by electrospray mass spectrometry (Section 2.5). The binding of HNE to myoglobin samples were also confirmed using mass spectrometry.

Table 1
Effects of species and pH on non-enzymatic metmyoglobin reduction at 25 °C.

Species	pH 5.6	pH 7.4	Standard error[1]	P-value for pH comparison[2]
Equine	$3850^{a,x}$	$1369^{a,y}$	245	0.0001
Bovine	$15{,}000^{b,x}$	$2956^{b,y}$	1450	0.0001
Porcine	$450^{c,x}$	$108^{c,y}$	26	0.0001

Least square means in each column with different letters (a–c) are significantly different ($P < 0.05$).
Least square means in each row with different letters (x, y) are significantly different ($P < 0.05$).
Non-enzymatic metmyoglobin reduction was expressed as nanomoles of metmyoglobin reduced per minute.
[1] Standard error for pH comparison.
[2] P-value for pH comparison within a species.

2.5. Mass spectrometry

Samples were analyzed by infusion into a hybrid LTQ-OrbitrapXL mass spectrometer (Thermo Fisher Scientific, San Jose, CA) coupled to a New Objective PV-550 nanoelectrospray ion source. For infusion, samples were mixed with one-third volume of acetonitrile/0.01% formic acid and loaded into New Objective metal-coated capillaries, and mass spectra were recorded for 60 s using the Orbitrap sector at a nominal resolution of 100,000.

2.6. Statistical analysis

The experimental design was a completely randomized design and each experiment was replicated six times ($n = 6$). For the first objective, fixed effects include pH, species, and their interactions. Various studies have reported the pH effect on HNE binding to myoglobin. Hence, pH was not included in the model statement for the second objective (the effects of HNE on non-enzymatic MRA). For both objectives, Type-3 tests of fixed effects were performed using the MIXED Procedure of SAS (Version 9.1, SAS Institute Inc., Cary, NC). Least square means for protected F-tests ($P < 0.05$) were separated by using the pdiff option

Table 2
Effects of pre-incubation of bovine, porcine, and equine myoglobins (0.05 mM) with HNE (0.35 mM) on non-enzymatic metmyoglobin reduction at 25 °C.

pH	Species	Mb + ethanol	Mb + HNE	Standard error	P-value[1]
a) 5.6	Equine	4337^{a}	3429^{a}	204	0.0001
	Bovine	$14{,}850^{b}$	9355^{b}	1890	0.0001
	Porcine	422^{c}	281^{c}	22	0.0010
b) 7.4	Equine	1420^{a}	1146^{a}	98	0.0400
	Bovine	3200^{b}	2468^{b}	200	0.0010
	Porcine	110^{c}	67^{c}	15	0.0020

Least square means in each column within a pH with different letters (a–c) are significantly different ($P < 0.05$).
Non-enzymatic metmyoglobin reduction was expressed as nanomoles of metmyoglobin reduced per minute.
Mb = myoglobin; HNE = 4-hydroxy-2-nonenal; ethanol = a volume of ethanol vehicle similar to that used to deliver HNE.
[1] P-value for control and HNE treatment within a species.

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