Meat Science 95 (2013) 224–228

Contents lists available at SciVerse ScienceDirect

Meat Science

journal homepage: www.elsevier.com/locate/meatsci

The effects of HNE on ovine oxymyoglobin redox stability in a microsome model

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ARTICLE INFO

Article history:
Received 19 December 2012
Received in revised form 15 April 2013
Accepted 18 April 2013

Keywords:
Myoglobin
Lipid oxidation
4-Hydroxy-2-nonenal
Ovine
Meat color
Microsome

ABSTRACT

The effect of 4-hydroxy-2-nonenal (HNE), a secondary lipid oxidation product, on ovine myoglobin (Mb) redox stability was investigated. HNE increased oxymyoglobin (OxyMb) oxidation under all pH/temperature conditions studied. Mono-, di- and tri-HNE adducts were detected by ESI-Q-TOF MS analysis. Sites of adduction, His 120, His 25 and His 65, were determined by ESI–CID–MS/MS analysis. The relationship between ovine Mb (with/without HNE) and lipid oxidation was also studied in a microsome model in the presence of α-tocopherol. Surprisingly, preincubation of Mb with HNE did not affect subsequent Mb redox stability in the microsome model ($P < 0.05$). Microsomes with elevated concentrations of α-tocopherol delayed lipid and Mb oxidations relative to controls. HNE-treated ovine Mb caused greater lipid oxidation compared to control ovine Mb in control microsomes ($P < 0.05$). This study demonstrated an interaction between ovine Mb oxidation and lipid oxidation.

1. Introduction

4-Hydroxy-2-nonenal (HNE) is a reactive chemical species which belongs to the α, β-unsaturated aldehyde group of secondary lipid oxidation products (Witz, 1989). HNE has been identified in meat (Gasc et al., 2007; Sakai, Yamauchi, Kuwazuru, & Gotoh, 1998; Surh & Kwon, 2005) and used as a model secondary oxidation product for the study of the relationship between lipid oxidation and myoglobin (Mb) redox stability. HNE decreases bovine Mb redox stability in vitro by forming covalent adducts with histidine (His) residues via Michael addition (Alderton, Faustman, Liebler, & Hill, 2003). The redox stability of oxymyoglobins (OxyMbs) from different meat-producing animals was investigated using an HNE model; decreased Mb redox stability was observed in all species investigated (Alderton et al., 2003; Joseph et al., 2010; Lee, Faustman, Liebler, & Phillips, 2003; Lee, Joo, Alderton, Hill, & Faustman, 2003; Maheswarappa et al., 2010). The effect of HNE on ovine OxyMb redox stability was not previously reported.

HNE and its potential interaction with Mb in meat would occur in the presence of oxidizing membrane lipids. Microsomes have been routinely used for studies of lipid oxidation in food and are appropriate models for protein: lipid interactions because they provide biologically relevant cellular and sub-cellular membranes (Albert, Lewis, & Roberts, 1989; Vladimirov, Olenev, Suslova, & Cheremisina, 1980). Microsome model systems provide a means to study lipid:heme protein interactions at a level that is less complex than meat (Faustman, Sun, Mancini, & Suman, 2010).

Microsomes have also been used to study the effect of antioxidants, specifically α-tocopherol, on Mb and lipid oxidation; microsomes with differing concentrations of α-tocopherol can be obtained from carcass tissues of livestock supplemented with vitamin E (Arnold, Williams, Schaefer, Arp, & Scheller, 1993). α-Tocopherol is a fat-soluble vitamin that delays polyunsaturated fatty acid (PUFA) oxidation by neutralizing lipid peroxy-radicals (Buttriss & Diplock, 1984). Yin and Faustman (1994) studied the relationship between Mb and lipid oxidation in a microsome model and found that microsomes with greater PUFA levels and lower α-tocopherol concentrations resulted in greater OxyMb oxidation. The relationship between porcine Mb and microsomal lipid stability, as influenced by α-tocopherol, was also investigated (Lee, Faustman, et al., 2003). While lipid oxidation was decreased by increased concentrations of α-tocopherol, a Mb redox-stabilizing effect was not observed.

The effect of vitamin E on lamb meat quality has also been investigated. Dietary supplementation of this antioxidant increased its tissue concentration in lamb muscle and also inhibited lipid oxidation in lamb meat; however, improved color stability was not consistently observed among related studies (Fuente et al., 2007; Guidera, Lynch, Morrissey, Kerry, & Buckley, 1997; Lauzurica et al., 2005; Strohecker, Faustman, Furr, Hoagland, & Williams, 1997).

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http://dx.doi.org/10.1016/j.meatsci.2013.04.055

The objectives of the current study were to (1) investigate the effect of HNE on redox stability of ovine OxyMb; and (2) investigate the relationship between Mb and lipid oxidation in an ovine microsome model.

2. Materials and methods

2.1. Materials and chemicals

Sodium bicarbonate, sodium citrate, sodium chloride, sodium hydrosulfite, ethanol, tris hydroxymethyl aminomethane hydrochloride (Tris–HCl), sephacryl 200-HR, ammonium sulfate, EDTA, 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). PD-10 columns were obtained from Amersham Biosciences (GE Healthcare; Piscataway, NJ). All chemicals were of reagent grade or greater purity.

2.2. Mb isolation and purification

The major muscles from the ovine leg (i.e., semimembranosus, semitendinosus, biceps femoris, quadriceps) were obtained from a local slaughterhouse, and Mb was isolated according to Faustman and Phillips (2001). Briefly, muscle samples were trimmed of visible fat and connective tissue, cut into small pieces and ground through an 8 mm plate. Three vol of homogenization buffer (10 mM Tris–HCl, 1 mM EDTA buffer, pH 8.0, 4 °C) were combined with 1 vol of ground ovine muscle and homogenized. The homogenates were centrifuged at 5000*g* for 10 min at 4 °C. Supernatants were passed through a double-layer of cheesecloth and then brought to 70% ammonium sulfate saturation. After stirring for 1 h at 4 °C, the mixture was centrifuged at 18,000*g* for 20 min, 4 °C. The resulting supernatants were brought to 100% ammonium sulfate saturation, followed by 1 h stirring and centrifugation at 20,000*g* for 1 h at 4 °C. The pellets were collected, re-suspended in 10 mM Tris–HCl, 1 mM EDTA buffer (pH 8.0, 4 °C), and dialyzed against 10 vol 5 mM Tris–HCl, 1 mM EDTA buffer (pH 8.0, 4 °C) for 24 h with 3 buffer changes to remove ammonium sulfate. 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. The purity of isolated ovine Mb was evaluated by SDS-PAGE with confirmation of a single protein band at 17,000 Da.

2.3. Reaction with HNE

Sodium dithionite was used to reduce the isolated Mb to deoxymyoglobin (DeoxyMb) which was subsequently converted to OxyMb by oxygenation (Brown, Harris, & Olcott, 1963). Excess sodium dithionite was removed by PD-10 columns (GE Healthcare, Piscataway, NJ) pre-calibrated with 50 mM sodium citrate (pH 5.6, 4 °C). OxyMb (0.15 mM) was incubated with HNE (1.05 mM) or with a volume of ethanol (control) equivalent to that used to deliver HNE at four different temperature/pH/time conditions (1) 25 °C and pH 5.6 for 6 h, (2) 4 °C and pH 5.6 for 96 h, (3) 25 °C and pH 7.4 for 6 h, and (4) 37 °C and pH 7.4 for 4 h. Samples were scanned spectrophotometrically from 650 to 450 nm using a UV–vis spectrophotometer (Shimadzu UV-2101PC spectrophotometer, Kyoto, Japan) at specific time points of incubation. Metmyoglobin (MetMb) percentages were calculated based on absorbance values at 503, 557, and 582 nm (Tang, Faustman, & Hoagland, 2004). Samples treated with HNE were passed through a PD-10 desalting column pre-calibrated with deionized water to remove unreacted HNE at the end of incubation and stored at −80 °C for further analysis.

2.4. ESI-Q-TOF MS

Ovine Mb samples with HNE treatment (0.07 mM, 50 μL) were mixed with methanol:distilled water (1:1, 100 μL) and 0.1% acetic acid to enhance protonation. An electrospray ionization-Q-TOF mass spectrometer (ESI-Q-TOF MS, Model: QSTAR Elite, Applied Biosystems/MDS SCIEX, Ontario, Canada) was used for sample analysis. The instrumentation software was used to transform the ESI-MS raw data into a true mass scale (10,000 to 60,000 Da) to determine the molecular mass of Mb and Mb:HNE adducts.

2.5. LC–MS/MS analysis

LC–MS/MS analysis was conducted as previously described (Maheswarappa et al., 2010). Briefly, HNE-treated ovine Mbs collected at the last time point under all temperature/pH conditions were analyzed by SDS-PAGE under reducing conditions using a mini-gel electrophoresis unit (model: Mini Protean II, Bio-Rad Laboratories, Inc., Richmond, CA). The Coomassie-stained protein bands of HNE-treated ovine Mbs were excised, destained and digested with sequencing-grade trypsin at 37 °C for 18 h. The digested peptides were extracted according to the protocol of Shevchenko, Wilm, Vorm, and Mann (1996) and sequenced using a high throughput LCQ ESI ion trap mass spectrometer (Thermo-Finnigan, Palo Alto, CA) equipped with a commercial nano-electrospray device. The acquired MS/MS spectra were searched against UniProt ovine protein database (2008) using the SEQUEST algorithm (Keller, Nesvizhskii, Kolker, & Aebersold, 2002). The SEQUEST output files were filtered with the use of the interface software tool INTERACT to identify peptides and proteins.

2.6. Ovine microsome preparation

Livers were obtained from control and vitamin E-supplemented lambs (VE; n = 3) (MacGlaflin, Zajac, Rego, & Petersson, 2011). Microsomes isolated from control and vitamin E livers were identified as control microsomes and VE microsomes, respectively (Guengerich, 1977). Microsomal protein concentration was determined using a BCA protein assay, and the α-tocopherol concentration of microsomal membranes was determined by reverse phase HPLC (Buttriss & Diplock, 1984).

2.7. Ovine OxyMb oxidation with/without HNE in ovine microsomes

Purified ovine Mbs were pre-incubated with HNE (1:7) at 37 °C for 2 h or with ethanol (control). Pre-incubated Mbs were reduced to DeoxyMb by sodium dithionite. Unreacted HNE and excess sodium dithionite were removed by passing through PD-10 columns pre-calibrated with 50 mM sodium citrate (pH 5.6, 4 °C). The resulting OxyMbs (0.15 mM) were incubated with VE microsomes and control microsomes (1 mg protein/mL) at 25 °C for 6 h. MetMb accumulation was monitored as previously described. Thiobarbituric acid reactive substances (TBARS) were measured as an indicator of oxidation of microsomal lipids according to Yin and Faustman (1993).

2.8. Statistical analysis

A completely randomized block with repeated measures (n = 3) was used for this study. A completely randomized design was used to determine the pH and temperature effects on ovine Mb redox stability with/without HNE. The data for myoglobin redox stability at pH 5.6 and 7.4 were analyzed separately. The microsomes isolated from one liver served as a block. The microsomes within block were randomly assigned to treatments (Mbs with/without HNE), and samples were measured repeatedly at different times of incubation. 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 square means were generated for significant F-tests ($P < 0.05$) and the diff option was used to separate least square means.