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Supranutritional Supplementation of Vitamin E Influences Myoglobin Post-Translational Modifications in Postmortem Beef *Longissimus Lumborum* Muscle

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Abstract: Post-translational modifications (PTM) in myoglobin (Mb) can influence fresh meat color stability. Dietary supplementation of vitamin E improves beef color stability by delaying lipid oxidation–induced Mb oxidation and influences proteome profile of postmortem beef skeletal muscles. Nonetheless, the influence of vitamin E on Mb PTM in postmortem beef skeletal muscles has yet to be investigated. Therefore, the objective of the current study was to examine the effect of dietary vitamin E on Mb PTM in postmortem beef *longissimus lumborum* muscle. Beef *longissimus lumborum* muscle samples (24 h postmortem) were obtained from the carcasses of 9 vitamin E–supplemented (VITE; 1,000 IU vitamin E diet/heifer·d^{-1} for 89 d) and 9 control (CONT; no supplemental vitamin E) heifers. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to separate Mb from other sarcoplasmic proteins of beef *longissimus lumborum* muscle. Tandem mass spectrometry identified multiple PTM (phosphorylation, acetylation, 4-hydroxynonenal alkylation, methylation, dimethylation, trimethylation, and carboxymethylation) in the protein bands (17 kDa) representing Mb. The amino acids susceptible to phosphorylation were threonine (T) and tyrosine (Y), whereas lysine (K) residues were prone to other PTM. The same sites of phosphorylation (T34, T67, Y103), carboxymethylation (K77, K78), and 4-hydroxynonenal alkylation (K77, K78, K79) were identified in Mb from CONT and VITE samples, indicating that these PTM were not influenced by the vitamin E supplementation in cattle. Nonetheless, differential occurrence of acetylation, methylation, dimethylation, and trimethylation were identified in Mb from CONT and VITE samples. Overall, a greater number of amino acids were modified in CONT than VITE, suggesting that the supplementation of vitamin E decreased the numbers of post-translationally modified residues in Mb. Additionally, PTM at K87, K96, K98, and K102 were unique to CONT, whereas PTM at K118 were unique to VITE. These findings suggested that dietary supplementation of vitamin E in beef cattle might protect amino acid residues in Mb—especially those located spatially close to proximal histidine—from undergoing PTM, thereby improving Mb redox stability.

Key words: beef color stability, myoglobin, post-translational modifications, vitamin E
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Introduction

Meat discoloration is due to the conversion of cherry-red oxymyoglobin (OxyMb) to brownish metmyoglobin and adversely affects consumer perception of quality (Suman et al., 2014; Neethling et al., 2017). The secondary products of lipid oxidation can accelerate OxyMb oxidation through alkylation of myoglobin (Mb) leading to meat discoloration (Faustman et al., 1999). Pre-harvest applications of dietary antioxidants in animal production can minimize lipid oxidation–induced OxyMb oxidation (Faustman et al., 2010).

Vitamin E (α-tocopherol) is a lipid-soluble antioxidant that protects highly oxidizable polyunsaturated fatty acids from oxidation by reactive oxygen species and free radicals (Buttriss and Diplock, 1988). Dietary supplementation of vitamin E demonstrated a dual protective effect for both lipid and Mb oxidation in beef (Faustman et al., 1989, Arnold et al., 1992; Sanders et al., 1997; Lynch et al., 1999; Zerby et al., 1999; Delosiere et al., 2020) and lamb (Wulf et al., 1995; Guidera et al., 1997; Strohecker et al., 1997; Gonzalez-Calvo et al., 2015; Belles et al., 2018; Leal et al., 2020). Vitamin E also retards lipid and OxyMb oxidation in microsomes (Yin et al., 2013) and liposome (Yin et al., 1993) models *in vitro*. In addition, dietary delivery of vitamin E improved pigment and lipid stability more efficiently than addition of this ingredient to postmortem muscles (Mitsumoto et al., 1993). The effect of vitamin E on meat color stability is believed to be through a direct protective effect for lipid and to be an indirect effect for minimizing OxyMb oxidation (Faustman et al., 2010; Ramanathan et al., 2020a, 2020b).

Post-translational modifications (PTM) refer to the covalent changes that proteins undergo after translation (Lodish, 1981; Han and Martinage, 1992; Mann and Jensen, 2003). PTM determine protein structure and modulate the protein properties by addition or removal of modifying groups to amino acids (Mann and Jensen, 2003; Seo and Lee, 2004). PTM play a fundamental role in regulating biological processes as they determine protein's functionality, localization, turnover, and interactions with other biomolecules (Seo and Lee, 2004; Müller, 2017). Diverse PTM such as phosphorylation (Stewart et al., 2004; Huang et al., 2011; Hohenester et al., 2013), acetylation (Livingston et al., 1985; Jiang et al., 2019), carboxymethylation (Ray and Gurd, 1967; Hugli and Gurd, 1970), and oxidation (Bostelaar et al., 2016; Lindsay et al., 2016) have been reported to regulate the structure and functionality of mammalian Mb. Furthermore, 4-hydroxynonenal (HNE) alkylation accelerated *in vitro* OxyMb oxidation by covalently binding to histidine residues (Faustman et al., 1999; Alderton et al., 2003; Suman et al., 2006, 2007; Yin et al., 2013; Nair et al., 2014; Elroy et al., 2015; Viana et al., 2020).

Li et al. (2018a, 2018b) documented that the phosphorylation level of sheep Mb was inversely related to the color stability of *longissimus* muscle. Moreover, Wang et al. (2021) indicated that PTM (including phosphorylation, acetylation, methylation, carboxymethylation, and HNE alkylation) compromised beef Mb redox stability and color stability. These observations suggested that PTM play a critical role in Mb functionality and fresh meat color stability.

The effect of vitamin E on fresh beef color has been extensively studied from the standpoint of lipid oxidation–induced Mb oxidation. Recent studies indicated that dietary supplementation of vitamin E influenced the mitochondrial (Zhai et al., 2018) and sarcoplasmic (Kim et al., 2021) proteome profile of postmortem beef *longissimus lumborum* (LL) muscle. Nonetheless, investigations were not undertaken on the potential effect of vitamin E on Mb PTM in beef skeletal muscle. Therefore, the objective of the current study was to examine the influence of dietary vitamin E supplementation on the Mb PTM in postmortem beef LL muscle.

Materials and Methods

Animal production and muscle sample collection

The muscle samples were obtained from a feeding study (Harsh et al., 2018) completed at the University of Illinois. All protocols were approved by the University of Illinois Institutional Animal Care and Use Committee (Protocol #15008). Eighteen Angus × Simmental heifers were used in a randomized complete block design with treatment factors including daily dietary inclusion of no supplemental vitamin E (CONT) or 1,000 IU vitamin E/heifer·d^{-1} (VITE). Heifers were managed as a group on a common diet prior to trial initiation and were administered an implant of 140 mg trenbolone acetate and 14 mg estradiol (Component TE-H; Elanco Animal Health, Greenfield, IN). After being weighed on days −1 and 0, heifers were stratified by bodyweight ($n = 9$ heifers per treatment). Diets were the same for the 2 dietary treatments with the exception of vitamin E inclusion as feed supplement. Diets were formulated to meet or exceed NRC (2000) recommendations and contained 20% corn silage, 35% modified wet distillers grains with solubles, 35% dry rolled corn, and 10% supplement (dry matter basis). Dietary vitamin E (dl-alpha-tocopheryl acetate) was provided to VITE heifers, and individual feed intakes of all heifers were collected with a GrowSafe feeding system (GrowSafe Systems Ltd., Airdrie, AB, Canada).

Heifers were weighed at 28-d intervals and fed *ad libitum* intake daily for a total of 89 d on feed. Heifers were housed in 4.88 m × 4.88 m pens in a confinement barn with slatted, concrete floors covered with interlocking rubber matting. On day 90, heifers were

slaughtered humanely under USDA inspection at a commercial slaughter facility. At 24 h postmortem, a 2.54-cm section of LL was excised from between the 12th and 13th rib section of the carcasses, immediately vacuum packaged, frozen at −80°C, and shipped in dry ice to the University of Kentucky. The results of growth performance, carcass quality, color attributes, and lipid oxidation are discussed in Harsh et al. (2018), which reported that steaks from VITE heifers exhibited greater redness and lower visual discoloration than those from CONT counterparts during 16-d retail display.

Isolation of sarcoplasmic proteome

The sarcoplasmic proteomes from beef LL muscle were extracted according to the method of Joseph et al. (2012). Frozen samples were thawed overnight at 2°C. Five grams of muscle tissue devoid of any visible fat and connective tissue from every sample was homogenized in 25 mL of ice-cold extraction buffer (40 mM Tris, 5 mM ethylenediaminetetraacetic acid [pH = 8]) using a Waring blender (Model No. 51BL32; Waring Commercial, Torrington, CT). The homogenate was then centrifuged at 10,000 × *g* for 15 min at 4°C. The supernatant consisting of sarcoplasmic proteome extract was filtered through Whatman No.1 paper (GE Healthcare, Little Chalfont, UK) and used for subsequent analysis.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The sarcoplasmic proteomes from beef LL muscle were separated based on molecular weight using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gels as described by Laemmli (1970). A stacking gel with 3% acrylamide and a resolving gel with 13% acrylamide were used. The diluted protein samples (3 mg/mL) were dissolved in the SDS-PAGE sample buffer (10% [w/v] SDS, glycerol, 0.1% [w/v] bromophenol blue, 0.5 M Tris-HCl [pH 6.8]), and then were boiled with 10% β-mercaptoethanol in the water bath for 5 min. Aliquots of 20 μg of protein per well were loaded to SDS-PAGE gels in a mini PROTEAN Tetra cell system (Bio-Rad Laboratories Inc.). The molecular weight standard (Bio-Rad Laboratories Inc.) used consisted of myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), lysozyme (14 kDa), and aprotinin (6.5 kDa). The gels were stained with Colloidal Coomassie Blue for 48 h and destained until sufficient background was cleared.

Liquid chromatography-electrospray ionization-tandem mass spectrometry

The protein gel bands (17 kDa) representing Mb in the SDS-PAGE gels were excised and subjected to dithiothreitol reduction, iodoacetamide alkylation, and in-gel trypsin digestion using a standard protocol (Shevchenko et al., 2006). The resulting tryptic peptides were extracted, concentrated, and subjected to shotgun proteomics analysis as previously described in Kamelgarn et al. (2018). Nano-liquid chromatography (LC)–tandem mass spectrometry (MS/MS) analysis was performed using an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) coupled with an Eksigent Nanoflex cHiPLC™ system (Eksigent, Dublin, CA) through a nano-electrospray ionization source. The peptide samples were separated with a reversed-phase cHiPLC™ column (75 μm × 150 mm) at a flow rate of 300 nL/min. Mobile phase A was water with 0.1% (v/v) formic acid, whereas B was acetonitrile with 0.1% (v/v) formic acid. A 50-min gradient condition was applied: initial 3% mobile phase B was increased linearly to 40% in 24 min and further to 85% and 95% for 5 min each before it was decreased to 3% and re-equilibrated. The mass analysis method consisted of 1 segment with 10 scan events. The first scan event was an Orbitrap MS scan (300–1,800 m/z) with 60,000 resolution for parent ions followed by data-dependent MS/MS for fragmentation of the 10 most intense multiply charged ions with the collision induced dissociation method.

Identification of post-translational modifications in myoglobin

The LC-MS/MS data were submitted to a local mascot server for MS/MS protein identification via Proteome Discoverer (version 1.3; Thermo Fisher Scientific, Waltham, MA) against a custom database containing only beef Mb protein [MYG_BOVIN] downloaded from UniProt (https://www.uniprot.org/uniprot/P02192). Typical parameters used in the MASCOT MS/MS ion search were as follows: trypsin digestion with a maximum of 2 miscleavages; 10-ppm precursor ion and 0.8-Da fragment ion mass tolerances; methionine oxidation; lysine acetylation; lysine carboxymethylation; lysine mono-, di-, and tri-methylation; arginine mono- and di-methylation;

serine, threonine, and tyrosine phosphorylation; and HNE alkylation at histidine and lysine.

Results and Discussion

Influence of vitamin E supplementation on beef myoglobin post-translational modifications

Tandem mass spectrometry identified multiple PTM (such as phosphorylation, methylation, dimethylation, trimethylation, carboxymethylation, acetylation, and HNE alkylation) in protein bands (17 kDa) representing Mb. PTM in Mb from CONT and VITE beef are presented in Table 1. The amino acids susceptible to phosphorylation were threonine (T) and tyrosine (Y), whereas lysine (K) residues were prone to other PTM. While Mb from CONT and VITE exhibited the same number of PTM, a greater number of amino acids were modified in CONT than VITE (16 vs. 13). Mb from CONT and VITE demonstrated similar patterns in phosphorylation (T34, T67, Y103), carboxymethylation (K77, K78), and HNE alkylation (K77, K78, K79) sites, suggesting that these PTM were not influenced by the vitamin E supplementation in beef animals. However, it should be noted that the degree of the modification could be different even in the cases in which a PTM was observed in both diets. On the other hand, differential occurrence of acetylation, methylation, dimethylation, and trimethylation were identified in Mb from CONT and VITE samples (Table 1; Figure 1), and these are discussed subsequently.

Table 1. Impact of supranutritional supplementation of vitamin E on myoglobin post-translational modifications in postmortem beef *longissimus lumborum* muscle

Post-translational modifications[a]	CONT[b]	VITE[b]
Phosphorylation	34 Threonine	34 Threonine
	67 Threonine	67 Threonine
	103 Tyrosine	103 Tyrosine
Carboxymethylation	77 Lysine	77 Lysine
	78 Lysine	78 Lysine
HNE alkylation	77 Lysine	77 Lysine
	78 Lysine	78 Lysine
	79 Lysine	79 Lysine
Acetylation	50 Lysine	50 Lysine
	63 Lysine	63 Lysine
	77 Lysine	77 Lysine
	78 Lysine	78 Lysine
	79 Lysine	79 Lysine
	87 Lysine[c]	**118 Lysine**[d]
	147 Lysine	147 Lysine
Methylation	31 Arginine	31 Arginine
	42 Lysine	42 Lysine
	98 Lysine[c]	**118 Lysine**[d]
Dimethylation	–	**118 Lysine**[d]
		133 Lysine[d]
Trimethylation	**96 Lysine**[c]	**118 Lysine**[d]
	102 Lysine[c]	
	133 Lysine[c]	
Total number of post-translationally modified residues	16	13

[a]Position and residue are listed for the post-translational modifications (PTM).

[b]CONT: non–vitamin E diet; VITE: 1,000 IU vitamin E diet/heifer·d^{-1} for 89 d.

[c]PTM unique to myoglobin from CONT.

[d]PTM unique to myoglobin from VITE.

Differential PTM are listed in boldface.

HNE = 4-hydroxynonenal.

Seven lysine residues were acetylated in Mb from both CONT and VITE animals. While 6 acetylation sites (K50, K63, K77, K78, K79, and K147) were observed in both CONT and VITE, acetylation at K87 and K118 were unique to CONT and VITE, respectively (Table 1). The addition of the acetyl group neutralizes the positive charge of lysine, thereby disrupting the ionic network and Mb tertiary structure. The acetylation at K87 could cause the displacement of the F-helix, which has been associated with altering oxygen affinity and heme-globin linkage in a manner that can decrease Mb redox stability (Nguyen et al., 2000). In agreement, the acetylation-induced conformational changes in horse apomyoglobin have been reported to result in an increased tendency for unfolding, compromising the heme stability and oxygen affinity (Azami-Movahed et al., 2018). Compared with K118, K87 is in closer proximity to the proximal histidine (H93), which is bound to heme moiety (Figure 1). Thus, the acetylation of distantly located K118 could have much less impact on the hydrophobic heme pocket than the acetylation in K87. In partial agreement, Suman et al. (2006) documented that the HNE alkylation at H88, which is adjacent to K87, appeared to compromise Mb redox state owing to potential interference with proximal histidine. In general, the lysine acetylation in VITE occurring at residues far from proximal histidine may result in minimum damage of Mb redox stability, which could also explain the better color stability in vitamin E–supplemented beef (Faustman et al., 1989).

The ε-amine moiety of lysine can be methylated up to 3 times from unmodified lysine to mono-, di-, and trimethylated forms, altering the biophysical properties (i.e., pKa value and size) of this residue (Luo, 2018).

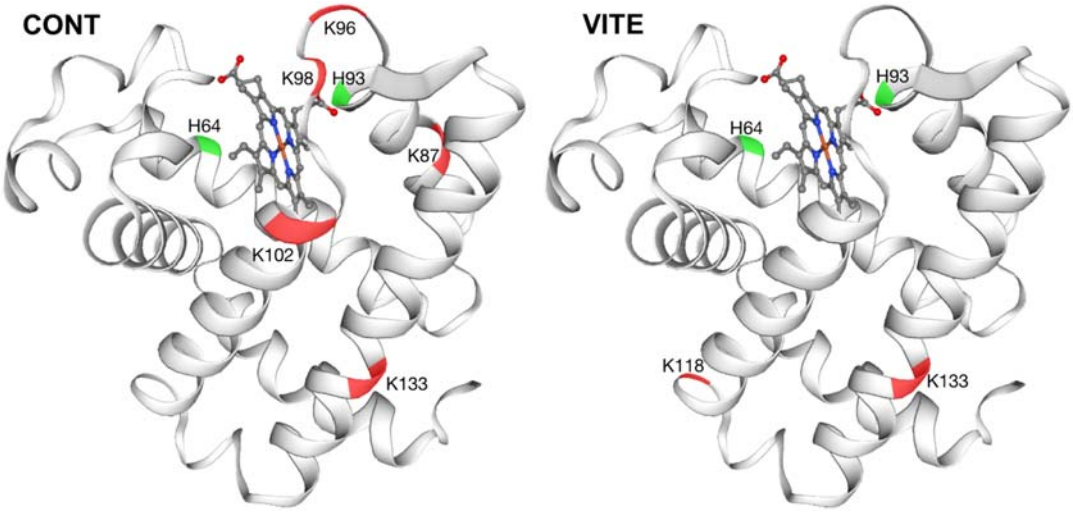

Figure 1. Differential post-translational modifications at lysine (K) residues in myoglobin from *longissimus lumborum* muscle of CONT (non–vitamin E diet) and VITE (1,000 IU vitamin E diet/heifer·d^{-1} for 89 d) supplemented heifers. Differential post-translational modifications are indicated in red, whereas distal (H64) and proximal (H93) histidines in beef myoglobin are in green. The three-dimensional homology model of beef myoglobin was downloaded from SWISS-MODEL (Waterhouse et al., 2018).

Differential methylation, di-methylation, and tri-methylation sites were detected in Mb from CONT and VITE samples (Table 1). While K31 and K42 were methylated in both CONT and VITE Mb, methylation at K98 and K118 were unique to CONT and VITE, respectively. The addition of methyl groups increases the overall size and hydrophobicity of the lysine side-chains (Bremang et al., 2013; Luo, 2018), which in turn could induce conformational changes and compromise Mb redox stability. The K98 lies in the closer vicinity of proximal histidine (H93) compared with the K118 (Figure 1). Consequently, methylation at K98 in CONT could compromise heme stability and heme affinity to a greater degree than the methylation at K118 in VITE (Smerdon et al., 1993; Hargrove et al., 1996). Additionally, lysine di-methylation at K118 and K133 were only detected in VITE beef. While K96, K102, and K133 were tri-methylated in CONT, only K118 were tri-methylated in VITE. As lysine methylation progressed, the adduction of increased number of methyl groups could lead to a decrease in lysine's hydrophilicity and the residue's capability to form hydrogen bonds (Luo, 2018); this in turn could compromise protein hydrophilicity and stability (Hamamoto et al., 2015). Therefore, a tri-methylated lysine could contribute to a greater level of hydrophobicity to proteins than a di-methylated lysine. Accordingly, the 3 tri-methylated lysine residues (K96, K102, and K133) observed in CONT could be more deleterious to Mb tertiary structure than the 2 di-methylated (K118 and K133) and 1 tri-methylated (K118) lysine residues in VITE samples; thus, Mb from VITE samples might have better redox stability than its counterpart from CONT samples. Furthermore, K96 and K102 are closer to proximal histidine (H93) spatially compared with K118 and K133 within Mb tertiary structure (Figure 1), so that the observed tri-methylation at K96 and K102 in CONT could have a greater impact on the hydrophobic heme pocket and Mb redox stability (Smerdon et al., 1993; Hargrove et al., 1996) compared with the di-methylation at K118 and K133 in VITE. Overall, the location and number of mono-, di-, and tri-methylated lysine residues observed in CONT might be more detrimental to Mb redox stability than those in VITE, which could lead to the lower color stability of beef from non–vitamin-E-fed animals than the beef from vitamin E–supplemented cattle (Faustman et al., 1989).

Previous investigations documented that dietary supplementation of cattle with vitamin E (i.e., α-tocopherol) could improve OxyMb stability (Faustman et al., 1989; Chan et al., 1996) and beef color stability (Faustman et al., 1989; Arnold et al., 1992; Lanari et al., 1993; Sherbeck et al., 1995). The observed color-stabilizing effect of α-tocopherol was believed to be achieved by direct inhibition of lipid oxidation, thereby indirectly delaying OxyMb oxidation (Faustman et al., 2010; Ramanathan et al., 2020a). Secondary products of lipid oxidation have been shown to accelerate OxyMb oxidation through alkylation of Mb (Witz, 1989; Faustman et al., 1999). HNE, a well-documented secondary product of linoleic acid oxidation, was observed to covalently adduct with histidine residues

in beef Mb via Michael addition, exposing heme and subsequently accelerating Mb oxidation (Faustman et al., 1999; Alderton et al., 2003; Suman et al., 2006, 2007). Therefore, α-tocopherol, the lipid-soluble and chain-breaking antioxidant, was expected to decrease HNE generation from lipid oxidation and in turn improve Mb stability. Nonetheless, the current study observed HNE alkylation at 3 lysine residues (K77, K78, and K79) in Mb from both CONT and VITE groups (Table 1), suggesting that this PTM was not influenced by the dietary supplementation of vitamin E.

Interestingly, our results indicated the supplementation of vitamin E seems to exert its protective effect on Mb by influencing several other PTM such as acetylation, methylation, di-methylation, and trimethylation. Additionally, the supplementation of vitamin E decreased the numbers of post-translationally modified residues in Mb. PTM at K87, K96, K98, and K102 were unique to CONT, whereas PTM at K118 were unique to VITE. These observations indicated that dietary supplementation of vitamin E in beef cattle might protect residues in Mb—especially those located spatially close to proximal histidine—from undergoing PTM, thereby improving Mb redox stability.

Conclusions

Dietary supplementation of vitamin E decreased the number of post-translationally modified residues in Mb from beef LL. While phosphorylation, carboxymethylation, and alkylation of Mb were not influenced by vitamin E supplementation, differential acetylation, methylation, dimethylation, and trimethylation sites were identified in Mb from CONT and VITE beef cattle. The unique PTM in CONT Mb (K87, K96, K98, and K102) were spatially closer to proximal histidine compared with the unique PTM (K118) in Mb from VITE samples, and thus could compromise Mb redox stability owing to the potential interference with proximal histidine. Vitamin E might have minimized the occurrence of PTM at residues located spatially close to proximal histidine in Mb, contributing to the improved beef color stability. Future research should examine the PTM sites in Mb from early postmortem muscles, quantify the PTM in Mb from both diets, and determine the kinetics of PTM formation at various susceptible residues to explain the impact of vitamin E–induced differential Mb PTM in beef color stability.

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