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Reverse electron transport effects on NADH formation and metmyoglobin reduction

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ABSTRACT

The objective was to determine if NADH generated via reverse electron flow in beef mitochondria can be used for electron transport-mediated reduction and metmyoglobin reductase pathways. Beef mitochondria were isolated from bovine hearts (n = 5) and reacted with combinations of succinate, NAD, and mitochondrial inhibitors to measure oxygen consumption and NADH formation. Mitochondria and metmyoglobin were reacted with succinate, NAD, and mitochondrial inhibitors to measure electron transport-mediated metmyoglobin reduction and metmyoglobin reductase activity. Addition of succinate and NAD increased oxygen consumption, NADH formation, electron transport-mediated metmyoglobin reduction, and reductase activity ($p < 0.05$). Addition of antimycin A prevented electron flow beyond complex III, therefore, decreasing oxygen consumption and electron transport-mediated metmyoglobin reduction. Addition of rotenone prevented reverse electron flow, increased oxygen consumption, increased electron transport-mediated metmyoglobin reduction, and decreased NADH formation. Succinate and NAD can generate NADH in bovine tissue postmortem via reverse electron flow and this NADH can be used by both electron transport-mediated and metmyoglobin reductase pathways.

1. Introduction

Meat discoloration influences consumer purchasing and results in approximately 1 billion dollars of lost revenue each year (Smith, Belk, Sofos, Tatum, & Williams, 2000). Myoglobin is the protein responsible for meat color and can exist in 3 redox states, including deoxymyoglobin, oxymyoglobin, and metmyoglobin (Mancini & Hunt, 2005). Deoxymyoglobin is purplish-red and associated with the color of vacuum packaged beef. Conversely, oxymyoglobin is responsible for the consumer-preferred bright cherry-red color commonly seen in retail stores. Formation of metmyoglobin on the surface of beef products results in brown discoloration due to oxidation of ferrous deoxymyoglobin and oxymyoglobin.

Postmortem muscle is biochemically active and has the ability to reduce metmyoglobin by the addition of an electron to oxidized ferric myoglobin. More specifically, electrons involved in metmyoglobin reduction are generated either from NADH or mitochondria-mediated electron transfer (Faustman & Cassens, 1990; Tang, Faustman, Mancini, Seyfert, & Hunt, 2005). Hence, processes associated with mitochondrial NADH regeneration can influence the color stability of beef. Mitochondria-mediated metmyoglobin reduction can occur between complexes III and IV of the electron transport chain via the transfer of electrons to metmyoglobin (complex III is coenzyme Q–cytochrome *c* reductase; complex IV is cytochrome *c* oxidase). Chance and Hollunger (1961) suggested that the addition of succinate (a complex II substrate) and NAD to mitochondria can regenerate NADH via reversed electron flow from complex II to complex I in rat tissue (complex I is NADH:ubiquinone oxidoreductase; complex II is succinate dehydrogenase). Low, Krueger, and Ziegler (1961) demonstrated that NADH can be generated via reverse electron flow in beef heart. However, no recently published studies have determined if the resulting NADH can be used for metmyoglobin reduction. Therefore, our objective was to determine if NADH generated from reverse electron flow in beef mitochondria can be used for electron transport-mediated reduction and metmyoglobin reductase.

2. Materials and methods

2.1. Materials and chemicals

Bovine hearts from market age cattle were obtained from a local abattoir within 30 min of exsanguination, placed on ice, and transported to the laboratory for mitochondrial isolation. Sodium succinate, sodium hydroxide (NaOH), magnesium chloride ($MgCl_2$), β-nicotinamide adenine dinucleotide (NAD), rotenone, antimycin A, potassium chloride (KCl), monopotassium phosphate (KH_2PO_4), sucrose, maleic acid, *N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid] (HEPES), ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), potassium ferrocyanide,

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bovine serum albumin (BSA), equine cardiac metmyoglobin, nagarase protease, and Bicinchoninic Acid Protein Assay Kit were purchased from Sigma Chemical Co. (St. Louis, Missouri); 0.2 μm syringe filter units were purchased from Fisher Scientific (Fair Lawn, New Jersey); 10 mL syringes were purchased from Becton Dickinson and Company (Franklin Lakes, New Jersey). All chemicals used in the experiment were of reagent grade or greater purity.

2.2. Bovine mitochondria isolation

Mitochondria were isolated from bovine cardiac muscle according to Smith (1967). Minced cardiac muscle (100 g) was washed twice with 250 mM sucrose and suspended in 200 mL of mitochondrial isolation buffer (250 mM sucrose, 10 mM HEPES, 1 mM EGTA, and 0.1% BSA, pH 7.2). The suspension was hydrolyzed for 20 min with nagarase protease (protease/tissue, 0.5 mg/g) and the pH was maintained between 7.0 and 7.2. The suspension was diluted with mitochondrial isolation buffer and homogenized using a Kontes Duall grinder (Vineland, New Jersey), passed through a Wheaton Potter-Elvehjem grinder (Millville, New Jersey), centrifuged for 20 min at 1200 ×*g* in a Sorvall refrigerated RC-5B centrifuge (Thermo Fisher Scientific, Waltham, MA), and the resulting supernatant was centrifuged for 15 min at 26,000 ×*g*. The pellet was washed twice and suspended in 250 mM sucrose and 10 mM HEPES. All steps were performed at 0–4 °C. Mitochondrial protein content was determined using a bicinchoninic protein assay (Ramanathan, Maheswarappa, & Mancini, 2010).

2.3. Oxygen consumption measurement

Isolated mitochondria were reacted with substrates according to Tang et al. (2005). Mitochondria were suspended in reaction buffer (250 mM sucrose, 5 mM KH_2PO_4, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1% BSA and 20 mM HEPES). To measure mitochondrial oxygen consumption, all treatments (Table 1) were added to mitochondria through a 1 mm port in a Clark electrode incubation chamber (polarizing voltage of 0.6 V and 8 mL incubation chamber) attached to a Rank Brothers digital model 20 oxygen controller (Cambridge, England) and connected to a personal computer and data logger. The incubation chamber was maintained at 25 °C and pH 5.6 by a Lauda RE120 circulating water bath (Westbury, NY) and stirred at 600 rpm with a 10 mm Teflon-covered bar. The oxygen consumption rate of isolated mitochondria was calculated according to Estabrook (1967).

2.4. Estimation of NADH formation

NADH formation was estimated using absorbance at 340 nm (Bergmeyer, 1974). The reaction was initiated by the addition of succinate (40 mM) and NAD (40 mM) per treatment descriptions listed in Table 1. Temperature was kept at 25 °C by a circulating water bath. Fifty μl of NaOH was added and samples were centrifuged in an Eppendorf Centrifuge 5415 D (Hamburg, Germany) at 16,000 ×*g* for 5 min and passed through a 0.2 μm syringe filter to decrease turbidity. Absorbance of the supernatant was recorded at 340 nm using a Shimadzu UV-2102PC spectrophotometer (Shimadzi Inc., Columbia, MD) at 0 min and 60 min.

Table 1
Treatment combinations added to bovine cardiac mitochondria (MT) and myoglobin used to measure oxygen consumption[a], metmyoglobin reduction[b], and NADH formation[a].

	Substrate/Inhibitors[c]				
	Treatments	Succinate (40 mM)	NAD (40 mM)	Antimycin A (20 mM)	Rotenone (40 mM)
1	Control	–	–	–	–
2	Succinate	+	–	–	–
3	Succinate + NAD	+	+	–	–
4	NAD	–	+	–	–
5	Succinate + NAD + antimycin A	+	+	+	–
6	Succinate + NAD + rotenone	+	+	–	+

[a] Oxygen consumption and NADH formation treatments included incubation buffer and mitochondria.
[b] Metmyoglobin reduction treatments included cardiac metmyoglobin, incubation buffer, and mitochondria.
[c] Substrate/inhibitors present (+) or absent (–).

2.5. Electron transport-mediated metmyoglobin reduction

The reaction was initiated by the addition of succinate (40 mM) and NAD (40 mM) per treatment descriptions listed in Table 1. The reaction was kept at 25 °C by a circulating water bath. Following incubation, samples were centrifuged in an Eppendorf Centrifuge 5415 D (Hamburg, Germany) at 16,000 ×*g* for 5 min and passed through a 0.2 μm filter to decrease turbidity. Absorbance of the supernatant was recorded from 600 nm to 500 nm using a Shimadzu UV-2101PC spectrophotometer (Shimadzu Inc., Columbia, MD) at 0 and 60 min. Relative proportions of metmyoglobin, oxymyoglobin, and deoxymyoglobin were calculated according to Tang et al. (2005). Decreased ferrous myoglobin indicates less metmyoglobin reduction (less mitochondria-mediated metmyoglobin reducing activity).

2.6. Enzymatic metmyoglobin reductase activity

Mitochondria (2 mg) were incubated with antimycin A (0.02 mM) and metmyoglobin (0.15 mM) at 37 °C and pH 7.4 to differentiate between metmyoglobin reduction associated with reductase and the electron transport chain. After incubation, potassium ferrocyanide (3 mM) and EDTA (5 mM) were added to each treatment. Succinate (40 mM) and NAD (40 mM) were added to treated samples while control samples received no additional substrate. Absorbance at 580 nm (the wavelength at which the difference in absorbance for oxymyoglobin and metmyoglobin is maximal) was measured for 2 min using the kinetic option in the spectrophotometer. Metmyoglobin reduction rate was calculated according to Faustman, Cassens, and Greaser (1988) and recorded as μM of metmyoglobin reduced per minute per mg of mitochondria.

2.7. Statistical analysis

The overall experiment was replicated on five separate occasions (using n = 5 hearts). A randomized complete block design was used to assign treatments to isolated mitochondria within a heart; each treatment was assigned once per heart. Duplicate subsamples were used for oxygen consumption analysis.

The primary objective was to assess the effects of reverse electron transport on NADH formation and metmyoglobin reduction. Fixed effects for all analyses had a 1-way treatment structure. Type 3 tests of fixed effects were performed using the MIXED procedure of SAS (version 9.1, SAS Institute Inc. Cary, NC). For all analyses, random terms included heart (block) and unspecified residual error. Least square means for protected F-tests ($p < 0.05$) were separated using the diff option (least significant differences) and were considered significant at $p < 0.05$. The results were expressed as the least squares mean values of five independent trials.

3. Results

3.1. Mitochondrial oxygen consumption rate

The addition of succinate to isolated mitochondria resulted in greater oxygen consumption ($p < 0.05$) when compared with control mitochondria without added substrate (Fig. 1). Addition of both succinate and NAD further increased ($p < 0.05$) oxygen consumption compared with succinate-treated mitochondria. However, addition of