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Effects of pyruvate on bovine heart mitochondria-mediated metmyoglobin reduction

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ABSTRACT

Pyruvate can regenerate NADH through a variety of biochemical processes. However, no meat science research has determined if NADH produced via pyruvate can be used for mitochondria-mediated metmyoglobin reduction. Thus, our objectives were to assess the effects of pyruvate on mitochondria isolated from bovine cardiac muscle: oxygen consumption and metmyoglobin reduction at pH 5.6 and 7.4, 25 °C in vitro. Both mitochondria and myoglobin were isolated from fresh bovine hearts (n = 5). Mitochondria were reacted with pyruvate (50 mM), succinate (positive control; 50 mM), and antimycin A (mitochondrial inhibitor; 0.01 mM) and oxygen consumption was measured using a Clark oxygen electrode. Mitochondria (3 mg/mL) and metmyoglobin (0.15 mM) were reacted with either pyruvate, succinate, or antimycin A for 3 h. Addition of succinate and pyruvate increased oxygen consumption and metmyoglobin reduction at pH 5.6 and 7.4 (succinate > pyruvate, $P<0.05$). Addition of a complex III inhibitor (antimycin A) decreased ($P<0.05$) oxygen consumption as well as metmyoglobin reduction associated with pyruvate and succinate. Results from the current study suggest that pyruvate can increase the ability of mitochondria to consume oxygen and reduce metmyoglobin.

1. Introduction

Myoglobin is the major sarcoplasmic protein responsible for the bright cherry red color in fresh meat. The redox form of myoglobin determines meat purchasing decisions as consumers often associate metmyoglobin accumulation with microbial spoilage. In general, metmyoglobin reducing activity (MRA) in muscle is an important intrinsic property that prolongs the color stability of meat during storage (Ledward, 1985). More specifically, metmyoglobin reducing activity relies on the production of either NADH (by dehydrogenase enzymes) or electrons (by mitochondria), both of which can be used to reduce metmyoglobin (Bekhit & Faustman, 2005; Faustman, Cassens & Greaser, 1988; Giddings, 1974).

Saleh and Watts (1968) reported that addition of glycolytic and tricarboxylic acid (TCA) intermediates to ground beef increased metmyoglobin reduction via production of NADH. These authors suggested that NADH in conjunction with sarcoplasmic diaphorase enzymes results in metmyoglobin reduction. Although, pyruvate can regenerate NADH through the TCA cycle, no research has assessed the potential role of pyruvate in metmyoglobin reduction.

Tang, Faustman, Mancini, Seyfert and Hunt (2005) highlighted the role of mitochondria in metmyoglobin reduction and reported a correlation between oxygen consumption and metmyoglobin reduction. More specifically, Tang et al. (2005) concluded that the addition of succinate to isolated mitochondria increased oxygen consumption, which in-turn decreases partial pressure and creates an environment that favors the transfer of available electrons from cytochrome c to metmyoglobin. Ramanathan, Mancini and Konda (2009) reported that addition of pyruvate to isolated beef heart mitochondria resulted in oxygen consumption. Although the role of pyruvate in mitochondrial oxygen consumption has been reported, no research has assessed the potential role of pyruvate in electron transport chain mediated metmyoglobin reduction. Hence, the objective of this study was to assess the effects of pyruvate on metmyoglobin reduction in mitochondria isolated from bovine cardiac muscle.

2. Materials and methods

2.1. Materials and chemicals

Bovine hearts from market age cattle were obtained locally from an abattoir within 0.5 h of exsanguination, placed on ice, transported to the laboratory, and used for isolating mitochondria and myoglobin. Magnesium chloride ($MgCl_2$), bovine serum albumin (BSA), sucrose, tris [hydroxymethyl] aminomethane hydrochloride (Tris–HCl), potassium phosphate monobasic (KH_2PO_4), ethylene glycol-bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid] (HEPES), nagarase protease (10.5 U/mg), sodium pyruvate, antimycin A, Sephacryl 200-HR, ammonium sulfate, 2-(N-morpholino) ethanesulfonic acid (MES), Tris–HCl, EDTA, and Bicinchoninic Acid Protein Assay Kit were purchased from Sigma Chemical Co. (St. Louis, MO); sodium succinate

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was purchased from Fisher Scientific (Fair Lawn, New Jersey). PD-10 columns were obtained from GE Healthcare (Piscataway, NJ). All chemicals were of reagent grade or greater purity.

2.2. Bovine myoglobin isolation and purification

Myoglobin was purified via ammonium sulfate precipitation and gel filtration chromatography according to Faustman and Phillips (2001). Briefly, cardiac muscle devoid of fat and connective tissue was homogenized in buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0, 4 °C) using a Waring table-top blender (Dynamics Corp. of America, New Hartford, CT) 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 later centrifuged at 18,000 *g* for 20 min. The resulting supernatant was saturated with ammonium sulfate (100%) and centrifuged at 20,000 *g* for 1 h. The precipitate was resuspended 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 200HR gel filtration column (2.5 × 100 cm). The elution buffer contained 5 mM Tris–HCl, 1 mM EDTA at pH 8.0, and the flow rate was 60 mL/h.

2.3. Bovine mitochondria isolation

Mitochondria were isolated from bovine cardiac muscle according to Smith (1967) with minor modifications. Briefly, 100 g of ground cardiac muscle was washed twice with 250 mM sucrose and suspended in 200 mL of mitochondria isolation buffer (250 mM sucrose, 10 mM HEPES, 1 mM EGTA, and 0.1% BSA, pH 7.2). The suspension was stirred slowly and hydrolyzed with nagarase protease (protease/tissue, 0.5 mg/g) for 20 min; the pH was maintained between 7.0 and 7.2. After proteolytic digestion, the suspension was diluted to 1000 mL with mitochondria isolation buffer and subjected to two homogenization processes. The first of these was accomplished in a Kontes Duall grinder (Vineland, New Jersey) with three passes, whereas the second homogenization was performed using a Wheaton Potter-Elvehjem grinder (Millville, New Jersey) with three passes. Pestles for these grinders were driven by a heavy duty drill at 1400 rpm. The homogenate was centrifuged (1200 *g*) for 20 min with a Sorvall refrigerated RC-5B centrifuge (Thermo Fisher Scientific, Waltham, MA), and the resulting supernatant was again centrifuged (26,000 *g*) for 15 min. The pellet was washed twice with isolation buffer and suspended in mitochondria suspension buffer (250 mM sucrose, 10 mM HEPES, pH 7.2). All steps were performed at 0–4 °C. Mitochondrial protein content was determined using a bicinchoninic acid protein assay.

2.4. Oxygen consumption measurement

Mitochondrial oxygen uptake was measured using a Clark oxygen electrode (polarizing voltage of 0.6 V and an 8 mL incubation chamber). Reaction components were added to the incubation chamber, which was maintained at 25 °C by a water jacket and Lauda RE120 circulating water bath (Westbury, NY). The chamber was stirred with a 10 mm Teflon-covered bar at 600 rpm. The electrode was attached to a Rank Brothers digital model 20 oxygen controller (Cambridge, England) and connected to a personal computer and data logger. Oxygen consumption was recorded over time by suspending mitochondria either at pH 5.6 (250 mM sucrose, 5 mM KH_2PO_4, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1% BSA, and 20 mM maleic acid) or 7.4 (250 mM sucrose, 5 mM KH_2PO_4, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1% BSA, and 20 mM HEPES). Oxygen consumption rate (OCR) was calculated based on the method of Estabrook (1967). Oxygen consumption measures the OCR of isolated mitochondria in the presence of added substrate.

Methodology similar to that of Tang et al. (2005) was used for reacting isolated mitochondria with either succinate (positive control; 50 mM) or pyruvate at 50 mM. Substrate additions were made with Hamilton syringes through a 1 mm port in the incubation chamber of the Clark oxygen electrode. Mitochondria also were incubated with antimycin A (0.01 mM; complex III mitochondrial inhibitor). Oxygen consumption was expressed as nanomoles of oxygen consumed per min per mg of mitochondria.

2.5. Metmyoglobin reduction

Methodology similar to that of Tang et al. (2005) was used for assessing metmyoglobin reducing activity. In order to adjust the pH of the metmyoglobin solution, samples were passed through PD-10 columns pre-calibrated with either MES (pH 5.6; 50 mM) or phosphate buffer (pH 7.4; 50 mM) (Suman, Faustman, Stamer & Liebler, 2007). Metmyoglobin (2.5 mg/mL) reduction was conducted in a glass open top tube at pH 7.4 (120 mM KCl, 5 mM KH_2PO_4, 30 mM K_2HPO_4), or 5.6 (120 mM KCl, 5 mM KH_2PO_4, 30 mM maleic acid) and 25 °C. Bovine heart mitochondria (3 mg/mL) were combined with metmyoglobin and 1 of 6 treatments: 1 = no substrate, only mitochondria and metmyoglobin; 2 = 50 mM succinate; 3 = 50 mM pyruvate; 4 = no substrate + 0.01 mM antimycin A; 5 = 50 mM succinate + 0.01 mM antimycin A; and 6 = 50 mM pyruvate + 0.01 mM antimycin A. At specific time points, samples were removed and centrifuged (12,000 *g*) with an Eppendorf 5415D centrifuge (Westbury, NY) for 5 min. The resulting supernatant was scanned from 650 to 500 nm with a Shimadzu UV-2101PC spectrophotometer (Kyoto, Japan). The relative proportions of deoxymyoglobin, oxymyoglobin, and metmyoglobin were calculated according to Tang, Faustman and Hoagland (2004).

In order to assess the ability of substrates to reduce metmyoglobin without mitochondria, bovine metmyoglobin was incubated with either succinate (50 mM), pyruvate (50 mM), or antimycin A (0.01 mM) at 25 °C for 3 h. At specific time points, samples were removed and the change in spectra was assessed by scanning the metmyoglobin–substrate solution from 650 to 500 nm with a Shimadzu UV-2101PC spectrophotometer (Kyoto, Japan). Metmyoglobin content was calculated according to Tang et al. (2004).

2.6. Statistical analysis

Each experiment was replicated five times. The experimental design was a randomized complete block design where hearts served as blocks. Treatments were assigned to isolated mitochondria within a heart (each treatment assigned once per heart). Duplicate subsamples used for Clark oxygen electrode analyses were averaged for statistical analysis.

Fixed effects for oxygen consumption experiment had 1-way treatment structure and for metmyoglobin reduction assay had a 2-way treatment structure of substrate, time, and their interaction. Type-3 tests of fixed effects were performed using the MIXED procedure of SAS (Version 9.1, SAS Institute Inc. Cary, NC). For both experiments, random terms included heart (block) and unspecified residual error. Mitochondria-mediated metmyoglobin reduction measurements were analyzed with the addition of a Repeated Statement within Proc Mixed. Least square means for protected *F*-tests ($P<0.05$) were separated by 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 and discussion

3.1. Oxygen consumption

Addition of succinate and pyruvate to mitochondria resulted in measurable oxygen consumption at pH 5.6 and 7.4 (succinate>pyruvate, $P<0.05$; Fig. 1). Addition of a mitochondrial inhibitor (antimycin A) decreased ($P<0.05$) the oxygen consumption associated