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Study on the apoptosis mediated by apoptosis-inducing-factor and influencing factors of bovine muscle during postmortem aging

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ABSTRACT

This study investigated the apoptosis pathway mediated by apoptosis-inducing factors (AIF) and internal factors that influence the release of AIF. The results indicated that the AIF expression was decreased in mitochondria and increased in nucleus ($P < 0.05$). However, no significant differences were observed in the AIF expression in mitochondria with caspase inhibitor treatment. The optical density of nucleus and mitochondrial swelling was significant increased ($P < 0.05$). Reactive oxygen species (ROS) fell gradually within the first 6 h and increased in the next 24 h. Calpain I activity was decreased, Ca^{2+} concentration, cathepsin B and D activities was increased ($P < 0.05$). The results demonstrated that AIF-mediated caspase-dependent pathway was a new mitochondrial apoptosis pathway and that mitochondrial swelling, ROS content, Ca^{2+} concentration, calpain I, cathepsin B and cathepsin D activities are the key influencing factors in apoptosis in postmortem bovine muscle.

1. Introduction

Currently, three main pathways of cell apoptosis exist: the first pathway involves cytochrome *c* release and caspase-3 activation, and described as the intrinsic mitochondrial pathway; the second pathway is mediated by the activation of caspase-8 and caspase-10, called the extrinsic death receptor pathway; and the third pathway, known as endoplasmic reticulum stress pathway, is mediated by the activation of caspase-12 (Gupta & Gollapudi, 2007). Mitochondria are important in regulating apoptosis. In particular, different pro-apoptotic proteins that are present in the inner membrane of the mitochondria are released to the cytoplasm and lead to apoptosis (Orrenius, 2004). Among intrinsic mitochondrial pathways, the broad-spectrum inhibitor of caspase could not completely prevent apoptosis in certain apoptosis models. Thus, another caspase-independent mitochondrial apoptosis pathway has been discovered, the apoptosis-inducing factor (AIF) pathway (Susin et al., 1999).

The mitochondrial protein AIF was the first caspase-independent death effector. AIF can induce caspase-independent chromatin condensation and large-scale DNA fragmentation to approximately 50 KB. AIF that is released to cytoplasm can mediate apoptosis when special extracellular signals trigger the opening of mitochondrial membrane permeability transition pores. Moreover, AIF is a double-edged sword. It can participate in mitochondrial oxidative phosphorylation and the respiratory chain to maintain cell survival, proliferation, and mitochondrial integrity, as well as be released from the mitochondria and further initiate nuclear fragmentation (Norberg, Orrenius & Zhivotovsky, 2010). It progresses in its participation in the mitochondria in the mechanism of apoptosis, whereas the mechanism of the mitochondrial pathway of apoptosis is extremely complicated. After bleeding, skeletal muscle cells become deprived of nutrients and oxygen and rapidly undergo the apoptosis program. Oxygen deprivation-induced apoptosis is possibly dependent on the intrinsic mitochondrial pathway (Huang et al., 2016). Unfortunately, limited information is available on the AIF release pathways during the postmortem aging of bovine muscle.

No consensus currently exists on the AIF-mediated apoptosis and how it exerts this effect. Two arguments exist in the research on AIF pathway. The first hypothesis involves AIF-mediated caspase-independent pathway; the second hypothesis is that before releasing AIF, caspase must be initially activated, that is, the caspase-dependent pathway. Yu, Meng, Wang & Yang (2002) suggested that the pro-apoptotic activity of AIF protein is fully independent and can function without caspase-3 activation. Daugas, Nochy, et al. (2000); Daugas, Susin, et al. (2000) demonstrated that AIF protein injected into the cytoplasm could make mitochondrial trans-membrane potential

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disappear and agglutinate the chromosome, thereby leading to apoptosis. However, other studies reported that AIF can trigger the release of cytochrome *c* from isolated mitochondria in vitro. In several paradigms of cell death induction, AIF is released from the mitochondria before cytochrome *c*. Therefore, AIF is related to the cytochrome *c*-dependent caspase activation cascade (Cande, Cecconi, Dessen & Kroemer, 2002). However, whether mitochondria-released AIF-mediated apoptosis is related to the caspase pathway during postmortem aging of bovine muscle is unclear. Moreover, AIF is located in the gap between the inner and outer membranes of mitochondria and can be released from the mitochondria in the induction of apoptosis signs to the cytosol and cell nucleus, thereby leading to cell apoptosis. Then, whether internal factors influence the release of AIF and exertion of biological function remains unknown. Therefore, the objectives of the current study are to investigate whether the mitochondria release AIF mediated caspase-dependent or caspase-independent apoptosis pathway and factors that affect the release of AIF in bovine longissimus muscle during postmortem aging.

2. Materials and methods

2.1. Animals and muscle sampling

Three healthy Simmental crossbreeding local yellow cattle (average age: 3 yr; average body weight: 450 kg) under the same natural grazing condition were obtained randomly from Kangle County, Gansu Province, China. They were slaughtered humanely at a commercial meat processing company (Kang Mei Meat Co. Ltd, Gansu, China). This experiment was conducted according to the "Operating Procedures of Cattle Slaughter" of the National Standards of PR China including animal welfare and conditions. After slaughter, *Longissimus thoracic* (LT) muscles (from 12th thoracic vertebrae to 5th lumbar vertebrae) were removed from the carcasses immediately. Subsequently, 15 g of muscle samples from excised LT (after removing all visible fat and connective tissue) were rapidly frozen in liquid nitrogen, which was designated as 0 h sample. Another 90 g muscle samples were vacuum packed into pouches, and then transferred to the laboratory in ice bags within 90 min. The samples were aged at 4 °C for 6, 12, 24, 72, 120 and 168 h, which served as normal aging sample. At the end of each storage period, the samples were individually obtained and treated as 0 h samples. The expression of AIF (Zhang et al., 2017), hematoxylin-eosin (HE) staining (Wang, He, Piao & Han, 2016), mitochondrial swelling (Huang, Lv, Hu & Zhong, 2013), reactive oxygen species (ROS) content (Zhang, Humphreys, Sahu, Shi & Srivastava, 2008), calcium (Ca^{2+}) concentration (Hopkins & Thompson, 2001), calpain activity (Pomponio & Ertbjerg, 2012) and cathepsins activity (Tian, Han, Yu, Shi & Wang, 2013) were determined. In addition, approximately 10 g of muscle samples from excised LT were rapidly soaked in the specific inhibitor of caspase Z-DEVE-FMK (100 μmol/L) (Sigma) in the ratio 1:1 (w/v) (meat/buffer) (Wang et al., 2018), and kept for 0, 6, 12, 24, 72, 120 and 168 h at 4 °C. At each storage period, the sample were individually obtained and only used for determining the expression of AIF with treatment of inhibitor.

2.2. Reagents

Z-DEVE-FMK, Tris, HEPES, anti-AIF antibody, rabbit polyclonal anti-GAPDH, 4′, 6-diamidino-2-phenylindole (DAPI), 2′, 7′-dichlorohydrofluorescein diacetate (DCFH-DA), casein, EDTA, Brij®35, CHAPS, Z-Arg-Arg-7-amido-4-methylcoumarin hydrochloride, haemoglobin were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used in this research were also from commercial sources and of analytical grade.

2.3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis

Mitochondria proteins were obtained according to Zhang et al. (2017) with few modifications. 1 g minced samples were homogenized in 4 mL of precooled mitochondria isolated buffer (250 mmol/L sucrose, 20 mmol/L HEPES, 10 mmol/L KCl, 1.5 mmol/L MgCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, pH 7.4). The homogenate was centrifuged at 1000*g* for 10 min. The supernatant S1 was obtained and centrifuged at 15,000*g* for 20 min. The supernatant S2 resulting from this procedure was subsequently isolated and centrifuged at 15,000*g* for 20 min. The resulting supernatant contained the enriched protein fraction and was utilized to determine the expression of AIF.

Protein samples were mixed with the sampling treatment buffer (125 mmol/L Tris-HCl, 4% SDS, 15% glycerol, pH 6.8) by 1:1 (v/v). The mixture was heated to 90 °C for 4 min, and then stored at −80 °C until loading. 20 μL samples were run on 8 mL of 12.5% separation gel and 4 mL of 4% concentration gel. The electrophoresis was carried out at 80 V for 3 h at 4C (running buffer: 25 mmol/L Tris, 192 mmol/L glycine, 1 mmol/L EDTA, pH 8.3). The target protein was transferred onto polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA) using a wet transfer apparatus (Bio-Rad Laboratories). Membranes were blocked with TTBS solution (50 mmol/L Tris-HCl, 0.05% Tween-20, 150 mmol/L NaCl, 5 mmol/L KCl, pH 7.5) containing 5% skimmed milk powder for 1 h at 25 °C and then exposed to the primary antibody for 24 h at 4 °C: anti-AIF antibody (sc-9416) from Santa Cruz (Dallas, Texas, USA) and rabbit polyclonal anti-GAPDH (G9545) from Sigma-Aldrich (Oakville, Ontario, Canada). The primary antibodies were diluted by 1:500 for anti-AIF antibody and GAPDH with TTBS solution containing 5% skimmed milk powder. Appropriate (horse radish peroxidase – conjugated goat) anti-rabbit IgGs conjugated with Alexa Fluor 568 (111-165-144, West Grove, PA, USA) secondary antibody was diluted by 1:3000 with TTBS solution containing 5% skimmed milk powder. Membranes were then washed with TTBS three times and incubated for 1 h at 25 °C with the secondary antibody. The membranes were washed with TTBS thrice again. The bands intensity of immunoblot were detected using Quantity One Software (Bio-Rad Laboratories, Hercules, CA).

2.4. HE staining

This method was conducted as described by Wang et al. (2016). Approximately 15 g samples of each aging time were fixed in 10% phosphate-buffered formalin for 24 h. Samples fixed in formalin were dehydrated, buried in paraffin wax and then sliced into 5-μm-thick histologic sections. The sections of tissues were stained with hematoxylin-eosin, and light microscopic (IX71, Olympus Microsystems Ltd., Japan) examination of each section. Nucleus were labeled with blue fluorescence by adopting 4′, 6-diamidino-2-phenylindole (DAPI) dyes. Image Pro Plus 6.0 (Media Cybernetics Inc., USA) was used to determine the optical density of nucleus.

2.5. Mitochondrial swelling detection

The extraction method of mitochondria proteins according to the Section of 2.2. The concentration of the mitochondria protein was determined through the Biuret method. The detection of mitochondrial swelling was done as described as by Huang et al. (2013) with several modifications. Mitochondria (0.5 mg/mL of protein) was added in a standard medium containing 0.5 mmol/L $FeSO_4$ and 0.5 mmol/L ascorbic acid (final volume 4 mL). The mitochondrial swelling of LT muscle during postmortem aging was tested at 520 nm in a SP-756P spectrophotometer (Spectrum Instruments Co., Ltd, Shanghai, China).