PROGRAMMED CELL DEATH (APOPTOSIS) IS INDUCED IN BOVINE MUSCLE CELLS WHEN AVAILABLE OXYGEN IS REMOVED

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Abstract – Muscle cells undergo changes post-mortem during the process of converting muscle into meat, and this complex process is far from being understood. Recent reports have suggested programmed cell death (apoptosis) to be important in the very early period of converting muscle into meat. In this study, we used primary bovine skeletal muscle cells, cultured in monolayers *in vitro*, to investigate if apoptosis is induced when oxygen is removed from the growth medium. Primary bovine muscle cells were differentiated to form myotubes, and anoxia was induced for 6h. The amount of ATP was unchanged indicating that the muscle cells were metabolically active during anoxia. The mRNA expression of anti- and pro apoptotic markers (Aif, Bcl2, Bid and Bim) were increased significantly, and the protein expression of Hsp70 and PARK7 was transiently increased. We observed a loss of mitochondrial membrane potential, which is an early apoptotic event, as well as cytochrome C release from the mitochondria. Reorganization and degradation of cytoskeletal filaments were also detected. These results suggest that muscles will enter apoptosis rapidly when available oxygen in the muscle cells diminishes post-mortem.

Key Words – Anoxia, apoptosis, conversion of muscle into meat

I. INTRODUCTION

For the industry, a predictable meat quality is essential, with tenderness, juiciness, binding properties and shelf-life being the most important quality attributes. These quality traits are controlled by both genetic and environmental factors including genetic background, age, sex, dietary status and stress prior to slaughter, as well as processing conditions such as electrical stimulation, chilling rate, tender stretch and chiller ageing. Although meat tenderness is considered the major trait of beef quality by consumers, and have received much attention from meat scientists, the molecular mechanisms of the tenderization process remains to be fully determined. The meat tenderization process is enzymatic, involving several intracellular proteolytic systems that degrade cell constituents, including caspases, calpains, proteasome, cathepsins and matrix metalloproteinases [1]. Recent reports have in addition suggested programmed cell death (apoptosis) to be important in the very early period (before the onset of rigor mortis) of converting muscle into meat (reviewed in [1-3].

Apoptosis was first defined by Kerr et al [4] who observed morphological cell alterations and cell deletion during normal embryonic development. Since then apoptosis is recognized as a basic physiologic and defense process for the body to devoid any inflammation reaction and induce cell death. Apoptosis is important during normal development and aging, and to maintain cell populations in tissues. It is also an important defense mechanism during immune reactions and exposure to cytotoxic compounds, heat, radiation, hypoxia (loss of oxygen) or infections (reviewed in [5]).

After slaughter, the skeletal muscles are depleted of oxygen, and cell death is induced. Traditionally, this has been termed necrosis, however, no inflammation is observed, and apoptosis is a better term describing the cell death in post-mortem muscle [3]. Post-slaughter conditions will affect the eating quality of beef. After animal bleeding, muscle tissue enter an anoxic state which impacts all metabolic pathways, and muscle cells enter cell death/survival pathways [1]. Apoptosis is

believed to be the first step in the conversion of muscle into meat [6], but this prosess and its involvement in meat quality is still not fully characterized. We used primary bovine skeletal muscle cells to investigate this *in vitro*, mimicking slaughtering conditions by removing oxygen (anoxia).

II. MATERIALS AND METHODS

Samples: Bovine primary skeletal muscle cells were isolated as previously described [7]. *Anoxia:* Oxyrase was used to remove oxygen from the growth media (anoxia). Oxyrase-treated samples were incubated in a medium consisting of 89 % differentiation medium (DM), 10 % sodium lactate and 1 % oxyrase for 6h. *Viability assay:* Measured using CellTiter-Glo® Luminescent Cell Viability Assay (Promega). *RNA extraction and real-time PCR:* Cells were lysed and further purified using RNeasy minikit including DNase treatment. Real-time PCR was performed on a ABI Prism 7700 Sequencing detection system. Gene expression was normalized to TATA and ΔCt values calculated. Comparison of gene expression between two samples (oxygen removal and without oxygen removal) was derived from subtraction of ΔCt values between the two samples to give a ΔΔCt value, and relative gene expression calculated as $2^{-\Delta\Delta Ct}$. *Immunofluorescence analyses*: Cells were grown on coverslips, fixed with PFA before immunostaining with primary antibody, followed by an Alexa-conjugated secondary antibody. The cells were subsequently analysed by fluorescence microscopy. *Western blot:* Protein samples from cells were run on a 10 % Bis-tris gel. Each membrane was stained with primary antibodies against PARK7 and HSP70. Membranes were subsequently stained with secondary antibodies CY3 and CY5, and finally scanned using an Ettan DIGE Imager.

III. RESULTS AND DISCUSSION

Apoptosis is an energy-dependent cascade of molecular events that eventually leads to termination, including activation of caspases, DNA fragmentation, degradation of cytoskeletal and nuclear proteins, formation of apoptotic bodies and finally uptake by phagocytic cells [5]. Our experiments demonstrate that the amount of ATP was unchanged during anoxia indicating that the muscle cells were metabolically active (Fig.1), capable of driving the apoptosis process.

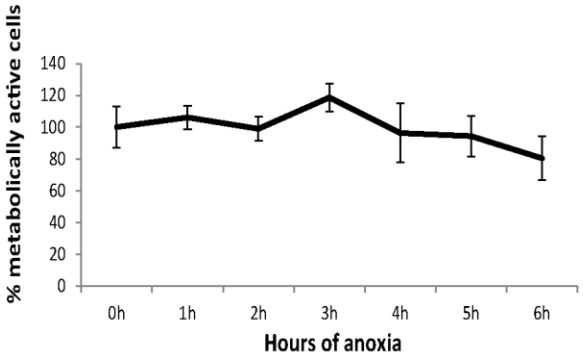


Fig. 1: The muscle cells were metabolically active during anoxia.

Cells exposed to stress try to suppress the apoptotic program, and they try to repair the damage. Members of the heat shock protein (HSP) family of molecular chaperone are capable of achieving this. Hsp70 has previously been shown to regulate the release of cytochrome *c* and pro-apototic factors from the mitochondria [8]. PARK7 is also shown to protect against apoptosis by decreasing the expression of pro-apoptotic factors and by inhibiting caspase activation [9]. We observed a transient expression of both Hsp70 and PARK7 (Fig. 2), suggesting a transient protecting mechanism.

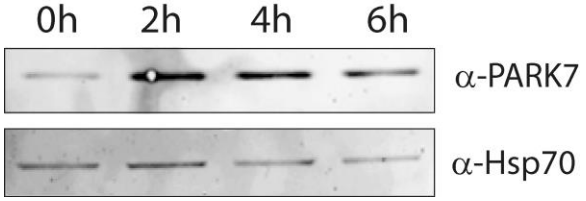


Fig. 2: Apoptosis inducers. The Bcl-2 family of pro-apoptotic molecules. The Bcl-2 family plays a pivotal role in deciding whether a cell will die or live. PARK7 and Hsp70 is observed during anoxia.

The muscle cells contain a number of pro- and anti-apoptotic factors, and many of these interact when apoptosis is initiated. The Bcl-2 proteins are central regulators of mitochondrial permeability and release of pro-apoptotic molecules. The Bcl-2 family plays a pivotal role in deciding whether a cell will die or live. Bcl-2 and Bcl-xL are anti-apoptotic members localized in the mitochondrial and endoplasmic

reticular membranes, as well as in the nuclear envelope. In the mitochondria, Bcl-2 and Bcl-xL preserve mitochondrial integrity and prevent the subsequent release of apoptotic molecules. The mRNA expression of anti- (Bcl-2) and pro-apoptotic markers (Aif, Bid and Bim) were increased significantly (Fig.3), indicating the onset of apoptosis.

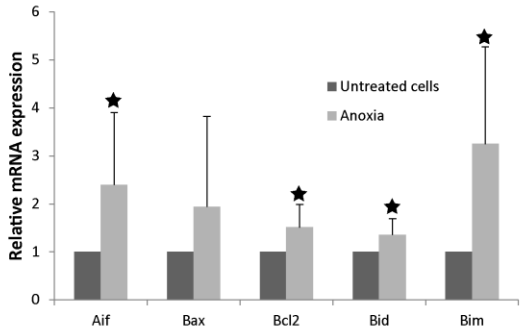
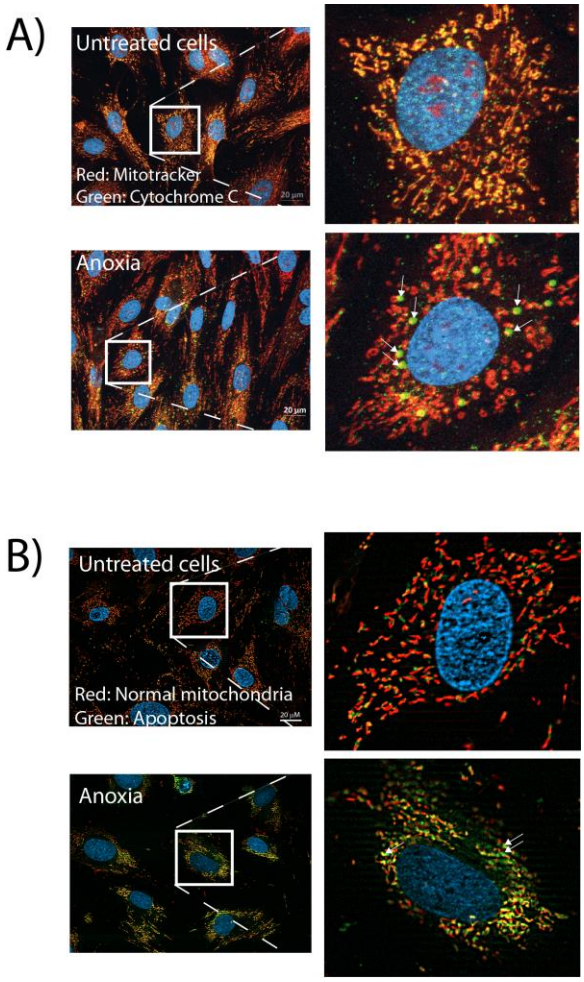


Fig. 3: The gene expression of anti- and pro apoptosis markers increased during anoxia 6h. Asterisk denote Asterisk denote significant differences between untreated cells and anoxic cells. (*p<0.05).

The ratio between Bcl-2 and Bax determines the susceptibility of the cells to undergo apoptosis. Downstream of this checkpoint are two major execution programs: the caspase pathway and mitochondrial dysfunction. Mitochondrial dysfunction includes a change in the mitochondrial membrane potential, production of reactive oxygen species (ROS), opening of the permeability transition pore (PTP), and the release of the intermembrane space protein, cytochrome *c*. Released cytochrome *c* activates Apaf-1, which in turn activates a downstream caspase program. We observed a loss of mitochondrial membrane potential, which is an early apoptotic event, as well as cytochrome *c* release from the mitochondria (Fig.4A and B). We did not observe an increase in caspase 3/7 or caspase 9, cysteine proteases normally active during apoptosis (data not shown). A possible reason for this could be that the caspases are shown to have a non-apoptotic role during muscle differentiation [10, 11]. There is also evidence showing that cells exposed to stress can initiate a suicide program that does not rely on caspase activation [12]. Finally, to date, ten major caspases have been identified [5], and other caspases than caspase 3/7 or 9 could be important in muscle cells. This remains to be examined further.



The breakdown of the cytoskeleton is an indicator of apoptosis. Actin and desmin are known

substrates for proteolytic enzymes, and microtubules are known to disassembly during early apoptosis. We did observe a re-organization of cytoskeletal filaments during anoxia (Fig.5).

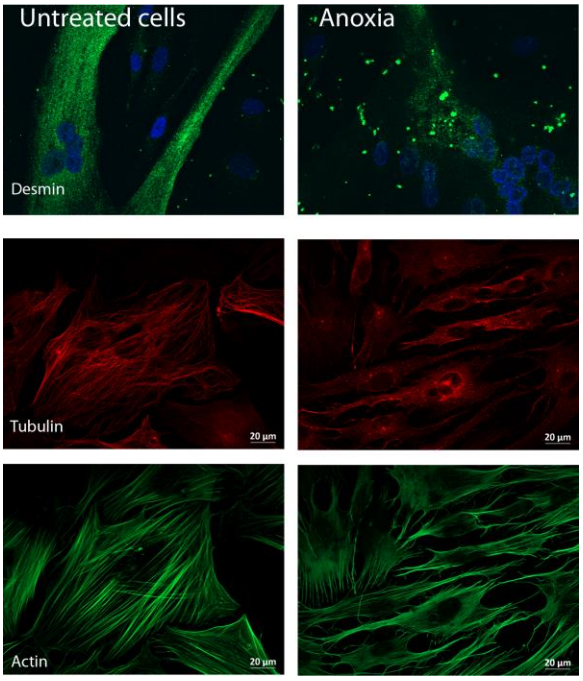


Fig. 5: Anoxia induced cytoskeletal re-organization during anoxia 6h. Measured using immunofluorescence with desmin ab (Abcam), tubulin ab (Sigma Aldrich) and Alexa-488 phalloidin (Life technologies).

IV. CONCLUSION

Altogether, these results suggest that muscles will enter apoptosis rapidly when available oxygen in the muscle cells diminishes post-mortem. The muscle cells try to protect themselves from the harmful environment post-anoxia by expressing protective markers such as Hsp70 and PARK7. We demonstrate an increased expression of pro- and anti-apoptotic markers, a loss of mitochondrial membrane potential, release of cytochrome *c*, and finally a re-organization of the cytoskeletal proteins desmin, tubulin and actin.

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