EFFECTS OF CAMPTOTHECIN AND ETOPOSIDE ON EXPRESSION OF CASPASE-3, CALPAIN AND CALPASTATIN OF CHICKEN DURING POSTMORTEM AGEING

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***Abstract*—Calpain activity is an important factor in the conversion of muscle into tender meat, and recently consideration has focused on the potential of a key protease of apoptosis, caspase-3, as being involved in the underlying mechanism for the postmortem tenderization of meat. As there are several common factors of meat ageing and apoptosis, we used apoptosis inducers Camptothecin and Etoposide to treat chicken muscle after slaughter and followed the outcome during 7 days of ageing. Our results showed that each treatments stimulated caspase-3/7 activity significantly during storage time ($p<0.01$). According to the results of Western blots, all the treatments enhanced the band intensity of calpain ($p<0.05$) but reduced calpastatin ($p<0.05$) during 7 days of meat ageing. The findings of this investigation revealed that caspase-3 possibly activates calpain via calpastatin degradation. We speculate that, through the calpain/calpastatin system, caspase-3 has the potential to contribute to the tenderisation of meat during the conversion of muscle tissue into meat.**

***Index Terms*—caspase-3, calpain, calpastatin, postmortem ageing**

I. INTRODUCTION

It is well established that that proteolysis of muscle is a major factor contributing to the postmortem tenderization of meat. Many reports have demonstrated that the calpain system plays a primary role in this proteolysis (see Koohmaraie & Geesink 2006). The proteolytic system of calpain has the ability to degrade cytoskeletal proteins, such as tropomyosin, troponin T, troponin I and desmin, which are responsible for the muscle fibers structural integrity (Koohmaraie 1992). The ubiquitous calpains, μ-calpain and m-calpain, are negatively regulated by interaction with their endogenous specific inhibitor calpastatin. Several studies have shown that tenderness of meat improved by the proteolytic action of the calpain-calpastatin system (Sentandreu, Coulis & Ouali 2002). Although there is considerable evidence to suggest that the activity of the calpain system early in the postmortem conditioning period influences the ultimate tenderness, overall meat tenderization depends on a multienzymatic system and the calpain system is not the sole proteolytic determinant of meat quality. Within a short time after slaughter when oxygen has been depleted from the tissues the muscle fibres will go to the condition of hypoxia-ischemia (HI), which will initiate an apoptotic–necrotic respone in the cells that become damaged. Recently, some meat scientists have proposed that an additional step should be considered befor rigor mortis, viz. apotosis (Herrera-Mendez, Becila, Boudjellal & Ouali 2006). Apoptosis is a common physiological phenomenon that occurs in proliferating and constantly renewing tissues, and caspase-3 is a member of the interleukin-1β-converting enzyme family of cysteine proteases, which trigger the execution phase of apoptosis (Chowdhury, Tharakan & Bhat 2008). Since the meat ageing and apoptosis have so many aspects in common, the question whether caspase-3 could be one candidate, or totally account for meat ageing become disputable. Recently, it has been demonstrated that caspase-3 activity was activated, and its activity at 0 to 32 h was negatively associated with Warner-Bratzler shear force after slaughter (Kemp, Bardsley & Parr 2006). This indicated that changes in caspase activity and caspase-mediated cleavage take place in muscle during the conditioning period, and this appears to be associated with the development of tender meat (Kemp, Parr, Bardsley & Buttery 2006). There is a more recent study which further investigates the potential role of caspases in meat tenderisation by determining if recombinant caspase-3 could degrade myofibrillar proteins (Kemp & Parr 2008). Huang, Huang, Xu & Zhou (2009) used caspase-3 selective inhibitor to show that it inhibited the degradation of muscle skeletal proteins significantly. Despite this, other researchers eliminate caspase-3 as not being involved in postmortem tenderization of beef (Underwood, Means & Du 2008).

Camptothecin is an alkaloid isolated from *Camptotheca acuminata* (family *Nyssaceae*) which has the ability to form intracellular DNA-protein adducts, and reversibly inhibits nuclear topoisomerase by binding to, and stabilizing the topoisomerase-DNA covalent complex (Murren, Beidler & Cheng 1996). Etoposide, a derivative of podophyllotoxin and is a topoisomerase II inhibitor and a potent inducer of DNA strand breaks by causing a cleavable complex formed between topoisomerase II and DNA (Floros, Thomadaki, Florou, Talieri & Scorilas 2006). In this investigation, we used these two types of topoisomerase inhibitors as apoptosis inducers to examine whether these treatments would effect the expression of caspase-3, calpain and calpastatin of chicken during postmortem ageing.

II. MATERIALS AND METHODS

A. Animals and treatment

Four Yellow-feathered chickens, a Chinese native breed (female, 45 days, 2.0-2.5 kg), from the animal experimental

station attached to Nanjing Agricultural University. After being sacrificed, the four chicken breast muscles (*Musculus pectoralis superficialis*) were rapidly removed and dissected into small pieces (0.5 g/piece). The 0 d sample was obtained within 20 min. Other samples were divided into three portions, one portion had no treatment and the other two were soaked in a treatment solution containing 40 mg/ml of either Camptothecin or Etoposide (meat : solution; 1:2 w/v), and was stored at 4℃ for 1, 3, 5 or 7 d.

B. Preparation of sarcoplasmic proteins

Finely minced meat samples (1.0 g) were homogenized with a polytron at a speed of 15,000 rpm for 10 s, six times, with a 10 s cooling interval between bursts; 1.0 g of chicken breast muscle was homogenized in 2 volumes (w/v) of extraction buffer containing 25 mM Tris–HCl (pH 7.6), 150 mM NaCl, 50 mM EDTA, 1mM DTT, 1mM PMSF, 1% Triton X-100 and protease inhibitor cocktail (10 ml/tablet). The homogenate was centrifuged at 14,000 g for 40 min at 4℃, and then the supernatant was collected and protein concentrations were determined using a BCA Protein Assay Kit.

C. Determination of caspases-3/7 activity

Caspase-3/7 activity was assessed by using the end-point Apo-One Homogeneous Caspase-3/7 Assay (Promega) according to the manufacturers' protocol. The caspase reagent was added to sarcoplasmic proteins of supernatant at a 1:1 ratio and incubated at 37℃ for 4 h. A fluorescence spectrometer (Tecan), at an excitation wavelength of 485 ± 20 nm and an emission wavelength of 530 ± 25 nm was used to determine activity.

D. SDS–PAGE and Western blot analysis

SDS-PAGE was performed on 4% stacking gel and 12.5% separating gel in a discontinuous buffer system. Gels for analysis of calpain and calpastatin were run on Mini-PROTEAN Tetra cell (Bio-Rad). Gels were loaded with 30 μg per well of protein for calpain and calpastatin, and run at a constant voltage of 80 V for 45 min, and then of 120 V for approximately 2 h. Then, the gels were immediately transferred to PVDF membranes (Millipore) using a Semi-Dry Transfer Cell (Bio-Rad). The electroblotted membrane was then blocked overnight at 4 ℃. After blocking in blocking buffer (TTBS: 0.1% Tween 20, 20 mM Tris, 137 mM NaCl, 5 mM KCl and 5% skim milk powder) for 90 min, membranes were rinsed by TTBS and incubated overnight at 4 ℃ with 1:500 diluted primary antibody in TTBS which containing 5% BSA and 0.1% Tween-20. Excess antibody was then removed by washing the membrane in TTBS and subsequently incubated for 1 h with secondary antibody diluted 1:2,500 in TTBS at room temperature. After several washes in TTBS, bands were visualized with DAB (Sigma-Aldrich). The membranes were scanned with a scanner, and then the intensities were quantified by Quantity One software (Bio-Rad) within the calibration range.

E. Statistical analyses

Data are showed as mean ± SD. The data were analyzed using the SPSS statistical package programmer by one-way ANOVA, and differences among the individual means were compared by Duncan's multiple range tests with $p < 0.05$ considered as the level for significance.

III. RESULTS AND DISCUSSION

Both Camptothecin and Etoposide treatments induced higher Caspase-3/7 activity compared with the control (Figure 1; $p<0.01$). There was a sharp decrease in caspase-3/7 activity after 0 day but levels did not much vary after 3 day (Figure 1). For Camptothecin, the major topoisomerase inhibitor, cytotoxicity is due to the trapping of cleavable complexes of topoisomerase I, thus increasing the steady-state concentration of topoisomerase I-DNA cleaved complexes (Kong & Rabkin 1999). Etoposide activity is mediated by its interaction with topoisomerase II, promoting the formation of a DNA-topoisomeraseetoposide complex (Stumpp, Sasso-Cerri, Freymüller & Miraglia 2004). When these permanent DNA breaks are present at sufficient concentration, a series of events occurs that culminates in apoptosis (Russell, Hunsicker, Johnson & Shelby 1998). Some apoptotic effects such as cell cycle arrest, membrane alterations, caspase-3 activation, etc. are derived from its action on different types of cells. Our results showed these two apoptosis chemicals did lead to acutely higher caspases-3/7 activity during the course of 7 days of ageing.

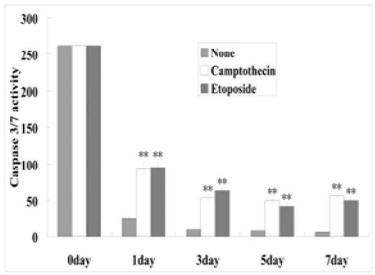

Fig. 1. Effect of Camptothecin and Etoposide treatments on caspase-3/7 activities.

In this study, western blots detected immunopositive bands of the large calpain subunit at 80 kDa. Camptothecin, Etoposide had similar effects on calpain, which made the intensity values of calpain extraordinary higher than control (Figure 2, 3). Statistically to use the 0 day as scale in each western picture, there were significant differences in 1 day ($p<0.05$), dramatically significant differences in 3, 5 day ($p<0.01$), and higher in 7 day (Figure 2, 3; $p=0.11$, $p=0.15$, respectively). Although caspase-3 has been identified as a key protease in the execution of apoptosis, calpain is activated in various necrotic and apoptotic conditions (Wood & Newcomb 1999). Calpains have been demonstrated to participate in caspase-dependent apoptosis pathways (Wang 2000). It is a controversial issue whether activation of calpain occurs upstream or downstream of caspase-3. Growing evidence suggest that calpains may play a main role in the execution of apoptosis either upstream or downstream of caspases in such as thymocytes and MCF-7 breast cancer cells (Pink et al. 2000; Squier & Cohen 1997). It should be noted that activation of calpain may occur downstream of caspase-3 in constitutive apoptosis of neutrophils and HL-60 cells as has been found in the U18666A-mediated neuronal apoptotic phase (Knepper-Nicolai, Savill & Brown 1998; Koh et al. 2006; Wood et al. 1999). However, it demonstrated the effect of simvastatin on apoptosis is association with activation of caspase-3 via calpain in vascular smooth muscle cells and (Cheng et al. 2003).

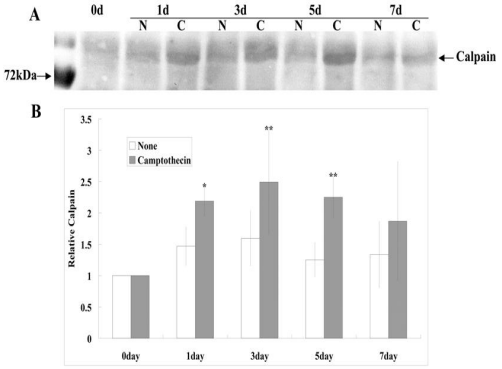

Fig.2 Effect of Camptothecin on calpain immunochemical detection.

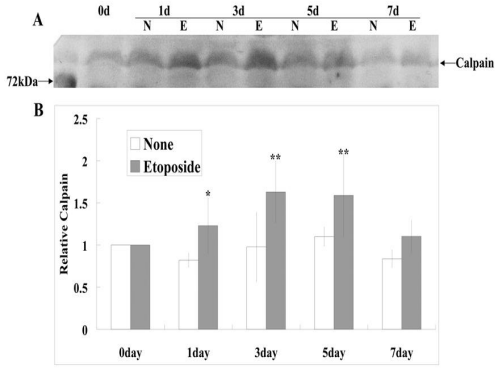

Fig.3 Effect of Etoposide on calpain immunochemical detection.

Western blots detected fragment bands of calpastatin, and the results confirmed both Camptothecin and Etoposide caused significant reduction in band intensity during 7 days postmortem ageing (Figure 4, 5). Relating amounts to 0 day, the difference of Etoposide was significant lower at 1 day ($p<0.05$, Figure 4) and at 3, 5 & 7 day ($p<0.01$, Figure 4), while the difference of Camptothecin were significant lower in 3 day ($p<0.05$, Figure 4) and at 3, 5 & 7 day ($p<0.01$, Figure 5). Calpastatin is present in most cells where calpain activation is low as a result of the expression of calpastatin (Melloni et al. 1998). Furthermore, it has been hypothesized that an increase in protein breakdown is regulated through an increase in calpain activity and/or a decrease in calpastatin activity, which would ultimately result in more tender meat (Therkildsen, Larsen, Bang & Vestergaard 2002). Shackelford et al. (1991, 1994) indicated that the activity of calpastatin postmortem provided an estimable predictor of postmortem tenderness. Other studies have also shown a similar relationship between calpastatin activity and shear force measurements in beef (Whipple, Koohmaraie, Dikeman & Crouse 1990) and lamb (Koohmaraie & Shackelford 1991).

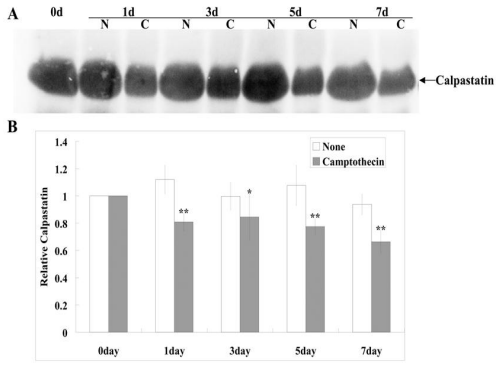

Fig.4 Effect of Camptothecin on calpastatin immunochemical detection.

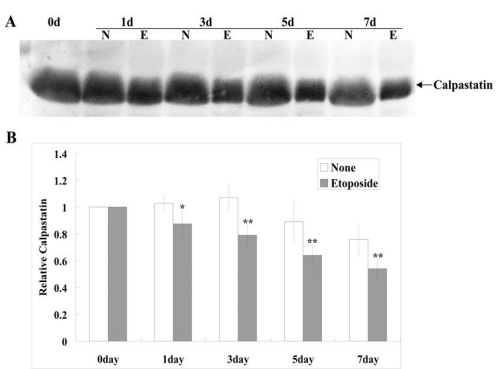

Fig.5 Effect of Etoposide on calpastatin immunochemical detection.

Execution of apoptosis may be mediated by the combined actions of calpains and caspases. Calpain activity is

regulated by the endogenous inhibitor, calpastatin. Interestingly, in several experimental models of apoptosis it has been shown that calpastatin is cleaved by caspase-3 and that this cleavage is essential for the regulation of calpain activity during cell death (Rami, Agarwal, Botez & Winckler 2000). Neumar, Xu, Gada, Guttmann & Siman (2003) report that during the early phase, calpain down regulates caspase-3 activity, whereas subsequent calpain activity facilitated by caspase-mediated degradation of calpastatin.

IV. CONCLUSION

After animal slaughter, the muscle cell will go through the hypoxia-ischemia (HI) condition and inevitably, apoptosis or necrosis. Given our current knowledge of the mechanism of apoptosis or necrosis, we used typical apoptosis inducer chemicals Camptothecin and Etoposide on chicken muscle sarcoplasmic proteins after slaughter. From our results we speculate that activation of caspase-3 induced calpastatin cleavage leading to an enhancement of calpain activity during postmortem ageing. This work indicates an association of caspase-3 with meat tenderization.

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