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Effects of Inhibitors on the Synergistic Interaction between Calpain and Caspase-3 during Post-mortem Aging of Chicken Meat

Lin Chen, Xian Chao Feng,[§] Wan Gang Zhang, Xing Lian Xu, and Guang Hong Zhou*

Key Laboratory of Meat Processing, Quality Control, Ministry of Education, Nanjing Agricultural University, Nanjing 210095, China

ABSTRACT: Calpain has been considered to be the most important protease involved in tenderization during the conversion of muscle into meat. However, recent evidence suggests the possible involvement of the key apoptosis protease, caspase, on post-mortem tenderization. This study used inhibitors of calpain and caspase-3 to treat chicken muscle immediately after slaughter and followed the changes in caspase-3 and calpain activities together with their expression during 5 days of aging. Addition of calpain inhibitors to the system resulted in significantly higher caspase-3 activities ($p < 0.01$) during storage. Western blot analysis of pro-caspase-3 and α-spectrin cleavage of the 120 kDa peptide (SBDP 120) showed that the addition of calpain inhibitors resulted in the formation of higher amounts of the active form of caspase-3 compared with the control ($p < 0.01$). Inclusion of inhibitors of caspase-3 led to lower calpain activities ($p < 0.01$) and dramatically reduced the expression of calpain-1 and calpain-2 ($p < 0.01$). Concomitantly, this inhibition resulted in greater calpastatin expression compared with the control ($p < 0.01$). The findings of this investigation show that calpain prevented the activation of caspase-3, whereas caspase-3 appeared to enhance the calpain activity during post-mortem aging through inhibition of calpastatin. It is therefore suggested that there is a relationship between caspase-3 and calpain which contributes to the tenderizing process during the conversion of muscle tissue into meat.

KEYWORDS: *calpain, caspase-3, aging, apoptosis, calpastatin, inhibitor*

INTRODUCTION

The tenderizing of meat during the aging process is highly complex, and although there remains some controversy regarding the actual mechanism, it is generally accepted that the major components affected by proteolysis are the key myofibrillar proteins.[1] Several studies suggest that some of the ultrastructural changes occurring in mammalian muscle during aging are associated with the action of calpains; moreover, there is much evidence that the calpain system plays a primary role in proteolysis.[1] Early studies on meat focused on post-mortem tenderization by calpain-1 and calpain-2. These two ubiquitous calpains are negatively regulated by interaction with their endogenous specific inhibitor, calpastatin.[2] Many studies have shown that the tenderness of meat is improved by the proteolytic action of the calpain−calpastatin system.[3] Although there is large amount of data showing that the calpain system has the major effect on the tenderness of meat during post-mortem aging, there is evidence that other proteolytic systems are involved in a complex multienzyme system.[4,5] Soon after animal slaughter, the tissues become exhausted of oxygenated blood by exsanguination, leading to hypoxia−ischemia (HI), which initiates an apoptotic−necrotic response.[6] Recently, it has been proposed that apoptosis should be considered as a step prior to the development of rigor mortis.[7] Apoptosis is a common physiological event that occurs in proliferating and regenerating tissues. Caspase-3, a member of the interleukin-1β-converting enzyme family of cysteine proteases, plays an important role during the execution phase of apoptosis.[8] It has been demonstrated that in post-rigor meat, caspase-3 activity is stimulated and its activity is negatively associated with Warner−Bratzler shear force, thus suggesting an improvement in tenderness.[9] Furthermore, the same group found that recombinant caspase-3 degraded myofibrillar proteins. A recent study that further investigated the potential role of caspases in meat tenderization by using a caspase-3 selective inhibitor, DEVD-CHO, showed that the inhibitor significantly reduced the degradation of skeletal muscle proteins.[10] Also, in our work with chicken meat, we showed that the three apoptosis inducers used increased myofibrillar dissociation and proteolysis during the first 3 days of aging.[11]

Caspase-3, like calpain, is a cytosolic cysteine protease, but does not require Ca^{2+} for activity as does calpain. Caspases have a central role in transducing the apoptosis signal, and caspase-3 activation is a distinctive characteristic of apoptosis. In contrast, necrotic cell death is, almost without exception, associated with massive Ca^{2+} influxes, thus resulting in calpain activation. Calpains have been implicated in various necrotic[12] and apoptotic conditions,[13] whereas caspase-3 has been identified as a key protease in the execution of apoptosis. Furthermore, there is growing evidence indicating a significant synergistic interaction and functional connections between calpains and caspases in the regulation of apoptosis.[14,15] Because the calpain system has been considered to be most important in the initial tenderizing process of meat, the recent implication of caspase-3 as playing an additional role has prompted questions regarding the actual relationships or interactions between these two proteolytic enzymes. On the basis of the available evidence, calpains have the potential to both positively and negatively modulate the caspase cascade during apoptosis, thus determining the relative roles of the two proteolytic systems in meat aging.

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MDL-28170 (amino acid sequence Z-Val-Phe-CHO) is a potent membrane-permeable cysteine protease inhibitor that inhibits calpain. This cell-permeable compound inhibits oxidative damage-induced apoptosis in PC12 cells[16] and capsaicin-induced apoptosis in dorsal root ganglion neurons.[17] Calpeptin (*N*-benzyloxycarbonyl-L-leucylnorleucinal), another effective and cell-permeable calpain inhibitor, can inhibit both calpain-1 and calpain-2.[18,19] Calpeptin inhibits calpain activation and prevents apoptosis in cultured cells. DEVD-CHO (amino acid sequence N-acetyl-Asp-Glu-Val-Asp-CHO) is recognized as a highly specific, powerful, and reversible inhibitor of caspase-3.[20] We have designed this investigation to examine interactions between the calpain and caspase protease systems in post-mortem chicken meat by using the inhibitors MDL-28170 and calpeptin to prevent the function of calpain and DEVD-CHO to inhibit caspase-3 activity. The objective of this study was an attempt to demonstrate the possibility of a synergistic interaction between the two proteolytic systems, calpain and caspase-3, as well as the three-way interaction with calpastatin. Moreover, we provide further evidence regarding the role of caspase in the tenderization of chicken meat.

MATERIALS AND METHODS

Chemicals. The following chemicals were purchased from Amresco Inc. (Solon, OH, USA): phenylmethanesulfonyl fluoride (PMSF), sodium dodecyl sulfate (SDS), 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris), Triton X-100, DL-dithiothreitol (DTT), calcuim chloride ($CaCl_2$), dimethyl sulfoxide (DMSO), and ethylenediaminetetraacetic acid disodium salt, dihydrate (EDTA Na_2).

The following chemicals were sourced as indicated: MDL-28170, calpeptin, DEVD-CHO, Ac-DEVD-AFC, Suc-LLVY-AMC (Enzo Life Sciences International, Plymouth Meeting, PA, USA); complete protease inhibitor cocktail tablets in EASYpack (Roche Diagnostics GmbH, Mannheim, Germany); BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA); rabbit polyclonal to caspase-3, mouse monoclonal to calpain-1, mouse monoclonal to calpain-2, mouse monoclonal to calpastatin, and mouse monoclonal to GAPDH (Abcam Inc., Cambridge, MA, USA); anti-Fodrin (spectrin) monoclonal antibody (Millipore, Temecula, CA, USA); anti-rabbit IgG, HRP-linked antibody, anti-mouse IgG, HRP-linked antibody (Cell Signaling Technology, Inc., Danvers, MA, USA); DAB (Sigma-Aldrich, St. Louis, MO, USA); ECL Western blotting detection reagents (GE Healthcare U.K. Ltd.).

All other chemicals and reagents were obtained from commercial sources at the highest grade of purity available.

Animals and Treatment. Four Yellow-feathered chickens, a Chinese native breed (female, 45 days, 2.0−2.5 kg), from the animal experimental station of Nanjing Agricultural University, were cared for and slaughtered as outlined in the guide for the care and use of experimental animals (Animal Experimental Special Committee of NAU). Chickens were slaughtered and breast muscles (Musculus pectoralis superficialis) from each bird were rapidly removed, cut into small pieces (about 0.2 g/piece), and thoroughly mixed. The chicken breasts were excised within 15 min after bleeding. A sample from each chicken was immediately (0 day) snap-frozen in liquid nitrogen (<10 min) and stored until required for subsequent analysis. The remainder of each of the individual batches of chopped muscle was then subdivided into four fractions and allocated to treatments as follows: one portion received no treatment (control), and the three other samples were placed in solutions containing either 100 μM MDL-28170, calpeptin, or DEVD-CHO in the ratio 1:2 (meat/solution; w/v). After addition of inhibitors, the solutions were divided in three parts and stored at 4 °C for either 1, 3, or 5 days as previously described.[11] During storage, we investigated the pH of all samples. The pH values were all about 5.65−5.80, and there was no apparent difference between the control and treated samples. At the end of each storage period the meat samples were taken individually and stored in liquid nitrogen until required for analysis.

Preparation of Sarcoplasmic Protein. Preparation of sarcoplasmic proteins was performed according to the procedure reported by Koumura et al.[21] with some modifications. The control and inhibitor-treated meat samples were homogenized (S10, Ningbo China) in 2.0 mL/g tissue in ice-cold lysis buffer containing 25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 50 mM EDTA, 1 mM DTT, 1 mM PMSF, 5 mM sodium fluoride (NaF), 1% Triton X-100, and protease inhibitor cocktail (10 mL/tablet) at a speed of 25000 rpm for six periods of 10 s, with a 10 s cooling period between bursts. The homogenates were then centrifuged at 18000*g* for 30 min at 4 °C, and the supernatants were collected and adjusted to equal protein concentrations following determination of protein concentrations using a BCA Protein Assay Kit.

Determination of Caspase-3 and Calpain Activities. Assay of caspase-3 activity was performed according to the previously described method.[22] The sarcoplasmic samples were incubated for 120 min in a reaction buffer (50 mM HEPES, pH 7.5, 10% sucrose, 10 mM dithiothreitol, 0.1% CHAPS) at 37 °C with 25 μM Ac-DEVD-AFC. Each reaction mixture contained 100 μL of the volumes of the sarcoplasmic samples and 100 μL of the substrate solution. Additional samples contained the inhibitor of caspase-3, DEVD-CHO at a concentration of 1 μM (λ_{ex} = 400 nm; λ_{em} = 505 nm). For the assessment of calpain,[23] the sample was mixed in calpain assay buffer, consisting of 115 mM NaCl, 1 mM KH_2PO_4, 5 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 25 mM HEPES, pH 7.4, supplemented with 60 μM Suc-LLVY-AMC (λ_{ex} = 380 nm; λ_{em} = 460 nm) and incubated at 37 °C in the absence or presence of 50 μM calpeptin. Fluorescence was measured using a MD spectrofluorometer (Spectramax M2), and activity was calculated as the difference between the substrate utilization velocity in the samples with and without inhibitor.

Protein concentration was determined using a BCA Protein Assay Kit.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis. SDS-PAGE was performed on 4% stacking gel and 12.5% separating gel in a discontinuous buffer system as described by Huang et al.[10] An appropriate aliquot of each sarcoplasmic protein sample was diluted to 1.5 mg/mL using treatment buffer containing 0.5% β-mercaptoethanol, 10% glycerol, and 0.02% bromophenol blue. Samples were well mixed and heated at 50 °C for 20 min. The analyses of α-spectrin, caspase-3, calpain-1, calpain-2, and calpastatin degradation were determined on a Mini-PROTEAN Tetra cell (Bio-Rad Laboratories, Hercules, CA, USA), and the gels (9 cm wide × 8 cm length) were loaded with 40 μg of protein per well. The degradation of α-spectrin, caspase-3, calpain-1, calpain-2, and calpastatin was analyzed by Western blot analysis, and gels were immediately transferred to PVDF membranes (Millipore) using a Semi-Dry Transfer Cell (Bio-Rad Laboratories) at a constant current of 2.5 mA/cm^2 for 50 min. The electroblotted membrane was then blocked overnight at 4 °C in blocking buffer, TTBS (0.1% Tween 20, 20 mM Tris-HCl, 137 mM NaCl, 5 mM KCl and 5% skim milk powder, pH 7.4) for 90 min. Membranes were rinsed in TTBS and then incubated overnight with gentle shaking at 4 °C in TTBS containing a 1:500 dilution of primary antibody together with 5% BSA and 0.1% Tween-20. Excess antibody was then removed from the membrane by washing in TTBS. The membrane was subsequently incubated for 1 h at room temperature with anti-mouse IgG HRP-conjugated or anti-rabbit IgG HRP-linked secondary antibody diluted 1:2500 in TTBS. After several washes in TTBS, the bands of α-spectrin and calpastatin were visualized with DAB, and the bands of caspase-3, calpain-1, calpain-2, and GAPDH were detected by ECL Western blotting detection reagents.

The membranes showing caspase-3, calpain-1, calpain-2, and GAPDH were photographed with a Gel Doc XR System (Bio-Rad Laboratories), the membranes showing α-spectrin and calpastatin were scanned (ScanMaker 4100, MICROTEK, Shanghai China), and then the intensities of the bands were quantified using Quantity One software (Bio-Rad Laboratories) within the calibration range.

Statistical Analyses. Data are shown as the mean ± SD. The data were analyzed using the SPSS statistical package program by one-way ANOVA, and differences among the individual means were compared by Duncan's multiple-range tests with $p < 0.01$ as the level for significance.

RESULTS AND DISCUSSION

Effect of Calpain on Caspase-3 Activity. The conversion of the substrate Ac-DEVD-AFC into free AFC was used as a measure of caspase-3 activity in the presence and absence of a calpain inhibitor. Compared with the initial caspase-3 activity on day 0, there was a significant increase in the control and treatment groups by day 1 (Table 1). By day 3 caspase-3 activities had commenced to fall, and by day 5 of aging the activities of all samples had reduced by about 33−61% compared with that measured on day 1. The caspase-3 activities in the presence of MDL-28170 or calpeptin were significantly higher ($p < 0.01$) on each of the 5 days post-mortem compared to the control (Table 1).

Table 1. Caspase-3 Activities of Chicken Meat in the Absence (Control) and Presence of Calpain Inhibitors, Calpeptin and MDL-28170, during Storage at 4 °C for 0, 1, 3, or 5 Days[a]

	caspase-3 activity		
days of storage	control	MDL-28170	calpeptin
0	48.34 ± 3.32		
1	61.76 ± 1.99a	177.26 ± 4.33b	140.62 ± 2.40b
3	34.41 ± 3.82a	51.33 ± 3.66b	67.56 ± 2.32b
5	20.53 ± 0.75a	82.32 ± 2.16b	85.80 ± 4.50b

[a]Activity was based on the conversion of the substrate Ac-DEVD-AFC to free AFC. Values are expressed as absorbance (arbitrary units) per milligram of protein. Each value represents the mean ± SD, $n = 4$. Means within a row with different letters are significantly different ($p < 0.01$).

Caspase-3 is synthesized as a precursor molecule, which is approximately 32 kDa in size and is activated by intrachain proteolytic cleavage by conversion into two active subunits,[24] and the protein is processed and activated by caspase-8, -9, and -10[8]. Using immunoblotting, we have demonstrated that the pro-form of caspase-3 decreased during post-mortem storage (Figure 1). When the calpain inhibitors were present, it can be seen that both MDL-28170 and calpeptin resulted in significantly lower amounts of the pro-form of caspase-3 compared with the control relative to day 0 ($p < 0.01$, Figure 1). The caspase-3-mediated cleavage of α-spectrin generates 120 kDa degradation products (SBDP120), but α-spectrin can also be cleaved by calpains, producing a 150 kDa peptide (SBDP150).[25] In this study Western blots probed with anti-α-spectrin detected immunopositive bands of intact 240 kDa, α-spectrin, and cleavage products of 150 and 120 kDa (Figure 2A). Treatment of muscle with MDL-28170 or calpeptin each significantly ($p < 0.01$) reduced the degradation of α-spectrin, resulting in lesser amounts of the 150 kDa fragment. These differences, compared with the controls, were significant for each of the storage days when using the 150 kDa of day 0 as a marker for each of the Western blot images ($p < 0.01$) (Figure 2B). However, the calpain inhibitors increased the 120 kDa fragment (Figure 2A), showing very high intensity values of the SBDP120 in the treated groups during 5 days of post-mortem aging (Figure 2C). These results show that the inhibitors of calpain, MDL-28170 and calpeptin, both enhance the level of caspase-3 cleavage. The two inhibitors have similar enhancing effects on the activity of caspase-3 during the tenderization of chicken.

Complete understanding of the biochemical basis of meat tenderization requires identification of the enzymes associated with this process. A series of peptidase families have been studied, and it is considered that the calpain system is predominantly responsible for improvement in meat tenderness during aging, as a result of its ability to degrade cytoskeletal

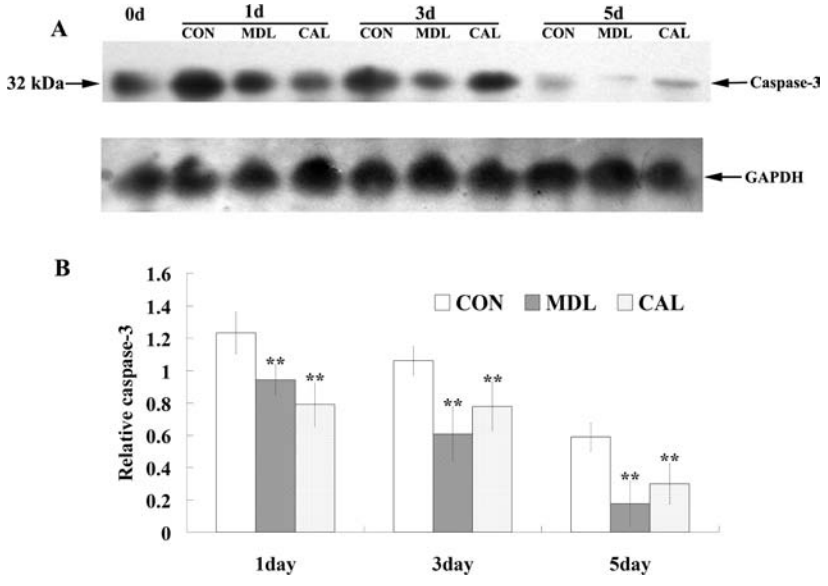

Figure 1. (A) Cleavage of caspase-3 in chicken breast muscle preparations in the absence (CON) and presence of 100 μM MDL-28170 (MDL) or 100 μM calpeptin (CAL) when stored at 4 °C for 0, 1, 3, or 5 days as shown by Western blots with anti-caspase-3. Forty micrograms of protein from each sample was loaded onto each lane. (B) Relative values of caspase-3 during 5 days of storage at 4 °C. Values were calculated as the blot intensity of the full-length caspase-3 at 0 days. Mean ± SD ($n = 4$). ** = $p < 0.01$ versus control. GAPDH showed that equal amounts of protein were applied to each lane.

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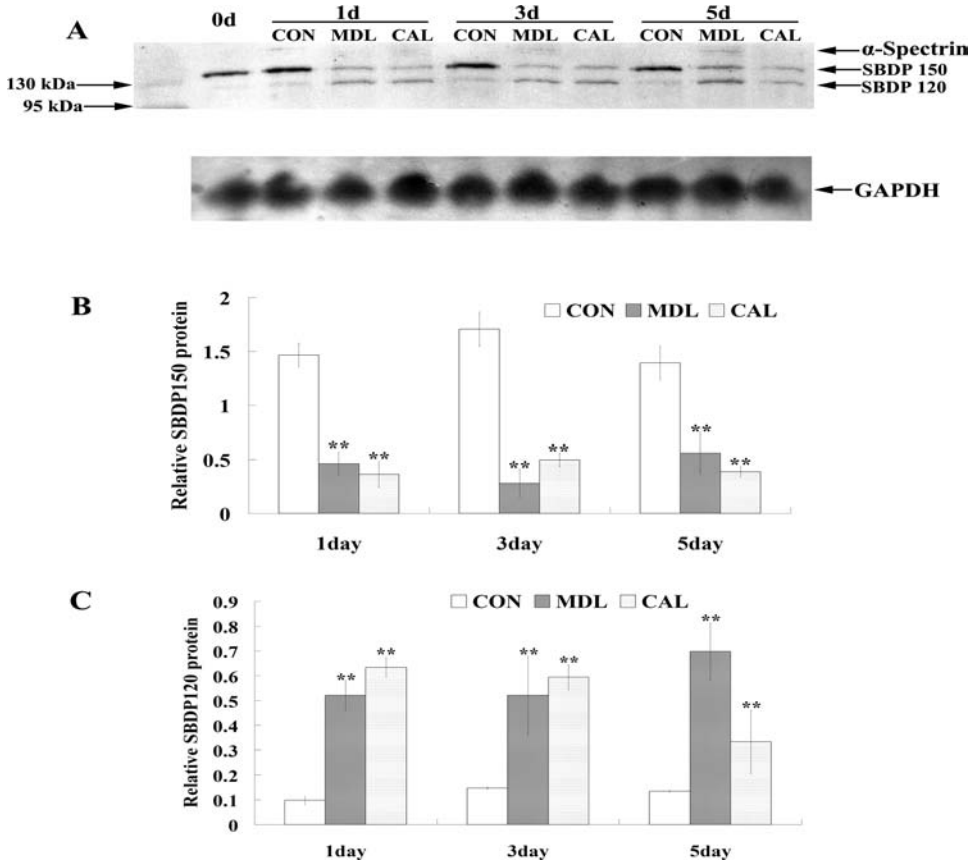

Figure 2. (A) Cleavage of α-spectrin of chicken breast muscle preparations in the absence (CON) and in the presence of 100 μM MDL-28170 (MDL) or 100 μM calpeptin (CAL) when stored at 4 °C for 0, 1, 3, or 5 days as shown by Western blots with anti-α-spectrin. Forty micrograms of protein from each sample was loaded onto each lane. (B, C) Relative values of 150 kDa (B) and 120 kDa (C) cleavage products of α-spectrin (SBDP150 and SBDP120) during 5 days of storage at 4 °C. Relative values were calculated as the blot intensity of 150 kDa fragment of α-spectrin at 0 day. Mean ± SD (n = 4). ** = p < 0.01 versus control. GAPDH showed that equal amounts of protein were applied to each lane.

Table 2. Calpain Activities of Chicken Meat in the Absence (Control) and Presence of Caspase-3 Inhibitor, DEVD-CHO, Using the Fluorogenic Suc-LLVY-AMC as Substrate following Storage at 4 °C for 0, 1, 3, or 5 Days[a]

	calpain activity	
days of storage	control	DEVD-CHO
0	55.76 ± 1.22	
1	26.75 ± 1.53a	16.26 ± 1.81b
3	19.41 ± 0.81a	12.79 ± 0.52b
5	13.46 ± 1.57a	2.57 ± 0.81b

[a]Values are expressed as absorbance (arbitrary units) per milligram of protein. Each value represents the mean ± SD, n = 4. Means within a row with different letters are significantly different (p < 0.01).

proteins responsible for the structural integrity of muscle fibers. As indicated previously, recent evidence suggests that caspase-3 also plays a significant role in the tenderization of meat post-mortem.[11] In a previous study, we used Ca^{2+}, camptothecin, and etoposide to act as apoptosis inducers to examine their effect on the activity of caspase-3 and thus improve the tenderness of chicken meat during post-mortem aging.[11] In the current investigation, we used inhibitors of calpain and caspase-3 to study the relationships between these two protease systems. Calpain is a Ca^{2+}-dependent proteinase, and others have shown that the membrane-permeable calpain inhibitor MDL-28170 can block Ca^{2+} and the Ca^{2+}-ionophore to inhibit the activity of calpain.[26,27] Calpeptin was the most potent of the synthesized inhibitors in terms of preventing the Ca^{2+}-ionophore-induced activity of calpain.[28] There are reports of cell-permeable calpain inhibitors, including MDL-28170 in thymocytes and metamyelocytes and leupeptin in T cells, inhibiting calpain activation and preventing apoptosis.[13,29] Spectrin is a main subplasmalemmal cytoskeleton component of the cell, which regulates cellular morphology by sustaining the integrity of cell membrane. Growing evidence suggests that α-spectrin is degraded in cells that undergo apoptosis, the degradation of which during apoptosis could extensively compromise the membrane permeability as well as cytoskeletal integrity. This could lead to structural changes that are associated with meat tenderization.[30] This cytoskeletal protein, spectrin, appears to be one of the substrates for caspases and calpain in cells undergoing apoptosis. The product of caspase-3-mediated spectrin breakdown has been observed during cell death, suggesting that caspase-3-mediated proteolysis may play a key part in the dismantling of cytoskeletal architecture associated with apoptosis. The actual breakdown pattern of α-spectrin into products (SBDP) is regarded as indicative for either caspase or calpain activation in cell death. There appears to be a positive correlation between the accumulation of spectrin breakdown products and activation of caspase-3; that is, α-spectrin cleavage is temporally related to caspase-3 activation, and caspase-3 specifically is responsible for the production of SBDP120.[31]

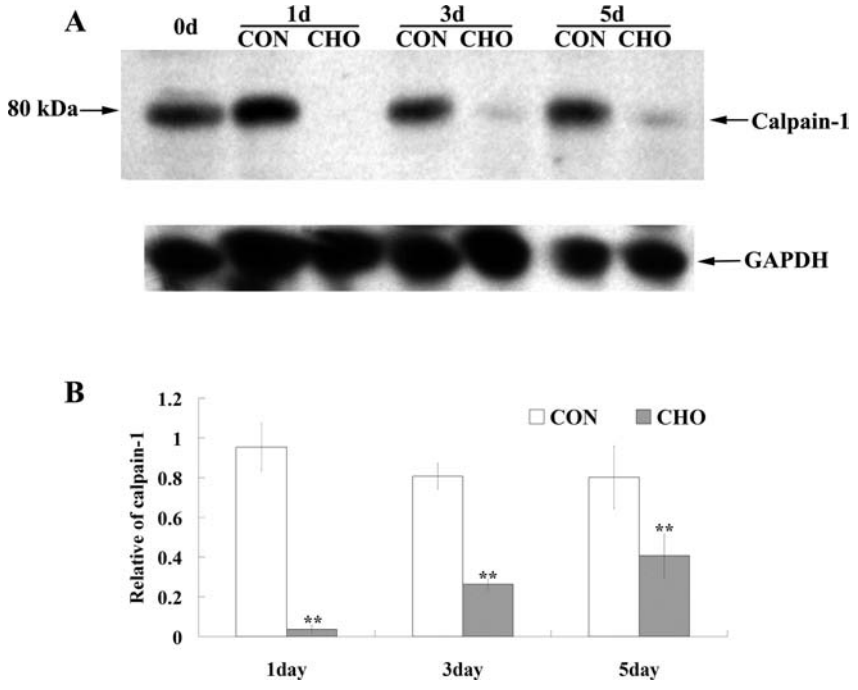

Figure 3. (A) Cleavage of calpain-1 by chicken breast muscle preparations in the absence (CON) and in the presence 100 μM DEVD-CHO (CHO) when stored at 4 °C for 0, 1, 3, or 5 days as shown by Western blots with anti-calpain-1. Forty micrograms of protein from each sample was loaded onto each lane. (B) Relative values of calpain-1 during 5 days of storage at 4 °C. Values were calculated as the blot intensity of calpain-1 at 0 day. Mean ± SD (n = 4). ** = p < 0.01 versus control. GAPDH showed that equal amounts of protein were applied to each lane.

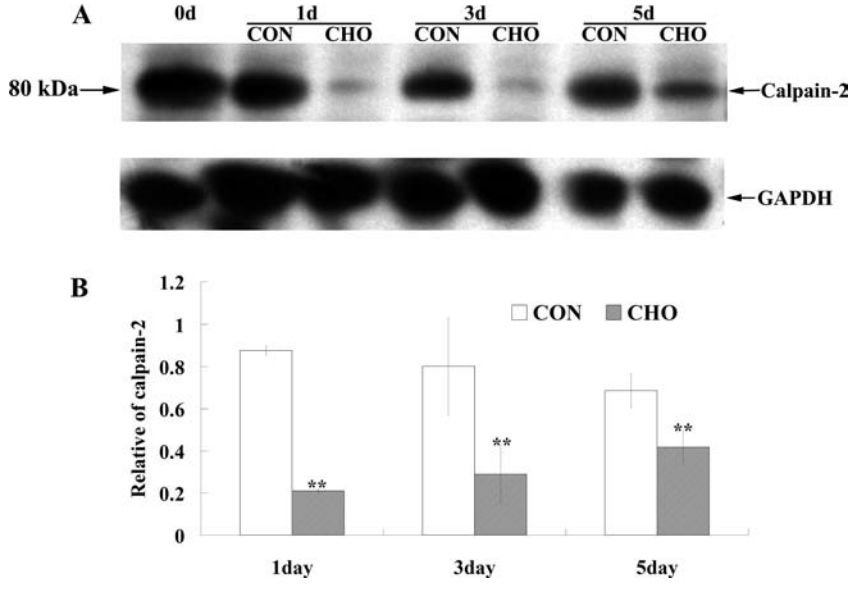

Figure 4. (A) Cleavage of calpain-2 by chicken breast muscle preparations in the absence (CON) and in the presence of 100 μM DEVD-CHO (CHO) when stored at 4 °C for 0, 1, 3, or 5 days as shown by Western blots with anti-calpain-2. Forty micrograms of protein from each sample was loaded onto each lane. (B) Relative values of calpain-2 during 5 days of storage at 4 °C. Values were calculated as the blot intensity of calpain-2 at 0 day. Mean ± SD (n = 4). ** = p < 0.01 versus control. GAPDH showed that equal amounts of protein were applied to each lane.

Pro-caspase-3 can be split by two distinct pathways: one by receptor-intermediate caspase-8 and the other by activation of mitochondrial−cytochrome c−intermediate caspase-9. On activation of the protease cascade, the caspase-3 proenzyme is cleaved into p20 and p11 subunits, which then heterodimerize to form an active enzyme.[32] It has been indicated that calpains cleave caspases, and usually this cleavage inactivates caspase function, suggesting that calpains may act as negative regulators of caspase processing.[33] Calpain cleaves caspase-7 at sites that are distinct from those cleaved by the upstream caspases, generating inactive fragments. In a similar manner, pro-caspase-8 and pro-caspase-9 can be cleaved by calpains, and the truncated caspase-9 is unable to activate pro-caspase-3.[33,34] Direct cleavage of caspase-9 by calpain blocks dATP and cytochrome c induced caspase-3 activation.[35] In a neuronal cell-free apoptosis system, calpain-1 prevented the ability of cytochrome c to activate the caspase cascade by inhibiting the processing of pro-caspase-3 and -9 into their active subunits.

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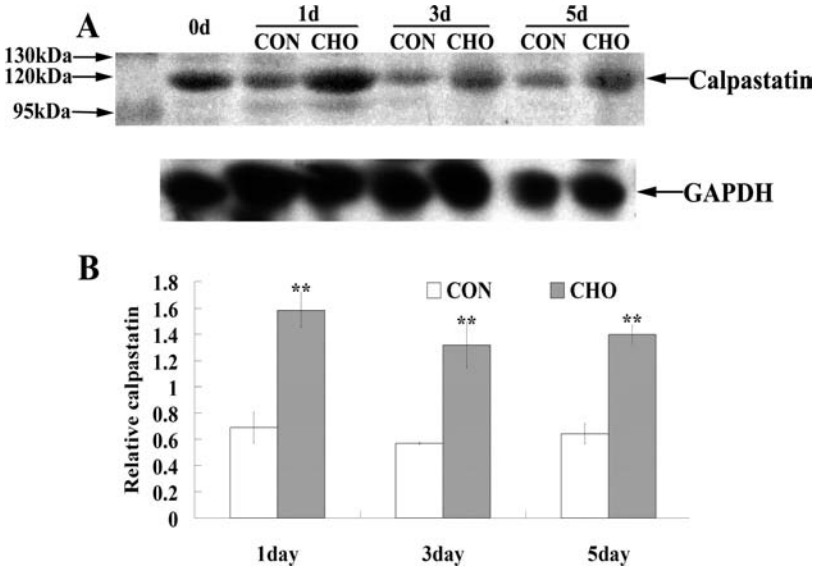

Figure 5. (A) Cleavage of calpastatin by chicken breast muscle preparations in the absence (CON) and in the presence of 100 μM DEVD-CHO (CHO) when stored at 4 °C for 0, 1, 3, or 5 days as shown by Western blots with anti-calpastatin. Forty micrograms of protein from each sample was loaded onto each lane. (B) Relative values of calpastatin during 5 days of storage at 4 °C. Values were calculated as the blot intensity of calpastatin at 0 day (B). Mean ± SD (n = 4). ** =p < 0.01 versus control. GAPDH showed that equal amounts of protein were applied to each lane.

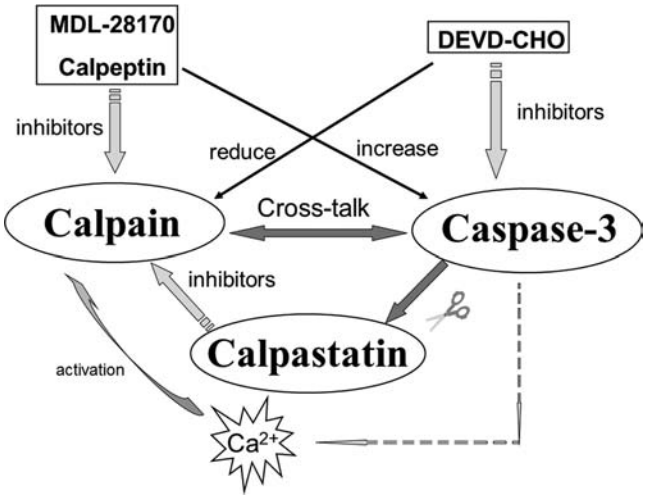

Figure 6. Significant cross-talk and functional connections between calpain and caspase-3 in the regulation of apoptosis during post-mortem aging of chicken meat.

Inhibition of calpain activity restored caspase-3-like protease activity.[36] It has been reported that calpain inhibition increases taxol-induced apoptosis in a caspase-3-dependent manner.[37] Also, the inhibitor of calpain may increase the expression and activation of caspase-3 in apoptotic neurons.[38] There is increasing evidence that activation of calpains can directly inactivate caspases, in both cell-free and cell systems.[39] Studies have demonstrated that calpain may act upstream of caspase-3 and induce apoptosis in both caspase-3-dependent and -independent ways.[34,39] Moreover, growing evidence suggests that calpains may play a key role in either upstream or downstream regulation of caspase during apoptosis. Examples are glucocorticoid-treated and irradiated thymocytes, β-lapachone-treated MCF-7 breast cancer cells, and neuronal cells exposed to various stresses.[40−42]

Effect of Caspase-3 on Calpain and Calpastatin Activities. The fluorogenic substrate Suc-LLVY-AMC was used to determine the activity of calpain in the presence and absence of a caspase-3 inhibitor. In control samples, calpain activity was highest on day 0 and decreased progressively over the 5 days of storage (Table 2). When the caspase-3 inhibitor DEVD-CHO was added, there was a significant (p < 0.01) reduction in calpain activity at each of the storage times investigated compared with the control. In this study, Western blots detected immune-positive bands of calpain-1 and calpain-2. DEVD-CHO had similar effects on calpain-1 and calpain-2, resulting in high levels of cleavage compared with the control (Figures 3 and 4). Relative to the amounts of calpain-1 and calpain-2 at day 0 there were highly significant differences at 1, 3, and 5 days (Figures 3B and 4B; p < 0.01). Western blots detected fragment bands of calpastatin, and the results confirmed that DEVD-CHO caused a significant enhancement in band intensity during 5 days of post-mortem aging (Figure 5). Relating amounts to day 0, the difference of DEVD-CHO and control was significant at 1, 3, and 5 days (p < 0.01, Figure 5B).

DEVD-CHO was chosen as the inhibitor of caspase-3 as it is a well-established specific inhibitor of caspase-3, thus allowing analysis of the selective effect of inhibiting one protease family when both are present and active. The caspase-3-selective inhibitors such as Ac-DEVD-CHO almost completely protect against any forms of apoptosis, and caspase-3 appears to be involved centrally in apoptotic cascades in various cell types. In our previous study, the apoptosis inducers camptothecin and etoposide both resulted in increased activity of caspase-3 and caused a large increase in the protein intensity values of calpain compared with the control. At the same time, their addition resulted in a significant reduction in the intensity of calpastatin during the 5 days of post-mortem aging. Calpains belong to a family of Ca^{2+}-dependent cysteine proteases that have been hypothesized to participate in several models of apoptosis and have been identified as a target of Ca^{2+}-dependent activation.[2] It has also been reported that caspase-3 cleaves Ca^{2+}-ATPase located in the plasma membrane and, therefore, inactivates the plasma membrane Ca^{2+} pump during apoptosis.[43] However,

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Figures 3 and 4 show that the addition of DEVD-CHO results in very low amounts of protein for both calpain-1 and calpain-2. The calpains may have already autolyzed and therefore are unable to bind to the monoclonal antibodies. A number of studies have reported that autolysis and activation are separate events,[44] and both unautolyzed calpain-1[45,46] and unautolyzed calpain-2[45,47] are also active proteases. Therefore, it now seems likely that pro-enzymes need not require autolysis for their activation and the unautolyzed calpains are capable of proteolytic activity.[2]

Calpastatin is present in most cells, and when calpain activity is low, and it is a result of the greater expression of calpastatin.[48] It has been hypothesized that an increase in myofibrillar-associated protein breakdown is regulated through an increase in calpain activity and/or a decrease in calpastatin activity, which would ultimately result in tenderer meat. Furthermore, execution of apoptosis is mediated by the combined actions of calpains and caspases, whereas calpain activity is regulated by the endogenous inhibitor calpastatin. Several investigators have demonstrated caspase-mediated cleavage of calpastatin, both in vitro and in cell culture models of apoptosis, and the functional consequence appeared to be a decreased ability of the cleaved calpastatin to inhibit calpain. Calpastatin was found to be very sensitive to the proteolysis of caspases-1 and -3 during early apoptosis, such as in staurosporine-treated SY5Y cells, anti-FAS-treated Jurkat cells, and TNF-treated U937 monocytic leukemic cells.[49−51] It has been demonstrated that caspase inhibitors VAD-cmk and IETD-fmk caused a decrease in calpastatin breakdown, which is important for the regulation of calpain activity.[52] In our work, calpastatin was susceptible to caspase-mediated fragmentation; thus, by inhibiting caspase activity though use of DEVD-CHO, the calpastatin level would be expected to increase, which in turn should attenuate calpain activity. Therefore, caspases have the potential to indirectly up-regulate calpain activity. Many papers have demonstrated that degradation of calpastatin by caspase-3 might have yet unidentified functions to facilitate the synergistic interaction between caspases and calpains during apoptosis.[52] Implementation of apoptosis may be mediated by the combined actions of calpains and caspases, and it might be expected that there would be a significant link between the caspase and calpain proteolytic pathways. Neumar et al.[15] reported that, during the early initiation phase, calpain down-regulates caspase-3 activity, whereas subsequent calpain activity is facilitated by the caspase-mediated degradation of calpastatin. Furthermore, the mechanism of apoptosis requires activation of cysteine proteases such as calpains and caspases, which work independently and also co-operatively to cause apoptosis.[15,30]

The synergistic effects of caspases and calpain inhibitors suggest that there is a synergistic interaction between caspases and calpains during apoptosis. Our results demonstrate a complex interaction of the two protease systems in which calpains down-regulate caspase-3 activity and caspase-3 indirectly up-regulates calpain activity through calpastatin degradation. Figure 6 shows the proposed interactions between calpain and caspase-3 and their modulation by specific inhibitors. However, it is still unknown whether calpain functions upstream or downstream of caspase-3 in apoptosis. Nevertheless, a better understanding of the interactions between calpains and caspases will help to reveal the molecular mechanisms of cell death and offer a new insight for a number of biological phenomena, including meat aging.

AUTHOR INFORMATION

Corresponding Author

*E-mail: ghzhou@njau.edu.cn. Phone: +86-25-84396928. Fax: +86-25-84396937.

Author Contributions

§Co-first author.

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Notes

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