Participation of the conventional calpains in apoptosis

Tao Lu, Ying Xu, Maura T. Mericle, Ronald L. Mellgren*

The Department of Pharmacology and Therapeutics, Medical College of Ohio, 3035 Arlington Avenue, Toledo, OH 43614-5804, USA

Received 4 December 2001; received in revised form 29 January 2002; accepted 7 March 2002

Abstract

The conventional calpains, m- and μ-calpain, are suggested to be involved in apoptosis triggered by many different mechanisms. However, it has not been possible to definitively associate calpain function with apoptosis, largely because of the incomplete selectivity of the cell permeable calpain inhibitors used in previous studies. In the present study, Chinese hamster ovary (CHO) cell lines overexpressing μ-calpain or the highly specific calpain inhibitor protein, calpastatin, have been utilized to explore apoptosis signals that are influenced by calpain content. This approach allows unambiguous alteration of calpain activity in cells. Serum depletion, treatment with the endoplasmic reticulum (ER) calcium ATPase inhibitor thapsigargin, and treatment with calcium ionophore A23187 produced apoptosis in CHO cells, which was increased in calpain overexpressing cells and decreased by induced expression of calpastatin. Inhibition of calpain activity protected h-spectrin, but not α-spectrin, from proteolysis. The calpains seemed not to be involved in apoptosis triggered by a number of other treatments. Calpain protected against TNF-α-induced apoptosis. In contrast to previous studies, we found no evidence that calpains proteolyze IκB-α in TNF-α-stimulated cells. These studies indicate that the conventional calpains participate in some, but not all, apoptotic signaling mechanisms. In most cases, they contributed to apoptosis, but in at least one case, they were protective. © 2002 Published by Elsevier Science B.V.

Keywords: Calpain; Apoptosis; Thapsigargin; A23187; TNF-alpha; CHO cell

1. Introduction

Cell death can either be the consequence of a passive, degenerative process, or the consequence of an active process. The former type of cell death is termed necrosis, the latter apoptosis, or programmed cell death (PCD). Apoptosis was originally identified morphologically [1]. It represents a mode of death that is actively driven by the cell. Necrosis represents a passive consequence of gross injury to the cell. It is morphologically distinct from apoptosis, and its physiological consequences at the tissue, organ and organism levels are also different.

Calpains are non-lysosomal cysteine proteases that are present in the cells of many different eukaryotic single-celled and multicellular organisms [2,3]. While there are a number of members of the calpain gene family, the best characterized have been named “conventional calpains”: m- and μ-calpain, one or both of which is present in all mammalian cells. The possible involvement of calpains in apoptosis was first suggested in 1993 [4,5], and has been reported for several cells including thymocytes, hippocampal cells and hepatocytes [6–9]. Both α-spectrin and β-spectrin breakdown by caspase-3 and calpain were reported during apoptosis in neural cells [10,11]. The spectrin breakdown product (SBDP) SBDP120 of caspase-3 is a very typical hallmark of apoptosis, on the other hand, calpain-mediated SBDPs are generally found in necrotic as well as in apoptotic neuronal death [12,13]. And the relative contribution to SBDP from calpain is quite cell type specific [14].

Although it is generally accepted that calpains are involved in cell death processes, their exact role is unknown, and little has been done to sort out their degree of participation in any of the more common apoptotic models. Moreover, the specificity of the calpain inhibitors used for such experiments is crucial in interpreting results, and to date, no satisfactorily selective calpain inhibitors have been developed. Thus, there is still some confusion as to the true targets of these tools vis-à-vis apoptosis, and it is difficult to rule out effects on proteasome and other intracellular proteases.

In this study, CHO cell-derived clones were developed that overexpressed either active μ-calpain or calpastatin. Unlike the synthetic calpain inhibitors, calpastatin specifi-
cally inhibits activity of the conventional calpains in vivo. By unambiguously increasing or decreasing calpain activity in the cells, it has been possible to demonstrate a clear-cut difference in participation of the conventional calpains in the various apoptosis models in a single cell type: CHO cells.

2. Materials and methods

2.1. Materials

SHI cells were selected from a CHO cell line by challenging with the calpain inhibitor, ZLLY-DMK, as previously described [15]. They possess one-third to one-half of the \( \mu \)-calpain content of the parental CHO cells. Thapsigargin, A23187, staurosporine, \( \text{H}_2\text{O}_2 \), EGTA and (3-[4,5-yl]-...otherwise described [15]. They possess one-third to one-half of the conventional calpains in vivo. The calpain small subunit cDNA was a gift from Dr. John S. Elce at Queen’s University, Kingston, Ontario, Canada. The full-length human calpastatin cDNA was a gift from Dr. Kevin K.W. Wang at Pfizer, Ann Arbor Laboratory, USA. Anti-mouse IgG, biotinylated species-specific whole antibody (from sheep), and streptavidin-alkaline phosphatase were purchased from Amersham Pharmacia Biotech. Is-B-\( \alpha \) polyclonal antibody was obtained from Santa Cruz. Mouse anti-human \( \mu \)-calpain large subunit monoclonal antibody, 1A-11; mouse anti-human calpastatin monoclonal antibody 5-8A; mouse anti-calpain small subunit monoclonal antibody, P-1; and a rabbit immune serum against bovine m-calpain, which also recognized the hamster antigen, were made in-house. Fibroblasts derived from calpain small subunit knockout [Capn4 (−/−)] mouse embryos, or from wild-type littermates [16], were kindly provided by Dr. John S. Elce.

2.2. Human calpain overexpressing clones

Human \( \mu \)-Calpain large subunit cDNA, and rat p21 truncated calpain small subunit cDNA [17], were separately inserted into pSBC-B and pSBC-A plasmids, respectively. These plasmids were cut and ligated as previously described [18], to form a dicistronic construct, pSBC/S-L, having the calpain small subunit upstream of the large subunit, and separated by an internal ribosomal entry sequence. SHI cells were co-transfected with pSBC/S-L and NEO vectors, and G418 was used for selection. A4 cells were derived by transfection with pSBC-B containing \( \mu \)-calpain large sub-unit alone. However, a heterodimeric \( \mu \)-calpain appeared to be expressed in A4 clone, because the human \( \mu \)-calpain immunoreactivity could be co-immunoprecipitated from A4 cell lysates using P-1 small subunit antibody (not shown). Several human calpain producing cell lines were established, and the two that expressed high levels of active human \( \mu \)-calpain, SL18 and SL225, were chosen for this study. Twenty cell lines with no detectable human \( \mu \)-calpain expression by Western blot were pooled together randomly, and early passages of this cell pool were used as mock-transfected cells, SHI-NEO.

2.3. Human full-length calpastatin overexpressing clones

The full-length human calpastatin cDNA was inserted into the polylinker EcoRI site of pIND (SP1)/V5-His C vector. EcR-CHO cells, which already constitutively express ecdyson receptor, were transfected with the calpastatin construct using electroporation, and selected in the presence of G418 (1.4 mg/ml) and zeocin (0.25 mg/ml). We identified two stable cell lines, clone 83 and 106, which overexpressed human calpastatin when induced with ponasterone. Clone 83 was chosen for most of our studies, because its morphologic and growth characteristics were very similar to the parental EcR-CHO cells. Clone 106 displayed a slightly larger size and elongated appearance. Mock-transfected cells, which did not express the human calpastatin, were obtained by transfecting the EcR-CHO cells with empty pIND (SP1)/V5-His C vector.

2.4. Protease and inhibitor assays

Calpain activity was determined by measuring the release of trichloroacetic acid (TCA)-soluble fragments from \( ^{14}\text{C}\)-methylcasein [19]. CHO cells or clones were lysed in a hypotonic buffer: 50 mM imidazole–\( \text{HCl} \), 1 mM EGTA, 1 mM dithiothreitol, 70 mM NaCl, pH 7.4. Under these conditions, calpains are extracted, but much of the calpastatin remains sedimentable [20, unpublished observations]; allowing assay of calpain activity in 10,000 × g supernatants of the lysate. Calpastatin activity was determined by its ability to inhibit a defined amount of purified human erythrocyte \( \mu \)-calpain (1 ng/\( \mu \)l) in the standard \( ^{14}\text{C}\)-caseinolytic assay. The inhibition activity was calculated by subtracting the percentage of calpain activity remaining from 100%. Proteasome was assayed fluorometrically, using Succinyl-LLVY-MCA as the substrate, as previously described [21].

2.5. Cell culture and treatments

SL clone cells were cultured in 5% \( \text{CO}_2 \) and 95% humidified air atmosphere at 37 °C in complete IMDM medium supplemented with heat-inactivated 10% bovine serum, 100 units/ml penicillin, and 100 \( \mu \)g/ml streptomycin. Calpastatin overexpressing clones were cultured in complete
Ham’s F12 medium containing 10% heat-inactivated fetal bovine serum and the same antibiotics. For induction of calpastatin expression, clone 83 cells were cultured until 95–100% confluent, and pre-treated for 3 days with 5 μM ponasterone A before any other treatments. Mock-transfected EcR-CHO cells were subjected to the same pre-treatment protocol. Cells were either further treated with 100 nM thapsigargin for 3 days, 5 μM A23187 for 2 days, 1 μM staurosporine for 24 h, 3 mM EGTA for 24 h, or 3–10 mM H2O2 in serum-free medium for 2 h, then changed back to normal medium for another 24 h before harvest.

2.6. MTT assay

Cell viability was estimated by using the MTT assay method [22]. Cells were cultured in 96-well plates until 90–100% confluent, and treated by different cell death stimuli for various durations. Adding 0.5 mM MTT to the plates, followed by incubation at 37 °C for at least 4 h. Isopropanol—0.1 N HCl, 100 μl, was added to dissolve the reduced tetrazolium dye. OD595 was determined using the Softmax software provided with a Thermomax microplate reader from Molecular Device Corp. For serum deprivation experiments, cells were cultured in the absence of serum for 24 h before assay. UV light irradiation experiments were performed as described [23]. Briefly, culture medium was exchanged for a PBS/Mg2+ solution, and the cells were exposed to 254 nm radiation at 5 mJ/cm2 for different times. They were then changed back to the usual growth medium, and further cultured for another 2 days before performing MTT assays. Irradiation was carried out by using the Stratagene product Stratalinker® UV cross-linker.

2.7. Extraction of proteins, immunoblots, and densitometric analysis

Cells were cultured in 12-well plates until about 100% confluent, and treated by different stimuli at different concentrations for different times as described in Section 2.5. Cells were collected, washed with ice-cold PBS, and lysed in the cell lysis buffer (1% Triton X-100, 20 mM Tris, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 100 μM Na3VO4, 100
nM microcystin-LR, 200 μM phenylmethylsulfonyl fluoride, 10 μM pepstatin A, and 1 mM dithiothreitol, pH 7.4) on ice for 10 min, then homogenized for about eight strokes, and centrifuged at 4 °C, 10,000 × g for 10 min. Quantitation of supernatant protein was carried out with the BCA reagent [24]. Supernatants were treated with SDS-PAGE sample buffer, and equal amounts of sample protein were subjected to 7.5% polyacrylamide SDS-PAGE. The gels were subsequently transferred to polyvinylidene difluoride membranes by electroblotting at 4 °C for 3 h at 70 V. Immunoblotting was performed with the following first antibodies: mouse anti-spectrin (nonerythroid) monoclonal antibody (1:2000); mouse anti-β-spectrin monoclonal antibody (1:700); rabbit

Fig. 2. Regulated expression of human calpastatin in clone 83 CHO cells. Clone 83 cells stably transfected with pIND vector expressing human full-length calpastatin were cultured minus or plus 5 μM ponasterone A for 3 days. Cell lysates were subjected to protein immunoblot analysis using a human-specific calpastatin antibody (panel A), or heat-treated as described in Materials and methods and assayed for calpastatin activity (panel B).

Fig. 3. Calpastatin overexpression inhibited calpain autoproteolysis. Clone 83 cells or mock-transfected EcR-CHO cells were cultured for 3 days with or without 5 μM ponasterone A in the culture medium. Where indicated, 100 nM thapsigargin or 3 μM A23187 were then added to the medium. After 2 more days in culture (A23187) or 3 more days (thapsigargin), cell lysates were prepared and subjected to protein immunoblot analysis for calpain small subunit.

Fig. 4. Calpastatin overexpression increased viability of clone 83 cells exposed to thapsigargin or A23187. Clone 83 cells were cultured plus (filled bars) or minus ponasterone, and then exposed to 100 nM thapsigargin (panel A) or 3 μM A23187 (panel B). After 1, 2 or 3 days, cell survival was measured by MTT reductase activity as described in Materials and methods. Where indicated, values were significantly different from the minus ponasterone sample at the same time point; * = P < 0.01, ** = P < 0.001.
anti-m-calpain large subunit (1:1000); mouse anti-calpain small subunit monoclonal antibody, P-1, (2 μg/ml); mouse anti-human μ-calpain large subunit monoclonal antibody, 1A-11 (2 μg/ml); mouse anti-human calpastatin monoclonal antibody 5-8A (2 μg/ml). Immunodetection was carried out with alkaline phosphatase second antibody, and BCIP substrate with nitro-blue tetrazolium development [25]. Densitometry was performed with a Bio-Rad bioscanner and Molecular Analyst software.

2.8. Cell morphology

Cells were cultured until about 100% confluence, and treated by the indicated stimuli for the indicated times as described in Section 2.5. Phase-contrast photomicrographs were taken using a Nikon N 5005 inverted phase-contrast microscope and camera at 100 × magnification.

2.9. DNA fragmentation assay

Cells were cultured in 60 mm petri dishes, and treated by different stimuli at the indicated conditions. Cells were lysed in 500 μl of DNA extraction solution (50 mM Tris–HCl, 10 mM EDTA, 0.5% Triton, and 100 μg/ml proteinase K, pH 8.0). After incubating overnight, Dnase-free Rnase A was added to 100 μg/ml. The samples were incubated at 37 °C for 2 h, extracted with an equal volume of phenol/chloroform (1:1 v/v), and centrifuged at 20,000 × g for 10 min at 4 °C. The aqueous phase was re-extracted with chloroform. DNA was collected by precipitating the aqueous supernatant.

Fig. 5. Calpastatin overexpression attenuated cell rounding associated with thapsigargin and A23187-induced cell death. Clone 83 and mock-transfected cells were treated with 100 nM thapsigargin or 3 μM A23187 as described in Materials and methods, then observed by phase contrast microscopy at 100 × magnification.
with 2 volumes of cold absolute ethanol in the presence of 1/10 volume of 3 M NaAc. After washing with 70% ethanol, the DNA pellet was dissolved in 40 μl of 10 mM Tris–HCl and 1 mM EDTA, pH 8.0. DNA was separated on 1.8% agarose gel containing 0.5 μg/ml ethidium bromide, and DNA fragments were visualized by exposing the gels to UV light.

2.10. Statistical analysis

Where indicated, samples were analysed by Students unpaired t-test. Results are expressed as mean ± standard deviation.

3. Results

3.1. Characterization of clones overexpressing calpain or calpastatin

Calpain overexpressing clones were established by co-transfecting Neo vector and the pSBC-S/L construct, which had both the μ-calpain large subunit and calpain small subunit, into SHI cells. Seven cell lines expressing human μ-calpain were established (Fig. 1). S/L18 and S/L225 cells were chosen for further study, because they expressed intermediate and high levels of μ-calpain, respectively (Fig. 1A). Moreover, they displayed morphologies and growth characteristics comparable to the mock-transfected

![Mock](image1)

![Clone 83](image2)

![Thapsigargin](image3)

![Ponasterone](image4)

Fig. 6. Calpastatin overexpression decreased DNA ladder formation in A23187 and thapsigargin-treated cells. Clone 83 and mock-transfected cells were exposed to thapsigargin or A23187 as described in Materials and methods. DNA was prepared and subjected to electrophoretic analysis for fragmentation as described in Materials and methods.

![Fig. 7](image5)

![A](image6)

![B](image7)

Fig. 7. Calpastatin overexpression inhibited β-spectrin proteolysis in thapsigargin-treated cells. Clone 83 and mock-transfected cells were treated with thapsigargin as described in Materials and methods. Cell lysates were subjected to SDS-PAGE and protein immunoblotting using anti-α-spectrin antibody (panel A) or anti-β-spectrin antibody (panel B), as described in Materials and methods. The asterisk in panel A shows the location of the immunoreactive band at slightly higher mobility than α-spectrin that was consistently protected by calpastatin overexpression.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Cells</th>
<th>Ponasterone</th>
<th>Degradation of β-spectrin ± SD relative to untreated samples (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thapsigargin</td>
<td>mock</td>
<td>–</td>
<td>72 ± 1</td>
</tr>
<tr>
<td></td>
<td>mock</td>
<td>+</td>
<td>70 ± 5</td>
</tr>
<tr>
<td></td>
<td>clone 83</td>
<td>–</td>
<td>74 ± 1</td>
</tr>
<tr>
<td></td>
<td>clone 83</td>
<td>+</td>
<td>42 ± 6*</td>
</tr>
<tr>
<td>A23187</td>
<td>mock</td>
<td>–</td>
<td>65 ± 10</td>
</tr>
<tr>
<td></td>
<td>mock</td>
<td>+</td>
<td>67 ± 11</td>
</tr>
<tr>
<td></td>
<td>clone 83</td>
<td>–</td>
<td>76 ± 8</td>
</tr>
<tr>
<td></td>
<td>clone 83</td>
<td>+</td>
<td>62 ± 7**</td>
</tr>
</tbody>
</table>

* P<0.01 vs. minus ponasterone sample.
** P<0.05 vs. minus ponasterone sample.
cultured in the presence of A23187 or thapsigargin, both of which activated calpains, as evidenced by increased autoproteolysis of calpain small subunit (Fig. 3; lanes 1, 2, 5, and 6). Overexpression of calpastatin decreased autoproteolysis (Fig. 3; lanes 3, 4, 7, and 8). No protection was observed when mock-transfected cells were exposed to ponasterone A.

3.2. Effect of altering calpain or calpastatin content on survival of cells exposed to A23187- or thapsigargin-induced apoptosis

Initial studies of the effect of calpain content on cell survival were carried out utilizing the calpastatin-overexpressing clone 83. The divalent cation ionophore A23187, and thapsigargin, an inhibitor of the endoplasmic reticulum calcium pump, were used as apoptogenic agents. As previously noted, both of these reagents activate calpains in CHO cells (Fig. 3). Treatment with either A23187 or thapsigargin resulted in time-dependent cell death, which was inhibited by calpastatin overexpression (Fig. 4). Light microscopic examination of cells exposed to A23187 or thapsigargin confirmed that calpastatin overexpression inhibited cell damage, as evidenced by decreased rounding and lifting from the culture surface (Fig. 5). No protection of apoptosis was observed in mock-transfected cells treated with ponasterone (Fig. 5; top).

The MTT reductase assay utilized to measure cell survival cannot distinguish between apoptosis and necrosis. Therefore, further studies were carried out to confirm that A23187 and thapsigargin trigger apoptosis in the cell lines utilized in the present investigation. Both treatments produced DNA fragmentation into a multi-nucleosomal “DNA ladder” (Fig. 6), typical of apoptosis. Overexpression of calpastatin attenuated DNA breakdown (Fig. 6; panel B).

Spectrin proteolysis has been utilized as a marker for apoptosis, especially generation of the 120 kDa α-spectrin fragment by caspase 3 [26]. Exposure of clone 83 cells to either thapsigargin or A23187 resulted in a substantial

### Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Calpastatin overexpressing</th>
<th>Calpain overexpressing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mock – Ponasterone</td>
<td>Ponasterone</td>
</tr>
</tbody>
</table>
| Serum       | 62.1 ± 2.5 | 58.3 ± 3.7 | 60.0 ± 3.1 | 78.1 ± 5.4* | 53.1 ± 1.4 | 50.7 ± 2.1 | 29.4 ± 1.8
| A23187      | 26.6 ± 1.4 | 25.0 ± 0.8 | 23.6 ± 1.5 | 37.9 ± 2.4* | 51.9 ± 1.4 | 50.8 ± 2.5 | 41.3 ± 0.7
| Thapsigargin| 66.2 ± 2.4 | 68.0 ± 2.4 | 65.0 ± 2.6 | 86.9 ± 3.3* | 49.9 ± 2.0 | 42.0 ± 0.8* | 40.6 ± 1.1
| Staurosporine| 35.7 ± 4.4 | 37.0 ± 1.3 | 35.3 ± 6.8 | 39.9 ± 1.5 | 36.2 ± 1.1 | 34.2 ± 1.0 | 37.0 ± 0.3
| EGTA        | 66.0 ± 1.7 | 65.4 ± 1.4 | 68.0 ± 1.6 | 66.8 ± 2.5 | 64.2 ± 1.7 | 65.5 ± 0.8 | 64.6 ± 1.7
| H₂O₂        | 30.3 ± 6.7 | 29.9 ± 4.4 | 31.3 ± 3.3 | 30.2 ± 4.0 | 47.4 ± 1.5 | 47.1 ± 3.3 | 43.7 ± 1.7
| UV          | 54.7 ± 2.7 | 53.6 ± 2.0 | 56.1 ± 2.6 | 53.1 ± 2.3 | 46.6 ± 0.6 | 44.7 ± 0.9 | 45.4 ± 2.0

*P < 0.001 compared with – ponasterone samples.

#P < 0.001 compared with mock-transfected cells.
increase in spectrin immunoreactivity at 120 kDa on protein blots (Fig. 7; panel A). However, increased expression of calpastatin had no apparent effect on generation of the 120 kDa fragment. In contrast, a minor immunoreactive band slightly smaller than α-spectrin was consistently spared (Fig. 7; panel A, asterisk). Utilization of a selective antibody demonstrated that this band was β-spectrin, and that its proteolysis was reduced upon calpastatin overexpression (Fig. 7; panel B). Overexpression of calpastatin in clone 83 cells resulted in a significant reduction of β-spectrin proteolysis induced by thapsigargin or A23187 treatment, under the conditions of our studies (Table 1).

### 3.3. Contribution of the conventional calpains to cell death produced by other apoptotic models

The availability of CHO cells overexpressing μ-calpain or calpastatin allowed a systematic survey of calpain involvement in other commonly studied apoptotic models (Table 2). The MTT reductase assay demonstrated that cell death produced upon serum withdrawal appeared to have a calpain component, as evidenced by its exacerbation by calpain overexpression, and the protective effect of calpastatin overexpression. Of the other apoptotic triggers studied, none except A23187 and thapsigargin appeared to be influenced by calpain/calpastatin balance. Further experiments were carried out to determine the mechanism of cell death in these studies. DNA fragmentation and spectrin proteolysis analysis confirmed that the conditions in Table
2, except exposure to H₂O₂, resulted in apoptosis (data not shown). H₂O₂ produced necrotic cell death at all concentrations studied (Fig. 8), with accompanying non-distinct DNA fragmentation, and proteolysis of α-spectrin without production of the distinct 120 kDa fragment.

3.4. Calpain protected against TNF-α triggered apoptosis

In contrast with the results presented in Table 2, calpain overexpression was found to protect against cell death signaling by TNF-α (Fig. 9A). Assessment of DNA ladder formation demonstrated apoptosis as the mechanism for cell death, and confirmed the protective effect of calpain overexpression (Fig. 9B). To assess the generality of calpain protection against TNF-α-stimulated apoptosis, fibroblasts derived from calpain small subunit knockout [Capn4 (-/-)] mouse embryos were utilized. Compared with wild-type mouse fibroblasts, the Capn4 (-/-) fibroblasts were more sensitive to TNF-α induced apoptosis (Fig. 10). In contrast, the knockout fibroblasts were less sensitive to apoptosis produced by addition of A23187 to the culture medium, or serum deprivation.

Previous studies in isolated hepatocytes have indicated that calpains can proteolyze IκB-α, thereby releasing NF-κB [27]. This is consistent with a protective effect of calpain in apoptosis, because it would allow expression of anti-apoptotic genes by NF-κB. However, in the present investigation, there was no detectable change in IκB-α stability in the calpain overexpressing cell lines (data not shown). To further investigate calpain participation in IκB-α degradation, the effects of lactacystin and SJA6017 were explored. The former is a selective proteasome inhibitor [28,29], while the latter is a potent calpain inhibitor having no detectable inhibitory activity on proteasome (Fig. 11A). IκB-α degradation in response to TNF-α was nearly completely abolished by lactacystin pre-treatment (Fig. 11B), while SJA6017 did not noticeably protect IκB-α (Fig. 11C). Therefore, the protective influence of calpain did not appear to be related to IκB-α proteolysis.

4. Discussion

By manipulating expression levels of μ-calpain and calpastatin, it has been possible to alter the sensitivity of CHO cells to apoptotic stimuli. These results indicate that the conventional calpains (μ- and m-calpain), which are inhibited by calpastatin, participate in some apoptotic pathways. Preliminary studies with calpain small subunit knockout mouse fibroblasts confirm this observation (Fig. 10). Notably, of the different systems studied, only apoptosis associated with A23187, thapsigargin, or serum depletion was shown to be increased by a positive calpain/calpastatin balance (Table 2). Cell death following exposure of CHO cells to staurosporine, EGTA, or ultraviolet light was not detectably influenced by calpain content. Calpain did not influence cell death upon exposure to H₂O₂. In some cell types, H₂O₂ produces apoptotic cell death [30–32]. However, it only produced necrotic death of CHO cells over a range of concentrations. Therefore, the influence of calpain on apoptosis produced by H₂O₂ could not be addressed by the present studies. Other well-characterized apoptotic stimuli could not be studied in the CHO cell system, because significant cell death was not observed. For example, silica fibers, which produce apoptosis in some cells [33,34], simply did not kill the CHO cells (data not shown).

Calpain protected against apoptosis produced by TNF-α. Other investigators have found that calpain can cleave IκB-α in hepatocytes stimulated with TNF-α [27]. In principle, this would lead to increased levels of free NF-κB transcription factor, which could translocate to the nucleus and increase expression of anti-apoptosis genes.
However, IkB-α did not appear to be a calpain target in the CHO cells used in the present studies. Very recently, it has been reported that TNF-α-dependent NF-κB activation in dermal fibroblasts is TRAF2- and NIK-independent, but requires sphinomelysin and calpain activity [35]. Thus, calpain appears to act through a mechanism separate from the NIK/IκB-α phosphorylation pathway. Further investigation will be required to elucidate the mechanism by which calpains protect against TNF-α-stimulated apoptosis.

A further surprising result from the present studies was the apparent lack of effect of calpain on α-spectrin proteolysis. Although calpastatin overexpression partially protected against apoptosis secondary to A23187 or thapsigargin treatment, α-spectrin was not noticeably protected from proteolysis (Fig. 7). Furthermore, the predominant breakdown product was the 120 kDa band expected from caspase cleavage of α-spectrin [26], suggesting that caspases are responsible for the majority of α-spectrin breakdown in these studies. Interestingly, there was significant protection of β-spectrin proteolysis upon calpastatin overexpression (Fig. 7B and Table 1). Although calpainolysis of α-spectrin has been most extensively studied, both α- and β-spectrins have been shown to be calpain substrates [36,37], and cleavage of both is required to fully dismantle the cortical cytoskeleton [11]. It was previously shown that in erythrocytes, only β-spectrin is cleaved by calpains [38]. If α-spectrin is predominantly proteolyzed by caspases in some cell types, while calpains degrade β-spectrin, this would provide a rational framework for utilizing combinations of caspase and calpain inhibitors to inhibit apoptotic death in these cell types.

In conclusion, the present studies demonstrate that direct alteration of the calpain/calpastatin balance in CHO cells influences some apoptotic systems, but not others. In no case did abrogation of conventional calpain activity lead to complete protection against apoptosis. Therefore, these calpains do not appear to be indispensable for this process. For a complex function like programmed death, it would be surprising if compromising one part of the signaling system would completely abolish the overall event. It is important to note that the changes in calpain and calpastatin expression levels displayed by the CHO cell clones described in this study are within the physiologic range observed in various cell types. For example, the amount of μ-calpain in the S/L 225 clone is similar to the level measured in blood platelets [39], and the maximum content of calpastatin in clone 83 cells after 3 or more days of induction with ponasterone A is only 2 or 3 times the level of endogenous hamster calpastatin (Fig. 2). Some of the apoptotic protocols utilized in these studies were clearly pharmacological in nature. However, two of the calpain-responsive apoptotic stimuli, TNF-α treatment, and abrogation of the serum survival pathway, represent physiologic apoptotic pathways. It will be important to determine which signal transduction events in these latter pathways are influenced by calpains.

Acknowledgements

This work was supported in part by NIH grant HL36573. The authors gratefully acknowledge Dr. Kevin K.W. Wang for providing valuable advice during these studies, as well as making available SJA6017 calpain inhibitor and antibodies against spectrins. We thank Dr. John Elce for the kind gift of Capn4 (−/−) and Capn4 (+/+) fibroblasts, and Dr. Masatoshi Maki for providing calpastatin cDNA.

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