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Procaspase-3 and Poly(ADP)ribose Polymerase (PARP) Are Calpain Substrates

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We demonstrate here that both procaspase-3 (32 kDa) and PARP are calpain substrates. In calcium-channel opener maitotoxin-treated cells, a 30 kDa caspase-3 fragment is produced in a time and concentration-dependent manner. Formation of this fragment is prevented by calpain inhibitors but not by the pancaspase inhibitor, carbobenzoxy-Asp-CH_2OC(O)-2,6-dichlorobenzene (Z-D-DCB) nor the selective proteasome inhibitor lactacystin. In maitotoxin-treated cells, PARP (113 kDa) is also cleaved into a 40 kDa immunoreactive fragment, in a calpain-inhibitor-sensitive manner. Both procaspase-3 and PARP are also cleaved *in vitro* by purified μ-calpain to a 30 kDa fragment and a 40 kDa fragment, respectively. Finally, we show that staurosporine-mediated caspase-3 activation is interrupted by maitotoxin pretreatment. © 1999 Academic Press

The cysteine protease caspase-3 is thought to be one of the most common mediators of mammalian apoptosis. After apoptotic injury, the 32 kDa caspase-3 proenzyme is cleaved by activated caspase −9 or −8 to 17 kDa and 12 kDa fragments, which form the active heterodimer (1). This proteolytic activation of procaspase-3 and the subsequent caspase activity are only observed in apoptotic cell death but not necrotic cell death (2). Caspase-3 also has a finite number of cellular protein substrates, although the list is rapidly growing (1). The most important specificity determinant is the Asp (D) in both the P1 and P4. Caspase-3 tends to produce "limited fragments" of its substrates, leaving them as fingerprint for caspase-3 activity. The first identified caspase-3 substrate was poly(ADP-ribose) polymerase (PARP), the major cleavage site was found to be DEVD*G, which conforms to the DXXD-specificity (3). Nonerythroid α-spectrin (280 kDa) is sensitive to caspase-3 degradation, producing both a 150 kDa and a 120 kDa spectrin breakdown product (SBDP) (4–5). Other caspase substrates include (i) cytoskeletal proteins (such as actin, tau and GAS-2), (ii) signal transduction enzymes (protein kinase C delta and theta isoforms, calmodulin-dependent protein kinase (CaMPK)-II and -IV, phospholipase C, P21-activated kinase, MEKK1), and (iii) cell cycle proteins (PITSLRE kinase; Rb, Kip21), and (iv) nuclear substrates DNA-PKcs, PARP, U1-70K and NuMA (for review see Ref. 1, 6).

Another cysteine protease calpain is also activated in certain forms of apoptosis (4, 6–10). Calpain activation is also very prominent in necrosis (4). Calpain preferred Val, Leu or Ile in the P2 position whereas the P1 restriction is rather loose. Like caspase-3, calpain also have a subset of cellular protein targets). Ironically, the caspase-3 substrate α-spectrin is also one of the most well known calpain substrate. It is degraded to two breakdown products of 150 kDa and 145 kDa (SBDP150, SBDP145). Other calpain substrates include cytoskeletal proteins (e.g., β-spectrin, talin, neurofilaments H and M), plasma membrane-associated proteins (EGF receptor, PDGF receptor) and signal transduction enzymes (protein kinase C isoforms) and calmodulin-binding proteins (11–12). Calpain also degrades a number of nuclear substrates, including a number of transcription factors (c-FOS, c-JUN, c-MYC, c-MOS and NF-κB). Interestingly, PARP has also suggested to be cleaved by a calpain-liked activity (13–15).

Previously, we have shown that a calcium channel opener maitotoxin (MTX, 0.1–1 nM) is highly effective in activating calpain and produces a necrotic response selectively (4, 16). No caspase-3 activation or apoptosis is detected with maitotoxin treatment. In the current

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studies, through studying calpain activation and the susceptibility of PARP to calpain-mediated attack in maitotoxin-treated human neuroblastoma SH-SY5Y cells, we accidentally identified that procaspase-3 was also a substrate of calpain.

MATERIALS AND METHODS

Cell culture. SH-SY5Y human neuroblastoma cells were grown on 12-well plates to confluence (roughly 2 million/well) at 37°C, 5% CO_2 in a humidified atmosphere with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 unit/ml penicillin and 100 μg/ml streptomycin, as previously described (4). Cells were washed with serum-free medium before challenged with various concentrations of maitotoxin or 0.5 μM staurosporine as described before (4).

Protein extraction and Western blotting. Total cellular proteins were extracted with a Triton X-100 buffer, as previously described (17). Protein concentration was determined with a modified Lowry (Bio-Rad D-C protein assay kit). Equal amounts of protein were loaded on each lane and run on SDS/PAGE (4–20% acrylamide gradient gel; Novex) with a Tris/glycine running buffer and transferred to a polyvinyldifluoride membrane (0.2 μM) by semi-dry electrotransfer for 2 h at 20 V. The blots were probed with antibodies to cytochrome *c* (human protein as antigen, monoclonal, Pharmingen), alpha-spectrin, poly(ADP-ribose) polymerase (polyclonal C-2-10, Biomol) or (goat polyclonal (N-20) Santa Cruz), or caspase-3 (polyclonal, Pharmingen) and a biotinylated secondary antibody and avidin conjugated with alkaline phosphatase (Amersham Pharmacia). The blots were developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma).

Cell death measurement. Cell death was assessed by measuring the release of the cytosolic enzyme, lactate dehydrogenase (LDH), into the culture medium (25 μl aliquots) using the Cytotox 96 colorimetric LDH assay kit (Promega) according manufacturer's directions (18).

Caspase activity assay. SH-SY5Y cell lysates were prepared by Triton X-100 extraction as previously described (4). Caspase-3-like activity was determined by monitoring proteolysis of the fluorogenic substrate Ac-DEVD-AMC (acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin, Peptide International). Whole cell lysate was added to a buffer containing 100 μM peptide substrate, 100 mM Hepes, 10% glycerol, 1 mM EDTA and 10 mM dithiothreitol. Fluorescence (excitation at 380 nm and emission at 460 nm) was measured every 30 min up to 2 h using a Millipore Cytoflor 2300 fluorescence plate reader (4).

Purified calpain or caspase-3 digestion of cell extract. Total protein was extracted from untreated confluent SH-SY5Y cells by the Triton X-100 method (4). SH-SY5Y cell extract (30 μg of protein) was digested with 1 μg of purified calpain or 1 μg recombinant caspase-3 (from BASF) in 100 mM Hepes buffer (pH 7.4 at RT), 10 mM DTT, 10% (v/v) glycerol, 1 mM Ca^{2+} (for calpain) or 1 mM EGTA (for caspase-3) for indicated times. The digestion was halted by the addition of SDS-containing sample buffer for PAGE. Samples were subjected to SDS–PAGE, transferred to PVDF membrane, and probed with the indicated antibody.

RESULTS

Procaspase-3 Is Truncated in Maitotoxin-Treated SH-SY5Y Cells

We first measured loss of viability by monitoring LDH release over 24 h in maitotoxin-treated SH-SY5Y cells (Fig. 1A). At the lowest MTX concentrations tested (0.01 and 0.05 nM) there was no increase in LDH release over control through 24 h. At concentrations of 0.1, 0.5 and 1.0 nM, there was a concentration-dependent increase in LDH release. We then examined proteolytic processing of alpha-spectrin in cells treated with increasing concentrations of maitotoxin for 24 h (Fig. 1B). Consistent with the LDH release data (Fig. 1A), there was a concentration-dependent formation of the 150 and 145 kDa α-spectrin breakdown product (SBDPs), starting at 0.1 nM maitotoxin. In contrast to

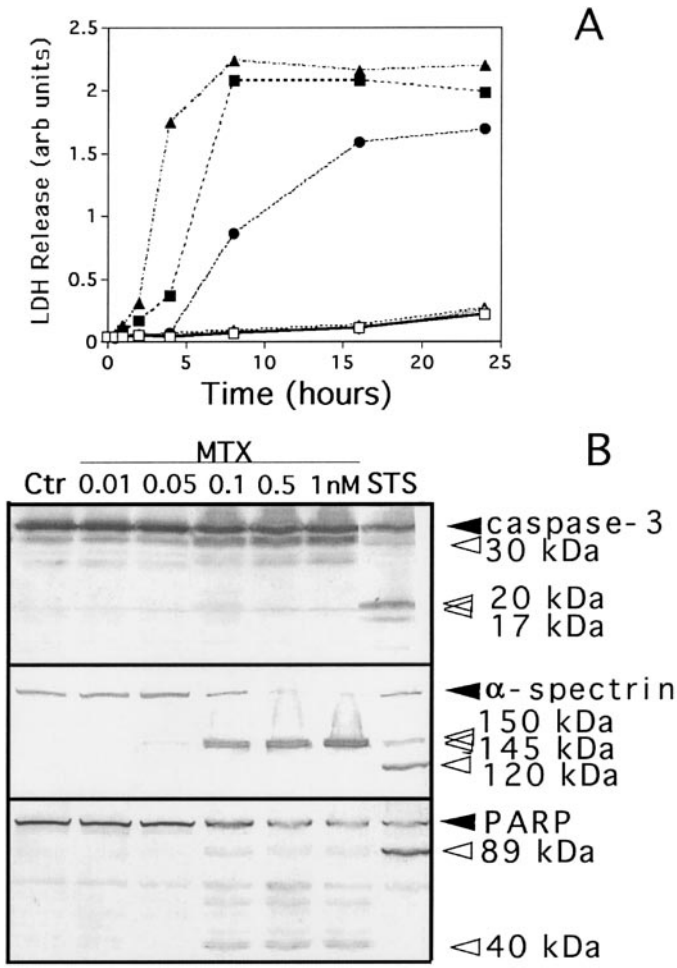

FIG. 1. Maitotoxin-treatment mediates cell death and proteolysis of caspase-3, α-spectrin, and PARP in a concentration-dependent manner. SH-SY5Y cells were treated for 24 h with the indicated concentration of maitotoxin. (A) Aliquots (25 μl) of maitotoxin-treated cell culture medium was collected at the indicated time and assayed for LDH activity. Maitotoxin concentrations: (open squares), none; (open triangle), 0.01 nM, (open circles), 0.05 nM; (solid circles), 0.1 nM; (solid squares), 0.5 nM; (solid triangles), 1 nM. (B) SH-SY5Y cells were treated with indicated concentration of maitotoxin or 0.5 μM staurosporine (STS) for 24 h. Whole cell lysate was prepared by TCA precipitation and subjected to SDS–PAGE (4–20%, 20 μg protein/lane), electrotransfered to PVDF and probed for caspase-3, α-spectrin, or PARP immunoreactivity. Intact protein (solid arrows) and major breakdown products (open arrows) are indicated.

staurosporine-induced apoptosis, no caspase-dependent 120 kDa SBDP appeared at any concentration of MTX.

PARP, a 113 kDa protein, is a well established caspase substrate, which fragmented to a N-terminally-truncated 89 kDa form during apoptosis. But recent literature also suggests that another calpain-like protease might also degrade PARP in necrotic conditions. We thus probe for the integrity of PARP in MTX treated cells. Indeed, we found that PAPR is partially degraded to a 40 kDa (using the C-2-10 antibody that detect the end of the N-terminal DNA-binding domain). This contrast to the 89 kDa fragment generated in the staurosporine treatment (Fig. 1B). This observations prompted us to look for changes in procaspase-3 processing. The active caspase-3 fragments do not appear at any concentration of MTX (Fig. 1B), again indicating that lower concentrations of maitotoxin do not lead to caspase activation. Interestingly, there is a dose-dependent procaspase-3 truncation in maitotoxin-treated SH-SY5Y cells. A 30 kDa fragment appears in cells treated with as less as 0.1 nM maitotoxin. This truncation pattern contrast to the processing of procaspase-3 to the 20 kDa and 17 kDa form in apoptosis (Fig. 1B).

Pro-caspase-3 Is a Calpain Substrate

Since calpain is highly activated in MTX-treated cells, we suspected that it might be responsible for procaspse-3 truncation and PARP cleavage. To confirm that the formation of the 30 kDa caspase-3 breakdown product (BDP) was due to calpain, we pretreated SH-SY5Y cells with 20 μM calpain inhibitor II (CI) or 50 μM Z-D-DCB, a pan-caspase inhibitor before treating with 1 nM of MTX. Formation of the 30 kDa caspase-3 fragment was blocked by calpain inhibitor II, but not Z-D-DCB (Fig. 2A). In parallel, we did immunoblots for PARP. Again, we found that calpain inhibitor II completely block the formation of the 40 kDa PARP fragment while Z-D-DCB had no effects (Fig. 2A). Since calpain inhibitor II is also known to inhibit proteasome, we wanted to examine its potential involvement. In addition to calpain inhibitor I and calpain inhibitor II, we also used lactacystin (1 and 10 μM), which is a selective inhibitor for proteasome (19). In this case, we found that both calpain inhibitors blocked caspase-3 truncation while lactacystin was ineffective (Fig. 2B). Lactacystin was also unable to block the calpain-mediated SBDP formation, confirming its selectivity (Fig. 2B).

We then digested control SH-SY5Y lysate with purified μ-calpain for 15-120 min. A 30 kDa caspase-3 fragment accumulated in a time-dependent manner starting at 15 min of *in vitro* calpain digestion (Fig. 3A). Formation of the calpain-dependent 150 kDa and 145 kDa SBDPs were used to monitor calpain activity.

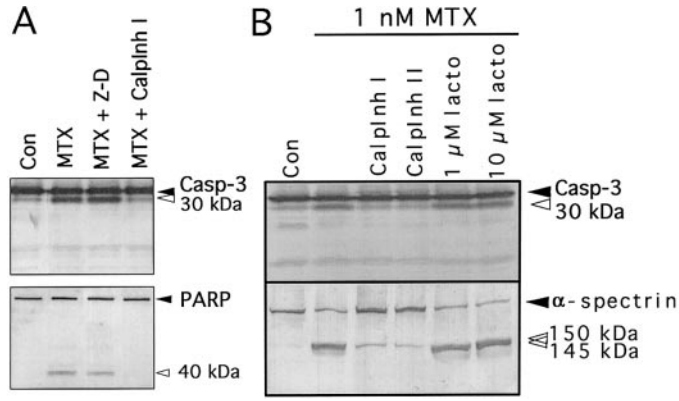

FIG. 2. Effect of protease inhibition on maitotoxin-mediated caspase-3 truncation. (A) SH-SY5Y human neuroblastoma cells were untreated (Con) or pretreated for 1 h with 50 μM Z-D-DCB (Z–D), or 10 μM CalpInh-I before challenged with 1 nM MTX (6 h) (as indicated). (B) SH-SY5Y cells were untreated (Con) or pretreated for 1 h with 10 μM CalpInh I, 20 μM CalpInh II, or 1 or 10 μM lactacystin and then treated for 6 h with 1 nM maitotoxin. Whole cell lysate was subjected to Western blot analysis with caspase-3, α-spectrin, or PARP antibodies. Intact proteins (solid arrows) and major breakdown products (open arrows) are indicated.

α-Spectrin is much more sensitive to *in vitro* calpain digestion than caspase-3: the SBDPs can be seen after 5 min and intact α-spectrin has been completely degraded at 1 h (Fig. 3A). Both procaspase-3 truncation and α-spectrin proteolysis were completely blocked in the presence of CalpInh II. Similarly, when control SH-SY5Y cell lysate were subjected to μ-calpain digestion for 20 min, a 40 kDa PARP was detected (when the polyclonal C-2-10 antibody was used) (Fig. 3B). This contrast to the 89 kDa fragment generated by caspase-3 digestion. The 40 kDa PARP fragment was also detected by a N-terminally directed antibody (N-20) (results not shown). The 40 kDa PAPR fragment formation was blocked by calpain inhibitor II (Fig. 3B).

Maitotoxin Pretreatment Disrupts Staurosporine-Mediated Caspase Activation in SH-SY5Y Cells

We investigated the effects of maitotoxin pretreatment on caspase activity with subsequent staurosporine treatment in SH-SY5Y cells. Caspase-3-like activity was monitored by Ac-DEVD-MCA hydrolysis (Fig. 4A) and by SBDP120 formation (Fig. 4B). SH-SY5Y cells were either untreated, treated with 3 h MTX alone (1 nM) for 6 h, staurosporine (0.5 μM) for 6 h; 30 min MTX pretreatment followed by 5.5 h staurosporine treatment. There is no detectable caspase-3 activity in the MTX-treated cells, while caspase-3 activity is readily detected in staurosporine treatment (Fig. 4A). Similarly, the caspase-3-produced SBDP120 was detected only in staurosporine-treated cells but not in MTX-treated cells. Intriguingly, MTX treatment abrogated the subsequent staurosporine-mediated

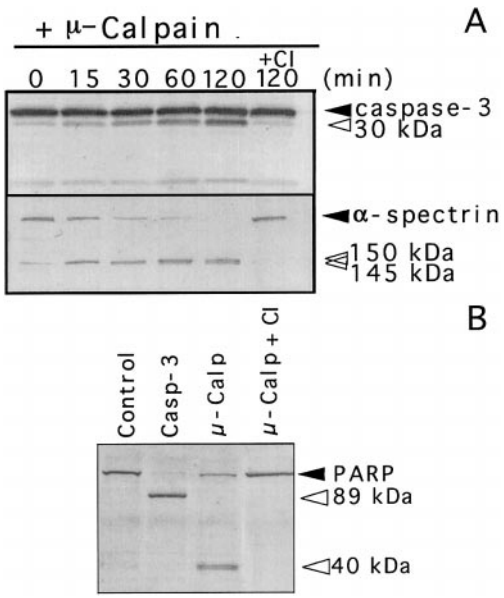

FIG. 3. Digestion of control SH-SY5Y lysate with purified calpain. (A) Whole cell lysate (30 μg) from untreated SH-SY5Y cells was collected by triton extraction as described under Materials and Methods and digested with purified porcine μ-calpain (1 μg) for indicated time (1, 15, 30, 60, or 120 min). In the last lane, lysate was incubated with μ-calpain for 120 min in the presence of CalpInh II (20 μM). (B) Whole cell lysate (30 μg) from untreated SH-SY5Y cells was incubated with buffer alone (Control), recombinant human caspase-3 (1 μg), μ-calpain (1 μg) in the absence or presence of CalpInh II (20 μM) The incubation was halted with the addition of 20 μl SDS sample buffer. The lysates were subjected to SDS–PAGE (4–20%) and analyzed by Western blotting with caspsase-3, PARP, or α-spectrin antibodies. Intact protein and major breakdown products are indicated.

caspase activation (Fig. 4A). Caspase-3-mediated SBDP120 upon staurosporine-treatment was also abolished by MTX pretreatment (Fig. 4B). Surprisingly, the addition of calpain inhibitor I during MTX pretreatment, which prevented caspase-3 truncation (Fig. 2B), still did not allow caspase-3 to be fully activated by subsequent staurosporine treatment (Figs. 4A and 4B).

DISCUSSION

In this study, we reported that procaspase-3 is an endogenous calpain substrate during a necrotic challenge of SH-SY5Y cells with maitotoxin. We showed that procaspase-3 was not processed to its activated dimeric form (20/17 kDa + 12 kDa), but rather was truncated to a 30 kDa BDP by calpain (Figs. 1, 3). The fragmentation of procaspase-3 was completely blocked by calpain inhibitors but not caspase inhibitors (Fig. 2). Finally, caspase-3 activity in staurosporine-treated SH-SY5Y cells is suppressed by maitotoxin pretreatment (Fig. 4).

Previously we demonstrated that cells treated with maitotoxin (0.1–1 nM) gave a rather robust morphologic necrosis (4). Under maitotoxin challenge, we demonstrated a calpain-dependent cleavage of procaspase-3. This cleavage is distinct from the caspase-8 or −9 processing of procaspase-3, leading to the 20/17 kDa + 12 kDa form. In fact, with 1 nM maitotoxin treatment, caspase-3 activity was never elevated (Fig. 4A), One might argue that cells die too quickly to allow the initiation of the apoptotic pathway. We addressed this issue by using lower doses of maitotoxin (Fig. 1A), but at no concentration of MTX was caspase-3 activity detectable.

Since the anti-caspase-3 antibody we used detect the 17 kDa domain of capasae-3, we concluded that the

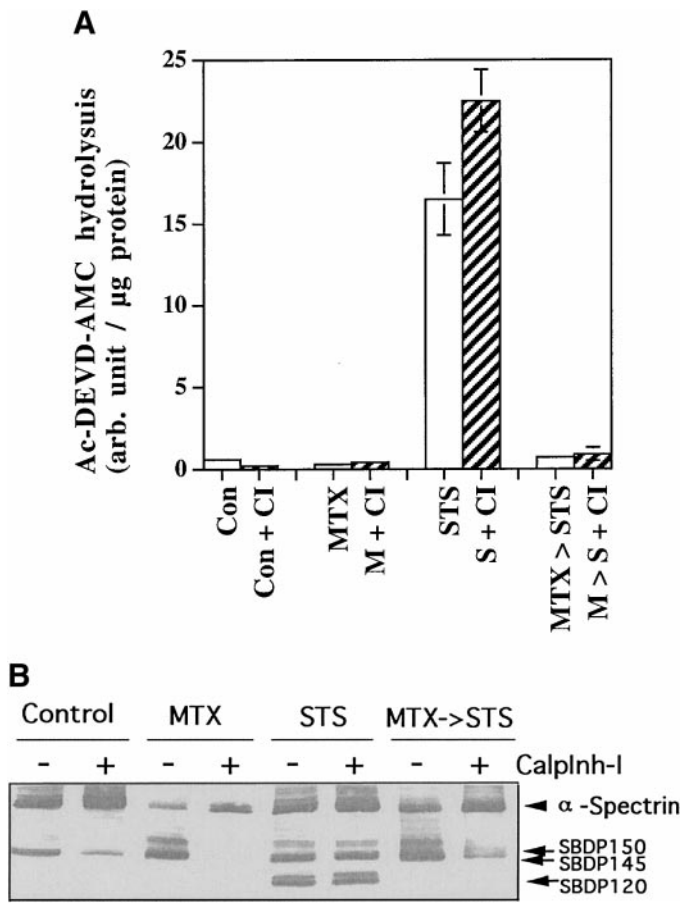

FIG. 4. Effect of maitotoxin on staurosporine-mediated caspase activation. (A) SH-SY5Y cells were either untreated (Con) or treated for 6 h with maitotoxin (MTX, 1 nM); 6 h with staurosporine (STS, 0.5 μM); 30 min with MTX then 5.5 h with STS (MTX > STS) with or without the presence of calpain inhibitor I (CI, 20 μM). Whole cell lysate treated SH-SY5Y cells was collected by Triton X-100 extraction at indicated time. Caspase-3-like activity was assayed by monitoring hydrolysis of the fluorogenic substrate Ac-DEVD-MCA as described under Materials and Methods. Data are mean ± SEM expressed as fluorescence unit per μg of protein. (B) SH-SY5Y cells were treated as in (A) and whole cell lysate was probed for α-spectrin immunoreactivity. Lane 1, control; lane 2, control with CalpInh I (20 μM); lane 3, MTX; lane 3, MTX with CalpInh I (20 μM); lane 4, STS; lane 5, STS with CalpInh I; lane 6, MTX 30 min then STS 5.5 h; and lane 7, MTX-STS with CalpInh I.

calpain mediated procaspase-3 truncated occurs at the N-terminal pro-domain rather than the C-terminal region. Recently, Wolf *et al.* (20) also had a preliminary report showing that upon platelet activation, both procapase-3 and procasapse-9 are truncated in the prodomain region by calpain with activating either caspase. Recently, several studies emerged that showed that both calpain and caspases are activated in a variety forms of apoptosis such as dexamethasone-treated thymocytes, TNF-α-treated Jurkat cells. constitutive apoptosis in neutrophils and potassium deprivation in cerebellar granule neurons (4, 21–24). Interestingly, while BCL-2 is a substrate for casapse-3 (25), BAX is reported to be a substrate for calpain (26–27). Interestingly, calpain inhibitor protein calpastatin is a substrate for caspases (28–29).

α-Spectrin II (α-fodrin), β-spectrin II, calmodulin-dependent protein kinase IV, tau, actin are among a growing list of cellular proteins are that dually sensitive to degradation by calpain and caspases (4, 30–31). PARP is the first identified substrate for caspase-3 (3). During classic apoptosis, the 113 kDa PARP is degraded by caspase to a distinct 89 kDa fragment and 24 kDa fragment (3). Yet recently, PARP have been found to be cleaved at alternative site(s), generating fragments from 70 kDa to 40 kDa during necrosis (13–14). Consistent with that, here we reported that beside casapse-3 truncation, PARP is also cleaved by calpain to a 40 kDa fragment by in SH-SY5Y cells challenged with maitotoxin (Figs. 1, 2). Since the 40 kDa PARP BDP was detected by a N-terminally directed antibody (N-20), we conclude that the calpain cleavage site was 40 kDa from the N-terminal. Another recent study described the purification of a non-caspase-protease that cleaves bovine PARP (15). They also identified the protease as bovine m-calpain.

Maitotoxin-pretreatment interrupts staurosporine-mediated activation of caspase-3 (Fig. 4), This could probably occur at several different levels: calpain-dependent processing of BAX may prevent the cytochrome c-release; truncation of caspase-9 hampers it ability to process caspase-3 and the direct truncation of caspase-3 prevents its activation. However, while calpain inhibitor I co-treatment with maitotoxin prevents the 30 kDa caspase-3 BDP formation (Fig. 2), no caspase-3 activity recovers in subsequent staurosporine treatment (Fig. 4). Therefore, it is likely that maitotoxin-pretreatment produces a number of cellular changes, including but not limited to calpain overactivation. The combination of these events disrupts the apoptotic cascade and promotes an shift to a calpain-driven necrotic pathway. The balance between necrosis and apoptosis may be tied to mitochondrial function, with acute insult to the mitochondria leading to necrosis while a mild insult would lead to apoptosis (32). Our data here suggest that there are cellular pathways that guide the cell exclusively into either apoptosis or necrosis. These pathways need to be further explored.

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