

Cytochrome *c* translocation does not lead to caspase activation in maitotoxin-treated SH-SY5Y neuroblastoma cells

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Abstract

Cytosolic cytochrome *c* elevation has been associated with activation of caspase-3-like proteases. In this study, we demonstrate that treatment with the neurotoxin and potent calcium channel opener maitotoxin (MTX) induces cytochrome *c* release from the mitochondria that is not accompanied by caspase activation. Cytochrome *c* translocation in MTX-treated SH-SY5Y cells was readily apparent after 30 min and peaked at 2.5 h. We assayed caspase activity by acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) hydrolysis and by immunoblotting for caspase-3 processing and proteolysis of α II-spectrin and PARP. In contrast, treatment with pro-apoptosis agent staurosporine (STS) induced both cytochrome *c* release and caspase-3 activation after 2 h. In addition, with MTX treatment, we found no evidence of caspase activation at any time point or MTX concentration used. Instead, we observed that caspase-9, Apaf-1 and caspase-3 were all partially truncated by calpain under these conditions. These combined effects likely contribute to the lack of caspase activation cascade in MTX-treated cells, despite the presence of cytochrome *c* in the cytosol.

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1. Introduction

Changes at the mitochondria are point of convergence for different apoptotic signaling pathways. Cytochrome *c* released from mitochondria is a necessary co-factor for activation of caspase-3-like proteases (Kluck et al., 1997; Liu et al., 1996). Direct microinjection of cytochrome *c* is sufficient to activate caspases and induce apoptosis in some systems (Zivotovsky et al., 1998; Li et al., 1997). The mechanism by which cytochrome *c* is released from the mitochondria is unclear. Cytochrome *c* release in some cell types corresponds with the opening of the MPT pore. However, several groups have demonstrated that the release of cytochrome *c* occurs prior to the change in mi-

tochondrial membrane permeability (Krohn et al., 1999; Bossy-Wetzel et al., 1998). We demonstrated that inhibition of the MPT does not prevent cytochrome *c* release in apoptotic SH-SY5Y cells (McGinnis et al., 1999a).

MTX is a potent marine toxin that stimulates Ca^{2+} influx through multiple types of calcium channels (both ligand- and voltage-gated calcium channels (Gusovsky and Daly, 1990; Daly et al., 1995). It has been documented previously reported that MTX could drastically elevate intracellular calcium levels to high micromolar range (Gusovsky and Daly, 1990; Daly et al., 1995; Wang et al., 1996). At above 1 nM MTX intracellular calcium concentration essentially would reach an equilibrium with the calcium concentration in the culture medium (i.e. 800 μ M in the case of DMEM medium used for SH-SY5Y cells). As a result of the sustained calcium increase, intense calpain activation has been observed (Wang et al., 1996; McGinnis et al., 1999b). We have previously demonstrated that MTX evokes a robust necrotic response in SH-SY5Y cells (Wang et al., 1996). We report here that cytochrome *c* is released into the cytosol in MTX-treated SH-SY5Y cells. Yet, this release is not accompanied by caspase-3 activation.

Abbreviations: AIF, apoptosis inducing factor; MPT, mitochondrial permeability transition; STS, staurosporine; MTX, maitotoxin; Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin; BDP, breakdown product; SBDP, α II-spectrin breakdown product; Z-D-DCB, carbobenzoxy-Asp-CH₂OC(O)-2,6-dichlorobenzene

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2. Materials and methods

2.1. Cell culture

SH-SY5Y human neuroblastoma cells were grown on 12-well plates to confluency (roughly 2 million per well) at 37 °C, 5% CO₂ in a humidified atmosphere with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin.

2.2. Protein extraction and fractionation

Cells were collected at the end of the treatment period by scraping and centrifugation at 3600 × *g*. Cell pellets were washed 2× with Tris buffered saline/EDTA. Pellet were re-suspended in 5 volumes of homogenization buffer: 20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EGTA, 1 mM dithiothreitol, protease inhibitor cocktail (Calbiochem), and 250 mM sucrose. Cells were homogenized in a Dounce Teflon glass homogenizer with 15 strokes. Homogenate was centrifuged at 500 × *g* for 5 min to pellet out nuclei and intact cells. Supernatants were centrifuged at 10,000 × *g* for 30 min. The 10,000 × *g* mitochondria-rich heavy membrane pellet was re-suspended in 100 µl homogenization buffer and represents the mitochondrial fraction. The 10,000 × *g* supernatant is used as a crude cytosol. Some experiments were performed using a 100,000 × *g* cytosol and were not different from the 10,000 × *g* supernatant. Samples were frozen at –70 °C until use.

2.3. Western blotting

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 polyvinylidene difluoride membrane (0.2 µM) by semi-dry electrotransfer for 2 h at 20 V. All gels were loaded with 15 µg of proteins and gel loading was even for all the gel used for Western blot analysis in this study. The blots were probed with antibodies to cytochrome *c* (human monoclonal, Pharming), αII-spectrin, poly(ADP-ribose) polymerase (PARP, human monoclonal, Biomol), or caspase-3 (human 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).

2.4. 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 color-

metric LDH assay kit (Promega) according manufacturer's directions.

2.5. Caspase activity assay

SH-SY5Y cell lysates were prepared by Triton X-100 extraction as previously described. Caspase-3-like activity was determined by monitoring proteolysis of the fluorogenic substrate Ac-DEVD-AMC, 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 Cytofluor 2300 fluorescence plate reader.

3. Results

3.1. Maitotoxin treatment evokes cytochrome *c* translocation

SH-SY5Y cells were treated with 0.1 nM MTX for various times. We have previously shown that this concentration of MTX shows toxicity as measured lactate dehydrogenase release 4–6 h after initiation of treatment (Nath et al., 1996a,b). Cytosolic and mitochondrial fractions were prepared and subjected to Western blot analysis for cytochrome *c*. The control cytosol has low level of cytochrome *c* due to the presence of newly synthesized non-heme pool (which does not trigger apoptosis) or minor contamination of mitochondrial fraction during preparation (Fig. 1A). An increase in cytosolic cytochrome *c* was evident after 1 h (Fig. 1A) and peaked after 2.5 h of MTX treatment (492 ± 29% of levels at 0 h) (Fig. 1B). Loss of mitochondrial cytochrome *c* was not apparent until 2 h after start of treatment, but was clearly discernible at 3 h (Fig. 1A). The inability to detect changes in mitochondrial cytochrome *c* levels at earlier times may be attributable to the initial high levels of mitochondrial cytochrome *c*. At 2.5 h of MTX treatment, mitochondrial cytochrome *c* levels had fallen to 63.3 ± 12.7% of levels at 0 h (Fig. 1B).

We previously reported translocation of endogenously expressed Bax from the cytosol to the mitochondrial fraction in SH-SY5Y cells and rat cerebellar granule neurons undergoing apoptosis (McGinnis et al., 1999a). Using the same samples as in Fig. 1A we Western blotted for Bax and consistently found that there was little or no apparent loss of Bax from the cytosol with a small increase in mitochondrial Bax after 3 h of MTX-treatment (Fig. 1C).

3.2. Maitotoxin treatment does not induce caspase-3-like activity

Because increases in cytosolic cytochrome *c* and mitochondrial Bax are associated with caspase-3-like protease

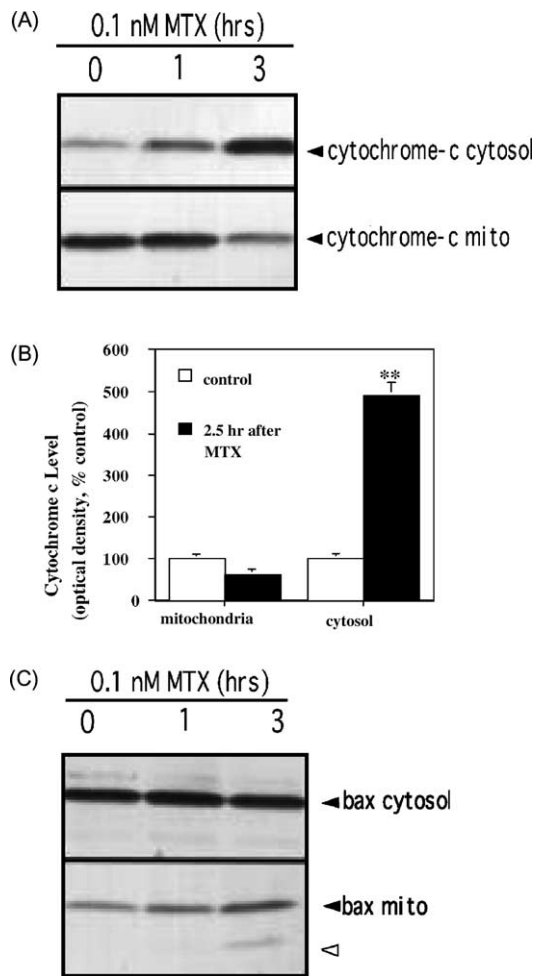


Fig. 1. Cytochrome *c* localization in cytosolic and mitochondrial fractions in MTX-treated SH-SY5Y. SH-SY5Y cells were fractionated and electrophoresed as described in Section 2. SH-SY5Y cells were untreated or treated for indicated times with 0.1 nM MTX. (A) Western blotting with cytochrome *c* antibody was performed on cytosolic (top, 40 μ g protein per lane) and mitochondrial (bottom, 20 μ g protein per lane) fractions. (B) Densitometric analysis of changes in levels of mitochondrial and cytosolic cytochrome *c* in SH-SY5Y treated with 1 nM MTX for 0 or 2.5 h. Data are mean \pm S.E.M. (** $P < 0.001$ vs. control cytosol, Student's *t*-test, $n = 3$). (C) Western blotting with Bax antibody was performed on cytosolic (top, 40 μ g protein per lane) and mitochondrial (bottom, 20 μ g protein per lane) fractions. Open arrow indicates 18 kDa calpain-dependent Bax BDP.

activity, we investigated whether caspase-3 is activated in MTX-treated SH-SY5Y cells. As a positive control for caspase-3-like activity, SH-SY5Y cells were treated with the well-characterized pro-apoptotic agent STS (500 nM) (Posmantur et al., 1997). Cleavage of the DNA repair enzyme PARP by caspase-3-like proteases to an 85 kDa BDP is an early marker of caspase-3-like activity in mammalian cells undergoing apoptosis (Lazebnik et al., 1994; Nicholson et al., 1995). Similarly, formation of the 120 kDa SBDP is solely due to caspase-3 activity (Wang et al., 1998). In STS-treated cells, both the 85 kDa PARP BDP and the

120 kDa SBDP appear after 4 h of treatment (Fig. 2A). With 1 nM MTX-treated SH-SY5Y cells, no 85 kDa PARP BDP nor 120 kDa SBDP was formed after up to 4 h of treatment. Note the appearance of 150 and 145 kDa SBDPs: these fragments were produced by the activation of the Ca^{2+} -dependent cysteine protease calpain (McGinnis et al., 1999a).

We examined the processing of pro-caspase-3 to its active fragments (Fig. 2B). Upon treatment with STS, caspase-3 is proteolyzed to fragments of 17 kDa, corresponding to the large subunit of the active enzyme and 11 kDa. A 20 kDa species is most likely the 17 kDa fragment plus the precursor pro-peptide (Fernandes-Alnemri et al., 1994; Schlegel et al., 1996). The antibody we used was unable to detect the 11 kDa fragment. In contrast, no active caspase-3 fragments were detected with up to 3 h of MTX treatment. Interestingly, there does appear to be some truncated pro-caspase-3 in MTX treated SH-SY5Y cells (see Fig. 3). STS treatment was again used as a positive control for caspase-3 activation. The appearance of the 17 kDa fragment was apparent by 4 h after STS treatment. Also note the loss of intact pro-caspase-3 in STS-treated SH-SY5Y cells.

We directly assayed for caspase-3-like activity by measuring hydrolysis of the fluorogenic caspase-3-like protease peptide substrate Ac-DEVD-AMC (Nath et al., 1996a,b; Posmantur et al., 1997) (Fig. 2C). Consistent with the data presented in Fig. 2A and B, in MTX treated cells, no hydrolysis of Ac-DEVD-AMC occurred up to 4 h after initiation of challenge (Fig. 2C). In contrast, lysate from SH-SY5Y cells treated with STS showed a significant increase in Ac-DEVD-AMC hydrolysis after 2 h of treatment.

To demonstrate that MTX treatment induced cytotoxicity in SH-SY5Y cells within the time frame of interest, we monitored release of lactate dehydrogenase (LDH) into the culture medium (Fig. 2D). In SH-SY5Y cells treated with 1 nM MTX, LDH release was significantly elevated above control within 1 h. LDH release continued to increase sharply throughout the 4 h of MTX treatment. In STS-treated SH-SY5Y cells, LDH release was not significantly elevated until 4 h after the start of treatment.

3.3. Lower concentrations of maitotoxin do not lead to caspase activation

Since reagents that mediate oncotic necrosis at high concentrations may induce an apoptotic response at lower concentrations, we investigated whether lower concentrations of MTX would elicit cytotoxicity and caspase activation. We examined proteolytic processing of α II-spectrin and pro-caspase-3 in cells treated with increasing concentrations of MTX for 24 h (Fig. 3). There was a concentration-dependent formation of the 150 and 145 kDa SBDPs (SBDP150 and SBDP145). No caspase-dependent α II-spectrin 120 kDa BDP (SBDP120) appeared at any concentration. No α II-spectrin processing occurred until

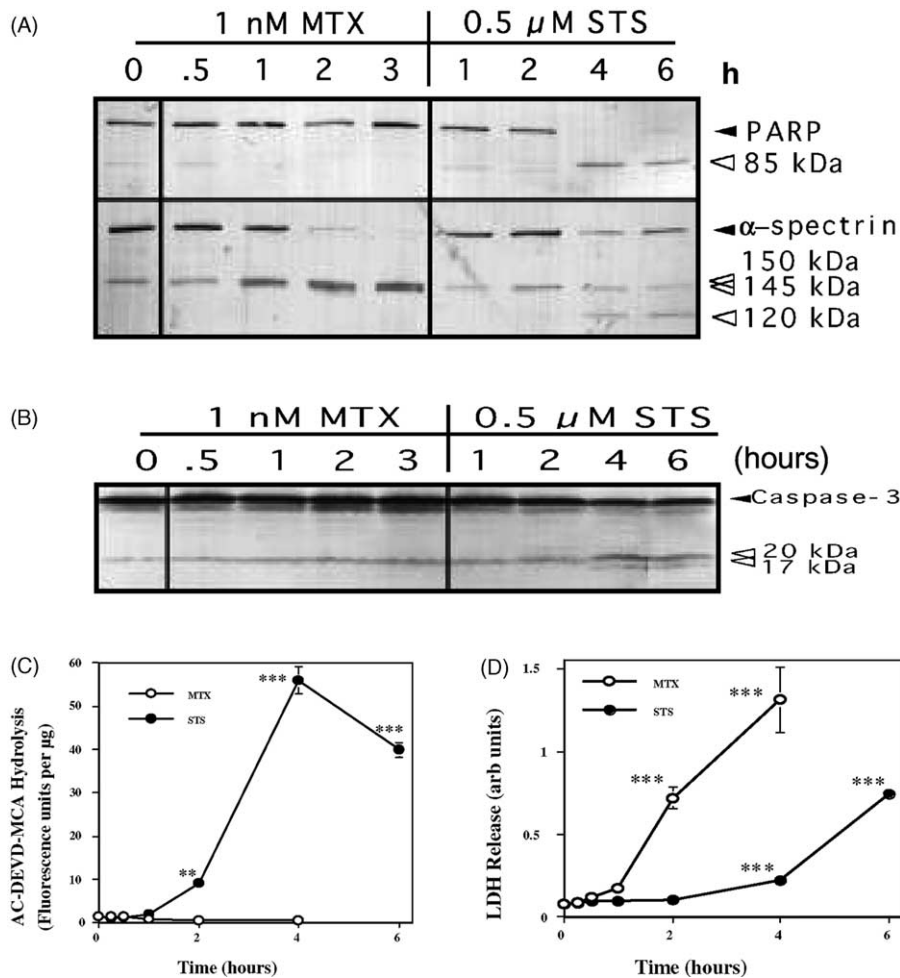


Fig. 2. Caspase-3 activities in STS- and MTX-treated SH-SY5Y cells. (A) SH-SY5Y cells were either untreated or treated with 0.5 μ M STS or 1 nM MTX. Whole cell lysate (20 μ g) was subjected to Western blot analysis with PARP or α II-spectrin antibodies. Open arrowheads indicate the 85 kDa PARP BDP and 120 kDa SBDP. (B) Caspase-3 processing to p20 and p17 (open arrowheads) was monitored by immunoblotting whole cell lysate from MTX- and STS-treated SH-SY5Y cells. (C) Whole cell lysate from 0.5 μ M STS or 1 nM MTX-treated SH-SY5Y cells was collected by Triton extraction at indicated time. Caspase-3-like activity was assayed by monitoring hydrolysis of the fluorogenic substrate Ac-DEVD-AMC as described in Section 2. Data are mean \pm S.E.M. ($n = 6$) expressed as fluorescence unit per mg of protein (** $P < 0.01$, *** $P < 0.001$, ANOVA with post-hoc Tukey–Kramer comparison between MTX-treatment vs. initial fluorescence values at time zero or STS-treatment vs. initial fluorescence values at time zero, $n = 4$). (D) LDH released into the culture medium from STS- or MTX-treated SH-SY5Y cells. Data are mean \pm S.E.M. ($n = 6$), (***) $P < 0.001$, ANOVA with post-hoc Tukey–Kramer comparison between MTX- or STS-treatment vs. controls). For (C) and (D), MTX data and STS data are represented by open and closed circles, respectively.

exposure to 0.1 nM MTX. We also looked for changes in pro-caspase-3 processing. The active caspase-3 fragments do not appear at any concentration, again indicating that lower concentrations of MTX do not lead to caspase activation. Interestingly, there is a dose-dependent pro-caspase-3 processing in MTX-treated SH-SY5Y cells. A 30 kDa fragment appears in cells treated with as little as 0.1 nM MTX. Previously, we have demonstrated that the truncation of pro-caspase-3 was due to calpain proteolysis (McGinnis et al., 1999b). The appearance of α II-spectrin and caspase-3 BDPs at 0.1 nM MTX is consistent with our previously published report that below 0.1 nM MTX is not toxic to SH-SY5Y cells (McGinnis et al., 1999b).

3.4. Calpain activation inhibits caspase activation cascade in maitotoxin treated cells

We further examined why caspase-3 was not activated despite that fact the cytochrome *c* was released from the mitochondria. We subjected SH-SY5Y cells to either MTX (1 nM) or STS (0.5 μ M) in the presence of either calpain inhibitor SJA6017 (25 μ M) or caspase inhibitor Z-D-DCB (50 μ M). We first confirmed that the 30 kDa fragment of pro-caspase-3 generated in MTX treatment (Fig. 3) was indeed produced by calpain as it was sensitive to calpain inhibitor but not caspase inhibitor (Fig. 4D). In contrast, STS leads to autolyzed and activated form (p17) of caspase-3,

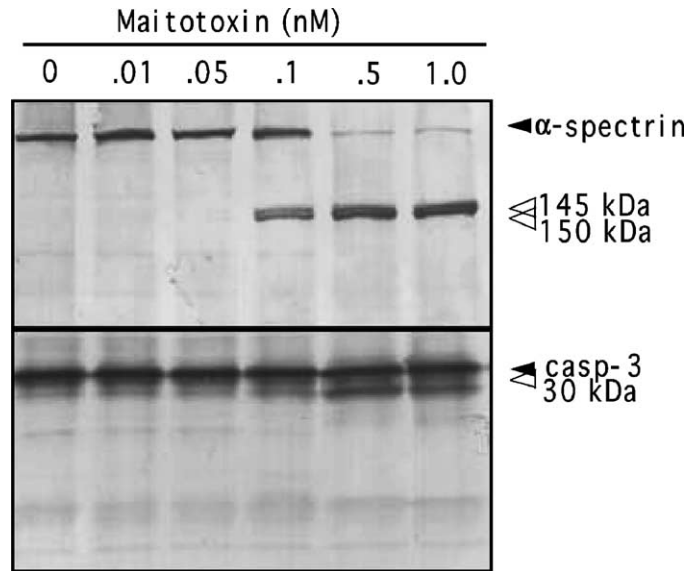


Fig. 3. Concentration/response for caspase-3-like activity in MTX-treated SH-SY5Y cells. SH-SY5Y cells were treated for 6 h with varying concentrations of MTX, as indicated. Whole cell lysate was subjected to Western blot analysis with α II-spectrin or caspase-3 antibodies. Immunoblots are representative of three experiments.

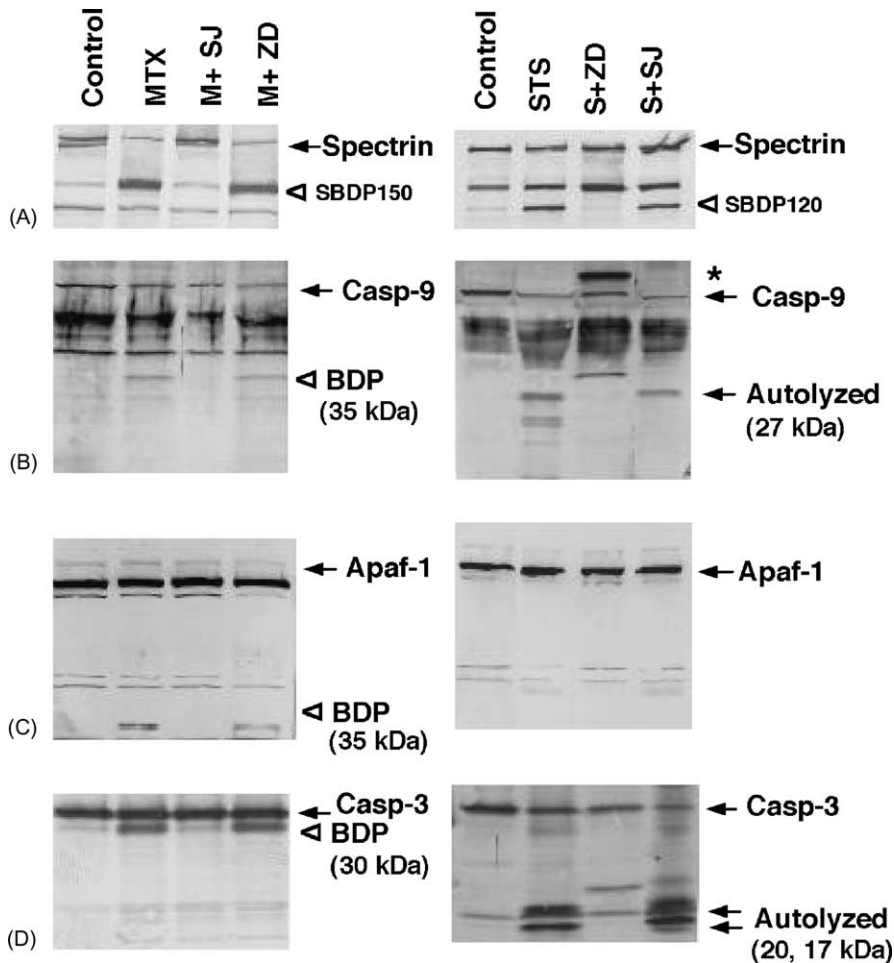


Fig. 4. Integrity of caspase-3, caspase-9 and Apaf-1 in STS- and MTX-treated SH-SY5Y cells. SH-SY5Y cells were either untreated or treated with 0.5 μ M STS or 1 nM MTX, in the presence or absence of 25 μ M calpain inhibitor SJA6017 or 50 μ M caspase inhibitor Z-D-DCB. Whole cell lysate (20 μ g) was subjected to Western blot analysis with α II-spectrin, 280 kDa (A), caspase-3, 32 kDa (B), caspase-9, 48 kDa (C) and Apaf-1, 140 kDa (D) antibodies. Arrowheads indicate the intact proteins or the autolyzed forms while triangles indicated their BDP. Symbol (*) indicates the caspase-9/Z-D-DCB complex. Immunoblots are representative of three experiments.

which was sensitive only to caspase inhibitor (Fig. 4D). As controls, we showed that under the same conditions, the α II-spectrin was degraded to calpain-inhibitor sensitive fragment SBDP150 and to caspase-inhibitor sensitive fragment SBDP120 in MTX- and STS-treated cells, respectively (Fig. 4A). Since caspase-9 and Apaf-1 were previously reported to be sensitive to calpain degradation under certain conditions (Wolf et al., 1999; Chua et al., 2000) we examined the integrity of these proteins. Caspase-9, as expected, was autolyzed to the active form (about 27 kDa). Formation of this form was blocked only by the addition of the caspase-inhibitor Z-D-DCB. Interestingly, Z-D-DCB apparently formed a covalent complex with full-length caspase-9, which has a slightly higher molecular weight (Fig. 4B). In contrast, in MTX-treated cells, we found that caspase-9 was largely degraded to a fragment (35 kDa), which was previously shown to be incapable of activating caspase-3 (Chua et al., 2000) (Fig. 4B). The formation of the 35 kDa form of caspase-9 was prevented by calpain inhibitor SJA6017 (Fukiage et al., 1997). Lastly, we also looked at integrity of Apaf-1, which complexes with cytochrome *c* to activate caspase-3. In STS-treated cells, Apaf-1 (140 kDa) remained intact. In contrast, after MTX treatment, partial fragmentation of Apaf-1 to a small BDP (35 kDa) was observed (Fig. 4C). This fragment was sensitive to calpain inhibitor SJA6017 and is consistent with previously studies that showed that the Aapf-1 BDP was incapable of activating caspase-3 (Chua et al., 2000).

4. Discussion

In this study, we sought to determine whether cytochrome *c* release from the mitochondria is invariably associated with caspase-3-like activity. We found that even in the face of a five-fold increase in cytosolic cytochrome *c*, there was no evidence of caspase-3-like activity in MTX-treated SH-SY5Y cells. The inability of cytochrome *c* release to activate caspase-3 in MTX-treated SH-SY5Y may be due to the absence of other necessary co-factors that are released during apoptosis. Microinjection of superior cervical ganglion cells with cytochrome *c* did not alter the rate of apoptosis (Neame et al., 1998). This is in contrast to other reports in non-neuronal cells that showed injection of cytochrome *c* alone was sufficient to induce apoptosis (Li et al., 1997; Zhivotovsky et al., 1998).

An AIF was found released from the mitochondria upon MPT and is a required co-factor for caspase-3-like activity (Susin et al., 1997). The necessity of AIF in SH-SY5Y cells undergoing apoptosis has not been evaluated. AIF and cytochrome *c* are released from mitochondria exposed to excess Ca^{2+} (Petit et al., 1998). In addition, after Ca^{2+} -mediated induction of MPT, mitochondrial extracts induced pro-caspase-3 processing in cytosolic extracts (Yang and Cortopassi, 1998). Cells treated with MTX may

die too quickly to allow the initiation of the apoptotic pathway. We addressed this issue by using low doses of MTX (Fig. 3), but at no concentration was caspase-3-like activity detectable. Cytochrome *c* may be released through different mechanisms in apoptotic versus necrotic neurons. In STS-treated SH-SY5Y cells Bax translocated to the mitochondria simultaneously with release of cytochrome *c* release (McGinnis et al., 1999a). In MTX treated cells, Bax translocation is not as profound and is not apparent until after cytochrome *c* release (Fig. 1C). Bax translocation to the mitochondria during apoptosis may form channels that release apoptosis-inducing co-factors other than cytochrome *c*.

The balance between oncotic necrosis and apoptosis may be tied to mitochondrial function, with acute insult to the mitochondria leading to oncotic necrosis (Green and Kroemer, 1998; Zamzami et al., 1997; Ankarcrona et al., 1995). Nicotera et al. (1998) demonstrated that ATP is important in determining whether a cell undergoes apoptosis or oncotic necrosis. At low ATP levels, neurons are more prone to undergo oncotic necrosis (Leist et al., 1997). While we did not measure ATP level or mitochondrial membrane potentials, it is expected that MTX would induce a huge calcium-mediated energy crisis (and ATP loss). This could be one of the reasons that the caspase pathway was not activated under these conditions. Calpain is activated during apoptosis in a variety of cell types (Nath et al., 1996a,b) and is required for apoptosis in some systems (Knepper-Nicolai et al., 1998). However, we (McGinnis et al., 1999a,b) and others (Lankiewicz et al., 2000; Wolf et al., 1999) described the processing of caspase-3 by calpain. Although this processing is not necessarily associated with the inactivation of caspase-3 (Lankiewicz et al., 2000; Wolf et al., 1999). More importantly, Chua et al. (2000) showed that caspase-9 is also sensitive to calpain cleavage and the truncated caspase-9 is incapable of activating caspase-3. They also showed that Apaf-1 can be cleaved by calpain. The cleaved Apaf-1 is rendered incapable of forming a complex with cytochrome *c* and caspase-9 (Chua et al., 2000). Indeed, in our MTX-treated cells, we found that both caspase-9 and to a lesser extent, Apaf-1 were fragmented by calpain. Taken together, we propose that acute robust calpain activation can at least in part direct the death pathway towards oncotic necrosis via inactivation of the caspase cascade. Previously, we have tested to see if the presence of calpain inhibitor would allow for caspase-3 activation following MTX treatment. Somewhat to our surprise, caspase-3 activity as measured by ac-DEVD-AMC was still not detected in this condition (Fig. 4 of McGinnis et al., 1999b). It thus argued that other biochemical mechanisms might be involved in preventing caspase-3 activation in the MTX-induced cytochrome *c* release paradigm. For example, regulation of caspases activation by inhibitor of apoptosis proteins (IAPs, XIAP) (Yang and Li, 2000) might be involved. Further investigation is needed to address these mechanisms.

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