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Neuroprotective effects of *Mycoplasma hyorhinis* against amyloid-β-peptide toxicity in SH-SY5Y human neuroblastoma cells are mediated by calpastatin upregulation in the mycoplasma-infected cells

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ABSTRACT

Mycoplasmas are frequent contaminants of cell cultures. Contamination leads to altered synthetic and metabolic pathways. We have found that contamination of neuroblastoma SH-SY5Y cells by a strain of *Mycoplasma hyorhinis* derived from SH-SY5Y cell culture (NDMh) leads to increased levels of calpastatin (the endogenous inhibitor of the Ca^{2+}-dependent protease, calpain) in NDMh-infected cells. We have now examined effects of amyloid-β-peptide (Aβ) (central to the pathogenesis of Alzheimer's disease) on uncontaminated (clean) and NDMh-infected SH-SY5Y cells. Aβ was toxic to clean cells, resulting in necrotic cell damage. Aβ treatment led to activation of calpain and enhanced proteolysis, cell swelling, cell membrane permeability to propidium iodide (PI) (without nuclear apoptotic changes), and diminished mitochondrial enzyme activity (XTT reduction). Aβ-toxicity was attenuated in the high calpastatin-containing NDMh-infected cells, as shown by inhibition of calpain activation and activity, no membrane permeability, normal cell morphology, and maintenance of mitochondrial enzyme activity (similar to attenuation of Aβ-toxicity in non-infected cells overexpressing calpastatin following calpastatin-plasmid introduction into the cells). By contrast, staurosporine affected both clean and infected cells, causing apoptotic damage (cell shrinkage, nuclear apoptotic alterations, caspase-3 activation and caspase-promoted proteolysis, without PI permeability, and without effect on XTT reduction). The results indicate that mycoplasma protects the cells against certain types of insults involving calpain. The ratio of calpastatin to calpain is an important factor in the control of calpain activity. Exogenous pharmacological means, including calpastatin-based inhibitors, have been considered for therapy of various diseases in which calpain is implicated. Mycoplasmas provide the first naturally occurring biological system that upregulates the endogenous calpain inhibitor, and thus may be of interest in devising treatments for some disorders, such as neurodegenerative diseases.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder, clinically manifested by progressive dementia. The neuropathological alterations include the accumulation of amyloid-β-peptide (Aβ, derived from the amyloid precursor protein, APP), extracellular neuritic plaques containing Aβ, intracellular neurofibrillary tangles that are composed mainly of the protein tau, and neuronal cell loss. Aβ plays a major role in the pathogenesis of AD and is toxic to neurons (Hardy and Selkoe, 2002). Aβ leads to increased cellular Ca^{2+}, mitochondrial dysfunction, enhanced generation of reactive oxygen species, and may cause neuronal cell death by apoptosis or necrosis (Demuro et al., 2010; Fifre et al., 2006; LaFerla, 2002).

Abbreviations: Aβ, amyloid-β-peptide; AD, Alzheimer's disease; APP, the amyloid precursor protein; BSA, bovine serum albumin; DTT, dithiothreitol; FCS, fetal calf serum; GM, growth medium; IB, immunoblotting; NDMh, neuroblastoma-derived *Mycoplasma hyorhinis*; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; sAβ, Aβ25–35; XTT, sodium 3′-[1-(phenyl-aminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate.

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The Ca^{2+}-dependent proteases, μ- and m-calpain are implicated in the pathogenesis of AD. Activation of calpain and depletion of calpastatin (the endogenous inhibitor of calpain) have been observed in brains of AD patients (Nixon, 2000; Rao et al., 2008; Saito et al., 1993). We have found that in normal mice, calpastatin levels are low in the hippocampus and frontal cortex, and high in the cerebellum. In Tg2576 mice (transgenic for a human APP mutant, providing a model for AD), the low-calpastatin brain regions exhibit calpain activation and proteolysis, and accumulation of Aβ; in the high-calpastatin brain region, calpain activity is not promoted and Aβ does not accumulate (Vaisid et al., 2007). These results suggest that calpastatin levels and their localization in regions of the normal brain are important in the development of AD.

Calpain is also activated in neurons exposed to Aβ (Fifre et al., 2006; Kelly and Ferreira, 2006). We have found that in differentiated PC12 neuron-like cells, Aβ causes calpain activation, protein degradation and membrane dysfunction (Vaisid et al., 2008a). Overexpression of calpastatin in the PC12 cells inhibits the Aβ-promoted alterations, thus substantiating the conclusion of calpain involvement in Aβ-toxicity (Vaisid et al., 2008b). In addition, we have found that caspase-8, which is implicated in AD (Matsui et al., 2006), is activated by calpain; inhibition of calpain (by a pharmacological inhibitor and by overexpression of calpastatin) prevents the conversion of procaspase-8 to active caspase-8 in the Aβ-treated PC12 cells (Vaisid et al., 2009). Calpain activation may also promote the expression of the β-site APP-cleaving enzyme 1 (BACE1, β-secretase), the enzyme responsible for Aβ production, and the BACE1 increase is inhibited by calpastatin overexpression (Liang et al., 2010). Overall, the results point to the importance of calpastatin in the response of the cells to Aβ, and possibly in diminishing the processing of APP to Aβ (Liang et al., 2010; Vaisid et al., 2007).

During a study on the effects of amyloid-β-peptide (Aβ) on the calpain–calpastatin system in the human SH-SY5Y neuroblastoma cell line, we found that contamination of the cells by a strain of *Mycoplasma hyorhinis* led to increased cellular calpastatin, resulting in inhibition of Ca^{2+}-induced calpain activation and inhibition of calpain-promoted proteolysis in the mycoplasmal-infected cells (Elkind et al., 2010). It was therefore of interest to study the effects of Aβ on infected cells. In the present study, we show that Aβ-toxicity is inhibited in infected cells. Staurosporine-toxicity is different from Aβ-toxicity in its manifestations and in that both clean and contaminated cells are affected. The results are relevant to studies on the modulation of host cells by mycoplasmas, and of importance in evaluating the response of contaminated cells to certain insults.

2. Experimental procedures

2.1. Cell culture and infection of SH-SY5Y cells with M. hyorhinis

A mycoplasma-free SH-SY5Y cells (obtained from Dr. Talia Han, Kaplan Medical Center, Rehovot, Israel) were grown in RPMI-1640 supplemented with 2 mM L-glutamine, 10% FCS, 100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (pen-strep solution) (growth medium, GM) in 25 cm^2 plastic culture flasks. To obtain a contaminated cell cultures, SH-SY5Y cells were infected with the neuroblastoma-derived *M. hyorhinis* (NDMh) strain (Elkind et al., 2010) at a multiplicity of infection of 50. Cell cultures were subcultured twice a week for one to three weeks

2.2. Amyloid-β-peptide, Ca^{2+} and staurosporine treatments

Aliquots of clean and contaminated cells ($(2–3) \times 10^6$ cells in 60 cm^2 petri dishes) were cultured in GM for 48 h. The medium was replaced by Dulbecco's modified Eagle's medium (DMEM), supplemented with 2 mM L-glutamine, 1% FCS, and pen-strep solution prior to the reagent treatments. Amyloid-β-peptide 25–35 (sAβ) (Bachem, Bubendorf, Switzerland) was used in the present study; sAβ retains the physical and biological properties and toxicity of the full length of Aβ, and is widely used to delineate degenerative changes in neuronal cells (Morishima et al., 2001). sAβ was suspended in sterile double distilled water (DDW) at a concentration of 1.0 mM, and preincubated for 48 h at 37 °C prior to addition to the cell cultures (Morishima et al., 2001); sAβ was added to clean and NDMh-infected cells at a final concentration of 25 μM. Cultures were continued for additional 24 h. To study the effects of Ca^{2+} on clean and infected cells, $CaCl_2$ (Sigma, St. Louis, MO, USA) (100 mM stock solution in double distilled water), and ionomycin (Calbiochem, La Jolla, CA, USA), (0.5 mM stock solution in DMSO) were added to the cell cultures at a final concentrations of 5 mM and 0.5 μM, respectively, and cultures continued for additional 4 h. Staurosporine (Sigma) was dissolved in DMSO at a concentration of 0.5 mM, added to cultures at a final concentration of 0.5 μM and cultures continued for additional 2 h.

2.3. Cell morphology and staining

The cells were cultured on cover slips placed in Petri dishes. The cultures were washed in PBS and the medium was replaced with DMEM without phenol red, containing 1% FCS. The cells were stained with the fluorescence dyes Hoechst 33342 and propidium iodide (PI) (Sigma), using 20 μg/mL of Hoechst and 10 μg/mL of PI added to the cultures for 10 min. The cells were then washed in PBS, fixed in 4% paraformaldehyde and washed in PBS. The cover slips were mounted on glass microscope slides and examined by tissue culture phase microscopy and by fluorescence microscopy (Olympus BX-4CB), using the same fluorescence intensity and exposure conditions for all samples. Multiple fields were examined and photographed, using the appropriate filters for PI and Hoechst fluorescence.

2.4. XTT reduction assay

XTT (sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) reduction to formazan was estimated using the XTT-based cell proliferation assay kit (Biological Industries, Israel), according to the manufacturer's protocol. The XTT reaction solution was added to cell cultures for 1 h prior to the end of reagent treatment, and level of the XTT formazan determined in the medium by absorbance measured with a spectrophotometer at the wavelength of 467 nm.

2.5. Preparation of cell extracts for SDS-PAGE, and immunoblotting analyses

SH-SY5Y cell lysates were prepared using 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 2.5 mM EDTA, 2.5 mM EGTA, and 1:200 protease inhibitors cocktail set III (Calbiochem). Lysates were kept on ice for 30 min and centrifuged. Protein

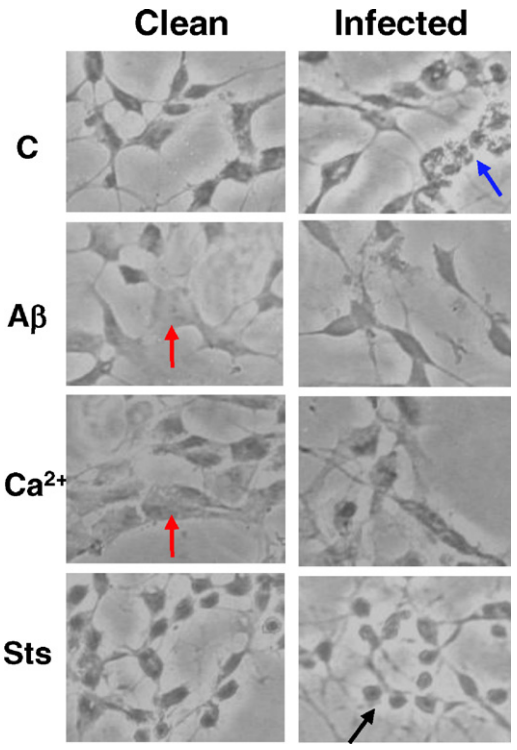

Fig. 1. Phase microscopy of clean and mycoplasma-infected SH-SY5Y cells. The cells were cultured, treated with sAβ, Ca^{2+}, and staurosporine, fixed, and mounted on microscope slides, as described in Section 2. C, control cells; Aβ, sAβ; Sts, staurosporine; blue arrow indicates mycoplasma-infected cells; red arrows indicate swollen cells; black arrow indicates cell rounding and shrinking. Representative of two experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

concentration in the supernatants was determined by the method of Bradford (1976), using bovine serum albumin (BSA) as standard. Aliquots of supernatants were mixed with Laemmli sample buffer for SDS-PAGE. SDS-PAGE was carried out according to standard procedures (using 15% acrylamide for caspase-3, 10% acrylamide for calpain, calpastatin and poly(ADP-ribose) polymerase (PARP), and 6.5% acrylamide for fodrin). Samples containing 20–40 μg of cell proteins were electrophoresed, then transferred to nitrocellulose membranes. Immunoblotting (IB) was carried out as previously described (Vaisid et al., 2008a), using polyclonal anti-PARP antibody (Cell Signaling Technology, Danvers, MA, USA) (1:1000); monoclonal anti-μ-calpain antibody (1:1000); polyclonal anti-calpastatin antibody (H-300): Sc-20779 (Santa Cruz) (1:500); polyclonal anti-caspase-3 antibody: Sc-7148 (Santa Cruz) (1:200); monoclonal anti-non-erythroid spectrin antibody (Chemicon International, Temecula, CA, USA) (1:1000). The appropriate peroxidase-conjugated secondary antibodies were used, and detection of bands carried out with ECL (KPL, USA), according to published methods (Vaisid et al., 2008a). Membranes were reprobed with monoclonal anti-β tubulin antibody (Sigma) (1: 80,000) for estimation of loading. Bands were quantified by densitometry.

2.6. Statistical analysis

Data are expressed as mean ± SEM. For the comparison of means of two groups, *t*-test for independent samples was performed; when control was considered as 100%, one sample *t*-test was performed (where the test value = 100). All comparisons were two tailed; $p < 0.05$ was considered as significant. Data were analyzed with SPSS 17.0 (SPSS, Chicago, IL, USA).

3. Results

3.1. sAβ-induced altered morphology and membrane permeability in SH-SY5Y cells: comparison with Ca^{2+} and with staurosporine effects

SH-SY5Y cells were treated with sAβ, Ca^{2+} and staurosporine, as described in Section 2. Examination by phase contrast microscopy showed that clean cells treated with sAβ or with Ca^{2+} appeared swollen, whereas the morphology of NDMh-infected cells treated with sAβ and Ca^{2+} was similar to that of the untreated infected cells, and did not show cell swelling (Fig. 1). As shown in Fig. 2, both untreated clean cells and NDMh-infected cells were labeled by Hoechst, and not labeled by PI. Uptake of PI was clearly observed in the sAβ-treated clean cells, whereas very little uptake was observed in the sAβ-treated NMDh-infected cultures. PI uptake and some chromatin condensation were observed in the Ca^{2+}-treated clean cells, but not in Ca^{2+}-treated infected cells. Under the conditions used here, Hoechst 33342 penetrates all cells, while PI penetrates only cells with damaged membranes (Grace et al., 2002; Willingham, 1999).

In order to further probe the mode of action of Aβ on the clean and mycoplasma-infected SH-SY5Y, the effects of sAβ were compared to effects of staurosporine. As seen in Fig. 1, the morphology of both clean and NDMh-infected cells was altered by treatment with staurosporine, showing cell rounding and shrinkage. As shown in Fig. 2, nuclear fragmentation was apparent in both clean and mycoplasma-infected, staurosporine-treated cells stained with Hoechst. There was no uptake of PI into the staurosporine-treated clean or infected cells. The results indicate a clear difference between the mode of action of sAβ and staurosporine in respect to effects on cell morphology and permeability. The alterations in morphological features and PI permeability induced by Aβ and Ca^{2+} in the clean cells are those ascribed to necrosis, whereas the morphological alterations induced by staurosporine indicate damage due to apoptosis (Kroemer et al., 2009). The results also show that Aβ affected clean cells but not mycoplasma-infected cells, whereas staurosporine affected both clean and infected cells.

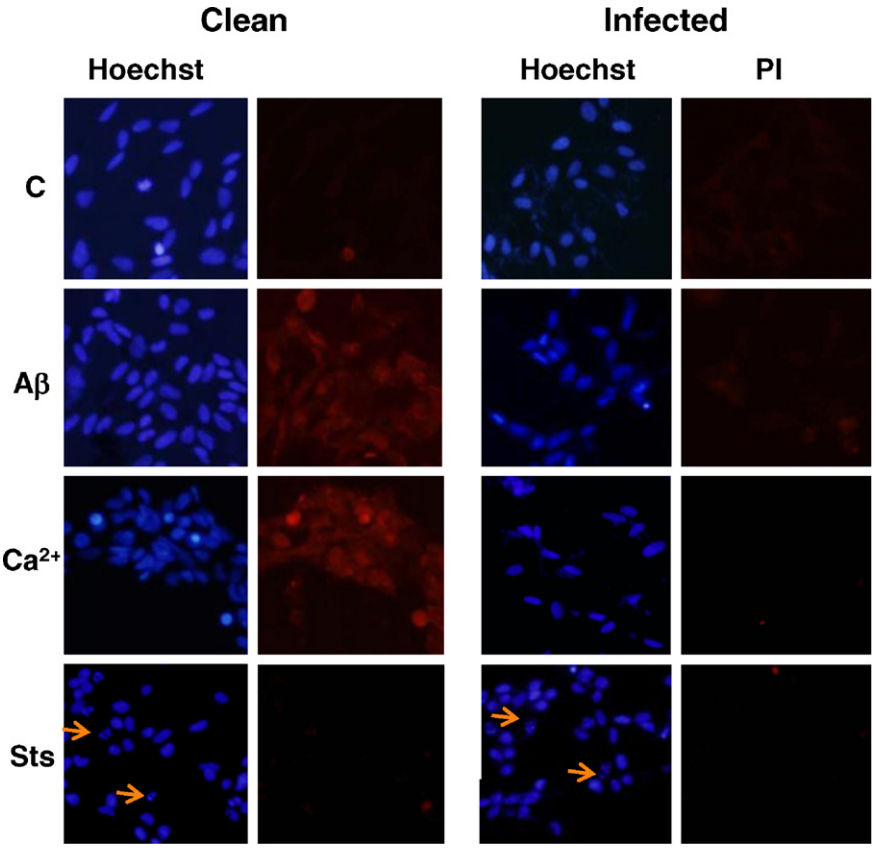

Fig. 2. Fluorescence microscopy of clean and mycoplasma-infected SH-SY5Y cells. The cells were cultured, treated with sAβ, Ca^{2+}, and staurosporine, stained with Hoechst and PI, fixed, and mounted on microscope slides, as described in Section 2. C, control cells; Aβ, sAβ; Sts, staurosporine; PI, propidium iodide. Orange arrows point to fragmented, apoptotic nuclei. Representative of three experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

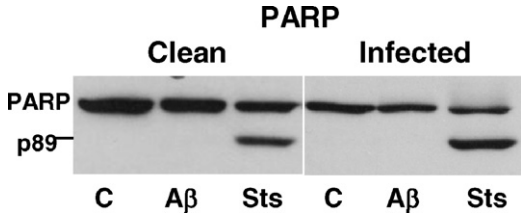

Fig. 3. Immunoblot of poly(ADP-ribose) polymerase (PARP) in clean and mycoplasma-infected SH-SY5Y cells. The cells were cultured, treated with sAβ or with staurosporine, cell lysates prepared and analyzed by western blot, as described in Section 2. p89, cleaved PARP; C, control cells; Aβ, sAβ; Sts, staurosporine. Representative of two experiments.

3.2. Effects of sAβ and staurosporine on PARP in SH-SY5Y cells

Cleavage of PARP serves as an indication for apoptotic processes (Soldani and Scovassi, 2002). As shown in Fig. 3, cleavage of PARP was not detected after sAβ treatment of either clean or mycoplasma-infected cells. By contrast, PARP degradation was observed in both clean and infected cell cultures exposed to staurosporine. These results are consistent with the apoptotic changes observed in the staurosporine-treated cells (altered cell size and nuclear morphology) and indicate that under the experimental conditions used here, sAβ does not cause apoptotic alterations in SH-SY5Y cells.

3.3. Metabolic activity in clean and mycoplasma-infected SH-SY5Y cells exposed to sAβ, Ca^{2+} and staurosporine

XTT is reduced to water-soluble colored formazan by dehydrogenase enzymes of metabolically active cells (Roehm et al., 1991). As shown in Fig. 4, formazan formation was significantly decreased in clean cells exposed to sAβ or to Ca^{2+}, indicating diminished metabolic activity of these cells. Formazan levels in staurosporine-treated clean cells were similar to the levels in the control cells, indicating that under the conditions used here, staurosporine had no effect on the metabolic activity of the clean cells. In the NDMh-infected cells, formazan levels were increased as compared with the levels in the clean cells, with the added formazan reflecting the ability of mycoplasmas to reduce tetrazolium salts (Kirchhoff et al., 1992). Little or no change in formazan formation was observed in the infected cells treated with sAβ, Ca^{2+} and staurosporine, compared with the formazan levels in the control infected cells (Fig. 4). Under the experimental conditions used, staurosporine did not alter the viability of NDMh, as exhibited by a similar growth of staurosporine-treated isolated mycoplasma to that of control mycoplasma (data not shown). The results indicate that in contrast to clean cells, the metabolic activity was not diminished in the infected cells exposed to sAβ, Ca^{2+} and staurosporine.

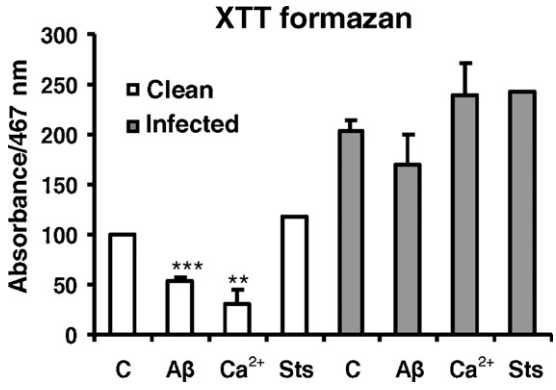

Fig. 4. XTT formazan formation in clean and mycoplasma-infected SH-SY5Y cells. The cells were cultured, and treated with sAβ, Ca^{2+}, and staurosporine. The XTT reaction solution was added to the cell cultures for 1 h, and formazan levels determined in the medium, as described in Section 2. C, control; Aβ, sAβ; Sts, staurosporine. For control, Aβ- and Ca^{2+}-treated clean cells and infected cells, the graph represents means ± SEM ($n = 3$); $^{**}p < 0.01$, $^{***}p < 0.001$ vs. clean control cells (considered 100%). For Sts, graph represents means of two experiments.

3.4. Calpastatin levels in sAβ-treated clean and infected SH-SY5Y cells

We previously found that in differentiated SH-SY5Y cells, calpastatin levels in the infected cells were significantly increased compared with levels in the clean cells (Elkind et al., 2010). As shown in Fig. 5, a similar difference between calpastatin levels in infected SH-SY5Y and clean cells was observed in dividing cells (cells cultured in GM). Treatment of clean SH-SY5Y cells with sAβ led to a diminution in calpastatin, with some diminution in calpastatin also observed in the sAβ-treated infected cells. The calpastatin levels in the sAβ-treated infected cells remained significantly higher than those in the clean cells (Fig. 5).

3.5. Calpain and caspase activation and activity in clean and infected SH-SY5Y cells

μ-Calpain activation is generally considered to be associated with autolysis of the 80 kDa subunit to 76 kDa band (Baki et al., 1996). As shown in Fig. 6, the ratio of the 76 kDa band to that of the 80 kDa band was greater in the sAβ-treated clean cells than in the control clean cells, whereas in the NDMh-infected cells exposed to sAβ, the ratio of the calpain 76 kDa to the 80 kDa band was similar to that in the control, untreated infected cells (Fig. 6A and B).

Fodrin is a known substrate for calpain and caspase, with fodrin fragment of 150 kDa indicative of calpain and caspase activities, 145 kDa considered to be due to calpain activity and 120 kDa considered to be due to caspase activity (Wang, 2000). As shown in Fig. 6C and D, fodrin degradation to 150/145 kDa fragments was significantly enhanced in the sAβ-treated clean cells, but was not enhanced in the NDMh-infected cells treated with sAβ. Little or no 120 kDa fragment was observed in the clean or infected cells.

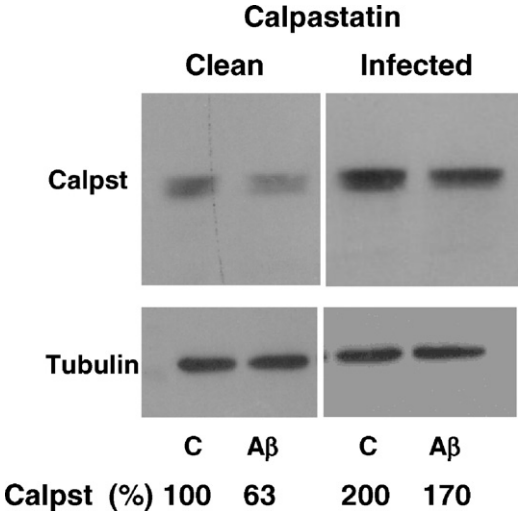

Fig. 5. Immunoblot of calpastatin in clean and mycoplasma-infected SH-SY5Y cells. The cells were cultured, treated with sAβ, cell lysates prepared and analyzed by western blot, as described in Section 2. C, control cells; Aβ, sAβ; Calpst, calpastatin; % calpast denotes the calpastatin levels obtained by densitometry, using tubulin bands for the estimation of loading. Representative of two experiments (levels in the second experiment were 60%, 188%, and 152% for calpastatin levels in Aβ-treated clean cells, control infected cells and Aβ-treated infected cells, respectively, vs control clean cells, considered 100%).

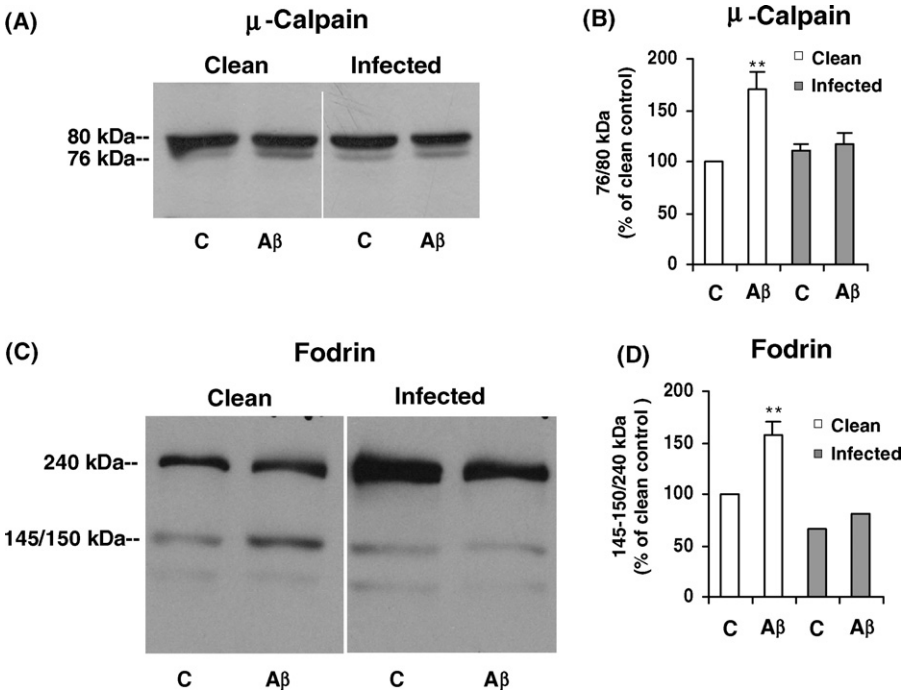

Fig. 6. Immunoblots of calpain and of fodrin in clean and mycoplasma-infected SH-SY5Y cells treated with sAβ. Cell lysates were prepared and analyzed by western blot, as described in Section 2. C, control cells; Aβ, sAβ. (A) Immunoblot of μ-calpain. (B) Ratios of calpain 76 kDa/80 kDa. Graph represents means ± SEM ($n = 4$ for clean cells and $n = 3$ for infected cells); $^{**}p < 0.01$ vs. clean control cells (considered 100%). (C) Immunoblot of fodrin. (D) Ratio of fodrin 145–150 kDa/240 kDa. For clean cells, graph represents means ± SEM ($n = 3$); $^{**}p < 0.01$ vs. clean control cells (considered 100%). For infected cells, graph represents means of two experiments.

Overall, the results indicate that sAβ-treatment led to enhanced calpain activation and activity in the clean cells, but not in the infected cells.

Staurosporine treatment led to caspase-3 activation (Fig. 7A), known to be associated with cleavage of the 32 kDa procaspase to a 17 kDa fragment (Lavrik et al., 2005), whereas no activation of caspase-3 was observed in either clean or infected cells exposed to sAβ (Fig. 7A). A significant degradation of fodrin to 120 kDa fragment, typical of caspase-promoted fodrin degradation (Wang, 2000), was observed in both clean and NDMh-infected cells treated with staurosporine (Fig. 7B). Overall, the results indicate that in contrast to the effects of sAβ, staurosporine led to caspase-3 activation in both the clean and the infected cells, with similar fodrin degradation in both clean and NDMh-infected cells.

4. Discussion

4.1. Mycoplasmas

We begin with a brief summary of the field of mycoplasmas. Mycoplasmas are the smallest self-replicating prokaryotes widely distributed in nature. They have limited biosynthetic abilities and

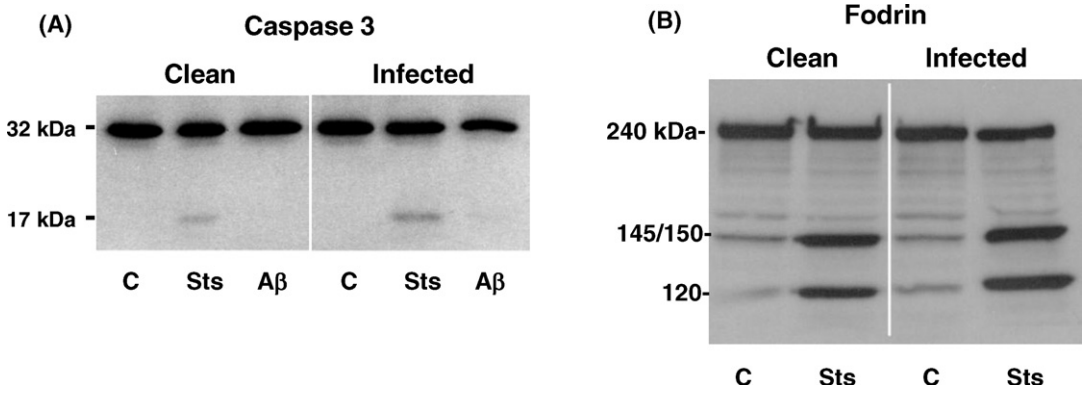

Fig. 7. Immunoblots of caspase-3 and of fodrin in clean and mycoplasma-infected SH-SY5Y cells. The cells were treated with sAβ, or with staurosporine. Cell lysates were prepared and analyzed by western blot, as described in Section 2. C, control cells; Aβ, sAβ; Sts, staurosporine. (A) Immunoblot of caspase-3. (B) Immunoblot of fodrin. Representative of two experiments.

most are parasites. Almost all of the mycoplasmas adhere to the surface of eukaryotic cells. Adherence of these organisms to the cells is essential for tissue colonization and the subsequent development of disease (Rottem, 2003). Some species may invade cells (Diaz-Garcia et al., 2006; Rottem, 2003; Yavlovich et al., 2004). Mycoplasmas frequently contaminate cultured cells, and is detected in 15–35% of cell cultures, with rates reaching 65–80% in some surveys (Drexler and Uphoff, 2002). Contamination leads to a variety of alterations in the cells, including alterations in gene expression, protein synthesis, cell membrane composition and changes in signal transduction (Drexler and Uphoff, 2002; Rottem, 2003). Contamination is often undetected, since the culture medium remains clear and the cellular morphological changes may not be obvious. Thus, mycoplasma-induced alterations in cell components and metabolism may not be recognized, unless specifically studied.

M. hyorhinis is one of the most common *Mycoplasma* species that contaminate various cell lines (Drexler and Uphoff, 2002; Timenetsky et al., 2006). *M. hyorhinis* affects membrane properties and cellular functions related to the immune system, including proliferation of lymphocytes, secretion of the tumor necrosis factor alpha (TNF-α) and proinflammatory cytokines (Rottem, 2003). It may provide a ligand for cell membrane receptors, as shown in the case of the interaction of *M. hyorhinis* with the CD99 receptor in contaminated melanoma cells (Gazit et al., 2004). *M. hyorhinis* has also been shown to promote cancer cell invasiveness through activation of the matrix metalloproteinase-2 (Gong et al., 2008).

4.2. Calpain–calpastatin

We have previously found that the calpain–calpastatin system is modulated in *M. hyorhinis*-infected SH-SY5Y cells. Mycoplasmal infection leads to increased levels of cellular calpastatin, and diminution in calpain activation and activity (Elkind et al., 2010). Calpain and calpastatin are widely distributed in a variety of biological systems. Activated calpain causes a limited degradation of cytoskeletal and membrane integral proteins, certain enzymes, transcription factors, components in cell adhesion and signaling pathways. The calpain–calpastatin system has been implicated in a variety of cellular physiological and pathological processes such as cell motility, myoblast fusion, signal transduction pathways, neurotoxicity, apoptosis and necrosis (Barnoy et al., 1998; Das et al., 2006; Goll et al., 2003; Liu et al., 2008; Orrenius et al., 2003; Vaisid et al., 2005). The ratio of calpastatin to calpain varies among tissues and species and is an important factor in the control of calpain activity within the cell. Overexpression of calpastatin is known to interfere with cellular physiological processes, such as cell motility, cell growth, myoblast fusion (Barnoy et al., 2005; Goll et al., 2003; Xu and Mellgren, 2002), and to inhibit pathological processes such as dystrophy of dystrophin-deficient muscles and Aβ-induced cell damage (Spencer and Mellgren, 2002; Vaisid et al., 2008b, 2009).

4.3. Protection against Aβ-toxicity

We show here that contamination of the neuronal-like SH-SY5Y cells with a strain of *M. hyorhinis* (NDMh) protects the cells from toxicity induced by Aβ. Exposure of uninfected (clean) SH-SY5Y cells to Aβ results in activation of calpain and enhanced proteolysis, cell swelling, membrane permeability to PI (without nuclear apoptotic changes), and mitochondrial dysfunction (diminished XTT reduction). The alterations caused by Aβ indicate a necrotic type of damage, according to the cell death classification by Kroemer et al. (2009). We previously found that Aβ-toxicity was attenuated in PC12 cells overexpressing calpastatin following calpastatin-plasmid introduction into the cells (Vaisid et al., 2008b). Similarly, Aβ-toxicity was attenuated in clean SH-SY5Y cells overexpressing calpastatin (E. Elkind, T. Vaisid, S. Barnoy and N.S. Kosower, unpublished results). The results presented here show that a similar inhibition of Aβ-toxicity occurs in the NDMh-infected cells, as indicated by the inhibition of calpain activation and proteolysis, lack of cell swelling, no PI permeability, and inhibition of XTT reduction.

By contrast, treatment of SH-SY5Y cells with staurosporine results in apoptotic damage that affects both uninfected and NDMh-infected cells, as evidenced by cell shrinkage, nuclear apoptotic changes (fragmentation, PARP cleavage), maintenance of cell membrane integrity, caspase-3 activation and caspase-induced fodrin degradation. The XTT formazan formation was not inhibited, suggesting that at this stage of the staurosporine-induced apoptosis, mitochondria were not significantly damaged, consistent with the occurrence of only minor modifications of cytoplasmic organelles in apoptosis (Kroemer et al., 2009). The fact that staurosporine treatment does not affect the viability of the mycoplasma excludes the possibility that a loss of live, functional mycoplasma is the reason that the response of the contaminated cells to staurosporine is similar to that of the clean cells.

Overall, the results indicate that the response of the mycoplasma-infected cells to insults is not universal, in that mycoplasmal-contamination suppresses certain types of insult-induced damage, but does not inhibit others. Toxins such as staurosporine may affect the cells via different mechanisms and pathways that are not influenced by mycoplasma. It is of interest to note that phosphoprotein phosphatase 5 activity inhibits Aβ toxicity to neurons, but does not prevent neurotoxicity induced by staurosporine (Sanchez-Ortiz et al., 2009). Our findings on the difference between the effects of Aβ/Ca^{2+} and those of staurosporine on the mycoplasma-infected cells strengthen the notion that the pathway responsible for staurosporine-induced cell damage is different from that induced by Aβ/Ca^{2+}.

In the case of certain ligands such as Aβ, the inhibition of cell damage by NDMh may be due to interference with ligand binding and signal transduction and/or effects on intracellular pathways. The resistance of NDMh-infected cells to Aβ-toxicity mimics that of clean cells overexpressing calpastatin. It is thus reasonable to conclude that the high calpastatin found in the infected cells is responsible for the protective effects of the mycoplasma against Aβ-toxicity. Inhibition of calpain activity by exogenous pharmacological means, including calpastatin-based inhibitors, has been considered for therapy of various diseases, in which calpain is involved (Anagli et al., 2009; Pietsch et al., 2010; Ray et al., 2002). Mycoplasmas provide the first naturally occurring biological system that upregulates the endogenous calpain inhibitor, and thus may be of interest in devising treatments for some disorders, such as neurodegenerative diseases and neural damage produced by trauma.

5. Conclusions

- *M. hyorhinis* raises calpastatin levels in host cells.
- Increased calpastatin protects the cells against Aβ toxicity.
- Mycoplasma protects the cells against certain types of insults, but not all.
- Increased calpastatin in mycoplasma-infected cells may be responsible for suddenly altered behavior of cultured cells.
- Thus, mycoplasmas may be of interest in devising treatments for some disorders.

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