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Calpastatin upregulation in *Mycoplasma hyorhinis*-infected cells is promoted by the mycoplasma lipoproteins via the NF-κB pathway

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Summary

Mycoplasma hyorhinis **frequently contaminates cultured cells, with effects on synthetic and metabolic pathways. We demonstrated for the first time that contamination of cells by a strain of *M. hyorhinis* (NDMh) results in increased levels of calpastatin (the endogenous inhibitor of the ubiquitous Ca^{2+}-dependent protease calpain). We now show that the calpastatin upregulation by NDMh in neuroblastoma SH-SY5Y cells resides in the NDMh lipoprotein fraction (LPP), via the NF-κB transcription pathway. NF-κB activation requires dissociation of the cytoplasmic NF-κB/IκB complex followed by NF-κB translocation to the nucleus. NDMh-LPP induced translocation of the NF-κB RelA subunit to the nucleus and upregulated calpastatin. RelA translocation and calpastatin elevation were prevented when dissociation of the NF-κB/IκB complex was inhibited either by transfection with the non-phosphorylatable IκB mutant ΔNIκBα, or by using PS1145, an inhibitor of the IκB kinase (IKK complex). Increased calpastatin levels attenuate calpain-related amyloid-β-peptide and Ca^{2+}-toxicity (these are central to the pathogenesis of Alzheimer's Disease). LPP-induced elevation of calpastatin provides an example of effects on non-inflammatory intracellular proteins, the outcome being significant alterations in host cell functions. Since calpastatin level is important in the control of calpain activity, mycoplasmal LPP may be of interest in treating some pathological processes involving excessive calpain activation.**

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Introduction

Mycoplasmas (class Mollicutes) are widely distributed in nature. They form a large group of prokaryotic microorganisms with over 200 species, distinguished from ordinary bacteria by their small size, minute genome and total lack of a cell wall. They have limited biosynthetic abilities. Most mycoplasmas are parasites, and depend on host cell adhesion for infection. Mycoplasmas frequently contaminate cultured cells, and contamination 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. Lipoproteins (LPP) are extremely abundant in the cell membrane of mycoplasmas, and 15–46 open reading frames encoding putative LPP genes have been identified in a variety of species sequenced so far (Yogev *et al.*, 1995; Chambaud *et al.*, 1999; Rottem, 2003; Kornspan *et al.*, 2011; Wise *et al.*, 2011). Information on the functional importance of the LPP is well established with respect to their ability to modulate the host immunity system, mainly to activate macrophages to produce and secrete cytokines (Rottem, 2003). *Mycoplasma 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 tumour

necrosis factor alpha (TNF-α) and pro-inflammatory cytokines (Rottem, 2003). P37, the major immunogen of *M. hyorhinis*, has been shown to promote cancer cell invasiveness through activation of the matrix metalloproteinase-2 (Gong *et al*., 2008).

The intracellular Ca^{2+}-dependent protease calpain and its specific endogenous inhibitor calpastatin are widely distributed in biological systems. The two major calpain isozymes are μ-calpain and m-calpain, activated by micromolar and millimolar Ca^{2+} respectively. Activated calpain causes a limited degradation of a variety of proteins, including cytoskeletal proteins, membrane integral proteins, certain enzymes, components in cell adhesion and in signalling pathways. 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. 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 and neurodegenerative diseases, apoptosis and necrosis (Barnoy *et al*., 1997; Goll *et al*., 2003; Liu *et al*., 2008).

We have shown for the first time that contamination of the neuroblastoma SH-SY5Y cells by a neuroblastoma-derived *M. hyorhinis* strain (to be referred to as NDMh) (Elkind *et al*., 2010) results in increased levels of calpastatin. When challenged by high cellular Ca^{2+}, the mycoplasma-infected cells exhibit lower calpain activation and diminished calpain-promoted proteolysis, compared with the non-infected (clean) cells (Elkind *et al*., 2010). Infection of SH-SY5Y cells with NDMh protects the cells from toxicity induced by Aβ/Ca^{2+} (Elkind *et al*., 2011).

The initial findings raised questions as to the mycoplasmal fraction(s) involved and the mechanism responsible. In the present study, we show that the LPP of NDMh are responsible for the calpastatin upregulation in NDMh-infected cells. The LPP-induced increase in calpastatin leads to resistance of the cultured cells to amyloid-β-peptide and Ca^{2+} (Aβ/Ca^{2+})-toxicity, in a manner similar to the effects of viable mycoplasma. The LPP promote the upregulation of calpastatin via activation of the transcription factor NF-κB. LPP are known initiators of inflamation reactions in mycoplasmal infections, involving cytokine production and secretion (Into *et al*., 2004; Gerlic *et al*., 2007). Calpastatin is the first intracellular protein shown to be upregulated in non-immune cells by mycoplasmal LPP via the NF-κB pathway.

Results

Calpastatin level is elevated in SH-SY5Y cells exposed to NDMh fractions

NDMh were sonicated and membrane and LPP fractions obtained, as described in *Experimental procedures*. As shown in Fig. 1, calpastatin levels were significantly elevated in SH-SY5Y cells that were exposed to viable NDMh, sonicated NDMh, NDMh membranes and NMDh LPP fraction. These results indicate that live mycoplasma are not required for the increased cellular calpastatin in the treated cells, and that the mycoplasmal LPP induce a significant calpastatin elevation (Fig. 1A and B). The response to LPP was dependent on LPP concentration and on the duration of treatment (Fig. 1C and D).

Amyloid-β-peptide (Aβ) and Ca^{2+}/ionophore-induced damage to SH-SY5Y cells is attenuated in cells treated with NDMh lipoproteins

Previously we have shown that SH-SY5Y cells exposed to Aβ or to increased cellular Ca^{2+} exhibit cellular damage, as indicated by enhanced activation of calpain, fodrin degradation, cell membrane permeability and diminished XTT formazan formation; infection of the cells by viable NDMh protect SH-SY5Y cultured cells from the Aβ/Ca^{2+} toxicity (Elkind *et al*., 2011). As shown in Fig. 2, exposure of the clean cells to Aβ led to enhanced activation of calpain. μ-Calpain activation is generally considered to be associated with autolysis of the 80 kDa subunit to 76 kDa band (Baki *et al*., 1996); the ratio of the 76 kDa band to that of the 80 kDa band was greater in the Aβ-treated cells than in the clean, control cells. In the LPP-treated cells exposed to Aβ, the ratio of the calpain 76 kDa to the 80 kDa band was similar to that in the control cells (Fig. 2A and B).

Fodrin is an endogenous cellular substrate of calpain, and serves as a marker for calpain activity, with fodrin fragments of 145/150 kDa indicative of calpain-induced degradation (Wang, 2000). Fodrin degradation to 145/150 kDa fragments was enhanced in the Ca^{2+} and Aβ-treated clean cells, whereas degradation was inhibited in the LPP-treated cells exposed to Ca^{2+} and to Aβ (Fig 2C and D).

XTT is reduced to water-soluble coloured formazan in metabolically active cells (Roehm *et al*., 1991). Formazan formation was significantly decreased in control cells exposed to high Ca^{2+}, indicating diminished metabolic activity of these cells. In contrast, XTT formazan was not diminished in the LPP-treated, high Ca^{2+} cells (Fig. 2E). The results indicate that the mycoplasmal LPP has protective effects on the cellular mitochondrial activity, similar to the effects of viable mycoplasma on cells exposed to Aβ and to high Ca^{2+}.

Overexpression of calpastatin by transfection with calpastatin-plasmid attenuates Aβ and Ca^{2+}/ionomycin-induced damage

SH-SY5Y cells were stably transfected with calpastatin-plasmid, resulting in increased cellular calpastatin levels

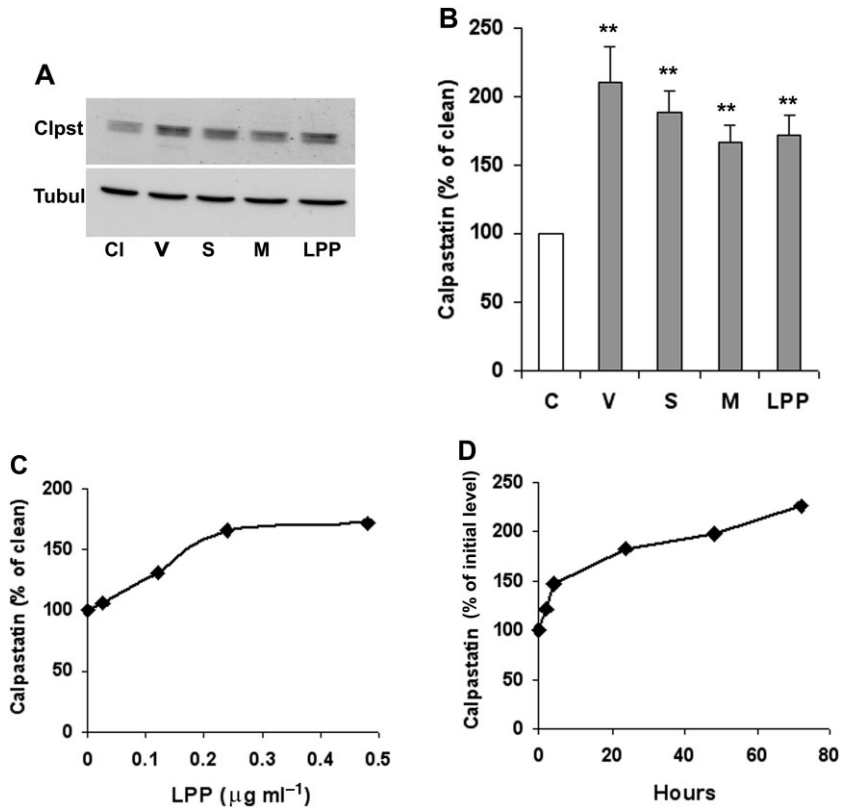

Fig. 1. *Mycoplasma hyorhinis* induce the elevation of calpastatin in SH-SY5Y cells. Cells were infected with viable *M. hyorhinis* (NDMh) at a multiplicity of infection of 1–2 and cultured for 2 weeks, or treated with sonicated NDMh (2.4 μg ml^{-1}), NDMh membranes (0.6 μg ml^{-1}) or NDMh LPP (0.1–0.5 μg ml^{-1}) for 72 h. Cl, clean cells; V, viable NDMh; S, sonicated NDMh; M, NDMh membranes; LPP, lipoproteins; Clpst, calpastatin; Tubul, tubulin.
A. Immunoblot of calpastatin.
B. Graph represents means ± SEM (V, S, $n = 3$; M, LPP, $n = 4$); $^{**}P < 0.01$ versus clean cells (considered 100%).
C. Cultured cells treated with 0.1–0.5 μg ml^{-1} of LPP. Average of densitometries of calpastatin immunoblots of two experiments.
D. Cultured cells treated with 0.3 μg ml^{-1} of LPP. Average of densitometries of calpastatin immunoblots of two experiments.

(Fig. 3A). Calpain activation was promoted in the control cells (containing empty plasmid) treated with Aβ, whereas calpain activation was inhibited in the Aβ-treated, high calpastatin-containing cells (Fig. 3B). Fodrin degradation, promoted in control cells exposed to Aβ, was attenuated in cells overexpressing calpastatin (Fig. 3C). XTT formazan was diminished in control cells exposed to Aβ or to Ca^{2+}/ionomycin, but not in the calpastatin-overexpressing cells treated with Aβ or with Ca^{2+} (Fig. 3D). Thus, the response of the cells overexpressing calpastatin to Aβ and to high Ca^{2+} was similar to the response of the NDMh LPP-treated cells (Fig. 2).

Involvement of NF-κB in the mycoplasmal LPP-induced calpastatin upregulation

Calpastatin mRNA levels were significantly elevated in NDMh-treated cells (Fig. 4A), pointing to the possibility of mycoplasma-induced enhanced transcription of calpastatin mRNA. To probe the possibility that NF-κB was involved in the overexpression of calpastatin, cultured SH-SY5Y cells were treated with viable NDMh, as well as with sonicated mycoplasma, isolated mycoplasmal membranes and LPP. Activation of NF-κB requires its translocation from the cytoplasm to the nucleus. The NF-κB RelA subunit was significantly elevated in nuclei of the LPP-treated cells (Fig. 4B–D). In contrast, RelA was not translocated to the nuclei in cells overexpressing calpastatin as a consequence of transfection with calpastatin-plasmid (Fig. 4D). The results indicate that NF-κB is activated in LPP-treated cells, resulting in elevated calpastatin, but that high cellular calpastatin per se (achieved by calpastatin-plasmid transfection) is not responsible for NF-κB activation.

NF-κB is present in the cell cytoplasm in association with the inhibitor IκB. Translocation of NF-κB from the cytosol to the nucleus is made possible by dissociation of NF-κB from IκB. The dissociation requires phosphorylation and degradation of IκB (Karin and Ben-Neriah, 2000). In order to further probe the involvement of NF-κB in the upregulation of calpastatin in LPP-treated cells, we transfected cultured SH-SY5Y cells with ΔNIκBα (IκB mutant that lacks the phosphorylation sites at the N-terminal domain of IκB, and is not degraded (Eldor *et al.*, 2006)), resulting in inhibition of NF-κB RelA subunit translocation into the nucleus. As shown in Fig. 5, LPP treatment led to translocation of RelA to the nuclei. RelA translocation was inhibited in cells transfected with the IκB mutant ΔNIκBα and treated with LPP. Calpastatin level was elevated in the LPP-treated cells, but not in the LPP-treated, ΔNIκBα mutant cells. The results indicate that activation of NF-κB is involved in the calpastatin upregulation in the SH-SY5Y cells.

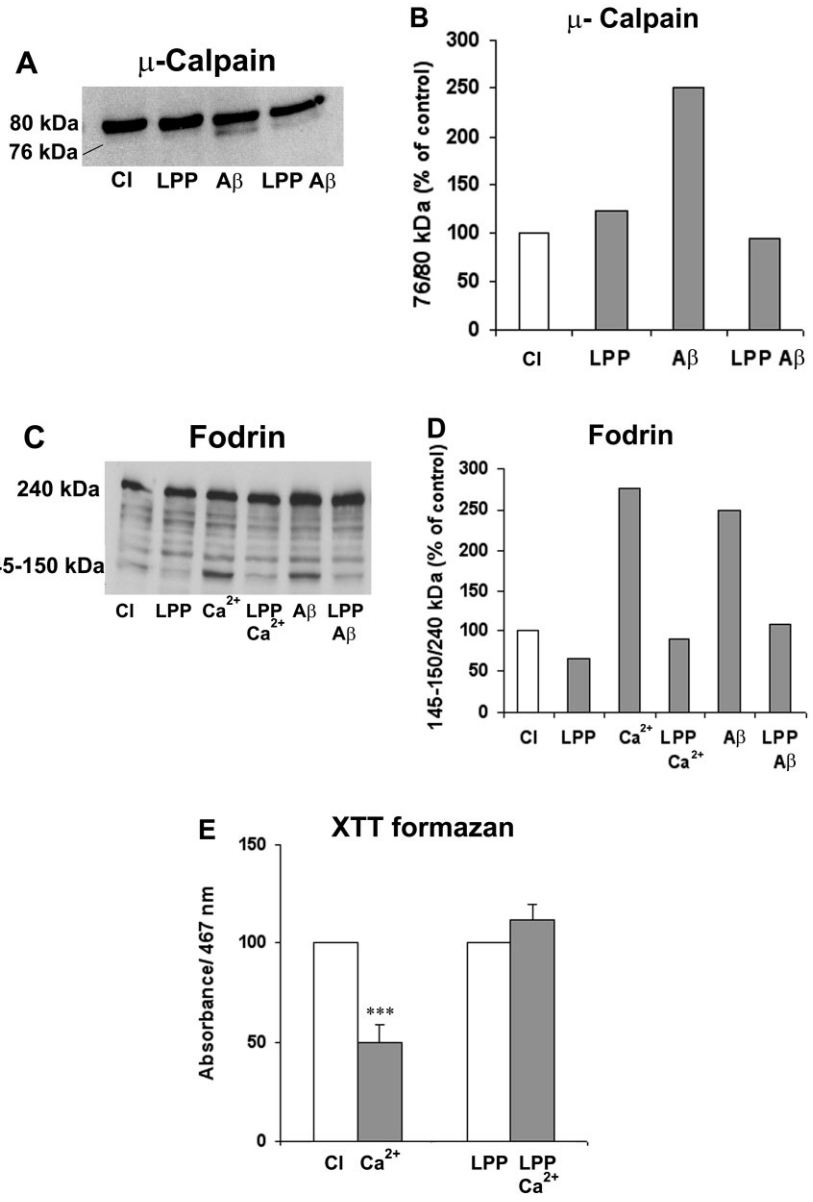

Fig. 2. NDMh-LPP attenuates amyloid-β-peptide (Aβ) and Ca^{2+}/ionophore-induced cell damage.
A–D. Cells were cultured with and without 0.3 μg ml^{-1} LPP for 48 h, then treated with 30 μM Aβ for additional 24 h; other cell aliquots were cultured with and without 0.3 μg ml^{-1} LPP for 72 h, then with added 5.0 mM Ca^{2+}/0.5 μM ionomycin for 4 h. Cl, clean control cells.
A. Immunoblot of μ-calpain.
B. Ratios of μ-calpain 76 kDa/80 kDa. Graph represents means of two experiments.
C. Immunoblot of fodrin.
D. Ratio of fodrin 145–150 kDa/240 kDa. Graph represents means of two experiments.
E. XTT formazan formation. Cells were cultured with and without 0.3 μg ml^{-1} LPP for 72 h, Then 5.0 mM Ca^{2+}/0.5 μM ionomycin added for 4 h, followed with the addition of XTT reaction solution for 1 h. Graph represents means ± SEM ($n = 3$) of absorbance at 467 nm; $^{***}P < 0.001$ versus control cells (considered 100%).

IκB is phosphorylated by the IKK kinase complex. IκB phosphorylation is inhibited by the IKK selective inhibitor PS1145, resulting in inhibition of NF-κB translocation from the cytosol to the nucleus (Yemelyanov *et al.*, 2006). SH-SY5Y cells were cultured in the presence and absence of PS1145, and treated with LPP. In PS1145-treated cells, the translocation of RelA to the nucleus in cells exposed to LPP was inhibited, as compared with the translocation in LPP-treated cells in the absence of the inhibitor (Fig. 6A and B). Calpastatin levels in the PS1145/LPP-treated cells were similar to control levels, as compared with the high calpastatin levels in the cells treated with only LPP (Fig. 6C and D). The results further substantiate the conclusion that LPP promotes the activation of NF-κB, and that activated NF-κB is involved in the calpastatin upregulation in the treated cells.

Discussion

We show here that treatment of cultured SH-SY5Y cells with NDMh LPP induces increase in cellular calpastatin. Endogenous calpastatin levels vary among species, age of animals, tissues and cells (Murachi *et al.*, 1981; Kosower and Barnoy, 2000; Wingrave *et al.*, 2004; Vaisid *et al.*, 2007; Sato *et al.*, 2011), and influence various physiological processes and the cell ability to resist certain pathology-promoting events and harmful agents. Cellular calpastatin levels can be elevated in vivo in calp-

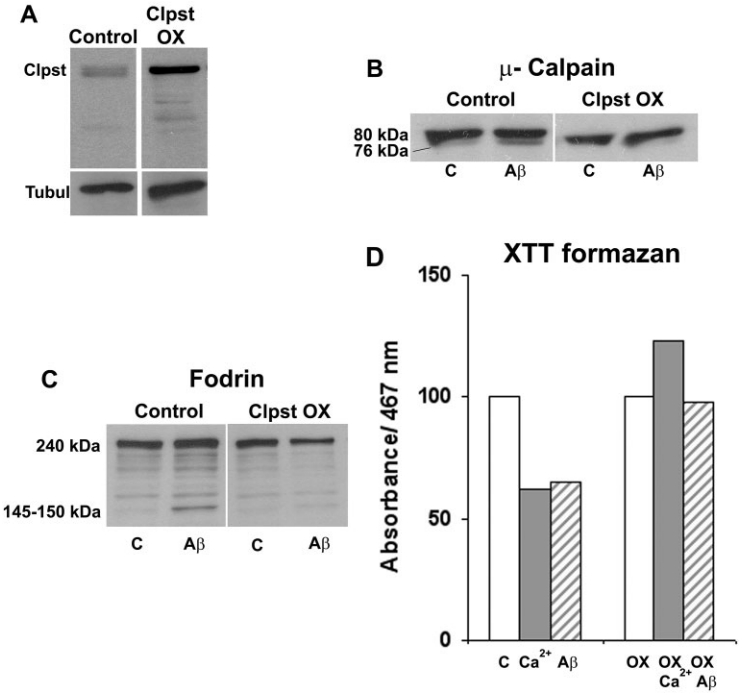

Fig. 3. Calpastatin overexpression in calpastatin-plasmid transfected SH-SY5Y cells attenuates cell damage induced by amyloid-β-peptide (Aβ) and by Ca^{2+}/ionomycin.
A–C. Cells, stably transfected with calpastatin-plasmid (Calpst OX) or empty plasmid (Control), were treated with 30 μM Aβ for 24 h.
A. Immunoblot of calpastatin. Calpst, calpastatin; Tubul, tubulin.
B. Immunoblot of μ-calpain.
C. Immunoblot of fodrin.
D. XTT formazan formation. Cells, stably transfected with calpastatin-plasmid (OX) or empty plasmid (C), were treated with 30 μM Aβ/Ca^{2+} for 24 h, or with 5.0 mM Ca^{2+}/0.5 μM ionomycin for 4 h. XTT reaction mixture was added and incubation continued for 1 h. Graph represents absorbance at 467 nm in control, Ca^{2+} and Aβ-treated cells compared with untreated control; in cells overexpressing calpastatin (OX), Ca^{2+} and Aβ-treated cells compared with untreated OX. Means of two experiments.

astatin overexpressing mice, and in cultured cells by transfection with calpastatin gene-containing vector. Calpastatin overexpression interferes with some cellular physiological processes, such as cell growth, myoblast fusion (Xu and Mellgren, 2002; Goll *et al*., 2003; Barnoy *et al*., 2005, Stifanese *et al*., 2008), and inhibits a variety of pathological processes such as dystrophy of dystrophin-deficient muscles, neuronal excitotoxic death, and Aβ-induced cell damage (Spencer and Mellgren, 2002; Bano *et al*., 2005; Higuchi *et al*., 2005; Vaisid *et al*., 2008a; 2009).

Calpain is implicated in the pathogenesis of Alzheimer's disease (AD). Activation of calpain and depletion of calpastatin have been observed in brains of AD patients (Rao *et al*., 2008). In normal mice, calpastatin level varies among brain regions; it is significantly higher in the cerebellum than in the hippocampus, frontal and temporal cortex (Vaisid *et al*., 2007; Sato *et al*., 2011). We have found that in Tg2576 mice (transgenic for a human APP mutant, providing a model for AD), the low-calpastatin brain regions (especially the hippocampus) exhibit calpain activation and proteolysis, and accumulation of Aβ; in the high-calpastatin brain region (cerebellum), calpain activity is not promoted and Aβ does not accumulate (Vaisid *et al*., 2007). Overall, the results suggest that calpastatin levels in regions of the normal brain are important in allowing or preventing the pathology in AD.

Aβ plays a major role in the pathogenesis of AD (Hardy and Selkoe, 2002) and is toxic to neurons. Aβ leads to increased cellular Ca^{2+}, enhanced generation of reactive oxygen species, mitochondrial dysfunction, and may cause neuronal cell death by apoptosis or necrosis (Fifre *et al*., 2006; Demuro *et al*., 2010). Calpain is activated in various cells exposed to Aβ (Fifre *et al*., 2006; Kelly and Ferreira, 2006; Vaisid *et al*., 2008b; Elkind *et al*., 2011). We have previously found that Aβ is toxic to the neuronal-like PC12 and SH-SY5Y cells, as indicated by enhanced calpain activation, proteolysis, diminished XTT formazan formation (indicative of mitochondrial dysfunction), and enhanced membrane permeability to propidium iodide (PI) (Vaisid *et al*., 2008b; Elkind *et al*., 2011). Here we show that the Aβ toxicity is attenuated in NDMh LPP-treated cells. The inhibitory effects of LPP are similar to the effects of calpastatin overexpression (achieved by introducing calpastatin gene vector into the cells) in inhibiting the Aβ-promoted alterations, thus substantiating the conclusion that the LPP-promoted increased calpastatin is responsible for the attenuation of Aβ toxicity.

Limited information is available on the mechanisms of calpastatin regulation. Calpastatin is synthesized from a single gene that contains multiple promoters, generating several different transcripts; these are alternatively spliced to multiple mRNAs, resulting in multiple protein isoforms (Lee *et al*., 1992; Goll *et al*., 2003). Certain promoter sequences act to stimulate and some others to inhibit calpastatin transcription (Sensky *et al*., 2006). Transcription of calpastatin is elevated by β-adrenergic agonists and activation of cAMP/PKA-dependent pathways (Goll *et al*., 2003; Sensky *et al*., 2006) and by hypoxia (Blomgren *et al*., 1999; Lin *et al*., 2004). Myco-

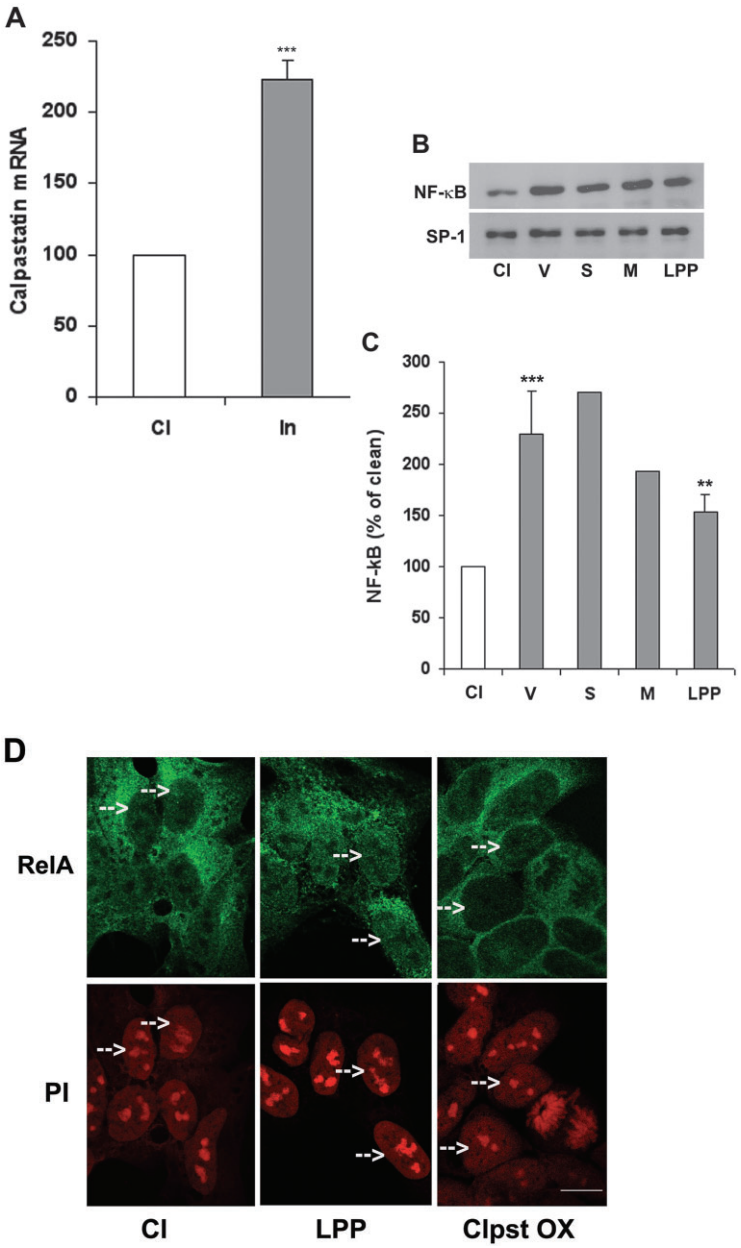

Fig. 4. Calpastatin mRNA is elevated in *Mycoplasma hyorhinis* (NDMh)-infected cells, and NF-κB is activated in NDMh-infected cells, and in mycoplasmal fractions-treated cells.
A–C. Cultured SH-SY5Y cells were infected with viable *M. hyorhinis* (NDMh) for 2 weeks or treated with mycoplasmal fractions for 72 h: sonicate (2.4 μg ml^{-1}), membranes (0.6 μg ml^{-1}) and LPP (0.3 μg ml^{-1}). Cl, clean cells; In, infected cells; V, viable NDMh; S, sonicate; M, membranes; LPP, lipoproteins.
A. Calpastatin mRNA levels in NDMh-infected cells, Means ± SEM ($n = 3$, each one in triplicate); ***$P < 0.001$ versus control cells (considered 100%).
B. Immunoblot of nuclear NF-κB RelA; SP-1 is used as a nuclear marker for estimation of loading.
C. Graph represents means of NF-κB RelA levels ± SEM (V, LPP, $n = 3$; S, M, $n = 2$); **$P < 0.01$, ***$P < 0.001$ versus clean control cells (considered 100%).
D. RelA is translocated to the nuclei in LPP-treated SH-SY5Y cells, but not in cells overexpressing calpastatin, as a result of transfection with calpastatin-plasmid. Cells were cultured on coverslips. For LPP treatment, 0.3 μg ml^{-1} LPP was added to cultures for 72 h. Cells were fixed with paraformaldehyde, then permeabilized and stained with antibody to RelA and with propidium iodide (for details, see *Experimental procedures*). Cl, clean cells; Clpst OX, cells overexpressing calpastatin; PI, propidium iodide. The nuclear RelA fluorescence intensity in the LPP-treated cells was 1.58, and intensity in the Clpst OX was 0.76 of the control values (considered 1.0). Arrows point to nuclei. Scale bar = 10 μm.

plasma is the first naturally occurring biological system that is shown to upregulate mammalian cell calpastatin. Since mycoplasmas and mycoplasmal LPP are known to activate NF-κB (Sacht *et al*., 1998; Shimizu *et al*., 2005; Logunov *et al*., 2008; Lai *et al*., 2010), we probed the possibility that calpastatin upregulation by LPP involves NF-κB.

NF-κB consists of dimers (homo- and heterodimers) of RelA (p65), RelB, c-Rel, p50 and p52. NF-κB is kept inactive in the cytosol by inhibitory IκB proteins. Activation of NF-κB involves phosphorylation of the IκB molecules by the IκB kinase complex (IKK), followed by ubiquitination and proteasomal degradation. The degradation of the inhibitory molecules allows the release of NF-κB dimers. The dimers then translocate to the nucleus to regulate the transcription of a large number of genes involved in cell survival/apoptosis, proliferation, inflammatory and immune responses, and in neuronal physiological activity (Karin and Ben-Neriah, 2000; Meffert and Baltimore, 2005; Hayden and Ghosh, 2008). Two major receptor-activated NF-κB pathways are considered to be responsible for NF-κB activation. The canonical NF-κB signalling is initiated by ligand binding to a variety of receptors, present on diverse types of cells, leading to the release of NF-κB RelA, p50 and c-Rel; the noncanonical NF-κB pathway is activated by a limited set of receptors in a few

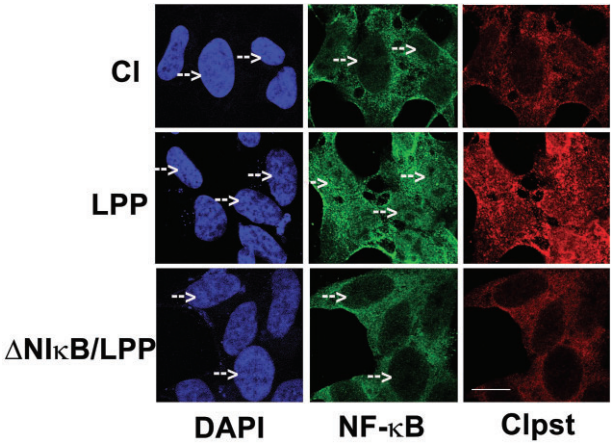

Fig. 5. NF-κB is activated and calpastatin level is elevated in LPP-treated SH-SY5Y cells, but not in cells transfected with the IκB mutant ΔNIκBα, and treated with LPP. Cells were transiently transfected with ΔNIκBα, cultured for 24 h, then treated with 0.5 μg ml^{-1} LPP for 24 h. Cells were fixed, permeabilized and stained with antibodies to NF-κB RelA and calpastatin, then stained with DAPI (for details, see *Experimental procedures*). Cl, control, untreated cells; ΔNIκBα/LPP, transfected cells treated with LPP; Calpst, calpastatin. Arrows point to nuclei. Scale bar = 10 μm.

types of cells and leads to the release of p52-RelB complex (Hayden and Ghosh, 2008). In the present study, using RelA antibody, we found that NDMh LPP promoted the translocation of RelA to the nucleus. The translocation of RelA to the nucleus indicates that the LPP trigger the common, canonical pathway. The involvement of other NF-κB proteins in the LPP effect remains to be studied.

The fact that high cellular level of calpastatin per se (as a result of calpastatin-plasmid transfection) does not result in RelA translocation to the nucleus, indicates that it is the LPP-induced activation of NF-κB that leads to upregulation of calpastatin. It is of interest to note that in the high calpastatin-expressing cells due to calpastatin-plasmid transfection, there may even be a partial inhibition of the basic, normal levels of RelA in the nucleus. Such an effect is consistent with published results on inhibition of NF-κB in cells overexpressing calpastatin or treated with calpain inhibitor (by inhibiting IκB degradation) (Chen *et al*., 2000; Lee *et al*., 2005; Nozaki *et al*., 2011). The fact that lack of IκB phosphorylation (by using ΔNIκBα or pharmacological phosphorylation inhibitor) prevented the LPP-promoted calpastatin upregulation substantiates the conclusion that NF-κB is responsible for the upregulation of calpastatin triggered by LPP interaction with the cells.

LPP are mostly integral membrane proteins and are very abundant in mycoplasmal membranes (Mühlradt *et al*., 1998; Chambaud *et al*., 1999; Rottem, 2003). As in the case of other *Mycoplasma* species, *M. hyorhinis* LPP and lipopeptides are potent lymphocyte and macrophage activators, and stimulate the release of various cytokines (Mühlradt *et al*., 1998; M. Zur and T. Brenner, pers. comm.). Mycoplasmas and mycoplasmal LPP also interact with non-immune cells (Into *et al*., 2004; Gerlic *et al*.,

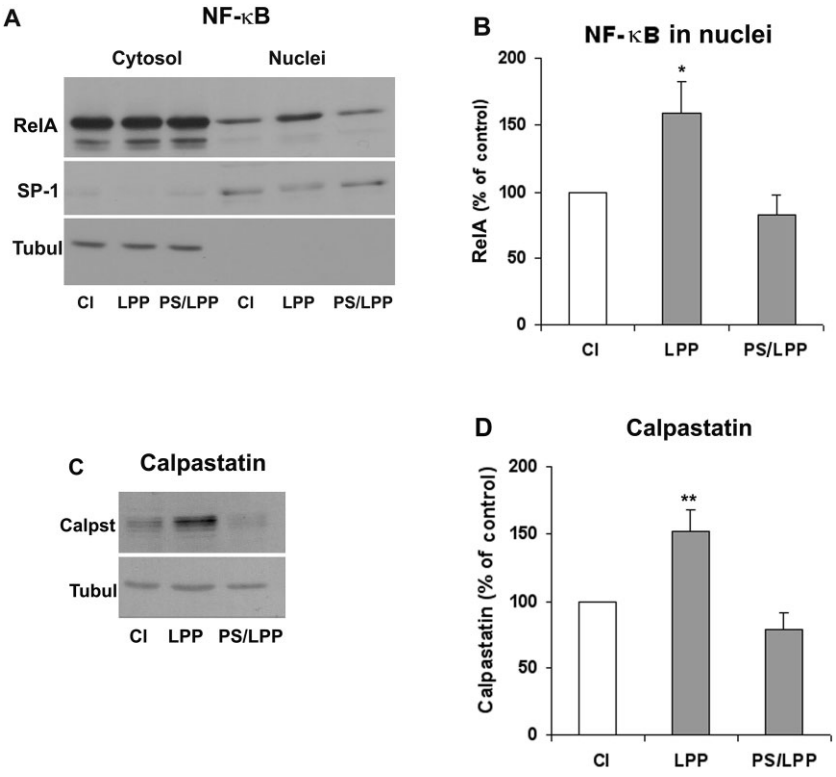

Fig. 6. NF-κB activation and calpastatin upregulation in LPP-treated cells are inhibited by the IKK kinase inhibitor PS1145. Cells were cultured in the presence and absence of 10 μM PS1145 for 24 h; medium was changed, and cultures continued for additional 72 h with and without 10 μM PS1145 and 0.3 μg ml^{-1} LPP. Cl, control cells; PS, PS1145; PS/LPP, treated with both PS1145 and LPP. For RelA, cytosol and nuclear fractions were obtained. For calpastatin, cell extract was used (for details, see *Experimental procedures*).
A. Immunoblot of RelA in cytosol and nuclei. SP-1, a marker for nuclear proteins, used for estimation of loading; Tubul, tubulin, a marker for cytosolic proteins.
B. Graph represents means of RelA levels in the cell nuclei ± SEM ($n = 3$); *$P < 0.05$ versus control cell nuclei (considered 100%).
C. Immunoblot of calpastatin in cell extracts. Calpst, calpastatin; Tubul, tubulin.
D. Graph represents means of calpastatin levels ± SEM ($n = 3$); **$P < 0.01$ versus control cell extracts (considered 100%).

2007; Zeiman *et al*., 2008; Kornspan *et al*., 2010), with little information available on cellular proteins affected by them. The LPP-induced elevation of calpastatin provides an example of LPP effects on intracellular proteins in non-immune cells, resulting in important alterations in the host cell functions.

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 (Rottem, 2003; Yavlovich *et al*., 2004), and invasiveness can be modulated by altering the lipoprotein profiles (Zeiman *et al*., 2008). *M. hyorhinis* usually propagates as a cell surface parasite, but under some conditions may penetrate into cells (Kornspan *et al*., 2010). In preliminary experiments we found that cultured SH-SY5Y cells were invaded by the NDMh and by the NDMh LPP (J.D. Kornspan, T. Vaisid, S. Rottem and N.S. Kosower, unpubl. obs.). It is not clear at present whether the LPP binding to the cultured cells suffices to trigger the upregulation of calpastatin or whether LPP uptake and intracellular presence is required for the increase in calpastatin levels.

Isolated mycoplasmal LPP are known to stimulate the production of cytokines by binding to Toll-like receptors (TLRs) on the surface of immune cells, particularly TLR 2 and TLR 6 (Into *et al*., 2004; Okusawa *et al*., 2004; Takeda and Akira, 2004). The specific receptors and associated proteins responsible for LPP-triggering of calpastatin overexpression remain to be studied. In a recent study, calpastatin upregulation has been demonstrated in macrophages activated by LPS and several other TLR-ligands (Huang *et al*., 2011). The possibility that NF-κB is also involved in LPS-stimulated calpastatin upregulation should be studied. The fact that calpastatin upregulation has now been found for both LPP and LPS stimulation may be of general importance in the response of non-immune and immune cells.

In conclusion, the mycoplasmal LPP provide a new and useful means for studying the regulation of cellular calpastatin. The results presented here may help to clarify the reasons for unexpected response of mycoplasmal-contaminated cells to certain insults (e.g. high Ca^{2+}), and may be relevant to the role of mycoplasmal-promoted NF-κB in some diseases (Baldwin, 2001). Since the level of cellular calpastatin is an important factor in the control of calpain activity, mycoplasmal LPP may be of interest in devising treatments for some pathological processes involving excessive calpain activation.

Experimental procedures

Preparation of mycoplasmal fractions

Mycoplasma hyorhinis strain NDMh was used throughout this study. The organisms were grown for 48 h at 37°C in a modified Hayflick's medium (Hayflick and Stinebring, 1960) supplemented with 10% heat-inactivated fetal calf serum (Biological Industries, Beit Haemek, Israel). The organisms were harvested at the mid-exponential phase of growth (A_{595} of 0.08–0.12; pH 6.8) by centrifugation for 20 min at 12 000 *g*, washed once, and resuspended in PBS. *M. hyorhinis* suspensions were sonicated by ultrasonic treatment for 2 min at 4°C in W-350 Heat Systems sonicator operated at 50% duty cycles. Membranes were collected by centrifugation at 34 000 *g* for 30 min, washed once and resuspended in PBS. The lipoprotein fraction of *M. hyorhinis* membranes was obtained by the Triton X-114 fractionation method (Bordier, 1981). In brief, membrane suspensions containing 0.5-0.8 mg ml^{-1} proteins were incubated in a PBS solution containing 1% Triton X-114 at 4°C for 1 h with gentle agitation. After centrifugation at 4°C for 30 min at 12 000 *g*, the supernatant containing the soluble proteins was subjected to three cycles of phase fractionation including incubation at 37°C for 5 min for micelle formation, followed by centrifugation at room temperature for 3 min at 12 000 *g* for phase separation, resulting in an upper aqueous phase and a lower detergent phase. The detergent phase was collected and the Triton X-114 was removed from the samples by gentle agitation of the detergent phase with beads (Bio-Beads SM-2; Bio-Rad) at 4°C for 2 h (Zeiman *et al*., 2008). Aliquots were kept at −80°C until use.

SH-SY5Y cell culture, infection of the cells with M. hyorhinis *and cell treatment with mycoplasmal fractions*

A mycoplasma-free SH-SY5Y cells were grown in RPMI-1640 supplemented with 2 mM L-glutamine, 10% FCS (growth medium, GM), with added 40 μg ml^{-1} gentamicin (g-GM) in 25 cm^2 plastic culture flasks. For infection with NDMh and treatment with NDMh fractions, SH-SY5Y cells were placed in Petri dishes in GM with added 100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (ps-GM). Cells were infected with the NDMh strain at a multiplicity of infection of 1–2, and subcultured twice a week for 2 weeks. For treatment with mycoplasmal fractions, cells were cultured in ps-GM medium for 24–72 h in the presence of sonicate, membranes and LPP. The cells were then washed in PBS, lysed and analysed by immunoblotting (see below).

Cell treatments with amyloid-β-peptide, Ca^{2+}/ionomycin and PS1145

Amyloid-β-peptide 25–35 (Aβ) (Bachem, Bubendorf, Switzerland) was suspended in sterile double distilled water (DDW) at a concentration of 1.0 mM, and pre-incubated for 48 h at 37°C (Morishima *et al*., 2001); amyloid-β-peptide 25–35 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; Vaisid *et al*., 2008a). Cells were cultured in ps-GM for 48 h in the presence and absence of LPP, as described above. To study the effects of Aβ on control and LPP-treated cells, pre-incubated Aβ was added to the cell cultures at a final concentration of 30 μM. Cultures were continued in ps-GM supplemented with 1.0 mM Ca^{2+}, for additional 24 h. To study the effects of Ca^{2+} on the control and LPP-treated cells, $CaCl_2$ 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 3–4 h. For studying the effects of the IKK selective inhibitor PS1145, cells were cultured in the presence and absence of PS1145 (Sc-301621) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a final concentration of 10 μM. Medium and PS1145 was replenished at 24 h, and incubation continued for 72 h, without and with added LPP (0.3 μg ml^{-1}). The cells were then washed in phosphate-buffered saline (PBS), lysed and analysed by immunoblotting (see below).

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 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 10% acrylamide for calpain, calpastatin, NF-κB, SP-1 and tubulin, 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*., 2008b), using monoclonal anti-μ-calpain antibody (1:1000); polyclonal anti-calpastatin antibody (H-300): Sc-20779 (Santa Cruz) (1:500); monoclonal anti-non-erythroid spectrin antibody (Chemicon International, Temecula, CA, USA) (1:1000); polyclonal anti-NF-κB RelA antibody (RB-1638) (Thermo Scientific, Fremont, CA, USA) (1:500). The appropriate peroxidase-conjugated secondary antibodies were used, and detection of bands carried out with ECL (Pierce, Rockford, IL, USA), according to published methods (Vaisid *et al*., 2008b). Membranes were reprobed with monoclonal anti-β tubulin antibody (Sigma-Aldrich, Saint Louis, MO, USA) (1:80 000) for estimation of loading.

Cytoplasmic and nuclear extracts of cultured SH-SY5Y cells were prepared using the ProteoJET cytoplasmic and nuclear protein extraction kit (MBI Fermentas, St. Leon-Rot, Germany), according to the manufacturer's protocol. The extracts were mixed with Laemmli sample buffer for SDS-PAGE, electrophoresed and immunoblotted, as described above. Membranes were reprobed, using polyclonal anti-SP-1 antibody (A300-134A) (Bethyl Laboratories, Montgomery, TX, USA) (1:10 000), as a marker for nuclear fractions, and anti-β tubulin antibody (see above) as a marker for cytosol fractions. Bands were quantified by densitometry.

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, and level of the XTT formazan determined in the medium by absorbance measured with a spectrophotometer at the wavelength of 467 nm.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from 5×10^6 cells by using GenElute Mammalian total RNA Miniprep kit (Sigma-Aldrich), and cDNA was synthesized from 1 μg of total RNA by using the iScript Synthesis Kit (Bio-Rad Laboratories, USA). PCR was performed on a Step One Real-Time PCR System (Applied Biosystems, Warrington, UK), using taqman Gene Expression Assays (Kit 4331182, containing Hs 00156280-m1 primaries for calpastatin, and Hs 99999905-m1 for GAPDH), according to the manufacturer's protocol.

Stable transfection of SH-SY5Y cells with calpastatin-plasmid, and confocal analysis of RelA in calpastatin-transfected cells and in LPP-treated cells

Stable SH-SY5Y cell lines overexpressing calpastatin were generated in cells cultured in g-GM medium. Cells were transfected with a plasmid containing the full length of human calpastatin cDNA, or with the empty plasmid, as previously described (Barnoy *et al*., 2005; Vaisid *et al*., 2008a). The cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The transfected cells were grown for four weeks in the presence of 800 μg ml^{-1} of G418 [Gibco (Invitrogen), Carlsbad, CA, USA], added to the medium every 48 h. The cells that survived the four weeks selection were defined as stably transfected cells. G418-resistant clonal cell colonies were cultured in g-GM in the presence of 400 μg ml^{-1} of G418.

For NF-κB confocal analysis, cells were cultured on coverslips in Petri dishes in ps-GM medium. For LPP treatment, 0.3 μg ml^{-1} LPP was added to cultures for 72 h. The cells were washed in PBS, fixed in 4% paraformaldehyde at room temperature for 10 min, and washed in PBS. Cells were permeabilized by incubation for 3 min with 0.2% Triton X-100 in PBS containing 1% bovine serum albumin (PBS-BSA solution), washed with PBS, and blocked for 20 min with 2% horse serum (HS). The cultured coverslips were overlaid with polyclonal anti-RelA antibody (RB-1638) (Thermo Scientific, Fremont, CA, USA) (1:100) in 4% HS for 40 min, and washed. Coverslips were then overlaid with FITC-conjugated anti-rabbit antibodies in 2% HS for 20 min, and washed in PBS. The coverslips were then stained with 10 μg ml^{-1} propidium iodide (PI) for 10 min, and washed in PBS. The slides were analysed by a Leica SP-5 laser scanning confocal microscope, using the excitation wave lengths for FITC and for PI. The nuclear RelA fluorescence intensity was quantified by using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Transient transfection of SH-SY5Y cells with ΔNIκBα, treatment with LPP, and confocal analysis of cellular RelA and calpastatin

Cells were cultured on coverslips in Petri dishes in ps-GM medium. A transient transfection of the cultured cells was carried out using a plasmid containing ΔNIκBα, the IκB mutant that lacks the N-terminal phosphorylation sites and is degradation-resistant (a gift from Prof. Yinon Ben-Neriah, Departments of Immunology and Genetics and Biotechnology, Hebrew University–Hadassah

Medical School, Jerusalem, Israel) (Lavon *et al*., 2000; Eldor *et al*., 2006). The cells were transfected using Lipofectamine 2000 (Invitrogen). After 24 h, LPP was added to the cells (0.5 μg ml^{-1} medium) for additional 24 h. The coverslips were washed, fixed in paraformaldehyde and permeabilized, as described above, and overlaid with polyclonal anti-RelA antibody (RB-1638) (Thermo Scientific) (1:100), and monoclonal anti-calpastatin antibody (PI –11): Sc-32324 (Santa Cruz Biotechnology) (1:50) in 4% HS for 40 min, and washed. Coverslips were then overlaid with FITC-conjugated anti-rabbit antibodies and Rhodamine Red-X-conjugated anti-mouse antibody (Jackson ImmunoResearch Laboratories, Baltimore Pike, PA, USA) (1:200) in 2% HS for 20 min, and washed in PBS. The coverslips were then stained with DAPI (Invitrogen) (100 ng ml^{-1}) in PBS for 2 min, washed and mounted on microscope slides. The slides were analysed by a Leica SP-5 laser scanning confocal microscope, using excitation at the wave lengths for DAPI, FITC and Rhodamine Red-X.

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 analysed with SPSS 17.0 (SPSS, Chicago, IL, USA).

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