A Correlative Study on A β and CD95 Pathway Independent to Ca²⁺ Dependent Protease and Activation of Caspase Activation

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Abstract

Amyloid- β -peptide (A β) is important in the pathogenesis of Alzheimer's disease (AD). Calpain (Ca²⁺-dependent protease) and caspase-8 (the initiating caspase for the extrinsic, receptor-mediated apoptosis pathway) have been implicated in AD/A β toxicity. We found that A β promoted degradation of calpastatin (the specific endogenous calpain inhibitor); calpastatin degradation was prevented by inhibitors of either calpain or caspase-8. The results implied a cross-talk between the two proteases and suggested that one protease was responsible for the activity of the other one. In neuron-like differentiated PC12 cells, calpain promotes active caspase-8 formation from procaspase-8 via the A β and CD95 pathways, along with degradation of the procaspase-8 processing inhibitor caspase-8 (FLICE)-like inhibitory protein, short isoform (FLIPS). Inhibition of calpain (by pharmacological inhibitors and by overexpression of calpastatin) prevents the cleavage of procaspase-8 to mature, active caspase-8, and inhibits FLIPS degradation in the A β -treated and CD95-triggered cells. Increased cellular Ca²⁺ per se results in calpain activation but does not lead to caspase-8 activation or FLIPS degradation. The results suggest that procaspase-8 and FLIPS association with cell membrane receptor complexes is required for calpain-induced caspase-8 activation. The results presented here add to the understanding of the roles of calpain, caspase-8, and CD95 pathway in AD/A β toxicity. Calpain-promoted activation of caspase-8 may have implications for other types of CD95-induced cell damage, and for nonapoptotic functions of caspase-8. Inhibition of calpain may be useful for modulating certain caspase-8-dependent processes.

Key words: A β , Alzheimer's Disease, Calpain, Caspase, Calcium, α -CD95, FLIP, DISC

1. Introduction

Alzheimer's disease (AD) is clinically manifested by progressive dementia. The neuro pathological alterations include the accumulation of amyloid- β -peptide (A β), a neurotoxic peptide derived from the amyloid precursor protein (APP), extracellular neuritic plaques containing A β , intracellular neurofibrillary tangles (NFT) that are composed mainly of the protein tau, and neuronal cell loss. A β plays a major role in the pathogenesis of AD and is toxic to neurons^[1]. A β leads to increased cellular Ca²⁺, mitochondrial dysfunction, enhanced generation of reactive oxygen species, and may cause neuronal cell death by apoptosis or necrosis^[2,3]. The intracellular Ca²⁺-dependent protease calpain and

its specific endogenous inhibitor calpastatin have been implicated in the pathogenesis of AD. Activated forms of μ -calpain and m-calpain (activated by μ M Ca²⁺ and mM Ca²⁺, respectively) and decreased levels of calpastatin have been found in some regions in the brains of AD patients^[4,5], and in Tg2576 mice (transgenic for a human APP mutant) in brain regions that exhibit accumulation of A β ^[6].

Caspases have also been implicated in AD. Partial activation of the caspase pathways may occur early in AD, whereas full activation of caspase-cascades and apoptosis may occur in the more advanced stages of the disease^[7]. Activated caspase-8, caspase-9 and caspase-3 were shown to be associated with neuritic plaques and NFT^[8]. The involvement of the caspase and/or calpain systems in A β - toxicity is also indicated by results of in vitro studies. In some cases, caspase activity (caspase-8, -3) was shown to be associated with A β -induced apoptosis of cultured neuronal cells^[9,10,11,12]. In other cases, calpain^[13], or calpain and caspase-12^[5], or cal-

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pain, caspase-9 and caspase-3^[14], were shown to be involved in A β -induced proteolysis and cytotoxicity. We previously found that in differentiated, neuron-like PC12 cells, A β induced the activation of calpain and caspase-8, and promoted degradation of calpastatin. The A β -promoted calpastatin degradation was prevented by inhibitors of calpain and of caspase-8^[15]. Calpastatin is a caspase substrate (including caspase-1, caspase-3 and caspase-8)^[16,17]. Under certain conditions, calpastatin is also degraded by calpain^[18,19]. Assuming independent activation and activities of calpain and caspase-8, inhibition of one protease should still allow calpastatin degradation by the other protease. The fact that pharmacological inhibitors of either protease inhibited calpastatin diminution suggested a crosstalk between the two proteases^[20], and pointed to the possibility that one protease was responsible for the activity of the other one. Caspase-8 is the initiator caspase of the extrinsic, receptor induced apoptosis pathway. The activation of caspase-8 via this pathway requires association with certain membrane receptors, such as CD95. CD95 (also called APO-1 and Fas) is a member of the tumor necrosis factor (TNF) super family of receptors. Binding of natural ligand or agonistic antibodies to CD95 initiates trimerization of CD95, recruitment of the adapter molecule Fas-associated death domain (FADD) and procaspase-8 to form the death-inducing signaling complex (DISC). The recruitment of procaspase-8 leads to its activation^[21,22]. Caspase-8 activation is blocked by the association of the inhibitor proteins FLIPS (short) and FLIPL (long) with the DISC^[23].

In order to elucidate the interaction between calpain and caspase-8, we studied calpain and caspase-8 activation in differentiated PC12 cells exposed to A β , to anti-CD95 antibody (α -CD95) and to high external Ca²⁺. In the present study, we show for the first time that calpain is involved in caspase-8 activation via the A β and the CD95 pathways. Inhibition of calpain (by a pharmacological inhibitor and by overexpression of calpastatin) prevents the conversion of the procaspase-8 to active caspase-8 and inhibits FLIPS degradation in cells exposed to A β and to α -CD95, exposing the cells to high external Ca²⁺ results in calpain activation but not caspase-8 activation, nor FLIPS degradation. The results suggest that the association of procaspase-8 and FLIPS with cell membrane receptor complexes is required for calpain-induced caspase-8 activation.

2. Experimental Section

2.1. PC12 Cell Cultures and Differentiation

PC12 were grown in Petri dishes in Dulbecco's modified Eagle's medium (DMEM), supplemented by 8% fetal calf serum (F.C.S.), 8% horse serum (H.S.), 2 mM glutamine and 100 Units/ml penicillin + streptomycin (growth medium, GM). Cells were induced to differentiate, by plating 2.5 $\times$ 10⁶ cells on collagen-coated 90 mm petri dishes, according to published procedures^[50], and cultured in DMEM, supplemented by 1% H.S., 2 mM glutamine, 50 Units/mL of penicillin + streptomycin, and 50 ng/mL nerve growth factor (NGF) (Alomone Labs, Jerusalem, Israel) (differentiation medium, DM). The time of cell replating in DM is defined as 0 h. The DM was replaced every 48 h.

2.2. Calpastatin Overexpression in Differentiated PC12 Cells

Stable PC12 cell lines over expressing calpastatin were generated in PC12 cells cultured in GM medium. Cells were transfected with a plasmid containing the full length human calpastatin cDNA (calpastatin plasmid, Pst), or with the empty plasmid (control plasmid, Pc), as previously described^[50]. The cells were transfected using Lipofectamine 2000 (Invitrogen). The transfected PC12 cells were grown for 4 weeks in the presence of 800 μ g/mL of G418 (Gibco), added to the medium every 48 h. The cells that survived the 4 weeks selection were defined as stably transfected cells. G418-resistant clonal cell colonies were cultured in GM in the presence of 400 μ g/mL of G418. For differentiation, cells were transferred to DM, as described above.

2.3. Treatment of Differentiated PC12 Cells with sA β , Anti-CD95 Antibody, Ca²⁺, and with Protease Inhibitors

Amyloid- β -peptide 25–35 (sA β) (purchased from BioSight Ltd., Karmiel, Israel and from Bachem, Bubendorf, Switzerland) was suspended in sterile double distilled water (DDW) at a concentration of 1.0 mM, and preincubated for 48 h prior to addition to the cell cultures^[55]. The sA β suspension was added to the cultures at 96 h of differentiation to a final concentration of 50 μ M. Control and sA β -treated cells were cultured in DM for additional 24 h. CaCl₂ (Sigma, St Louis, MI, USA) was dissolved in DDW (100 mM stock solution),

added to the DM at 96 h of differentiation at a final concentration of 5 mM, and cultures continued for additional 24 h. Anti-CD95 antibody (α -CD95) (Upstate cell signaling, Lake Placid, NY, USA, clone CH11) was added to the DM at 120 h of differentiation at a final concentration of 50 ng/mL, and cultures continued for additional 3 h. To study effects of protease inhibition on the treated cells, the cell permeable selective calpain inhibitor calpeptin (Calbiochem, La Jolla, CA, USA), was added to the DM at 72 h and 96 h of differentiation at a final concentration of 50 μM . The cell membrane permeable Ca^{2+} chelator BAPTA-AM (Sigma, St Louis, MI, USA) was added at 96 h of differentiation at a final concentration of 3.5 μM . To study effects of calpastatin overexpression, differentiated Pc cells and Pst cells were treated with sAb or α -CD95, as described above.

2.4. Preparation of Cell Extracts for SDS-PAGE, and Immunoblotting Analyses

PC12 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^[50]. 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-8 and FLIP, 10% acrylamide for calpain). Samples containing 20–40 μg of PC12 cell proteins were electrophoresed and then transferred to nitrocellulose membranes (Schleicher & Schuell, Maidstone, UK). Immunoblotting was carried out as previously described^[50], using polyclonal anti-caspase-8 antibody p18 (H-134): Sc-7890 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:500); monoclonal anti- μ -calpain antibody (1:1000)^[50]; polyclonal anti-FLIPS/L antibody (H-202): Sc-8347 (Santa Cruz (1:500); monoclonal anti- β tubulin antibody (Sigma, St Louis, MO, USA); The appropriate peroxidase-conjugated secondary antibodies were used, and detection of bands carried out with ECL (KPL, Gaithersburg, MD, USA), as previously described^[50]. Membranes were stripped off and reprobed with monoclonal anti- β tubulin antibody (Santa Cruz Biotechnology) (1:2000) for estimation of loading. Bands were quantified by densitometry.

2.5. Measurement of Calpain and Caspase Activities in PC12 Cell Extracts

For estimation of calpain activity, PC12 cells were differentiated for 72 h, and cultures continued for 48 h without and with added calpeptin. Cells were then cultured for additional 3 hr in the absence or presence of α -CD95, as described above. The measurement of calpain activity was carried out in cell extracts, prepared as previously described^[51]. Calpain activity was estimated by using FITC-casein (Sigma) as a substrate, according to published procedure^[10], as previously described^[51]. m-Calpain was isolated from a PC12 cell extract by column chromatography, according to published methods^[10] and served as a positive control. For estimation of caspase activity, Pc and Pst cells were differentiated for 120 h, then cultures continued for 3 h in the presence and absence of α -CD95, as described above. The cells were harvested, washed with PBS, and centrifuged at 700 $\times$ g. Cells were lysed in 10 mM Tris-HCl, pH 7.4, 130 mM NaCl, 10 mM Na pyrophosphate, 1% Triton X-100, 10 mM $\text{NaH}_2\text{PO}_4/\text{NaHPO}_4$, pH 7.4. Lysates were kept on ice for 30 min, centrifuged and the protein concentration in the supernatants measured^[6]. Supernatants were kept at -20°C prior to the estimation of caspase activity. Caspase activity in the supernatants was estimated according to published methods^[48] in 20 mM Hepes, pH 7.4, 2 mM dithiothreitol (DTT), 10% glycerol, using the caspase-8 fluorogenic substrate Ac-IETD-AMC (Alexis Biochemicals, Lausen, Switzerland), in the absence and presence of the selective caspase-8 inhibitor Z-IETD-FMK (ZIET). Fluorescence levels of the released AMC were estimated by spectrofluorimetry (excitation at 380 nm, emission at 460 nm).

3. Results and Discussion

3.1. Activation of Caspase-8 in Differentiated PC12 Cells Exposed to sAb_i is Mediated by Calpain

Caspase-8 in control and in sAb_i-treated cells was analyzed by immunoblotting (IB), as described in Materials and methods. A band of 18 kDa, indicative of activation of the procaspase (~55 kDa band), was observed in control cells, with significantly increased levels of the 18 kDa band seen in sAb_i-treated cells (Fig. 1A and B). A significant inhibition in caspase-8 activation was observed in the cells treated with the selective calpain inhibitor calpeptin and exposed to sAb_i, as compared to

Fig. 1. A β -treated differentiated PC12 cells enhance Caspase-8 activation and inhibited by calpeptin and by calpastatin overexpression. (A) and (B) Caspase-8 in control and sA β -treated cells in the absence and presence of calpeptin. C, control; Calpep, calpeptin. (A) Immunoblot of caspase-8. (B) Ratios of caspase-8 18 kDa/55 kDa. Graph represents means $\pm$ SEM ($n = 4$). * $p < 0.05$ vs. control (considered 100%); # $p < 0.05$ vs. sA β . (C) and (D) Caspase-8 in control and sA β -treated cells transfected with control plasmid (Pc) and with calpastatin plasmid (Pst). (C) Immunoblot of caspase-8. (D) Ratios of caspase-8 18 kDa/55 kDa. Graph represents means $\pm$ SEM ($n = 4$). * $p < 0.05$ vs. Pc (considered 100%); # $p < 0.05$ vs. Pc + sA β .

cells treated only with sA β (Fig. 1A and B). The inhibition of caspase-8 activation by calpeptin indicated that calpain was responsible for the appearance of the 18 kDa caspase-8. In order to substantiate this conclusion, we used PC12 cells overexpressing the calpain specific inhibitor, calpastatin (Pst cells). The Pst cells used expressed about 140–220% of calpastatin levels as compared to levels in control cells transfected with empty plasmid (Pc cells)^[51]. Caspase-8 activation was significantly inhibited in sA β -treated calpastatin-over express-

ing cells (Fig. 1C and D). Thus the results show that calpain is involved in caspase-8 activation in the sA β -treated PC12 cell.

3.2. Activation and Activity of Calpain and Caspase-8 in Differentiated PC12 Cells Treated with Anti-CD95 Antibody

In order to further examine the relationship between calpain and caspase-8, we used an agonist antibody (α -CD95) as a ligand for triggering the receptor CD95. We

assessed calpain and caspase-8 activation in cells exposed to α -CD95, and examined the effects of calpeptin and calpastatin overexpression on the proteases in the α -CD95-treated cells. Calpain and caspase-8 activation was assessed by immunoblotting (IB) and activity estimated in cell extracts, using the appropriate fluorogenic substrates, as described in Materials and methods. IB analysis of μ -calpain showed that the ratio of the 76 kDa band to the original 80 kDa band (calpain

autolysis) was significantly greater in the α -CD95-treated cells than in the control; the increased calpain autolysis is indicative of enhanced μ -calpain activation in the treated cells. The α -CD95-promoted calpain activation was inhibited by calpeptin (Fig. 2A and B). Calpain activity (using FITC-casein as a substrate) was significantly increased in extracts made of α -CD95-treated cells as compared with the control cells; calpain activity was inhibited in cells treated with both calpeptin

Fig. 2. μ -Calpain activation is enhanced in α -CD95-treated differentiated PC12 cells, and activation is inhibited by calpeptin and by calpastatin overexpression. (A)–(C) μ -Calpain in control and α -CD95-treated cells in the absence and presence of calpeptin. C, Control; Calpep, calpeptin. (A) Immunoblot of μ -calpain. (B) Ratios of μ -calpain 76 kDa/80 kDa. Graph represents means $\pm$ SEM ($n = 4$). ** $p < 0.01$ vs. control (considered 100%); # $p < 0.05$ vs. α -CD95. (C) Calpain activity in control and in α -CD95-treated cells cultured in the absence and presence of calpeptin. Activity of calpain was estimated in cell extracts, using FITC-casein as a substrate, without and with calpeptin (0.2 mM) added to the reaction mixture samples. The activity exhibited by the cell extract reaction mixtures containing calpeptin represents cleavage of FITC-casein in the extracts by proteases other than calpain. The activity in the reaction mixtures containing calpeptin was subtracted from the total activity measured without calpeptin addition to the reaction mixture, to obtain values for calpain activity. Calpain activity was normalized for equal protein content in the cell extracts. For control and α -CD95-treated cells, the graph represents means $\pm$ SEM ($n = 3$). ** $p < 0.01$ vs. control. For α -CD95-treated cells cultured in the presence of calpeptin (α -CD95 + calpep), average of two experiments is shown. (D) Ratios of μ -calpain 76 kDa/80 kDa in control and α -CD95-treated cells transfected with control plasmid (Pc) and with calpastatin plasmid (Pst). Graph represents means $\pm$ SEM ($n = 3$). * $p < 0.05$ vs. Pc (considered 100%); # $p < 0.05$ vs. Pc + α -CD95.

and α -CD95 as compared with the α -CD95-treated cells (Fig. 2C). The results are consistent with the results on calpain activation presented in Fig. 2A and B, and indicate that calpain activity is enhanced by α -CD95. Calpain was activated in α -CD95-treated Pc cells, and inhibited in α -CD95-treated Pst cells (Fig. 2D), further substantiating the conclusion that calpain is activated by triggering the CD-95. Caspase-8 is known to be activated at the CD95 death-inducing signalling complex upon triggering of the cell CD95^[24,25]. As shown in Fig. 3, caspase-8 was activated in the cells

exposed to α -CD95, as indicated by the significant increase in the 18 kDa band in the treated cells. Caspase-8 activation was inhibited by calpeptin (Fig. 3A and B), and by calpastatin overexpression (Fig. 3C and D). Caspase-8 activity (using Ac-IETDAMC as a substrate) was significantly increased in extracts made of α -CD95-treated Pc cells as compared with the control Pc cells. In contrast, caspase-8 activity in extracts of the α -CD95-treated Pst cells was not different from that of the control cells (Fig. 3E). These results indicated that overexpression of calpastatin inhibited the α -CD95-pro-

Fig. 3. Caspase-8 activation in α -CD95-treated differentiated PC12 cells is inhibited by calpeptin and by calpastatin overexpression. (A) and (B) Caspase-8 in control and α -CD95-treated cells in the absence and presence of calpeptin. C, control; Calpep, calpeptin. (A) Immunoblot of caspase-8. (B) Ratios of caspase-8 18 kDa/55 kDa. Graph represents means $\pm$ SEM ($n = 4$). ** $p < 0.01$ vs. control (considered 100%); ### $p < 0.01$ vs. α -CD95. (C) and (D) Caspase-8 in control and α -CD95-treated cells transfected with control plasmid (Pc) and with calpastatin plasmid (Pst). (C) Immunoblot of caspase-8. (D) Ratios of caspase-8 18 kDa/55 kDa. Graph represents means $\pm$ SEM ($n = 4$). ** $p < 0.01$ vs. Pc (considered 100%); # $p < 0.05$ vs. Pc + α -CD95. (E) Activity of caspase-8 in extracts made of α -CD95-treated Pc and Pst cells, using Ac-IETD-AMC as a substrate, in the absence and presence of the caspase-8 inhibitor Z-IETD-FMK (ZIET) added to the reaction mixture samples. The activity exhibited by the samples containing ZIET represents cleavage of Ac-IETD-AMC by proteases other than caspase-8. The activity in the samples containing ZIET was subtracted from the total activity measured in the absence of ZIET to obtain values for caspase-8 activity. Caspase-8 activity was normalized for equal protein content in the cell extracts. Graph represents means $\pm$ SEM ($n = 3$). ** $p < 0.01$ vs. Pc; ### $p < 0.01$ vs. Pc + α -CD95.

moted caspase-8 activity, supporting the results of the IB analysis (Fig. 3C and D). Overall, the results indicated that calpain was activated by stimulation of CD95, and that calpain was involved in caspase-8 activation via the CD95 signalling pathway.

3.3. Intracellular Ca²⁺ Involvement in Calpain and Caspase-8 Activation in α CD95 and sA β Treated PC12 Cells

Calpain activation implies an increase in intracellular Ca²⁺. We previously found that calpain was activated in differentiated PC12 cells exposed to sA β ^[26]. In the present study, μ -calpain was analyzed in cells treated with the cell membrane permeable Ca²⁺ chelator BAPTA-AM (BAPTA) and exposed to A β and to α -CD95. As shown in Fig. 4, a decrease in the ratio of the 76 kDa band to the 80 kDa band was observed in BAPTA-treated cells, as compared with BAPTA-

untreated cells exposed to A β or to α -CD95 (Fig. 4A–C). The results indicated that BAPTA inhibited calpain activation induced by A β and by α -CD95. Under the same conditions there was also less caspase-8 activation in BAPTA treated cells than in cells without BAPTA, when exposed to sA β or to α -CD95 (Fig. 4D and E). These results are consistent with the results described above, showing that in both the A β -treated and α -CD95-treated cells, Ca²⁺-promoted calpain activation leads to caspase-8 activation.

3.4. Increased Cellular Ca²⁺ per se Leads to Calpain Activation but not to Caspase-8 Activation

Calpain was activated under these conditions; calpain activation was prevented when BAPTA was added to the cells (Fig. 5A and B). In contrast, caspase-8 was not activated in cells cultured in Ca²⁺-rich medium (Fig. 5C and D). Caspase-8 activity, using Ac-IETD-AMC as a

Fig. 4. BAPTA-AM inhibits activation of μ -calpain and of caspase-8 in cells exposed to sA β , and to α -CD95. (A) μ -calpain in cells exposed to A β . Immunoblot, representative of two experiments. (B) and (C) μ -calpain in cells exposed to α -CD95. (B) Immunoblot of μ -calpain. (C) Ratios of μ -calpain 76 kDa/80 kDa. Graph representing means $\pm$ SEM ($n = 4$). ** $p < 0.01$ vs. control (considered 100%); # $p < 0.05$ vs. α -CD95. (D) and (E) Caspase-8 in cells exposed to sA β , and to α -CD95. (D) Caspase-8 immunoblots. (E) Ratios of caspase-8 18 kDa/55 kDa. Graph represents means $\pm$ SEM ($n = 4$). \$ $p < 0.02$; # $p < 0.05$ vs. A β -treated and α -CD95-treated cells, respectively.

Fig. 5. μ -Calpain and caspase-8 in cells exposed to high Ca^{2+} in the medium. (A) and (B) μ -Calpain in cells exposed to high Ca^{2+} in the presence and absence of BAPTA-AM (BAPTA). (A) Immunoblot of μ -calpain. (B) Ratios of μ -calpain 76 kDa/80 kDa. Graph represents means $\pm$ SEM ($n = 4$). $**p < 0.01$ vs. control (considered 100%); $\#p < 0.05$ vs. Ca^{2+} . (C) And (D) Caspase-8 in cells exposed to high Ca^{2+} . (C) Immunoblot of caspase-8. (D) Ratios of caspase-8 18 kDa/55 kDa. Graph represents means $\pm$ SEM ($n=6$) of Ca^{2+} -treated cells vs. control (considered 100%).

substrate, was not enhanced in cells cultured in Ca^{2+} -rich medium (data not shown). The results indicate that calpain activation leads to enhanced caspase-8 activation under conditions where caspase-8 is recruited to the membrane (i.e., upon binding of $\text{A}\beta$ or of α -CD95 to the cell membrane), but not when caspase-8 is free in the cytoplasm.

3.5. FLIPS is Degraded in Differentiated PC12 Cells Exposed to $\text{sA}\beta$ and to α CD95, but not When Exposed to High Ca^{2+}

When recruited to the DISC in CD95-stimulated cells, FLIPS prevents caspase-8 activation by inhibiting the initial cleavage step of procaspase-8, whereas FLIPL allows the initial cleavage step of procaspase-8 but blocks further processing to caspase-8^[27]. As shown in Fig. 6, FLIPS levels were decreased in $\text{sA}\beta$ -treated cells; calpeptin inhibited the $\text{sA}\beta$ -induced decrease in

FLIPS levels (Fig. 6A and B). α -CD95 treatment also led to decreased FLIPS levels and calpeptin inhibited this decrease (Fig. 6C and D). In contrast, increased Ca^{2+} per se had no effect on the levels of FLIPS (Fig. 6E). The lack of effect of high Ca^{2+} in the medium on FLIPS suggests that increasing cellular Ca^{2+} per se, though it activates calpain, does not affect FLIPS, similar to the lack of effect on caspase-8. No changes were seen in FLIPL under any of the treatments described here (data not shown).

4. Conclusion

Active calpain promotes the cleavage of several procaspases, resulting in either inactivation or activation of the caspases^[20]. Calpain cleaves and inactivates caspase-9 in cell lysate and in intact cells, and may decrease caspase-3 by cleavage of the active caspase-3^[41,21]. Cal-

Fig. 6. FLIPS levels in cells exposed to sAβ, α-CD95 and to high Ca²⁺ in the medium, in the presence and absence of calpeptin. C, control; Calpep, calpeptin; Tubul, Tubulin. (A) And (B) Cells treated with sAβ. (A) Immunoblot of FLIPS. (B) Graph represents means±SEM (*n* = 4). ****p*<0.001 vs. control (considered 100%); ###*p*<0.001 vs. sAβ. (C) And (D) Cells treated with α-CD95. (C) Immunoblot of FLIPS. (D) Graph represents means±SEM (*n* = 4). ****p*<0.001 vs. control (considered 100%); ###*p*<0.001 vs. α-CD95. (E) FLIPS levels in cells exposed to high Ca²⁺ in the medium. Graph represents means±SEM (*n* = 4).

pain promotes the activation of the endoplasmic reticulum-associated caspase-12^[22], and has been implicated in the activation of caspase-7^[22] and caspase-3 during certain insults^[23]. Caspase-8 has been shown to be cleaved by calpain in cell lysate, but the functional consequences are not known and the effects of calpain on caspase-8 in intact cells have not been studied^[24]. Here we demonstrate that in differentiated PC12 cells, calpain is involved in caspase-8 activation via the Aβ-induced and the CD95-triggered pathways. Calpain is known to be activated in several types of neuronal cells exposed to Aβ^[15,19,25], whereas calpain activation via the CD95 pathway has not been previously described.

This report shows that calpain is activated upon binding of α-CD95 to differentiated PC12 cells, and that the α-CD95-induced activation is inhibited in cells treated with calpeptin, with Ca²⁺ chelator, and in cells overexpressing calpastatin. Caspase-8 is known to be activated in cells treated with α-CD95^[26] and with Aβ^[28,37].

Unexpectedly, the enhanced caspase activation via both the Aβ and CD95 pathways was inhibited under conditions of calpain inhibition and Ca²⁺ chelation. These results, based on the use of pharmacological inhibitors, indicated that calpain activity led to caspase-8 activation. To further probe the effect of calpain on caspase-8, we studied differentiated PC12 cells overexpressing calpastatin. The fact that overexpression of calpastatin inhibits Aβ and α-CD95-induced caspase-8 activation substantiates the conclusion that calpain is involved in the caspase-8 activation in both pathways. FLIP has been identified as a protein capable of blocking CD95-mediated apoptosis^[29]. FLIP associates with procaspase-8 in the DISC; when present in excess to procaspase-8, FLIP inhibits the processing of the procaspase to active caspase-8. Both FLIP isoforms, FLIPS (short) and FLIPL (long) inhibit the generation of active caspase-8. FLIPS prevent the initial cleavage step of procaspase-8, so that the full length of procaspase-8 is

detected at the DISC. In contrast, FLIPL allows the initial cleavage step but blocks further processing to active caspase-8^[30]. FLIP can be degraded by caspase^[31], but there is no information on whether it is a substrate for calpain. We show here that FLIPS (but not FLIPL) is degraded in cells exposed to A β or to α -CD95, and that the degradation is inhibited by inhibition of calpain. These results indicate that calpain is responsible for FLIPS degradation under the conditions used here.

Taken together, the results are consistent with published data on the activation of procaspase-8 at the cell membrane^[32]. In the case of the CD95 pathway, it has generally been assumed that when associated with the CD95-death-inducing signalling complex (DISC), procaspase-8 is dimerized. Subsequently, two sequential cleavage events generate mature stable active caspase-8 that is released into the cytosol, where it can activate a cascade of other caspases^[33,47,56]. The results presented here indicate that calpain promotes the proteolysis of the α -CD95-induced, DISC-associated, dimerized procaspase-8. In the case of A β , binding of the peptide to the cell membrane appears to be required for its toxicity^[42]. A β binds to membrane lipids and to a variety of membrane proteins^[34,53]. A β binding has been shown to lead to the formation of A β -APP complexes, and to the recruitment and activation of caspase-8^[35]. Thus, though the exact identity of the membrane receptors and membrane-associated proteins involved in the A β -induced toxicity and caspase-8 activation is not known at present, the overall mechanism responsible for the A β -induced caspase-8 activation may be similar to that involved in the CD95 pathway, i.e., A β -binding to the membrane triggers the recruitment of procaspase-8 to the membrane, followed by activation to caspase-8. It is of interest to note that the CD95 pathway and FLIP have been implicated in AD, and that A β toxicity may involve the CD95 pathway. Increased levels of the death receptor CD95 have been observed in postmortem AD brain^[36], and FLIP has been found to be decreased in AD as compared to age-matched control brains^[37]. A β -mediated neuronal death is prevented by caspase-8 inhibition, or in dominant negative FADD-cells^[38].

A β may also participate in apoptosis of neurons via the activation of c-Jun and induction of CD95 ligand, leading to activation of the CD95 pathway^[39]. In addition, a tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) contributes to A β -toxicity;

caspase-8 inhibition and FADD deficiency prevent the TRAIL and A β -induced neurotoxicity^[40]. Thus A β may, either directly or via forming protein complexes upon binding to other membrane sites, interact with the CD95 pathway, leading to calpain activation and calpain-dependent caspase-8 activation. It should be noted that caspase-8 can be activated by interchain proteolysis via other triggered apoptotic pathways, such as the mitochondrial pathway^[31,41]. A possible role of calpain in these pathways remains to be studied. Ca²⁺ is increased in cells exposed to A β ^[42], and A β plaques impair Ca²⁺ homeostasis in vivo, resulting in disruption of neuronal networks^[43]. A β -induced calpain activation is inhibited by Ca²⁺-chelation, as documented in published studies^[10,44], and confirmed in the present study. Ca²⁺ is also increased in cells exposed to CD95 ligands^[45]. In addition, it has been shown that Ca²⁺ mobilization is impaired in cells deficient in FADD^[46]. Since increased cellular Ca²⁺ is required for promoting calpain activation, we studied calpain and caspase-8 activation under conditions of increased cellular Ca²⁺ per se (i.e., by placing differentiated PC12 cell cultures in medium containing high Ca²⁺). The fact that under these conditions, calpain activation is promoted whereas caspase-8 activation is not which indicates that calpain leads to enhanced caspase-8 activation when procaspase-8 is associated with the membrane protein complexes formed by α -CD95 or by A β binding.

Our results in the present study may cast light on the published results, i.e., that in the intact cell calpain is involved in caspase-8 activation when a membrane site is triggered, whereas free caspase-8 is not activated by ionophore-induced Ca²⁺ increase. As shown here, FLIPS is also not affected by active calpain in cells exposed to high Ca²⁺ per se. The fact that in the absence of membrane binding ligands (i.e., in the absence of A β or CD95 triggering) FLIPS is not degraded by calpain is consistent with the notion that FLIPS is degraded when associated with procaspase-8 at the cell membrane. It is not clear at present whether the dimerization of caspase-8 at the DISC promotes its cleavage by calpain, or whether the calpain-promoted degradation of FLIPS allows autoproteolysis of procaspase-8. Additional study is necessary to further clarify this point. Involvement of additional factors (e.g., binding to phospholipids, extracellular signal-regulated kinase (ERK) activation)^[8,22] remains to be studied. Caspase-8, in

addition to being an initiator of receptor-induced apoptosis^[47], plays a role in nonapoptotic processes^[23,48]. In some processes, caspase-8 activity is required. Specific examples of participation of active caspase-8 in nonapoptotic processes include NF- κ B activation in lymphocytes^[49], placental trophoblast differentiation and fusion^[50], T-cell activation^[51]. In such cases, calpain may be involved in promoting caspase-8 activation.

In contrast to nonapoptotic functions, caspase-8 may play a role independently of its proteolytic activity. Inactive procaspase-8 protein or its death effector domains (DED) are involved in cell motility, via association with the integrin-dependent focal adhesion complex (FAC) proteins, including calpain/calpastatin^[9,17,44,52]. The FAC-associated inactive caspase-8 appears to promote calpastatin dissociation from calpain, thereby facilitating calpain activation^[55]. Calpain cleaves certain FAC components and plays a role in the regulation of cell adhesion and motility^[53]. Other nonapoptotic processes involving caspase-8 include embryonic development^[54] and transformation suppression^[55]; however, it is not yet clear whether the presence of the protein suffices or active caspase-8 is required in these processes. It is possible that in all cases, membrane protein complex-associated caspase-8 protein promotes dissociation of calpain from its inhibitor calpastatin, allowing calpain activation. In some cases, calpain helps to transform procaspase-8 to active caspase-8, resulting in caspase and calpain-induced protein degradation. In cases where caspase-8 formation is precluded, calpain-promoted protein degradation still occurs.

To highlight the output, contrasting the effects of calpain on caspase-8 and FLIPS via CD-95 and A β -associated membrane complexes with lack of effects of calpain on free caspase-8 and FLIPS, is presented in Fig. 7. In addition, a possible procaspase-promoted dissociation of calpain/calpastatin is indicated, as proposed by^[56] for FAC-recruited caspase-8. In sum, calpain is shown here to be involved in the processing of procaspase-8 to caspase-8. The results suggest that recruitment of procaspase-8 to the membrane is required for calpain-promoted activation to caspase-8. The results presented here add to the understanding of the roles of calpain, caspase-8 and the CD95 pathway in AD and in A β -toxicity. Calpain-promoted activation of caspase-8 may have implications for other types of CD95-induced cell damage, and for certain nonapoptotic

Fig. 7. Schematic representation of the proposed pathways involved in calpain induced caspase-8 activation. Triggering of the CD95 receptors leads to DISC formation (including FADD, procaspase-8 and FLIP). Similarly, binding of A β to the cell membrane leads to the formation of membrane-associated protein complex, incorporating procaspase-8/FLIP. In both cases, cellular Ca^{2+} is increased; the membrane-associated procaspase-8 may enhance calpastatin dissociation from calpain for the focal adhesion complex-associated caspase-8. As a result, calpain activation is facilitated in the CD95-triggered and in A β -treated cells; in turn, calpain activates caspase-8 (by direct cleavage of procaspase-8, or possibly allowing procaspase-8 auto proteolysis by degradation of FLIPS). A significant increase in Ca^{2+} per se leads to calpain activation without effect on procaspase-8/FLIPS.

functions of caspase-8. Increased expression of calpastatin or FLIP and/or decrease in calpain and caspase-8 may serve as means for the prevention of A β -toxicity and CD95-induced cell death.

The abbreviations used are: AD, Alzheimer's disease; α -CD95, anti-CD95 antibody; A β , amyloid- β -peptide;

APP, amyloid precursor protein; BAPTA, BAPTA-AM (1,2-Bis-(oaminophenoxy) ethane-N,N,N',N'-tetraacetic acid tetraacetoxymethyl ester); DISC, death-inducing signalling complex; DMEM, Dulbecco's modified Eagle's medium; DM, differentiation medium; ERK, extracellular signal-regulated kinase; FAC, focal adhesion complex; FADD, Fas-associated death domain; FLIP, caspase-8 (FLICE)-like inhibitory protein; GM, growth medium; H.S., horse serum; IB, immunoblotting; NFT, neurofibrillary tangles; NGF, nerve growth factor; Pc, control plasmid; Pst, calpastatin plasmid; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; sA β , A β 25-35; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis inducing ligand.

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