α-Secretase-derived Fragment of Cellular Prion, N1, Protects against Monomeric and Oligomeric Amyloid β (Aβ)-associated Cell Death*5

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Background: Cellular prion undergoes α-secretase cleavage, yielding N1. We examined whether N1 protects against Aβ monomers and oligomers.

Results: N1 protects against Aβ monomers and oligomers prepared from APP-London-expressing human cells and Alzheimer disease-affected brains.

Conclusion: N1 could protect from Aβ-associated toxicity at the early asymptomatic phase of Alzheimer disease.

Significance: These data emphasize the cross-talk between PrPc and βAPP catabolites.

In physiological conditions, both β-amyloid precursor protein (βAPP) and cellular prion (PrPc) undergo similar disintegrin-mediated α-secretase cleavage yielding N-terminal secreted products referred to as soluble amyloid precursor protein-α (sAPPα) and N1, respectively. We recently demonstrated that N1 displays neuroprotective properties by reducing p53-dependent cell death both in vitro and in vivo. In this study, we examined the potential of N1 as a neuroprotector against amyloid β (Aβ)-mediated toxicity. We first show that both recombinant sAPPα and N1, but not its inactive parent fragment N2, reduce staurosporine-stimulated caspase-3 activation and TUNEL-positive cell death by lowering p53 promoter transactivation and activity in human cells. We demonstrate that N1 also lowers toxicity, cell death, and p53 pathway exacerbation triggered by Swedish mutated βAPP overexpression in human cells. We designed a CHO cell line overexpressing the London mutated βAPP (APP_LDN) that yields Aβ oligomers. N1 protected primary cultured neurons against toxicity and cell death triggered by oligomer-enriched APP_LDN-derived conditioned medium. Finally, we establish that N1 also protects neurons against oligomers extracted from Alzheimer disease-affected brain tissues. Overall, our data indicate that a cellular prion catabolite could interfere with Aβ-associated toxicity and that its production could be seen as a cellular protective mechanism aimed at compensating for an sAPPα deficit taking place at the early asymptomatic phase of Alzheimer disease.

The β-amyloid precursor protein (βAPP)5 and cellular prion are central to two neurodegenerative diseases, namely Alzheimer and prion diseases (1, 2). In Alzheimer disease (AD), anatomical and genetic clues directed research toward the investigation of βAPP proteolysis, and it emerged relatively rapidly that this protein could undergo various physiological or potentially pathological alternative cleavages via distinct proteolytic entities. Most of the normal cleavage of βAPP occurs in a constitutive or regulated manner by two disintegrins belonging to the a disintegrin and metalloprotease (ADAM) family, ADAM10 and ADAM17, respectively (3). This α-cleavage has been proved to be very important for at least two main reasons. First, the cleavage occurs within the Aβ domain of βAPP and thereby prevents the production, accumulation, and aggregation of Aβ. Second, the α-secretase-mediated breakdown of βAPP gives rise to a secreted product referred to as sAPPα that is cytotoxic and neuroprotectant and has the remarkable property to protect against Aβ-related toxicity both in vitro and in vivo (4–6). Because, α- and β-secretases apparently compete for βAPP substrate (7, 8), it has been speculated that part of Alzheimer disease pathology could be linked to a deficiency in sAPPα-associated protection against Aβ-related toxicity.

The cellular prion is a glycosylphosphatidylinositol-anchored protein (9). It is known that glycosylphosphatidylinositol-linked protein is mainly released from cellular membranes

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5 The abbreviations used are: βAPP, β-amyloid precursor protein; Aβ, amyloid β; sAPPα, soluble amyloid precursor protein-α; PrPc, cellular prion; APP_LDN, London mutated βAPP; AD, Alzheimer disease; ADAM, a disintegrin and metalloprotease; STS, staurosporine; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; APPsw, Swedish mutated APP; a.u., arbitrary units; LRP1, low density lipoprotein receptor-related protein 1.
by the action of the well described phosphatidylinositol-specific phospholipase C (10). Unexpectedly, as is the case for βAPP, we have established that PrP<sup>C</sup> can undergo both constitutive and regulated proteolysis by ADAM10 and ADAM17, respectively (11, 12), although the ADAM family of enzymes was thought to target only transmembrane proteins (13, 14). The striking similarity between the enzymatic machineries responsible for the physiological processing of βAPP and PrP<sup>C</sup> led us to pursue our investigation of PrP<sup>C</sup> cleavage regulation and the putative biological function harbored by disintegrin-mediated PrP<sup>C</sup> catabolites. Data concerning the muscarinic control of the regulated processing of PrP<sup>C</sup> (15) and the nature of the protein kinase C and downstream kinases involved delineate striking similarities, although a few differences remain (13, 16, 17).

Converging phenotypes conveyed by sAPPα and N1, the α-secretase-derived product yielded by ADAM-dependent PrP<sup>C</sup> cleavage, also exist. Thus, as had been documented for sAPPα (4–6), we established that N1 protects various cells, including primary cultured neurons, from various proapoptotic challenges (18). Most interesting is that N1 also protects neurons in vivo in a pressure-induced ischemia model of rat retina (18). In both in vitro and in vivo approaches, N1 triggers its protective phenotype by down-regulating the p53-dependent pathway (18). Interestingly, a previous study suggested that Aβ peptides could elicit cell death by exacerbating the p53 pathway (19). Altogether, this led us to postulate that N1 could potentially protect cells from Aβ-induced toxicity. Here we show that recombinant N1 and sAPPα similarly protect human cells from staurosporine (STS)-induced cell death by reducing p53 pathway activation. Interestingly, N1 reduces the toxicity and p53 pathway activation in cells expressing familial AD-linked mutations in βAPP and PS2 (i.e. engineered to overproduce Aβ). Finally, we establish that N1 protects cells from Aβ oligomers recovered in the secretions of cells expressing BAPP bearing the London mutation or prepared from pathogenic AD-affected brain extracts. Altogether, our study is the first demonstration that a PrP<sup>C</sup> catabolite could interfere with Aβ toxicity. We speculate on the possibility that such a mechanism could be part of the compensatory mechanisms likely taking place during the early asymptomatic phase of AD pathology.

**Materials and Methods**

**Stable Transfection Procedure and Cells—Wild-type (βAPPWT) or Swedish mutated (βAPPsw) βAPP-overexpressing human embryonic kidney (HEK) 293 cells were obtained and maintained in culture in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) as reported previously (20–22).** APP/APLP1/APLP2 triple knock-out mouse embryonic fibroblasts were cultured as described (23). LRP1<sup>++</sup> and LRP1<sup>−/−</sup> CHO cell lines were grown in F-12 medium (Invitrogen) with 10% FBS (24). Primary cultured neurons from mouse embryos were prepared as detailed previously (25). 3·10<sup>6</sup> cells were seeded in 35-mm diameter dishes precoated with polylysine (10 μg/ml; Sigma) and kept for 4 days before performing assays. For experiments in 96-well plates, 1·10<sup>5</sup> cells were seeded per well.

Mock-transfected and APP695WT- or APP695<sub>LDN</sub>-expressing CHO cells were obtained by stable transfection of pcDNA<sub>4</sub> empty vector and hAPP695WT or hAPP695<sub>LDN</sub> cDNA subcloned in pcDNA<sub>4</sub> vector. Cells were maintained in DMEM containing 10% FBS, sodium hypoxanthine-thymidine supplement, and 300 μM proline. cDNA encoding APP695<sub>LDN</sub> was obtained by site-directed mutagenesis of APP695WT cDNA as described below. Cells were stably transfected with 2 μg of cDNA constructs according to Lipofectamine protocols reported previously (26). Clones were selected with 250 μg/ml Zeocin (Invitrogen).

**Conditioned Media from CHO Cell Lines—Mock-transfected, APP695WT, or APP695<sub>LDN</sub> CHO cells were grown in 150-mm-diameter dishes until reaching 80% confluence, then washed with PBS, and allowed to secrete for 24 h into 15 ml of Neurobasal medium (Invitrogen). Secretions were centrifuged (1000 × g for 10 min) and then concentrated into Amicon Ultra-15 3000 filters (4000 × g for 30 min). One-milliliter aliquots of concentrates were stored at −80 °C until use (27).

**Site-directed Mutagenesis of βAPP—** The βAPP sequence harboring the London V642I mutation was obtained by a site-directed mutagenesis kit (QuikChange, Stratagene, La Jolla, CA) by converting the hAPP695WT nucleotide sequence ATGTCATC into ATCATCATC (28) by means of the following set of primers: hAPP695V642I-S, 5′-GGCACAGT-GATCATCATCACCTTGTTG-3′ (forward primer) and hAPP695V642I-aS, 5′-CACAAGGTGATGATGATCAC-TGTCGC-3′ (reverse primer). The construct was confirmed by sequencing.

**Recombinant Fragments—** N1 and N2 recombinant fragments were produced as described previously (18, 29). Briefly, the pGEX-KG glutathione S-transferase-N1- or -N2-expressing vectors were transformed into the BL21-Gold strain of Escherichia coli. Bacteria were grown in Luria broth medium, and then the fusion protein was induced with 0.5 mM isopropyl-1-thio-β-d-galactopyranoside for 4 h at 37 °C. Cells were pelleted, resuspended in PBS with Complete protease inhibitor mixture and lysozyme, incubated on ice for 30 min, and then solubilized by 1% Triton X-100, 10 mM MgCl₂, and 5 μg/ml DNase I. Debris was pelleted, and then glutathione-Sepharose beads were added to the lysate and swirled for 1 h at 4 °C. Peptides were cleaved with thrombin. Thrombin was removed using Sepharose-benzamidine beads.

sAPP695 was purified as described (29). A strain of Pichia pastoris expressing sAPP695 (kindly provided by Dr. R. Cappai, University of Melbourne, Melbourne, Australia) was grown at 30 °C in 1% yeast extract (Invitrogen), 2% peptone (Invitrogen), 2% d-glucose (Sigma). Protein expression was induced during 48 h in BMMY (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base without amino acids (Sigma), 4 × 10<sup>−3</sup>% biotin (Sigma), 2% methanol (Merck)). Purification was carried out on ice using a modification of the method of Henry et al. (30). Yeast cultures (0.5–2 liters) were centrifuged at 16,000 × g for 10 min at 4 °C, and supernatants were filtered (0.45 μm; Whatman). The supernatant was diluted to ionic strength 0.2 in 20 mM imidazole, 5 mM EDTA, 10 mg/liter phenylmethylsulfonyl fluoride (PMSF), pH 5.5 and applied at a flow rate of 7 ml/min onto a Q-Sepharose column (HR 26-10, GE Health-
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Immunoprecipitation of Aβ40 and Aβ42 species or total Aβ from human brain tissue homogenates was performed according to Shankar et al. (32) using FCA3340, FCA3542, or 6E10 antibody, respectively. Immunoprecipitates were then subjected to Tris-Tricine 16.5% polyacrylamide gels. The same gel analysis conditions were used for CHO conditioned medium concentrates.

Proteins were transferred onto nitrocellulose and incubated overnight with the 2H3 or 6E10 monoclonal antibody at a 1:1000 dilution. Immunological complexes were detected with a goat anti-mouse peroxidase-conjugated antibody (1:2000 dilution). Chemiluminescence was recorded using a LAS-3000 Luminescence Image Analyzer (Raytest, Courbevoie, France), and quantifications were performed using the AIDA analyzer software.

Antibodies and Pharmacological Agents—The following antibodies were used to detect corresponding proteins: SAF32 antibody directed against PrP residues 79–92 (generously provided by J. Grassi, Commissariat à l’Energie Atomique/Saclay, Gif sur Yvette, France), 6E10 anti-APP (provided by Dr. M. Goedert, Cambridge, UK), 2H3 antibody raised against the 1–12 sequence of Aβ (provided by Dr. D. Schenk, San Francisco, CA), 22C11 anti-APP antibody (Roche Applied Science), anti-N-terminal fragment PS1 and anti-loop PS2 antibodies (kindly provided by Dr. Thinakaran (34)), anti-nicastrin (Sigma), anti-APH1aL (O2C2) and anti-PEN-2 (PNT2, rabbit polyclonal; Calbiochem; 1:1000); anti-BACE1 (kindly provided by Dr. R. Vassar); and anti-actin and anti-tubulin (Sigma). Corresponding anti-mouse and anti-rabbit secondary antibodies were purchased from Beckman Coulter, Inc. Staurosporine was purchased from Sigma.

Western Blot Analysis—Recombinant N1 and N2 (16.5% gel), Aph1 (12%), and Pen2 (14%) were separated on Tris-Tricine acrylamide gels. bAPP, BACE1, nicastrin (8%), and PS2 and PS1 (12%) were separated on Tris-glycine acrylamide gels. Proteins were transferred onto nitrocellulose and incubated overnight with the appropriate antibody and correspondent peroxidase-conjugated secondary antibody. Chemiluminescence was recorded using a LAS-3000 Luminescence Image Analyzer (Raytest), and quantifications were performed using the AIDA analyzer software.

BACE1 and γ-Secretase Assays—For BACE1, cells were lysed with 10 ml Tris-HCl, pH 7.5, and then homogenates were monitored for their BACE1 activity by means of a fluorometric assay described previously (35, 36).

The in vitro γ-secretase assay was performed according to Seville et al. (31). Briefly, “solubilized membranes” were obtained from HEK293 cells resuspended in solubilization buffer and diluted to yield a 1 mg/ml final protein concentration; then diluted with an equal volume of sodium citrate buffer, 1 mg/ml egg phosphatidylcholine, and 50 μg/ml recombinant C100-FLAG; and assayed as described.

Cell Viability and Toxicity Assays—Cells viability was assayed in 96-well plates by a 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide-based colorimetric assay as described previously (38).

Membrane integrity was evaluated by measuring the lactate dehydrogenase activity in the culture medium using a Cyto-
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Tox-ONE™ kit (Promega Corp., Madison, WI). Cells were grown in 96-well plates and allowed to reach 80% confluence. Assays were performed according to the manufacturer’s recommendations for 40 μl of medium transferred in 96-well dark plates, and fluorescence was recorded with excitation and emission wavelengths of 560 and 590 nm, respectively.

TUNEL Analysis—Cells were fixed for 30 min with 4% paraformaldehyde, rinsed in phosphate-buffered saline, permeabilized for 10 min in 0.01% Triton X-100, and then processed for the dUTP nick-end labeling TUNEL technique according to the manufacturer’s recommendations (Roche Applied Science). Fragmented DNA labeling corresponds to green spots. A second labeling with DAPI (1:20,000 in PBS) was carried out to visualize the total number of nuclei.

Caspase-3-like Activity Measurements—Cells were grown in 6-well plates and incubated with STS (2 μM for 16 h for HEK293 cells and primary cortical neurons, 1 μM for 2 h for fibroblasts, and 1 μM for 16 h for CHO cells) after they reached confluence. Samples were processed for caspase-3-like activity assay as described previously (37). Caspase-3-like activity is calculated from the linear part of fluorimetry recorded and expressed in units/h/mg of proteins (established by the Bio-Rad procedure).

Samples were processed for caspase-3-like activity assay as described previously (37). Caspase-3-like activity is calculated from the linear part of fluorimetry recorded and expressed in units/h/mg of proteins (established by the Bio-Rad procedure). One unit corresponds to 4 nmol of 7-amino-4-methylcoumarin released.

p53 Transcriptional Activity and Promoter Transactivation—Reporter constructs PG13-luciferase and p53 promoter-luciferase (pp53) (provided by Dr. B. Vogelstein, Baltimore, MD and by Dr. M. Oren, Rehovot, Israel, respectively) were used to measure p53 transcriptional activity and p53 promoter transactivation as has been extensively described elsewhere (38–40).

Statistical Analysis—Statistical analysis was performed with PRISM software (GraphPad Software, San Diego, CA) using the Newman-Keuls multiple comparison tests for one-way analysis of variance and t test.

RESULTS

HEK293 Cells Expressing Swedish Mutated βAPP Exhibit Enhanced STS-stimulated Caspase-3 Activation That Is Down-regulated by Recombinant sAPPα and N1—Both βAPP and cellular prion undergo α-secretase cleavage, generating sAPPα and N1, respectively (3, 13). We have compared the potential of recombinant human sAPPα (29) and N1 (18), respectively, as a protector against STS-induced caspase-3 activation in HEK293 cells. In agreement with our previous study, STS-evoked caspase-3 activation in HEK293 cells was significantly reduced by recombinant N1 (−45.1 ± 4.5% of control STS-treated cells, p < 0.05, n = 4). The extent of inhibition was comparable with that displayed by recombinant sAPPα (−37.1 ± 3.4%, p < 0.05, n = 4), whereas the N2 fragment remained biologically inert in this paradigm (supplemental Fig. 1A). Interestingly, recombinant sAPPα lowered p53 activity (−40.3 ± 3% of control cells, p < 0.05, n = 4; supplemental Fig. 1B) and p53 promoter transactivation (−36.6 ± 5%, p < 0.05, n = 4; supplemental Fig. 1C) as did N1 (−35 ± 3.9%, p < 0.05, n = 4 and −22.05 ± 3.75%, p < 0.05, n = 4, respectively), whereas here again, N2 remained inactive. Altogether, these results indicate that sAPPα and N1 harbor similar abilities to protect human cells against STS-evoked cell death likely by down-regulating p53 promoter transactivation and activity.

We examined whether N1 and sAPPα could protect cells against Aβ-associated cell death. In this context, we used HEK293 cell lines overexpressing either APPWT or APPswe (Fig. 1A). As expected, the latter cell system displayed exacerbated production of Aβ peptides (Fig. 1A). Fig. 1B shows that mock-transfected HEK293 cells displayed enhanced caspase-3 activity in response to STS treatment (24.0 ± 2.5 × 10³ versus 60.6 ± 5.1 × 10³ arbitrary units (a.u.), Δ = 36.6 ± 5.0, n = 11 in control and STS-stimulated conditions, respectively). As has been reported previously (41), APPWT expression reduced STS-stimulated caspase-3 activation (26.5 ± 2.3 × 10³ versus 41 ± 7.4 × 10³ a.u., Δ = 14.5 ± 7.1, n = 11), whereas APPswe transfectants exhibited enhanced STS responsiveness (34.4 ± 6.5 × 10³ versus 86.6 ± 9.4 × 10³ a.u., Δ = 52.2 ± 11.2, n = 11).
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Interestingly, recombinant sAPPα and N1, but not N2, drastically reduced STS-stimulated caspase-3 activation in both APPWT- (−46 ± 4.9%, p < 0.01 and −52.2 ± 5.1%, n = 3, p < 0.05, respectively; Fig. 1C) and APPswe (−41.8 ± 3.4%, n = 3, p < 0.05 and −51.53 ± 4.87%, n = 3, p < 0.05; Fig. 1D)-expressing HEK293 cells. This lowering of STS-induced caspase-3 activation was corroborated by the reduced number of TUNEL-positive apoptotic cells observed upon recombinant N1 treatment (Fig. 1E) in both mock-transfected (38 ± 13.1% reduction versus untreated cells, n = 9, p < 0.05) and APPswe-expressing (58.57 ± 9.64% reduction, n = 20, p < 0.01) cell lines.

To confirm our data in another cell system, we used a stably transfected HEK293 cell line that overexpresses N141I-mutated presenilin-2 (PS2N141I; see Fig. 2A). This cell system was chosen first because it leads to increased production of Aβ (mainly Aβ42 (42)) and second because we previously established that this mutation triggers a drastic exacerbation of both STS-stimulated caspase-3 activation and p53-dependent cell death (38). Fig. 2B confirms an enhanced basal and STS-stimulated caspase-3 activity in N141I-PS2-expressing HEK293 cells (48.2 ± 7.6% increase when compared with mock-transfected cells, n = 4, p < 0.05) and further documents a protective effect of N1, but not N2, that reduces STS-induced caspase-3 activity (−30.5 ± 3.3%, n = 4, p < 0.05; Fig. 2C). This set of data indicates that N1 could protect against cell death triggered by various familial AD mutations responsible for enhanced formations of either total Aβ (βAPP Swedish mutation) or more selectively Aβ42 (N141I-PS2 mutation).

We previously suggested that the N1 effect could be mediated through its binding to a yet unknown receptor (18). It has been shown that PrP0 interacts with low density lipoprotein receptor-related protein 1 (LRP1) by an N-terminal domain that overlaps with the N1 sequence (43, 44). Therefore, we examined the contribution of LRP1 in the N1-mediated effect. In line with previous data showing that LRP1 promoted an anti-apoptotic phenotype (45), we confirmed in CHO cells that LRP1 depletion increased STS-induced caspase-3 activation (817.3 ± 47.3 versus 1034 ± 32 a.u., Δ = 217.0 ± 58.9, n = 7 for CHO-LRP1+/+ versus CHO-LRP1−/−, respectively; see supplemental Fig. 2). Interestingly, the N1-associated reduction of STS-induced caspase-3 activation (817.3 ± 47.3 versus 628.5 ± 14.1 a.u., n = 7, for control (Ct) versus N1-treated LRP1+/+ cells) was abolished in LRP1−/− cells (supplemental Fig. 2), suggesting that LRP1 could mediate the N1-associated protective phenotype.

We also examined the protective phenotype of N1 in fibroblasts devoid of βAPP and its family members APLP1 and APLP2 (triple KO). This cell system was used to abolish a putative protective effect of endogenous sAPPα (absent in cells lacking βAPP) competing with that of N1. Furthermore, this cell system allows the examination of a putative cell-specific dependent phenotype associated with N1, N1, but not N2, similarly protected wild-type and triple KO fibroblasts from STS-induced caspase-3 activation (supplemental Fig. 3).

HEK293 Cells Expressing Swedish Mutated βAPP Exhibit Enhanced p53-dependent Pathway That Is Down-regulated by Recombinant N1—We found that unlike the case for wild-type βAPP the expression of APPswe in HEK293 cells drastically potentiated p53 activity (36.5 ± 8.71 versus 136.8 ± 40.5 a.u. (n = 10) in mock-transfected and APPswe-expressing cells, respectively; Fig. 3A) and promoter transactivation (32.6 ± 9.5 versus 185 ± 3 a.u. (n = 10); Fig. 3D). Importantly, both p53 transcriptional activity (Fig. 3, B and E) and promoter transactivation (Fig. 3, C and F) were lowered by N1 treatment in APPWT-expressing cells (−30.7 ± 18.3%, n = 6, p > 0.05 and −11.5 ± 4.9%, n = 6, p < 0.05 reduction for p53 activity (Fig. 3B) and promoter transactivation (Fig. 3E), respectively) as well as in APPswe-expressing cells (−31.2 ± 19.2, n = 6, p > 0.05 and −18.7 ± 8.6, n = 6, p < 0.05 for p53 activity (Fig. 3C) and p53 promoter transactivation (Fig. 3F), respectively). Thus, the above data overall suggest that N1 likely protects HEK293 cells expressing familial AD-linked mutations from STS-induced
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caspase-3 activation by reducing p53 activity and promoter transactivation.

N1 Modifies Neither β- and γ-Secretase Expression nor Activities in Human Cells—The exacerbation of both caspase-3 activation and p53 pathway in HEK293 cells engineered to overexpress a familial mutation in βAPP or PS2 strongly suggest that cell death is triggered by overload of Aβ. Therefore, the N1 protective effect could indeed account for a late effect on Aβ-associated toxicity but also theoretically for an early upstream interference with Aβ production. In this context, we

FIGURE 3. N1 lowers p53 activity and promoter transactivation in APPswe-expressing HEK293 cells. PG13-luciferase (A–C) or p53 promoter-luciferase (D–F) reporter constructs were transiently transfected in the indicated cell lines together with β-galactosidase-expressing vector as detailed under “Materials and Methods.” Twenty-four hours after transfection, APPWT (B and E) and APPswe HEK293 cells (C and F) were treated with recombinant N1 (1 μM for 16 h) or control buffer. The luciferase and β-galactosidase activities were measured as described under “Materials and Methods.” Values corresponding to the ratios of luciferase/β-galactosidase activities represent the means of four independent experiments (performed in triplicates) and are expressed as percentage of control (control buffer-treated cells (Ct) were taken as 100). Error bars = S.E. *, p < 0.05; **, p < 0.01.

FIGURE 4. N1 does not affect Aβ secretion, β- and γ-secretase expression, and in vitro activity. A–C, stably mock-transfected or APPWT- and APPswe-expressing HEK293 cells were treated for 4 h with recombinant N1 (1 μM) or an equivalent volume of control buffer. At the end of the incubation, the treatment was repeated, and then cells were allowed to secrete for 16 h. A, βAPP expression (upper panel) and Aβ secretion (lower panel) were monitored by Western blot with 22C11 antibody and by immunoprecipitation with 6E10 antibody, respectively, as described under “Materials and Methods.” Expressions of the members of γ-secretase complex (B), BACE1 (C), and tubulin (B and C) were detected in the indicated cell line as described under “Materials and Methods.” D, an in vitro γ-secretase activity assay was performed by means of recombinant C100 as described under “Materials and Methods.” Solubilized membrane fractions were prepared from the indicated cell line 16 h after treatment with 1 μM recombinant N1 or an equivalent volume of control buffer. C100 and Aβ expressions were monitored as described under “Materials and Methods.” E, endogenous BACE1 activity was fluorometrically monitored as described under “Materials and Methods.” Values correspond to BACE1 activity per microgram of protein (prot) per minute and are the means of four independent determinations. Error bars = S.E. ***, p < 0.001; ns, not statistically significant. NCT, nicastrin; Ct, control; fl, full-length.
examined the putative effect of N1 on the expression of secretases in both APPWT- and APPswe-expressing cells. Fig. 4A first shows that N1 treatment did not influence the expression of APPWT and APPswe or the recovery of Aβ in culture media (compare Ct and N1 lanes). Furthermore, N1 did not modify the endogenous expressions of PS1 or PS2, Aph1, Pen2, and nicastrin (Fig. 4B), the four members of the biologically active γ-secretase complex (46). Finally, N1 did not affect the endogenous expression of the β-secretase BACE1 (Fig. 4C) (7). This set of data was corroborated by the demonstration that γ-secretase activity in reconstituted membranes (47) prepared from APPWT- and APPswe-expressing HEK293 cells and BACE1 activity were not altered by N1 (Fig. 4D, respectively). Thus, this set of data indicates that N1 does not interfere with the proteolytic machineries responsible for the production of Aβ in APPWT and APPswe cells and therefore that the N1 protective effect is not due to its propensity to reduce the load of Aβ production.

**N1 Protects Primary Cultured Mouse Cortical Neurons from Apoptosis Induced by Aβ Oligomer-enriched Conditioned Medium Prepared from APP<sub>LDN</sub> CHO Cells**—Several lines of evidence recently suggested that at least part of the AD-related neurodegenerative process could be due to Aβ multimers of high molecular weights (48–50). More recently, it was suggested that Aβ dimers and trimers could account for most of the Aβ-related toxicity (51). To examine whether N1 could protect against Aβ oligomer-related toxicity, we developed CHO cell lines stably expressing either wild-type APP<sub>695</sub> or its homolog APP<sub>LDN</sub> harboring the London mutation (28) that is known to yield and secrete high levels of Aβ oligomers (27). Fig. 5A shows that cell extracts and secretion medium from APP<sub>LDN</sub>-expressing CHO cell lines were analyzed for APP<sub>695</sub> expression and monomeric Aβ species, respectively. βAPP and tubulin expressions were monitored by Western blot with 22C11 antibody, and Aβ40 and Aβ42 were immunoprecipitated using FCA3340 or FCA3542 antibody, respectively, as described under “Materials and Methods.” B, 20 μl of conditioned medium were loaded on a Tris-Tricine 16.5% acrylamide gel, then transferred onto nitrocellulose membrane, and blotted with 6E10 antibody as described under “Materials and Methods.” C, primary cultured neurons from mouse cortices were prepared and maintained in culture as described under “Materials and Methods” and exposed to Aβ oligomer-enriched conditioned medium for the indicated time periods, and then cellular toxicity was assessed using an lactate dehydrogenase assay as described under “Materials and Methods.” Bars are the means of four independent determinations. Error bars = S.E. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
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**FIGURE 6.** N1 protects primary cultured mouse cortical neurons from Aβ oligomer-induced toxicity and cell death. A, primary cultured mouse cortical neurons (seeded on 96-well plates) were treated (24 h) with recombinant N1, N2 (1 μM), or an equivalent volume of control buffer (Ct) with Aβ oligomer-enriched conditioned media (4-fold dilution). Twenty-four hours after incubation, cells were treated as above for an additional 24-h period, and then neuronal viability was determined by 2,3-bis[2-(methoxy-4-nitro-5-sulophenyl)]-2H-tetrazolium-5-carboxanilide assay as described under “Materials and Methods.” Values are the means of three independent experiments (carried out in triplicates) and are expressed as percentage of control untreated cells (taken as 100). Error bars = S.E. B, representative pictures of mouse primary cultured neurons seeded on glass coverslips treated as in A and then processed for TUNEL as describe under “Materials and Methods.” C, TUNEL-positive nuclei were counted in six independent optical fields. Bars correspond to the percentage of labeled nuclei of total DAPI-stained nuclei. *, p < 0.05; **, p < 0.01; ns, not statistically significant.

and 45.8 ± 2.1 versus 64.4 ± 4.8%, n = 4, p < 0.05 after 72 h; Fig. 5C) and elicited a dose-dependent effect on triple KO fibroblasts (for dilution 10 versus dilution 4: 42.1 ± 5.3 versus 65.1 ± 6.9% cell death, n = 4, p < 0.05; for dilution 4 versus dilution 2: 65.1 ± 6.9 versus 91.8 ± 6.8% cell death, n = 4, p < 0.05; supplemental Fig. 4).

We subsequently examined the potential of N1 to protect primary cultured mouse cortical neurons from Aβ oligomer-associated toxicity. Fig. 6 shows that Aβ oligomer-enriched conditioned medium from APP<sub>LDN</sub> but not APP<sub>WT</sub>, cells reduced neuronal viability (Fig. 6A) and increased the number of TUNEL-positive apoptotic neurons by about 2-fold (Fig. 6, B and C). Interestingly, N1, but not N2, abolished Aβ oligomer-associated toxicity (Fig. 6A) and cell death (14.7 ± 1.8% (n = 6) versus 6.7 ± 2.1 (n = 6) of apoptotic nuclei per optical field in N1-treated and control neurons, respectively; Fig. 6, B and C). Therefore, our data demonstrate for the first time that N1 protects neurons from Aβ oligomer-induced cell death.

**N1 Protects Primary Cultured Mouse Cortical Neurons from Apoptosis Induced by Brain Extracts from Patient with AD**—

We prepared extracts from cortical tissues of AD-affected patient diagnosed with Braak stage VI. We characterized the Aβ species that were immunoprecipitable from extracts of control and AD samples. The AD sample was characterized by an increase in both Aβ40 and Aβ42 accounting for an overall augmentation of Aβ as well as increased high molecular weight oligomers (Fig. 7A). AD extracts increased the number of TUNEL-positive primary cultured neurons (Fig. 7B) by about 50% (28.70 ± 1.60% (n = 6) versus 20.8 ± 1.3% (n = 6) of apoptotic nuclei in AD-treated and control cells, respectively). Interestingly, apoptosis associated with AD extract was abolished by N1 treatment (28.70 ± 1.6%, n = 6 versus 20.75 ± 1.7%, n = 8) but not by N2 (27.4 ± 1.9%, n = 8). This study demonstrates that N1 can protect primary cultured neurons from Aβ-induced apoptosis triggered by pathogenic brain extracts.

**DISCUSSION**

We show here that the α-secretase-derived fragments of βAPP and PrP<sup>C</sup>, namely sAPPα and N1, respectively, similarly protect human cells from STS-induced caspase-3 activation by interfering with the p53-dependent pathway. Furthermore, we establish that N1 protects cells from toxicity associated with the overload of Aβ derived from the overexpression of Swedish mutated βAPP and N1411-PS2, two proteins involved in familial cases of Alzheimer disease. We show that these mutations exacerbate STS-induced caspase-3 activation and p53, the extent of which is lowered by both recombinant N1 and sAPPα. Interestingly, several studies have demonstrated that intracellular Aβ42 can trigger toxicity via p53 and Bax in primary cultured human neurons (52) likely by directly activating the transactivation of p53 promoter (19). Later, the tight link between Aβ-associated toxicity and the p53 pathway was reinforced by the identification of the late effectors of Aβ toxicity that corresponded to the p53 downstream targets RNA-dependent-protein kinase and mammalian target of rapamycin (53). Furthermore, similar to the present study, it was reported that wild-type βAPP confers resistance to p53-induced apoptosis and that this phenotype was abolished by familial AD mutated βAPP (41). Finally, mutated PS1 and PS2 trigger p53-dependent apoptosis (26, 38, 54) that could be prevented by γ-secretase inhibitors (54). Combined with our present results, we propose that N1 protects cells expressing familial AD-linked APP or PS2 by down-regulating Aβ-associated p53-dependent cell death.

The similar protective effect of sAPPα and N1 fragments does not per se prove that they lower Aβ-related toxicity via identical cellular pathways. A line of data suggests that it could be indeed the case. Thus, both sAPPα and N1 signaling could involve the phosphatidylinositol 3-kinase/Akt cellular pathway (18, 55, 56). However, close examination of the literature and our present data could counter such a hypothesis. It was reported that sAPPα interacts with the LRP1 and that this interaction triggers sAPPα endocytosis and subsequent degradation (57). Therefore, sAPP-LRP1 interaction ultimately leads to the inactivation of the sAPPα-associated phenotype. At first sight, this concurs with the proposal of LRP1 as a pathogenic trigger in AD. However, in the case of N1, it appears that LRP1 deficiency abolishes the N1-mediated protective function and therefore that LRP1 could overall trigger a protective function. This agrees fairly well with another study showing that LRP1...
triggers a neuronal antiapoptotic phenotype by promoting the Akt survival pathway as N1 does (18, 45). However, it is unlikely that N1 signaling needs LRP1-mediated endocytosis because we previously showed that the N1 protective function did not require internalization/endocytosis (18). This set of data indicates that although sAPPα and N1 could display similar phenotypes and both protect against p53-dependent cell death, the two fragments could elicit their effects via distinct cellular pathways.

Recent advances led to the proposal that the Aβ-associated toxicity may due to soluble Aβ oligomers instead of Aβ monomers that could display a protective function (47). Thus, Shankar et al. (38) showed that oligomers prepared from human brain pathogenic extracts indeed alter long term potentiation and therefore affect cognitive functions linked to memory and learning. This prompted us to assess whether N1 also protected against Aβ oligomer-associated toxicity from two sources, i.e. conditioned medium from cells engineered to produce oligomers and pathogenic human brain extracts. Our data show that in both cases N1 protects primary cultured neurons against Aβ oligomer-associated cell death.

How N1 can protect against Aβ oligomer toxicity can be deduced from recent works showing that PrPc could be necessary for Aβ oligomer-associated deleterious effects. Thus, it has been suggested that PrPc could act as a potential receptor for Aβ oligomers (59, 60). The functional consequence of the physical interaction between Aβ oligomers and PrPc is still under extensive debate (61–64), but the validity of the protein-protein interaction is less controversial. Two PrPc regions bind to soluble Aβ42 oligomers: the 95–110 sequence and the 23KKRPK27 N-terminal basic residues of PrPc (59, 65). Indeed, a partial loss of Aβ binding was observed for Δ101–110 deleted huPrP fragment, whereas removal of the 23–90 segment resulted in a complete loss of interaction between PrPc and Aβ42 oligomers (65). Strikingly, both domains implicated in PrPc-Aβ oligomer binding are included in the N1 sequence. Interestingly, we previously demonstrated that the mutation of the N-terminal KKRPK sequence or its deletion led to the loss of the neuroprotective function of N1 (18). Whether N1 could directly bind Aβ peptide oligomers remains to be established, but one can envision that the activation of α-secretase cleavage and subsequent production of N1 may be a potential way to deplete extracellular media of Aβ toxic oligomeric species. In this context, interestingly, a recent study reports that Aβ oligomers recruit PrPc to the neuronal cell surface (66). These results could be seen as an attempt for neurons to enhance N1 availability to protect themselves from Aβ toxicity.

The various possible cellular mechanisms underlying the N1 protective function toward Aβ or Aβ oligomer-associated neurotoxicity, i.e. 1) the activation of the intracellular cell survival pathway by modulating the Akt/p53 pathway, 2) the depletion of toxic Aβ species by direct binding to N1, 3) the obstruction by direct competition of the N-terminal PrPc-dependent binding site necessary for Aβ-mediated cell death, and 4) a combination of the three previous possibilities, reinforce the view that a strong and intimate functional dialogue between PrPc and β-amyloid precursor protein and their catabolites exists. If one considers that in AD the α-secretase levels are slightly affected, then this means that the potential of formation of N1 remains and that the formation of this PrPc catabolite could, at least at the early stages of the disease, contribute to the cellular mechanisms aimed at compensating for sAPPα deficiency. Thus, our
data reinforce the interest in a strategy aimed at stimulating the α-secretase pathway that has been envisioned as a therapeutic track for Alzheimer disease (67–69). Furthermore, this could open new therapeutic avenues for the design of drug candidates that could potentially prevent Aβ-associated toxicity to delay the early symptoms of Alzheimer disease.

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REFERENCES


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