

## Ceramide Stabilizes $\beta$ -Site Amyloid Precursor Protein-cleaving Enzyme 1 and Promotes Amyloid $\beta$ -Peptide Biogenesis\*

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**The lipid second messenger ceramide regulates several biochemical events that occur during aging. In addition, its level is highly elevated in the amyloid-burdened brains of Alzheimer's disease patients. Here, we analyzed the impact of aberrant ceramide levels on amyloid  $\beta$ -peptide ( $A\beta$ ) generation by using a cell-permeable analog of ceramide, C6-ceramide, and several biochemical inhibitors of the sphingomyelin/glycosphingolipid biosynthetic pathway. We found that C6-ceramide increased the biogenesis of  $A\beta$  by affecting  $\beta$ - but not  $\gamma$ -cleavage of the amyloid precursor protein. Similarly to C6-ceramide, increased levels of endogenous ceramide induced by neutral sphingomyelinase treatment also promoted the biogenesis of  $A\beta$ . Conversely, fumonisin B1, which inhibits the biosynthesis of endogenous ceramide, reduced  $A\beta$  production. Exogenous C6-ceramide restored both intracellular ceramide levels and  $A\beta$  generation in fumonisin B1-treated cells. These events were specific for amyloid precursor protein and were not associated with apoptotic cell death. Pulse-chase and time-course degradation experiments showed that ceramide post-translationally stabilizes the  $\beta$ -secretase BACE1. Taken together, these data indicate that the lipid second messenger ceramide, which is elevated in the brains of Alzheimer's disease patients, increases the half-life of BACE1 and thereby promotes  $A\beta$  biogenesis.**

Alzheimer's disease (AD)<sup>1</sup> affects ~15 million individuals worldwide. The prevalence of the disease doubles every 5 years

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<sup>1</sup> The abbreviations used are: AD, Alzheimer's disease; APP, amyloid precursor protein;  $\beta$ -APP-CTF,  $\beta$ -APP-C terminal fragment;  $\alpha$ -APP-CTF,  $\alpha$ -APP-C terminal fragment;  $A\beta$ , amyloid  $\beta$ -peptide; BACE,  $\beta$ -site APP-cleaving enzyme; SM, sphingomyelin; C6-cer, C6-ceramide; FB1, fumonisin B1; nSMase, neutral sphingomyelinase; NB-DGJ, N-butyldeoxygalactonojirimycin; PARP, poly(ADP-ribose) polymerase; f. l.,

after age 65 and approaches 50% by age 85. Because of the ongoing increase in life expectancy, the number of people affected by this disease is rapidly increasing. The major risk factor for late-onset AD is aging (1). The molecular events that mediate the effect of aging on AD are the subjects of intensive study.

The main pathogenic event that occurs in all forms of AD is the abnormal accumulation of amyloid  $\beta$ -peptide ( $A\beta$ ) into senile (or amyloid) plaques (2).  $A\beta$  is a 39–43-amino acid peptide proteolytically derived from the amyloid precursor protein (APP). APP is first cleaved by  $\beta$ -site APP-cleaving enzyme 1 (BACE1) at the N terminus of  $A\beta$  ( $\beta$ -cleavage), producing a C-terminal fragment ( $\beta$ -APP-CTF) of ~12 kDa, and subsequently in the transmembrane domain ( $\gamma$ -cleavage) by a presenilin-harboring protease complex. The two major sites of  $\gamma$ -cleavage are located at positions 40 and 42 of  $A\beta$ , generating  $A\beta_{40}$  and  $A\beta_{42}$ , respectively.

The membrane lipid ceramide is the backbone of all complex sphingolipids and acts as a second messenger in many biological events. In addition, it regulates several biochemical and genetic events that occur during aging/senescence, including inhibition of phospholipase D and c-Fos-dependent signaling pathways, retinoblastoma protein dephosphorylation, arrest of the serum/growth factor-mediated activation of protein kinase C, and arrest of DNA synthesis (3, 4). Endogenous ceramide can be generated by either *de novo* synthesis or hydrolysis of sphingomyelin (SM) at the cell surface, the latter being the most important source of the active pool of ceramide (5, 6). The intracellular levels of ceramide increase progressively during aging in both cultured cells and the whole organ (7–10). In addition, brains from AD patients contain approximately three times more ceramide when compared with age-matched controls (11).

In this study, we investigated the role of the second messenger ceramide in the regulation of  $A\beta$  generation through several biochemical approaches. We found that  $A\beta$  biogenesis is strictly regulated by the endogenous pool of ceramides, implicating the sphingomyelin, but not the glycosphingolipid, biosynthetic pathway in  $A\beta$  generation. We also found that the ceramide-dependent regulation of  $A\beta$  biogenesis is achieved via control of BACE1 steady-state levels, is specific for APP, and is not associated with cell death.

### EXPERIMENTAL PROCEDURES

**Cell Culture and Treatments**—Chinese hamster ovary (CHO) and H4 (human neuroglioma) cell lines were grown as described previously (12). Cells were grown in either six-well plates or 100-mm tissue culture dishes (BD Biosciences). nSMase, FB1, NB-DGJ, and C6-ceramide (C6-cer) were obtained from either Sigma or Calbiochem. Pharmacological

full-length; m-, mature; im-, immature; CHO, Chinese hamster ovary; MES, 4-morpholineethanesulfonic acid; ELISA, enzyme-linked immunosorbent assay; ZVAD, benzyloxycarbonyl-Val-Ala-Asp.

treatment was for three (nSMase), four (FB1), or two (C6-cer and NB-DGJ) days.

**Lipid Labeling and Extraction**—Labeling of glycosphingolipids was performed using [9,10-<sup>3</sup>H]palmitic acid (60 Ci/mmol) (PerkinElmer Life Sciences). Cells were incubated in the presence of [<sup>3</sup>H]palmitic acid for at least 3 days (*ad equilibrium*) to allow steady-state labeling of palmitic-containing lipids. At the end of each treatment (see above), cells were washed twice in phosphate-buffered saline, scraped, and extracted in chloroform:methanol (2:1, v/v). After Folch extraction, the lipid phase was dried, resuspended in chloroform, and applied together with standards to a Silica Gel-G (EM Science, Gibbstown, NJ) thin layer chromatography (TLC) plate. Plates were developed in either chloroform, methanol, acetic acid, formic acid, water (70:30:12:4:2, v/v/v/v/v) (13) or chloroform, methanol, 9.8 mM CaCl<sub>2</sub> (60:35:8, v/v/v) (14). Spots were scraped and counted in a liquid scintillation counter.

**Antibodies and Western Blot Analysis**—Polyclonal antibodies C7 and C8 against the C terminus of APP were a generous gift from Dr. Dennis J. Selkoe (Harvard Medical School, Boston, MA). The polyclonal antibodies against caveolin-1 came from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and those against BACE1 were from Abcam (Cambridge, UK). Monoclonal antibodies against poly(ADP-ribose) polymerase (PARP) (C-2-10) were obtained from Clontech Laboratories, Inc. (Palo Alto, CA), and those against tumor necrosis factor- $\alpha$  (M32255a) were from Fitzgerald Industries International, Inc. (Concord, MA). The hybridoma antibody against Notch developed by S. Artavanis-Tsakonas was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD, National Institutes of Health and maintained by the University of Iowa, Department of Biological Sciences (Iowa City, IA).

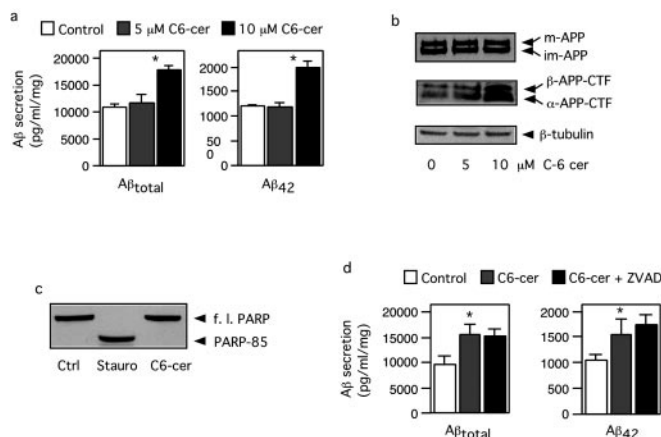
For Western blot analysis, total proteins (50–100  $\mu$ g/lane) were separated by NuPage 4–12% BisTris-polyacrylamide gel electrophoresis (Invitrogen) using MES running buffer (Invitrogen) and then blotted on Immun-Blot™ polyvinylidene difluoride membranes (Bio-Rad). Proteins were visualized using the LumiGLO™ protein detection kit (KPL, Gaithersburg, MD) as described by the manufacturer.

**A $\beta$  Concentration Determinations**—For A $\beta$  determination, APP<sub>751</sub> stably transfected CHO cell lines were grown in 6-well plates (BD Biosciences). When ~80–90% confluent, cells were washed in phosphate-buffered saline and incubated in 1 ml of fresh medium for 24 h (12). Secreted A $\beta_{total}$  and A $\beta_{42}$  were quantitated by standard sandwich ELISA (A $\beta$  ELISA Core Facility, Center for Neurological Diseases, Harvard Institutes of Medicine, Harvard Medical School).

## RESULTS

**C6-ceramide Promotes A $\beta$  Generation**—The role of ceramide in APP processing and A $\beta$  generation was initially analyzed using C6-ceramide, a cell-permeable and active analog of ceramide. CHO cells, stably transfected with APP<sub>751</sub>, were treated with 10  $\mu$ M C6-ceramide for 2 days. C6-ceramide increased the secretion of both A $\beta_{total}$  and A $\beta_{42}$  by ~60% (Fig. 1*a*). This increase was accompanied by elevated steady-state levels of both  $\alpha$ - and  $\beta$ -APP-CTFs (Fig. 1*b*), produced by  $\alpha$ - and  $\beta$ -cleavage of APP, respectively. In addition, C6-ceramide increased the release of the secreted form of APP into the conditioned media (data not shown). C6-ceramide treatment did not affect CHO cell viability, as assessed by trypan blue uptake and by lactate dehydrogenase release into the media (Table I). It also did not activate the apoptotic cascade, as indicated by the absence of the 85-kDa apoptosis-related isoform of PARP (Fig. 1*c*). PARP-85 is an early indicator of apoptosis; it is produced by CPP32/Mch2 $\alpha$ -mediated cleavage of the 116-kDa native form of PARP (*f. l. PARP*), which follows activation of the apoptotic cascade (Fig. 1*c*). Finally, to assess whether the C6-ceramide-dependent increase in A $\beta$  secretion was due to caspase activation, we used ZVAD, a downstream inhibitor of caspase activity, in conjunction with C6-ceramide treatment. The increase in A $\beta$  generation induced by C6-ceramide was not reversed by ZVAD (Fig. 1*d*), indicating that C6-ceramide up-regulates A $\beta$  generation in CHO cells in the absence of apoptosis.

To confirm the above results in a different cell type and to assess whether the changes in the rate of A $\beta$  generation were determined by changes in only  $\beta$ - or in both  $\beta$ - and  $\gamma$ -cleavage of APP, we used H4 (human neuroglioma) cells stably trans-



**FIG. 1. C6-ceramide, a cell-permeable analog of ceramide, increases A $\beta$  generation.** CHO cells stably transfected with APP<sub>751</sub> were treated with increasing concentrations of C6-cer, a cell-permeable and metabolically active analog of ceramide, for 2 days. *a*, A $\beta$  secretion in the conditioned media was analyzed by sandwich ELISA. C6-cer at 10  $\mu$ M concentration increased the secretion of both A $\beta_{total}$  and A $\beta_{42}$  by ~60 to 70%. *b*, Western blot showing that C6-cer (10  $\mu$ M) increased the steady-state levels of both  $\beta$ - and  $\alpha$ -APP-CTFs without any apparent effect on APP expression or maturation. *c*, C6-ceramide (10  $\mu$ M) treatment did not induce CPP32/Mch2 $\alpha$ -mediated cleavage of the 116-kDa native form of PARP (*f. l. PARP*), which follows activation of the apoptotic cascade. Cleavage and generation of the 85-kDa apoptosis-related isoform of PARP was instead induced by staurosporine (*Stauro*)-mediated activation of the apoptotic cascade. *Ctrl*, control. *d*, inhibition of the apoptotic cascade with ZVAD (100  $\mu$ M), a specific downstream inhibitor of caspase activity, did not reverse the C6-cer (10  $\mu$ M) effect on A $\beta$  generation. Results are expressed as the means  $\pm$  S.D. of at least three different determinations. Asterisks (\*) indicate a significant difference from control at  $p < 0.05$ . One representative immunoblot (of at least three) is shown.

TABLE I

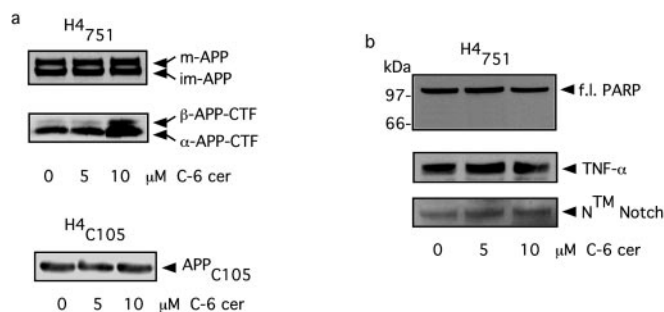
C6-ceramide did not affect the release of the cytosolic enzyme lactate dehydrogenase into the conditioned media

Stable transfected CHO cell lines were treated with 10  $\mu$ M C6-ceramide up to 6 days. Lactate dehydrogenase (LDH) release in the conditioned media was analyzed using the enzymatic assay from Sigma, as described by the manufacturer.

	Days		
	0	2	6
LDH (units/liter)	29.3 $\pm$ 1.1	31.3 $\pm$ 2.0	29.1 $\pm$ 2.6

fecting with either full-length APP<sub>751</sub> or the C-terminal 105 amino acids of APP (APP<sub>C-105</sub>), which mimic  $\beta$ -APP-CTF (12). C6-ceramide increased the steady-state levels of both  $\alpha$ - and  $\beta$ -APP-CTFs in H4 cells expressing full-length APP<sub>751</sub> without any evident effect on APP expression or maturation (Fig. 2*a*). Significantly, no effect was observed on the steady-state levels of APP<sub>C105</sub> (Fig. 2*a*), indicating that the changes in A $\beta$  secretion were due to changes in  $\beta$ - but not  $\gamma$ -cleavage of APP. Again, we did not detect any effect on cell viability (data not shown) or caspase-mediated cleavage of full-length PARP (Fig. 2*b*). Finally, C6-ceramide did not stimulate the “ $\alpha$ -like” cleavage of tumor necrosis factor- $\alpha$  by tumor necrosis factor- $\alpha$ -converting enzyme (TACE) or the furin-dependent cleavage of full-length Notch (Fig. 2*b*).

Taken together, the above results suggest that the second messenger ceramide regulates the rate of A $\beta$  generation by affecting  $\beta$ -, but not  $\gamma$ -cleavage of APP. Reduced  $\alpha$ -cleavage of APP is unlikely to lower A $\beta$  levels. Instead, most reports indicate that it would elevate A $\beta$  under normal cellular conditions (15). Finally, low levels of C6-ceramide did not induce apoptotic cell death of CHO and H4 cell lines under our experimental conditions.



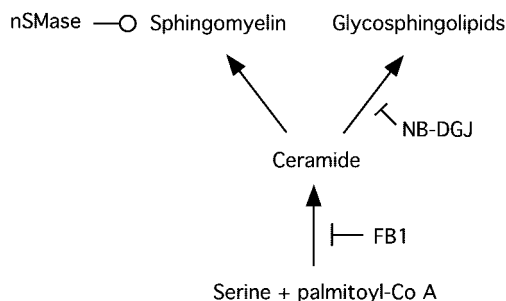
**FIG. 2. C6-ceramide increases the rate of A $\beta$  biosynthesis by promoting  $\beta$ - but not  $\gamma$ -cleavage of APP.** H4 (human neuroglioma) cells were stably transfected with either full-length (H4<sub>751</sub>) or the C-terminal 105 amino acids (H4<sub>C105</sub>) of APP. APP<sub>C105</sub> is a good substrate for  $\gamma$ - but not  $\alpha$ - or  $\beta$ -secretase and mimics  $\beta$ -APP-CTF (12). Cells were treated with increasing concentrations of C6-cer for 2 days. *a*, Western blot showing that 10  $\mu$ M C6-cer increased the steady-state levels of  $\alpha$ - and  $\beta$ -APP-CTFs but not those of APP<sub>C105</sub>. *b*, C6-cer treatment did not activate the apoptotic cascade, as assessed by PARP activation. *f. l. PARP* indicates the 116-kDa native form of PARP. The 85-kDa apoptosis-related cleavage product of PARP, which is generated upon activation of the apoptotic cascade (see Fig. 1c), could not be detected. C6-cer treatment did not affect  $\alpha$ -like cleavage of tumor necrosis factor- $\alpha$  or full-length Notch (*f. l. Notch*) cleavage by furin. *N<sup>TM</sup> Notch* indicates the mature transmembrane domain of Notch produced by furin-cleavage of *f. l. Notch*. *f. l. Notch* could only be detected at high exposures, and its levels were not affected by ceramide treatment (data not shown). One representative immunoblot (of at least three) is shown.

**Endogenous Ceramide Levels Regulate A $\beta$  Generation**—To confirm that endogenous ceramide and not only cell-permeable analogues can modulate A $\beta$  production, we used additional biochemical approaches known to regulate the endogenous pool of active ceramide. The different approaches used in these studies are schematically described in Fig. 3. nSMase increases the intracellular pool of ceramide by hydrolysis of cell surface SM. In contrast, FB1 inhibits ceramide-synthase, preventing the biosynthesis of ceramide and all the other glycosphingolipids beyond the ceramide moiety. In addition to nSMase and FB1, we also used NB-DGJ, a biochemical inhibitor of the ceramide-specific glycosyltransferase. NB-DGJ blocks the glycosphingolipid, but not the SM, biosynthetic pathway and does not affect the levels of the signaling-active ceramide (16, 17).

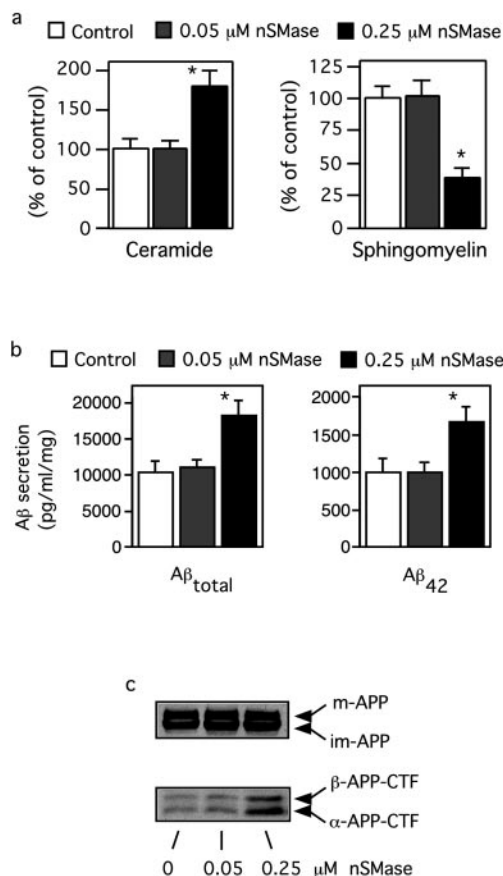
Neither nSMase nor FB1 affected cell viability under the conditions used in our studies, as assessed by the uptake of trypan blue and by the release of the cytosolic enzyme lactate dehydrogenase into the media (data not shown). When used at 0.25  $\mu$ M, nSMase produced a  $\sim$ 60% decrease of SM levels (Fig. 4a). Similar to C6-ceramide, nSMase increased both ceramide levels (Fig. 4a) and A $\beta$  secretion (Fig. 4b) by  $\sim$ 2-fold. The increase in A $\beta$  secretion was accompanied by increased steady-state levels of both  $\alpha$ - and  $\beta$ -APP-CTFs in the absence of any evident effect on APP expression or maturation (Fig. 4c).

As seen with nSMase, FB1 treatment reduced SM by  $\sim$ 50% (Fig. 5a). However, in contrast with C6-ceramide and nSMase, FB1 reduced ceramide levels by  $\sim$ 60–70% (Fig. 5a). Decreased endogenous ceramide levels were paralleled by a corresponding reduction in A $\beta_{total}$  and A $\beta_{42}$  secretion into the media by  $\sim$ 50% (Fig. 5b). Steady-state levels of  $\alpha$ - and  $\beta$ -APP-CTF also decreased, with no apparent effect on APP expression or maturation (Fig. 5c). Most importantly, exogenous C6-ceramide could recover A $\beta$  levels reduced by FB1 (Fig. 5d), confirming that decreased ceramide levels were responsible for lowering A $\beta$  in the first place. In contrast to nSMase and FB1, NB-DGJ, which did not affect the endogenous pool of the signaling active ceramide (Fig. 6a), was not able to produce any effect on A $\beta$  secretion (Fig. 6b) or APP processing (Fig. 6c).

Finally, we assessed the effect of nSMase, FB1, and NB-DGJ on  $\beta$ - and  $\gamma$ -secretase cleavage of APP by using H4



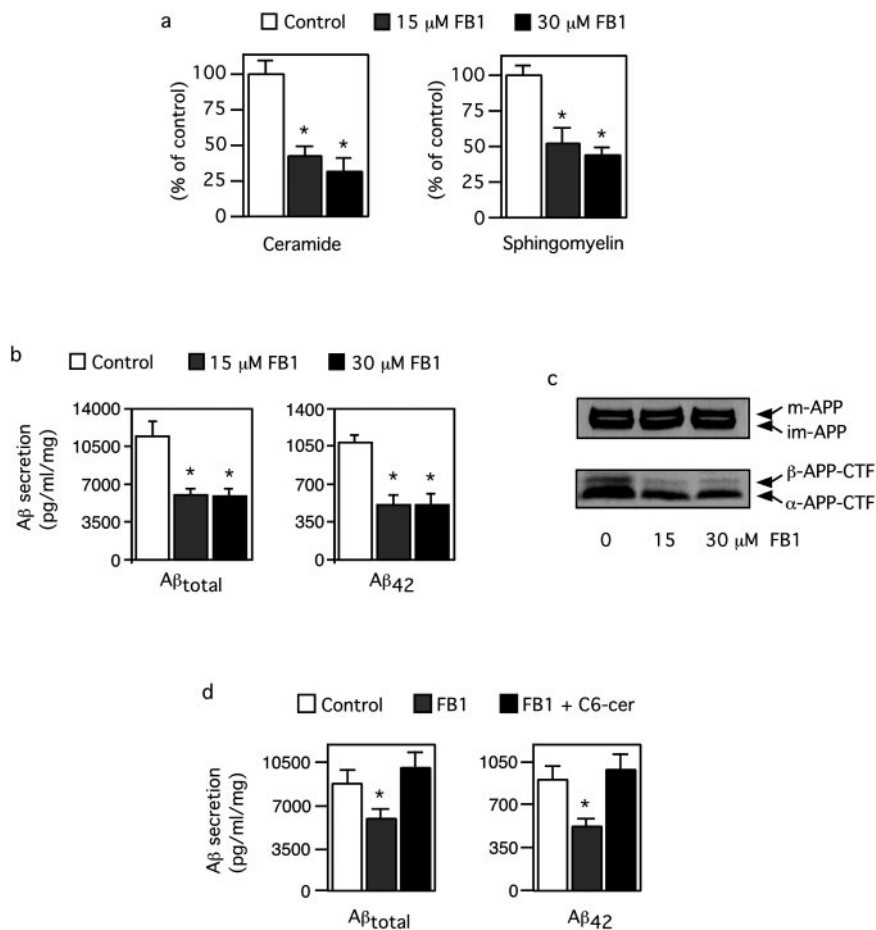
**FIG. 3. Schematic view of the sphingomyelin and glycosphingolipid biosynthetic pathways with the inhibitors used in our studies.**



**FIG. 4. Hydrolysis of cell surface sphingomyelin by nSMase releases active endogenous ceramide and increases A $\beta$  generation.** CHO cells stably transfected with APP<sub>751</sub> were treated with 0.25  $\mu$ M nSMase for 3 days to release ceramide. *a*, cells were labeled with [<sup>3</sup>H]palmitic acid before nSMase treatment. nSMase decreased the levels of sphingomyelin but increased those of ceramide. Results are expressed as percentages of control values. *b*, A $\beta$  secretion in the conditioned media was analyzed by sandwich ELISA. nSMase increased the secretion of both A $\beta_{total}$  and A $\beta_{42}$  by  $\sim$ 80%. *c*, Western blots showing that nSMase increased the steady-state levels of both  $\beta$ - and  $\alpha$ -APP-CTFs without any apparent effect on APP expression or maturation. Results are expressed as the means  $\pm$  S.D. of at least three different determinations. Asterisks (\*) indicate a significant difference from control at  $p < 0.05$ . One representative immunoblot (of at least three) is shown.

(human neuroglioma) cells stably transfected with either full-length APP<sub>751</sub> or with APP<sub>C105</sub>. As seen in CHO cells, both nSMase and FB1 reduced SM levels while producing opposite effects on ceramide levels. nSMase increased, whereas FB1 reduced ceramide levels by  $\sim$ 30 and  $\sim$ 50%, respectively (data not shown). Again, the changes in ceramide production were paralleled by similar changes in the

**FIG. 5. FB1, an inhibitor of endogenous ceramide biosynthesis, reduces A $\beta$  generation.** CHO cells stably transfected with APP<sub>751</sub> were treated with FB1, a general inhibitor of ceramide-synthase, for 4 days. *a*, cells were labeled with [<sup>3</sup>H]palmitic acid during FB1 treatment. FB1 reduced the biosynthesis of both ceramide and sphingomyelin by ~50 to 65%. Results are expressed as percentage of control values. *b*, A $\beta$  secretion in the conditioned media was analyzed by sandwich ELISA. FB1 treatment reduced the secretion of both A $\beta$ <sub>total</sub> and A $\beta$ <sub>42</sub> by ~50%. *c*, Western blot showing that FB1 reduced the steady-state levels of both  $\beta$ - and  $\alpha$ -APP-CTFs without any apparent effect on APP expression or maturation. *d*, cells were first treated with FB1 (15  $\mu$ M) alone and then with FB1 plus C6-ceramide (10  $\mu$ M). A $\beta$  secretion in the conditioned media was analyzed by sandwich ELISA. C6-ceramide reversed the reduction of A $\beta$  secretion produced by FB1 treatment, confirming that ceramide was responsible for such effect. Results are expressed as the means  $\pm$  S.D. of at least three different determinations. Asterisks (\*) indicate a significant difference from control at  $p < 0.05$ . One representative immunoblot (of at least three) is shown.



steady-state levels of both  $\alpha$ - and  $\beta$ -APP-CTFs; nSMase increased, whereas FB1 reduced APP CTFs (Fig. 7, *a* and *b*). Significantly, no effect was observed on the steady-state levels of APP<sub>C105</sub> (Fig. 7, *a* and *b*), indicating that the changes in A $\beta$  secretion were only due to changes in  $\beta$ - and not  $\gamma$ -cleavage of APP. As already observed in CHO cells, NB-DGJ did not affect ceramide levels or APP processing (Fig. 7*c*).

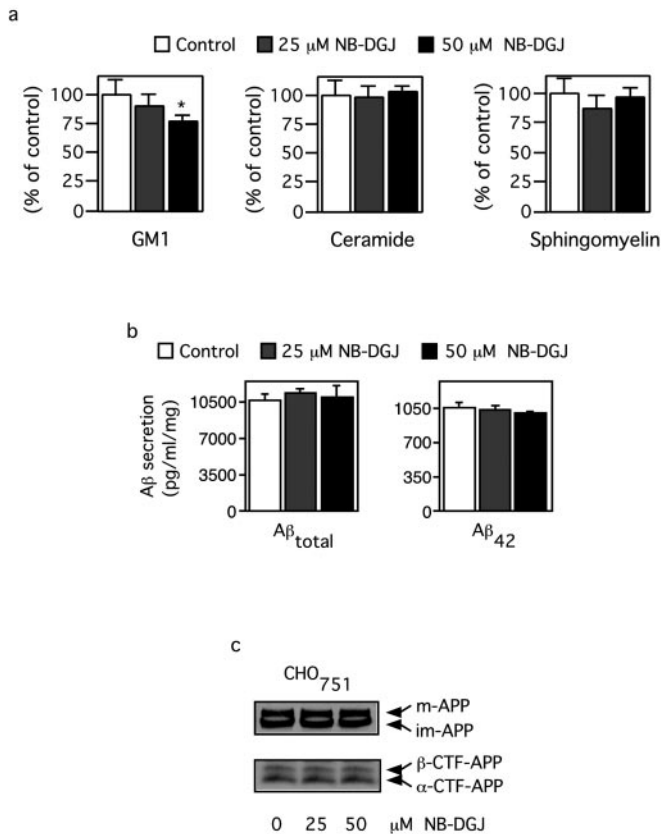
Overall, our results indicate that both cell-permeable analogues and endogenous ceramides regulate A $\beta$  generation by affecting  $\beta$ -, but not  $\gamma$ -cleavage of APP. Although ceramide levels also modulate  $\alpha$ -cleavage of APP, this clip does not directly contribute to A $\beta$  generation and could be regulated by separate cellular events. Moreover, our data argue for a direct effect of ceramide on BACE1 activity instead of APP trafficking, because  $\gamma$ -cleavage of APP is not affected by ceramide in APP<sub>C105</sub>-expressing H4 cells. Finally, biotinylation of cell surface proteins, subcellular fractionation studies, and analysis of APP mature/immature ratios revealed that ceramide levels do not significantly affect APP trafficking or localization (data not shown).

**C6-ceramide Reduces the Turnover Rate of BACE1**—To assess the effect of altered ceramide levels on BACE1, we asked whether C6-ceramide regulates either the subcellular/membrane distribution or the steady-state levels of this enzyme. Endogenous BACE1 was detected in H4 cells as a double band at around 65–70-kDa (Fig. 8*a*). H4 cells were grown in the presence or absence of C-6 ceramide for up to 6 days and then analyzed for subcellular/membrane distribution or steady-state levels of BACE1. C6-ceramide did not affect the overall distribution of BACE1 among intracellular membranes or membrane microdomains (data not shown). However, C6-ceramide progressively increased BACE1 levels, reaching a plateau after

approximately 4 days of treatment (Fig. 8*a*). No effect was observed on the steady-state levels of BACE2, a BACE1 homologue, or TACE (tumor necrosis factor- $\alpha$ -converting enzyme), a regulated form of  $\alpha$ -secretase (data not shown).

To assess whether post-transcriptional events lead to increased BACE1 protein levels, we used a CHO cell line stably expressing C-terminally Myc-tagged human BACE1 under the control of a cytomegalovirus promoter. When these cells were treated with C6-ceramide, the steady-state levels of both native and epitope-tagged BACE1 increased progressively, reaching a plateau after 4 days, at which point they were ~3-fold higher than control (Fig. 8*b*). Most importantly, as observed for the rate of A $\beta$  secretion (see Figs. 4 and 5), steady-state levels of BACE1 also paralleled changes in endogenous ceramide induced by FB1 and nSMase. Fig. 8*c* shows that FB1 reduced, whereas nSMase increased, the steady-state levels of BACE1, strongly suggesting that ceramide levels regulate A $\beta$  generation by modulating the amount of enzyme available for  $\beta$ -secretase cleavage of APP.

Finally, we performed pulse-chase analysis and cycloheximide degradation assays to directly study changes in the turnover rate of BACE1. CHO cells stably expressing BACE1 were treated with C-6 ceramide followed by pulse-chase with radiolabeled methionine/cysteine to calculate the half-life of BACE1. Ceramide treatment increased the half-life of BACE1 from ~16–20 h to ~30 h (Fig. 8*d*). It is worth noting that the levels of newly synthesized BACE1 found in ceramide-treated cells after 56 h of chase were very similar to those found in control cells after only 24 h of chase (Fig. 8*d*). Very similar results were obtained when the same cells were treated with cycloheximide to inhibit protein synthesis. Ceramide treatment significantly reduced the turnover rate of BACE1 and increased the half-life



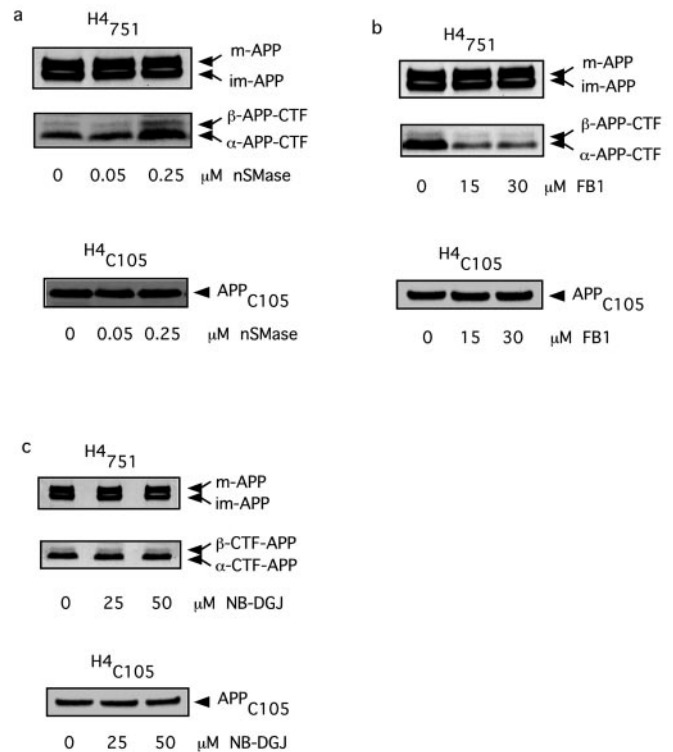
**FIG. 6. NB-DGJ does not affect the cellular pool of active ceramide or the rate of A $\beta$  generation.** CHO cells stably transfected with APP<sub>751</sub> were treated with increasing concentrations of NB-DGJ for 2 days. NB-DGJ inhibits the ceramide-specific glycosyltransferase without affecting the intracellular pool of active ceramide (16, 17). *a*, cells were incubated in the presence of [<sup>3</sup>H]palmitic acid during NB-DGJ treatment. Results are expressed as percentages of control values. NB-DGJ reduced the incorporation of palmitic acid into the glycosphingolipid GM1 but did not affect the biosynthesis of either ceramide or sphingomyelin. *b*, after NB-DGJ treatment, media was subjected to sandwich ELISA for A $\beta$  quantitation. No effect was observed on the secretion of either A $\beta_{total}$  or A $\beta_{42}$ . *c*, immunoblot showing that the steady-state levels of  $\beta$ - and  $\alpha$ -APP-CTFs were not affected by NB-DGJ treatment. Results are expressed as means  $\pm$  S.D. of at least three different determinations. Asterisks (\*) indicate a significant difference from control at  $p < 0.05$ . One representative immunoblot (of at least three) is shown.

of preformed BACE1 from ~20 to ~56 h (Fig. 8e).

Taken together, the above data indicate that ceramide regulates A $\beta$  generation by affecting the steady-state levels of BACE1, the rate-limiting enzyme in the biogenesis of A $\beta$ . They also suggest that the increase in BACE1 levels is, at least in part, the result of post-transcriptional stabilization of BACE1.

#### DISCUSSION

Our results show for the first time that intracellular levels of ceramide regulate A $\beta$  generation by modulating  $\beta$ -secretase cleavage of APP. C6-ceramide, a cell-permeable and active analog of ceramide, increased the rate of A $\beta$  biosynthesis by affecting  $\beta$ -, but not  $\gamma$ -, cleavage of APP. nSMase, FB1, and NB-DGJ, general inhibitors of the SM/glycosphingolipid metabolic pathway, caused changes in ceramide levels, which were consistently paralleled by changes in A $\beta$  generation. Additionally, we found that ceramide controls the processing of APP by affecting the molecular stability of the  $\beta$ -secretase, BACE1. These effects occur under physiological conditions that do not perturb cell viability and do not activate apoptosis. Finally, we found that ceramide levels do not affect  $\gamma$ -cleavage of APP, as



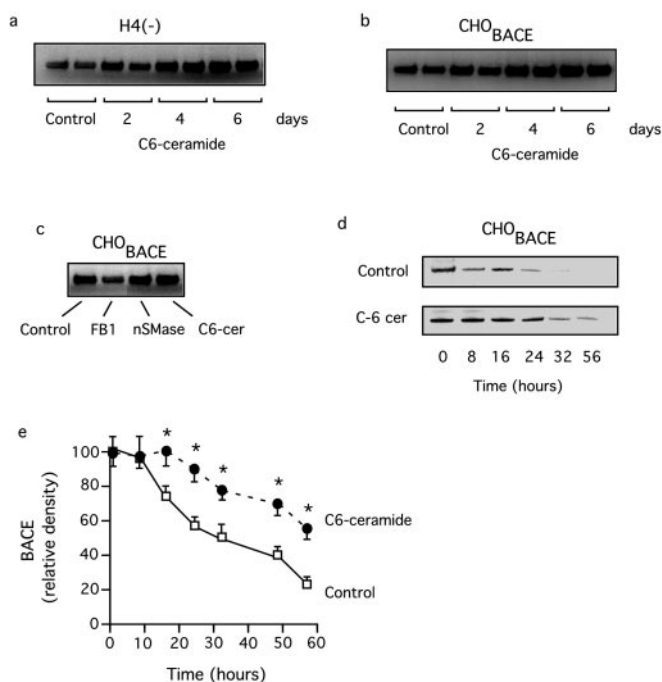
**FIG. 7. Endogenous ceramide regulates  $\alpha$ - and  $\beta$ -, but not  $\gamma$ -cleavage of APP.** H4 (human neuroglioma) cells were stably transfected with either full-length APP<sub>751</sub> (H4<sub>751</sub>) or the C-terminal 105 amino acids of APP (H4<sub>C105</sub>). APP<sub>C105</sub> is a good substrate for  $\gamma$ -, but not  $\alpha$ - or  $\beta$ -secretase, and mimics  $\beta$ -APP-CTF (12). Cells were treated with nSMase, FB1, or NB-DGJ, as described in Figs. 4–6, respectively. *a* and *b*, Western blots showing that nSMase (at 0.25  $\mu$ M) increased, whereas FB1 reduced the steady-state levels of  $\beta$ - and  $\alpha$ -APP-CTFs. No apparent effect on APP expression or maturation was observed. Neither FB1 nor nSMase affected the steady-state levels of APP<sub>C105</sub>, indicating that ceramide levels do not regulate  $\gamma$ -cleavage of APP. *c*, immunoblots showing that NB-DGJ, which did not affect endogenous ceramides, did not induce any change in  $\alpha$ -,  $\beta$ -, or  $\gamma$ -cleavage of APP. One representative immunoblot (of at least three) is shown.

shown by the absence of changes in A $\beta_{42}$ /A $\beta_{total}$  ratios and in the steady-state levels of APP<sub>C105</sub>, a short C-terminal fragment of APP that mimics  $\beta$ -APP-CTF.

Ceramide is a lipid second messenger involved in many biological events that regulate terminal differentiation of neurons, cellular senescence, proliferation, and death (7, 14, 18, 19). Depending on the cell type and the doses used, exogenously added ceramide has been shown to either activate or inhibit apoptosis (3, 18). Intracellular levels of ceramide increase during aging in both cultured cells and the whole organ (7–10). In addition, senescent-like doses (~10  $\mu$ M) of ceramide are able to induce a senescent phenotype in young cultured cells (7, 9, 10). Under those conditions, ceramide has been shown to promote outgrowth and survival of cultured neurons (14, 18, 20).

In apparent contrast to the above studies, a chronic increase in intracellular ceramide can inhibit axonal elongation and receptor-mediated internalization of nerve growth factor and activate cell death (13, 21). In addition, it also reduces receptor-mediated internalization of lipoprotein-associated cholesterol (13), which is involved in the regulation of synaptogenesis (22). These effects may be part of a delicate set of events that occur during aging. Very recently, Han *et al.* (11) also reported that ceramide levels are increased more than 3-fold in the brains of AD patients when compared with age-matched controls.

In this study, we showed that ceramide levels control A $\beta$  biosynthesis, the first pathogenic event in the generation of senile (or amyloid) plaques. It is worth noting that our results



**FIG. 8. Ceramide regulates the molecular stability of the  $\beta$ -secretase, BACE1.** *a*, H4 (human neuroglioma) cells were treated with 10  $\mu$ M C6-ceramide for different periods of time and then analyzed for BACE1 expression. *b*, the same experiment was performed with CHO cells stably transfected with BACE1 (CHO<sub>BACE1</sub>). *c*, CHO<sub>BACE1</sub> cells were treated with either FB1 (15  $\mu$ M, for 4 days), nSMase (0.25  $\mu$ M, for 3 days), or C6-cer (10  $\mu$ M, for 2 days). FB1 reduced, whereas nSMase and C6-cer increased the steady-state levels of BACE1. *d*, CHO<sub>BACE1</sub> cells were grown in the presence or absence of 10  $\mu$ M C6-cer for 4 days, pulsed with radiolabeled methionine/cysteine, and then chased for different periods of time. C6-ceramide treatment increased the half-life of newly synthesized BACE1. *e*, CHO<sub>BACE1</sub> cells were first grown in the presence or absence of 10  $\mu$ M C6-ceramide for 4 days and then treated with 0.5 mg/ml of cycloheximide for increasing periods of time to inhibit protein synthesis. BACE1 expression was analyzed by SDS-PAGE followed by immunoblotting. Band intensities were quantitated and expressed as relative densities. C6-ceramide increased the half-life of pre-formed BACE1. Results are expressed as the means  $\pm$  S.D. of at least three different determinations. Asterisks (\*) indicate a significant difference from control at  $p < 0.05$ . One representative immunoblot (of at least three) is shown.

with exogenous C6-ceramide (Figs. 1, 2, and 8) were observed at 10  $\mu$ M concentration, which is known to produce a cellular concentration of active ceramide very close to that observed in senescent cells (7). Additionally, the signaling function of ceramide is likely to be required for its effect on A $\beta$  generation, because the signaling inactive analog dehydroceramide did not produce any effect on A $\beta$  production in our CHO cells (data not shown). Dehydroceramide is a naturally occurring ceramide that lacks the 4–5 *trans* double bond, which is required for the signaling activity but retains the stereochemical configuration of ceramides and is metabolized very similarly to ceramides (13, 18).

Cell surface SM is mostly found in cholesterol-rich domains, which are specialized membrane microdomains highly enriched in SM, cholesterol, and the glycosphingolipid GM1 (23). Hydrolysis of SM has been reported to reduce the clustering of cholesterol into cholesterol-rich domains and to induce retro-transport of cholesterol from the plasma membrane to the endoplasmic reticulum (23). In the endoplasmic reticulum excess cholesterol activates the enzyme acyl-coenzyme A:cholesterol acyltransferase, which we have recently implicated with A $\beta$  generation (12). However, acyl-coenzyme A:cholesterol acyltransferase activation follows retro-transport of cholesterol,

which occurs only in the presence substantial SM hydrolysis and massive sterol mobilization from cholesterol-rich domains (23). In our study, both nSMase and FB1 reduced the clustering of cholesterol into cholesterol-rich domains (data not shown), but they only induced a modest mobilization of sterols, which was not accompanied by acyl-coenzyme A:cholesterol acyltransferase activation (data not shown). In addition, although FB1 and nSMase had similar effects on SM levels and on cholesterol distribution between membrane microdomains, they produced opposite effects on A $\beta$  generation. Those effects paralleled the changes in ceramide levels and could be reproduced by C6-ceramide. Finally, the reduction in A $\beta$  biosynthesis produced by FB1 treatment was reversed by C6-ceramide, confirming that indeed ceramide was responsible for the changes observed in A $\beta$  generation.

BACE1 is a type I integral membrane protein with an aspartyl protease motif in its luminal domain that fulfills most of the requirements expected for a candidate  $\beta$ -secretase (24). It is highly expressed in brain and neurons and colocalizes with Golgi and endosomal markers. BACE1 is the primary brain  $\beta$ -secretase and is highly increased in both protein levels and enzymatic activity in the neocortex of AD patients (25, 26). Disruption of BACE1 in AD transgenic mice almost completely abolished  $\beta$ -cleavage of APP together with the ability to generate A $\beta$  (27), further confirming that BACE1 is indeed the long-sought APP  $\beta$ -secretase. Even if much is known about intracellular trafficking and post-translational modifications of BACE1, very little or nothing is known about regulation of BACE1 expression/activity. Very recently Tamagno *et al.* (28) have shown that oxidative stress is able to increase the expression of BACE1 together with  $\beta$ -cleavage of APP. In this previous work, BACE1 activation involved cell damage and most likely required transcriptional activation of BACE1. In contrast, our results indicate that the ceramide-dependent regulation of BACE1 expression occurs, at least in part, at the level of protein degradation. Whether ceramide also affects transcription and/or translation of BACE1 remains to be further analyzed. However, it is worth mentioning that for the stable transfection of CHO<sub>BACE1</sub> cells we only used the coding region of BACE1, eliminating potential 5' and 3' regulatory elements.

In conclusion, our study implicates for the first time the lipid second messenger ceramide in the generation of A $\beta$  and proposes ceramide as a potential novel link between AD and aging. It also shows that ceramide regulates  $\beta$ -secretase activity at the level of BACE1 stabilization. Identification of the downstream molecules that mediate the ceramide-dependent regulation of BACE1 turnover may provide novel targets for the therapeutic treatment of AD patients.

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