A Distinct ER/IC γ-Secretase Competes with the Proteasome for Cleavage of APP[†]

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ABSTRACT: The deposition of amyloid-β peptides (Aβ) in senile plaques (SPs) is a central pathological feature of Alzheimer's disease (AD). Since SPs are composed predominantly of Aβ1−42, which is more amyloidogenic in vitro, the enzymes involved in generating Aβ1−42 may be particularly important to the pathogenesis of AD. In contrast to Aβ1−40, which is generated in the trans-Golgi network and other cytoplasmic organelles, intracellular Aβ1−42 is produced in the endoplasmic reticulum/intermediate compartment (ER/IC), where it accumulates in a stable insoluble pool. Since this pool of insoluble Aβ1−42 may play a critical role in AD amyloidogenesis, we sought to determine how the production of intracellular Aβ is regulated. Surprisingly, the production of insoluble intracellular Aβ1−42 was increased by a putative γ-secretase inhibitor as well as by an inhibitor of the proteasome. We further demonstrate that this increased generation of Aβ1−42 in the ER/IC is due to a reduction in the turnover of Aβ-containing APP C-terminal fragments. We conclude that the proteasome is a novel site for degradation of ER/IC-generated APP fragments. Proteasome inhibitors may augment the availability of APP C-terminal fragments for γ-secretase cleavage and thereby increase production of Aβ1−42 in the ER/IC. Based on the organelle-specific differences in the generation of Aβ by γ-secretase, we conclude that intracellular ER/IC-generated Aβ1−42 and secreted Aβ1−40 are produced by different γ-secretases. Further, the fact that a putative γ-secretase inhibitor had opposite effects on the production of secreted and intracellular Aβ may have important implications for AD drug design.

Alzheimer's disease (AD)[1] is characterized by the progressive accumulation of amyloid fibrils in senile plaques (SPs), which are composed principally of amyloid-β peptides (Aβ). The accumulation of Aβ has been strongly linked to AD pathogenesis by studies showing that mutations in the Aβ precursor protein (APP) gene and in the presenilin 1 (PS1) and presenilin 2 (PS2) genes cause early-onset familial AD (FAD) (reviewed in ref *1*). These FAD-associated mutations may be pathogenic by altering the normal processing of APP, since their expression both in vitro and in vivo results in increased Aβ production or increased production of Aβ1−42 relative to Aβ1−40 (*2*−*4*). Further, while cerebral spinal fluid (CSF) contains 10-fold more Aβ1−40 than Aβ1−42, SPs are composed largely of Aβ1−42 (*5*−*8*). Consistent with the preponderance of Aβ1−42 in SPs, in vitro studies indicate that Aβ1−42 is more prone to aggregation than Aβ1−40 (*9*−*12*). The selective enhancement of Aβ1−42 production by FAD-causing mutations combined with the preferential accumulation of the more amyloidogenic Aβ1−42 in SPs supports the importance of Aβ1−42 production in the pathogenesis of AD.

Aβ1−40 and Aβ1−42 are generated by the posttranslational proteolytic cleavage of APP. APP is cleaved by β- and γ-secretase activities to generate the NH_2 and COOH termini of Aβ, respectively (*13*, *14*). These cleavages can occur in at least three subcellular locations: the endoplasmic reticulum/intermediate compartment (ER/IC) (*15*−*18*); the trans-Golgi network (TGN) and post-Golgi organelles (*18*); and the endosomal/lysosomal system (*19*). While secreted Aβ may be produced in later compartments such as post-Golgi vesicles, the Aβ generated in the ER/IC accumulates intracellularly in a stable insoluble pool that is not secreted (*20*). Interestingly, this stable pool of intracellular Aβ is composed almost exclusively of Aβ1−42. While the relative importance of intracellular Aβ in the pathogenesis of AD remains to be determined, intracellular Aβ could contribute to disease pathology if it served as a nidus for plaque formation or if it were directly toxic to neurons. Thus, it is important to understand the mechanisms underlying production of both intracellular and secreted Aβ.

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[1] Abbreviations: Aβ, amyloid-β peptide; Aβ1−40 and Aβ1−42, 40 and 42 amino acid long forms of Aβ, respectively; AD, Alzheimer's disease; FAD, familial Alzheimer's disease; APP, amyloid-β precursor protein; APPwt, wild-type human APP 695 protein; APPΔKK, APP 695 which has been modified to contain a dilysine ER retention motif; APPΔNL, APP 695 containing the KM/NL FAD Swedish mutation; SFV, Semliki Forest virus; ELISA, enzyme-linked immunosorbent assay; CHO cells, Chinese hamster ovary cells; NT2N neurons, neurons derived from the human embryonal carcinoma cell line NTera2/D_1; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; RIPA buffer, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP40, 5 mM EDTA in TBS (pH 8.0); TGN, trans-Golgi network; ER, endoplasmic reticulum; IC, intermediate compartment; mAb, monoclonal antibody.

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To gain better insights into the proteases that generate Aβ, the effects of peptide−aldehyde protease inhibitors on Aβ secretion have been examined (*21*−*23*). The selective inhibition of secreted Aβ1−40 but not Aβ1−42 by peptide−aldehyde γ-secretase inhibitors suggests that distinct enzymes may generate these two species of secreted Aβ. However, the effects of these compounds on the production of intracellular Aβ have not been examined, nor have their effects on Aβ production in neurons been determined. This prompted us to compare the effects of a peptide aldehyde inhibitor of Aβ secretion (MG132, carbobenzoxy-Leu-Leu-leucinal) on the production of intracellular and secreted Aβ species in both neuronal and nonneuronal cells. Here, we demonstrate that the production of Aβ1−42 in the ER/IC was increased by MG132 as well as lactacystin and this increase may have been caused by inhibition of the turnover of APP C-terminal fragments by the proteasome. These data further suggest that distinct mechanisms underlie the production of secreted Aβ1−40 and intracellular Aβ1−42.

EXPERIMENTAL PROCEDURES

Cell Culture. NT2 cells derived from a human embryonal carcinoma cell line (Ntera2/cl.D1) were grown and passaged as described previously (*24*, *25*). Cells were differentiated by biweekly retinoic acid treatments (10 μM) for 5 weeks, and were replated (replate-2 cells) in the presence of mitotic inhibitors to yield nearly pure NT2N neurons (*24*). To obtain 99% pure neurons (replate-3 cells), replate-2 cells were removed enzymatically and mechanically, and were replated in 10 cm dishes (*24*). Cultures of replate-3 NT2N neurons were used for experiments when they were 2−3 weeks old unless otherwise indicated. CHO Pro5 cells were grown and passaged 3 times per week in Alpha-MEM (Life Technologies, Inc., Gaithersburg, MD) containing 10% FBS and penicillin/streptomycin.

Preparation of Semliki Forest Virus and Infection of Cultured Cells. Semliki Forest virus (SFV) vectors expressing β-galactosidase (SFV-lacZ), wild-type APP 695 (SFV-APPwt), an FAD-associated APP mutant in which the two amino acids immediately flanking the amino terminus of the Aβ domain were mutated to asparagine and leucine (APPΔNL), an APP mutant in which the third and fourth amino acids from the carboxyl terminus of APP have been changed to lysines (SFV-APPΔKK), or the Aβ-containing C-terminal fragment of APP (SFV-C99) were prepared and titered as previously described (*15*, *16*, *26*). CHO-Pro5 and NT2N cells were infected in serum-free medium at a multiplicity of infection of 10. After 1 h, complete growth medium was replaced, and infection was allowed to proceed for 12 h.

Metabolic Labeling, Immunoprecipitation, and Gel Electrophoresis. Cultured NT2N neurons were methionine-deprived by incubation in methionine-free DMEM (Life Technologies, Inc.) for 30 min before adding [^{35}S]methionine (500 μCi/mL in methione-free DMEM + 5% dialyzed FBS; DuPont-NEN, Boston, MA). For steady-state experiments, cells were labeled for 12 h; for pulse−chase experiments, label was removed after 30 min (for full-length APP labeling), 1 h (for C99 labeling), or 3 h (for APPwt and APPΔKK C-terminal fragment labeling) and replaced with growth medium for the indicated times. Cells were washed twice in PBS and lysed in 600 μL of RIPA buffer (0.5% sodium deoxycholate, 0.1% SDS, 1% NP40, 5 mM EDTA in TBS, pH 8.0) with a cocktail of protease inhibitors (1 μg/mL each of pepstatin A, leupeptin, TPCK, TLCK, and STI; and 0.5 mM PMSF). After brief sonication, cell lysates were centrifuged at 40000*g* for 20 min at 4 °C, and the supernatant was subjected to immunoprecipitation with 6E10 (a monoclonal antibody specific for Aβ1−17) (*27*), 2493 (a rabbit anti-APP antibody raised to the last 40 amino acids of APP), or Karen (a goat anti-APP extracellular domain antibody) as previously described (*28*). For direct extraction into formic acid, cells were scraped in 1 mL of PBS, pelleted by centrifugation, and lysed in 100 μL of 70% formic acid with sonication. Formic acid was removed by vacuum centrifugation for 40 min, and the resulting dry pellet was resuspended in 100 μL of 60% acetonitrile. RIPA buffer (1.9 mL) was added to each of the samples before they were subjected to immunoprecipitation with 6E10. Immunoprecipitated Aβ and APP C-terminal fragments were resolved on 10/16.5% step gradient Tris−Tricine gels. Immunoprecipitated full-length APP and secreted APP (sAPP) were resolved on 7.5% Tris−glycine gels. Gels were fixed in 60% methanol, dried, and placed on PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA) plates for 72 h.

Lysis of Cells and Sandwich-ELISA. For extraction in RIPA, cells were washed twice in PBS and then lysed in 600 μL of RIPA buffer and centrifuged for 20 min at 40000*g* at 4 °C. Supernatant was subjected directly to sandwich-ELISA. For direct extraction into formic acid, cells were scraped in PBS after washing twice with PBS. Cells were pelleted by centrifugation at 2000*g* for 2 min, and were then lysed in 100 μL of formic acid. Insoluble material was pelleted by centrifugation at 40000*g* at 4 °C for 20 min, and the supernatant was neutralized by adding 1.9 mL of 1 M Tris base and diluted 1:3 in H_2O before quantification of Aβ by sandwich-ELISA.

Sandwich-ELISA was performed as described previously using mAbs specific for different species of Aβ (*28*, *29*). BAN-50 (a mAb specific for the first 10 amino acids of Aβ) was used as a capturing antibody, and horseradish peroxidase-conjugated BA-27 (a mAb specific for Aβ1−40) and horseradish peroxidase-conjugated BC-05 (a mAb specific for Aβ1−42) were used as secondary antibodies. To calibrate the sensitivity of the ELISA for detecting Aβ after formic acid extraction and neutralization, synthetic Aβ1−40 and Aβ1−42 peptides (Bachem Bioscience Inc., King of Prussia, PA) used to generate the standard curves were treated with formic acid and neutralized in the same manner as the cell lysates. Under these conditions, the sandwich-ELISA had a detection limit of <1 fmol of synthetic Aβ per sample. The BAN-50, BA-27, and BC-05 mAbs were prepared and characterized as described previously (*29*).

MG132, Lactacystin, BFA, and Cycloheximide Treatment. For experiments involving drug treatments, NT2N cells were incubated in media containing 50 μM MG132 (Peptides Intl.), 20 μM lactacystin (Calbiochem), 20 μg/mL BFA (Sigma) or 150 μg/mL cycloheximide (Sigma), for the indicated times unless otherwise stated. MG132 and lactacystin were diluted from 10 and 20 mM DMSO stocks, respectively. BFA was diluted from a 20 mg/mL EtOH stock. Control cells for experiments involving MG132 or lactacystin were treated with DMSO alone.

RESULTS

MG132 Inhibits Neuronal Aβ Secretion. Several studies have examined the inhibition of Aβ secretion by structurally related di- and tripeptide aldehydes. Higaki et al. (1995) and Citron et al. (1996) have shown that the modified dipeptide aldehyde MDL 28170 inhibits γ-secretase, leading to a selective reduction in Aβ1−40 secretion and the accumulation of intracellular APP C-terminal fragments. In addition, calpeptin (carbobenzoxyl-Leu-norleucinal), ALLN (calpain inhibitor I; *N*-acetyl-Leu-Leu-norleucinal), and MG132 inhibit the secretion of Aβ1−40 to a greater extent than Aβ1−42 (*22*). However, none of these studies examined intracellular Aβ, and all utilized nonneuronal cell lines engineered to overexpress APP. Nonneuronal cells process APP predominantly by the α-secretory pathway, thus generating mostly the nonamyloidogenic p3 fragment (which begins at Aβ residue 17 and extends to the C-terminus of APP) rather than Aβ. In contrast, postmitotic neurons preferentially use the β-secretory pathway, and thus generate a much higher proportion of Aβ (*15*, *26*, *28*). Since this may reflect differences in neuronal and nonneuronal secretases, we asked whether the γ-secretase responsible for secretion of Aβ from human neurons is also inhibited by MG132.

Human NT2N neurons were treated with increasing concentrations of MG132 for 14 h, and levels of secreted Aβ1−40 and Aβ1−42 were measured using a highly specific sandwich-ELISA (*29*). Aβ1−40 secretion was decreased by MG132 in a dose-dependent manner while Aβ1−42 secretion was decreased only at high concentrations of MG132 (Figure 1A). Thus, MG132 inhibited Aβ1−40 secretion with a lower half-maximal concentration as compared to Aβ1−42 secretion (5 μM versus 30 μM). At 25 μM MG132, Aβ1−40 secretion was reduced by nearly 70% while Aβ1−42 secretion was not affected. Therefore, while MG132 diminished secretion of both Aβ species in neurons, it impaired secretion of Aβ1−40 to a much greater extent than Aβ1−42, particularly at lower drug concentrations.

MG132 Does Not Inhibit Aβ1−42 Production in the ER/IC. To determine the effects of MG132 on intracellular Aβ levels, NT2N neurons were treated with MG132 for 14 h, and soluble and insoluble pools of intracellular Aβ were sequentially extracted using RIPA followed by formic acid. Aβ1−40 and Aβ1−42 levels in each pool were quantitated by sandwich-ELISA (Figure 1B). While intracellular Aβ1−40 levels were not significantly affected by MG132 treatment, total Aβ1−42 levels increased by approximately 42%. Interestingly, MG132 preferentially increased Aβ levels in the insoluble (formic acid solubilized) pool rather than in the soluble pool. Since our previous studies have shown that the bulk of intracellular Aβ1−42, including the insoluble pool, is generated in the ER/IC in NT2N neurons (*16*, *20*), this increase in intracellular Aβ production likely occurs by this pathway. Indeed, we found that MG132 treatment led to a similar increase in intracellular Aβ1−42 levels in NT2N neurons expressing an APP mutant that is retained in the ER/IC due to the presence of the dilysine ER retrieval sequence (data not shown). Thus, MG132 does not inhibit the ER/IC-associated γ-secretase, but in fact increases recovery of Aβ1−42 generated by this pathway.

To further demonstrate that the material produced in the ER/IC and measured by sandwich-ELISA was indeed Aβ,

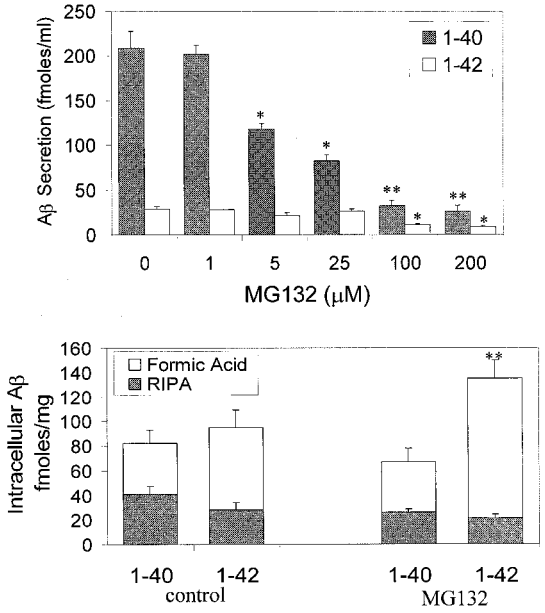

FIGURE 1: Carbobenzoxy-Leu-Leu-leucinal (MG132) selectively inhibits Aβ1−40 secretion in human NT2N neurons. (A) Following treatment with 0, 1, 5, 25, 100, or 200 μM MG132 for 14 h, the amounts of Aβ1−40 and Aβ1−42 secreted by NT2N neurons were quantitated by sandwich-ELISA. Mean results and standard errors of three separate experiments (each done with duplicate samples) are shown where * = $p < 0.05$ and ** = $p < 0.005$ compared to control cells (paired *t*-test). (B) NT2N neurons treated with 50 μM MG132 for 14 h were extracted in RIPA buffer. The remaining insoluble material was solubilized by formic acid and neutralized. Intracellular Aβ levels in the RIPA-soluble and formic acid solubilized pools were quantitated by sandwich-ELISA. Mean results and standard errors are shown (three separate experiments, each done in duplicate). (**) $p < 0.005$ (paired *t*-test).

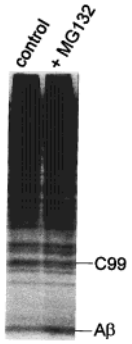

FIGURE 2: MG132 increases intracellular Aβ1−42 generated in the ER/IC. NT2N neurons were infected with SFV-APPΔKK to restrict APP to the ER/IC. Lysates were immunoprecipitated with 6E10 following 12 h steady-state metabolic labeling in the presence or absence of MG132 to recover intracellular Aβ. Bands corresponding to the apparent molecular weights of Aβ and C99 are labeled.

NT2N neurons expressing ER/IC-retained APP (APPΔKK) through the use of a Semliki Forest virus (SFV) vector were metabolically labeled with [^{35}S]methionine for 12 h in the presence and absence of MG132, and Aβ was immunoprecipitated using the mAb 6E10. As shown in Figure 2, immunoprecipitation of Aβ was increased in the presence of MG132 even though we have previously shown that immunoprecipitation of Aβ from cell lysates recovers only a fraction of intracellular Aβ (*20*). As APPΔKK expressing cells generate exclusively Aβ1−42, the increased yield of

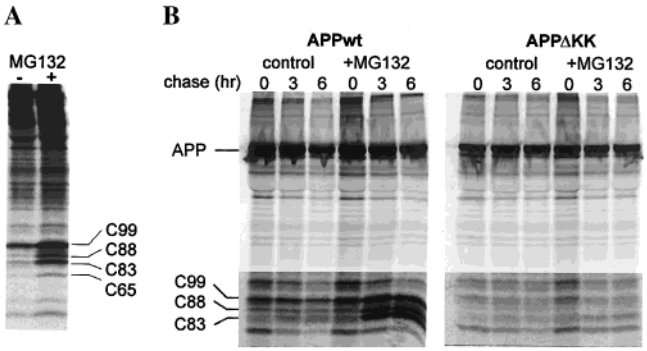

FIGURE 3: Effects of MG132 on the generation of APP C-terminal fragments. (A) SFV-APPΔNL-infected NT2N neurons were metabolically labeled for 12 h in the presence (+) or absence (−) of MG132. APP C-terminal fragments were immunoprecipitated with 2493 from cell lysates and resolved on a 10/16.5% step gradient Tris−Tricine gel. Bands labeled C65, C83, C88, and C99 (corresponding to the last 65, 83, 88, and 99 amino acids of APP, respectively) were excised and identified by radiosequencing. A representative example from five experiments is shown. (B) SFV-APPwt- and SFV-APPΔKK-infected NT2N neurons were pulse-labeled for 3 h and chased in the presence or absence of MG132, followed by immunoprecipitation with 2493. Contrast was enhanced on the lower portion of the image to emphasize APP C-terminal fragments. Bands corresponding to the apparent molecular weights of APP, C99, C88, and C83 are labeled.

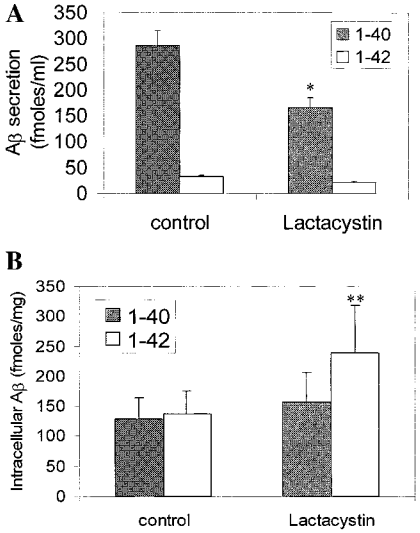

FIGURE 4: Effects of lactacystin on secreted and intracellular Aβ. (A) NT2N neurons were treated with 20 μM lactacystin for 14 h, and secreted Aβ1−40 and Aβ1−42 were quantitated by sandwich-ELISA. Mean results and standard errors are shown (three separate experiments, each done in duplicate). (*) $p < 0.05$ (paired *t*-test). (B) NT2N neurons treated with 20 μM lactacystin for 14 h were lysed in formic acid, and intracellular Aβ1−40 and Aβ1−42 levels were quantitated by sandwich-ELISA. Mean results and standard errors are shown (three separate experiments, each done in duplicate). (**) $p < 0.005$ (paired *t*-test).

Aβ1−42 in the presence of MG132 is in agreement with the ELISA data.

MG132 Inhibits Turnover of a Fraction of APP C-Terminal Fragments. Since MG132-inhibited secretion of Aβ1−40 but did not inhibit production of intracellular Aβ, we sought to confirm that in neurons, MG132 inhibited secretion of Aβ1−40 by acting at the step of γ-secretase cleavage. While inhibition of Aβ1−40 secretion could result from interference in any step of APP processing or Aβ secretion, inhibition of γ-secretase would lead to the accumulation of APP C-terminal fragments containing all or part of the Aβ sequence (*21*). Thus, we examined the levels of these fragments by steady-state metabolic labeling followed by immunoprecipitation (Figure 3A). We found that C-terminal fragments generated by both α-secretase (C83) and β-secretase (C99, which contains the whole Aβ domain) accumulated after MG132 treatment. In addition, a major band corresponding to the last 88 amino acids of APP (C88) also accumulated with MG132 treatment. A minor band corresponding to the last 65 amino acids of APP (C65) was also detected. The identities of C65, C88, and other APP C-terminal fragments were confirmed by radiosequencing of immunoprecipitated labeled fragments (data not shown). Thus, C83, C88, and C99 are the three major neuronal substrates for γ-secretase cleavage, and MG132 specifically inhibits their turnover by γ-secretase.

Since MG132 inhibited Aβ1−40 secretion but not the production of Aβ1−42 generated in the ER/IC, we hypothesized that the C-terminal APP fragments that accumulate upon MG132 treatment are generated in post-ER/IC compartments. To test this, we compared the C-terminal fragments generated by cleavage of APPΔKK and APPwt in the presence and absence of MG132. As shown in Figure 3B, we used a pulse−chase metabolic labeling paradigm to follow the turnover of APP C-terminal fragments. While MG132 caused a dramatic accumulation of APP C-terminal fragments generated by APPwt (similar to that seen in Figure 3A), it had no effect on the levels of C-terminal fragments generated by APPΔKK (Figure 3B). In contrast, the level of APP recovered by these immunoprecipitations was unchanged by MG132 treatment. The failure of MG132 to induce accumulation of APP C-terminal fragments in the ER/IC combined with the continued production of Aβ1−42 suggests that this compound does not inhibit the γ-secretase responsible for the production of intracellular Aβ1−42.

Increased Production of Intracellular Aβ1−42 Is Due to Proteasome Inhibition. The experiments described above indicated that not only did MG132 fail to inhibit the ER/IC γ-secretase, but also it increased recovery of Aβ1−42 generated by this pathway (Figures 1B and 2). Since one of the effects of MG132 is inhibition of the proteasome (*30*), we sought to determine if proteasome inhibition alone could account for the effects of MG132 on APP processing. Therefore, we examined Aβ generation in NT2N neurons treated with lactacystin, a compound structurally unrelated to MG132. Lactacystin is a highly specific, irreversible inhibitor of the proteasome (*31*). Unlike MG132, which specifically inhibited Aβ1−40 secretion by up to 70%, lactacystin only moderately reduced secretion of both Aβ1−40 and Aβ1−42 from NT2N neurons (Figure 4A). In contrast, lactacystin treatment increased endogenous intracellular Aβ1−42 levels by 73% in neurons (Figure 4B), suggesting that proteasome inhibition may be important for the increased recovery of ER/IC-produced Aβ1−42 seen after MG132 treatment. Indeed, we found that lactacystin, like MG132, increased recovery of Aβ1−42 from NT2N neurons expressing APPΔKK (data not shown).

Proteasome Inhibition Reduces C99 Turnover and Increases Aβ1−42 Production. Since the proteasome degrades many misfolded and tightly regulated ER proteins, we hypothesized that the effects of MG132 and lactacystin on intracellular Aβ1−42 might be due to decreased proteasomal

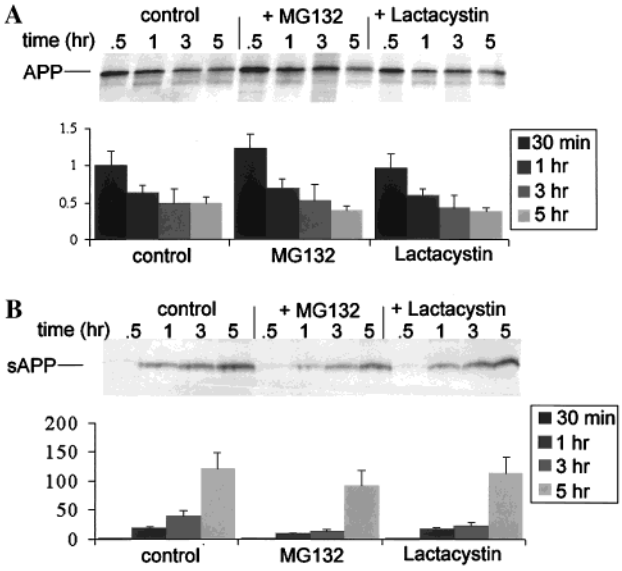

FIGURE 5: Lactacystin and MG132 do not affect APP production, secretion, or turnover. NT2N neurons were metabolically labeled for 30 min, and chased in the presence or absence of MG132 or lactacystin for the indicated times. (A) Intracellular and (B) secreted APP were immunoprecipitated using Karen, a polyclonal antiserum directed against APP, and resolved on 7.5% glycine gels. Means and standard errors of quantitations from three experiments are shown below each representative gel.

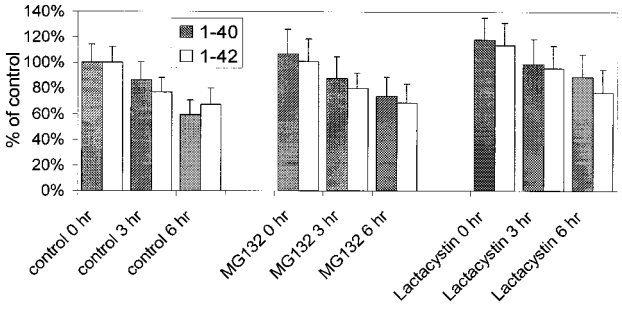

FIGURE 6: Lactacystin and MG132 do not inhibit turnover of Aβ. NT2N neurons were pretreated with cycloheximide for 3 h to inhibit protein translation and allow APP turnover. Cells were then treated with cycloheximide or with cycloheximide plus lactacystin or MG132 for the indicated times. Cells were lysed with formic acid, and Aβ1−40 and Aβ1−42 levels were quantitated by sandwich-ELISA. Mean results and standard errors are shown (three separate experiments, done in either single sets or duplicates).

turnover of APP itself or APP-derived species in the ER. To this end, we examined the effects of MG132 and lactacystin on APP production, turnover, and secretion as well as on turnover of Aβ itself. We found that neither lactacystin nor MG132 affected the turnover of full-length APP or secretion of APP, indicating that the proteasome was not involved in the processing or degradation of these species (Figure 5A,B). To test the involvement of the proteasome in turnover of Aβ, we treated NT2N neurons with cycloheximide to prevent protein synthesis, and measured endogenous levels of Aβ in the presence and absence of MG132 or lactacystin over time. This approach was needed (rather than a standard pulse−chase analysis) because immunoprecipitation of Aβ after formic acid extraction is not quantitative (*20*). Figure 6 shows that neither lactacystin nor MG132 significantly affected Aβ turnover over a 6 h period, suggesting that the proteasome is not a major pathway for degradation of intracellular Aβ.

Generation of Aβ requires cleavage of C99 by γ-secretase. If the proteasome also degrades C99, proteasomal inhibition could increase the availability of C99 for γ-secretase cleavage and thus increase Aβ production. To test this hypothesis, we directly expressed C99 fused to a signal sequence using SFV-C99. Overexpression of this construct, rather than APP, was required for accurate quantitation of turnover since after pulse-labeling of APP, C-terminal fragments are simultaneously produced (from the large pool of previously labeled APP) and turned over. Direct expression of C99 overcomes this problem since during the chase interval no additional labeled C99 is generated. SFV-C99-infected neurons were metabolically labeled for 1 h and chased for up to 8 h in the presence or absence of lactacystin, prior to immunoprecipitation of C99 from cell lysates. As shown in Figure 7A, expression of SFV-C99 increased recovery of C99 by approximately 8-fold over endogenous levels (compare lanes 1 and 2). Lactacystin treatment did not significantly alter the synthesis of endogenous or overexpressed C99 (data not shown). Significantly, lactacystin treatment decreased turnover of C99 over 8 h (compare lanes 6 and 12). After 8 h of chase in the absence of lactacystin, only 11% (±4%) of C99 remained, while after 8 h of chase in the presence of lactacystin, 45% (±5%) of C99 remained. The slower turnover of C99 in the presence of lactacystin suggests that C99 may be degraded by a proteasome-dependent pathway. As expected, C99 is partially turned over even during lactacystin treatment, since lactacystin does not inhibit the γ-secretase(s) that cleave C99. Thus our data predict that both the proteasome and the cellular γ-secretase(s) play a role in turnover of intracellular C99.

Since proteasome inhibition increased generation of Aβ1−42 from ER/IC-restricted APP (Figure 2), we hypothesized that the proteasome might be a specific site for the turnover of C99 produced in the ER/IC. To test this hypothesis, we restricted C99 expression to the ER using Brefeldin A (BFA), which causes a redistribution of the Golgi into the ER (*32*−*34*). As shown in Figure 7B, BFA treatment slowed the turnover of C99. While only 11% (±4%) of C99 was recovered after 8 h of chase in the absence of BFA (Figure 7A, lane 6), 30% (±5%) of C99 was recovered after 8 h of chase in the presence of BFA (Figure 7B, lane 6). This suggests that a fraction of the activity that turns over C99 is localized in post-ER compartments. γ-Secretase cleavage of C99 to generate Aβ1−40 in the TGN (*13*) may account for a fraction of the post-ER turnover of C99 that we have observed. Since BFA treatment slowed but did not inhibit turnover of C99, there must be mechanisms for turnover of C99 within the ER. Indeed, we know that γ-secretase cleavage of C99 in the ER is one such mechanism. To test if the proteasome also turns over C99 from the ER, we tested the effects of lactacystin in the presence of BFA. Interestingly, lactacystin further slowed the turnover of C99 in the presence of BFA, and after 8 h of chase 57% (±8%) of C99 could be recovered (Figure 7B, lane 12). The slowed turnover of C99 in the presence of lactacystin and BFA indicates that C99 in the ER is indeed turned over by a proteasome-dependent mechanism.

Since Aβ1−42 production from endogenous APP was increased by the proteasome inhibitors lactacystin and MG132 (Figures 1B and 4B), we asked if lactacystin also increased Aβ1−42 production from overexpressed C99. NT2N neurons were infected with SFV-C99 and treated with

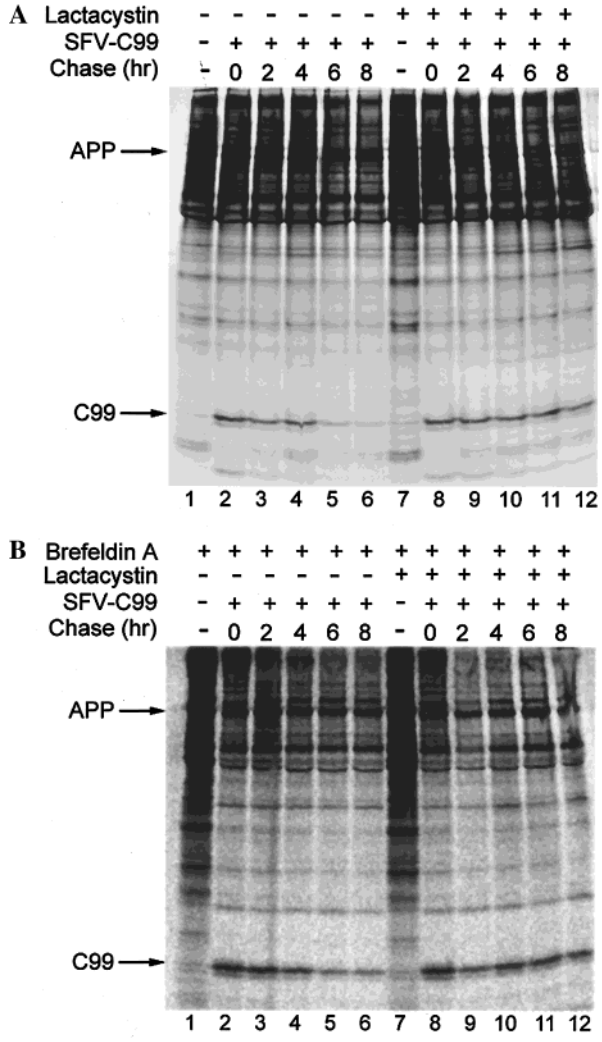

FIGURE 7: Lactacystin decreases turnover of C99 in the ER/IC. (A) SFV-C99-infected NT2N neurons were metabolically labeled for 1 h and chased for the indicated times in the absence (lanes 2−6) or presence (lanes 8−12) of lactacystin. Uninfected NT2N neurons metabolically labeled for 2 h in the absence or presence of lactacystin were included as controls (lanes 1 and 7, respectively). APP C-terminal fragments were immunoprecipitated with 2493 from cell lysates and resolved on a 10/16.5% step gradient Tris−Tricine gel. A representative example from three experiments is shown. (B) SFV-C99-infected NT2N neurons were metabolically labeled for 1 h in the presence of BFA and chased for the indicated times in the presence of BFA and absence (lanes 2−6) or presence (lanes 8−12) of lactacystin. Uninfected NT2N neurons metabolically labeled for 2 h in the presence of BFA and absence or presence of lactacystin were included as controls (lanes 1 and 7, respectively). APP C-terminal fragments were immunoprecipitated as before, and a representative example from three experiments is shown.

or without lactacystin for 8 h, and Aβ1−40 and Aβ1−42 levels in formic acid extracted cell lysates were quantitated by sandwich-ELISA. Lactacystin increased Aβ1−42 levels by 32% (±4%) in SFV-C99-infected neurons (data not shown). Similarly, in C99-expressing BFA-treated neurons, lactacystin increased Aβ1−42 levels by 40% (±8%) over 8 h (data not shown). Lactacystin treatment did not significantly alter Aβ1−40 levels. Since lactacystin increased Aβ1−42 production and concomitantly decreased turnover of C99, we hypothesize that upon proteasome inhibition a new equilibrium is reached in which increased levels of ER/IC-resident APP C-terminal fragments are degraded by γ-secretase rather than by the proteasome.

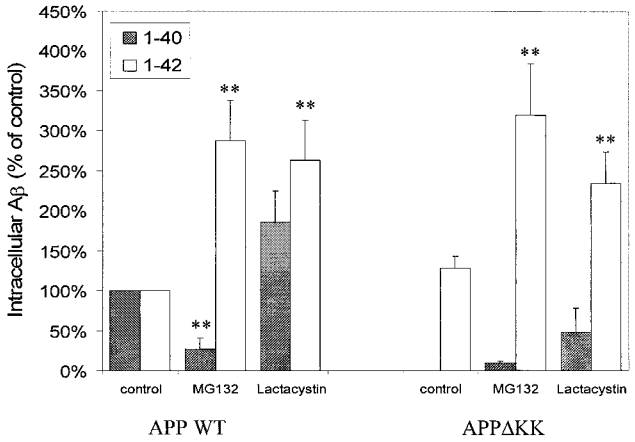

FIGURE 8: Effects of MG132 and lactacystin on Aβ production in CHO cells. CHO-Pro5 cells were infected with SFV-APP or SFV-APPΔKK and incubated in the presence or absence of 50 μM MG132 or 20 μM lactacystin for 14 h. Cells were extracted in formic acid, and intracellular Aβ1−40 and Aβ1−42 were quantitated by sandwich-ELISA. Mean results and standard errors are shown (four separate experiments, each done in duplicate). (**) $p < 0.005$ (paired t-test).

Effects of MG132 and Lactacystin on Aβ Production in CHO Cells. Since neurons and nonneuronal cells process APP differently, we asked if nonneuronal cells also generate Aβ in the ER/IC via a distinct γ-secretase which competes with the proteasome for substrate avaibility. However, unlike neurons, nonneuronal cells do not express high enough levels of APP to reliably detect Aβ. Thus, we expressed APPwt and APPΔKK in CHO cells using recombinant SFV vectors and tested the effects of MG132 and lactacystin on these cells. As in neurons, MG132 selectively inhibited secretion of Aβ1−40 from CHO cells while resulting in increased levels of intracellular Aβ1−42 (Figure 8 and data not shown). As in neurons, lactacystin did not have a significant effect on Aβ secretion from CHO cells (not shown) but did lead to increased levels of intracellular Aβ1−42 (Figure 8). These results suggest two qualitative similarities between APP processing in CHO cells and in neurons. First, in both cell types, a MG132-sensitive protease generated secreted Aβ1−40, while an MG132-insensitive protease generated Aβ1−42 in the ER/IC. Second, proteasome inhibition increased recovery of Aβ1−42 produced in the ER/IC. However, while MG132 caused only a modest, nonsignificant decrease in intracellular Aβ1−40 in neurons, it decreased recovery of Aβ1−40 from CHO cells by 80%. The greater effect of MG132 on intracellular Aβ1−40 levels in CHO cells compared to neurons may reflect the use of different secretases by these two cell lines to produce intracellular Aβ1−40 or may result from a faster turnover or secretion of intracellular Aβ in CHO cells than in neurons.

DISCUSSION

Increased production of Aβ1−42 relative to Aβ1−40 may play a key role in the pathogenesis of a subset of FAD cases. Although the role of abnormal Aβ production in the etiology of sporadic AD remains unclear, modulation of Aβ generation may nonetheless be a useful therapeutic intervention in many AD patients. Thus, several recent studies have examined the effects of peptide−aldehyde protease inhibitors on Aβ secretion (*22*, *23*). MG132 and related compounds

effectively inhibit secretion of Aβ1−40, but the effects on Aβ1−42 secretion vary from moderate decreases to moderate increases depending on cell type and concentration of the inhibitor used. These data suggest that secreted Aβ1−40 and Aβ1−42 are generated by secretases with distinct properties. However, these studies did not examine the effects of the peptide−aldehyde protease inhibitors on the production of intracellular Aβ. Neurons as well as many nonneuronal cell lines uniquely generate Aβ1−42 in the ER/IC, with much of this material entering an insoluble, intracellular pool. Interestingly, this compartment is also the site where PS1 and PS2 are localized (*35*, *36*). Since mutations in these genes account for the majority of early-onset FAD cases, and expression of FAD-associated PS1 and PS2 mutations results in increased ratios of Aβ1−42/1−40 (*2*−*4*), colocalization of the presenilins with a major site of constitutive Aβ1−42 production raises the possibility that alterations in Aβ production by the ER/IC pathway may play an important role in AD pathogenesis.

We examined the effects of MG132 on the production of secreted and intracellular Aβ species in NT2N neurons, and found that it inhibited secretion of Aβ1−40 nearly 2-fold more than secretion of Aβ1−42 from human NT2N neurons. Further, inhibition of Aβ1−42 secretion was only observed at high concentrations of MG132, while inhibition of Aβ1−40 secretion occurred at much lower concentrations. Concomitant to decreasing secretion of Aβ1−40, MG132 led to the accumulation of APP C-terminal fragments. Levels of C83, C88, and C99 were all increased to similar extents, suggesting that all three of the major APP C-terminal fragments are substrates for the same or similar γ-secretase(s). Further, the bulk of these fragments were generated in post-ER/IC compartments. The accumulation of C99 (which contains the entire Aβ domain) in the context of selective inhibition of Aβ1−40 secretion suggests that cleavage to generate secreted Aβ1−40 accounts for at least a fraction of the turnover of this fragment. These results are consistent with previous studies that have reported selective inhibition of Aβ1−40 (relative to Aβ1−42) by MG132 and related peptide aldehydes in nonneuronal cells (*22*). However, in contrast to these studies, we did not observe an increase in Aβ1−42 secretion at any concentration of MG132 tested. This may reflect use of γ-secretases with different properties by neuronal versus nonneuronal cells.

In contrast to its effects on secreted Aβ, MG132 did not significantly decrease levels of intracellular Aβ1−40 in NT2N neurons. This may be due to the fact that a large fraction of intracellular Aβ is present in a stable, insoluble pool. Since this pool is turned over very slowly, decreases in intracellular Aβ levels may be difficult to detect, even in the absence of Aβ production. Indeed, MG132 decreased Aβ1−40 in the RIPA-soluble pool by 37% while levels of Aβ1−40 in the RIPA-insoluble (formic acid extracted) pool remained unchanged (Figure 1B). Thus, we cannot rule out the possibility that MG132 may inhibit production of intracellular Aβ1−40 in neurons, but due to the slow turnover of the insoluble pool of Aβ this effect may not have been detected during the time course of our experiments. This idea is further supported by the dramatic reduction of intracellular Aβ1−40 in CHO cells upon treatment with MG132.

Interestingly, we found that MG132 actually increased the recovery of intracellular Aβ1−42. Since overproduction of Aβ1−42 is implicated in the pathogenesis of AD, we investigated the mechanism by which MG132 had this effect. We examined the role of the proteasome in Aβ generation and found that a more specific proteasome inhibitor, lactacystin, induced a similar increase in recovery of ER/IC-generated Aβ. Moreover, this increase in intracellular Aβ1−42 levels was not caused by inhibition of Aβ turnover but was rather due to increased Aβ1−42 production. While previous studies found that lactacystin treatment modulated α-secretase activity in 293 cells (*37*, *38*), we found no effect of lactacystin or MG132 on α-secretase activity in NT2N neurons. Since we controlled for any confounding effects of proteasome inhibition on early stages of APP processing, including APP production and turnover, as well as α- and β-secretase cleavage, we infer that proteasome inhibition increased Aβ1−42 production at the step of γ-secretase cleavage, and that the γ-secretase in the ER/IC is not inhibited by MG132.

How might inhibition of the proteasome increase production of Aβ1−42? Potentially, the proteasome is involved in the normal metabolism of γ-secretase (or a γ-secretase cofactor) itself. If so, proteasome inhibition should lead to increased γ-secretase activity and, as a consequence, increased APP C-terminal fragment turnover. However, lactacystin treatment decreased the turnover of C99, making this hypothesis unlikely. Rather, our data support a role for the proteasome in degradation of C99 and other APP C-terminal fragments. Indeed, a degradative turnover of an APP transmembrane probe has been observed in the ER of 293 cells (*39*). Inhibition of this process would increase the availability of these fragments for γ-secretase cleavage, and thus increase Aβ production. Since proteasome inhibition increased production of Aβ1−42 at the step of γ-secretase cleavage in the ER/IC, while simultaneously decreasing turnover of C99, we hypothesize that the proteasome may degrade this fragment of APP. Thus, the involvement of the proteasome in APP processing may have important consequences for regulation of intracellular Aβ production and the pathogenesis of AD.

To test if nonneuronal cells also utilize a distinct γ-secretase to generate Aβ1−42 in the ER/IC, we examined the effects of MG132 on Aβ production in CHO cells. As in neurons, MG132 inhibited Aβ1−40 secretion and increased intracellular Aβ1−42 levels in CHO cells. However, we detected several notable differences. First, we observed incomplete inhibition of Aβ secretion even at very high concentrations of MG132 in neurons, while Aβ secretion was completely abolished by these doses in nonneuronal cells. This may reflect only partial inhibition of neuronal γ-secretase activity by MG132 or the presence of multiple γ-secretases involved the production of secreted Aβ by neurons, only a subset of which are inhibited by MG132. Second, MG132 decreased intracellular Aβ1−40 levels to a much greater extent in CHO cells than in neurons. This may reflect the faster turnover or secretion of Aβ in CHO cells than in neurons. Thus, intracellular Aβ1−40 levels drop quickly in CHO cells after Aβ production is blocked. Similarly, the greater effect of MG132 on intracellular Aβ1−42 levels in CHO cells as compared to neurons may also reflect differences in the metabolic rate. Since APP is processed more rapidly in CHO cells than in neurons, any change in APP

production in CHO cells would be reflected by a greater change in steady-state Aβ levels.

Our results highlight the differential distribution of γ-secretases in human neurons and in nonneuronal cells. We found that while MG132 inhibited the γ-secretase that produced intracellular and secreted Aβ1−40, it only partially inhibited the production of secreted Aβ1−42 and increased the production of intracellular Aβ1−42. Thus, the ER/IC compartment contained a γ-secretase that utilized C99 as its predominant substrate, produced only Aβ1−42, and was insensitive to MG132. We further showed that this γ-secretase competed with the proteasome for turnover of APP C-terminal fragments and was highly sensitive to substrate concentration since increased availability of C99 dramatically increased production of Aβ1−42. In contrast, later organelles contained γ-secretase(s) that cleaved C99, C88, and C83, produced mainly Aβ1−40 rather than Aβ1−42, and were sensitive to MG132. While these γ-secretase(s) generated the bulk of secreted Aβ, p3, and other amino-truncated Aβ fragments, the Aβ1−42 generated by the ER/IC γ-secretase remained entirely intracellular. Thus, our data raise the possibility that compounds designed to inhibit Aβ secretion may have unpredicted effects on intracellular Aβ production, and suggest the ER/IC Aβ1−42 producing γ-secretase as a novel target for pharmacological intervention in AD.

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