Cell-type and Amyloid Precursor Protein-type Specific Inhibition of Aβ Release by Bafilomycin A1, a Selective Inhibitor of Vacular ATPases*

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Jeroen Knops, Susanna Suomensaaari, Michael Lee, Lisa McConlogue, Peter Seubert, and Sukanto Sinha
From Athena Neurosciences, Inc., South San Francisco, California 94080

Treatment of human 293 cells transfected with amyloid precursor protein (APP)K650N-M506L (the "Swedish" mutation) with a specific inhibitor of the vacuolar H+-ATPases, bafilomycin A1 (baf A), leads to a potent inhibition of the release of the Aβ peptide. This is accompanied by a selective inhibition of β-secretase activity. Surprisingly, baf A did not inhibit the production of Aβ from either wild-type APP (WT APP) or from APPV717I (the "Hardy" mutation), expressed in the same cell type. In contrast, the robust production of Aβ from a human neuroglioma-derived cell line (H8065) transfected with WT APP, or from primary human mixed brain cultures (HMBC) expressing genomic WT APP, were also effectively inhibited by baf A. The inhibition of Aβ production from the HMBC was also accompanied by the inhibition of s-APP release. No inhibition of a-secretase release was seen in any of the cell types tested. These results indicate that intracellular acidic processes are rate-limiting for β-secretase cleavage and Aβ production from SW APP, but not WT APP, in the peripheral 293 cell line. Furthermore, such acidic processes also play a rate-limiting role in Aβ release from human central nervous system-derived cells, including HMBC. Differential trafficking of the SW APP into an acidic compartment conducive to β-secretase cleavage and Aβ release could be one explanation for the increased production of Aβ observed on expression of this mutation.

The pathogenesis of Alzheimer's disease involves the deposition of the Aβ peptide as an early, and perhaps causative, event in the subsequent maturation of such deposits into senile or neuritic plaques (1). The secretion of Aβ by human mixed brain cultures (HMBC) (2), as well as a number of APP-transfected cell lines in culture (3, 4), suggests that Aβ is produced by a normal metabolic pathway of the cell. However, the cellular mechanisms that lead to the production of Aβ from APP are not well understood. One metabolic fate of newly synthesized APP appears to be constitutive secretion of the bulk of its ectodomain as α-secretase (5), after an endoproteolytic non-amyloidogenic cleavage by an unidentified enzyme, "α-secretase" (6). However, other cellular processing pathways must also contribute to APP metabolism, since a substantial proportion of APP metabolism results in lysosomal degradation of the mature, full-length protein (7–9). Although intermediates of APP degradation containing the entire Aβ peptide region accumulate in the cell upon treatment with lysosomal protease inhibitors (8), such inhibitors do not have any effect on Aβ production (10). Considerable doubt is thus cast upon the relevance of such "potentially amyloidogenic" intermediates to the formation of Aβ. More relevant to Aβ formation, however, are observations on the analysis of a truncated secreted form of APP that appears to terminate at Met-596 (β-s-APP) (11). These forms, produced at a relatively low level by the 293 cell line transfected with WT APP, form a larger proportion of the total s-APP produced by HMBC, which correlates well with the higher level of Aβ production in the primary cultures. This suggests that an alternate enzymatic activity, dubbed "β-secretase," is responsible for the production of the truncated secreted form, and, by inference, a cell-associated carboxyl-terminal fragment starting at Asp-597. Subsequent cleavage at Val-637 or Ala-639 in the transmembrane domain of such a fragment by a second protease would then release Aβ into the extracellular medium.

We have utilized 293 cells stably transfected with either WT APP751 or APP-NL, incorporating the so-called "Swedish" mutation (APPK590N-M690L), as a system for investigating the role of such an alternative secretory cleavage in the cellular production of Aβ peptide. Tissue culture expression of this double mutation, identified as pathogenic for Alzheimer's disease in the affected kindred (12), results in a substantial stimulation of Aβ production (13), without any significant effect on total s-APP released into the CM. Direct sequencing of the Aβ produced by these cells shows that the primary product is Aβ 1–40 (14), indicating that the presumptive site of β-secretase cleavage has not been altered by the presence of the double mutation. Preliminary data indicated that in comparison to wild-type APP751, transient transfections incorporating the Swedish mutation produce more of a truncated s-APP (data not shown). This form was not immunoprecipitable with antibodies that recognize epitopes specific to the amino-terminal region of the Aβ peptide, consistent with the previously described truncated cleavage. The relatively high levels of Aβ produced by stable transfecants incorporating the Swedish mutation allowed the design of experiments that tested the ability of specific compounds to alter or affect the relative balance between normal secretase cleavage and β-secretase cleavage. We were especially interested in identifying whether α-secretase and β-secretase activity are separate manifestations of the same enzymatic process, or whether they occur in different intracellular compartments. Although acidotropic amines have previously been shown to have an inhibitory effect on Aβ production (10), the lack of specificity of such compounds precluded a clear identification of cellular mechanisms that contributed to Aβ production. Since the vacuolar-type H+-ATPase(s) are thought to be ubiquitously involved in the maintenance of the acidic milieu in diverse intracellular acidic organelles (16), we de-
FIG. 1. Immunoprecipitation of Aβ from conditioned medium of bafylomicin A1-treated 293 cells. 293 cells (transformed human embryonic kidney cells) overexpressing APP751 wild-type (APP-WT) or APP751(C99,MM1G) (APP-NL) were grown to semiconfluence and subsequently metabolically labeled with [35S]methionine for 4 h in the absence or presence of 1 μM bafylomicin A1. Aliquots of supernatants equivalent to 100 μg of cell lysate protein were immunoprecipitated with 5 μl of R1282 antisera.

Inhibited test bafylomicin A1 (baf A), a potent and selective inhibitor of the vascular-type II′-ATPase(s) (17) as to its effect on APP metabolism, including Aβsecretion.

EXPERIMENTAL PROCEDURES

293 cells (transformed human embryonic kidney cells) overexpressing either APP751 wild-type (APP-WT) or APP751(C99,MM1G) (APP-NL) were grown to semiconfluence and subsequently metabolically labeled in the absence or presence of 1 μM baf A. Human fetal cortical cultures were prepared as described previously (2). Cultures were grown at least 30 days in vitro and were subsequently metabolically labeled for 16 h in the absence or presence of 1 μM baf A. Bafylomicin A1 was obtained from Dr. K. Altendorf (Osnabrück, Germany). Aβ was immunoprecipitated from aliquots of conditioned medium (CM) using R1282 antisera (obtained from Dr. D. Selkoe (Brigham and Women’s Hospital, Boston, MA)). Total s-Aβ was similarly determined by immunoprecipitating aliquots of the CM with α-5 (5). α-s-Aβ was separately immunoprecipitated with 6C8 (11), and β-s-Aβ immunoprecipitated with 92 (wild-type APP) (10) or SW192 (APP-NL) antibodies. The SW192 polyclonal was produced essentially according to the method described for the wild-type 92 antibody (11), with the exception that the synthetic pentapeptide antigen ended with NL rather than KM. Cell-associated forms of APP were immunoprecipitated from aliquots of the cell lysate protein with α-5 (5).

Metabolic labeling and immunoprecipitations were carried out as described before (5). Labeling was carried out for the indicated period of time with 100 μCi/ml L-[35S]methionine in methionine-free medium containing 10% heat-inactivated, dialyzed fetal bovine serum. After labeling, CM were gently removed and the cells lysed in 1 ml of lysis buffer (50 mm Tris pH 8.0, 0.15 M NaCl, 20 mm Na-EDTA, 1% sodium deoxycholate, 1% Triton X-100, and 0.1% SDS) and the crude lysates were cleared of debris by centrifugation at 100,000 × g for 4 h at 5°C. Approximately 100 μg of total cell lysate or the equivalent amount of supernatant were immunoprecipitated with 5 μl of anti-6C8 or 5 μg of antibody and 10 μl of protein A-Sepharose beads (Pharmacia Biotech Inc.). Immunoprecipitated protein was solubilized from the beads after boiling in reducing Laemmli buffer. Solubilized protein was separated by SDS-polyacrylamide gel electrophoresis on 10–20% Tricine gradient gels or 6% Tris-glycine gels. The gels were fixed, soaked in Amplify (Amersham), dried, and exposed to Kodak X-AR radiography film.

RESULTS AND DISCUSSION

Treatment of cells stably transfected with SW-APP with baf A at 1 μM leads to a virtually complete inhibition of Aβ release (-92%) from the transfected cells (Fig. 1; Table 1) as determined by the loss of total Aβ (Fig. 1A) and immunoprecipitable by R1282. In contrast, there appears to be a stimulation of secretion of the 3-kDa APP-derived fragment (+234%), previously determined to start at either Leu-17 or Val-18 (18). This fragment is presumed to derive from the cell-associated ~9–10-kDa APP carboxyl-terminal segment generated as a result of α-secretory cleavage.

Immunoprecipitation of s-Aβ from the CM with two separate APP antibodies revealed that although total s-Aβ production was somewhat diminished (-9%) from the SW APP-transfected cells (Fig. 2A; Table 1), there was actually a modest increase in α-s-Aβ secretion (+11%) (Fig. 2B; Table 1). In contrast, there is a dramatic inhibition of β-s-Aβ release (-55%) (Fig. 2C; Table 1) in the presence of baf A. The less dramatic inhibition of total s-Aβ appears to result as a consequence of this as well, since, on careful examination of the immunoprecipitated total s-Aβ, which resolves into a closely spaced doublet, it appears that bafylomicin treatment abolishes the shorter (β-s-Aβ) component, accompanied by an increase in intensity of the longer (α-s-Aβ) species (data not shown). Treatment with baf A thus does not inhibit α-s-Aβ, but selectively inhibits β-s-Aβ secretion, along with Aβ secretion.

Investigation of the fate of full-length APP in the cell lysates revealed that there is a selective increase in the mature APP full-length protein, but no detectable change in the immature holoprotein (Fig. 3). The mobilities of either species are not altered on SDS-polyacrylamide gel electrophoresis, suggesting that the effects observed with baf A are probably not owing to effects on protein glycosylation, for example.

Surprisingly, baf A failed to inhibit Aβ production from the
WT APP (Fig. 1), expressed in the same cell type. In fact, increased Aβ production (+48%) was actually observed (Table I). Treatment with baf A, however, stimulated α-s APP production (+125%) (Fig. 2, A and B; Table I) and increased levels of mature holoprotein (Fig. 3) detected in the cell lysates. These results were similar to that observed with the SW APP. The lack of inhibition of Aβ was paralleled by an undiminished secretion of WT β-s APP (+100%) (Fig. 2C). A slightly decreased electrophoretic mobility of the β-s APP also indicated baf A-induced alterations in post-translational processing events.

The differential effect of baf A on Aβ production from the modified APP in comparison to the WT APP prompted us to investigate the effect of the drug on a separate pathogenetic APP mutation, that at codon 717 (19). As with WT APP, treatment of 293 cells stably transfected with APP695V717I with baf A did not lead to any detectable inhibition of Aβ production (Fig. 4); again, as with the WT APP, a small increase was seen. Unlike the Swedish mutation, the cellular expression of this mutation does not lead to an overproduction of the Aβ peptide. It has been reported, instead, that the expression of this mutation leads to a selective increase in the longer, 1–42 form of the Aβ peptide (20), which is known to be more prone to aggregation than the shorter 1–40 peptide. It thus appears that the baf A inhibitory effect observed with the SW APP in these cells is specific to the SW mutation and is not shared by the Hardy mutation.

We were interested in knowing whether the insensitivity to baf A treatment observed with Aβ produced from WT APP in 293 cells represents an universal phenomenon. To this end, we evaluated a human neuroglioma-derived cell line, HS683, stably transfected with APP695. Immunoprecipitation from the CM of this cell line revealed very robust production of a ~4-kDa Aβ band, comparable to the level of secretion from 293 cells transfected with the SW APP (Fig. 4). Treatment with baf A resulted in a significant inhibition of production of the Aβ fragment, sparing the 3-kDa fragment. Thus, the cell type apparently determines whether or not Aβ is produced from APP in an acidic milieu.

To investigate these puzzling observations further, HMBC, which also secrete Aβ as a result of endogenous APP processing, were treated with 1 μM baf A. CM was collected overnight and evaluated using an enzyme-linked immunosorbent assay that specifically detects Aβ in the CM from these cells (2). Greater than 75% inhibition of Aβ production was observed in these cultures (Table II). As in the 293 cells expressing the Swedish mutation, this was also accompanied by a dramatic inhibition of the β-s APP, whereas no significant effect was observed with α-s APP. At the levels of baf A tested, no cytotoxicity was evident. Removal of the drug, in fact, led to a reversal of the inhibitory effect over a 48-h recovery period (data not shown). Immunoprecipitation with R1282 from the CM of HMBC treated with baf A for 24 h confirmed the selective inhibition of release of the 4-kDa Aβ peptide (Fig. 5), without loss of the 3-kDa fragment. Therefore, as in the 293 cells expressing the SW APP, and the HS683 cells expressing WT APP, Aβ production from the primary cultures expressing genomic WT APP is thus effectively inhibited by baf A as well.

As selective and potent inhibitors of the V-ATPas, the bafylomycins are capable of inhibiting the acidification of diverse intracellular vesicles. Indeed, baf A has been shown in a number of different studies to have dramatic effects on specific cellular processes, such as phagosomal acidification, lysosomal acidification and intracellular protein degradation, endosomal carrier vesicle formation, and synaptic vesicle catecholamin uptake (21–24). The susceptibility to baf A has been used as diagnostic for the rate-limiting involvement of the V-ATPas in these various processes. The experimental observations in this report therefore strongly suggest that an intracellular acidic compartment plays a critical role in the generation and secretion of Aβ from SW APP, but not WT APP, in 293 cells. The observed increased production of Aβ with the Swedish APP may, at least in part, be due to the mutant protein being differentially trafficked into an acidic intracellular compartment in the 293 cells, much more conducive to both β-secretase cleavage and release of Aβ. Differential intracellular trafficking of the SW APP metabolites has been recently suggested to occur in polarized cells (25). By this analogy, increased trafficking of APP into more acidic compartments in HMBC and the neuroglioma cell line may explain the sensitivity to baf A inhibition of Aβ release. An alternative explanation might be that the SW-APP is much more prone to β-secretase cleavage in an acidic compartment, and the increase in pH as a consequence of baf A treatment thus has a more dramatic inhibitory effect on this mutation, but not on WT. However, one would then have to additionally posit that the β-secretase protease has different pH optima toward SW as compared to the WT
APP. In the absence of an isolated β-secretase enzymatic activity, it may not be possible to choose between these separate explanations.

These observations, additionally, also directly link Aβ production to the occurrence of the β-secretase cleavage, reflected in the release of β-s-APP. In both the SW APP-transfected 293 cells, as well as in the HMBC, the inhibition of Aβ release by baf A also strongly inhibited the release of the β-s-APP as well.

The lack of any negative effect of the drug on either α-s-APP secretion or the production of the 3-kDa APP fragment in all of the cell types tested also suggests that α-secretase activity, in contrast to β-secretase, does not require an acidic environment.

In one study (26), treatment with 500 nM baf A led to the inhibition of maturation and targeting of two separate lysosomal enzymes, processes known to involve acidic compartments such as the endosome and lysosome; yet, no effect on the concurrent processing and secretion of either proalbumin, or proC3 was detected. The latter events, mediated presumably by the constitutive secretory pathway involving the trans-Golgi network, appear to be insensitive to alterations of intravesicular pH. The insensitivity of α-secretase activity toward baf A also confirms previous conclusions (15) regarding the likely cellular locus for this constitutive secretory cleavage.

In both SW and WT APP-transfected 293 cells, treatment with baf A is accompanied by a significant increase in α-s-APP (Fig. 2), as well as an increase in the level of cell-associated mature holoprotein (Fig. 3). Lysosomal degradation of APP has been suggested previously to be an important component of total APP metabolism in 293 cells expressing WT APP (8). The inhibition of such degradative processes by baf A may lead to the recycling of both WT and SW APP holoprotein into the constitutive secretory pathway. Contrary to its effect on the 293 cells, baf A affected only relatively modest changes on either mature holoprotein levels or on α-secretase cleavage in either HMBC or the HS683 cells (data not shown).

The identities of the processes sensitive to bafilomycin treatment, apparently crucial to Aβ/β-secretase APP formation, are not clear from these experiments. It is also unclear whether the presumed elevation of intravesicular pH that leads to the β-s-APP and Aβ release inhibition is owing to a direct effect on the enzyme(s) involved, or owing to indirect effects, mediated perhaps by inhibition of vesicle fusion events, for example. In any case, the availability of compounds such as baf A stimulate further experimental work aimed at pinpointing the cellular compartments which mediate Aβ production in different cells.

Knowledge gained from such studies would lead to increased understanding of the cellular enzymology involved in the formation of Aβ, which would be in the critical path to designing effective therapeutic agents for Alzheimer’s disease.

REFERENCES