Insensitivity to Aβ42-lowering Nonsteroidal Anti-inflammatory Drugs and γ-secretase Inhibitors Is Common among Aggressive Presenilin-1 Mutations*

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Aβ42-lowering nonsteroidal anti-inflammatory drugs (NSAIDs) constitute the founding members of a new class of γ-secretase modulators that avoid side effects of pan-γ-secretase inhibitors on NOTCH processing and function, holding promise as potential disease-modifying agents for Alzheimer disease (AD). These modulators are active in cell-free γ-secretase assays indicating that they directly target the γ-secretase complex. Additional support for this hypothesis was provided by the observation that certain mutations in presenilin-1 (PS1) associated with early-onset familial AD (FAD) change the cellular drug response to Aβ42-lowering NSAIDs. Of particular interest is the PS1-ΔExon9 mutation, which provokes a pathogenic increase in the Aβ42/Aβ40 ratio and dramatically reduces the cellular response to the Aβ42-lowering NSAID sulindac sulfide. This FAD PS1 mutant is unusual as a splice-site mutation results in deletion of amino acids Thr291–Ser319 including the endoproteolytic cleavage site of PS1, and an additional amino acid exchange (S290C) at the exon 8/10 splice junction. By genetic dissection of the PS1-ΔExon9 mutation, we now demonstrate that a synergistic effect of the S290C mutation and an additional amino acid exchange (S290C) at the exon 8/10 splice junction. By genetic dissection of the PS1-ΔExon9 mutation, we now demonstrate that a synergistic effect of the S290C mutation and the lack of endoproteolytic cleavage is sufficient to elevate the Aβ42/Aβ40 ratio and that the attenuated response to sulindac sulfide results partially from the deficiency in endoproteolysis. Importantly, a wider screen revealed that a diminished response to Aβ42-lowering NSAIDs is common among aggressive FAD PS1 mutations. Surprisingly, these mutations were also partially unresponsive to γ-secretase inhibitors of different structural classes. This was confirmed in a mouse model with transgenic expression of the PS1-1166P mutation, in which the potent γ-secretase inhibitor LY-411575 failed to reduce brain levels of soluble Aβ42. In summary, these findings highlight the importance of genetic background in drug discovery efforts aimed at γ-secretase, suggesting that certain AD mouse models harboring aggressive PS mutations may not be informative in assessing in vivo effects of γ-secretase modulators and inhibitors.

Compelling evidence indicates that aberrant production and accumulation of amyloid β (Aβ)3 peptides is a central event in the pathology of familial and sporadic forms of Alzheimer disease (AD), the most common age-related neurodegenerative disorder, with around 18 million patients worldwide (1, 2). Ongoing efforts to develop disease-modifying therapies mainly aim to block production, to hinder aggregation, or to enhance clearance of Aβ peptides (3). Because Aβ peptides are generated through sequential proteolysis of the amyloid precursor protein (APP) by β- and γ-secretase, these aspartyl proteases are prime drug targets for pharmacological intervention in AD. γ-Secretase is a multiprotein complex consisting of four essential proteins, presenilin (PS), nicastrin, Aph-1, Pen-2; the PS proteins seem to harbor the active site of the enzyme (4). For γ-secretase, a large number of highly potent inhibitors have been developed, and few have been evaluated in phase I clinical trials (5, 6). However, preclinical studies have also revealed substantial mechanism-based toxicity, which can be largely attributed to disruption of processing and function of the γ-secretase substrate NOTCH (5, 6). More recently, some nonsteroidal anti-inflammatory drugs (NSAIDs) including ibuprofen and sulindac sulfide were shown to selectively lower production of the highly amyloidogenic Aβ42 peptide (7–12), which has been proposed to be the disease-causing agent in AD (13). These compounds induce a shift in γ-secretase activity and concomitantly increase shorter Aβ species such as Aβ38, but they spare...
processing and function of other γ-secretase substrates including NOTCH (7, 9, 11, 12, 14). Although these findings suggested a novel explanation for epidemiological observations that chronic intake of NSAIDs lowers the risk of developing AD (12), the more significant implication seems to be that these compounds have originated a new class of γ-secretase modulators that could provide a safer alternative to conventional γ-secretase inhibitors (7, 8).

Although the molecular details are far from resolved, several arguments strongly support the hypothesis that Aβ42-lowering NSAIDs act by direct modulation of γ-secretase activity (8). First, similar to pan-γ-secretase inhibitors, Aβ42-lowering NSAIDs are active in cell-free γ-secretase assays (7, 9, 11, 15, 16). Second, studies using fluorescence lifetime imaging have provided indirect evidence that Aβ42-lowering NSAIDs change the conformation of PS1 (17). Finally, some familial early-onset AD (FAD) PS1 mutations, which induce a pathogenic increase in the Aβ42/Aβ40 ratio, have been shown to modulate the response of cultured cells to Aβ42-lowering NSAIDs (16). In this respect, overexpression of the PS1-M146L mutation profoundly enhances Aβ42 reduction after sulindac sulfide and ibuprofen treatment as compared with wild type PS1. Intriguingly, the exact opposite is observed with the PS1-ΔExon9 mutation, which strongly diminishes Aβ42 reductions after sulindac sulfide treatment (16). Remarkably similar findings have been reported with the well characterized transition-state γ-secretase inhibitor L-685,458 (18). The ability of L-685,458 to reduce Aβ production from cells expressing PS1-ΔExon9 is also substantially attenuated as compared with wild type PS1, and this effect is even more pronounced for Aβ42 levels with a maximal reduction of 50% at high inhibitor concentrations (18). However, the structural or mechanistic basis for these observations has not been determined.

The PS1-ΔExon9 mutant is unusual as a splice-site mutation results in deletion of a whole exon comprising amino acids Thr291–Ser319 and an amino acid exchange (S290C) at the exon 8/10 splice junction, whereas virtually all other FAD PS1 mutations are point mutations (19). Subsequent studies in cultured cells suggest that the S290C mutation rather than the deletion Δ291–319 mediates the pathogenic increase in Aβ42 production, as overexpression of PS1 harboring only the Δ291–319 deletion results in normal Aβ42 production (20). The Δ291–319 region further includes the endoproteolytic cleavage site of PS1 and the PS1-ΔExon9 mutant accumulates as full-length protein (21). The site of endoproteolysis was originally mapped at amino acids Thr291–Ala299 (22), but further analysis has demonstrated that a single point mutation at position 292 (M292D) prevents endoproteolytic cleavage of PS1 (23). By genetic analysis of the PS1-ΔExon9 mutation, we now show that the attenuated response to the Aβ42-lowering NSAID sulindac sulfide is partially because of the lack of endoproteolytic cleavage. A strongly diminished response to sulindac sulfide is partially because of the lack of endoproteolysis.

**EXPERIMENTAL PROCEDURES**

**Drugs and Antibodies**—The NSAID sulindac sulfide was purchased from Biomol (Plymouth Meeting, PA), γ-secretase inhibitors L-685,458 and DAPT were from Merck Biosciences (Nottingham, UK), and γ-secretase inhibitor LY-411575 was synthesized by standard chemical techniques as described (47, 48). All other chemicals were from Sigma-Aldrich except when otherwise indicated. Monoclonal antibody PSN2 raised against a synthetic peptide corresponding to amino acids 31–56 of human PS1 was kindly provided by Dr. Hiroshi Mori (24). Monoclonal antibody 26D6 recognizing residues 1–12 of the human Aβ sequence and polyclonal antibody CT-15 against the C-terminal 15 amino acid residues of human APP have been described (12). Biotinylated and nonbiotinylated versions of monoclonal antibody 6E10 recognizing amino acids 1–17 of the human Aβ sequence were purchased from Signet Laboratories (Dedham, MA).

**cDNAs Constructs**—cDNAs encoding mutants PS1-S290C, PS1-M292D, PS1-G384A, PS1-S290C/M292D, and PS1-M292D/M146L were generated with a two-step PCR mutagenesis strategy using the wild type human PS1 cDNA as template. The final PCR products were gel-purified, digested with Xhol and NotI, and cloned into the pLPCX retroviral shuttle plasmid (Clontech, Mountain View, CA). The constructs encoding PS1-P117L, PS1-L166P, and PS1-Δ291–319 were generated using the QuickChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) using plasmids pLPCX-PS1wt and pLPCX-PS1ΔExon9 as templates. Information about PCR primers and conditions is available upon request. All constructs were sequenced to verify successful mutagenesis.

**Generation of Retroviral Vectors**—For generation of pseudotyped retroviral vectors, GP2-293 packaging cells (Clontech) were transiently co-transfected with retroviral shuttle plasmids and plasmid encoding VSV-G envelope glycoprotein at a 1:1 ratio using Genejuice transfection reagent (Merck Biosciences). The medium was changed 48 h after transfection. Retroviral particles were collected for another 24 h and stored at −80°C.

**Cell Lines and Cell Culture**—Chinese hamster ovary cells with stable overexpression of wild type human APP751 (APP CHO cells) have been described previously (16) and are the parental cells for all stably transfected CHO cell lines used in this study. APP CHO cells were infected with retroviral vectors encoding PS1-S290C, PS1-M292D, PS1-P117L, PS1-L166P, PS1-G384A, PS1-Δ291–319, PS1-S290C/M292D, and PS1-M292D/M146L. All constructs were maintained in α-minimum essential medium.
supplemented with 10% fetal bovine serum, 1 mM sodium-pyruvate, 2 mM l-glutamine, and 100 units/ml penicillin/streptomycin (Invitrogen). Comparable PS1 and APP expression in all cell lines was verified by Western blot analysis. Cells were lysed in Nonidet P-40 buffer (1% Nonidet P-40, 50 mM Tris, pH 8.0, 150 mM NaCl), and total protein was separated on 10% bis-Tris gels and analyzed by Western blotting with antibodies PNS2 or CT-15.

**Dose-Response Experiments and Statistical Analysis**—Aβ40 and Aβ42 secretion of individual cell lines after sulindac sulfide or γ-secretase inhibitor treatments were compared in dose-response experiments. All cell lines intended for comparison were cultured and treated in parallel at similar cell densities. Cells were cultured in serum-containing medium and treated for 24 h with increasing concentrations of the NSAID sulindac sulfide, γ-secretase inhibitors or Me₂SO vehicle. Aβ40 and Aβ42 levels in conditioned media were then analyzed by Aβ liquid phase electrochemiluminescence assay. Duplicate measurements from each drug concentration were averaged and normalized to Me₂SO control condition. These experiments were repeated 3–5 times, and results were analyzed by one-way ANOVA with Dunnett’s post tests and drug concentration as categorical variable. For calculation of EC₅₀ values, cells were treated with 10 increasing concentrations of LY-411575, and sigmoidal curve-fit with variable slope was applied. Statistics were performed using GraphPad Prism software (GraphPad Software, San Diego).

**Aβ Liquid Phase Electrochemiluminescence Assay (LPECL)—** Aβ levels were analyzed by LPECL assay. The biotinylated Aβ-specific antibody 6E10 was used as capture antibody. The C-terminal-specific Aβ antibodies BAP24 and BAP15 were generated as described previously (25) and labeled with TAG electrochemiluminescent label according to the manufacturer’s protocol (Bioveris, Gaithersburg, MD). Labeled antibodies were purified from unincorporated label using a PD-10 column (GE Healthcare) and stored in phosphate-buffered saline containing 0.1% sodium azide at –80 °C. Culture media were collected following conditioning for 24 h, and cell debris was removed by centrifugation. Complete protease inhibitor mixture (Roche Diagnostics) was added, and supernatants were stored at –80 °C until quantification by LPECL. Before use, M-280 paramagnetic beads (Invitrogen) were diluted with assay buffer (50 mM Tris, 60 mM NaCl, 0.5% bovine serum albumin, 1% Tween 20, pH 7.4). For detection of Aβ peptides, conditioned media (10 µl for Aβ40 and 50 µl for Aβ42) were incubated with 50 µl of beads and 25 µl of each labeled antibodies 6E10-bio and BAP24-TAG (for Aβ40) or BAP15-TAG (for Aβ42). Incubation was performed in a final volume of 250 µl for 3 h with gentle shaking. Synthetic Aβ40 and Aβ42 peptides (Bachem AG, Bubendorf, Switzerland) were used to generate standard curves. These Aβ peptides were solubilized in Me₂SO at a concentration of 1 mg/ml, aliquoted, and stored frozen at –80 °C. Immediately before use, peptides were diluted in culture media to 16–2000 pg/ml. Electrochemiluminescence was quantified using an M-Series M8 analyzer (Bioveris).

**Animals and Dosing—** Tg2576 mice expressing “Swedish” mutant APP (KM670/671NL) under the prion protein promoter (26) were purchased from Taconic (Hudson, NY).

**APP**P51 and human PS1-L166P under the control of a neuron-specific Thy-1 promoter element have been described (27). Five-month-old Tg2576 mice or 2-month-old APPP51 mice were orally dosed with 10 mg/kg γ-secretase inhibitor LY-411575 dissolved in 0.5% tylose or vehicle alone. Mice were sacrificed 3 h post-administration, and brains were homogenized in Tris buffer containing 0.2% Triton X-100. After centrifugation for 1 h at 200,000 × g, soluble Aβ42 levels in the supernatant were quantified by sandwich enzyme-linked immunosorbent assay using commercial kits from BIO-SOURCE International (Camarillo, CA) according to the manufacturer’s protocol. For enzyme-linked immunosorbent assay determination of soluble Aβ40 levels, monoclonal antibody 6E10 (Sigma) was employed as capture antibody and combined with an alkaline phosphatase-coupled Aβ40-specific detection antibody. All animal studies were performed according to German animal welfare law.

**RESULTS**

**Genetic Dissection of the PS1-ΔEoxon9 Mutation**—The PS1-ΔEoxon9 mutation has been shown to dramatically attenuate the response of cells to the Aβ42-lowering NSAID sulindac sulfide (16), but the structural basis of this effect is unknown. We reasoned that three different scenarios could explain the attenuated response and generated mutant PS1 cDNAs representing individual features of the complex PS1-ΔEoxon9 mutation. See “Results” for details.

**Figure 1. cDNA constructs for dissection of the PS1-ΔEoxon9 mutation.** The PS1-ΔEoxon9 mutation consists of a deletion comprising amino acids Thr₂⁹¹–Ser²¹⁹ and an additional amino acid exchange (S290C) at the border of exon 8 to exon 10. Shown are PS1 cDNA constructs that represent individual features of the complex PS1-ΔEoxon9 mutation. See “Results” for details.
The increased Aβ42/Aβ40 ratio of PS1-ΔExon9 is caused by a synergistic effect of the S290C mutation and the deficiency in endoproteolytic cleavage. A, retroviral vectors for the PS1 cDNA constructs shown in Fig. 1 were employed to infect CHO cells with stable overexpression of wild type APP (APP CHO cells), and stable mass cultures were generated. PS1 expression was analyzed by Western blotting with monoclonal antibody PSN2. PS1-WT and the point mutation PS1-S290C displayed endoproteolytic cleavage with detection of full-length protein and the N-terminal fragment of PS1 (PS1 N TF) as expected. Mutants PS1-ΔExon9, PS1-Δ291–319, PS1-M292D, PS1-S290C/M292D, and PS1-M292D/M146L, which had a deleted or mutated endoproteolytic cleavage site, did not undergo endoproteolysis, and only full-length PS1 was detected. B, Aβ40 and Aβ42 levels in conditioned media of cell pools with stable expression of PS1 mutants were measured and the Aβ42/Aβ40 ratio was determined. Expression of PS1-ΔExon9 induced a 3-fold increase in the Aβ42/Aβ40 ratio as compared with PS1-WT. PS1-Δ291–319, PS1-S290C, and PS1-M292D displayed a normal Aβ42/Aβ40 ratio comparable with PS1-WT. Only PS1-S290C/M292D mimicked the effect of PS1-ΔExon9 and caused an increase in the Aβ42/Aβ40 ratio, indicating that the pathogenic effect of PS1-ΔExon9 results at least partially from a synergistic effect of the S290C mutation and the lack of endoproteolytic cleavage. PS1-M292D/ M146L displayed a lesser but significant increase in the Aβ42/Aβ40 ratio as expected. n = 5; one-way ANOVA, **, p < 0.01 Dunnett’s post tests.

were infected with retroviral vectors for each mutant, and stable pools were selected and used for all further investigations. Previously described cell lines with stable expression of PS1-WT and PS1-ΔExon9 were employed as controls (16).

The Pathogenic Increase in the Aβ42/Aβ40 Ratio Induced by PS1-ΔExon9 Is Caused by a Synergistic Effect of the S290C Point Mutation and the Lack of Endoproteolytic Cleavage—Stable expression of the PS1 constructs in APP CHO cells was verified using the human-specific monoclonal antibody PSN2 (Fig. 2A). As expected, three of the mutant proteins, PS1-ΔExon9, PS1-Δ291–319, and PS1-M292D, failed to undergo endoproteolytic cleavage, and only full-length PS1 was detected. In contrast, PS1-WT and PS1-S290C showed endoproteolytic cleavage with detection of full-length protein and the N-terminal fragment of PS1 (PS1 N TF) (Fig. 2A). We then measured the levels of Aβ40 and Aβ42 in conditioned media of the stable cell pools to determine the cause for the pathogenic increase in the Aβ42/Aβ40 ratio associated with the PS1-ΔExon9 mutant. In a previous study, stable overexpression of PS1-Δ291–319 in HEK293 cells had failed to change the Aβ42/Aβ40 ratio as compared with PS1-WT (20). This suggested that the increased Aβ42/Aβ40 ratio of PS1-ΔExon9 might result from the S290C point mutation rather than from the Δ291–319 deletion or the lack of endoproteolytic cleavage. APP CHO cells with stable overexpression of PS1-ΔExon9 demonstrated a robust 3-fold increase in the Aβ42/Aβ40 ratio as compared with PS1-WT control cells, PS1-Δ291–319 cells, or PS1-M292D cells as expected (Fig. 2B). Surprisingly, cells expressing PS1-S290C likewise displayed an unchanged Aβ42/Aβ40 ratio as confirmed in three independently generated mass cultures (Fig. 2B). Because these mutants did not clarify the reason for the elevated Aβ42/Aβ40 ratio of PS1-ΔExon9, another PS1 cDNA was constructed in which we combined the S290C mutation with the M292D mutation (Fig. 1). Western blot analysis confirmed that this PS1-S290C/M292D mutant failed to be cleaved, and only full-length protein was detected (Fig. 2A). Intriguingly, PS1-S290C/M292D induced a significant increase in the Aβ42/Aβ40 ratio as compared with PS1-WT (Fig. 2B). From these results we conclude that a synergistic effect of the S290C point mutation and the deficiency in endoproteolytic cleavage were sufficient to cause a pathogenic change in Aβ42 production. However, the increase in the Aβ42/Aβ40 ratio of PS1-S290C/M292D was less pronounced as compared with PS1-ΔExon9, indicating that the Δ291–319 deletion may have further contributed to the strongly elevated Aβ42/Aβ40 ratio of PS1-ΔExon9.

The Diminished Response of PS1-ΔExon9 to the Aβ42-lowering NSAID Sulindac Sulfide Is Partially Caused by the Lack of Endoproteolytic Cleavage—To investigate the cause for the attenuated response of PS1-ΔExon9 to Aβ42-lowering NSAIDs, we next compared individual cell lines in their response to sulindac sulfide treatment by means of repeated dose-response experiments. For these experiments, CHO cell lines were treated with two increasing concentrations of sulindac sulfide (30–60 μM), and Aβ40 and Aβ42 levels in culture media were measured. The Aβ42 response of individual cell lines in five independent dose-response experiments was then compared by one-way ANOVA. We had previously shown that, in this concentration range, sulindac sulfide did not induce toxicity in CHO cells (12, 16), and measurements confirmed that Aβ40 levels were not significantly affected at these concentrations in any of the cell lines as compared with Me2SO control condition (data not shown). ANOVA corroborated our earlier findings that PS1-ΔExon9 cells display a substantially diminished Aβ42 response to sulindac sulfide as compared with PS1-WT control cells (16) (Fig. 3 and Table 1). In contrast, the Aβ42 response of PS1-S290C cells was not significantly different from PS1-WT control cells, excluding the possibility that the S290C point mutation was responsible for the attenuated response of PS1-ΔExon9. However, cells expressing the Δ291–319 deletion behaved similarly to PS1-ΔExon9 cells and exhibited a diminished Aβ42 response (Fig. 3 and Table 1). To further explore whether the loss of the endoproteolytic cleavage site within Δ291–319 or the deleted region itself was critical, we examined cells expressing the PS1-M292D point mutation. These PS1-M292D cells also displayed a significantly reduced response to the Aβ42-lowering NSAID sulindac sulfide (Fig. 3 and Table 1). When the M292D mutation was combined with the S290 splice-site mutation, a trend toward more pronounced attenuation was observed (supplemental Fig. 1 and Table 1). However, none of these mutants representing individual or combined features of PS1-ΔExon9 fully recapitulated the effect size of this complex FAD PS1 mutant. Interestingly, when the M292D mutation was combined with the FAD M146L mutation, which was shown to enhance the cellular response to Aβ42-lowering NSAIDs (16), the resulting M292D/M146L mutant behaved similar to PS1-WT and displayed a normal response to sulindac sulfide treatment (Fig. 3 and Table 1). Altogether, these results indicate that the M292D point mutation, which prevents endoproteolysis of PS1 but does not change the Aβ42/Aβ40 ratio, is able to significantly reduce the

FIGURE 2. The increased Aβ42/Aβ40 ratio of PS1-ΔExon9 is caused by a synergistic effect of the S290C mutation and the deficiency in endoproteolytic cleavage. A, retroviral vectors for the PS1 cDNA constructs shown in Fig. 1 were employed to infect CHO cells with stable overexpression of wild type APP (APP CHO cells), and stable mass cultures were generated. PS1 expression was analyzed by Western blotting with monoclonal antibody PSN2. PS1-WT and the point mutation PS1-S290C displayed endoproteolytic cleavage with detection of full-length protein and the N-terminal fragment of PS1 (PS1 N TF) as expected. Mutants PS1-ΔExon9, PS1-Δ291–319, PS1-M292D, PS1-S290C/M292D, and PS1-M292D/M146L, which had a deleted or mutated endoproteolytic cleavage site, did not undergo endoproteolysis, and only full-length PS1 was detected. B, Aβ40 and Aβ42 levels in conditioned media of cell pools with stable expression of PS1 mutants were measured and the Aβ42/Aβ40 ratio was determined. Expression of PS1-ΔExon9 induced a 3-fold increase in the Aβ42/Aβ40 ratio as compared with PS1-WT. PS1-Δ291–319, PS1-S290C, and PS1-M292D displayed a normal Aβ42/Aβ40 ratio comparable with PS1-WT. Only PS1-S290C/M292D mimicked the effect of PS1-ΔExon9 and caused an increase in the Aβ42/Aβ40 ratio, indicating that the pathogenic effect of PS1-ΔExon9 results at least partially from a synergistic effect of the S290C mutation and the lack of endoproteolytic cleavage. PS1-M292D/M146L displayed a lesser but significant increase in the Aβ42/Aβ40 ratio as expected. n = 5; one-way ANOVA, **, p < 0.01 Dunnett’s post tests.
PS1 Mutations Insensitive to NSAIDs and γ-Secretase Inhibitors

Inhibitors Is Common among Aggressive PS1 Mutations

To examine whether the attenuated Aβ42 response is specific to the PS1-ΔExon9 mutation, we chose to inspect three other features of this complex mutation, such as the deletion of amino acids 291–319 and the S290C splice-site mutation, also contribute to the observed attenuation phenotype.

Insensitivity to Aβ42-lowering NSAIDs and γ-Secretase Inhibitors Is Common among Aggressive PS1 Mutations

These results demonstrate that the attenuated Aβ42 response of PS1-ΔExon9 is not a unique feature of this mutant but rather a common one among aggressive PS1 mutations.

A previous study had further demonstrated that cells expressing PS1-ΔExon9 are only partially sensitive to the γ-secretase inhibitor L-685,458 suggesting that the lack of response to γ-secretase modulators and γ-secretase inhibitors could be mechanistically related (18). To reproduce these findings, we treated PS1-ΔExon9 cells and PS1-WT control cells with increasing concentrations of L-685,458 and compared the cellular response by measuring Aβ40 and Aβ42 levels in conditioned media. Confirming previous results, we observed a reduced response of PS1-ΔExon9 cells and gave rise to a 3-fold increase in the Aβ42/Aβ40 ratio comparable with PS1-ΔExon9 (supplemental Fig. 2). The Aβ42 response of individual cell lines to sulindac sulfide treatment was then compared in dose-response experiments as described above. All three mutants displayed a strongly reduced response to sulindac sulfide treatment (Fig. 4 and Table 2). The effect was comparable with PS1-ΔExon9 cells that were completely refractory to sulindac sulfide treatment (Fig. 4 and Table 2).

TABLE 1

The diminished Aβ42 response of PS1-ΔExon9 to sulindac sulfide is partially caused by the lack of endoproteolytic cleavage

| Cell line  | 30 μM | 60 μM |
|------------|-------|-------|
| PS1-WT     | 78.19 ± 2.37 | 61.65 ± 1.68 |
| PS1-S290C  | 83.76 ± 1.75 | 65.92 ± 1.24 |
| PS1-ΔExon9 | 99.70 ± 1.63** | 99.49 ± 0.93** |
| PS1-Δ291–319 | 88.71 ± 1.64** | 82.65 ± 0.89** |
| PS1-M292D  | 89.53 ± 1.06** | 73.90 ± 2.19** |
| PS1-M292D/M146L | 73.19 ± 0.65 | 58.45 ± 0.82 |

FIGURE 3. The diminished Aβ42 response of PS1-ΔExon9 to sulindac sulfide is caused in part by the lack of endoproteolytic cleavage. APP CHO cells with stable expression of mutant PS1 or PS1-WT were treated with increasing concentrations of the Aβ42-lowering NSAID sulindac sulfide, and Aβ42 levels in conditioned media were quantified. The results represent averages ± S.E. from five independent dose-response experiments analyzed by one-way ANOVA with PS1-WT cells as control group. Cells overexpressing the FAD mutants PS1-L166P, PS1-P117L, and PS1-G384A displayed a dramatically diminished Aβ42 reduction as compared with PS1-WT control cells (Table 2), n = 5; one-way ANOVA, ***; p < 0.001 Dunnett’s post tests.

FIGURE 4. Insensitivity to Aβ42-lowering NSAIDs is common among aggressive FAD PS1 mutations. APP CHO cells with stable expression of FAD PS1 mutants or PS1-WT were treated with increasing concentrations of sulindac sulfide, and Aβ42 levels in conditioned media were quantified. Dose-response experiments were analyzed by one-way ANOVA with PS1-WT cells as control group. Cells overexpressing the FAD mutants PS1-L166P, PS1-P117L, and PS1-G384A displayed a dramatically diminished Aβ42 reduction as compared with PS1-WT control cells (Table 2), n = 5; one-way ANOVA, ***, p < 0.001 Dunnett’s post tests.

TABLE 2

Insensitivity to the Aβ42-lowering NSAID sulindac sulfide is common among aggressive FAD PS1 mutations

Dose-response experiments were performed as described in the text and analyzed by one-way ANOVA with PS1-WT cells as control group. n = 5; **, p < 0.01 Dunnett’s post tests.

| Cell line  | 30 μM | 60 μM |
|------------|-------|-------|
| PS1-WT     | 79.19 ± 2.37 | 61.65 ± 1.68 |
| PS1-ΔExon9 | 99.70 ± 1.63** | 99.49 ± 0.93** |
| PS1-L166P  | 99.45 ± 1.50** | 99.16 ± 2.51** |
| PS1-P117L  | 99.95 ± 2.76** | 88.53 ± 1.98** |
| PS1-G384A  | 95.13 ± 1.35** | 90.34 ± 0.93** |
PS1 Mutations Insensitive to NSAIDs and γ-Secretase Inhibitors

FAD PS1 mutants are partially insensitive to γ-secretase inhibitors of different structural classes. A, APP CHO cells with stable expression of PS1-ΔExon9, PS1-L166P or PS1-WT were treated with increasing concentrations of transition state γ-secretase inhibitor L-685,458, and Aβ40 and Aβ42 levels were observed by one-way ANOVA with PS1-WT cells as control group. A strongly diminished reduction in Aβ40 (left panel) and Aβ42 (right panel) was observed at all concentrations tested (Table 3). B, the same cell lines as in A were treated with increasing concentrations of the benzodiazepine γ-secretase inhibitor LY-411575, and dose-response experiments were analyzed in an identical fashion. Similar to the results with L-685,458, a significantly attenuated reduction in Aβ40 (left panel) and Aβ42 (right panel) levels was observed at all concentrations tested (Table 3). C, the same cell lines as in A were treated with increasing concentrations of the benzodiazepine γ-secretase inhibitor LY-411575, and dose-response experiments were analyzed in an identical fashion. Similar to the results with L-685,458, a significantly attenuated reduction in Aβ40 (left panel) and Aβ42 (right panel) levels was observed at all concentrations tested (Table 3).

Table 3

| Treatment | Aβ40 levels | Aβ42 levels |
|-----------|-------------|-------------|
| L-685,458 | % control ± S.E. | % control ± S.E. |
| 100 nM    | 8.1 ± 1.14 | 98.33 ± 8.55** |
| 250 nM    | 3.95 ± 0.31 | 54.05 ± 7.86* |
| 500 nM    | 1.92 ± 0.28 | 33.37 ± 5.76** |
| LY-411575 | % control ± S.E. | % control ± S.E. |
| 0.1 nM    | 64.23 ± 3.87 | 92.56 ± 1.62** |
| 0.2 nM    | 15.79 ± 1.81 | 72.89 ± 0.85** |
| 0.5 nM    | 5.6 ± 0.62 | 26.74 ± 2.05** |

For both Aβ40 and Aβ42 as compared with PS1-WT cells. After treatment with 100 nM L-685,458, Aβ40 and Aβ42 levels were reduced by almost 90% from PS1-WT control cells, whereas they were reduced less than 15% from PS1-ΔExon9 cells (Fig. 5 and Table 3). To examine whether these findings could be extended to a second PS1 mutation with diminished response to Aβ42-lowering NSAIDs, we treated cells expressing the PS1-L166P mutation with L-685,458. PS1-L166P cells likewise demonstrated a reduced response as compared with PS1-WT control cells. For Aβ40, the response of PS1-L166P cells was significantly attenuated at the lowest concentration with a nonsignificant trend at higher concentrations, whereas for Aβ42 the response was significantly diminished at all concentrations tested (Fig. 5 and Table 3). L-685,458 was designed as a transition-state inhibitor for aspartyl proteases, and photoactivable derivatives of this compound directly interact with the N- and C-terminal fragments of PS1 (31). To investigate whether the results with L-685,458, both PS1-ΔExon9 and PS1-L166P cells demonstrated a substantially reduced response to DAPT treatment as compared with PS1-WT cells. With the PS1-L166P mutation, the attenuation seemed to be more pronounced for Aβ42 than for Aβ40, and DAPT treatment promoted an increase in the Aβ42/Aβ40 ratio at all concentrations tested (supplemental Fig. 3 and Table II). One of the drawbacks of DAPT is its comparatively low potency, and high doses were required to significantly reduce brain Aβ levels in an APP-transgenic mouse model (33). Therefore, to enable in vivo experiments, we next investigated the benzodiazepine γ-secretase inhibitor LY-411575, which is reported to have an EC_{50} value of 119 pm for reduction of Aβ40 in HEK293 cells (34). Both PS1 mutations displayed a significantly reduced response to this highly potent γ-secretase inhibitor (Fig. 5 and Table 3), and more extended dose-response experiments defined strongly increased EC_{50} values for Aβ40 and Aβ42 reduction with PS1-ΔExon9 cells (EC_{50}Aβ40 = 358 pm, EC_{50}Aβ42 = 352 pm) as compared with PS1-WT cells (EC_{50}Aβ40 = 114 pm, EC_{50}Aβ42 = 135 pm). In summary, FAD PS1 mutations displayed reduced sensitivity to three γ-secretase inhibitors of increasing potency that belong to two different structural classes and are known to interact with different sites of PS1.

The Highly Potent γ-Secretase Inhibitor LY-411575 Failed to Reduce Brain Aβ42 Levels in an Alzheimer Disease Mouse Model with Transgenic Expression of the PS1-L166P Mutation—To examine whether the attenuated response of PS1 mutations to γ-secretase inhibitors has consequences for the evaluation of such drugs in transgenic mouse models of AD, we compared two animal models in their response to γ-secretase inhibitor LY-411575: single-transgenic mice expressing Swedish mutant APP (Tg2576 mice (26)) and...
PS1 Mutations Insensitive to NSAIDs and γ-Secretase Inhibitors

![Figure 6]

**FIGURE 6.** γ-Secretase inhibitor LY-411575 failed to reduce Aβ42 levels in brain of PS1-L166P transgenic mice. Single-transgenic mice expressing Swedish mutant APP (Tg2576 mice, n = 4–6) or double-transgenic mice expressing Swedish mutant APP and human PS1-L166P (APPPS1 mice, n = 6) were orally dosed with 10 mg/kg of the benzodiazepine γ-secretase inhibitor LY-411575 or vehicle. 3 h post administration the animals were sacrificed and Aβ levels in brain were determined by enzyme-linked immunosorbent assay. A, in Tg2576 mice, LY-411575 treatment reduced brain levels of Aβ40 (left panel) and Aβ42 (right panel) by ≥ 85%. In sharp contrast, in APPPS1 mice LY-411575 reduced brain Aβ40 levels (left panel) to similar levels as in Tg2576 mice (≥80% reduction) but failed to significantly reduce Aβ42 levels (right panel) (Table 4).

**TABLE 4**

| γ-Secretase inhibitor LY-411575 failed to reduce Aβ42 levels in brain of PS1-L166P transgenic mice |
| Drug treatments of mice were performed as described in the text. n = 4–6; ***, p < 0.001 paired t-test. |

|                      | Tg2576 (APP “Swedish”) | APPPS1 (APP “Swedish”/PS1-L166P) |
|----------------------|------------------------|----------------------------------|
|                      | Aβ40 pmol/g            | Aβ42 pmol/g                      |
|                      | Aβ40 pmol/g            | Aβ42 pmol/g                      |
| Vehicle              | 8.28 ± 0.62            | 1.93 ± 0.17                      |
| LY-411575            | 1.23 ± 0.13            | 0.21 ± 0.05                      |
| **% reduction**      | 85***                  | 89***                            |

**DISCUSSION**

γ-Secretase is a multi-subunit aspartyl protease with the presenilin proteins PS1 or PS2 at its catalytic core (4). Current evidence indicates that PS has a nine-transmembrane domain (TMD) topology, and two critical aspartate residues in TMD6 and TMD7 form the active center of γ-secretase. PS proteins are endoproteolytically cleaved during assembly and maturation of the γ-secretase complex and are incorporated together with three accessory proteins, nicastrin, Aph-1, Pen-2, into high molecular weight complexes that correlate with the bulk of enzymatic activity (4). Because of its essential role in the cellular production of Aβ, γ-secretase constitutes a principal drug target for AD, and a large number of highly potent inhibitors for this enzyme have been identified (5, 6). These inhibitors belong to various structural classes and target three different binding sites in the γ-secretase complex that all seem to be present within the PS proteins (31, 32, 36–39). Compounds like L-685,458 that were modeled after transition-state inhibitors for aspartyl proteases interact with both the N- and C-terminal fragments of PS1, consistent with the notion that the active site is located at the interface of the two PS fragments and is composed of one aspartyl residue in each subunit (31, 37). These transition-state inhibitors bind the γ-secretase complex in a noncompetitive fashion with respect to substrate, and blocking the active site does not prevent binding of substrate to the complex (40, 41). This indicates that γ-secretase contains an exosite for substrate binding or docking that functions in initial recognition of the substrate prior to movement to the catalytic site. α-Helical peptides based on the transmembrane domain of APP interact with this docking site and are potent inhibitors of γ-secretase (38). A third class of small molecule inhibitors was identified from library screens and includes aryalkylamides, dipeptidic compounds like DAPT, and benzodiazepines like LY-411575. This class contains highly potent compounds with drug-like properties that seem to interact with a binding site located in the PS1 C-terminal fragment (32).

In addition to these indiscriminate γ-secretase inhibitors, a new class of γ-secretase modulators that selectively lower Aβ42 production but spare NOTCH processing has been discovered (12). These compounds, like the Aβ42-lowering NSAID sulindac sulfide, display activity in cell-free γ-secretase assays, indicating that they target yet another allosteric binding site within the γ-secretase complex (7, 9, 11, 15, 16). This idea was further supported by the observation that certain FAD PS1 mutations change the cellular drug response to Aβ42-lowering NSAIDs, and a dramatic reduction in the response to sulindac sulfide is apparent in cells with stable expression of the PS1-ΔExon9 mutant (16). In contrast to the majority of FAD missense mutations, the complex PS1-ΔExon9 mutation is characterized by three separable features: a point mutation (S290C), a deletion (Δ291–319), and lack of endoproteolytic cleavage. The cause for the pathogenic increase in the Aβ42/Aβ40 ratio associated with PS1-ΔExon9 remained uncertain, although a previous study points to the S290C mutation, as expression of the Δ291–319 deletion alone does not result in abnormal Aβ42 production (20). However, these authors did not investigate the effect of the S290C mutation in a wild type PS1 background. Our studies, using PS1 cDNA constructs that represented individual features of the PS1-ΔExon9 mutation, now have clearly demonstrated that the S290C point mutation was not responsible for the increased Aβ42/Aβ40 ratio of this complex FAD mutation. In addition, we could confirm that the Δ291–319 deletion or the M292D point mutation, which prevented endoproteolytic cleavage (23), did not cause an elevated Aβ42/Aβ40 ratio. This showed that any single feature of the PS1-ΔExon9 mutant did not result in a pathogenic Aβ42/Aβ40 ratio. Surprisingly, when we combined the S290C and M292D point mutations, an increased Aβ42/Aβ40 ratio was observed, indicating that the
PS1 Mutations Insensitive to NSAIDs and γ-Secretase Inhibitors

S290C mutation when placed into a noncleavable PS1 background became pathogenic. This finding further demonstrated that two point mutations, which were not pathogenic by themselves, could synergistically force a conformational change in PS1 that resulted in an increased Aβ42/Aβ40 ratio.

Consistent with these results, we also found that the S290C mutation was not responsible for the attenuated response of PS1-ΔExon9 to the Aβ42-lowering NSAID sulindac sulfide. In contrast, cells expressing either the Δ291–319 deletion or the M292D point mutation exhibited a significantly diminished response to sulindac sulfide. Interestingly, when the M292D mutation was combined with the FAD M146L mutation, which previously was shown to enhance the cellular response to Aβ42-lowering NSAIDs (16), the M292D/M146L mutant behaved similar to PS1-WT and displayed a normal response to sulindac sulfide treatment. Our interpretation of these findings is that the attenuated response of PS1-ΔExon9 was at least partially caused by the deficiency in endoproteolysis and the resulting structural changes within the γ-secretase complex. Furthermore, introduction of the M146L mutation seemed to overcome these structural changes and rendered the M292D mutant more susceptible to the Aβ42-lowering activity of sulindac sulfide.

Importantly, a subsequent screen of FAD missense mutations that are associated with very early onset of AD quickly discovered three additional mutations with a similarly attenuated response to sulindac sulfide. This demonstrated that insensitivity to Aβ42-lowering NSAIDs was not confined to the PS1-ΔExon9 mutant but was rather common among aggressive PS1 mutants. A previous publication had further suggested that the PS1-ΔExon9 mutant is partially unresponsive to the transition-state γ-secretase inhibitor L-685,458 (18). We were able to confirm these findings and to extend them to two non-transition-state γ-secretase inhibitors, the dipeptidic compound DAPT and the highly potent benzodiazepine LY-411575. With a second FAD PS1 mutation, PS1-L166P, the lack of response was even more pronounced for Aβ42 production. Consequently, in cells expressing PS1-L166P, all three γ-secretase inhibitors, while lowering overall Aβ production, induced a dose-dependent increase in the Aβ42/Aβ40 ratio.

How these PS1 mutants simultaneously attenuate the response to two different classes of γ-secretase inhibitors and to Aβ42-lowering γ-secretase modulators remains uncertain, as all these molecules are presumed to target pharmacologically distinct binding sites within the γ-secretase complex. One possibility is that these binding sites are located in close proximity and are therefore similarly perturbed by conformational changes in PS1 induced by specific FAD mutations. Photolabeling studies with α-helical peptides and mutagenesis studies indicate that the active site and the docking site are indeed spatially close together and may even overlap (38, 42). It has further been speculated that small molecule inhibitors like DAPT interfere with substrate movement from the docking to the catalytic side (39), which would place their binding site in between the docking site and the active site. The identity of the allosteric binding site for γ-secretase modulators is unknown. However, the remarkable finding that NSAIDs also modulate proteolytic cleavage by signal peptide peptidase, which is homologous to PS but functions on its own and does not require accessory proteins, strongly favors a location within PS (43). Furthermore, in radioligand binding studies Aβ42-lowering NSAIDs were able to displace transition-state and benzodiazepine γ-secretase inhibitors by noncompetitive antagonism, providing further evidence for an allosteric interaction between the binding sites of these compounds (7, 36). An alternative explanation is that the binding sites for the various γ-secretase inhibitors and modulators are not in close proximity but that substantial conformational changes induced by aggressive FAD PS1 mutations may even affect binding sites that are structurally far apart. In both scenarios, these conformational changes might simply lower the affinity of inhibitors and modulators for their respective binding sites. In support of this argument, photolabeling of PS1-L166P by a transition-state inhibitor was almost completely abolished as compared with PS1-WT (38). In contrast, PS1-ΔExon9, which in our study appeared even less responsive to γ-secretase inhibitors than PS1-L166P, was readily labeled by photoactivable derivatives of DAPT and L-685,458 in other studies (31, 32). Even so, subtle changes in binding affinity beyond the sensitivity of photolabeling studies cannot be excluded. In any case, high resolution structural data on PS will likely be required to further characterize the effects of FAD mutations on the binding sites of γ-secretase inhibitors and modulators.

Besides these mechanistic aspects, our findings have important implications for the use of cell lines and animals models in drug discovery efforts aimed at γ-secretase. Cell lines with overexpression of APP are routinely used to identify and optimize inhibitors and modulators of γ-secretase, and many of these cell lines further express PS1 FAD mutations to facilitate easy detection of Aβ42 levels. More importantly, newer APP-transgenic mouse models frequently incorporate aggressive PS1 mutations to accelerate the development of pathological changes. Even within the limited number of FAD PS1 mutations that we have investigated, three animal models with transgenic expression of PS1-ΔExon9, PS1-P117L, and PS1-L166P have been described (27, 44, 45). Consistent with our in vitro data, the γ-secretase inhibitor LY-411575 also failed to lower brain Aβ42 levels in double-transgenic mice expressing Swedish mutant APP and PS1-L166P. Although confirmation in other models is required, these results clearly raise the issue that in vivo studies to evaluate the potency and efficacy of γ-secretase inhibitors and modulators could be confounded by the FAD PS transgenes expressed in certain mouse models of AD.

Finally, our results may also have relevance for future clinical studies of γ-secretase inhibitors or modulators if these were to include FAD patients with PS mutations. In our tissue culture model, overexpression of exogenous mutant PS1 resulted in displacement of endogenous wild type PS1 because of a limiting cellular supply of the γ-secretase accessory proteins (4). Accordingly, it is expected that γ-secretase complexes in our cell lines contained predominantly mutant PS. This situation is clearly different from heterozygous FAD patients that should
PS1 Mutations Insensitive to NSAIDs and γ-Secretase Inhibitors

express mutant and wild type PS1 in an approximately equal ratio. Therefore, it is possible but not certain that the observed insensitivity to γ-secretase inhibitors or modulators would not apply to heterozygous FAD PS1 patients. In fact, recent studies in mutant PS1 knock-in mice suggest that the presence of an endogenous wild type allele modulates γ-secretase activity and ameliorates pathological changes (46). Unfortunately, primary cells from FAD patients with specific PS1 mutations, which would constitute appropriate cellular models to address this question, are not available in public cell line repositories. Nevertheless, in future clinical trials it might be prudent to evaluate the potential differences in the efficacy of compounds targeting the γ-secretase complex in sporadic AD versus FAD PS1 patients.

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