Evidence That Nonsteroidal Anti-inflammatory Drugs Decrease Amyloid β42 Production by Direct Modulation of γ-Secretase Activity*

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Chronic use of nonsteroidal anti-inflammatory drugs (NSAIDs) is associated with a lower risk of developing Alzheimer’s disease. Recent evidence indicates that some NSAIDs specifically inhibit secretion of the amyloidogenic Aβ42 peptide in cultured cells and mouse models of Alzheimer’s disease. The reduction of Aβ42 peptides is not mediated by inhibition of cyclooxygenases (COX) but the molecular mechanism underlying this novel activity of NSAIDs has not been further defined. We now demonstrate that NSAIDs efficiently reduce the intracellular pool of Aβ42 in cell-based studies and selectively decrease Aβ42 production in a cell-free assay of γ-secretase activity. Moreover, we find that presenilin-1 (PS1) mutations, which affect γ-secretase activity, differentially modulate the cellular Aβ42 response to NSAID treatment. Overexpression of the PS1-M146L mutation enhances the cellular drug response to Aβ42 lowering NSAIDs as compared with cells expressing wild-type PS1. In contrast, expression of the PS1-DExon9 mutation strongly diminishes the Aβ42 response, showing that PS1 mutations can modulate the cellular drug response to NSAID treatment both positively and negatively. Enhancement of the NSAID drug response was also observed with overexpression of the APP V717F mutation but not with Swedish mutant APP, which affects β-secretase cleavage. In sum, these results strongly suggest that NSAIDs represent a founding group of compounds that lower Aβ42 production by direct modulation of γ-secretase activity or its substrate.

Despite considerable advances in the understanding of Alzheimer’s disease (AD) pathology, therapeutic interventions that may halt or reverse the underlying disease process are not available (1, 2). Numerous epidemiological studies support the finding that chronic intake of nonsteroidal anti-inflammatory drugs (NSAIDs) can decrease the risk for AD by more than 50% (3–6). This protective effect of NSAIDs has generally been ascribed to a diminution of deleterious inflammatory processes in the AD brain (3). However, recent findings suggest a direct impact of some NSAIDs on the amyloid pathology in AD. Treatment of various cultured cells with the NSAIDs sulindac sulfide, ibuprofen, indomethacin, and flurbiprofen specifically inhibited the release of the amyloidogenic Aβ42 peptide (7, 8). Aβ42 is a proteolytic fragment derived from the β-amyloid precursor protein (APP) by β- and subsequent γ-secretase cleavage activities and is believed to play a central role in AD pathology (9, 10). Short term administration of ibuprofen to APP transgenic mice lowered brain levels of Aβ42 and chronic high dose ibuprofen treatment significantly reduced amyloid plaque numbers and plaque-associated pathology in aging APP transgenic mice (7, 11). The reduction in Aβ42 levels was achieved without affecting other APP processing pathways, like secretion of the soluble APP ectodomain (APPS), and is not the result of enhanced degradation or cell-mediated clearance of Aβ42. Importantly, within the concentration range tested, Aβ42-lowering NSAIDs did not inhibit processing of the NOTCH receptor and presumably other substrates of γ-secretase (7).

NSAIDs are the first small molecules reported to specifically target Aβ42 without apparent overall inhibition of γ-secretase activity, and it will be important to elucidate the molecular mechanism of this activity if more active compounds are to be developed. The NSAID effect on Aβ42 levels is fully retained in cells deficient in cyclooxygenase-1 (COX1) and COX2 enzymatic activity, thereby excluding the primary pharmacological targets of NSAIDs as mediators of the Aβ42 reduction (7). COX-independent mechanisms for NSAID activity are well established (12). For example, NSAIDs have emerged as potentially valuable drugs in the chemoprevention of certain cancers, and they affect several molecular pathways regulating cellular proliferation and apoptosis (13, 14). Many of these mechanisms seem to be COX independent, including nuclear factor κB activation (15), inhibition of lipoxigenases (16) and modulation of peroxisome proliferator-activated receptor signaling (17, 18) and potentially any of these signaling pathways could contribute to the observed NSAID effect on Aβ42 secretion.

Alternatively, NSAIDs might directly alter γ-secretase function. This possibility is attractive because the reduction in Aβ42 level is accompanied by an increase in shorter Aβ species, particularly Aβ38. This suggests a subtle alteration of γ-secretase cleavage pattern rather than a selective inhibition of Aβ42 generation (7, 19). γ-Secretase is a multiprotein complex consisting of at least four membrane-bound proteins: presenilin...
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FIG. 1. Analysis of intracellular Aβ levels after treatment with the Aβ42-lowering NSAID sulindac sulfide. CHO cells overexpressing WT-APP and PS1-M146L were treated with 60 μM sulindac sulfide or MeSO vehicle. Aβ was immunoprecipitated from cell lysates and analyzed by bicine/urea SDS-PAGE. Sulindac sulfide treatment decreased intracellular Aβ42 to undetectable level but had essentially no effect on Aβ40 from a representative experiment. Aβ(1–40) and Aβ(1–42) peptide standards, used to identify corresponding intracellular Aβ species, are shown on the right.

DMSO 60 μM

Aβ40

Aβ42

EXPRESSING APP695NL+1 as described (23, 34). Samples were incubated for 2h at 37 °C in the presence of selected compounds or MeSO vehicle to allow in vitro production of Aβ and the APP intracellular cytoplasmic domain (AICD, CTFγ) and subsequently stored at −80 °C. Aβ40 and Aβ42 levels were then quantified by ELISA. Net activity during the 2-h incubation period was defined by subtracting background values obtained from samples immediately frozen at time 0. Corrected values were then normalized to the MeSO control condition and expressed as % control.

Dose Response Experiments and Statistical Analysis—The Aβ42 response of individual cell lines to NSAID treatment was 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 pretreated overnight with increasing concentrations of sulindac sulfide, ibuprofen, indomethacin, or MeSO vehicle. Medium was changed and treatment was continued for another 24 h. Aβ40 and Aβ42 levels in conditioned medium were then analyzed by ELISA. Duplicate Aβ42 measurements from each drug concentration were averaged and normalized to the MeSO control condition. These experiments were repeated 6–10 times, and results were analyzed by two-way ANOVA with Bonferroni post-tests using cell line and drug concentration as categorical variables. Calculations were performed with GraphPad Prism software (GraphPad Software).

Matrix-assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry—MALDI-TOF was performed on Aβ peptides immunoprecipitated from conditioned medium of CHO cells as described (35) with the following modifications. P3, phosphoramidon, and a synthetic Aβ1–22 peptide that served as an internal control were added, and all 42-lowering NSAIDs have been shown to reduce secretion of Aβ peptides from a variety of cell lines, and this effect is not caused by enhanced degradation or clearance of Aβ42 from tissue culture media (7, 8). These changes in the secreted pool of Aβ42 if NSAIDs affect Aβ42 production by direct modulation of γ-secretase activity. We therefore analyzed intracellular Aβ42 levels after treatment of CHO cells stably transfected with both APP751 (wild-type APP, WT-APP) and the PS1 mutant M146L (PS1-M146L) with the Aβ42-lowering NSAID sulindac sulfide. Aβ was immunoprecipitated from cell lysates and separated on a gel system that permits resolution of individual Aβ species. Treatment of cells with 60 μM sulindac sulfide consistently reduced intracellular Aβ42 levels and strongly decreased the Aβ42/Aβ40 ratio with minimal effects on Aβ40 levels (Fig. 1).

Aβ42-lowering NSAIDs Inhibit Aβ Production in a Cell-free Assay of γ-secretase Activity—We next examined the effect of several NSAIDs on Aβ production in a cell-free assay of γ-secretase activity. If Aβ42-lowering NSAIDs act through di-
...rect modulation of γ-secretase activity, they would be expected to inhibit Aβ42 production in cell-free assays, as demonstrated previously for well characterized γ-secretase inhibitors (23, 36). As predicted, the γ-secretase inhibitors z-IL-CHO and Compound E both inhibited γ-secretase activity, with significant reductions in both Aβ40 and Aβ42 production. NSAIDs, such as indomethacin and sulindac sulfide, that have been shown to affect Aβ42 production in cell-based assays (7) displayed very similar activities in the cell-free γ-secretase assay (Fig. 2). At these doses, even when Aβ42 production is virtually abolished as seen with sulindac sulfide and indomethacin, no measurable effect was observed on AICD/CTFγ production (data not shown). Naproxen, an NSAID without Aβ42-lowering properties (7), had no effect on Aβ42 production (Fig. 2).

**The PS1-M146L Mutation Enhances the Cellular Aβ42 Response to NSAID Treatment**—In our previous study, the NSAID effect on Aβ42 was observed across multiple cell lines and cell types, including those expressing various PS mutations. In view of the in vitro γ-secretase assay results, we now asked whether different PS1 mutants might subtly influence the cellular Aβ42 response to NSAID treatment. We choose to first analyze the PS1-M146L mutation as preliminary observations suggested that the level of Aβ42 reduction was different from wild-type PS1 cells. For these experiments, CHO cell lines were treated with three increasing concentrations of sulindac sulfide (20–60 μM) and Aβ40 and Aβ42 levels in culture media were measured by ELISA. The Aβ42 response of individual cell lines was then compared by two-way ANOVA (see “Experimental Procedures” for details). Total Aβ levels (Aβ40 + Aβ42) were not significantly affected at these concentrations of sulindac sulfide and treatment did not cause toxicity as reported previously (7). ANOVA demonstrated that overexpression of wild type, human PS1 resulted only in minimal, non-significant changes in the Aβ42 response as compared with parental cells expressing endogeneous, hamster PS1 (Fig. 3 and Table I). However, overexpression of the PS1-M146L mutation strongly enhanced the cellular Aβ42 response to sulindac sulfide treatment. ANOVA showed a highly significant statistical difference in the Aβ42 reduction as compared with either parental WT-APP CHO cells expressing endogeneous PS1 or WT-APP WT-PS1 CHO cells overexpressing wild-type, human PS1 (p < 0.001, Fig. 3 and Table I). At all three concentrations tested, the reduction in Aβ42 levels was almost 20% greater in PS1-M146L cells than that seen in the other two cell lines. Similar trends toward enhanced Aβ42 reduction with the PS1-M146L mutant as compared with WT-PS1 were also observed with indomethacin and ibuprofen and, importantly, in HS683 neuroglioma cells expressing the same PS constructs (data not shown). These results indicated that the enhanced Aβ42 reduction is distinctively associated with the PS1-M146L mutant, and is not limited to the NSAID sulindac sulfide or to a specific cell type.

**The PS1-ΔExon9 Mutation Diminishes the Cellular Aβ42 Response to NSAID Treatment**—We next examined CHO cells overexpressing the PS1-ΔExon9 mutation to assess whether another PS1 mutation would similarly alter the cellular drug response to NSAID treatment. We chose this mutation because it results in an exon deletion rather than a missense substitution as seen in virtually all other FAD PS1 mutations and because the clinical manifestation of this mutation is varied (37). Surprisingly, we found that the PS1-ΔExon9 mutation strongly diminished the Aβ42 response to sulindac sulfide treatment. Two-way ANOVA analysis of 10 independent dose-response experiments showed a highly significant attenuation of the Aβ42 response at all three concentrations tested as compared with parental WT-APP CHO cells expressing endogeneous PS1 or WT-APP WT-PS1 CHO cells overexpressing wild-type, human PS1 (p < 0.001, Fig. 4, panel A, and Table II). This result was confirmed with HS683 neuroglioma cells overexpressing the PS1-ΔExon9 mutation (Fig. 4, panel B, and Table II). In sum, these findings strongly indicated that PS1 mutations that alter Aβ42 generation are able to modulate the cellular Aβ42 response to NSAID treatments.

It has recently been demonstrated that inhibition of Aβ production by the transition state analogue γ-secretase inhibitor L-685,458 was also attenuated in cells expressing the PS1-ΔExon9 mutant (38). Furthermore, in contrast to cells expressing wild type PS1, cells expressing PS1-ΔExon9 produced significant amounts of Aβ1–43 peptides and, paradoxically, secretion of Aβ1–43 was increased in a dose-dependent manner by inhibitor treatment. Our ELISA for detection of Aβ42 does not efficiently discriminate between Aβ42 and Aβ43 peptides (39). Hence, our results could be confounded by the Aβ3 effects of the ΔExon9 mutation. We therefore investigated the profile of Aβ peptides secreted by WT-APP CHO cells overexpressing PS1-ΔExon9 treated with 60 μM sulindac sulfide or Me2SO vehicle by MALDI-TOF mass spectrometry (Fig. 5). We confirmed the prominent release of Aβ1–43 peptides in cells overexpressing PS1-ΔExon9. However, when compared with vehicle control, treatment with sulindac sulfide did not increase...
Dose-response experiments demonstrate enhanced Ap42 reduction in cells overexpressing PS1-M1461

### Table I

| Sulindac Sulfide (μM) | CHO WT-APP | CHO WT-APP WT-PS1 | CHO WT-APP PS1-M146L |
|-----------------------|------------|-------------------|----------------------|
| 20 μM                 | 79.28 ± 1.63 | 78.77 ± 2.01 | 63.13 ± 1.82*** |
| 40 μM                 | 70.56 ± 2.42 | 68.31 ± 1.52 | 50.76 ± 2.53*** |
| 60 μM                 | 56.36 ± 1.49 | 51.13 ± 2.96 | 32.80 ± 1.87*** |

**DISCUSSION**

Accumulation and aggregation of Aβ peptides in the cerebral cortex is believed to be an early and crucial event in the pathogenesis of AD. Aβ peptides are generated by sequential proteolytic cleavage of APP by β- and γ-secretase activities, which are therefore considered prime targets for therapeutic intervention (1, 2). A number of small molecule γ-secretase inhibitors have been identified, and they are able to block Aβ production with high potency in cultured cells and in APP transgenic mouse models of AD and have entered early stages of clinical testing (1, 42). However, γ-secretase activity also cleaves a number of additional substrates besides APP such as Notch and ErbB-4, and γ-secretase inhibitors suppress Notch processing in vivo (43–45). Consequently, it is unclear whether inhibition of γ-secretase activity can be used clinically without causing serious side effects (46).

NSAIDs that specifically inhibit Ap42 production do not appear to affect Notch processing (7). Elucidation of their mechanism of action therefore seems to be highly desirable not only for pharmacological intervention in Alzheimer’s disease but also for further understanding γ-secretase function. Since cyclooxygenases are the main pharmacological target of NSAIDs, inhibition of COX and suppression of prostaglandin synthesis seemed to be an obvious mechanism by which NSAIDs could reduce Aβ42 secretion. However, such a mechanism appeared less likely when it became apparent that only a subset of NSAIDs lower Aβ42 levels, whereas all NSAIDs by definition inhibit COX. Consistent with this interpretation, we subsequently showed that the NSAID effect on Aβ42 was preserved in cells lacking cyclooxygenase activity (7). Given these results and, in particular, our recent negative correlation of the NSAID Aβ42 effect with other known non-COX targets of NSAIDs (57), we therefore considered a more immediate effect of Aβ42-lowering NSAIDs on γ-secretase activity.

If NSAIDs were to inhibit γ-secretase activity, then perhaps predecessors can be found in the transition state analogs and other small molecule inhibitors that modulate or directly interact with the γ-secretase complex or its substrates. Three characteristics appear to define these γ-secretase inhibitors. First, these compounds have been shown to reduce Aβ production in both cell-based and cell-free in vitro γ-secretase assays. The latter use membrane preparations or solubilized microsomes for Aβ production and many γ-secretase inhibitors suppress Aβ formation in these in vitro assays with equal or higher potency as compared with cell-based assays (47). However, it is noteworthy that a group of Aβ-lowering isocoumarins reduced Aβ secretion only in cell-based assays but not in vitro assays (48, 49), suggesting that these compounds do not directly affect γ-secretase activity. Second, FAD mutations in the PS and APP proteins that alter γ-secretase cleavage (43) may affect γ-secretase activity. However, such a mechanism appeared less likely when it became apparent that only a subset of NSAIDs lower Aβ42 levels, whereas all NSAIDs by definition inhibit COX. Consistent with this interpretation, we subsequently showed that the NSAID effect on Aβ42 was preserved in cells lacking cyclooxygenase activity (7). Given these results and, in particular, our recent negative correlation of the NSAID Aβ42 effect with other known non-COX targets of NSAIDs (57), we therefore considered a more immediate effect of Aβ42-lowering NSAIDs on γ-secretase activity.

An APP Mutation That Affects γ-Secretase Cleavage (APV717F) Enhances the Cellular Aβ42 Response to NSAID Treatment—Finally, in view of the results from cells expressing PS1 mutations, we investigated whether FAD APP mutations would likewise alter the cellular Aβ42 response to NSAID treatment. WT-APP CHO cells, CHO cells overexpressing the APPV717F mutant, which affects γ-secretase cleavage (40), and CHO cells overexpressing Swedish mutant APP, which affects β-secretase cleavage (41), were treated with sulindac sulfide and analyzed as above. The Aβ42 response of CHO cells overexpressing Swedish mutant APP was not significantly different from WT-APP CHO cells. However, similar to the PS1-M1461 mutation, CHO cells overexpressing the APPV717F mutant displayed a significant enhancement in the Aβ42 reduction at 40–60 μM (p < 0.01) and a trend toward greater reduction at 20 μM as compared with WT-APP CHO cells. (Fig. 6 and Table III).
two notable ways. First NSAIDs lower Aβ42 both in cell-based assays and in vitro. Second, the ability of NSAIDs to reduce Aβ42 is altered by PS and APP mutations. Specifically, in regards to the first characteristic, we observed that Aβ42 lowering isocoumarins, NSAIDs and cell-based assays (24, 53–55). In contrast, neither of us detected any competitive inhibitor of Aβ42, which affects γ-secretase cleavage, enhances the Aβ42 response to sulindac sulfide treatment. CHO cells expressing wild-type APP (WT-APP), cells expressing the APPV717F mutation (APPV717F) or cells expressing Swedish mutant APP (APPswedish) were treated with increasing concentrations of sulindac sulfide and Aβ42 secretion was quantified by ELISA. Dose response experiments were analyzed by two-way ANOVA with WT-APP cells as control. APPV717F cells but not APPswedish cells exhibited significantly greater Aβ42 reduction at 40–60 μM (p < 0.01) and a trend toward greater reduction at 20 μM as compared with WT-APP CHO cells (n = 8–10, Table III); **, p < 0.01 Bonferroni post-tests.

**Fig. 6.** The APPV717F mutation, which affects γ-secretase cleavage, enhances the Aβ42 response to sulindac sulfide treatment. CHO cells expressing wild-type APP (WT-APP), cells expressing the APPV717F mutation (APPV717F) or cells expressing Swedish mutant APP (APPswedish) were treated with increasing concentrations of sulindac sulfide and Aβ42 secretion was quantified by ELISA. Dose response experiments were analyzed by two-way ANOVA with WT-APP cells as control. APPV717F cells but not APPswedish cells exhibited significantly greater Aβ42 reduction at 40–60 μM (p < 0.01) and a trend toward greater reduction at 20 μM as compared with WT-APP CHO cells (n = 8–10, Table III); **, p < 0.01 Bonferroni post-tests.

**Table II**

Dose response experiments demonstrate diminished Aβ42 reduction in cells overexpressing PS1-ΔExon9

Dose-response experiments were performed as described in the text and analyzed by two-way ANOVA with WT-APP CHO or WT-APP HS683 cells as control group. n = 10 (CHO); n = 6 (HS683). **, p < 0.01 Bonferroni post-tests. ***, p < 0.001.

| Sulindac sulfide | Aβ42 CHO WT-APP | Aβ42 CHO WT-APP WT-PS1 | Aβ42 CHO WT-APP PS1-ΔExon9 |
|------------------|-----------------|------------------------|---------------------------|
| 20 μM            | 79.28 ± 1.63    | 77.77 ± 2.01           | 103.99 ± 4.32***          |
| 40 μM            | 70.56 ± 2.42    | 68.31 ± 1.52           | 96.39 ± 4.29***           |
| 60 μM            | 56.36 ± 1.49    | 51.13 ± 2.96           | 89.02 ± 4.03***           |
|                  |                 |                       |                           |
| HS683 WT-APP     | 79.65 ± 3.48    | 77.82 ± 2.11           | 96.85 ± 4.18**            |
| 40 μM            | 76.40 ± 1.89    | 65.00 ± 1.63           | 83.28 ± 4.25***           |
| 60 μM            | 40.22 ± 1.83    | 43.05 ± 1.87           | 65.40 ± 4.20**            |

**Table III**

Dose-response experiments demonstrate enhanced Aβ42 reduction in cells overexpressing APPV717F but not in cells harboring Swedish mutant APP

Dose-response experiments were performed as described in the text and analyzed by two-way ANOVA with WT-APP CHO cells as control group. n = 8–10. **, p < 0.01 Bonferroni post-tests.

| Sulindac sulfide | Aβ42 CHO WT-APP | Aβ42 CHO APPswedish | Aβ42 CHO APPV717F |
|------------------|-----------------|---------------------|-------------------|
| 20 μM            | 58.80 ± 2.00    | 60.13 ± 3.56        | 53.71 ± 2.71      |
| 40 μM            | 49.34 ± 3.36    | 45.03 ± 2.45        | 38.54 ± 1.69***   |
| 60 μM            | 27.38 ± 0.73    | 32.84 ± 1.43        | 16.32 ± 0.82***   |
reported that production of the APP intracellular cytoplasmic domain (AICD) was similarly perturbed by sulindac sulfide in their in vitro assay system, again with an unusual biphasic response: a severalfold increase at low concentrations and almost complete inhibition at 100 μM. As before, we did not find any effects of sulindac sulfide on AICD/CTFγ production in cell-based assays (60 μM) or in our in vitro assay (58) (up to 400 μM). The apparent discrepancies between our results with sulindac sulfide and the results of Takahashi et al. may be related to the use of different γ-secretase in vitro assays. The experiments by Takahashi et al. (56) employed solubilized membrane preparations from transfected cells that contain both γ-secretase activity and full-length APP as substrate. Given that NSAIDs appear to induce a very subtle shift in γ-secretase activity, it is highly likely that differences in the in vitro assays, such as the cell type used as a source of γ-secretase activity, solubilization conditions, and source of substrate, could result in slightly different effects of NSAIDs on γ-secretase activity. Nevertheless, despite these differences, both studies strongly support the hypothesis that some NSAIDs directly modulate γ-secretase activity.

A second unexpected characteristic shared by NSAIDs and γ-secretase inhibitors was seen when cells expressing FAD mutations in the PS and APP proteins were treated with Aβ42-lowering NSAIDs. The PS1-M146L and the APPV717F “Indiana” mutations that alter γ-secretase cleavage to increase Aβ42 production, but not the “Swedish” APP mutation that affects β-secretase cleavage, significantly enhanced the Aβ42 reduction after NSAID treatment as compared with wild type PS1 or APP. In striking contrast, the PS1-ΔExon9 mutation significantly attenuated the Aβ42 response to sulindac sulfide indicating that PS1 mutations modulate the drug response both positively and negatively. The greatly reduced Aβ42 response in cells expressing PS1-ΔExon9 in this report is especially informative in light of a recent study with the γ-secretase inhibitor L-685,458 (38). It was shown that the ability of L-685,458 to reduce Aβ secretion, especially the Aβ42 species, from cells expressing PS1-ΔExon9 was substantially attenuated as compared with cells expressing wild-type PS1, a situation highly reminiscent of what we observed with the Aβ42-lowering NSAID sulindac sulfide. Finally, photoaffinity derivatives of the transition-state analog L-685,458 label the N- and C-terminal fragments of PS1, which form mature non-covalent heterodimers. L-685,458 does not bind to immature full-length protein but does bind to the PS1-ΔExon9 mutant, which lacks the endoproteolytic cleavage site and accumulates as full-length protein (36). Therefore, the prominent deficiency of the γ-secretase inhibitor L-685,458 and the NSAID sulindac sulfide to reduce Aβ42 production from cells expressing the PS1-ΔExon9 mutant indicates that these compounds are both conformationally affected by the same PS mutation. Importantly, this observation leads us to speculate that these compounds may share the same molecular target.

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