Journal of Neurochemistry, 2002, **83**, 1009–1012

RAPID COMMUNICATION

Selective inhibition of Aβ42 production by NSAID *R*-enantiomers

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Abstract
Non-steroidal anti-inflammatory drugs (NSAIDs) have been associated with reduced risk for Alzheimer's disease (AD) and selected NSAIDs racemates suppress β-amyloid (Aβ) accumulation *in vivo* and Aβ42 production *in vitro*. Clinical use of NSAIDs for preventing or treating AD has been hampered by dose-limiting toxicity believed to be due to cyclooxygenase (COX)-inhibition that is reportedly not essential for selective Aβ42 reduction. Profens have racemates and *R*-enantiomers were supposed to be inactive forms. Here we demonstrate that *R*-ibuprofen and *R*-flurbiprofen, with poor COX-inhibiting activity, reduce Aβ42 production by human cells. Although these *R*-enantiomers inhibit nuclear factor-κB (NF-κB) activation and NF-κB can selectively regulate Aβ42, Aβ42 reduction is not mediated by inhibition of NF-κB activation. Because of its efficacy at lowering Aβ42 production and low toxicity profile, *R*-flurbiprofen is a strong candidate for clinical development.

Keywords: Alzheimer's disease, β-amyloid, non-steroidal anti-inflammatory drugs (NSAIDs), nuclear factor-κB (NF-κB), *R*-flurbiprofen, *R*-ibuprofen.
J. Neurochem. (2002) **83,** 1009–1012.

β-Amyloid (Aβ) peptides of 40 and 42 amino acids are major constituents of plaque and vessel amyloid in Alzheimer's disease (AD). Most genetic mutations that cause early onset AD, including mutations in Aβ precursor protein (APP) and presenilins, appear to do so by increasing production of the longer Aβ peptide 1–42 (Aβ42) (Younkin 1995). These findings have led to intense research efforts to develop drugs that inhibit the production of Aβ peptides, in particular Aβ42 (Selkoe 1997). In principle, inhibition of Aβ42 production would prevent or delay the onset of AD.

Non-steroidal anti-inflammatory drugs (NSAIDs) are typically cyclooxygenase (COX) inhibitors and appear to prevent or delay the onset of AD (Rogers *et al*. 1996; Akiyama *et al*. 2000). It had been widely believed that NSAIDs main effect is protection against cell death which presumably would continue throughout disease progression and predict NSAID treatment efficacy. However, most anti-inflammatory clinical trials have been plagued by toxicity and efficacy issues. However, NSAIDs also appear to protect against amyloid pathology which is more specific to AD and precedes cell death. Further, at least some NSAIDs can limit the progression of Aβ plaque pathology in APP transgenic mouse models (Lim *et al*. 2000; Jantzen *et al*. 2002) and a subset of NSAIDs at high doses can selectively inhibit the production of Aβ42 (Weggen *et al*. 2001). A major concern with widespread use of NSAIDs at high doses for AD prevention is the potential NSAID toxicity associated with COX inhibition, most frequently gastric ulceration, but also liver and kidney damage. All NSAIDs reported to reduce Aβ42 (Weggen *et al*. 2001) also markedly inhibit COX activity at much lower doses and are therefore likely to have toxicity problems, particularly with chronic use in the elderly. NSAIDs are administered as a racemic *R*- and *S*-mix of enantiomers and the COX-inhibitory activity of common NSAIDs in the 2-arylpropionic or profen family (ibuprofen, ketoprofen, flurbiprofen, etc.) is caused by the *S*-enantiomers (Jerussi *et al*. 1998). In contrast, *R*-enantiomers are supposed to be inactive isoforms. The reduction of Aβ42 by a subset of NSAIDs racemates was shown to be independent of COX inhibition (Weggen *et al*. 2001). Therefore, the so-called inactive *R*-enantiomer might still be able to reduce Aβ42 production.

In this study, two supposedly inactive *R*-enantiomers were tested and found to reduce Aβ42 production. High doses of ibuprofen and both *S*- and *R*-enantiomers inhibit NF-κB (Scheuren *et al*. 1998; Tegeder *et al*. 2001a) and NF-κB has been reported to specifically induce Aβ42 production (Tomita *et al*. 2000). Therefore, we tested the ability of an NF-κB peptide inhibitor to inhibit Aβ42 production. We report that *R*-flurbiprofen, an NSAID with a favorable toxicity profile at high doses in humans can selectively reduce Aβ42 production, but more specific NF-κB inhibition leaves Aβ42 production unchanged. Part of this work has been in abstract form (Morihara *et al*. 2002).

Materials and methods

Materials

R- and *S*-ibuprofen was from Sigma (St Louis, MO, USA). *R*-ibuprofen and *R*-flurbiprofen were the generous gift of Dr Peter Andersch (PAZ Pharma, Germany). A 1000 × stock solution in ethanol (EtOH) was prepared. Cell-permeable NF-κB inhibitory peptide SN50 and its inactive control peptide, SN50M, were purchased from Biomol (Plymouth Meeting, PA, USA).

Cell culture

Human embryonic kidney (HEK293) cells stably transfected with human 'Swedish' mutant APP (HEK293 APPSw3, the generous gift of Dr S. Sisodia, University of Chicago, Chicago, IL, USA) were cultured in

Received August 12, 2002; accepted August 21, 2002.

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Abbreviations used: Aβ, amyloid β protein; AD, Alzheimer's disease; COX, cyclooxygenase; NF-κB, nuclear factor κB; NSAIDs, non-steroidal anti-inflammatory drugs.

Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum, 1 mM Na pyruvate, 2 mM GlutaMax-1 (Gibco-BRL, Grand Island, NY, USA), 16 mM HEPES pH 7.4, penicillin 100 U, streptomycin 100 μg/mL, and 200 μg/mL G418. Serum containing media was withdrawn from semiconfluent cultures and drugs added at the indicated doses in serum-free media with 0.1% EtOH. After 18 h, media were harvested, aliquoted, and assayed for LDH and Aβ by sandwich enzyme-linked immunosorbent assay (ELISA).

Sandwich ELISAs for Aβ

ELISA kits for Aβ40 and Aβ42 were purchased from Biosource International (Camarillo, CA, USA) and performed as per manufacturer's instructions. Our sandwich ELISA for total Aβ has been previously described (Lim *et al*. 2000). Briefly, the assay uses monoclonal 4G8 against Aβ17–24 (Senentek, Napa, CA, USA) as the capture antibody (3 μg/mL), biotinylated 10G4 against Aβ1–15 as the detecting antibody, and a reporter system using streptavidin–alkaline phosphatase and AttoPhos (JBL Scientific Inc, San Luis Obispo, CA, USA) as the substrate (excitation 450 nm/emission 580 nm). All ELISAs were carried out in duplicate.

Immunoblots for APP

Levels of full-length APP (rabbit polyclonal 681–695, Kang sequence) in cell lysates and secreted APP in media (22C11, Chemicon, Temecula, CA, USA) were determined on immunoblots. Blots were performed as previously described (Lim *et al*. 2001). The comparisons were done in triplicate, scanned with exposure in a linear range and the signal strength (relative optical density, OD) analyzed.

Results

Aβ42 was readily detectable in the 18 h conditioned media from HEK293Sw3 cells. The percentage of Aβ42 compared with vehicle control was effectively reduced by racemic ibuprofen (ibuprofen) at clinically achievable plasma levels, where side-effects frequently emerge particularly in the elderly population (50–250 μM; Fig. 1a). Inhibition of the percentage of Aβ42 was also obtained with *R*-ibuprofen with these doses (Fig. 1b). *R*-flurbiprofen had a larger effect, reducing Aβ42 by nearly 40% ($p = 0.003$) at 250 μM (Fig. 1c). In independent experiments, we confirmed that Aβ42/total Aβ ratios were similarly reduced by *R*-flurbiprofen and ibuprofen (not shown).

The results showing that the weak-COX inhibiting *R*-enantiomers of both ibuprofen and flurbiprofen also result in similar reductions in Aβ42 is consistent with Weggen *et al.*'s (2001, 2002) evidence that the Aβ42 reduction is COX-independent. One known COX-independent activity of both *R*-ibuprofen and *R*-flurbiprofen in the same range of Aβ42 reduction dosage is NF-κB inhibition (Scheuren *et al*. 1998; Tegeder *et al*. 2001a) and NF-κB has been reported to selectively induce Aβ42 production (Tomita *et al*. 2000). Therefore, we tested the Aβ42 lowering activity of a specific NF-κB inhibitor (SN-50) at doses which effectively inhibit translocation of the NF-κB active complex into the nucleus (Lin *et al*. 1995). Compared with the manufacturer's control peptide (SN-50M; Fig. 1d) or no drug control (not shown), SN-50 failed to decrease or even increased Aβ42 production. Given that 50 μg/mL SN-50 is able to inhibit NF-κB by 88% (Lin *et al*. 1995), these results with 50 μg/mL and even higher doses argue that NF-κB inhibition was not responsible for *R*-profen action in lowering Aβ42 production.

Another possible mechanism for profen activity lowering Aβ42 production could be a reduction in total APP production or APP secretion. However, at doses inhibiting Aβ42, there was also no drug effect on the levels of full-length APP protein in cell lysates assayed by

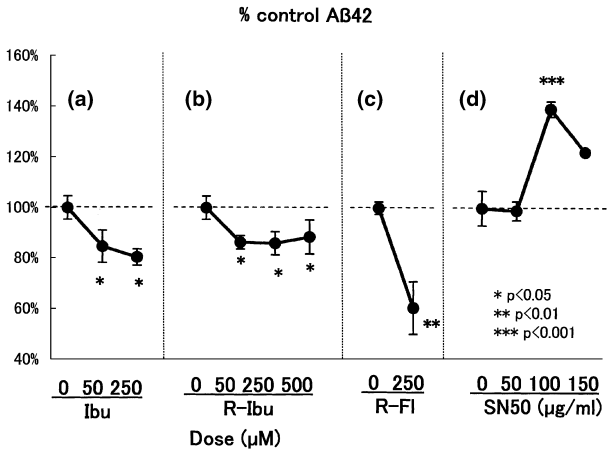

Fig. 1 Secreted Aβ42 levels from HEK293Sw3 were measured by ELISA. (a–c) Ibuprofen, *R*-ibuprofen and *R*-flurbiprofen significantly reduced Aβ42 production compared with vehicle control ($n = 3$). (d) NF-κB inhibitor SN50 failed to reduce Aβ42 production ($n = 3$–4 for 0–100 μg/mL $n = 1$ for 150 μg/mL). Error bars indicate SD.

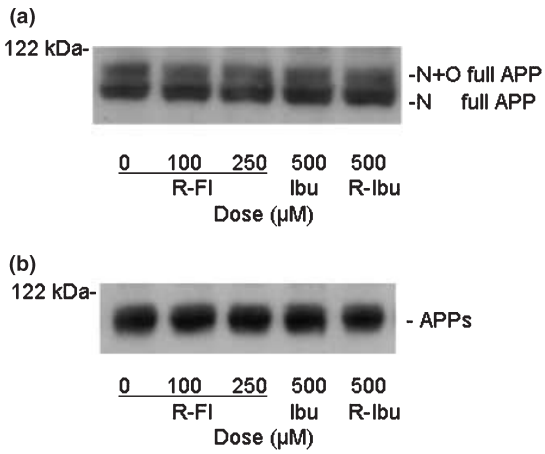

Fig. 2 APP western blotting. (a) N- and N + O-glycosylated full length APP in cell lysates were not changed by ibuprofen, *R*-ibuprofen or *R*-flurbiprofen. (b) Secreted APP in the medium was not affected by the drugs.

western analysis (Fig. 2a) or on APPs in the media (Fig. 2b). This result is consistent with the lack of cell loss or inhibition of APP production and maturation, but consistent with an effect on APP processing to Aβ.

As shown in Fig. 3, *R*-flurbiprofen significantly reduced Aβ42, but not Aβ40 at 100 and 250 μM ($p < 0.001$). The selective reduction in 42, but not 40 resulted in significant decreases in the Aβ42/Aβ40 ratios expressed as percent of control (vehicle-treated) levels ($p < 0.05$). No evidence of toxicity in lactate dehydrogenase (LDH) assays was evident with doses of *R*-flurbiprofen of 250 μM and below, but toxicity was observed with 150 μg/mL SN-50 (not shown). Along with the evidence that media levels of APPs and Aβ40 are unchanged, our data indicate a selective *R*-flurbiprofen effect on Aβ42 production.

Discussion

Unfortunately, inhibition of the Aβ-generating γ-secretase and genetic knockout for the secretase-related presenilin proteins result in not only

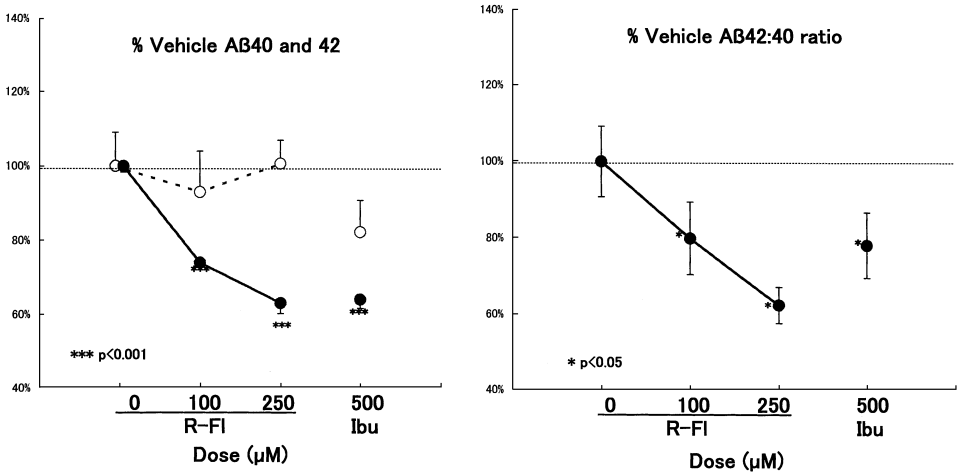

Fig. 3 Selective Aβ42 reduction by *R*-flurbiprofen. (a) %Vehicle levels of Aβ40 and 42 and (b) %Vehicle Aβ42 : 40 ratio in the medium. $n = 4$. Error bars indicate SD.

inhibition of both Aβ40 and Aβ42, but compromised Notch activity and immune function (De Strooper *et al*. 1999). In this study, supposedly inactive and safe *R*-enantiomers successfully reduced Aβ42 (Figs 1 and 3). Though both *R* and *S*-flurbiprofen can inhibit the NF-κB pathway at high doses and induction of the NF-κB pathway has been reported to selectively increase Aβ42 production, NF-κB inhibitior SN-50 did not reduce Aβ42 (Fig. 1). During the submission of this report, Koo's group presented additional evidence against involvement of NF-κB pathway in the reduction of Aβ42 by NSAIDs using p65/RelA knockout fibroblasts (Weggen *et al*. 2002). Though the mechanism of Aβ42 reduction is still unknown, the shared action of R and S NSAIDs is a clue toward its elucidation.

Most NSAID have optimized IC_{50} for COX inhibition in the nanomoler or low micromoler range. Their ability to lower Aβ42 production occurs at doses at least an order of magnitude higher than COX inhibition. For example, ibuprofen's IC_{50} for COX-1 is 2.1 μM (Neupert *et al*. 1997; see for review Tegeder *et al*. 2001b), well below dosing for Aβ42 reduction. This implies toxicity from near complete COX inhibition will sharply curtail their use for Aβ42 reduction. In contrast, *R*-flurbiprofen has similar Aβ42 lowering activity to *S*-flurbiprofen, but much less potent COX inhibitory activity. (see for review Tegeder *et al*. 2001b) Because HEK293 cells produce very low prostaglandin E_2 and have negligible activity of the enzymes involved in this cascade (Ueno *et al*. 2001), the prostaglandin E_2 levels in our HEK293 culture were undetectable, although readily measured in CHO cells (data not shown). While we could not confirm the absence of COX inhibition by *R*-flurbiprofen in HEK293 cells, previous reports show the IC_{50} for human COX-1 and COX-2 by *R*-flurbiprofen are more than 40 μM and 100 μM, while those of *S*-flurbiprofen are 0.03 μM and 0.9 μM, respectively (Geisslinger *et al*. 2000; see for review Tegeder *et al*. 2001b).

Unlike other *R*-enantiomers of profens which are rapidly inverted into their potent COX-inhibiting *S*-forms, *R*-flurbiprofen is very poorly bio-inverted and therefore has only weak *in vivo* COX-inhibitory activity (Geisslinger and Schaible 1996). The absence of enantiomer inversion has allowed a clinical trial with chronic doses of *R*-flurbiprofen resulting in sustained plasma levels of *R*-flurbiprofen well above levels required for significant Aβ42 inhibition in the absence of drug-related toxicity (Keegan and Loughman 2001). Because *R*-flurbiprofen readily enters the CNS (Geisslinger *et al*. 2000), it should also limit Aβ42 production in human CNS. Although low doses of *R*-flurbiprofen have been safely used in patients as half of flurbiprofen racemate for many years, whether chronic high dose *R*-flurbiprofen results in significant toxicity in AD patients can only be resolved by clinical trials.

We are currently carrying out chronic *in vivo* studies in APPsw transgenic animals to determine the impact of *R*-flurbiprofen on plaque pathogenesis. The probability of success is increased by a recent report showing plaque reduction in a transgenic model with chronic nitro-flurbiprofen, a drug that breaks down to produce both *R*- and *S*-flurbiprofen (Jantzen *et al*. 2002).

Acknowledgements

This work was supported by NIA-AG13471 (GMC), VA Merit, the Alzheimer Association and The Elizabeth and Thomas Plott Family Foundation. The authors would like to thank Dr S. Sisodia, U Chicago for HEK293Sw3 cells.

Note added in proof

Dr Todd Golde's group has also presented data demonstrating the reduction of Aβ42 by *R*-flurbiprofen at the 8th International Conference on Alzheimer's Disease and Related Disorders (Stockholm, July 20–25, 2002, Abstract 373).

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