Supplementary Information for "A subset of NSAIDs lower amyloidogenic Aβ42 independent of COX activity"

Methods for Supplementary Information

Antibodies.

Monoclonal antibodies 5A3 and 1G7 recognizing non-overlapping epitopes of APP between residues 380-665, polyclonal antibody CT15 against the C-terminal 15 amino acid residues of APP, and monoclonal antibody 26D6 against amino acid residues 1-12 of the Aβ sequence have been described [1-3]. The monoclonal antibody against COX-2 was purchased from BD Transduction Laboratories, the polyclonal antibody against COX-1 (M-20) was purchased from Santa Cruz Biotechnology.

Measurements of Prostaglandin E_2 levels in cell culture media

To determine PGE_2 levels in CHO cells and mouse fibroblasts after NSAID treatment, 2 x 10^5 cells were seeded per well in 24 well plates and treated overnight with the indicated concentrations of NSAIDs. Medium was replaced and COX-2 expression was induced with 100 nM PMA for 8h in the presence of NSAIDs or vehicle. PGE_2 levels were then analyzed with a commercial enzyme immunoassay (Amersham Pharmacia Biotech). To compare Prostaglandin E_2 secretion in COX-1 -/-, COX-2 -/- fibroblast versus littermate control fibroblasts, 2.5 x 10^5 cells were seeded per well in 24 well plates and grown for 24 h. Medium was changed and cells were incubated in the presence or absence of 100 nM PMA for 8 h. PGE_2 levels in culture medium were then quantified by enzyme immunoassay as above.

Analysis of APP processing after NSAID treatment

Expression of holo-APP and APP CTFs was examined by Western blot analysis with antibody CT-15. β-CTF levels were investigated by immunoprecipitation with antibody 26D6 recognizing the N-terminus of Aβ, followed by Western-blotting with antibody CT-15. APPs and APPs-α secretion was analyzed by Western blot analysis of culture medium with antibodies 5A3/1G7 or 26D6. APP turnover was investigated by metabolic labeling in CHO cells with 150 μCi [^{35}S]-methionine for 15 min and cold chase for up to 4h. Cell lysates were immunoprecipitated with antibody CT-15, subjected to SDS-PAGE and analyzed by phosphor imaging. APP surface expression and internalization was measured as described [1]. In brief, iodinated 1G7 antibody (~3-6 μCi/μg) was applied to confluent layers of CHO cells in binding medium (DMEM, 0.2% BSA, 20 mM HEPES) and incubated at 37°C for 30 min. After incubation, cells were rapidly chilled on ice, and the reaction was quenched by the addition of ice-cold binding medium. Chilled cells were washed multiple times with ice-cold Dulbecco's phosphate-buffered saline to eliminate unbound antibody. Residual antibody bound to surface APP was then detached from cell surfaces by two 5 min washes with ice-cold PBS, pH 2.0. Cells remaining in the culture dish were lysed in 0.2 M NaOH. Acid-labile and acid-resistant APP antibody counts were measured by γ-counting. The ratio of acid-resistant to acid-labile counts provided a measure of the internalized versus cell surface pools of APP.

Table 1 Summary of results with sulindac sulfide in various cell lines

Cell line	Transfected gene(s)	≥50% reduction in Aβ42 levels (80-100 μM)
CHO cell lines		
	APP751 wild type	+
	APP751 wild type and PS-1 wild type	+
	APP751 wild type and PS-1 M146L mutation	+
	APP751 wild type and PS-1 C410Y mutation	+
	APP751 wild type and PS-2 wild type	+
	APP751 wild type and PS-2 M239V mutation	+
	APP751 KM670/671 NL "Swedish" mutation	+
	APP751 with V717F "Indiana" mutation	+
Neuroglioma cell lines		
HS683	APP695 wild type	+
HS683	APP695 wild type and mutant PS-1 M146L mutation	+
H4	APP695 wild type	+
HEK293 cell lines		
	APP695 wild type	+
Mouse fibroblast cell lines		
	COX-1 +/+, COX-2 +/+ control fibroblasts	+
	COX-1 -/-, COX-2 -/- fibroblasts	+
	COX-1 -/-, COX-2 -/- fibroblasts and PS-1 M146L mutation	+

Table 2 Summary of effects on Aβ42 levels by various NSAIDs in cultured cells

NSAID	COX specificity	Highest concentration tested (μM)	Aβ42 reduction*
sulindac sulfide	non-selective	200	+
ibuprofen	non-selective	1000	+
indomethacin	non-selective	400	+
naproxen	non-selective	400	-
sulindac sulfone	no COX activity	400	-
aspirin	COX-1 specific	3000	-
SC-560	COX-1 specific	100	-
meloxicam	COX-2 preferential	100	-
celecoxib	COX-2 specific	20	-

* Reduction at least 50% in two or more cell lines.

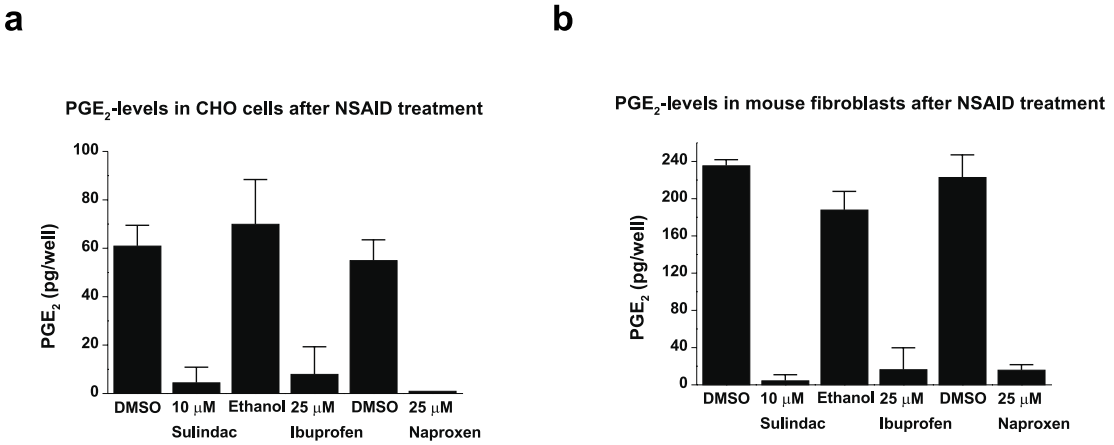

Figure 1 Prostaglandin E_2 levels in cell culture media after NSAID treatment. **a**, PGE_2 levels after NSAID treatment in CHO cells stably transfected with both APP751 and the PS1 M146L mutation (APP WT/ PS1 M146L). NSAID treatment dramatically reduced PGE_2 levels in culture media as expected. Treatment with 25 μM naproxen reduced PGE_2 production to undetectable levels. **b**. PGE_2 levels in mouse fibroblasts after NSAID treatment. Mouse fibroblasts showed high levels of PGE_2 secretion after PMA stimulation. Consistent with the results on CHO cells, NSAID treatment strongly reduced PGE_2 levels in culture media. Results show one representative experiment performed in duplicate (mean ± s.d.).

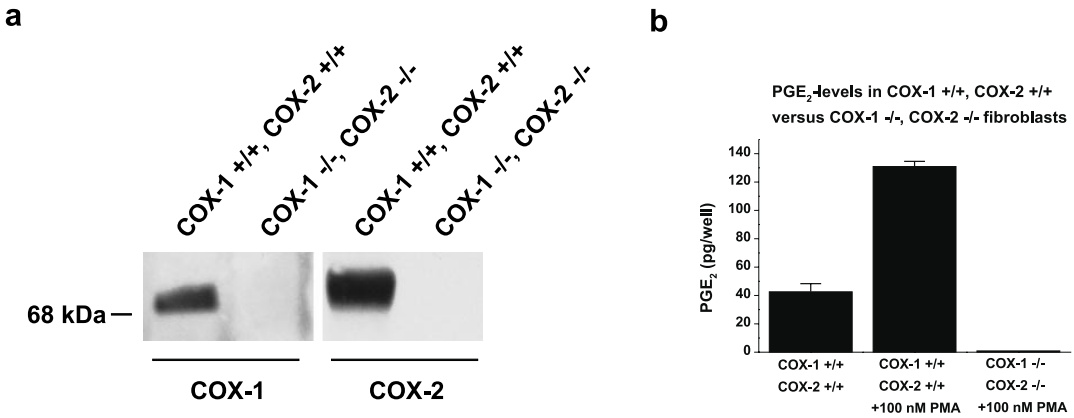

Figure 2 COX-1 -/-, COX-2 -/- fibroblasts are devoid of COX protein expression and enzymatic activity. **a**, Western blot analysis of COX protein expression in COX-1 -/-, COX-2 -/- fibroblasts and littermate control fibroblasts. The control fibroblasts showed expression of COX-1 and COX-2 protein whereas in the knockout fibroblasts, COX-1 and COX-2 proteins were absent. **b**, COX-1 +/+, COX-2 +/+ fibroblasts showed a threefold induction of PGE_2 levels after stimulation with PMA. Consistent with previously published data [4] PGE_2 levels in COX-1 -/-, COX-2 -/- fibroblast were undetectable even under induced conditions. Results show one representative experiment performed in triplicate (mean ± s.d.).

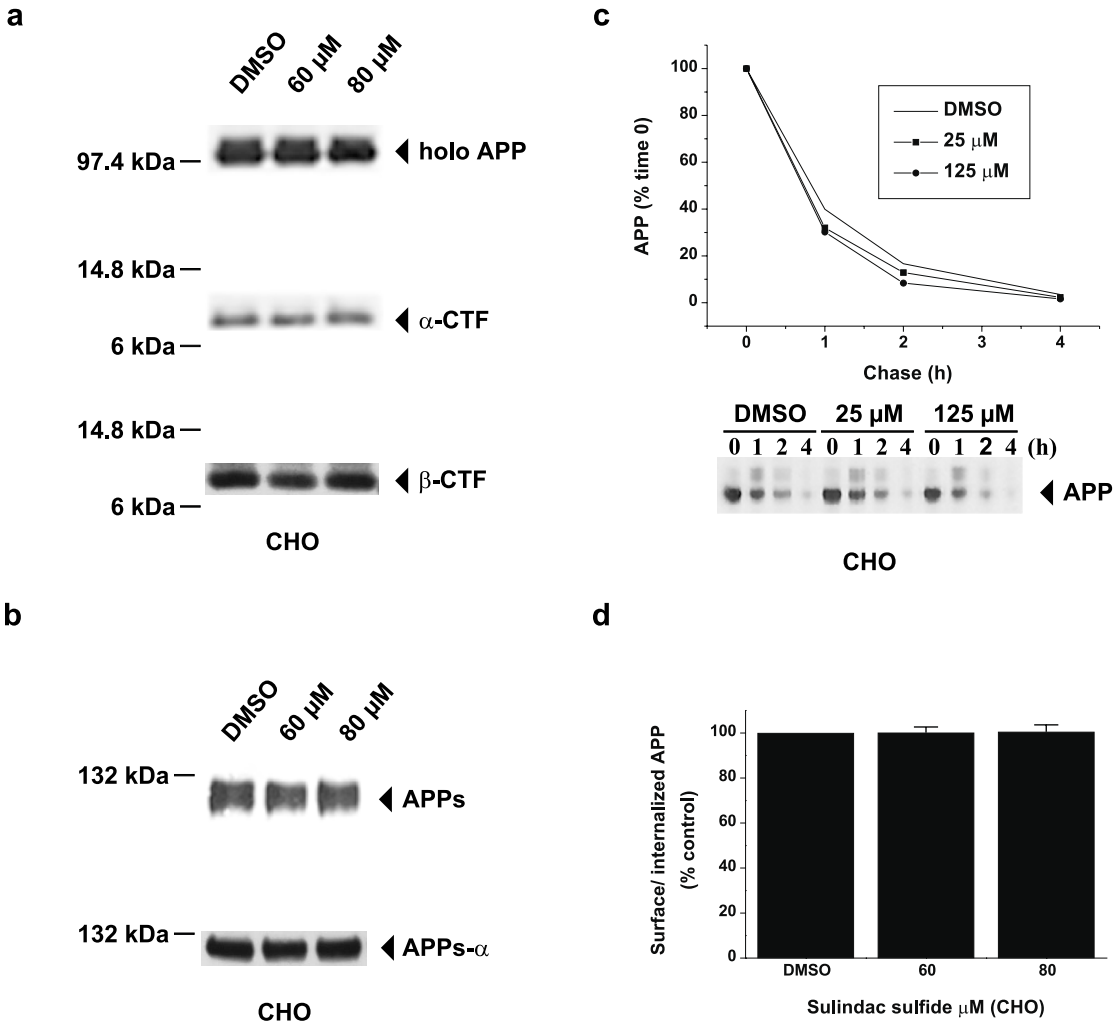

Figure 3 Analysis of APP processing in APP WT CHO cells after sulindac sulfide treatment. a, Steady-state APP levels detected by western blotting. No change was seen in either the levels of full length APP, α-CTFs or β-CTFs after sulindac sulfide treatment. Western blotting with antibody CT-15 detects full length APP and predominantly α-CTF while immunoprecipitation with antibody 26D6 followed by blotting with CT-15 detects β-CTF. b, Secretion of APPs and APPs-α was unaffected by sulindac sulfide treatment as examined by Western blot analysis of culture medium with antibodies antibodies 5A3/1G7 or 26D6. Antibody 26D6 against amino acid residues 1-12 of the Aβ sequence detects only APPs-α. c, APP turnover in the presence of sulindac sulfide. All values were normalized to the signal obtained at the beginning of the chase period (top panel). Representative autoradiogram is shown in the bottom panel. APP turnover was unaffected by sulindac sulfide. Similar results were obtained in HS683 neuroglioma cells (data not shown). d, APP internalization, expressed as the ratio of cell surface APP versus internalized APP, was unchanged after sulindac sulfide treatment. The mean ± s.d. of two independent experiments performed in duplicate is shown.

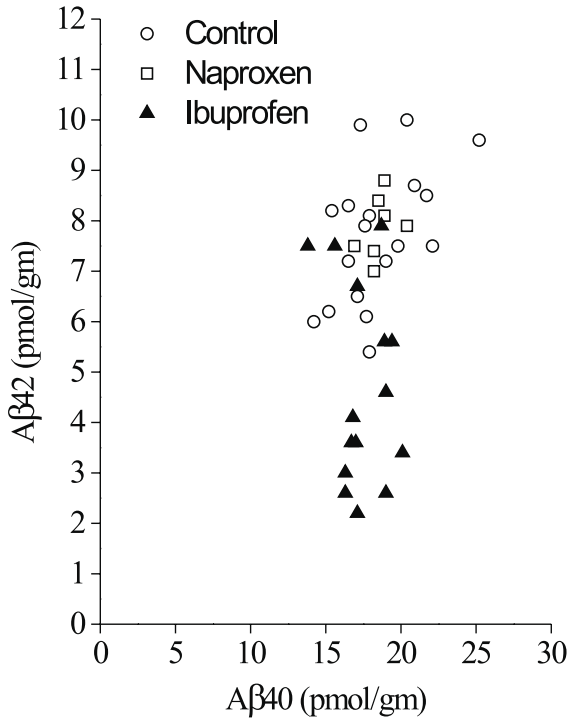

Figure 4 Scattergram of Aβ40 and Aβ42 levels in brain of Tg2576 mice after short-term ibuprofen treatment by ELISA. Three-month-old female mice were orally dosed with ibuprofen (n=15), naproxen (n=7) or mock treated (n=18). On average, treatment with ibuprofen induced a 39% decrease in brain levels of SDS-soluble Aβ42 without any changes in Aβ40. Consistent with our cell culture experiments, naproxen treatment did not induce any changes in Aβ40 and Aβ42 levels. Note that x- and y-axes represent Aβ40 and Aβ42 levels in pmol/gm brain tissue, respectively.

References for Supplementary Information

1. Perez, R. G. et al. Mutagenesis identifies new signals for beta-amyloid precursor protein endocytosis, turnover, and the generation of secreted fragments, including Abeta42. *J Biol Chem* 274, 18851-6 (1999).

2. Sisodia, S. S., Koo, E. H., Hoffman, P. N., Perry, G. & Price, D. L. Identification and transport of full-length amyloid precursor proteins in rat peripheral nervous system. *J Neurosci* 13, 3136-42 (1993).

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4. Zhang, X., Morham, S. G., Langenbach, R. & Young, D. A. Malignant transformation and antineoplastic actions of nonsteroidal antiinflammatory drugs (NSAIDs) on cyclooxygenase-null embryo fibroblasts. *J Exp Med* 190, 451-59 (1999).