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Ibuprofen Suppresses Interleukin-1β Induction of Pro-Amyloidogenic α_1-Antichymotrypsin to Ameliorate β-Amyloid (Aβ) Pathology in Alzheimer's Models

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Epidemiological and basic research suggests that nonsteroidal anti-inflammatory drugs (NSAIDs) should protect against the most common forms of Alzheimer's disease (AD). Ibuprofen reduces amyloid (Aβ) pathology in some transgenic models, but the precise mechanisms remain unclear. Although some reports show select NSAIDs inhibit amyloid production *in vitro*, the possibility that *in vivo* suppression of amyloid pathology occurs independent of Aβ production has not been ruled out. We show that ibuprofen reduced Aβ brain levels in rats from exogenously infused Aβ in the absence of altered Aβ production. To determine whether ibuprofen inhibits pro-amyloidogenic factors, APPsw (Tg2576) mice were treated with ibuprofen for 6 months, and expression levels of the Aβ and inflammation-related molecules α_1 antichymotrypsin (ACT), apoE, BACE1, and peroxisome proliferator-activated receptor γ) (PPARγ) were measured. Among these, ACT, a factor whose overexpression accelerates amyloid pathology, was reduced by ibuprofen both *in vivo* and *in vitro*. IL-1β, which was reduced in our animals by ibuprofen, induced mouse ACT *in vitro*. While some NSAIDs may inhibit Aβ42 production, these observations suggest that ibuprofen reduction of Aβ pathology may not be mediated by altered Aβ42 production. We present evidence supporting the hypothesis that ibuprofen-dependent amyloid reduction is mediated by inhibition of an alternate pathway (IL-1β and its downstream target ACT).
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INTRODUCTION

Although Alzheimer's disease (AD) is classified as a neurodegenerative rather than inflammatory disorder, the associated chronic inflammation of the brain is thought to play a role in pathogenesis and progression (Yasojima *et al*, 1999); see reviews (Akiyama *et al*, 2000; McGeer and McGeer, 2003). Although epidemiological data suggesting nonsteroidal anti-inflammatory drugs (NSAIDs) are protective against AD (Breitner *et al*, 1994; McGeer *et al*, 1996; Stewart *et al*, 1997; in t' Veld *et al*, 2001; Zandi *et al*, 2002) have been used to argue for the pathogenic significance of neuroinflammation in AD, it is not resolved whether the beneficial targets of NSAIDs are inflammation-related. While some studies argue for inflammation-independent pathways targeted by NSAIDs in AD prevention (Weggen *et al*, 2001), other studies support the argument that inflammatory responses (known to be induced by β-amyloid protein (Aβ), neurodegeneration, and Aβ-comprised neuritic plaques) may contribute to further neurodegeneration and induce additional amyloid pathology (Lim *et al*, 2000, 2001; Xiang *et al*, 2002).

We previously reported that ibuprofen treatment reduced amyloid pathology in the APPsw Tg2576 mice (Lim *et al*, 2000). Although ibuprofen has failed to impact plaques in some more aggressive models, others have confirmed ibuprofen reduction of plaques in APP transgenic (APP Tg) Alzheimer model mice (Jantzen *et al*, 2002; Yan *et al*, 2003). The major inflammatory target of NSAIDs, including ibuprofen, is cyclooxygenase (COX), and in our model ibuprofen-induced plaque reduction was associated with reductions in brain interleukin-1β, a downstream target of

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COX. Although this and other evidence suggests a possible role of inflammation in controlling plaque pathogenesis, this hypothesis has been challenged by discovery of an unexpected COX-independent function of a subset of NSAIDs (including ibuprofen) in reducing Aβ42 production *in vitro* (Weggen *et al*, 2001, 2003a, b; Morihara *et al*, 2002; Eriksen *et al*, 2003; Sagi *et al*, 2003; Takahashi *et al*, 2003; Zhou *et al*, 2003). Adding to the controversy whether the mechanism(s) of plaque reduction are inflammation-dependent or -independent, it is unclear whether the high ibuprofen concentrations required *in vitro* to reduce Aβ42 production can be attained in the human brain with doses shown epidemiologically to reduce AD risk. Further, epidemiological studies suggest that some of the NSAIDs that appear to show AD risk reduction cannot reduce Aβ42. In summary, it remains uncertain whether the suppression of Aβ42 production by γ-secretase is the main mechanism for the protective effects of NSAIDs in the epidemiological studies and for the reduction of amyloid plaques in chronic NSAID-treated APP Tg mice.

An alternative possible NSAID mechanism for suppressing Aβ production is targeting a key regulator of Aβ production in the brain: BACE1, the main enzyme that cleaves APP at the β-secretase site. BACE1 deficiency abolishes Aβ production in BACE1 knockout mouse (Cai *et al*, 2001; Luo *et al*, 2001; Roberds *et al*, 2001) and in cell cultures (Cai *et al*, 2001; Roberds *et al*, 2001). BACE mRNA and protein levels and β-secretase activities were reported to be induced by a combination of proinflammatory cytokines and suppressed by NSAIDs and peroxisome proliferator-activated receptor gamma (PPARγ) agonist *in vitro* (Sastre *et al*, 2003).

Besides suppressing Aβ42 production, anti-inflammatory effects of NSAIDs, particularly those achieved with lower NSAID doses, could exert protection from AD by reducing amyloid deposition without altering Aβ42 production. Amyloid pathology can be affected by multiple inflammation-driven pathways, notably those involving acute phase proteins. Inflammation-related astrocyte molecules like apolipoprotein E (apoE) (Bales *et al*, 1997) and α_1-antichymotrypsin (ACT) (Mucke *et al*, 2000; Nilsson *et al*, 2001) regulate amyloid pathology in APP Tg mice. ACT is an acute phase protein that accelerates amyloid pathology in APP Tg mice and is elevated in AD (Abraham *et al*, 1988; Pasternack *et al*, 1989; Koo *et al*, 1991; Shoji *et al*, 1991; Licastro *et al*, 1998), where it accumulates with amyloid plaques. ApoE, expressed primarily by astrocytes, can be induced during glial activation associated with cell damage or inflammation. ApoE has been shown to dose-dependently and isotype-specifically promote Aβ deposition in APP Tg mice (Bales *et al*, 1997; Fagan *et al*, 2002). Chronic proinflammatory LPS injection into APP Tg apoE wild-type mice has been reported to induce extensive GFAP-positive astrocytes and to accelerate amyloidosis, while LPS failed to increase amyloid deposition in APP Tg apoE-knockout mice (Qiao *et al*, 2001). Since ibuprofen treatment reduced GFAP in our APP trangenics (Lim *et al*, 2000), we reasoned that control of inflammation and gliosis might reduce apoE production. Although some reports show an association between increased apoE mRNA (or promoter polymorphisms promoting such increases) and AD risk or amyloid plaques (Diedrich *et al*, 1991; Yamada *et al*, 1995; Bullido *et al*, 1998; Lambert *et al*, 1998; Laws *et al*, 1999), other reports show that this relation is restricted to apoE4 cases (Lambert *et al*, 1997; Roks *et al*, 1998; Song *et al*, 1998; Pahnke *et al*, 2003).

Therefore in this study, we explored the potential of ibuprofen to alter post-amyloid production effects of NSAIDs using the Tg2576 mouse and *in vitro* model systems. We focused on the impact of chronic ibuprofen treatment on expression of a set of molecules related to both activated glia and amyloid pathology: BACE1, apoE, Il-1β, α1 ACT. We also evaluated the effect of ibuprofen on Aβ deposition from exogenously infused Aβ, independent of effects on production.

MATERIAL AND METHODS

Immunohistochemistry for Amyloid Burden in Aβ-infused Rats

Female Sprague–Dawley rats (19 months old) were fed chow with ($n=10$) or without ($n=10$) ibuprofen (375 ppm) for 60 days. They were then intracerebroventricularly infused bilaterally into the lateral ventricles with a mixture of Aβ40 (20 μg total) and Aβ42 (5 μg total) with HDL as carrier and 4 mM HEPES, pH 8.0 buffer for 30 days, as previously described (Frautschy *et al*, 2001). The 4 : 1 ratio used approximates the typical ratio of secreted Aβ40 : 42. Rats were then killed and brain sections prepared for histochemical analysis. Aβ deposits were labeled with 10G4 anti-Aβ antibody and detected by ABC ELITE-peroxidase kits (Vector) using metal diaminobenzidine (DAB, Pierce, Rockford, IL). Captured immunohistochemical images were analyzed by NIH Image software with custom macro subroutines, as previously described (Frautschy *et al*, 1999).

Mice for Chronic Ibuprofen Treatment

Tg2576 transgenic mice, 10-month-old huAPPsw line, were used. Transgene negative littermates (Tg−; $n=10$) were fed control chow and transgene positive (Tg+) mice were fed control chow ($n=12$) or chow with 375 ppm ibuprofen (Sigma St Louis, MO) for 6 months ($n=8$) or 3 months ($n=4$) as reported previously (Lim *et al*, 2000). Cortical tissue was snap frozen in liquid nitrogen and powderized for mRNA analysis. Half of the powdered tissue was used for protein analysis reported previously for Aβ and IL-1β (Lim *et al*, 2000) and half was analyzed for mRNA in this study. APPsw Tg+ ($n=7$) and Tg− control mice ($n=9$), 22-month old, on control chow were also analyzed. Positive controls for verification of CNS inflammation were made by injecting LPS (6 μg) into the right lateral ventricle of Tg− mice and killing after 24 h. The protocols using mice and rats were approved by the UCLA and VA IACUCs.

Real-Time PCR of mACT and apoE mRNA

Total RNA was prepared using RNAqueous kits (Ambion, Austin TX). The absence of RNA degradation was confirmed by gel electrophoresis and total RNA was DNase treated (DNA-free, Ambion). cDNA was generated from

1.4 μg total RNA in two reaction volumes of RETROscript (Ambion) using dT primer and then aliquoted for single uses. TaqMan real-time PCR assays for mACT and apoE mRNA were developed. Primers (Sigma; IDT) and fluorescent probes (Biosource, Camarillo CA; IDT, Coralville IA) were designed using Primer Express 1.1 (Applied Biosystems, Foster City CA). BACE1 and COX-2 was measured using Assay on Demand (Mm00478664_m1 and Mm00478374_m1, Applied Biosystems, Foster City, CA). PPARγ primer and probes were designed in Dr P Tontonoz's lab (UCLA). Sequences and optimized concentrations of primers and probes are shown in Table 1. The absence of nonspecific PCR was confirmed by gel electrophoresis using 45 cycle PCR products (data not shown). cDNA for relative standard curves was prepared from Tg− mouse brain with the same protocols with double RNA concentration in the reverse transcript step. Real-time PCR was performed by SDS7700 (Applied Biosystems) and TaqMan Universal PCR Master Mix (Applied Biosystems), using triplicates for samples and quadruplicates for standards. GAPDH was measured as the internal control using TaqMan Rodent GAPDH control (Applied Biosystems). Consistent GAPDH levels confirm the absence of RNA and/or cDNA degradation (data not shown). The absence of contamination was checked using RT products made without M-MLV reverse transcriptase. The R^2 of relative standard curves in TaqMan PCR was above 0.99. The R^2 of mRNA levels from the same samples but measured on different days was around 0.9. In spite of the great reproducibility and use of the same five-point standards in every plate, around 5% difference in average values between different PCR plates with same samples was observed (data not shown). For optimum reliable comparisons, only sample values from the same PCR plate were statistically analyzed and shown here.

Multiplex RT-PCR of mACT mRNA

Murine ACT (EB22/4, mACT) was amplified by PCR along with β-actin in the same tubes. The sequences of primers are shown in Table 1. cDNA (1 μl) was amplified by 1.5 U of thermostable *Taq* Polymerase (#0032 003.416 Eppendorf, Hamburg Germany) in 20 μl reaction volumes (1 μCi 32P dCTP, 0.2 mM dNTPs, 50 mM KCl, 10 mM Tris-HCl pH 8.3, and 1.5 mM MgCl). Since the expression levels of β-actin mRNA were much higher than those of mACT mRNA, mACT PCR amplification was distorted in the same PCR tubes. To avoid this PCR interruption, mACT primers alone were added at the beginning of PCR and β-actin primers were added into the PCR tubes after nine cycles of PCR. The optimized total PCR cycles were 24 for ACT. The absence of PCR interactions and the expected exponential amplification of both products were confirmed during these optimizations (data not shown). PCR products were analyzed by acrylamide gel electrophoresis and exposed autoradiogram and quantified using Molecular Imager system (BioRad, Hercules, CA).

ApoE Western Blotting

Cortex was homogenized in TBS and then spun down at 55 000 RPM (100 000 *g*) for 20 min at 4°C. The supernatant was used as TBS fraction. The pellet was solublized in 2% SDS and used as SDS fraction. Western blotting was

Table 1 Sequences and Optimized Concentrations of Primers and Probes

Molecules	Sequence	nM
mACT (α1-antichymotrypsin)		
Forward primer	GTCTATCCGCAGCATCTAGGACTAC	300
Reverse primer	GGAGGCAAAGCCTGGAGAA	900
Probe	FAM-TGTGCCCAGTCTGCCTCACTCTTTC-TAM	225
ApoE		
Forward primer	TCTGACCAGGTCCAGGAAGAG	300
Reverse primer	AGCTGTTCCTCCAGCTCCTTT	900
Probe	FAM-CACACAAGAGCTGACGGCACTGATGG-TAM	200
mACT (α1-antichymotrypsin multiplex)		
Forward primer	CCCAGACTTCTTGGTAGCAC	500
Reverse primer	CCTAGATGCTGCGGATAGAC	500
β Actin		
Forward primer	TCTACAATGAGCTGCGTGTG	500
Reverse primer	TACATGGCTGGGGTGTTGAA	500

Murine ACT (EB22/4, mACT) was amplified by PCR along with β-actin in the same tubes. cDNA (1 μl) was amplified by 1.5 U of thermostable *Taq* polymerase (#0032 003.416 Eppendorf, Hamburg Germany) in 20 μl reaction volumes (1 μCi 32P dCTP, 0.2 mM dNTPs, 50 mM KCl, 10 mM Tris-HCl pH 8.3, and 1.5 mM MgCl). mACT primers alone were added at the beginning of PCR and β actin primers were added into the PCR tubes after nine cycles of PCR. The optimized total PCR cycles were 24 for ACT. PCR products were analyzed by acrylamide gel electrophoresis and exposed autoradiogram and quantified using Molecular Imager system (BioRad, Hercules, CA).

performed using rabbit anti-rat apoE antibody R243 (gift of Dr Schonfeld, Wash. U., St Louis, MO, 1 : 1000) and anti-rabbit antibody conjugated to HRP (Pierce #31463, 1 : 10 000).

Human Astrocytoma U373 Cell Cuture and ACT Western Blotting

Human astrocytoma U373 cells were cultured to semiconfluence in MEM supplemented with 10% fetal bovine serum, 1 mM Na-pyruvate, 2 mM GlutaMax-1 (Gibco BRL, Grand Island NY), 16 mM HEPES pH 7.4, penicillin 100 U, and streptomycin 100 μg/ml. Cells were washed twice with serum-free media before the addition of test samples. Medium was replaced by serum-free media containing 0.1% EtOH with or without ibuprofen. After 1 h incubation, half the volume of media was replaced with the same media with or without recombinant human IL-1β (final concentration was 2–10 ng/ml) (Sigma St Louis MO) was added. After 24 h incubation, media were collected. Western analysis employed our standard protocol (Lim *et al*, 2000) using anti-ACT polyclonal antibody (Calbiochem, CA #178216) (1 : 2500) and anti-rabbit antibody conjugated to HRP (Pierce #31463) (1 : 10 000).

Mouse Primary Astrocytes

Primary astrocyte cultures were prepared from cortices of postnatal 1–2-day-old mice (McCarthy and de Vellis, 1980). Briefly, mixed glia cells were obtained from tissue suspensions after incubation with trypsin. Confluent cultures were plated into six-well plates. Microglia contamination was limited by shaking flasks every 2 days and washing off cells. This yields cultures of 98% pure astrocytes (by GFAP ICC; data not shown). Ibuprofen in EtOH (final EtOH was 0.1%) was added 24 h before mouse IL-1β (final concentration was 2–10 ng/ml (Sigma, St Louis, MO)) or LPS (final 5 μg/ml #L-8274 Sigma, St Louis, MO) stimulation. After 24 h stimulation, RNA was prepared using QIA shredder (QIAGEN, Valencia, CA) and an RNAqueous kit (Ambion, Austin, TX). One step QPCR was performed in triplicate from 10ng total RNA. LDH was assayed (Promega, Madison, WI) in the medium to measure toxicity.

RESULTS

Ibuprofen can Suppress Exogenous Amyloid Accumulation *In Vivo*

While ibuprofen at extremely high concentrations can reduce Aβ42 production *in vitro*, epidemiological studies suggest AD risk reduction is achieved at lower NSAID doses and with NSAIDs that cannot reduce Aβ42 production. Whether ibuprofen can suppress amyloid pathology beyond the level of Aβ production was tested in an Aβ CNS infusion rat model where infused exogenous Aβ accumulates in plaque-like deposits throughout the cortex and thalamus. Rats fed chow with ($n = 10$) or without ibuprofen (375 ppm) ($n = 10$) for 60 days were then infused with human Aβ using Alzet pumps cannulated into the ventricles for 30 days, and then killed. Diffuse Aβ deposits (Figure 1a and b) were analyzed by immunohistochemistry using image analysis.

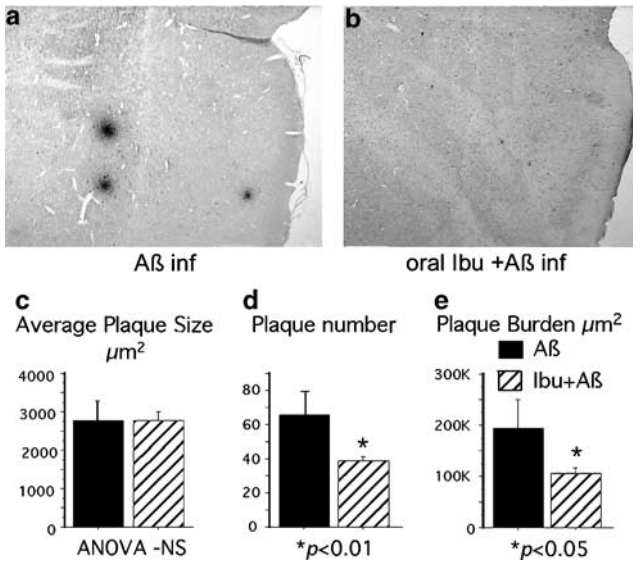

Figure 1 Amyloid deposition in Aβ-infused rats. Rats were fed with or without ibuprofen 3 month prior to and 1 month during intracerebroventricular infusion with Aβ (Aβ40 and Aβ42). Brain Aβ immunohistochemistry of rat without (a) and with ibuprofen (b). Average plaque size (c), number of Aβ deposits (d), and total Aβ-ir area (e) were analyzed.

While the average size of Aβ deposits was not changed with ibuprofen (Aβ: $2770 \pm 22\,\mu m^2$ *vs* Aβ/Ibu: $2788 \pm 498\,\mu m^2$; Figure 1c), the number of Aβ deposits (40.9% reduction, $p < 0.05$, Aβ: 65.3 ± 13.2 *vs* Aβ/Ibu: 38.6 ± 2.6; Figure 1d) and the total Aβ-ir area (45% reduction, $p < 0.05$, Aβ: $192\,800 \pm 57\,000\,\mu m^2$ *vs* Aβ/Ibu: $106\,000 \pm 10\,500\,\mu m^2$; Figure 1e) were significantly reduced by ibuprofen. These results show that ibuprofen can also suppress Aβ accumulation at a post Aβ-production step. This suggests the hypothesis that ibuprofen inhibits pro-amyloidogenic factors at this CNS-achievable dose.

ApoE Levels were not Significantly Reduced by Ibuprofen Treatment

ApoE levels were measured in three different ways because apoE is amyloid binding and apoE levels might be changed as a secondary effect of reduced plaques where apoE protein accumulates. ApoE levels potently control the rate of amyloidosis by modulating Aβ deposition in APP transgenics, and apoE expression is potentially subject to inflammatory control. Since ibuprofen treatment reduced the inflammation marker GFAP in our APP transgenics (Lim *et al*, 2000), we reasoned that control of inflammation and gliosis might reduce apoE production. However, apoE levels were not affected by ibuprofen treatment in APPsw mice (Figure 2a–c). ApoE protein levels in both SDS soluble fraction ($+3.3\%$, $p = 0.78$) and TBS soluble fraction (-17.5%, $p = 0.21$) were not significantly changed by ibuprofen (Figure 2a and b). ApoE mRNA levels, which avoids amyloid binding issues, were also measured using real-time PCR. ApoE mRNA levels were not significantly suppressed by ibuprofen (Figure 2c). Thus, ibuprofen treatment did not significantly impact levels of apoE protein or mRNA.

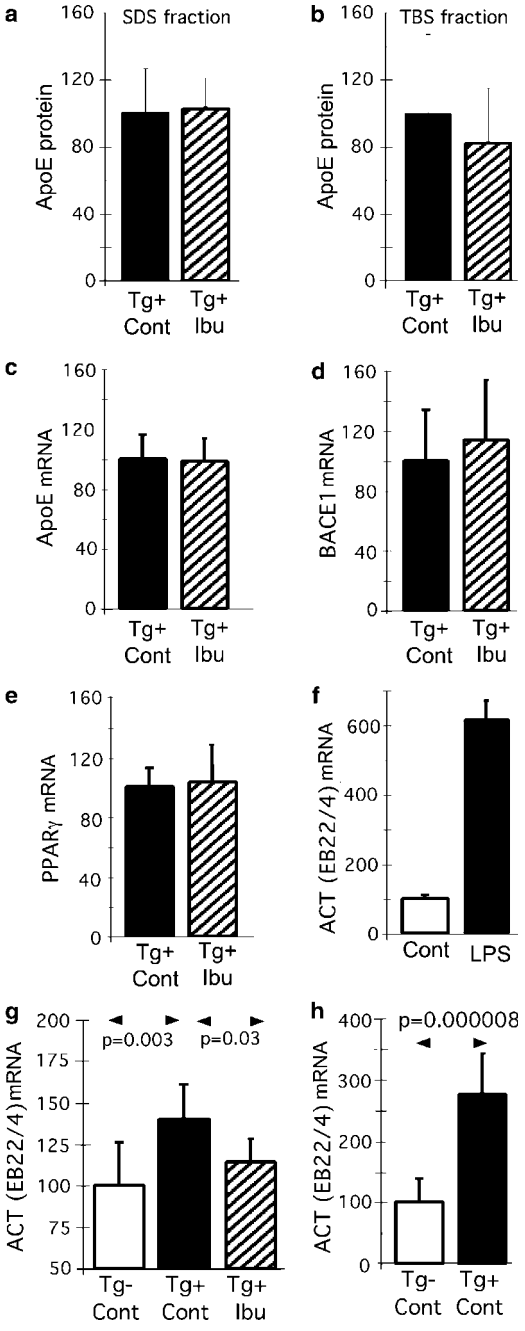

Figure 2 Relative mRNA expression and protein levels in mice cortex. mRNA levels were normalized to GAPDH and the average levels of control mice were set to 100. (e, g, and h) Open bars are Tg− mice with control diet ($n=10$), closed bars are APP Tg+ mice with control diet ($n=12$), and hatched bars are APP Tg+ mice with ibuprofen diet (375 ppm) for 6 months ($n=8$). ApoE protein in SDS and TBS fractions (a and b) and mRNA levels (c), BACE1 mRNA levels (d), and PPARγ mRNA levels (e) in APP Tg mice at the age of 16 months were not significantly changed by ibuprofen. mACT mRNA levels (f) in control (open bar) were increased by CNS LPS injection (closed bar) in mice. mACT mRNA (g and h) levels in mice at the age of 22 months (h) increased over levels at 16 months (g). Error bars indicate SD.

BACE mRNA was not Suppressed by Ibuprofen

Since BACE1 is critical for Aβ production and its expression is subject to inflammatory and NSAID control (Sastre *et al*, 2003), we measured BACE mRNA levels in ibuprofen-treated mice. Surprisingly, BACE mRNA was not reduced (Figure 2d). PPARγ, an ibuprofen target whose agonists suppress BACE (Sastre *et al*, 2003), was also not changed in its mRNA levels by ibuprofen treatment (Figure 2e); however, this does not rule out an effect of ibuprofen on PPARγ activity since PPARγ mRNA levels do not always reflect PPARγ activity.

A Murine Homologue of ACT, an Acute Phase Protein and Pro-Amyloidgenic Molecule, was Induced in APP Tg+ Mice and Suppressed by Ibuprofen

ACT is an acute phase protein induced by inflammation (Akiyama *et al*, 2000) as well as a pro-amyloidogenic molecule (Mucke *et al*, 2000; Nilsson *et al*, 2001). In our experiments, the analysis of ACT was performed in human and mouse material rather than in the rat infusion model, because a rat homolog of ACT has not been adequately identified. The murine ACT homologue mRNA (mACT, EB22/4) rather than protein was measured, because anti-mouse ACT antibody is not available and anti-human ACT antibody did not detect mouse ACT (data not shown). Measurement of ACT mRNA also has an advantage in that it can avoid the complication of ACT protein accumulation associated with Aβ deposits, which were reduced by ibuprofen.

The mACT mRNA was induced 5.02-fold by LPS injection, showing that inflammation induces mACT mRNA (Figure 2f). mACT mRNA was elevated in Tg+ mice compared to Tg− controls by 40% at 16 months of age, $p=0.003$ (ANOVA; $F(2,25)=6.21$, $p=0.0065$) and by 177% at 22 months of age, $p=0.000008$ (Figure 3g and h). The 40% elevated mACT mRNA in 16-month Tg+ mice was suppressed by 18% ($p=0.03$) by ibuprofen (Figure 3g). Since the changes in mACT mRNA levels were moderate, we remeasured mACT mRNA using two sets of independently prepared cDNA from the same mice and observed very similar results (data not shown). This mACT mRNA suppression by ibuprofen was also confirmed by carefully optimized multiplex RT-PCR with radioactivity using differently designed primers (Table 1), where we observed a 24% reduction ($p=0.02$). Using different internal controls (β-actin *vs* GAPDH), the two assays showed reasonable reproducibility ($R^2=0.797$, data not shown).

The Suppression of an ACT mRNA by Ibuprofen in the Absence of Aβ Plaques *In Vitro*

The reduction of mACT mRNA by ibuprofen could be an indirect effect of the loss of Aβ plaques that stimulate glial activation and ACT production. To address this issue, we used a cell culture system with no Aβ accumulation or plaques. Astrocytes were chosen since they are the major source of ACT in brain (Abraham *et al*, 1988; Pasternack *et al*, 1989; Koo *et al*, 1991). mACT mRNA was induced 100.9%, $p<0.001$ (ANOVA; $F(3,8)=68.6$, $p<0.0001$) by LPS in mouse primary astrocyte cultures. Ibuprofen suppressed the mACT mRNA induction moderately, but significantly (10.7%, $p=0.02$ at 20 μM and 20.7%, $p=0.0005$ at 100 μM ibuprofen; Figure 3a). These results suggested that ibuprofen can suppress inflammation-induced mACT independently of Aβ plaque reduction.

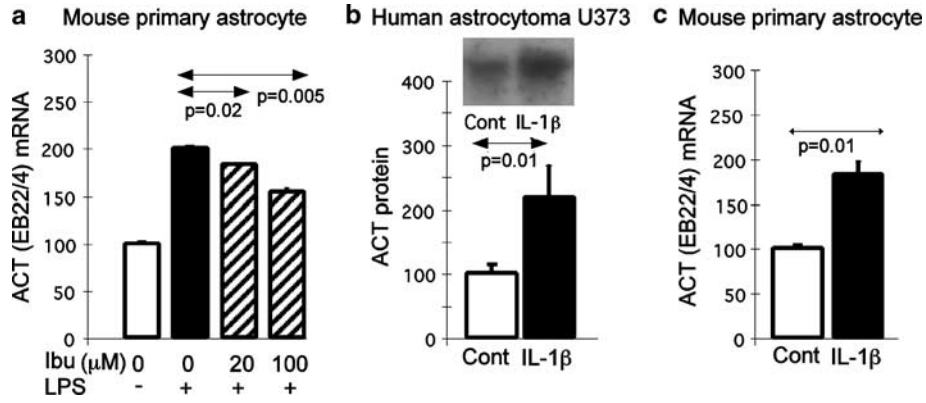

Figure 3 ACT levels in mouse and human astrocyte cell culture. The average levels of nontreated cells (open bars) were set to 100. Error bars indicate SD. ACT mRNA levels (a and c) were normalized to GAPDH. (a) mACT mRNA levels in mouse primary astrocyte culture ($n=3$). Incubation with ibuprofen (20 or 100 μM) or vehicle (0.1% EtOH) started 24 h prior to incubation with or without LPS (5 μg/ml) for the following 24 h. (b) ACT protein levels in human astrocytoma U373 measured by Western blotting ($n=3$). Cells were incubated with or without human IL-1β (2 ng/ml) for 24 h. (c) mACT mRNA levels in mouse primary astrocyte culture ($n=3$). Cells were incubated with or without mouse IL-1β (10 ng/ml) for 24 h.

We previously reported that IL-1β was reduced 64.7% by ibuprofen in the same mouse brains (Lim *et al*, 2000). IL-1β induces ACT in human astrocytes, but this has not been reported for any mouse ACT homolog. So we examined whether IL-1β can induce mouse and human ACT *in vitro*. We confirmed human ACT induction by human IL-1$\beta\beta$ using the human astrocytoma U373-MG, as previously reported (Gitter *et al*, 2000). ACT protein in medium from these cells was 2.2-fold induced ($p=0.01$; Figure 3b). In low passage, young, subconfluent murine primary astrocytes cultures, mouse IL-1β induced mACT (mouse homolog EB22/4) mRNA by 82.1% ($p=0.01$; Figure 3c). Confluent mouse primary astrocytes showed smaller (39%) inductions of mACT mRNA (data not shown). Aged astrocytes grown >40 days *in vitro* expressed much higher mACT mRNA (7–8-fold) compared to young cultures, and IL-1β did not induce mACT mRNA, even in subconfluent conditions (data not shown). Interestingly, similar confluence-dependent phenomena have been shown with human primary astrocytes (Das and Potter, 1995). Ibuprofen (10–500 μM) failed to suppress the ACT induction by IL-1β in both human astrocytoma and mouse astrocytes (data not shown). These *in vivo* and vitro data suggest that mACT suppression by ibuprofen is likely mediated by a reduction in IL-1β, rather than inhibition of the ACT response to IL-1β.

DISCUSSION

In APP Tg mice, we previously reported that 375 ppm ibuprofen in diet from 10 to 16 month of age suppressed amyloid pathology as measured by the number (−52.6%, $p=0.02$) and total area (−55.9%, $p=0.02$) of amyloid plaques and SDS-insoluble Aβ levels (−39%, $p=0.03$) (Lim *et al*, 2000). To evaluate possible mechanisms mediating this effect of ibuprofen on amyloid, we measured the effect of ibuprofen on several factors known to affect amyloid formation. Two factors that showed significant effects of ibuprofen were ACT (an acute phase protein) and IL-1β (an inflammatory cytokine known to regulate ACT). We show that in ibuprofen-treated mice with amyloid reduction, ibuprofen also reduced mACT mRNA by ~20% and reduced IL-1β levels by ~70%. These effects can be linked by the regulatory pathway of ibuprofen controlling COX and other targets which elicit anti-inflammatory effects including reduction of IL-1β and ACT; further, IL-1β can directly regulate ACT expression (as discussed below).

Using additional animals chronically fed with the same dose of ibuprofen (375 ppm), we measured ibuprofen levels in brain and plasma. The ibuprofen level in brain was 4.07 μM. The ibuprofen levels in plasma (47.0 μM) were comparable to high analgesic or the low end of the therapeutic range for rheumatoid patients (10–50 μg/ml = 48.5–242.4 μM) (Davies, 1998). Although the tolerance of these levels of ibuprofen for the vulnerable elderly might be an issue for some, the ibuprofen dose for our mice produces plasma values that are in a range that can be chronically achieved in many patients.

Three mechanisms that could account for amyloid pathology suppression by ibuprofen are: (1) the selective reduction of Aβ42 production independent of COX; (2) the reduction of total Aβ production dependent on PGE2; and, (3) the postproduction reduction of amyloid aggregation and plaque formation. Considering the first of these three mechanisms, high dose ibuprofen (100–500 μM) has been shown to reduce Aβ42 production *in vitro*, an effect also shown by other NSAIDs to be independent of their anti-inflammatory effects on regulating COX, PPAR, and NFκB (Weggen *et al*, 2001; Morihara *et al*, 2002; Sagi *et al*, 2003; Takahashi *et al*, 2003). Although the COX-dependency of ibuprofen's effects on Aβ42 suppression has not been tested, another NSAID sulindac has been more extensively analyzed and clearly reduces Aβ42 independent of COX (Weggen *et al*, 2001; Sagi *et al*, 2003; Takahashi *et al*, 2003). Aβ42 production was reduced in 3-month-old Tg2576 mice with a brain ibuprofen level of ~2 μM, which, surprisingly, was much lower than dosing required *in vitro* (Eriksen *et al*, 2003). Since the CNS ibuprofen levels (4.07 μM ± 1.292, $n=6$) achieved in our animals are comparable, the suppression of amyloid pathology we observed, could, at least in part, be due to a reduction of Aβ42 production.

In relation to the second mechanism, low dose ibuprofen (1 μM) can reduce total Aβ generation in COX-overexpressing cultured cells (Qin *et al*, 2003). The mechanism depends on prostaglandins and is supported *in vivo* by overexpression of neuronal COX-2, which increased amyloid pathology in APP Tg mice (Xiang *et al*, 2002). While in our mice PGE2 levels as a measure of COX activity were not measured, 375 ppm ibuprofen was likely to suppress locally elevated COX activity, for the following reasons: (1) Daily ibuprofen dosing (375 ppm in chow) was above the single dose ED50 (2.3 mg/kg) that inhibited PGE2 synthesis in *ex vivo* mouse brain (Ferrari *et al*, 1990), even when extrapolated based on 2.5 h ibuprofen plasma half life. (2) The ibuprofen levels in brain (4.07 μM) in mice consuming 375 ppm ibuprofen (are above the IC_{50} of COX-1 (2.1 μM) and COX-2 (1.6 μM) (Tegeder *et al*, 2001). (3) In general, NSAID doses at the IC_{50}, including ibuprofen, are known to reduce COX activity by blocking COX enzyme. However, ibuprofen can also reduce COX-2 mRNA levels (Kannan *et al*, 2004; Lo *et al*, 1998). COX-2 mRNA levels were measured in our ibuprofen treated mice, however, they were not significantly reduced (-13%, $n=8$, $p=0.38$; see supplemental data).

Taken together, these data argue that 375 ppm ibuprofen should limit any local elevation of COX and PGE2 caused by the APP Tg. However, global elevations in PGE2 have not been detected in APP Tg mice at 14 or 20 months of age (Quinn *et al*, 2003). Whether or not COX-2 mRNA or activity is locally elevated in APP Tg and suppressed by ibuprofen in selected neuronal layers or periplaque regions is worthy of further study.

These two mechanisms related to Aβ production are, however, unlikely to be the only mechanisms for amyloid pathology reduction by ibuprofen, because ibuprofen reduced exogenous Aβ deposition in Aβ infused rats (Figure 1), demonstrating ibuprofen's ability to limit Aβ deposition independent of Aβ42 or Aβ production. Other reported results also suggest mechanisms beyond reduction of Aβ42 production. First, there are no published epidemiological studies to suggest substantial differences in the apparent neuroprotective (antidementia) effects between ibuprofen and naproxen, the latter being a drug that does not reduce Aβ42 production (Breitner JC, personal communication, 17 May 2004). In fact, the Rotterdam Study (in t' Veld *et al*, 2001) found a five-fold reduction in AD incidence with 2 or more years' administration (at least 2 years before onset) of any NSAID, including diclofenac, naproxen, and other drugs without 'Aβ42 lowering' activity. Second, COX-independent Aβ42 reduction *in vitro* requires unrealistically high ibuprofen concentrations that are probably not relevant epidemiologically, while low dose ibuprofen has anti-inflammatory effects. For example, *in vitro*, ibuprofen and other NSAIDs can inhibit COX and suppress inflammation at lower doses than those for Aβ42 suppression (Weggen *et al*, 2001). It is thus likely that low dose ibuprofen can decrease amyloid pathology through IL-1β and ACT suppression based on anti-inflammation (our third mechanism), even when the ibuprofen dose is not high enough to suppress Aβ42 production. With high enough dosing, all three mechanisms should act coordinately to reduce amyloidogenesis.

ACT is not only an acute phase molecule but also a pro-amyloidogenic molecule. Overexpression of human ACT accelerates amyloid pathology in human APP Tg mice (Mucke *et al*, 2000; Nilsson *et al*, 2001). Although ACT functions remain unclear and some reports about biochemical interactions with Aβ are controversial, most evidence argues that ACT can play a causal role in amyloid aggregation and plaque formation, which is post-Aβ-production (Ma *et al*, 1994). mACT mRNA was reduced by ibuprofen both *in vivo* and *in vitro*. We confirmed that mACT mRNA was induced in brain by the inflammatory effect of LPS injection (Figure 2d) and showed that ibuprofen suppressed the induction of mACT mRNA by LPS in mouse primary astrocytes (Figure 3a). The mACT mRNA reduction by ibuprofen (Figure 2e) was associated with, but unlikely to be simply secondary to the reduction in amyloid plaques. First, in the astrocyte culture where amyloid plaques are absent, mACT mRNA levels were suppressed by ibuprofen (Figure 3a), and induced by IL-1β (Figure 3b and c). Second, we measured mACT mRNA to avoid the caveat that ACT protein accumulates with amyloid plaques.

In AD, both ACT levels and genetic polymorphisms could contribute to its effects on amyloid formation, since AD patients have higher ACT levels in plasma and/or CSF (Matsubara *et al*, 1990; Harigaya *et al*, 1995; Licastro *et al*, 1995; Lieberman *et al*, 1995) and polymorphisms differentially modify AD risk (Wang *et al*, 2002a; Wang *et al*, 2002b). ACT mRNA is greatly elevated in AD (Abraham *et al*, 1988; Pasternack *et al*, 1989; Koo *et al*, 1991). Our data showed mACT mRNA was elevated in Tg2576 mice compared to non-Tg mice by 2.8-fold at 22 months of age and by 1.4-fold at 16 months, even though Aβ levels are as high in these mice as those of late stage AD patients (Kawarabayashi *et al*, 2001). The larger percent increase in ACT in AD compared to mouse models may contribute to a more significant role of ACT in AD than in mouse models. In addition to ACT levels, variations in the association of ACT polymorphisms with increased AD risk are observed, with some haplotypes associated with increased and others with decreased AD risk (Wang *et al*, 2002b).

Human gene polymorphism and promoter studies suggest a relationship between IL-1β, ACT, and AD (Akiyama *et al*, 2000; Nicoll *et al*, 2000). The common TT IL-1β polymorphism, which shows higher plasma IL-1β levels in AD patients, interacts with one polymorphism in ACT to significantly increase AD risk (Licastro *et al*, 2000). This genetic polymorphism data support the participation of IL-1 in cytokine cascades previously linked to AD pathogenesis (Mrak and Griffin, 2000, 2001). The human ACT gene has enhancer elements responsive to IL-1-β-stimulated NFκB and AP-1 (Kordula *et al*, 2000) and human ACT mRNA expression is directly controlled by NFκB (Lieb *et al*, 1996). As ibuprofen is a weak NFκB inhibitor (Tegeder *et al*, 2001), it is possible that higher levels of ibuprofen or the less toxic R-flurbiprofen (Morihara *et al*, 2002) could directly reduce human ACT expression levels by inhibiting NFκB.

The contribution of ibuprofen's effects on ACT and IL-1β to the suppression of amyloid pathology is strengthened by the lack of effect of ibuprofen on several other candidate factors known to modulate amyloid formation (submitted).

Candidate amyloid-regulating molecules that can be controlled by NSAIDs include APP (Lim *et al*, 2000), BACE, apoE, TGF-β1, C1q, C3, CD11b, CD11c, iNOS, and PPARγ. APP and BACE relate to Aβ42 production, while apoE modulates amyloid deposition or clearance; no changes in these molecules were detected (Figure 2a–e) (Lim *et al*, 2000). Other molecules whose possible suppression by NSAIDs can interrupt the clearance of amyloid, for example, inflammation-related, phagocytic microglia markers, complement components (TGF-β1, C1q, C3, CD11b, CD11c, iNOS, PPARγ) were also not suppressed by ibuprofen in our mice (submitted). Among these markers, only CD11c (phagosytosis-related amyloid clearance) was increased by the APP transgene; its insensitivity to reduction by ibuprofen supports the importance of the ACT and IL-1β axis as a NSAID target that can directly modulate amyloid metabolism.

We found no reductions in apoE mRNA or protein levels in TBS soluble or insoluble fractions from cortical regions of ibuprofen-treated animals (Figure 2a–c). Although apoE is often increased by inflammatory stimuli, and this can be coordinate with upregulation of the canonical glial reactivity marker GFAP (Poirier *et al*, 1991; Laping *et al*, 1994a) (which was downregulated by ibuprofen in these mice (Lim *et al*, 2000)), in some inflammatory and neurodegenerative conditions apoE mRNA expression is not coordinately regulated with GFAP mRNA (Laping *et al*, 1994b; Schauwecker *et al*, 1998; Petegnief *et al*, 2001), including conditions related to amyloid plaques and AD (Shao *et al*, 1997; Zarow and Victoroff, 1998). In addition, apoE mRNA is uniquely downregulated by apoE and Aβ proteins (Soulie *et al*, 1999; Yamauchi *et al*, 2003).

In summary, ibuprofen specifically reduced pro-amyloidogenic ACT both *in vivo* and *in vitro*, an effect likely to be mediated by reduction of IL-1β. To our knowledge, this is the first report that suggests those molecules are involved in NSAID suppression of amyloid pathology *in vivo* and are regulated by ibuprofen through its anti-inflammatory effects, a mechanism that could apply to other AD-protective anti-inflammatory drugs that fail to lower Aβ42 production, including naproxen (Martin *et al*, 2002) and high dose aspirin.

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