Acute treatment with the PPARγ agonist pioglitazone and ibuprofen reduces glial inflammation and Aβ1–42 levels in APPV717I transgenic mice

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Summary
Neuritic plaques in the brain of Alzheimer’s disease patients are characterized by β-amyloid deposits associated with a glia-mediated inflammatory response. Non-steroidal anti-inflammatory drug (NSAID) therapy reduces Alzheimer’s disease risk and ameliorates microglial reactivity in Alzheimer’s disease brains; however, the molecular mechanisms subserving this effect are not yet clear. Since several NSAIDs bind to and activate the nuclear receptor peroxisome proliferator-activated receptor-γ (PPARγ) which acts to inhibit the expression of proinflammatory genes, this receptor appears a good candidate to mediate the observed anti-inflammatory effects. Recent data in vitro suggested that NSAIDs negatively regulate microglial activation and immunostimulated amyloid precursor protein processing via PPARγ activation. We report that an acute 7 day oral treatment of 10-month-old APPV717I mice with the PPARγ agonist pioglitazone or the NSAID ibuprofen resulted in a reduction in the number of activated microglia and reactive astrocytes in the hippocampus and cortex. Drug treatment reduced the expression of the proinflammatory enzymes cyclooxygenase 2 (COX2) and inducible nitric oxide synthase (iNOS). In parallel to the suppression of inflammatory markers, pioglitazone and ibuprofen treatment decreased β-secretase-1 (BACE1) mRNA and protein levels. Importantly, we observed a significant reduction of the total area and staining intensity of Aβ1–42-positive amyloid deposits in the hippocampus and cortex. Additionally, animals treated with pioglitazone revealed a 27% reduction in the levels of soluble Aβ1–42 peptide. These findings demonstrate that anti-inflammatory drugs can act rapidly to inhibit inflammatory responses in the brain and negatively modulate amyloidogenesis.

Keywords: Alzheimer’s disease; PPAR; inflammation; NSAID; neurodegeneration

Abbreviations: Aβ = amyloid β; APP = amyloid precursor protein; COX = cyclooxygenase; BACE-1 = β-secretase-1; GAPDH = glycerolaldehyde-3-phosphate dehydrogenase; GFAP = glial fibrillary acidic protein; IB4 = isolectin B4; iNOS = inducible nitric oxide synthase; NSAID = non-steroidal anti-inflammatory drug; PPARγ = peroxisome proliferator-activated receptor-γ; PCR = polymerase chain reaction


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Introduction

Inflammation plays an important role in the pathogenesis of a number of neurodegenerative diseases and arises as a consequence of the primary disease process. Alzheimer’s disease is clinically characterized by progressive memory loss and decline of cognitive functions. Its classical histopathological hallmarks include extracellular amyloid peptide (Aβ) deposition in neuritic plaques, and intracellular deposits of hyperphosphorylated tau, causing formation of neurofibrillary tangles and finally neuronal death. Aβ peptides are generated from the amyloid precursor protein (APP) by sequential actions of two proteolytic enzymes, the β-secretase 1 (BACE1) and the γ-secretase (Dingwall, 2001; Esler and Wolfe, 2001). Formation and aggregation of Aβ represent a key feature and possibly a triggering mechanism of Alzheimer’s disease. The importance of Aβ formation is supported by dominantly inherited familial forms of Alzheimer’s disease that are linked to APP mutations in or close to the β- and γ-secretase cleavage sites (Hardy and Allsop, 1991). This made it possible to generate transgenic mouse models of cerebral amyloidosis and Alzheimer-like histopathology, i.e. amyloid plaques and cerebral amyloid angiopathy (Hsiao et al., 1995; Sturchler-Pierrat et al., 1997; Lamb et al., 1999; Moechars et al., 1999; Van Dorpe et al., 2000).

Next to neurodegeneration, Alzheimer’s disease shows a significant inflammatory component that is characterized by the presence of abundant and activated glial cells associated with fibrillar deposits of Aβ that comprise the senile plaque (Akiyama et al., 2000; Wyss-Coray and Mucke, 2002). Once initiated, inflammatory processes may contribute independently to neural dysfunction and cell death, thereby establishing a self-perpetuating vicious cycle (Griffin et al., 1998), by which the inflammation may represent the primary cause of further neurodegeneration.

In the CNS, the cellular mediators of the innate immune response are the microglia cells whose activation results in the production of a diverse range of inflammatory molecules. Once activated, microglia release a diverse range of cytokines and other proinflammatory products, which contribute to neurodegeneration and exacerbation of the inflammatory reaction by recruiting and stimulating astrocytes. The recognition that a robust inflammatory response is a component of the disease process led to the discovery that long-term treatment with non-steroidal anti-inflammatory drugs (NSAIDs) dramatically reduced the risk for Alzheimer’s disease, delayed disease onset, ameliorated symptomatic severity and slowed cognitive decline (McGeer et al., 1996; Stewart et al., 1997; In ‘t Veld et al., 2001). In addition, NSAID treatment was positively correlated with a reduction of plaque-associated, activated microglia in humans (Mackenzie and Munoz, 1998; Alafuzaff et al., 2000). This observation is supported by two recent studies which demonstrate that the NSAID ibuprofen acts to reduce microglial activation and cytokine production in transgenic mice overexpressing APP (Lim et al., 2000; Yan et al., 2003). Importantly, a 6 month oral ibuprofen treatment also reduced the amount of Aβ deposited in the brains of these animals.

The mechanisms by which NSAIDs act to reduce inflammation in Alzheimer’s disease are controversial. The established targets of NSAIDs are cyclooxygenases (COXs) 1 and 2, and experimental data using a neuronal COX2 overexpression paradigm in APP transgenic mice support the hypothesis of a detrimental role for elevated COX2 expression in brains of Alzheimer’s disease patients (Xiang et al., 2002). While it has been shown that prostaglandin E2 levels are increased 5-fold in the CSF of probable Alzheimer’s disease patients (Montine et al., 1999), the role of COX products for Alzheimer’s disease pathophysiology has not been fully elucidated yet (Akiyama et al., 2000), raising the question of whether the COXs are indeed the biologically relevant target of NSAIDs responsible for the reduced risk of Alzheimer’s disease. Moreover, recent clinical trials of COX2 selective inhibitors in Alzheimer’s disease patients showed that they are ineffective in treating the disease (Aisen et al., 2003). Therefore, alternative targets of NSAID action have to be considered.

A novel action of a subset of commonly used NSAIDs was reported recently by Weggen et al. (2001) who found that these drugs selectively reduce the production of Aβ1–42 due to alteration of the preferred γ-secretase cleavage site in APP.

A subset of NSAIDs directly regulate gene expression through their ability to bind and activate the nuclear receptor peroxisome proliferator-activated receptor-γ (PPARγ) (Lehmann et al., 1997). In microglia and macrophages, PPARγ activation results in the inhibition of proinflammatory gene expression through silencing the action of the transcription factors nuclear factor-κB and AP-1 on the promoters of these genes (Jiang et al., 1998; Ricote et al., 1998; Daynes and Jones, 2002). We have demonstrated that PPARγ agonists reduce Aβ- and cytokine-mediated neuroinflammation and neurotoxicity both in vitro (Heneka et al., 1999; Combs et al., 2000) and in vivo (Heneka et al., 2000). Moreover, we were able to show in vitro that NSAIDs, such as indomethacin and ibuprofen, as well as several structurally different PPARγ agonists, reduce immunostimulated Aβ production in a PPARγ-dependent manner (Sastre et al., 2003). We report here that acute treatment of APP-overexpressing mice with ibuprofen or the specific PPARγ agonist, pioglitazone, results in reduction of microglial activation, inflammatory gene expression, BACE1 levels and Aβ deposition in the brain.

Material and methods

Animals

The transgenic mice used in this study were of the FVB/N genetic background and expressed APPV717I under the control of the mouse thy1 gene promoter. The generation of these APPV717I transgenic mice has been described previously (Moechars et al., 1999). Mice (n = 6 per group) of 10 months of age were used for experiments.
since APPV171 mice begin to deposit amyloid peptides in the subiculum at this age, with an accompanying profound inflammatory component becoming apparent. Mice were fed Purina 5002 rodent chow ad libitum supplemented with either 375 p.p.m. ibuprofen (Sigma, St Louis, MO) or 240 p.p.m. pioglitazone (Actos™ Takeda Pharmaceuticals, Osaka, Japan). The latter drug concentration was chosen since only ~18% of pioglitazone crosses the blood–brain barrier in mammals (Maeshiba et al., 1997), and a previous experiment using a lower drug dosage failed to show statistically significant results (Yan et al., 2003). Animals were treated for 7 days, since this time period was believed to allow the detection of both anti-inflammatory and amyloid-lowering effects. The final dosages of drug were computed to be 62.5 mg/kg/day of ibuprofen and 40 mg/kg/day of pioglitazone based on an average daily food consumption of 5 g of chow per mouse. There were no significant changes in body weight between or within groups of mice. Of note, pioglitazone did not affect blood glucose levels in APP mice as determined in a set of pilot experiments using the identical treatment protocol in accordance with previous observations in non-diabetic mice (Shiomi et al., 2002). During the experimental treatment, animals were housed singly to allow monitoring of drug intake by weighing the animal daily as well as weighing the remaining food pellets at the same time. In all instances, animals lived under standard conditions of 22°C with a 12 h light–dark cycle and with free access to food and water. At the time of sacrifice, animals received a short inhalation anaesthesia using isoflurane. Animals were transcardially perfused with heparinized sodium chloride (0.9%). The brains were removed with anaesthesia using isoflurane. Animals were transcardially perfused with heparinized sodium chloride (0.9%). The brains were removed with

**Biochemical analysis of transgenic mouse brain**

The individual hemispheres were homogenized in 6.5 volumes of ice-cold buffer containing 20 mM Tris–HCl (pH 8.5) and a cocktail of proteinase inhibitors (Roche Mannheim, Germany), using a small Potter-type mechanical homogenizer. After centrifugation (135 000 g for 1 h at 4°C), a portion of the supernatant was centrifuged again (200 000 g for 2 h at 4°C) and soluble amyloid peptides were quantified by enzyme-linked immunosorbent assay (ELISA).

**ELISA for soluble and plaque-associated amyloid peptides**

The extracted protein fractions were applied to small C18-Sep-Pack reverse phase columns (Waters, Massachusetts, MA) and washed with increasing concentrations of acetonitrile (5, 25 and 50%) containing 0.1% trifluoroacetic acid (TFA). The last fraction, containing the amyloid peptides, was dried under vacuum overnight. The samples were assayed using a sandwich ELISA for Aβ1–40 and Aβ1–42 peptides. The capture antisera were CFA3340 (Barelli et al., 1997) and 21F12. The reaction was developed with the biotinylated monoclonal antibody 6F3D.

**Immunohistochemistry**

Serial sagittal sections of paraffin-embedded or cryofixed tissue were cut (7 µm thick, Leica microtome 2155 or Leica Cryostat CM3050S) and mounted (poly-l-lysine-coated slides, Histobond adhesion slides, Marienfeld, Germany). Retrieval of antigen sites, blocking of endogenous peroxidase activity and blocking of non-specific binding sites were performed according to standard protocols. After washing in phosphate-buffered saline (PBS), sections were incubated overnight at 4°C with primary antibodies. The antibodies and dilutions used were: (i) rabbit anti-glial fibrillary acidic protein (GFAP) polyclonal antibody (pAb; Z 334; 1 : 800, DAKO, Hamburg, Germany); (ii) rabbit pAb against inducible nitric oxide synthase (iNOS) (32030; 1 : 150, Transduction Laboratories, Bio-science, Heidelberg, Germany); (iii) anti-COX2 affinity-purified antibody (160126; 1 : 1000; Cayman Chemical, Ann Arbor, MI); (iv) rabbit pAb against Aβ1–42 (#444-344; 1 : 50, Biosource International, Inc., USA); (v) a pAb 7520 directed against the C-terminal domain of BACE1 (a gift from Dr Christian Haass, Adolf-Butenandt-Institute, University of Munich); and (vi) a mouse monoclonal antibody against neuronal nuclei (neuN, #MAB 377; 1 : 500, Chemicon, Hofheim, Germany). Microglial cells were identified by means of biotin-labeled isoelectric B4 (IB4), from Bandeiraea simplicifolia as described by others (Naujoks-Manteuffel and Niemann, 1994) (1 : 50, Sigma Taufkirchen, Germany). Immunohistochemistry was performed using the avidin–biotin–peroxidase complex method (ABC-Kit, Vector Labs) with 3,3′-diaminobenzidine tetrahydrochloride as chromogen. All fluorescent double immunostaining was performed on cryo-sections cut (6 µm) and mounted as described above. Sections were dried at room temperature for 1 h and then fixed in 4% PFA or methanol for 15 min at room temperature. After washing with PBS, the double staining was performed by adding simultaneously both first antibodies, and followed by overnight incubation at 4°C. The goat secondary antibodies (fluorescein DTAF-conjugated anti-rabbit 1 : 150, Texas red-conjugated anti-mouse 1 : 80, Texas red-conjugated anti-rat 1 : 80, Jackson Immuno Research Laboratories, West Grove, PA) were applied sequentially after washing in PBS. Negative controls included non-specific IgG instead of primary antibodies, pre-absorption with the respective cognate peptides (150–200 µg of peptide/ml of antibody working solution), omission of the secondary antibody and absence of immunoreactivity in non-transgenic controls of the respective age.

**Confocal laser scanning microscopy**

Double-labelled specimens were analysed with a confocal laser scanning microscope (Multiprobe 2001; Molecular Probes, Inc., Eugene, OR) equipped with an Ar/Kr laser with balanced emission at 488, 568 and 647 nm. To achieve an optimal signal-to-noise ratio for each fluorophore, sequential scanning with 568 and 488 nm was used. The digitalized images were then processed with ImageSpace 3.10 software (Molecular Probes, Inc.) on a Silicon Graphics
RNA preparation and RT–PCR
Total RNA was extracted from brain samples using Trizol reagent according to the manufacturer’s procedures (Sigma, St Louis, MO), and RT–PCR was carried out as described (Heneka et al., 2002). The primers used were: iNOS forward 5'-TGG GCA CAG CAA TAT AG-3' and iNOS reverse 5'-ACA GTT TGG TGT GGT GTA GG-3'; GFAP forward 5'-TCC GGC GCA CGA ACG AGT C-3' and GFAP reverse 5'-CAC CAT CCC GCA TCT CCA CAG TCT-3'; COX2 forward 5'-AAC ATC CCC TTC CTG CGA AG-3' and COX2 reverse 5'-AAG TCC ACT CCA TGG CCC AG-3'.; BACE1 forward 5'-GCGGGGAGTGGTATTATGAGGTA-3' and BACE1 reverse 5'-TATTTGCTGGAGAAGGGATGGTA-3'; GAPDH forward 5'-TCA CCA GGG CTG CCA TTT GC-3' and GAPDH reverse 5'-GAC TCC ACG ACA TAC TCA GC-3'. PCRs were carried out on RNA prepared from the individual animals in each group, and representative gels of two different animals per group are shown. PCR conditions were 35 cycles of denaturation at 95°C for 30 s, annealing at 63°C for 45 s, and extension at 72°C for 45 s using a PX2 thermocycler (ThermoHybaid, Ulm, Germany). PCR products were separated by electrophoresis through 2% agarose containing 0.5 μg/ml ethidium bromide, imaged using an Alpha Inotech imaging system (Temecula, CA), and band intensities determined using ImageJ public domain software from NIH. The data were analysed by ANOVA with Tukey’s post-tests (Systat, Evanston, IL).

Results

Anti-inflammatory drug treatment reduces glial activation

The 10-month-old APPV717I transgenic mice received an oral treatment with either standard chow, or chow containing the PPARγ agonist pioglitazone or with ibuprofen for a period of 7 days. At 10 months of age, the APPV717I mice are starting to exhibit focal deposits of amyloid, accompanied by a glia-mediated inflammatory response. Analysis of sections of the hippocampus and frontal cortex revealed that treatment with ibuprofen or pioglitazone reduced the area covered by IB4-positive microglia by 30 and 40%, respectively. Additionally, both drugs reduced the integral staining intensity of immunopositive cells (Fig. 1A). Confocal immunolabelling showed that clusters of IB4-stained microglia were closely surrounded by GFAP-positive astrocytes (Fig. 1B). Within these clusters, arborized as well as rounded IB4-positive microglia, indicating different stages of inflammatory activation, were found. A subset of IB4-positive microglial cells also expressed COX2 as determined by confocal laser microscopy (Fig. 1C). The vast majority of COX2-positive microglial cells showed a round to oval morphology, indicating an inflammatory activated state. Quantitative counting of activated and COX2-positive microglial cells in the hippocampus and frontal cortex of placebo- and drug-treated APPV717I mice showed that both ibuprofen and pioglitazone treatment effectively reduced the number of COX2-positive microglia in both brain regions. In parallel, COX2 mRNA levels in hippocampal lysates were significantly decreased by ibuprofen and pioglitazone treatment (Fig. 1C).

To determine whether astrocyte activation is influenced by drug treatment, immunohistochemical analysis of GFAP, an astrocyte-specific intermediate filament component, was performed. GFAP expression previously has been reported to be upregulated in APP-overexpressing mice by ~50% (Lim et al., 2000) and is associated with astrocytosis accompanying amyloid deposition in these animals (Bondolfi et al., 2002). Evaluation of sections of the hippocampus and frontal cortex showed that the area covered by GFAP-positive astrocytes and the respective integral staining intensities were reduced by ibuprofen and pioglitazone treatment (Fig. 2A and B). Co-staining of GFAP and Aβ1–42 confirmed that astrocyte activation could be observed predominantly at sites of amyloid deposition (Fig. 2C). Confocal immunostaining for iNOS and GFAP showed that a subset of astrocytes expressed iNOS. These iNOS-positive cells were found in close contact with amyloid plaques (not shown). In addition, an increased immunostaining against nitrotyrosine, indicating NO-dependent peroxynitrite generation, was found near to amyloid deposits (not shown). Detection of GFAP mRNA levels showed that both ibuprofen and pioglitazone reduced GFAP mRNA by ~60%. Pioglitazone-treated animals exhibited a statistically significant 40% decrease in iNOS mRNA, whereas ibuprofen treatment did not reduce iNOS mRNA.
Fig. 1 Anti-inflammatory drug treatment reduces the number of reactive microglia in the hippocampus of APPV717I mice. APPV717I transgenic mice (10 months of age) received either control chow (Con), or chow supplemented with ibuprofen (Ibu) or pioglitazone (Pio) for 7 days. Activated microglia were visualized by staining with IB4 and are shown in the hippocampus (A). Arrows indicate clusters of microglia cells. For quantitation, the total area covered by IB4 and the integral IB4 staining intensity were determined in serial sections of the hippocampus (HC) and frontal cortex (FC) (A). Confocal analysis of IB4 and the astrocytic marker GFAP revealed that clusters of microglia were closely surrounded by GFAP-positive astrocytes (B). Further co-staining showed that a subset of IB4-positive microglial cells were also expressing cyclooxygenase 2 (COX2), and the number of COX2-positive cells was quantified in the HC and FC (C). COX2 mRNA levels were determined by RT–PCR in hippocampal lysates of all animal groups (displayed as triplicates) and subsequently analysed by densitometry. GAPDH (GDH) served as control (C). Asterisks indicate significant differences between control and drug-treated groups (*P < 0.05, **P < 0.01, SEM, ANOVA followed by a Tukey test). (A) Bar = 25 µm, (B) bar = 50 µm, (C) bar = 25 µm.
Fig. 2 Downregulation of GFAP expression in 10-month-old APPV717I transgenic mice by anti-inflammatory drug treatment. GFAP expression was analysed immunohistochemically in the hippocampus (HC) and frontal cortex (FC) of 10-month-old APPV717I transgenic mice treated with either control chow (Con), or chow supplemented with ibuprofen (Ibu) or pioglitazone (Pio) for 7 days (A). Arrows indicate clusters of GFAP-positive astrocytes in the FC. For quantitation, the total area covered by GFAP and the integral GFAP staining intensity were determined for both areas, the HC and FC, in serial sections (B). Representative picture of a section co-stained for GFAP (brown) and Aβ1–42 (blue), revealing that astrocytes assembled predominantly at sites of amyloid plaque deposition (C). Confocal staining of GFAP and inducible nitric oxide synthase (iNOS) showed that a subset of GFAP-positive cells expressed iNOS (C) and the number of iNOS positive astrocytes was quantified (D). iNOS and GFAP mRNA levels were then determined by RT–PCR in hippocampal lysates of all animal groups (displayed as triplicates) and subsequently analysed by densitometry. GAPDH (GDH) served as control (D). Asterisks indicate significant differences between placebo- and drug-treated groups (*P < 0.05, **P < 0.01, SEM, ANOVA followed by Tukey test). (A) Bar = 250 μm (HC) and 100 μm (FC), (C) bar = 50 μm (GFAP/Aβ1–42) and 25 μm (GFAP/iNOS).
levels to a comparable extent but tended to be lower than in control animals. The variation observed in the degree of GFAP and iNOS mRNA inhibition upon ibuprofen or pioglitazone treatment between single animals may be due to inter-animal variability in this particular assay but may also result from different drug concentrations at the time of sacrifice. Because chronic amyloid deposition will result in a constant inflammatory stimulus, the degree of inhibition may depend on the individual food intake, especially in food-based treatment protocols. However, immunohistochemical quantification of iNOS-positive cells found a corresponding reduction of the number of iNOS-positive astrocytes in response to drug treatment (Fig. 2D).

Reduced levels of BACE1 protein and mRNA in pioglitazone-treated APPV717I mice

We previously have reported that treatment of neuroblastoma cells with several proinflammatory cytokines resulted in an increased BACE1 expression and that ibuprofen and pioglitazone reduced immunostimulated BACE1 levels in vitro (Sastre et al., 2003). These data prompted us to perform an immunohistochemical analysis of BACE1 expression and detection of BACE1 mRNA levels. BACE1-positive cells were immunohistochemically detected in the frontal cortex (Fig. 3A) and hippocampus of 10-month-old APPV717I

Fig. 3 Pioglitazone decreases the level of BACE1 mRNA and protein in APPV717I-transgenic mice. Immunohistochemical detection of β-secretase (BACE1) in the frontal cortex (FC) of 10-month-old APPV717I transgenic mice treated with either control chow (Con), or chow supplemented with ibuprofen (Ibu) or pioglitazone (Pio) for 7 days (A). Confocal immunostaining of BACE1 and the neuronal marker neuN revealed that BACE1 was mostly expressed by neurons in 10-month-old APPV717I mice (B). However, co-staining of BACE1 and GFAP showed that a subset of astrocytes also expressed BACE1 (B). Quantification of BACE1-positive cells showed a significant reduction in response to both pioglitazone and ibuprofen treatment (B). BACE1 mRNA levels were determined in lysates of the hippocampus (HC) and subsequently analysed by densitometry (C). Asterisks indicate significant differences between the control and drug-treated groups (*P < 0.05, **P < 0.01, SEM, ANOVA followed by Tukey test). (A) Bar = 250 μm, (B) bar = 50 μm (BACE/neuN) and 25 μm (BACE1/GFAP).
transgenic mice. At this age, APPV717I mice expressed BACE1 mainly in neurons as determined by confocal immunohistochemistry for the neuronal marker neuN and BACE1 (Fig. 3B). In addition, co-staining of sections with GFAP revealed that activated astrocytes were found in focal clusters, with a subset of astrocytes also expressing BACE1 (Fig. 3B). Quantification of BACE1-positive neurons in ibuprofen- or pioglitazone-treated animals revealed a marked reduction in the frontal cortex and hippocampus when compared with non-treated APP mice (Fig. 3B). Analysis of BACE1 mRNA levels in whole brain lysates from the respective brain regions showed that pioglitazone, and to a lesser extent ibuprofen, downregulated BACE1 mRNA (Fig. 3C).

**Pioglitazone and ibuprofen drug treatment alters Aβ levels and deposition**

Anti-inflammatory drug treatment has been reported to reduce Aβ levels in the brain of APP-expressing mice (Lim et al., 2000, 2001; Weggen et al., 2001; Yan et al., 2003). The APPV717I-transgenic mice investigated in the present study start to exhibit Aβ deposition at 10 months of age (Moechars et al., 1999; Van Dorpe et al., 2000). Immunohistochemical analysis of Aβ deposits in the hippocampus and frontal cortex was performed using an antibody specific for Aβ1–42 (Fig. 4A). Compared with non-treated APP mice, pioglitazone reduced the percentage of Aβ1–42-stained plaque area by ~25 and 33% in the hippocampus and frontal cortex, respectively (Fig. 4B). Ibuprofen exerted a similar reduction in the hippocampus; however, it was less efficient in reducing amyloid deposition in the frontal cortex. In addition, there was a corresponding significant reduction of Aβ1–42 staining intensity in the pioglitazone-treated animals (Fig. 4B). We measured soluble levels of Aβ1–40 and Aβ1–42 by ELISA and found that treatment with pioglitazone significantly reduced cerebral levels of soluble Aβ1–42 by 27% (Fig. 4C). There was a smaller and statistically insignificant reduction in ibuprofen-treated mice. Soluble Aβ1–40 levels were not significantly altered by drug treatment over this interval (Fig. 4C). The Aβ42/40 ratio was reduced 22% in pioglitazone-treated animals (Fig. 4C).

**Discussion**

There has been an increased appreciation of the role that inflammation plays in the pathogenesis of Alzheimer’s disease that has arisen principally from epidemiological studies showing a dramatic effect of long-term NSAID treatment on Alzheimer’s disease risk. However, the molecular mechanisms by which NSAIDs intervene in the pathological processes that underlie cognitive decline and neuronal loss remain unclear (Stewart et al., 1997; In’t Veld et al., 2001).

We show that a brief and oral treatment of 10-month-old APPV717I-overexpressing mice with the PPARγ agonist pioglitazone and with the NSAID ibuprofen decreased amyloid-associated microglial and astroglial activation in the hippocampus and frontal cortex. In particular, microglial COX2 expression as well as astrocytic expression of iNOS were potently reduced by both treatments. BACE1, a key enzyme of the APP processing pathway, was detected predominantly in neurons and to a minor extent in clustered astrocytes, and found to be significantly reduced by both pioglitazone and ibuprofen treatment. In parallel to BACE1 reduction, pioglitazone, and to a lesser extent ibuprofen, were able to increase the total Aβ1–42-positive amyloid plaque area and the respective staining intensity. Additionally, soluble levels of Aβ1–42 were significantly attenuated by pioglitazone treatment.

Animal models of Alzheimer’s disease which develop extensive amyloid deposits in the brain also exhibit reactive astrocytosis and abundant plaque-associated, activated microglia with elevated levels of proinflammatory secretory products (Frautschy et al., 1998; Benzing et al., 1999; Stalder et al., 1999; Mehlhorn et al., 2000; Bornemann et al., 2001; Matsuoka et al., 2001). Two recent studies in Tg2576 animals have demonstrated that ibuprofen treatment over a period of several months dramatically reduces glial activation and proinflammatory gene expression (Lim et al., 2000; Yan et al., 2003). These studies suggest that anti-inflammatory treatment strategies may offer substantial clinical benefit. However, several recent clinical trials using COX2-specific inhibitors (Aisen, 2002; Aisen et al., 2003) and traditional NSAIDs alone or in combination (Aisen, 2002) have failed, suggesting that we do not yet fully understand the biology underlying the inflammatory processes in Alzheimer’s disease or the relevant mechanisms of protective drug actions, and suggesting that alternative mechanisms of NSAID drug action have to be considered.

It has been recognized recently that a subset of NSAIDs can bind to and activate the nuclear hormone receptor PPARγ (Lehmann et al., 1997; Jaradat et al., 2001). In myeloid lineage cells, including microglia, the principal effect of PPARγ activation is to transcriptionally silence proinflammatory gene expression (Jiang et al., 1998; Ricote et al., 1998; Daynes and Jones, 2002). We have argued that the anti-inflammatory effects of NSAIDs may be partly mediated through the activation of this transcription factor (Landreth and Heneka, 2001). Anti-inflammatory effects of PPARγ agonists and ibuprofen have been shown to protect neurons from cytokine-mediated death (Heneka et al., 1999) and were also observed following infusion of immunostimulants into rodent brain (Heneka et al., 2000). Studying Aβ-mediated microglial activation and neurotoxicity in vitro, Combs et al. reported that PPARγ agonists, including ibuprofen, were neuroprotective (Combs et al., 2000). These findings led us to investigate the effects of a highly specific PPARγ agonist and ibuprofen in APPV717I-overexpressing mice, a murine model of the amyloid pathology in Alzheimer’s disease (Van Leuven, 2000). We found that acute treatment of these animals with the PPARγ agonist pioglitazone resulted in a significant reduction in the number of IB4-positive microglia
and GFAP-positive astrocytes in the hippocampus and the frontal cortex. Moreover, the brief anti-inflammatory drug treatment inhibited the transcription and expression of COX2 and iNOS in both brain areas. COX2 was expressed predominantly in activated microglial cells, whereas iNOS was strictly co-localized with GFAP, indicating that activated astrocytes served as a major source of iNOS-dependent NO. Inhibition of both COX2 and iNOS has been proven beneficial in several other models of neurodegeneration and inflammation including amyotrophic lateral sclerosis, brain trauma

Fig. 4 Effect of anti-inflammatory drug treatment on Aβ levels in 10-month-old APPV717I transgenic mice. Immunohistochemical analysis of Aβ1–42 deposition in the hippocampus (HC) and frontal cortex (FC) was performed in 10-month-old APPV717I transgenic mice treated with either control chow (Con), or chow supplemented with ibuprofen (Ibu) or pioglitazone (Pio) for 7 days (A). Serial sections were evaluated for the total plaque surface (given as a percentage of Aβ1–42-stained surface/region) and for the integral Aβ1–42 staining density in the HC and FC (B). Levels of soluble Aβ1–42, Aβ1–40 and the ratio Aβ1–42/Aβ1–40 were quantitated by ELISA (C). Aβ levels are given as ng/g brain tissue. Asterisks indicate significant differences between the control and drug-treated groups (*P < 0.05, SEM, ANOVA followed by Tukey test). (A) Bar = 250 μm.
and experimental stroke (Zhang et al., 1996; Iadecola et al., 2001; Parmentier-Batteur et al., 2001; Hurley et al., 2002; Pompl et al., 2003; Manabe et al., 2004), suggesting that pioglitazone, and to a lesser extent ibuprofen, can exert neuroprotective effects. Similar results showing that ibuprofen treatment reduced activated, plaque-associated microglia as well as interleukin (IL)-1β levels were obtained after an oral treatment of APP-overexpressing mice for 6 months (Lim et al., 2000). One of the most important outcomes of the present study is that short intervals of drug treatment inhibit the expression of inflammatory markers and reduce the activation status of microglia and astrocytes. In this respect, it seems noteworthy that similarly to the findings in previous studies and the present study, evaluation of human pathological material indicates that NSAID treatment results in a reduction of glial activation that accompanies amyloid deposition and plaque formation in the brains of Alzheimer’s disease patients (Mackenzie and Munoz, 1998; Alafuozoff et al., 2000). While pioglitazone and ibuprofen share the pharmacological mechanism of PPARγ activation, it is not clear whether both drugs exert their beneficial effects through activation of this receptor. Pioglitazone and other thiazolidinediones have also been suggested to exert their anti-inflammatory effects through PPARγ-independent mechanisms (Chawla et al., 2001) and ibuprofen has been shown to inhibit protein kinases that are elements of inflammatory signalling cascades and nuclear factor-κB (Tegeder et al., 2001).

Inflammation plays a role in amyloidogenesis and Aβ deposition; however, the mechanistic relationship between these processes remains to be resolved. The capacity of inflammatory stimuli to promote the generation of amyloidogenic Aβ species has not been examined extensively; however, Blasko and colleagues have reported that neuronal Aβ secretion is enhanced by cytokine exposure (Blasko et al., 2001). These findings were recently extended by showing that several cytokines including IL-1β and tumour necrosis factor-α, alone or in concert, effectively increase the generation of the fibrillogenic Aβ1–40 and Aβ1–42 peptides in vitro (Sastre et al., 2003). The observed increase of amyloid secretion was paralleled in vitro by an upregulation of BACE1, one of two key secretases of the APP processing pathway. In the present study, BACE1 was mainly expressed by neurons but was also focally found in astrocytes as revealed by confocal immunostaining. Treatment of animals with pioglitazone and ibuprofen reduced the number of BACE1-positive neurons and astrocytes and BACE1 mRNA levels in both brain regions, the hippocampus and frontal cortex.

In the present study, a 7 day treatment with pioglitazone and ibuprofen decreased the area of focal Aβ1–42-positive amyloid deposits and their respective staining intensity in the hippocampus and frontal cortex. This latter finding confirms previous reports in ibuprofen- and indomethacin-treated animals. However, in all previous studies, treatment intervals were considerably longer (Lim et al., 2000; Jantzen et al., 2002; Quinn et al., 2003; Yan et al., 2003). While PPARγ-independent mechanisms need to be considered, in vitro data showing that ibuprofen and pioglitazone as well as other NSAIDs and structurally different PPARγ agonists share a common and PPARγ-dependent mechanism in reducing immunostimulated BACE1 and Aβ production (Sastre et al., 2003) suggest that at least one dimension of NSAID-mediated protection is mediated by PPARγ.

An unexpected finding was the observation that acute treatment with pioglitazone resulted in a significant 27% reduction of brain soluble Aβ1–42 levels. The only comparable data on acute effects of anti-inflammatory drugs on Aβ levels in transgenic mice are those of Weggen et al. (Weggen et al., 2001; Eriksen et al., 2003). These authors reported that oral treatment of 3-month-old Tg2576 mice with ibuprofen (50 mg/kg/day) for only 3 days resulted in a reduction of SDs-soluble Aβ1–42 levels by ~40% without a change in Aβ1–40. In the present study, we observed a reduction in soluble Aβ levels with 7 days of ibuprofen treatment; however, this effect did not reach statistical significance. Weggen and colleagues have shown that ibuprofen and other NSAIDs can alter APP processing, and this effect is correlated with the ability of ibuprofen to reduce Aβ1–42 levels acutely in APP-expressing transgenic mice (Weggen et al., 2001; Sagi et al., 2003; Yan et al., 2003). These findings support the view that these drugs act on γ-secretase to alter its specificity. The effects of pioglitazone on Aβ deposition are not likely to arise from a direct effect on γ-secretase, as postulated for ibuprofen, as we and others have been unable to demonstrate an effect of pioglitazone or other PPARγ agonists on γ-secretase-dependent processing using in vitro assay systems (Sastre et al., 2003). Importantly, the drug concentrations used to affect γ-secretase in vitro are far higher than those observed in human CSF samples after oral or intramuscular NSAID application (Bannwarth et al., 1989, 1995; Weggen et al., 2001). In contrast, the observed CSF levels of NSAIDs are exactly in the range (low micromolar concentrations) where they start to act as effective PPARγ activators (Jaradat et al., 2001; Kojo et al., 2003).

In a previous study using APP transgenic mice, pioglitazone had failed to reduce amyloid levels significantly, even when administered for a longer period (Yan et al., 2003). However, since only ~18% of orally administered pioglitazone crosses the intact blood–brain barrier in mammals (Maeshiba et al., 1989, 1995; Weggen et al., 2001), it seems likely that drug dosage, rather than treatment duration, is critical to observe drug effects in the brain. While Yan and colleagues observed a strong tendency of pioglitazone to reduce Aβ1–42 levels, we found a significant and strong reduction of soluble and deposited Aβ1–42 levels using twice the drug concentrations employed in the previous study.

The principal clinical usage of PPARγ agonists is for treatment of type II diabetes. The drugs act to enhance insulin sensitivity and normalize blood glucose levels (Patsouris et al., 2004; Yki-Jarvinen, 2004). It is possible that the reported salutary effects of PPARγ might arise from improved
brain glucose utilization in addition to the anti-inflammatory actions of these drugs (Watson and Craft, 2003). Since insulin resistance increases amyloidosis in APP transgenic mice (Ho et al., 2004), the amyloid-lowering effect of pioglitazone observed in this study may also be due to enhanced insulin sensitivity.

The data reported here support the use of PPARγ agonists in the treatment of Alzheimer’s disease. Recent studies have documented the salutary effects of PPARγ agonists in animal models of multiple sclerosis (Niino et al., 2001; Diab et al., 2002; Feinstein et al., 2002; Natarajan and Bright, 2002) and Parkinson’s disease (Breidert et al., 2002). The potent anti-inflammatory effects of PPARγ agonists suggest that they may have beneficial effects in treating other CNS diseases with an inflammatory component.

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Anti-inflammatory drugs in an Alzheimer’s disease model


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