European Journal of Neuroscience, Vol. 18, pp. 1–11, 2003

Non-steroidal anti-inflammatory drugs mediate increased *in vitro* glial expression of apolipoprotein E protein

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Keywords: Alzheimer's disease, aspirin, cyclooxygenase, indomethacin, rodent

Abstract

Epidemiological studies have shown that use of nonsteroidal anti-inflammatory drugs (NSAIDs) by the elderly is associated with a decreased relative risk and a delayed onset of Alzheimer's disease (AD). In contrast, the apolipoprotein E (apoE) gene has proven to be a risk factor for AD with apoE ε4 AD patients having been found to show lower levels of brain apoE. In the present study, treatment of primary rat mixed glial cell cultures with the common NSAIDs, indomethacin and aspirin, induced significant increases in extracellular apoE protein levels. Similarly, treatment of primary rat astrocyte cell cultures with aspirin and a cyclooxygenase (COX)-2-selective aspirin derivative also stimulated significant increases in apoE protein. However, astrocyte and mixed glial apoE protein levels were significantly reduced following exposure to COX-2-specific indomethacin amides and an inactive indomethacin derivative. ApoE protein modulation was observed at physiological and subphysiological concentrations well below the COX inhibition IC_{50} values of the NSAIDs used, suggestive of a COX-independent mechanism. In contrast to these results, indomethacin and aspirin treatment failed to induce any significant changes in apoE mRNA levels. The failure of NSAIDs to significantly alter apoE expression may have been indicative of a nontranscriptional mechanism of apoE protein induction. Consequently, NSAID-induced increases in apoE protein may enhance apoE-mediated immunosuppression and compensatory synaptic plasticity, potentially resulting in decreased AD risk and delay of disease onset.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the presence of neurofibrillary tangles, neuritic plaques composed of beta-amyloid (Aβ), dystrophic neurites and cortical atrophy (Cummings *et al*., 1998). It has been hypothesized that one key aspect of AD pathogenesis involves the apolipoprotein E (apoE) gene. In rodent brains, apoE has been found to be primarily synthesized by rat astrocytes and microglia (Poirier *et al*., 1991; Stone *et al*., 1997). Unlike in rodents, three separate human apoE isoforms have been identified, namely apoE ε2, ε3 and ε4 (Breslow *et al*., 1982; Rall *et al*., 1982). Among the Caucasian population, the allelic frequencies of the apoE ε2, ε3 and ε4 alleles have been reported to be 8, 78 and 14%, respectively (Utermann *et al*., 1980). In addition, studies have shown a general trend of higher serum levels of apoE protein among healthy apoE ε2 carriers than ε3 and ε4 carriers (Shen *et al*., 1998; Panza *et al*., 2003).

Individuals bearing two copies of the apoE ε4 allele have been found to be at significantly greater risk for AD (Strittmatter *et al*., 1993; Poirier *et al*., 1993a). In addition, apoE ε4 AD patients have been reported as showing lower levels of apoE in the brain (Bertrand *et al*., 1995; Beffert *et al*., 1999a; Glockner *et al*., 2002). It has been proposed that reduced levels of apoE may significantly inhibit apoE-mediated lipid transport and homeostasis, synaptic plasticity, and Aβ clearance (Mahley, 1988; Boyles *et al*., 1989; Poirier, 1994; Holtzman & Fagan, 1998).

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Received 2 April 2003, revised 23 June 2003, accepted 27 June 2003

doi:10.1046/j.1460-9568.2003.02869.x

In recent years, many researchers have focused their efforts on the role of the immune system in AD (Hull *et al*., 1996; Sheng *et al*., 1996). Studies have demonstrated high levels of microglial activation and clustering around AD plaques (Haga *et al*., 1989; Perlmutter *et al*., 1992; Saitoh *et al*., 1997), as well as elevated levels of complement proteins, interleukin (IL)-1 and IL-6 in human AD brains (Griffin *et al*., 1989; Walker & McGeer, 1992; Wood *et al*., 1993; Wisniewski *et al*., 1996). In addition, use of nonsteroidal anti-inflammatory drugs (NSAIDs) by the elderly has been associated with a decreased relative risk and a delayed onset of AD (McGeer *et al*., 1990; Breitner *et al*., 1994; Andersen *et al*., 1995; Breitner *et al*., 1995). NSAIDs include a variety of drugs whose main effect is to inhibit the enzymes cyclooxygenase-1 (COX-1) (E.C. 1.14.99.1) and cyclooxgenase-2 (COX-2) (Rome & Lands, 1975; Copeland *et al*., 1994; Kurumbail *et al*., 1996). Although the main effect of NSAIDs has been described as involving COX inhibition, alternative mechanisms of action have been proposed (Derham & Harding, 2002). For example, it has been suggested that NSAIDs may delay or prevent cataract formation in humans via acetylation of lysine residues found on lens proteins, thereby blocking the reaction of lysine with other cataract-forming modifiers (Derham & Harding, 2002).

Prior work has established a complex relationship between apoE and the immune system, with apoE showing potential immunosuppressive properties both *in vitro* (Laskowitz *et al*., 1997; Lynch *et al*., 2001) and *in vivo* (de Bont *et al*., 2000; Van Oosten *et al*., 2001), and inflammatory mediators showing significant apoE-regulatory effects (Brand *et al*., 1993; Duan *et al*., 1995; Starck *et al*., 2000). In addition, epidemiological data has revealed that the NSAID neuroprotective effect is stronger in subjects lacking an apoE ε4 allele (Breitner *et al*., 1995).

Based upon the clear relationship between apoE and the immune system, the epidemiological link between NSAIDs and apoE, and the potential benefits of apoE in immune and lipid regulation, we hypothesize that the benefits of NSAIDs in AD may be due to a modulation of glial apoE production, thereby influencing compensatory synaptogenesis and immunosuppression. The objective of the current study was to examine the effects of NSAIDs and NSAID derivatives on apoE protein and mRNA regulation in primary rat glial cell cultures.

Materials and methods

Cell culture solutions and supplies were purchased from Gibco (Grand Island, NY, USA), unless otherwise indicated. Indomethacin and acetylsalicylic acid (aspirin) were acquired from Sigma (St Louis, MO, USA). Indomethacin derivatives LM 4108, LM 4115 and LM 4192, as well as aspirin derivatives o-(acetoxyphenyl)hept-2-ynyl sulphide (APHS) and APHS phenol, were gifts from Dr L. Marnett, Department of Biochemistry and Chemistry, Vanderbilt University School of Medicine, Nashville, TN, USA. Hydrogen peroxide was from Fischer Scientific (Fair Lawn, NJ, USA) while acridine orange was from Eastman Kodak Comp. (Rochester, NY, USA). Goat antihuman apoE capture antibodies were from International Immunology Corporation (Murrieta, CA, USA). The HiTrap Protein G Kit was purchased from Amersham Pharmacia Biotech (Baie d'Urfe, QC, CAN). Biotin was from Boehringer Mannheim Corp. (Indianapolis, IN, USA) and recombinant apoE4 protein was from Panvera Quality Reagents (Madison, WI, USA). Alkaline phosphatase–streptavidin was acquired from Zymed Laboratories Inc. (San Francisco, CA, USA) and attophos reagent from Promega Corporation (Madison, WI, USA). All RNA extraction materials were included in the RNeasy Mini Kit purchased from Qiagen (Mississauga, ON, CAN). SYBR Green PCR Core Reagents and 1X SYBR Green PCR Master Mix were acquired from Molecular Probes Inc. (Eugene, OR, USA). [Q1]

Tissue cell culture models

Primary rodent cell cultures were prepared as previously described (Guillaume *et al*., 1996). All experimental protocols were granted ethics approval under the provisions of the McGill University Animal Care Committee and Canadian Council on Animal Care. Briefly, primary astrocyte cell cultures were obtained from the cortices of 1-day-old Sprague-Dawley rat pups (Charles River Laboratories Inc., St Constant, Quebec, CAN). [Q2] Isolated cortical tissue was enzymatically dissociated with dispase [10 mg/mL phosphate buffer solution (PBS)] and DNase 1 mg/mL (both from Boehringer Mannheim Corp., Indianapolis, IN, USA). The cell suspension was then filtered through a 70-μm nylon mesh (Becton Dickinson, Franklin Lakes, NJ, USA) and ultimately suspended in supplemented growth medium [Dulbecco's modified Eagle's medium-F12 containing 10% fetal bovine serum (FBS) (Immunocorp, Montreal, QC, CAN), 1% penicillin/streptomycin, and 0.1% amphotericin B (fungazone), pH = 7.6]. Cells were subsequently plated in 75-cm^2 flasks (Sarstedt, Newton, NC, USA), previously coated with poly D-lysine (Sigma, St Louis, MO, USA). The cell culture medium was replenished every 3–4 days until the astrocytes reached ≈70% confluence and microglia were visible. Microglia and oligodendrocytes were then removed via gentle shaking. Manual shaking was repeated 3–4 days later in order to purify the astrocyte cell cultures further. Astrocyte cell cultures were sustained with supplemented cell culture medium and assessed by immunocytochemistry for expression of glial fibrillary acidic protein (GFAP) in astrocytes, ED-1 antigen in microglia, and galactocerebroside (GALC) protein in oligodendrocytes. The results indicated that astrocyte cell cultures were composed of ≈95% astrocytes and 5% microglia.

Mixed cell culture samples were also utilized in order to create a more physiologically representative environment in which to measure potential changes in apoE protein levels. The mixed glial cell suspension in PBS, derived from initial shaking, was re-suspended in fresh supplemented growth medium and plated in 75-cm^2 flasks, previously coated with poly D-lysine. After 30 min, the cell culture medium was replaced so as to remove excess oligodendrocytes. Subsequently, the cell culture medium was replaced every 3–4 days until ≈85% confluence was achieved. Immunocytochemical labelling established that cell cultures were composed of ≈70% astrocytes, 25% microglia and 5% oligodendrocytes.

Upon reaching ≈85% confluence, the cell cultures were rinsed with PBS, treated with 0.1% trypsin and warmed at 37 °C for 10 min. The trypsin was then inactivated by adding supplemented cell culture medium in equal volume. All cell types were plated in 24-well cell culture plates, previously coated with poly D-lysine, at a density of ≈50 000 cells/well. The cell culture medium was refreshed every 3–4 days until ≈70% confluence was reached.

Drug treatment

Enriched primary rat astrocyte and mixed glial cell cultures were treated with the NSAIDs indomethacin and acetylsalicylic acid (aspirin). Stock solutions of each drug were made in 100% ethanol. Cells were subsequently treated with each drug at various concentrations dissolved in fresh cell culture medium and incubated for a treatment period of 48, 72 or 96 h. Each concentration was tested in triplicate. Upon completion of treatment, cells were visually assessed and the cell culture medium was collected and stored at −80 °C.

Indomethacin and aspirin are nonselective COX inhibitors whose ratios of COX-1 : COX-2 inhibitory activity vary (Meade *et al*., 1993); however, both have been found to show preferential inhibitory activity against COX-1 (Roth *et al*., 1975; Picot *et al*., 1994; Gierse *et al*., 1999). Consequently, in order to explore the individual role of each COX enzyme, COX-2-selective derivatives of indomethacin (Kalgutkar *et al*., 2000) and aspirin (Kalgutkar *et al*., 1998a) were used to treat primary rat astrocyte and mixed glial cell cultures. Indomethacin aromatic amide and indomethacin phenethyl amide (Kalgutkar *et al*., 2000), having been previously shown to mediate COX-2-selective inhibition, were utilized during a treatment regimen over a period of 24, 48, 72 or 96 h. In addition, an inactive indomethacin derivative (Kalgutkar *et al*., 2000), characterized by a 4-bromobenzyl group on the indole ring, was included as a negative control. All three indomethacin derivatives were initially dissolved in dimethylsulfoxide (5 mM) and subsequently in fresh supplemented cell culture medium for treatment of the cells.

In addition, rat astrocyte and mixed glial cell cultures were treated with APHS (Kalgutkar *et al*., 1998b), a COX-2-selective inhibitor, and APHS phenol (Kalgutkar *et al*., 1998a), an inactive hydrolysis product of APHS. Both aspirin derivatives were provided in aqueous solution and subsequently dissolved in fresh supplemented cell culture medium. Drug treatment was concluded following 24, 48, 72 or 96 h. Following treatment with indomethacin and aspirin derivatives, the cell culture medium was collected from each well and frozen at −80 °C for later analysis.

The concentrations used in the present experiments all fell within physiological concentrations and, often, well below reported IC_{50} values. For example, the concentrations of indomethacin and its derivatives used in the described experiments ranged from 10^{-6} to 10^{-19} M. Thus, most of these values fell well below the indomethacin IC_{50} value of 0.75 μM (Kalgutkar *et al*., 2000) as well as within the physiological concentrations described in human plasma and CSF, both of which lie in the nanomolar range (Bannwarth *et al*., 1990). In addition, the

derivative concentrations used typically fell well below the IC_{50} values of the COX-2-selective amides, listed as 0.12 μM and 0.060 μM (Kalgutkar *et al*., 2000).

Aspirin and APHS derivative treatment concentrations used in the present study ranged from 10^{-6} to 10^{-18} M. These values typically fell well below the IC_{50} values of both aspirin and APHS, reported to be 62.5 μM (Kalgutkar *et al*., 1998a) and 0.8 μM (Kalgutkar *et al*., 1998b), respectively. Furthermore, the values used were well within the physiological ranges of aspirin found in human plasma, reported to be in the millimolar range (Hansen *et al*., 1998).

Acridine orange staining

Cell viability was assessed using an acridine orange labelling protocol following 96 h of drug treatment. As a positive control, cells were treated with 0.01 and 0.001% hydrogen peroxide for 10 min prior to staining. Initially, the cell culture medium and hydrogen peroxide were removed and 100 μL of fresh supplemented cell culture medium was added. Subsequently, cells were incubated with a stock buffer solution of pH 3.5 (triton, 0.1%; sucrose, 0.2 M; disodium EDTA, [Q3] 10^{-4} M; citrate phosphate buffer, 0.02 M, pH 3.0) for 1 min. Cells were then treated with an acridine orange solution (2 mg/mL in water) diluted 1 : 100 with a second stock buffer solution (NaCl, 0.1 M; citrate phosphate buffer, 0.01 M, pH = 3.8 based on phosphate) for a duration of 5 min. Upon completion of incubation, removal of the acridine orange solution was followed by a rapid rinse with PBS. Coverslips were immediately mounted on noncoated slides with Vectishield (Vector Laboratories Inc., Burlingame, CA, USA) and observed with a Nikon Eclipse E600 fluorescent microscope (Nikon Inc., Melville, NY).

Apolipoprotein E protein enzyme-linked immunosorbent assay (ELISA)

Assessment of extracellular apoE protein levels in all collected cell culture medium samples was achieved using a protein-specific ELISA assay (Beffert *et al*., 1999a). The apoE ELISA assay was validated using 17-β-estradiol as a positive control, whereby treatment with 17-β-estradiol induced significant increases in apoE protein, as demonstrated previously (Stone *et al*., 1997). ELISA plates (Corning Costar E.I.A./R.I.A., Acton, MA, USA) were coated with goat antihuman apoE capture antibody, purified with a HiTrap Protein G Kit, in 10 mM sodium carbonate. Plates were sealed and stored overnight at 4 °C. The capture antibody was subsequently blocked with 0.1% bovine serum albumin (BSA) in PBS and stored overnight at 4 °C. The following day, each well was washed with 20 mM tris–base–salt–tween (TBS-T) between individual incubation periods of 2 h. Defrosted cell culture medium samples and recombinant apoE4 standards (50–2000 ng/mL in PBS) were incubated in triplicate. Goat antihuman apoE antibody labelled with biotin in 0.1% BSA in TBS-T was then added. Subsequently, alkaline phosphatase–streptavidin diluted 1 : 1000 in 0.1% BSA in TBS-T was added and the plates were incubated at room temperature for 1 h. Finally, wells were incubated with attophos reagent, warmed to room temperature. At 30 min and 60 min, measurements of emitted fluorescence were taken using a microplate fluorescence reader FL-600 (Bio-tek Instruments Inc., Winooski, VT, [Q4] USA), reading at a bandwidth of 450–50 nm. Detectable levels of apoE protein were between 50 and 2000 ng/mL.

Apolipoprotein E mRNA quantification

In order to assess whether NSAID treatment had transcriptional effects on apoE expression, primary rat astrocyte cell cultures were prepared and plated in 75-cm^2 flasks. Rodent astrocyte cell cultures (≈ 80% confluence) were then treated with indomethacin and aspirin, diluted in supplemented cell culture medium. Cells were incubated with the described compounds for 2, 4, 8, 16, 24 or 30 h. Cells were rinsed with PBS and manually detached by scraping the flask surface. The cell suspension in PBS was centrifuged for 10 min, upon which the supernatant was removed. Samples were then stored at −80 °C.

RNA extraction

Using an RNeasy Mini Kit, frozen cells were disrupted by the addition of a mixed buffer containing β-mercaptoethanol and subsequently homogenized using a QIAshredder column. Samples were then centrifuged for 2 min at 20 °C. Following centrifugation, one volume of 70% ethanol was added to the homogenized lysate and mixed. The lysate was then applied to an RNeasy mini spin column and centrifuged for 15 s. Subsequently, the mini spin column was successively washed with various buffers and centrifuged for between 15 s and 2 min. The RNeasy column membrane was then rinsed with RNase-free water, allowed to stand for ≈1 min, and centrifuged for 1 min. Extracted RNA samples were quantified using a spectrophotometer reading at 260 nm and then stored at −80 °C.

Real-time quantitative reverse transcriptase–polymerase chain reaction (RT-PCR)

Extracted RNA samples were amplified using a two-step RT-PCR reaction (PE Applied Biosystems, 1997; Winer *et al*., 1999). In a total reaction volume of 50 μL containing 1 × RT buffer, 5.5 mM $MgCl_2$, 500 μM of each deoxynucleotide triphosphate, 2.5 μM of random hexamers, 0.4 U/μL of RNase inhibitor and 1.25 U/μL of Multiscribe Reverse Transcriptase, 1 μg of extracted RNA from each individual sample was amplified. The reaction volumes were sequentially held at 25 °C for 10 min, 48 °C for 30 min and 95 °C for 5 min. Subsequently, the amplified cDNA templates were frozen at −20 °C.

ApoE mRNA quantification was achieved using SYBR Green PCR core reagents, designed for use with the GeneAmp 5700 sequence detector (PE Applied Biosystems, Foster City, CA, USA). Specific rat apoE and β-actin oligonucleotide primers, based on the rat apoE mRNA sequence (McLean *et al*., 1983; forward primer, nucleotides 921–940; reverse primer, nucleotides 978–996) and rat β-actin cDNA sequence (Bonaldo *et al*., 1996; forward primer, nucleotides 218–238; reverse primer, nucleotides 265–284), respectively, were designed using Primer Express software (PE Applied Biosystems). Each reac- [Q5] tion utilized a total reaction volume of 35 μL containing 1 × SYBR Green PCR Master Mix, 1000 nM of each primer and 3 μL of cDNA template, with completion to volume with RNase-free water. Each sample then underwent a thermal reaction cycle of 50 °C for 2 min, 95 °C for 10 min and 40 repetitions of 95 °C for 15 s and 60 °C for 1 min. Subsequently, the PCR product was stored at 4 °C. All samples were amplified using both apoE and β-actin primers.

Quantification of mRNA was achieved by measuring changes in fluorescent signal emitted by SYBR Green Dye upon binding to double-stranded DNA (PE Applied Biosystems, 1997; Bieche *et al*., 1999). Specifically, the threshold cycle (C_T) values of treated and non-treated samples were compared following normalization to the endogenous control, β-actin. The C_T value refers to the fractional PCR cycle number at which the amplified target reaches a fixed threshold, a condition indicated by the fluorescent signal emitted by the SYBR Green Dye.

Statistics

Statistical normality was initially assessed using Normcheck, version 1.0; J. Rochford, Montreal, QC, CAN. Statistical outliers were excluded at the 95% confidence level based upon the Dixon Test for Rejection of Outliers (Dixon, 1953). Statistical outliers were

established across drug trials and within time and drug concentration. Data were excluded from four of the reported analyses with the number of data points removed being $<1.7\%$ of the total number of data points included in each analysis. Subsequent statistical analyses were conducted utilizing Datasim, version 1.2; D.R. Bradley, Lewiston, ME, USA.

All individual data points were expressed relative to nontreated mean protein values within trial, drug and time. Protein levels of treated cell cultures were assessed using two-way independent-measures ANOVA; specifically ANOVAs were used to analyse the effects of drug concentration and duration of treatment across trials. *Post hoc* pairwise comparisons were completed as required using Tukey's Honestly Significant Difference Test. Significant differences between nontreated and treated cells were then assessed using multisample 95% confidence intervals with the nontreated mean protein value arbitrarily set as a population mean of 1.0. For those analyses determined to have a significant main effect of concentration or significant time $\times$ concentration interaction effect, confidence intervals were completed across and within time points, respectively.

In order to quantify sample apoE mRNA, a comparative C_T method (PE Applied Biosystems, 1997; Winer *et al*., 1999) was utilized for each individual trial. C_T values derived from amplification with rat β-actin primers were subtracted from the C_T values derived from amplification of the same samples with rat apoE primers, thereby producing a ΔC_T value. Consequently, the resulting data were normalized to an endogenous control. Subsequently, the ΔC_T values of treated samples were compared to those of nontreated samples within each time point. Ultimately, apoE mRNA quantities for each treatment condition were expressed relative to a nontreated value of 1.0 (Winer *et al*., 1999).

Across trials, the data were collated such that an independent-measures two-way ANOVA was conducted with duration of treatment and agent concentration as independent factors. Statistical differences between treated and nontreated cells were examined using multisample 95% confidence intervals with the nontreated mean apoE mRNA value set as a population mean of 1.0.

Results

Indomethacin treatment of primary rat mixed glial cell cultures significantly increased extracellular apoE protein levels

Results for all experiments were derived from a minimum of two separate cell culture trials. All apoE protein levels refer to those found in collected cell culture medium samples. Acridine orange staining of drug-treated cells, when used in the reported concentrations, displayed significant green fluorescence, indicative of viable cells. Rodent astrocyte and mixed glial cell cultures, following 96 h of drug treatment using the reported concentrations, displayed at least 95% cell viability.

Two-way independent-measures ANOVA of astrocyte data revealed a **[Q6]** significant main effect of time ($F_{2,259} = 4.38$, $P < 0.05$) with simple main effects testing demonstrating mean protein levels to be significantly higher at 72 h than at either 48 or 96 h, all $P < 0.01$. All significant main effects of time are described in Table 1. However, indomethacin treatment of astrocyte cell cultures resulted in no significant protein differences between nontreated and treated cells over a 96-h period (Data not shown).

Analysis of mixed glial data revealed a significant time $\times$ concentration interaction ($F_{18,287} = 3.95$, $P < 0.0001$). Subsequent simple main effects analysis revealed significant differences in apoE protein levels between time points of 48, 72 and 96 h, within specific concentrations (all $F_{2,287} \leq 21.76$, $P \leq 0.05$). As such, *post hoc* pairwise analysis established a general trend of increased apoE protein with increased duration of incubation within wells treated with indomethacin at 10^{-17}, 10^{-18} and 10^{-19} M, all $P \leq 0.05$. Specifically, mean apoE protein values at 48 and 72 h were significantly lower than those at 96 h ($P < 0.05$). In contrast, a significant reduction in apoE with time was detected at a concentration of 10^{-14} M with mean protein values at 48 h being greater than at 72 h: $P \leq 0.01$. Confidence interval analysis demonstrated that indomethacin treatment of mixed glia induced significant increases in extracellular apoE protein levels after 96 h relative to nontreated cells: all $P \leq 0.05$ (Fig. 1A).

Treatment of primary rat astrocyte and mixed glial cell cultures with indomethacin derivatives significantly reduced extracellular apoE protein levels in a dose-dependent manner

Three indomethacin derivatives, LM 4108, LM 4115 and LM 4192, were utilized in the current study. LM 4108, an indomethacin amide derivative, and LM 4115, an aromatic indomethacin amide derivative, have been characterized as COX-2-selective inhibitors (Kalgutkar *et al*., 2000). Kinetic analysis has demonstrated that LM 4108 behaves as a slow, tight-binding inhibitor with a much slower time course of COX-2 inhibition than indomethacin (Kalgutkar *et al*., 2000). Furthermore, both LM 4108 and LM 4115 have proven to be effective at inhibiting COX-2 activity in macrophage cell cultures, as well as *in vivo* in a rat footpad oedema model (Kalgutkar *et al*., 2000). In contrast, LM 4192 has been shown to be ineffective as a COX inhibitor and was, thus, used as a negative control in the current experiments (Kalgutkar *et al*., 2000).

Treatment of astrocytes with LM 4108 resulted in significant main effects of treatment duration ($F_{3,211} = 30.84$, $P < 0.0001$) and drug concentration ($F_{6,221} = 2.37$, $P < 0.05$). Main effects analysis revealed that mean apoE levels were significantly higher at 24 h than at 48, 72 or 96 h ($P < 0.01$). Moreover, significant decreases in apoE protein were observed in LM 4108-treated cells compared to control cells ($P < 0.05$; Fig. 1B). Similarly, LM 4108 treatment of mixed glial cell cultures induced significant main effects of time ($F_{3,221} = 5.37$, $P < 0.01$) and compound concentration ($F_{6,221} = 4.58$, $P < 0.001$). Unlike astrocyte cells, mean apoE protein levels were significantly elevated at 72 h relative to 48 or 96 h, irrespective of drug concentration ($P \leq 0.05$). In addition, when compared to nontreated cells, mixed glial apoE protein levels of treated cells were found to be significantly lower ($P < 0.05$; Fig. 1C).

[Q7] TABLE 1. Percentage change in apolipoprotein E levels across time among treated cell cultures showing a significant main effect of duration of treatment

	24 h		48 h		72 h		96 h	
Drug compund	Astrocyte	Mixed glia	Astrocyte	Mixed glia	Astrocyte	Mixed glia	Astrocyte	Mixed glia
Indomethacin	N/A	N/A	104.9 ± 4.4	N.S.	121.2 ± 5.5	N.S.	109.4 ± 4.4	N.S.
LM4108	105.2 ± 5.2	79.2 ± 2.7	61.7 ± 3.0	75.9 ± 2.7	62.7 ± 3.8	90.5 ± 3.2	60.8 ± 3.7	72.1 ± 4.8
LM4115	104.0 ± 7.4	79.7 ± 3.9	83.4 ± 5.0	53.1 ± 4.0	55.3 ± 4.0	76.2 ± 3.2	43.6 ± 3.1	87.3 ± 5.0
LM4192	94.5 ± 7.2	94.1 ± 4.5	78.6 ± 5.8	70.0 ± 4.2	70.0 ± 4.2	88.2 ± 3.5	81.3 ± 4.9	92.3 ± 3.6
Aspirin	N/A	N/A	113.4 ± 4.3	122.3 ± 4.7	122.3 ± 4.7	N.S.	82.5 ± 5.1	N.S.

Values are $\pm$SEM. All data points are expressed relative to the arbitrarily set nontreated population mean of 100%. N/A, ???; N.S., ???.

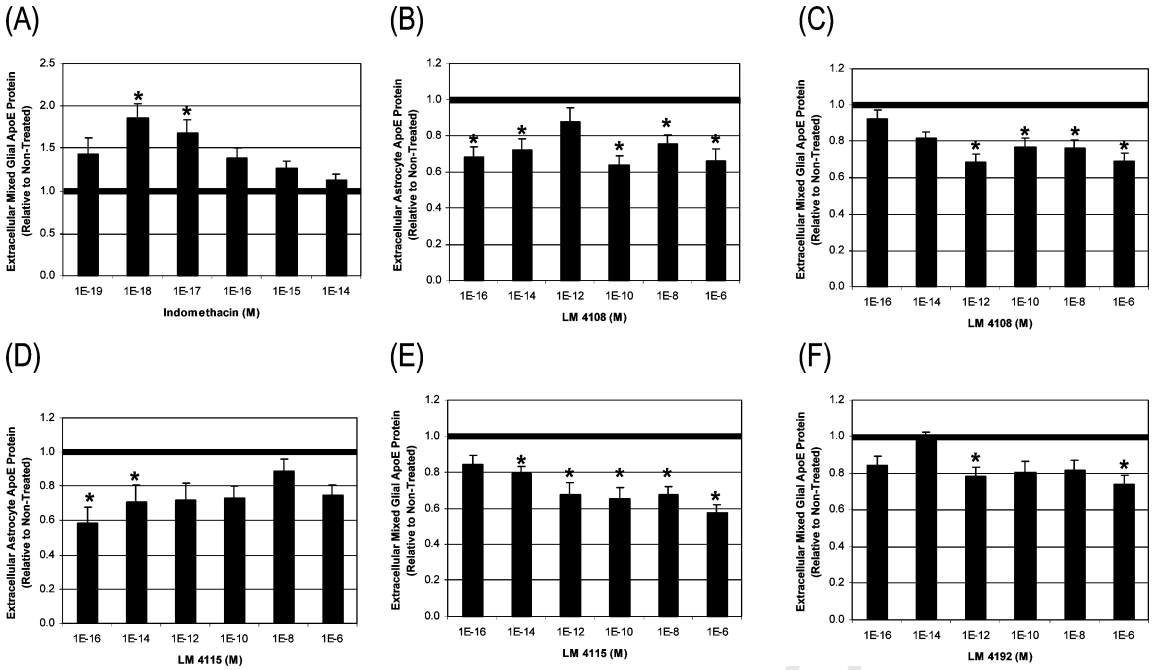

FIG. 1. Mean (+ SEM) extracellular apolipoprotein E (apoE) protein. Figures depict mean (A) mixed glial apoE protein levels following 96 h as a function of indomethacin treatment concentration; (B) astrocyte and (C) mixed glial apoE protein levels as a function of LM 4108 treatment concentration; (D) astrocyte and (E) mixed glial apoE protein levels as a function of LM 4115 treatment concentration; and (F) mixed glial apoE protein levels as a function of LM 4192 treatment concentration. All data points were expressed relative to the mean of nontreated (NT) cells. Mean protein values were derived from an average of (A) $n \geq 6$, (B and C) $n \geq 34$, (D) $n \geq 23$ and (E and F) $n \geq 33$ cell culture wells. The mean apoE protein values of treated cells were then compared via multiple 95% confidence intervals to the arbitrarily set NT population mean of 1.0, as depicted by the solid black line. Significant differences in extracellular apoE protein levels, relative to those of NT cells, are indicated by $^{*}P < 0.05$.

Use of another COX-2-selective indomethacin derivative revealed similar results. LM 4115 treatment induced significant main effects of time and drug concentration in both astrocytes (time, $F_{3,139} = 29.39$, $P < 0.0001$; concentration, $F_{6,139} = 2.23$, $P < 0.05$) and mixed glia (time, $F_{3,215} = 16.64$, $P < 0.0001$; concentration, $F_{6,215} = 7.28$, $P < 0.0001$). In astrocyte cells, apoE protein levels decreased with time as mean levels at 24 h were significantly greater than those at 48, 72 or 96 h ($P \leq 0.05$) and mean levels at 48 h were significantly higher than those at either 72 or 96 h ($P < 0.01$). In mixed glia, however, mean apoE levels at 48 h were significantly lower than mean levels at 24, 72 or 96 h ($P < 0.01$). Nevertheless, in both astrocyte and mixed glial cell cultures, treatment with LM 4115 proved to significantly reduce extracellular apoE protein levels ($P < 0.05$; Fig. 1D and E).

As a negative control, an indomethacin derivative with no COX inhibitory activity, LM 4192, was utilized. However, analysis revealed a significant main effect of time in both astrocytes ($F_{3,205} = 3.25$, $P < 0.05$) and mixed glia ($F_{3,211} = 6.98$, $P < 0.001$), as well as a significant main effect of LM 4192 concentration in mixed glia ($F_{6,211} = 5.39$, $P < 0.0001$). In astrocyte cell cultures, mean levels of apoE were found to be significantly higher at 24 h than at 72 h ($P < 0.05$). In contrast, mean levels at 48 h were significantly lower than at 24, 72 or 96 h in mixed glia ($P \leq 0.05$). In comparing treated to nontreated cells, subtle decreases in apoE protein were detected following treatment in mixed glia ($P < 0.05$; Fig. 1F).

Aspirin treatment of primary rat astrocyte and mixed glial cell cultures significantly increased extracellular apoE protein levels

Significant main effects of both duration of treatment ($F_{2,214} = 13.74$, $P < 0.0001$) and aspirin concentration ($F_{9,214} = 4.35$, $P < 0.0001$) on astrocyte apoE levels were identified. In fact, apoE protein levels decreased over time irrespective of concentration as protein levels at 96 h proved to be significantly lower than those at 48 or 72 h ($P < 0.01$). However, relative to nontreated cells, aspirin treatment was found to induce significant increases in astrocyte apoE protein at a concentration of 10^{-17} M ($P < 0.05$; Fig. 2A).

Following treatment with aspirin, mixed glia showed a significant main effect of drug concentration on extracellular apoE levels ($F_{9,329} = 6.35$, $P < 0.0001$). However, no effect of duration of treatment was observed. Statistical analysis revealed a significant increase in apoE levels upon treatment with aspirin at a concentration of 10^{-11} M ($P < 0.05$; Fig. 2B).

Treatment of primary rat astrocyte cell cultures with a COX-2-specific aspirin derivative induced significant increases in extracellular apoE protein levels in a time- and dose-dependent manner

The aspirin-like molecule o-(acetoxyphenyl)hept-2-ynyl sulphide (APHS) has been recently described as showing preferential acetylation and irreversible inactivation of COX-2 (Kalgutkar *et al*., 1998b). APHS has been reported to be ≈60 times more potent and 100 times more selective for COX-2 inhibition than aspirin (Kalgutkar *et al*., 1998b). The functional applicability of APHS has also been confirmed both *in vitro* and *in vivo*, whereby COX-2 activity in stimulated macrophages has been inhibited by APHS treatment (Kalgutkar *et al*., 1998b). Furthermore, using an *in vivo* rat air pouch model, significant reductions in prostaglandin synthesis have been observed following treatment with APHS (Kalgutkar *et al*., 1998b).

In the present study, a significant time × APHS concentration interaction effect was observed ($F_{18,137} = 3.34$, $P < 0.0001$). Subsequent testing established a significant effect of time at an APHS concentration of 10^{-10} M, whereby mean apoE levels at 24 h proved to be significantly greater than those at 48, 72 or 96 h ($P < 0.01$). A significant increase in

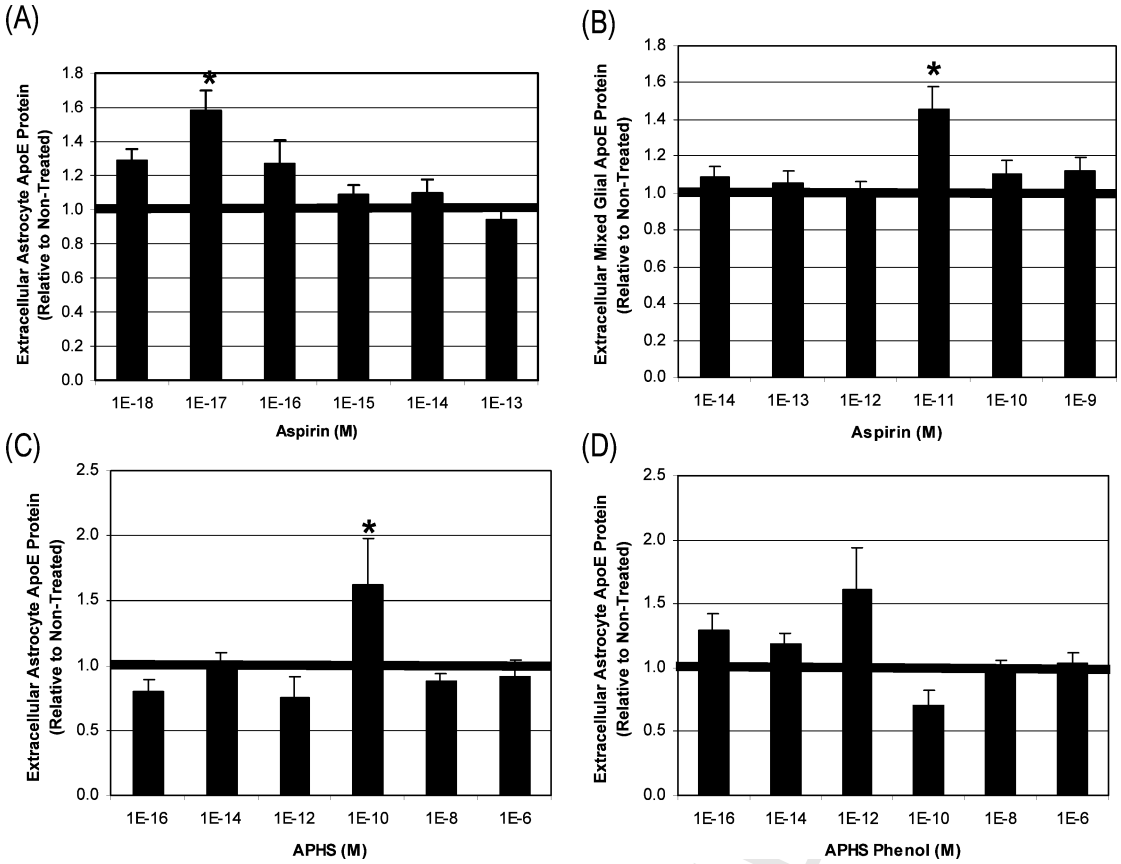

FIG. 2. Mean (+ SEM) extracellular apolipoprotein E (apoE) protein. Figures depict mean (A) astrocyte and (B) mixed glial apoE protein levels as a function of aspirin treatment concentration. Mean astrocyte apoE protein levels were determined after (C) 24 h of APHS and (D) 96 h of APHS phenol treatment. All data points were expressed relative to the mean of nontreated (NT) cells. Mean astrocyte and mixed glial protein values, following aspirin treatment, were derived from an average of $n \geq 8$ and $n \geq 25$ cell culture wells, respectively. Mean astrocyte protein values following APHS and APHS phenol treatment were derived from an average of $n \geq 5$ and $n \geq 6$ cell culture wells, respectively. The mean apoE protein values of treated cells were then compared via multiple 95% confidence intervals to the arbitrarily set NT population mean of 1.0, as depicted by the solid black line. Significant differences in apoE protein levels, relative to those of NT cells, are indicated by $^{*}P < 0.05$.

astrocyte apoE protein levels was also observed following 24 h of treatment, relative to nontreated cells ($P < 0.05$; Fig. 2C).

In contrast, the phenol derivative of APHS has been shown to be inactive with no inhibitory activity against either COX-1 or COX-2 (Kalgutkar *et al*., 1998a). ANOVA substantiated a significant time × APHS phenol concentration interaction ($F_{18,138} = 3.13$, $P < 0.0001$). Astrocyte apoE levels were generally found to increase with prolonged incubation at specific APHS phenol concentrations. Specifically, mean apoE levels at 24 h proved to be significantly lower than at 72 h at a concentration of 10^{-18} M ($P < 0.05$) and at 96 h at concentrations of 10^{-10}, 10^{-12}, 10^{-16} and 10^{-18} M ($P \leq 0.05$). Mean levels at 48 h proved to be significantly lower than those at 72 h at 10^{-16} M ($P \leq 0.05$) and at 96 h at both 10^{-12} and 10^{-16} M ($P \leq 0.05$). Finally, apoE protein levels at 72 h were found to be significantly lower than levels at 96 h at 10^{-12} M ($P \leq 0.05$). In contrast to the results shown for APHS treatment of astrocyte cell cultures, cell cultures treated with APHS phenol showed no significant differences in mean apoE levels compared to nontreated cells (Fig. 2D).

Treatment of primary rat astrocytes with indomethacin and aspirin failed to induce any significant changes in cellular apoE mRNA levels

ANOVA of apoE mRNA levels failed to show any significant effect of indomethacin or aspirin treatment ($P > 0.05$; Fig. 3A and B).

Discussion

NSAID and NSAID derivative treatment of primary rodent glia induced significant changes in apoE protein levels

In the present study, treatment of primary rat tissue cell cultures with indomethacin, aspirin and the COX-2-selective aspirin derivative APHS induced significant increases in extracellular apoE protein levels. Generally, astrocyte and mixed glial apoE protein levels were found to increase by 58–62 and 46–86%, respectively. In contrast, use of indomethacin derivatives with COX-2-selective inhibitory activity, as well as an inactive indomethacin derivative, significantly reduced apoE protein levels. The reported decreases in astrocyte and mixed glial apoE protein levels were between 24 and 42% and 21 and 43%, respectively. The failure of pure astrocyte cell cultures to show any significant protein level changes following indomethacin and LM 4192 treatment could have been indicative of the need for a mixed cellular environment, one which is more physiologically relevant. The presence of immune-responsive microglia and their potential interaction with astrocytes in mixed cell cultures may have facilitated the apoE protein changes detected in the reported experiments.

Similar results in terms of the differential response of pure astrocyte vs. mixed glial cell cultures have been reported in the context of apoE regulation (Stone *et al*., 1997). Mixed glial cell cultures, when treated with 17β-estradiol, were found to display increased levels of apoE

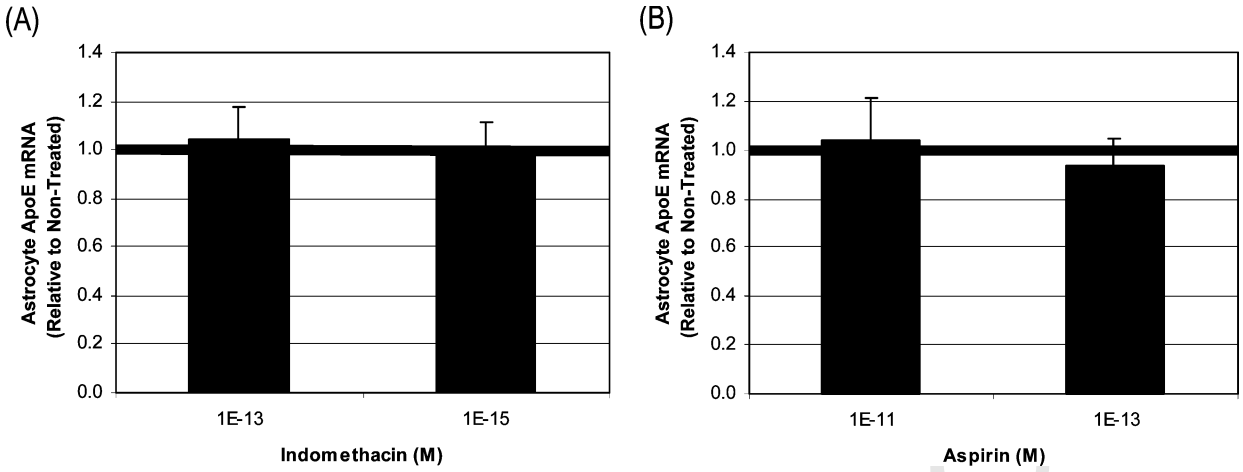

FIG. 3. Mean (+ SEM) rat astrocyte apolipoprotein E (apoE) mRNA as a function of (A) indomethacin and (B) aspirin treatment concentration. All data points were expressed relative to the mean of nontreated (NT) cells. Mean apoE mRNA values following indomethacin and aspirin exposure were derived from an average of $n \geq 12$ and $n \geq 14$ cell culture flasks, respectively. The mean apoE mRNA values of treated cells were then compared via multiple 95% confidence intervals to the arbitrarily set NT population mean of 1.0, as depicted by the solid black line.

mRNA (Stone *et al*., 1997). In contrast, monotypic cell cultures of astrocytes or microglia failed to show any significant response to oestrogen treatment (Stone *et al*., 1997). The authors similarly suggested that heterotypic cell–cell interactions were required to physiologically represent the effect of oestrogen on glial apoE production (Stone *et al*., 1997). Other studies have also demonstrated the need for heterotypic cell cultures in order to show a biological effect. For example, transforming growth factor-β1 treatment of pure astrocytes [Q9] has been found to decrease apolipoprotein J mRNA levels, in contrast to the *in vivo* phenotype. However, the same compound has been found to increase astrocyte apolipoprotein J production when astrocytes were cocultured with microglia and oligodendrocytes (Morgan *et al*., 1995). These studies support our hypothesis that a heterotypic cell culture environment is often required to show the true physiological effect of various compounds.

One must also note that the cell cultures used in the present experiments did not contain any neurons. It has been generally accepted that brain apoE expression occurs primarily in astrocytes and microglia (Poirier *et al*., 1991; Nakai *et al*., 1996; Stone *et al*., 1997). Thus, neurons were excluded from the present experiments in order to focus upon the effect of NSAIDs and their derivatives on primary apoE-producing brain cells. Nevertheless, in humans, apoE mRNA has been localized in selected cerebral cortical and hippocampal CA1–CA4 neurons and large neurons in the frontal lobe (Xu *et al*., 1999). In spite of low-level neuronal localization of apoE mRNA, it has been typically suggested that neuronal apoE is the result of apoE uptake via available apoE receptors (Beffert *et al*., 1999b). Thus, future experiments must consider the potential role of neuronal uptake on extracellular levels of apoE protein, the dependent variable in the present experiments.

Main effects analysis revealed increases in apoE protein levels with longer incubation time in both astrocyte and mixed glial cell cultures. These significant changes may have been the result, at least in part, of increasing cell number with time. In contrast, trends towards decreased apoE protein with time, as demonstrated by treatment with aspirin and APHS, may have been the product of an early peak in drug effect with increases in apoE protein appearing early during treatment and decreasing thereafter. Finally, exposure to indomethacin derivatives resulted in a trend towards decreased protein with time, a trend which may have reflected increasing drug effect with longer incubation time. Indomethacin derivatives generally induced significant decreases in apoE protein relative to nontreated cells; thus, drug effects at later time points may have resulted in the observed main effect of time with lower protein levels seen at 72 and 96 h.

NSAID-mediated neuroprotection in AD may not be exclusively the product of an immune-mediated pathway but rather the product of alternative mechanisms involving apoE

Published evidence suggests that commonly used NSAIDs, including [Q10] indomethacin and aspirin, have the capacity to affect the expression of proteins beyond those involved in inflammation. As such, the results of the current study implicate apoE modulation as a potential mechanism of NSAID neuroprotection in AD. The failure of recent NSAID clinical trials to demonstrate significant quantitative benefits for symptomatic AD patients has called into question the exclusivity of an inflammatory mechanism of NSAID action in AD. To date, the general hypothesis underlying the potential benefits of NSAIDs in AD has been one derived from an inflammatory perspective. The elevated levels of immune cell activation, complement, and cytokines observed in AD naturally led many to hypothesize that inhibition of these inflammatory mechanisms might provide some benefit by reducing chronic inflammation, immune-mediated cell damage and further Aβ pathology (Stewart *et al*., 1997; Kitamura *et al*., 1999).

However, more recent evidence suggests that NSAID neuroprotection may not be solely the result of anti-inflammatory processes but rather a collaboration of effects, not all of which are immune in nature. Epidemiological analysis has revealed that an inverse association between AD and NSAID use exists at both low and high drug dosages (Broe *et al*., 2000). In fact, it has been suggested that the low naproxen dose equivalent of < 500 mg/day used in the study would prove relatively ineffective at suppression of brain inflammation (Broe *et al*., 2000). Thus, the reported inverse association may not be the product of NSAID-mediated immunomodulation but rather the result of an as yet undefined alternative pathway, one which may involve NSAID-mediated effects on apoE regulation.

Furthermore, many studies to date have shown that NSAID use is associated with a protective effect, more so than a treatment effect following diagnosis. Various inconsistencies in NSAID benefit among diagnosed AD patients emphasize the potential importance of NSAID treatment in the period prior to diagnosis (Scharf *et al*., 1999). In fact, recent studies have found the reduction in AD risk to be most prevalent following use of NSAIDs during a critical latent stage of the

disease, prior to the appearance of disease symptoms (Zandi *et al*., 2002). Thus, the inability of NSAIDs to consistently provide cognitive benefit among AD patients may be a function of patient age and severity of the disease process already in progress. Studies indicate that, even in mild AD cases, a loss of neurons of up to 46% can be observed in the CA1 region (Price *et al*., 2001). Consequently, NSAID-mediated anti-inflammatory activity following AD diagnosis might be moot in the light of the cell loss already suffered. In the face of the severity of pathology typically present even upon initial diagnosis of AD, it seems likely that NSAIDs may be best used as preventive agents.

Although COX-1 and COX-2 protein levels significantly increase over time in AD (Pasinetti & Aisen, 1998; Kitamura *et al*., 1999) and may therefore present a progressively larger target for NSAID action, it is unlikely that NSAID inhibition of COX enzyme activity would account for the degree of protection reported. In spite of the fact that some reduction in inflammation is likely due to NSAID-mediated COX inhibition, various other chronic mechanisms may be more than capable of perpetuating brain inflammation. For example, existing cytokines could foster further cytokine and neurotoxin production via chronic activation of astrocytes and microglia. Although NSAIDs may function to alleviate the initiation of chronic AD inflammation during the preclinical phase, it seems unlikely that the degree of observed neuroprotection can be solely attributed to the suppression of relatively minor elevations in immune function prior to diagnosis.

NSAID-mediated neuroprotection may be, in part, the result of apoE-modulated changes in immune function, Aβ clearance and synaptic plasticity

The aforementioned evidence is consistent with an alternative mechanism of NSAID neuroprotection in AD, one that may involve apoE. NSAID-mediated increases in apoE, as demonstrated in the present study, may have implications in immune function, Aβ clearance and synaptic plasticity. Prior studies have established that apoE has the capacity to act as an immunosuppressive modulator via inhibition of glial cytokine secretion (Laskowitz *et al*., 1997), microglial activation (Laskowitz *et al*., 2001) and astrocyte activation (Overmyer *et al*., 1999). Consequently, increased levels of apoE in persons taking NSAIDs could potentially act to temper building levels of immune mediators preclinically, thereby reducing levels of cytokines and immune cell activation and, ultimately, limiting the chronic inflammatory cycle.

Similar apoE involvement in amyloid metabolism has been recently described in studies of mild AD patients treated with the cholesterol-lowering drugs probucol and lovastatin (Poirier & Panisset, 2002; Poirier, 2002). Specifically, in the case of a 6-month clinical trial of probucol, total Aβ levels were found to decline over time in an apoE-concentration-dependent manner whereby AD subjects with the highest induction of CSF apoE also exhibited the most pronounced reduction of CSF Aβ concentration (Poirier, 2002).

Although we postulate that apoE modulation may play a role in NSAID-mediated neuroprotection in AD, recent *in vitro* studies have also implicated NSAIDs in the direct metabolism of Aβ. In fact, studies have demonstrated that various NSAIDs including ibuprofen and indomethacin are capable of (i) reducing production of the $A\beta_{42}$ variant, a vital component of AD plaques (Weggen *et al*., 2001) and (ii) stimulating secretion of the nonamyloidogenic α-secretase form of the soluble amyloid precursor protein (sAPPα) (Avramovich *et al*., 2002). Consequently, it has been suggested that NSAIDs may influence Aβ metabolism and reduce subsequent AD plaque production.

Though it has been proposed that NSAID neuroprotection is the product of immune modulation, the up-regulation of apoE, as seen here, suggests that synaptic plasticity modulation may very well play a role in NSAID action. Previous work has demonstrated that apoE protein and mRNA levels are significantly increased in the regenerative phase that follows entorhinal cortex lesions (Poirier *et al*., 1993b), sciatic nerve crush injuries (Boyles *et al*., 1989) and forebrain ischemia (Ali *et al*., 1996). Furthermore, apoE has been shown to mediate a neurite extension process in an allelic-dependent manner with apoE3 and apoE4 having been proven to promote increases and decreases in neurite branching and extension, respectively (Nathan *et al*., 1994; Bellosta *et al*., 1995). In contrast, the absence of apoE in knockout mice completely prevents reinnervation and synaptic remodelling (Masliah *et al*., 1996; Veinbergs *et al*., 1999). Collectively, the evidence supports the idea that increased levels of apoE during synaptic remodelling following injury or during development may facilitate cholesterol transport for membrane and synapse formation, nerve regeneration and remyelination (Poirier *et al*., 1991; Poirier *et al*., 1995). Consequently, the reduced risk for AD associated with prolonged NSAID use may be, at least in part, the result of NSAID-mediated increases in apoE and, ultimately, a greater capacity for synaptic plasticity. Pre-clinically, heightened apoE levels may (i) provide a greater 'cognitive reserve' with which to face the potential AD neurodegenerative process and (ii) mediate enhanced recovery mechanisms against early neuronal injury.

COX-mediated pathways do not appear to underlie the changes in apoE protein levels

The precise mechanisms underlying NSAID-mediated apoE induction remain unclear. However, in an attempt to analyse the role of COX enzymes in this process, COX-2-selective and inactive NSAID derivatives were utilized. The results indicate that astrocyte treatment with the COX-2-selective aspirin derivative APHS and with its inactive phenol resulted in a significant increase in apoE protein and a trend towards increased protein, respectively. However, the concentrations at which these effects were seen fell well below the IC_{50} value of 0.8 μM for APHS-mediated inhibition of COX-2 (Kalgutkar *et al*., 1998b); consequently, it is unlikely that the apoE induction seen here was the result of COX-2-mediated processes. Moreover, the ability of the inactive APHS phenol compound to affect apoE levels suggests a COX-independent pathway. In the light of an aspirin IC_{50} value of 62.5 μM (Kalgutkar *et al*., 1998b), aspirin-mediated apoE increases at concentrations < 1 nM provide further evidence of an alternative mechanism.

In contrast, COX-2-selective and inactive indomethacin derivatives were found to induce significant decreases in astrocyte and mixed glial apoE protein levels. These decreases were observed over a large range of treatment concentrations, generally within the micromolar–picomolar range. However, the IC_{50} values for these COX-2-selective indomethacin amides have been listed as 0.12 μM and 0.060 μM (Kalgutkar *et al*., 2000), thereby suggesting that the concentrations exhibiting apoE-reducing effects could have involved some COX-inhibitory activity. However, the bulk of the data demonstrating reductions in apoE protein reflected treatment at concentrations far below the micromolar range, indicating the potential involvement of a COX-independent mechanism. Again, such a COX-independent mechanism would be consistent with the significant reductions in apoE levels induced by the inactive indomethacin derivative, as well as the significant increases produced by indomethacin at concentrations far below its own IC_{50} value of 0.75 μM (Kalgutkar *et al*., 2000).

Unlike aspirin, APHS and APHS phenol, the results show that indomethacin and its derivatives induced conflicting increases and decreases in apoE protein levels, respectively. The mechanisms underlying the opposing effects of these compounds may require

specific structural components of indomethacin, components which have been augmented in the COX-2-selective compounds. The substitution or addition of chemical side groups may have, in turn, altered subsequent structural interactions and, ultimately, the nature of the drug's effect on apoE regulation. Thus, the ability of NSAIDs and NSAID derivatives to induce significant changes in apoE expression at physiological and subphysiological concentrations well below their IC_{50} values, as well as the ability of inactive COX inhibitors to significantly affect apoE protein levels, lends to the hypothesis that these compounds mediate their apoE effects via a COX-independent pathway.

NSAID-induced apoE protein level changes may have been the result of nontranscriptional mechanisms

In an attempt to further explore the underlying mechanisms of apoE protein induction, additional quantitative RT-PCR experiments were completed. The failure of indomethacin or aspirin to induce any significant changes in apoE mRNA levels may have been indicative of a nontranscriptional mechanism of apoE protein induction. Increased mRNA stabilization or reduced apoE degradation rather than increased apoE mRNA quantity may have facilitated increased protein levels.

In fact, up-regulation of extracellular apoE protein may have been the product of various other metabolic mechanisms including changes in the rates of apoE recycling, secretion and degradation. For example, upon internalization of apoE via specific receptors and release of cholesterol, it has been observed that apoE may be degraded or resecreted for subsequent action (Fazio *et al*., 1999; Rensen *et al*., 2000; Swift *et al*., 2001). Consequently, elevated levels of extracellular apoE protein may have been the result of enhanced recycling of pre-existing apoE following release of lipid particles, as opposed to *de novo* protein synthesis.

In summary, these results indicate that NSAID neuroprotection in AD may be, at least in part, the product of increased apoE levels and, consequently, enhanced immunosuppression, Aβ clearance and synaptic remodelling. In addition, the failure of the present experiments to demonstrate any significant changes in apoE mRNA levels following NSAID treatment may have been reflective of a nontranscriptional mechanism of apoE protein induction. Nevertheless, these results indicate the need to investigate treatment strategies targeting apoE regulation in individuals at risk for AD, as well as those in the early stages of the disease process.

Acknowledgements

This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada (R.A.), the Canadian Institutes of Health Research (J.P.), Alcan Canada and the Bombardier Foundation. Many thanks to Dr Lawrence Marnett and Brenda Crews, Vanderbilt University School of Medicine, for their generous donation of the COX-2-specific and inactive NSAID derivatives. Our thanks also go to Caroline Petit-Turcotte for her input regarding the immunolabelling protocols, Dr Joseph Rochford, McGill University, for his statistical assistance, and to Drs Jack Antel and Josephine Nalbantoglu, McGill University, for their comments.

Abbreviations

Aβ, amyloid; AD, Alzheimer's disease; APHS, o-(acetoxyphenyl)hept-2-ynyl sulphide; apoE, apolipoprotein E; BSA, bovine serum albumin; COX, cyclooxygenase; CSF, cerebrospinal fluid; C_T, threshold cycle; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; GALC, galactocerebroside; GFAP, glial fibrillary acidic protein; IL, interleukin; NSAIDs, nonsteroidal anti-inflammatory drugs; PBS, phosphate buffer solution; RT-PCR, reverse transcriptase–polymerase chain reaction; TBS-T, tris–base–salt–tween.

[Q11]

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