Non-steroidal anti-inflammatory drugs mediate increased \textit{in vitro} glial expression of apolipoprotein E protein

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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\textsubscript{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 neurofilary tangles, neurofibrillary plaques composed of beta-amyloid (A\beta), dystrophic neurites and cortical atrophy (Cummings \textit{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 \textit{et al.}, 1991; Stone \textit{et al.}, 1997). Unlike in rodents, three separate human apoE isoforms have been identified, namely apoE ε2, ε3 and ε4 (Breslow \textit{et al.}, 1982; Rall \textit{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 \textit{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 \textit{et al.}, 1998; Panza \textit{et al.}, 2003).

Individuals bearing two copies of the apoE ε4 allele have been found to be at significantly greater risk for AD (Strittmatter \textit{et al.}, 1993; Poirier \textit{et al.}, 1993a). In addition, apoE ε4 AD patients have been reported as showing lower levels of apoE in the brain (Bertrand \textit{et al.}, 1995; Beffert \textit{et al.}, 1999a; Glockner \textit{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 \textit{et al.}, 1989; Poirier, 1994; Holtzman & Fagan, 1998).

In recent years, many researchers have focused their efforts on the role of the immune system in AD (Hull \textit{et al.}, 1996; Sheng \textit{et al.}, 1996). Studies have demonstrated high levels of microglial activation and clustering around AD plaques (Haga \textit{et al.}, 1989; Perlmutter \textit{et al.}, 1992; Saitoh \textit{et al.}, 1997), as well as elevated levels of complement proteins, interleukin (IL)-1 and IL-6 in human AD brains (Griffin \textit{et al.}, 1989; Walker & McGeer, 1992; Wood \textit{et al.}, 1993; Wśniiewski \textit{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 \textit{et al.}, 1990; Breitner \textit{et al.}, 1994; Andersen \textit{et al.}, 1995; Breitner \textit{et al.}, 1995). NSAIDs include a variety of drugs whose main effect is to inhibit the enzymes cyclooxygenase-2 (COX-2) (E.C. 1.14.99.1) and cyclooxygenase-2 (COX-2) (Rome & Lands, 1975; Copeland \textit{et al.}, 1994; Kurumbail \textit{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 catastrophic formation in humans via acetylation of lysine residues found on lens proteins, thereby blocking the reaction of lysine with other cathepsin-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 \textit{in vitro} (Laskowitz \textit{et al.}, 1997; Lynch \textit{et al.}, 2001) and \textit{in vivo} (de Bont \textit{et al.}, 2000; Van Oosten \textit{et al.}, 2001), and inflammatory mediators showing significant apoE-regulatory effects (Brand \textit{et al.}, 1993; Duan \textit{et al.}, 1995; Starck \textit{et al.}, 2000). In addition, epidemiological data has revealed that the NSAID neuroprotective effect is stronger in subjects lacking an apoE ε4 allele (Breitner \textit{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 acetysalicylic acid (aspirin) were acquired from Sigma (St Louis, MO, USA). Indomethacin derivatives LM4108, LM4115 and LM4192, as well as aspirin derivatives o-(acetoxyphe)nylhept-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 Amershams 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).

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). 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) (Inmunocorp, Montreal, QC, CAN), 1% penicillin/streptomycin, and 0.1% amphotericin B (fungazone), pH = 7.6]. Cells were subsequently plated in 75-cm² 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-cm² 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 acetysalicylic 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; Giere et al., 1999). Consequently, in order to explore the individual role of each COX enzyme, COX-2-selective derivatives of indomethacin (Kalugtukar et al., 2000) and aspirin (Kalugtukar et al., 1998a) were used to treat primary rat astrocyte and mixed glial cell cultures. Indomethacin aromatic amide and indomethacin phenethyl amide (Kalugtukar 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 (Kalugtukar et al., 2000), characterized by a 4-bromobenzyol 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 (Kalugtukar et al., 1998b), a COX-2-selective inhibitor, and APHS phenol (Kalugtukar 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 10⁻¹³ M. Thus, most of these values fell well below the indomethacin IC_{50} value of 0.75 µM (Kalugtukar 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
In order to assess whether NSAID treatment had transcriptional
apoE protein were between 50 and 2000 ng/mL.

In subsequent experiments, measurements of emitted
were taken using a microplate
assay (Beffert
apoE and
beta-mercaptoethanol and subsequently
extracted RNA samples were quanti-
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| Q4 | Q3 | Q5 |
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 Datavisim, 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 multiasample 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 × concentration interaction effect, confidence intervals were completed across and within time points, respectively.

In order to quantify sample apoE mRNA, a comparative C<sub>T</sub> method (PE Applied Biosystems, 1997; Winer et al., 1999) was utilized for each individual trial. C<sub>T</sub> values derived from amplification with rat β-actin primers were subtracted from the C<sub>T</sub> values derived from amplification of the same samples with rat apoE primers, thereby producing a ΔC<sub>T</sub> value. Consequently, the resulting data were normalized to an endogenous control. Subsequently, the ΔC<sub>T</sub> 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 multiasample 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 untreated cell cultures. All apoE protein levels for each treatment condition were expressed relative to nontreated and treated cells were then assessed using multiasample 95% confidence intervals with the nontreated mean apoE mRNA value set as a population mean of 1.0.

Two-way independent-measures ANOVA of astrocyte data revealed a significant main effect of time (F<sub>2,290</sub> = 4.38, P < 0.05) with simple main effects testing demonstrating mean protein levels to be significantly higher at 72h than at either 48 or 96h, 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 × concentration interaction (F<sub>18,287</sub> = 3.95, P < 0.0001). Subsequent simple main effects analysis revealed significant differences in apoE protein levels between time points of 48, 72 and 96h, within specific concentrations (all F<sub>2,287</sub> ≤ 21.76, P ≤ 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<sup>−17</sup>, 10<sup>−18</sup> and 10<sup>−19</sup>M; all P ≤ 0.05. Specifically, mean apoE protein values at 48 and 72h were significantly lower than those at 96h (P < 0.05). In contrast, a significant reduction in apoE protein with time was detected at a concentration of 10<sup>−14</sup>M with mean protein values at 48h being greater than at 72h: P ≤ 0.01. Confidence interval analysis demonstrated that indomethacin treatment of mixed glia induced significant increases in extracellular apoE protein levels after 96h relative to nontreated cells: all P ≤ 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, LM4108, LM4115 and LM4192, were utilized in the current study. LM4108, an indomethacin amide derivative, and LM4115, an aromatic indomethacin amide derivative, have been characterized as COX-2-selective inhibitors (Kalugutkar et al., 2000). Kinetic analysis has demonstrated that LM4108 behaves as a slow, tight-binding inhibitor with a much slower time course of COX-2 inhibition than indomethacin (Kalugutkar et al., 2000). Furthermore, both LM4108 and LM4115 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 (Kalugutkar et al., 2000). In contrast, LM4192 has been shown to be ineffective as a COX inhibitor and was, thus, used as a negative control in the current experiments (Kalugutkar et al., 2000).

#### Table 1. Percentage change in apolipoprotein E levels across time among treated cell cultures showing a significant main effect of duration of treatment

<table>
<thead>
<tr>
<th>Drug compound</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>96 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Astrocyte</td>
<td>Mixed glia</td>
<td>Astrocyte</td>
<td>Mixed glia</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>N/A</td>
<td>N/A</td>
<td>104.9 ± 4.4</td>
<td>N.S.</td>
</tr>
<tr>
<td>LM4108</td>
<td>105.2 ± 5.2</td>
<td>79.2 ± 2.7</td>
<td>61.9 ± 3.0</td>
<td>75.9 ± 2.7</td>
</tr>
<tr>
<td>LM4115</td>
<td>104.0 ± 7.4</td>
<td>79.7 ± 3.9</td>
<td>83.4 ± 5.0</td>
<td>53.1 ± 4.0</td>
</tr>
<tr>
<td>LM4192</td>
<td>94.5 ± 7.2</td>
<td>94.1 ± 4.5</td>
<td>70.0 ± 4.2</td>
<td>88.2 ± 3.5</td>
</tr>
<tr>
<td>Aspirin</td>
<td>N/A</td>
<td>N/A</td>
<td>113.6 ± 4.3</td>
<td>122.3 ± 4.7</td>
</tr>
</tbody>
</table>

Values are ±SEM. All data points are expressed relative to the arbitrarily set nontreated population mean of 100%. N/A, ??; N.S., ??.
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, $F_{3,205} = 29.39$, $P < 0.0001$; concentration, $F_{6,119} = 2.23$, $P < 0.05$) 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 either 72 or 96 h ($P < 0.05$) and mean levels at 48 h were significantly higher than those at 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 ($F_{6,211} = 6.98$, $P < 0.001$), 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 ($P < 0.05$). 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 ($F_{3,214} = 13.74$, $P < 0.0001$) and aspirin concentration ($F_{6,214} = 4.35$, $P < 0.0001$) 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 $10^{-11} M$ ($P < 0.05$; Fig. 2A).

Following treatment with aspirin, mixed glia showed a significant main effect of drug concentration on extracellular apoE levels ($F_{6,215} = 6.35$, $P < 0.0001$). 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-acetoxyphenylhept-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 $\approx 60$ 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 $\times$ APHS concentration interaction effect was observed ($F_{18,129} = 3.34$, $P < 0.0001$). 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
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.
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 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 (Poitier 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 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 NSAID use and AD pathology (Scharf et al., 1999). 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 result 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 pravastatin and lovastatin (Poirier & Panisset, 2002; Poirier, 2002). Specifically, in the case of a 6-month clinical trial of pravastatin, 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 Aβ42 variant, a vital component of AD plaques (Weggen et al., 2001) and (ii) stimulating secretion of the nonamyloidogenic 6-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), sciatric 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 allele-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 remodeling (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 (Kalugtak 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 (Kalugtak 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 (Kalugtak 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 (Kalugtak 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\textsubscript{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 rescерreted 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

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Abbreviations

Aβ, amyloid; AD, Alzheimer’s disease; APHS, o-(acetoxyphenyl)hept-2-yl sulphone; apoE, apolipoprotein E; BSA, bovine serum albumin; COX, cyclooxygenase; CSF, cerebrospinal fluid; C\textsubscript{p}, threshold cycle; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; GALT, 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.

References


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