THE CHOLESTEROL-LOWERING DRUG PROBUCOL INCREASES APOLIPOPROTEIN E PRODUCTION IN THE HIPPOCAMPUS OF AGED RATS: IMPLICATIONS FOR ALZHEIMER’S DISEASE

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The Abbreviations: AD, Alzheimer’s disease; apoE, apolipoprotein E; apoEKO, apolipoprotein E knockout; CSF, cerebrospinal fluid; GFAP, glial fibrillary acidic protein; HMGCoAr, 3-hydroxy-3-methylglutaryl coenzyme A reductase; LDLr, low density lipoprotein receptor; LRP, LDLr-related protein; OD, optical density; PFA, paraformaldehyde; PNS, peripheral nervous system; RT-PCR, reverse transcriptase-polymerase chain reaction; SNAP-25, synaptosomal associated protein of 25 kDa.

Abstract—Several recent epidemiological studies have proposed that cholesterol-lowering drug Statin may provide protection against Alzheimer’s disease (AD). Probucol is a non-Statin cholesterol-lowering drug and a potent inducer of apolipoprotein E (apoE) production in peripheral circulation. A recent clinical study using Probucol in elderly AD subjects revealed a concomitant stabilisation of cognitive symptoms and significant increases in apoE levels in the cerebral spinal fluid in these patients. To gain insight into the mechanisms underlying these effects, we treated a cohort of aged male rats (26-month-old) with oral dose of Probucol for 30 days.

Specifically, we examined the effects of Probucol on apoE production and its receptors (low density lipoprotein receptor [LDLr] and low density lipoprotein receptor-related protein [LRP]), astroglial marker of cell damage (glial fibrillary acidic protein [GFAP]), markers of neuronal synaptic plasticity and integrity (synaptosomal associated protein of 25 kDa [SNAP-25] and synaptophysin) as well as cholesterol biosynthesis (3-hydroxy-3-methylglutaryl coenzyme A reductase [HMGCoAr]) in the hippocampus. We report that Probucol induces the production of apoE and one of its main receptors, LRP, increases HMGCoAr (rate-limiting enzyme in cholesterol synthesis), substantially attenuates age-related increases in glial activation, and induces production of synaptic marker SNAP-25, a molecule commonly associated with synaptogenesis and dendritic remodeling.

These findings suggest that Probucol could promote neural and synaptic plasticity to counteract the synaptic deterioration associated with brain aging through an apoE/LRP-mediated system. Consistent with the beneficial effects of other cholesterol-lowering drugs such as the Statin, Probucol could also offer additional benefits based on apoE neurobiology. © 2003 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: astrocytes, Statin, synaptic plasticity, glial fibrillary acidic protein, LDLr receptor family, 3-hydroxy-3-methylglutaryl coenzyme A reductase.

Increasing interest in cholesterol-lowering drugs has been prompted by recent studies reporting that Statin may provide protection against Alzheimer’s disease (AD; Jick et al., 2000; Wolozin et al., 2000). A recent clinical study using Probucol, another well-known cholesterol-lowering drug, in elderly AD subjects revealed a concomitant stabilisation of cognitive symptoms and significant increases in apolipoprotein E (apoE) levels in the cerebrospinal fluid (CSF) in these patients. Probucol was also shown to influence the production of specific class of apolipoproteins in blood circulation as well as in certain organs. For instance, Probucol increased the production of apoE mRNA levels in the spleen and brain in rabbits (Aburutani et al., 1988) and apoE protein levels in cultured type 1 astrocytes (Poirier and Panisset, 2002b). Plasma (McPherson et al., 1991) apoE levels were also reported to increase following to Probucol treatment in human whilst other studies reported either decreases (Takegoshi et al., 1992) or no changes (Homma et al., 1991).

ApoE mediates the transport and distribution of cholesterol in the entire body, including the CNS (Mahley, 1988), and plays a key role in repair and plasticity in both peripheral nervous system (PNS; Boyles et al., 1990; Ignatius et al., 1986) and CNS (Pasinetti et al., 1993; Poirier, 1994; Poirier et al., 1991a). In humans, the apoE gene exists in three major alleles, i.e. ε2, ε3 and ε4 and three corresponding isofoms, i.e. E2, E3, and E4 (Davigon et al., 1988a). ApoE ε4 allele has been demonstrated to be associated with an increased risk of developing both sporadic (Poirier et al., 1993b; Saunders et al., 1993) and familial (Corder et al., 1993; Saunders et al., 1993) forms of AD. The mechanisms by which apoE ε4 exerts its detrimental effect are still unknown, but it appears that apoE levels may play a role in the pathology of AD. Although controversial, a majority of studies reported decreases in apoE levels in the CSF of AD patients (Blennow et al., 1994; Hesse et al., 2000; Kunicki et al., 1998; Landen et al., 1996; Lehtimaki et al., 1995; Skoog et al., 1997; Song et al., 1997), whilst few other found increases (Carlsson et al., 1991; Diedrich et al., 1991; Fukuyama et al., 2000; Song et al., 1997) or no changes (Lefranc et al., 1996; Rosler et al., 1996). Interestingly, whilst no obvious association between levels of apoE in CSF and apoE genotype were found, other studies investigating apoE levels in brain
tissue (mostly hippocampus) reported an inverse correlation between hippocampal apoE mRNA and protein levels and ε4 allele number in AD patients (i.e. apoE mRNA and protein levels decreases and ε4 allele copy number increases; Beffert et al., 1999; Bertrand et al., 1995; Glockner et al., 2002; Ramassamy et al., 2000), whilst few others reported either a decrease but no effect of apoE polymorphism (Hesse et al., 1999) or rises in apoE levels with increasing number of ε4 alleles (Yamada et al., 1995; Yamagata et al., 2001).

Despite discrepancies, it is noteworthy that most of the studies indicate that brain tissue levels of apoE are reduced in AD, and that apoE levels may be especially compromised in the hippocampus of ε4 carriers. These findings are in agreement with observations of: 1) more severe synaptic loss and increased neuropathological alterations (senile plaques and neurofibrillary tangles) in the hippocampal formation of ε4 carriers suffering from AD (Arendt et al., 1997; Miller et al., 1994), and 2) impaired plasticity and poor recovery displayed by apoE knockout (apoEKO) mice in response to several types of brain injuries (Chapman et al., 2000; Chen et al., 1997; Laskowitz et al., 1997b; Masliah et al., 1995a; Masliah et al., 1995b) reinforcing the hypothesis that improper (low) levels of apoE may severely compromise neuronal/synaptic integrity in AD (Poirier and Panisset, 2002b).

Increasing endogenous apoE levels in the CNS through pharmacological means may represent a way to enhance synaptic plasticity (e.g. synaptogenesis and terminal remoulding) and attenuate age- or disease-related alterations in vulnerable brain areas displaying substantial reduction in apoE levels in AD, such as the hippocampus and cortical areas (Beffert et al., 1999; Bertrand et al., 1995; Glockner et al., 2002). Furthermore, the eventuality of using apoE as a therapeutic target in the treatment of AD is of interest given the close relationship between apoE, cholesterol metabolism, synaptic plasticity and astroglia. First, both synaptic loss and cholesterol reduction (especially the hippocampus and frontal cortex) are prominent features of aging and are particularly accentuated in AD (Hoff et al., 1981; Poirier, 2002a; Scheff et al., 1980). Specifically, we examined the effect of Probucol on synaptic markers (synaptophysin and synaptosomal associated protein of 25 kDa [SNAP-25]), cholesterol content and biosynthesis (3-hydroxy-3-methylglutaryl coenzyme A reductase [HMGCoAr]), and astrocyte activation using GFAP, a marker of reactive astrocytes (Eng, 1985). We also examined the effects of Probucol on two key apoE receptors, namely the low density lipoprotein receptor (LDLr) and the LDLr-related protein (LRP), which have been shown to be central to apoE function in the CNS (Beffert et al., 1998; Herz, 2001; Herz and Beffert, 2000).

**EXPERIMENTAL PROCEDURES**

**Animals and Probucol administration**

Males Long-Evans rats were purchased from Charles River, St-Constant, Quebec, Canada. All animals were housed two per cage, had ad libitum access to food and water, and were maintained on a 12-h light/dark cycle (lights on at 7:00 a.m.) with conditions of constant humidity and temperature (22 °C). Twenty-six-month-old rats (total n=28; average weight=550 g) were used. Probucol was dissolved in chloroform and was evenly sprayed on standard rat chow (Aburatani et al., 1988). Chloroform was allowed to evaporate from the food pellets under a fume hood for a period of 72 h. Animals were either fed with Probucol- (1% weight/weight; n=14) or chloroform-treated chow (n=14) for 30 consecutive days. Based on studies using the Watanabe heritable hyperlipidemic rabbits and rodents, this particular dose was shown to yield plasmatic concentrations ranging from 43 to 58 μg/ml, which is consistent with the circulating levels of Probucol measured in humans receiving a standard oral dose of 1 g of Probucol per day (Aburatani et al., 1988; Davignon, 1994; Kita et al., 1988; Shankar et al., 1989).

At the end of the treatment, one half of each of the two groups of rats were killed and their brain and plasma were collected, frozen and stored at −80 °C and −20 °C, respectively. The other half were anaesthetized and transcardically perfused with a solution of paraformaldehyde (PFA) 4% 0.1 M sodium phosphate, pH 7.4, for 20–30 min. Brains were removed and cryoprotected overnight in a 30% sucrose/PFA 4% 0.1 M sodium phosphate solution, pH 7.4. All efforts were made to minimize the number of animals required and their suffering. All procedures were carried out in accordance with the Canadian Guidelines for Use and Care of
The real-time RT-PCR method consists of two steps, i.e. RT (first step) followed by the PCR (second step; Morrison et al., 1998; Powell and Kroon, 1992). The RT was performed in the PE Biosystems GeneAmp 5700 sequence detection system. The reverse transcription of RNA was performed in a final volume of 100 μl. Each reaction contained 10 μl of 10× RT buffer, 20 μl of deoxyNTPs mixture (500 μM each dNTP), 22 μl of 25 mM MgCl₂, 2 μl of Rnase inhibitor, 2.5 μl of MultiScribe Reverse Transcriptase (50 U/μl), 5 μl of random hexamers, and 2 μg of total RNA, and Rnase-Free water to 100 μl. The RT reactions were performed in a microAmp optical tubes. The samples (n=7 hippocampus per group) were incubated at 25°C for 10 min and 48°C for 30 min, and the reverse transcriptase was inactivated by heating at 95°C for 5 min. The PCR was performed in the PE Biosystems GeneAmp 5700 sequence detection system using SYBR green 1 PCR Kit, in triplicate in a final volume of 35 μl reaction. Each reaction contained 17.5 μl of the 10× SYBR green PCR Master Mix (200 nM dATP, dGTP, and dCTP; 400 nM dUTP; N 100 l), 5 μl of the cDNA; and Rnase-Free water to 35 l. The reactions were performed in MicroAmp 96-well plated capped with Micro-Amp optical caps. The reactions were incubated at 50°C for 2 min, 10 min at 95°C followed by 40 cycles of 15 min at 95°C and 1 min at 60°C. Dissociation curves were used to verify non-specific amplification products.

Using Primer Express software (PE Biosystems), primers were designed to recognise rat apoE, LDLr, HMGCoAr and β-actin mRNA sequences. Primers for each gene target were selected according to the manufacturer’s guidelines as previously described (Livak, 1999). PCR primers were synthesised and purified by the McGill University Sheldon Biotechnology Centre (Montreal, Canada). The sequence of the PCR forward and reverse primers, amplicon size and gene bank references are listed in Table 1.

### Table 1. Primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Amplicon size (pb)</th>
<th>Gene bank reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoE</td>
<td>5'-CCATCTGGCCTCCACACAGT-3'</td>
<td>5'-GGGGCTAGGTTAGGAGGTA-3'</td>
<td>56</td>
<td>J00705</td>
</tr>
<tr>
<td>LDLr</td>
<td>5'-CAAGGAGTGCAAGGCAAGCGA-3'</td>
<td>5'-TGGGAACAGCCACCATGTGTA-3'</td>
<td>51</td>
<td>X13722</td>
</tr>
<tr>
<td>HMG CoAr</td>
<td>5'-GCTGATCCGGACATCCTGCTAAC-3'</td>
<td>5'-GCTGACGGACAGGTTCGAGAA-3'</td>
<td>51</td>
<td>NM 013134</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5'-GGCCTGGGACCTGACAGACTAC-3'</td>
<td>5'-CCTCTCAGCTGCTGTTGTA-3'</td>
<td>74</td>
<td>NM 031144</td>
</tr>
</tbody>
</table>

Sequential for forward and reverse primers along with the amplicon size and gene bank references used to target rat apoE, LDLr, HMGCoAr and β-actin mRNAs are listed. Primers were designed according to the manufacturer’s guidelines using Primer Express software.

### Western blots

Hippocampi (n=7 per group) were homogenised in phosphate buffer (10 mM KPO₄/10 mM KCl, pH 7.4) and protein concentration was determined using the Bradford method (BCA protein assay reagent; Pierce, IL, USA). A quantity of 25 μg of hippocampal tissue per sample was loaded and electrophoresed on a 4–12% Bis–Tris NU-PAGE gel (Invitrogen, Carlsbad, CA, USA) at 125 V for 90 min in Tris–glycine running buffer (16.5 mM Tris, 0.135 M glycine, 0.1% SDS; pH 8.3), and transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) at 25 V for 2 h. The nitrocellulose membranes were incubated in a blocking solution (TBS, pH 7.5 with 5% skim milk and 0.1% Tween-20), for 60 min. The blots were then incubated 60 min at room temperature with appropriate dilutions of the primary antibody (1:500 for apoE; rabbit; Chemicon, Temecula, CA, USA), 1:10,000 for GFAP (rabbit; Dako, Mississauga, Ontario, Canada), 1:2500 for LDLr (mouse; Amersham Pharmacia Biotech, UK), 1:2500 for SNAP-25 (rabbit; Calbiochem, San Diego, CA, USA), and 1:1000 for synaptophysin (mouse; Sigma, St. Louis, MO, USA). The blots were washed in TBS buffer and then incubated for 60 min at room temperature with the appropriate secondary antibodies (anti-rabbit (1:2000) and anti-mouse (1:3000; Calbiochem Novabiochem, LaJolla, CA, USA)). The blots were developed using Western blot Enhanced Luminol Reagent (ECL) kit (NEN Life Sciences Products, Boston, USA) and exposed to X-ray film. The films were developed and the intensity of the immunoreactive labelling was analysed using a computerised image analysis system (MCID-4, Imaging Research, St-Catharines, Ontario, Canada). The results were expressed as relative optical density. To control the amount of protein loaded on the gel, the optical density measures obtained for each sample were compared with that of α-tubulin (Wagner et al., 1992). Each blot was stripped (in 62.5 mM Tris, 2% SDS and 100 mM β-mercaptoethanol (pH 6.7) for 30 min at 50°C) and re-probed with an anti-α-tubulin antibody (1:1000 mouse; Biode-sign International, Kennebunk, ME, USA). All Western blot experiments were run in duplicate and repeated twice independently. Optical density (OD) measurements were made according to the procedures described above. The values for each sample were expressed as the percentage of OD obtained using α-tubulin. The results were analysed using unpaired t-test analysis. For all tests probability level of 5% was used as the minimal criterion of significance (Winer, 1971).

### RNA isolation and RT-PCR analysis

Total RNA was isolated from brain tissues (25–30 mg/sample; n=7 hippocampus chunks per group) using the Rneasy Mini kit (Qiagen, Ontario, Canada), according to the manufacturer’s guidelines. Briefly, tissue was disrupted in buffer until a completely homogeneous lysate was obtained. The lysate was centrifuged and washed several times. In the final step, the lysate was eluted with 52 μl of Rnase-free water for 5 min and centrifuged (1 min). The concentration of each sample was estimated from A₂₆₀ measurements.

We used the comparative Cₚ method using arithmetic formulas to achieve results that are similar to those generated by the standard curve method (Biosystems, 1997; Pfaffl, 2001). The results were expressed as the fold change in the target (apoE, LDLr and HMGCoAr) relative to housekeeping (β-actin) genes (Schmittgen et al., 2000; Winer et al., 1999).

### Lipid extraction and cholesterol measurement

The experimental protocol used for lipid extraction and measurement of cholesterol in the brain was adapted from the technique of Svennerholm et al. (1991). Briefly, samples of 25–30 mg (n=7 hippocampus chunks per group) were mortar-ground on a cold plate (−20°C). Mortar-ground tissue was weighed and trans-
ferred to clean tubes. Tissue was suspended in 150 μl of H₂O using a vortex homogeniser. Five hundred microlitres of methanol and 250 μl of chloroform were added to each sample, which were gently shaken with a rotary shaker for 15 min and then centrifuged (5000 r.p.m.) for 10 min. The supernatants were transferred to new tubes and the residual pellets were kept for a second lipid extraction following the same protocol. The supernatants obtained from the second extraction were combined with those obtained during the first extraction. The supernatants were then allowed to evaporate (approximately 2 h) until dryness, using a speed vac. The pellets were suspended in a mixture containing 10 ml/l of Triton X-100, 6 ml/l Brij 96 and 0.1 M/l of HCl in isotonic NaCl. The samples were shaken with a rotary shaker overnight, until complete suspension. Total and free cholesterol from plasma and hippocampus was measured with appropriate in vitro enzymatic colorimetric method, according to the manufacturer’s protocol (Wako Chemicals USA, Richmond, VA, USA). Total and free cholesterol in each sample was determined by measurement of the absorbance at A₆₀₀ nm. Amount of cholesterol ester was derived from the following formula: Amount of cholesterol ester=[total cholesterol amount]–[free cholesterol amount].

Immunohistochemistry

Rats were perfused transcardiatically (n=7 rats per group), brains were collected and sliced with a freezing cryostat. Free-floating 25 μm sections were rinsed in PBS and incubated for 30 min in blocking solution (1% bovine serum albumin, 0.4% Triton X-100 and 4% normal goat serum). Sections were incubated overnight at 4 °C with antibodies against GFAP (astroglial marker; 1:5000; Sigma, St. Louis, MO, USA; Poirier et al., 1991). Immunoreactivity for GFAP appeared as a distinctive brown reaction product. Qualitative analysis of GFAP-positive cells on hippocampal sections was performed under high power bright field microscopy (Nikon, Mississauga, Ontario, Canada).

RESULTS

Body weight

Before the beginning of the experiment, rats were randomly allocated to either the control (n=14) or Probucol (n=14) groups, balancing the groups according to body weight. Body weight measurements were collected at the beginning and the end of the experiment. No significant differences were observed between body weight measurements taken at the beginning (672.73±35.06 versus 700.0±28.09) and the end (594.09±27.80 versus 618.33±28.67) of the experiment for control and Probucol-treated animals, respectively indicating that Probucol did not alter food intake.

Effect of Probucol on apoE production

The results obtained after 30 days revealed significant changes in apoE protein levels, with a marked increase in the hippocampus of Probucol-treated relative to control rats (P=0.0036; Figs. 1A and 2). ApoE mRNA level did not change in response to Probucol treatment (Fig. 1B).

Effects of Probucol on markers of cholesterol metabolism

Using hippocampi, we next examined the effect of Probucol treatment on other elements of brain cholesterol metabolism, namely cholesterol synthesis via its rate limiting step enzyme (HMGCoAr), and cholesterol internalization via two key receptors for apoE, i.e. LDLr and LRP (Figs. 2 and 3A). The results revealed a significant increase in protein levels of HMGCoAr (P<0.0001) in Probucol-treated animal as compared with control rats. Whilst no significant changes were detected in LDLr levels in Probucol-treated animals as compared with controls, Probucol treatment produced a significant increase in LRP protein levels (P=0.0004), paralleling our findings with HMGCoAr (Figs. 2 and 3A). No significant changes in either HMGCoAr or LDLr mRNA levels were observed in response to Probucol treatment, suggesting that observed changes in HMGCoAr protein levels occurred at the post-transcriptional level (Fig. 3B).

Effect of Probucol chronic treatment on synaptic markers

In order to determine the effect of Probucol administration on synaptic integrity, we measured protein levels of two
synaptic markers, i.e. synaptophysin, and SNAP-25 (Figs. 2 and 4). Both synaptic markers are closely associated with synaptic vesicles and participate in neurotransmission (Sollner and Rothman, 1994). The results showed a significant increase in SNAP-25 protein levels ($P=0.0092$) in the Probucol-treated animals relative to control rats. Synaptophysin protein levels also appeared to increase in Probucol-treated rats relative to controls, but this difference did not reach statistical significance.

**Effect of Probucol on astroglial reactivity**

Hippocampal GFAP protein levels and GFAP immunoreactivity (ir) were examined in sections of the hippocampus. Probucol produced a small, but significant decrease in GFAP protein levels relative to control rats ($P=0.041$; Figs. 2 and 5). Qualitative analysis of GFAP-ir cells in hippocampal sections revealed that Probucol treatment caused a substantial decrease in both the number of GFAP-ir cells and intensity of GFAP-ir in the molecular layer of the dentate gyrus of the hippocampus (Fig. 6B) as compared with controls (Fig. 6A). These changes in GFAP-ir were consistent with the observed decrease in GFAP protein levels in the hippocampus of Probucol-treated rats (Fig. 5).

**Effect of Probucol on hippocampal cholesterol levels**

Probucol is a potent cholesterol-lowering drug, at least in periphery. We examined the effect of Probucol on cholesterol levels in hippocampal tissue. There were no significant changes in total cholesterol, free cholesterol or cholesterol ester levels between control and Probucol-treated animals (Fig. 7).

**DISCUSSION**

The results of the present study show that chronic oral Probucol administration is associated with significant increases in hippocampal apoE levels. The increase is paralleled by an increase in the LRP protein concentration and HMGCoAr levels (rate-limiting enzyme in cholesterol synthesis). These effects were accompanied by a corresponding increase in synaptic marker SNAP-25,
and concomitant reduction of astroglial marker GFAP. These results are consistent with a proof-of-principle study that used Probucol to increase apoE levels in the CSF of mild-to-moderate AD elderly subjects (Poirier and Panisset, 2002b), and are discussed in turn in the following sections.

**Probucol increases ApoE production and receptor expression**

Concomitant increases in apoE and its receptors have been documented in response to PNS and CNS injuries (Boyles et al., 1990; Poirier, 1994; Poirier et al., 1993a), and are regarded as playing a key role in cholesterol transport and recycling during synaptic remodeling. It has been proposed that under situations of repair or ongoing neuronal remodeling, apoE–cholesterol–lipoprotein complexes are formed, released from astrocytes and targeted to cells undergoing membrane biosynthesis, dendritic sprouting and synaptogenesis through apoE receptor mediated endocytosis (Mauch et al., 2001; Poirier, 1994; Poirier et al., 1991). Our findings are consistent with this idea and suggest that the newly synthesised apoE molecules might form apoE–cholesterol–lipoprotein complexes that are internalised into cells in need of cholesterol via apoE receptor family, particularly through LRP. Although no changes in low density lipoprotein (LDL) receptor levels were detected following Probucol treatment, the possibility that LDLr are involved in these processes cannot be excluded. Entorhinal cortex lesions study showed an increase in neuronal LDLr binding activity that coincided with

![Figure 4](image1.png)

**Fig. 4.** Effect of Probucol on synaptic markers. Quantification of synaptophysin and SNAP-25 protein levels as measured by relative OD from Western blot analysis using hippocampal tissue. Note the significant increase in SNAP-25 in the Probucol-treated rats (n=7) relative to their age-matched controls (n=7) (P<0.01). A trend toward an increase in synaptophysin was observed in Probucol-treated animals, but did not reach statistical significance. (**P<0.01).**

![Figure 5](image2.png)

**Fig. 5.** Effect of Probucol on GFAP protein levels. Quantification of GFAP protein levels as measured by relative OD from Western blot analysis using hippocampal tissue. Note the significant decrease in GFAP levels in the Probucol-treated rats (n=7) relative to their age-matched controls (n=7; P<0.05). (*P<0.05).
the onset of reactive synaptogenesis in the granule cell layer of the dentate gyrus (Poirier et al., 1993a).

The time course as well as the nature of apoE induction is unclear and requires further studies. No significant changes were observed in apoE mRNA in response to Probucol, suggesting that changes observed in apoE protein levels resulted from post-translational regulatory processes. However, the possibility that Probucol may have initially affected apoE mRNA expression is plausible, since we observed a concomitant increase in apoE mRNA paralleled by an increase in apoE protein levels in rat type 1 astrocytes in culture shortly after probucol exposure (usually within 24–48 h; data not shown).

Probucol promotes dendritic growth/remodeling and synaptogenesis in aged rats

In addition to the concurrent increase in brain apoE and LRP seen in the Probucol-treated animals, we observed an increase in SNAP-25 protein levels, a membrane-associated protein, mainly localised in nerve terminals (Sollner and Rothman, 1994). In addition to its role in synaptic vesicle exocytosis, SNAP-25 has been shown to participate in constitutive axonal growth and dendritic formation in hippocampal neurons (Grosse et al., 1999). Blockage of SNAP-25 with specific toxin results in inhibition of axonal and dendritic growth (Grosse et al., 1999). Based on these facts and previous work by our group, it is conceivable that apoE–cholesterol–lipoprotein complexes are internalised through cell–surface LDLr and/or LRP present on the cell body and dendrites of reinnervating neurons. Both LDLr and LRP are expressed on neurons, but LRP appears to be particularly abundant in the somatodendritic portion of neurons (Beffert et al., 1998; Bu et al., 1994; Ishiguro et al., 1995). Our findings of simultaneous increases in apoE, LRP and SNAP-25 in response to Probucol treatment are in agreement with the contention that Probucol may pro-
mote plasticity in the hippocampus of aged rats by increasing synaptogenesis and/or dendritic growth/remodeling via apoE-LRP interactions, a well-described system known to play a major role in neurite outgrowth of CNS neurons (Fagan et al., 1996; Holtzman et al., 1995; Narita et al., 1997; Nathan et al., 2002; Poirier, 2002a; Poirier and Panisset, 2002b).

**Cholesterol level and biosynthesis**

We observed no significant changes in hippocampal levels of cholesterol in response to Probucol treatment. This effect is in contrast with a recent study showing a decrease in cholesterol levels in the CNS in response to supraphysiological levels (100 mg/kg/day) of Statin in female apoEKO mice (Eckert et al., 2001). Several differences including gender, species, lack of apoE, dose concentration and method of administration (oral gavage versus oral ingestion of Probucol-treated food, in the study by Eckert et al., 2001 and the present report, respectively) may account to explain the discrepant result. It is also possible that this divergence may be related to the mechanism of action of the two cholesterol-lowering drugs. Statins are well-known inhibitors of HMGCoAr (rate-limiting enzyme in cholesterol biosynthesis; Meier et al., 2000; Singer et al., 1984), which accounts for explaining the reported reduction in cholesterol levels in the brain. On the other hand, the mechanism by which Probucol lowers peripheral cholesterol does not appear to proceed through inhibition of HMGCoAr (Goldstein and Brown, 1990). Rather, Probucol cholesterol-lowering effects appear to be achieved through multiple ways, including apoE and apoA1 inductions. For instance, it has been suggested that Probucol enhances reverse cholesterol transport via an apoE-dependent mechanism that accelerates the movement of lipids between lipoproteins, and increases LDL catabolism (Beynen, 1986; Pfuetze and Dujovne, 2000; Tawara et al., 1986). Furthermore, the fact that cholesterol turnover in the rat brain is very slow (4–6 months half-life), it may have been impossible to detect changes in cholesterol levels resulting from treatment with Probucol within the time range (30 days) used in the present study.

Increase in HMGCoAr protein level in response to Probucol was observed in the present study. Similar increase in HMGCoAr protein level have also been reported to occur, as an adaptive reaction, in response to Statin treatment (Brown et al., 1978; Conde et al., 1999; Nakashishi et al., 1988). However, this increase in HMGCoAr protein is kept inactive by the local Statin concentration (HMGCoAr inhibitor; Bucher et al., 1960; Meier et al., 2000). Since no significant changes were observed in HMGCoAr mRNA in response to Probucol, post-translational regulation of HMGCoAr appears to underlie the increase in protein level. In the present study, it is assumed that the newly synthesised HMGCoAr molecules are fully active, since Probucol does not inhibit this enzyme (Davignon et al., 1988b; Pfuetze and Dujovne, 2000). However, we cannot exclude the possibility that the degradation rate of HMGCoAr protein through elements of the mevalonate pathway or via phosphorylation may also be influenced by Probucol (Gardner and Hampton, 1999).

The increased HMGCoAr protein levels may also reflect changes in intracellular cholesterol availability, synthesis or metabolism in specific cellular compartments. Alternatively, it may represent an increase in de novo cholesterol synthesis by reinnervating neurons (Bucher et al., 1960; Goldstein and Brown, 1990; Ness and Chambers, 2000), which would be consistent with synaptogenesis and dendritic remodeling, both relying heavily on cholesterol availability (Mauch et al., 2001; Poirier et al., 1993a).

**Probucol attenuates glial reactivity associated with aging**

Well-characterised changes occur in glia during aging and age-related disorders such as AD. Particularly, astrocytes hypertrophy, increase in number and display enhanced GFAP expression (Landfield et al., 1977; Morgan et al., 1999; Nichols et al., 1995). GFAP is the major intermediate filament protein of astrocytes and is used as a specific marker for reactive astrocytes (Eng, 1985). Age-related increases in GFAP are often seen in parallel with synaptic loss and neuronal death (Gordon et al., 1997). Changes in glial reactivity due to aging are widespread throughout the brain but also region-specific. For instance, the molecular layer of the dentate gyrus along with the hippocampal fields CA1 and CA3 of the hippocampus are particularly vulnerable to aging and glial reactivity (Day et al., 1993; Hansen et al., 1987; Pilegaard and Ladefoged, 1996).

Probucol chronic treatment seems to have attenuated the age-related increase in hippocampal glial reactivity normally seen in 26-month-old animals, as shown by the substantial decreases in GFAP-ir and protein levels in treated animals. However, since astrocytes are the major source of apoE, it may seem paradoxical that whilst apoE levels are up-regulated, the levels of the astrocytic marker GFAP (protein and ir) are down-regulated in response to Probucol treatment. Although concurrent increases in both apoE and GFAP expression are observed in the context of a variety of acute brain injuries (Laskowitz et al., 1998; Poirier et al., 1991), the regulation of these astrocytic genes was shown to differ in other contexts. For instance during aging, opposite direction of changes for apoE (decreases) and GFAP (increases) are observed in the hilus of the dentate gyrus (Morgan et al., 1999). Interestingly, this aged-related increase in GFAP levels was shown to be attenuated in response to caloric restriction regimen whereas apoE levels were found to be further decreased (Morgan et al., 1999).

The mechanism by which Probucol increases apoE and decreases GFAP is unknown and whether the consequences are harmful or beneficial still remain unclear. However, several studies reporting a similar phenomenon suggested that these changes optimise brain functions, and therefore may be viewed as beneficial (Morgan et al., 1999; Nichols et al., 1995; Stone et al., 1997, 1998). For example, oestrogen which was shown to promote synaptic plasticity and dendritic remodeling (Rozovsky et al., 2002;
CONCLUSION

In the present study, we report that Probucol, a well-known cholesterol-lowering drug that does not belong to the Statin family, is capable of inducing the production of apoE and its receptor LRP, increase HMGCoAr (perhaps reflecting cholesterol de novo synthesis in discrete compartment of the astrocytes and/or neurons), substantially attenuates age-related increases in astroglial activation, and induces the expression of synaptic markers normally associated with synaptogenesis and dendritic remodeling. These findings support our hypothesis that Probucol could promote neural and synaptic plasticity to counteract the synaptic deterioration associated brain aging through an apoE/LRP-mediated system. Consistent with the beneficial effects of other cholesterol-lowering drugs such as Statin, Probucol also appears to offer some therapeutic benefits based on apoE neurobiology.

Acknowledgements—This work was supported by the Canadian Institute of Health Research (CIHR), the Alzheimer Society of Canada, and Alcan Corporation. D. Champagne is the recipient of CIHR Doctoral Award. J. Rochford is supported by a Chercheur Boursier (senior) award from the Fonds pour la Recherche en Sante du Quebec, and J. Poirier is supported by a Scientist Award from CIHR. We also wish to thank the J. A. Bombardier and the Birk Foundation for their support.

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Arendt T, Schindler C, Krzywkowski P, Ramassamy C, Berrada F, Poirier J (2001) have been demonstrated to exert anti-inflammatory and anti-oxidant actions. It is thusly possible that the direct action of Probucol, or indirectly via apoE pathway, may have reduced pro-inflammatory and/or pro-oxidant processes that are normally present in aging brain.

Stone et al., 2000; Teter et al., 1999), acts as a potent inducer of apoE in the brain (Stone et al., 1997) and markedly reduces GFAP expression in the aging brain and/or in response to injury (Garcia-Estrada et al., 1993; Rozovsky et al., 2002; Stone et al., 2000). Furthermore, it is likely that Probucol and/or apoE may have contributed directly to this phenomenon. Both Probucol (Dujovne et al., 1994; Pfuetze and Dujovne, 2000) and apoE (LaDu et al., 1998; Lynch et al., 2001) have been published 7700 sequence detection system. User bulletin No. 2. Foster City, CA: PE Applied Biosystems.
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(Accepted 30 April 2003)