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An investigation into the mechanisms mediating plasma lipoprotein-potentiated β-amyloid fibrillogenesis

Lee Stanyer, D. John Betteridge, Christopher C.T. Smith*

Department of Medicine, Royal Free and University College Medical School, Sir Jules Thorn Institute, The Middlesex Hospital, Mortimer Street, London W1N 8AA, UK

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Abstract The toxicity of the β-amyloid (Aβ) peptide of Alzheimer's disease may relate to its polymerisation state (i.e. fibril content). We have shown previously that plasma lipoproteins, particularly when oxidised, greatly enhance Aβ polymerisation. In the present study the nature of the interactions between both native and oxidised lipoproteins and $A\beta_{1-40}$ was investigated employing various chemical treatments. The addition of ascorbic acid or the vitamin E analogue, trolox, to lipoprotein/Aβ coincubations failed to inhibit Aβ fibrillogenesis, as did the treatment of lipoproteins with the aldehyde reductant, sodium borohydride. The putative lipid peroxide-derived aldehyde scavenger, aminoguanidine, however, inhibited Aβ-oxidised lipoprotein-potentiated polymerisation, but in a manner consistent with an antioxidant action for the drug. Lipoprotein treatment with the reactive aldehyde 4-hydroxy-2-*trans*-nonenal enhanced Aβ polymerisation in a concentration-dependent fashion. Incubation of Aβ with lipoprotein fractions from which the apoprotein components had been removed resulted in extents of polymerisation comparable to those observed with Aβ alone. These data indicate that the apoprotein components of plasma lipoproteins play a key role in promoting Aβ polymerisation, possibly via interactions with aldehydes. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Plasma lipoprotein; β-Amyloid; Fibrillogenesis

1. Introduction

The cytotoxicity of the β-amyloid (Aβ) peptide of Alzheimer's disease (AD) appears to be related to its polymerisation state, i.e. fibril content. It could be assumed therefore that factors that promote Aβ polymerisation may be critical with respect to determining Aβ toxicity. A variety of factors have been shown to stimulate Aβ fibrillogenesis including heparan sulphate proteoglycans, α_1-antichymotrypsin, metal ions and apolipoprotein (apo) E [1–5]. The elucidation of the mechanisms mediating these pro-aggregatory actions on Aβ may prove vitally important with respect to the development of treatments that block Aβ polymerisation and possibly the pathogenesis of AD.

Cardiovascular risk factors including hypercholesterolaemia, hypertension and hyperhomocysteinaemia have been implicated in the aetiology of AD [6]. Blood plasma exhibits significant Aβ immunoreactivity and platelets liberate Aβ on activation [7–11]. We have reported that platelet Aβ efflux is enhanced in hypercholesterolaemia, which is characterised by raised plasma low-density lipoprotein (LDL) and is associated with increased platelet sensitivity [12]. We have also shown that LDL and the other plasma lipoproteins, very-low-density lipoprotein (VLDL) and high-density lipoprotein (HDL), greatly enhance Aβ fibril formation, particularly when oxidised [13]. Indeed, indices of lipoprotein oxidation, i.e. thiobarbituric acid-reactive substances (TBARS) and conjugated dienes, correlated positively with extents of lipoprotein-stimulated Aβ polymerisation. We have speculated that in hypercholesterolaemia interactions between plasma LDL (and particularly oxidised LDL which is elevated in this condition also) and Aβ released by platelets may contribute to tissue deposition of Aβ, especially in the cerebrovasculature, and the development of AD.

Having established that plasma lipoproteins profoundly influence Aβ polymerisation the present studies were undertaken with the aim of elucidating the chemical mechanisms underlying these interactions. The studies entailed examining how various experimental treatments influenced lipoprotein-potentiated fibrillogenesis. Thus, experiments were conducted employing antioxidant vitamins/vitamin analogues (i.e. ascorbic acid and trolox), 4-hydroxy-2-*trans*-nonenal (HNE), a reactive aldehyde which stimulates protein aggregation and alters LDL structure and function [14,15], sodium borohydride ($NaBH_4$), which reduces reactive aldehydes and aminoguanidine (AG), a drug which has been used to treat diabetic complications and is a putative reactive aldehyde scavenger [16]. The influence of lipoprotein deproteinisation, i.e. the removal of the apoprotein components of lipoprotein fractions, was also investigated. We believe that these studies may have implications for the treatment and prevention of AD.

2. Materials and methods

2.1. Chemicals

$A\beta_{1-40}$ was purchased from Biosource International (Nivelles, Belgium). HNE came from Alexis Biochemicals (Nottingham, UK). Ascorbic acid, trolox, $NaBH_4$, AG and all other chemicals were supplied by Sigma Chemical Co. (Poole, UK).

*Corresponding author. Fax: (44)-20-7679 9192.
E-mail address: christopher.smith@ucl.ac.uk (C.C.T. Smith).

Abbreviations: Aβ, amyloid-β peptide; AD, Alzheimer's disease; AG, aminoguanidine; AU, arbitrary units; HDL, high-density lipoprotein; HNE, 4-hydroxy-2-*trans*-nonenal; LDL, low-density lipoprotein; LPDA, lipid peroxide-derived aldehydes; PBS, phosphate-buffered saline; TBARS, thiobarbituric acid-reactive substances; Th-T, thioflavin T; VLDL, very-low-density lipoprotein

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2.2. Lipoprotein isolation

VLDL, LDL and HDL were isolated from normal human plasma by sequential density gradient ultracentrifugation employing the method of Hatch and Lees [17]. After isolation lipoproteins were dialysed against 0.9% w/v NaCl at 4°C.

2.3. Lipoprotein oxidation

Oxidation of lipoproteins was achieved via dialysis against 0.9% NaCl containing 1 μM $FeSO_4$ (three changes) and halted by dialysis versus saline containing 240 μM EDTA [13]. Non-oxidised (native) lipoproteins were prepared by dialysis against EDTA saline. All lipoproteins were dialysed versus saline before characterisation and experimentation.

2.4. Lipoprotein characterisation

2.4.1. Protein. The protein content of lipoprotein fractions was determined according to the method of Wang and Smith [18].

2.4.2. Cholesterol and triglyceride. The cholesterol and triglyceride contents of lipoprotein fractions were determined employing the CHOD–PAP and GPO–PAP methods, respectively (Infinity™ enzymatic test reagents, Sigma Diagnostics, Poole, UK).

2.4.3. Thiobarbituric acid-reactive substances. General products of lipoprotein peroxidation were determined by measuring TBARS according to the method of Schuh et al. [19].

2.4.4. Conjugated dienes. Specific lipid peroxidation products, i.e. conjugated dienes, were determined by second derivative UV spectroscopy [20]. Lipids were extracted from lipoprotein fractions employing a $CHCl_3$/MeOH extraction procedure. Lipoproteins (100 μl) were combined with $CHCl_3$/MeOH (10 ml, 2:1) and gently mixed at room temperature. The aqueous and organic phases were then separated by centrifugation (1600×*g*, 5 min). The upper phase was then discarded and the lower phase re-extracted with 1.2 ml $CHCl_3$/MeOH/saline (45:45:3). Samples were then re-centrifuged, the upper phase discarded and the lower phase dried down under nitrogen at 60°C. The isolated lipids were then dissolved in cyclohexane (2 ml) and the second derivative UV absorption spectrum determined between 212 and 300 nm, employing cyclohexane as a blank. Peaks of absorption corresponding to the *trans–trans* and *cis–trans* conjugated dienes were detected at 233 and 242 nm, respectively. The values obtained at these wavelengths were combined and sample concentrations calculated by reference to (±)-9-hydroxyoctadecadienoic acid standards.

2.4.5. Agarose electrophoresis. The electrophoretic mobilities of native and oxidised lipoprotein fractions were determined by one-dimensional agarose gel electrophoresis (LKB Bromma 2117 multiphor electrophoresis system attached to a LKB Bromma 2197 power supply). Lipoproteins (3 μl) were applied to 0.5% agarose plates (Paragon® Lipo Gel, Beckman Coulter, CA, USA) which were incubated at room temperature for 5 min to allow sample diffusion. Gels were electrophoresed in barbital buffer (50 mM sodium barbital, 10 mM barbital) at 100 V for 1 h. At the end of this period gels were placed in fixative (60% v/v ethanol, 30% v/v H_2O, 10% v/v glacial acetic acid) for 5 min, dried and then stained with Sudan black B stain (3 ml 7% w/w Sudan black B stain, 300 ml 55% v/v ethanol). Gels were destained in 45% v/v ethanol, dried and viewed.

2.5. Aβ fibril formation

Aβ fibril formation was assessed by thioflavin T (Th-T) fluorescence spectroscopy [21]. Stock $A\beta_{1-40}$ solutions (6 mg/ml) which had been prepared in distilled deionised (DD) water and stored at −85°C were diluted (1 mg/ml, final concentration) with phosphate-buffered saline (PBS; 20 mM sodium phosphate, 0.9% w/v NaCl, pH 7.4) on the day of experimentation. Aβ samples were then incubated at 37°C for up to 5 days. At specific intervals aliquots (20 μl) were removed and combined with 5 mM glycine, pH 8.75, containing 2 μM Th-T in a final volume of 2 ml. Fibril formation was then assessed by measuring the sample fluorescence (excitation and emission wavelengths 425 nm and 480 nm, respectively, and excitation and emission bandpass slits 5 and 10 nm) in a Perkin-Elmer fluorescence spectrophotometer.

2.6. Native and oxidised lipoproteins

The effects of native and oxidised lipoproteins on Aβ polymerisation was determined via their coincubation with soluble $A\beta_{1-40}$. Lipoproteins were diluted with PBS and combined with $A\beta_{1-40}$ (1 mg/ml, final concentration) to yield final concentrations for VLDL of 0.2 mg/ml and for LDL and HDL of 2 mg/ml. Samples were incubated at 37°C for up to 5 days with the lipoproteins incubated by themselves serving as controls, and Th-T fluorescence determined.

2.7. Antioxidant treatment

Ascorbic acid and trolox (prepared by dissolving in DD water and adjusting pH to 7.4 with 1 mM NaOH) were tested for their effects on lipoprotein-potentiated Aβ fibrillogenesis. Native or oxidised VLDL (0.2 mg/ml final concentration), LDL (2 mg/ml) and HDL (2 mg/ml) were treated with ascorbic acid or trolox at final concentrations of 500 μM and 1 mM, respectively, and the influence of these treatments on Aβ fibrillogenesis established by comparison with antioxidant-free samples. Samples were incubated at 37°C for 4 days and fluorescence readings taken at 0, 24 and 96 h.

2.8. Sodium borohydride

The influence of aldehydes upon $A\beta_{1-40}$ fibrillogenesis was investigated using the selective aldehyde reductant $NaBH_4$. Solid $NaBH_4$ was added to native and oxidised lipoprotein fractions (1 mg/mg protein) which were then incubated at room temperature for 1 h. Following extensive dialysis versus PBS, lipoprotein fractions were characterised and aldehyde reduction confirmed by the measurement of TBARS. $NaBH_4$-treated and untreated lipoprotein samples were then compared with respect to their ability to promote Aβ fibrillogenesis.

2.9. Aminoguanidine

The putative lipid peroxide-derived aldehydes (LPDA) scavenger AG was tested for its effects on Aβ fibrillogenesis. Isolated VLDL and LDL were oxidised in the presence or absence of 25 mM AG (0.25 M AG bicarbonate stock solution prepared in 0.6 M HCl and neutralised with NaOH) and then dialysed against saline/EDTA, followed by dialysis versus 0.9% saline. Following lipoprotein characterisation to determine the effects of AG on lipoprotein oxidation, AG-treated and untreated lipoproteins (native and oxidised) were incubated with $A\beta_{1-40}$ for 5 days and the extents of fibrillogenesis measured at 0, 6, 24, 96 and 120 h.

2.10. 4-Hydroxynonenal

A highly purified preparation of the reactive aldehyde HNE was used to modify native lipoprotein fractions employing a modified version of the method of Hoff and O'Neil [15]. Isolated lipoproteins were prepared and dialysed versus EDTA/saline and then diluted 1:1 with PBS. 900 μl of lipoprotein fraction was then combined with 100 μl HNE (prepared by evaporation of stock HNE solution in *n*-hexane under N_2, followed by dissolution of the neat oil in sterile filtered (0.2 μM) PBS, pH 7.2, to a concentration of 30 mM) to yield final concentrations of 1, 2 and 3 mM. Control lipoproteins were prepared by substituting 100 μl PBS for the HNE solution. Samples were then incubated with stirring at 37°C for 5 h. Following incubation lipoprotein samples were dialysed against PBS to remove unreacted HNE and the fractions characterised for cholesterol, triglyceride, protein, TBARS, conjugated dienes and electrophoretic mobility. Native and HNE-treated lipoprotein fractions were then compared for their effects on Aβ fibrillogenesis at 0, 3, 6 and 24 h.

2.11. Lipoprotein deproteinisation

The preparation of deproteinised lipoprotein extracts (i.e. the isolation of the lipid components of lipoproteins) was achieved by chloroform–methanol (2:1) extraction as employed for the preparation of conjugated diene samples [20], the extracts being evaporated to dryness under N_2 at 60°C. The resulting lipid-rich fractions were then resuspended in PBS and sonicated briefly, followed by chemical characterisation. The lipid extracts were then compared with intact lipoproteins with respect to their effects on Aβ fibrillogenesis.

3. Results

3.1. Ascorbic acid and trolox

VLDL, LDL and HDL in their native and oxidised forms markedly enhanced Aβ polymerisation as assessed by the Th-T fluorescence assay.

The addition of ascorbic acid or trolox to lipoprotein/$A\beta_{1-40}$ coincubations failed to influence lipoprotein-potenti-

Table 1
Characterisation of lipoproteins treated or untreated with $NaBH_4$

Lipoprotein class	Cholesterol (μmol mg^{-1})	Triglyceride (μmol mg^{-1})	TBARS (nmol mg^{-1})	Conjugated dienes (nmol mg^{-1})
VLDL native	2.34 ± 0.18	5.37 ± 0.16	0.78 ± 0.18	71.73 ± 14.47
VLDL native+$NaBH_4$	2.33 ± 0.18	5.38 ± 0.11	0.66 ± 0.12	71.73 ± 14.47
VLDL oxidised	2.4 ± 0.17	5.38 ± 0.14	13.03 ± 1.44	411.63 ± 26.77
VLDL oxidised+$NaBH_4$	2.37 ± 0.18	5.38 ± 0.17	2.95 ± 0.31*	402.26 ± 22.89
LDL native	3.52 ± 0.13	0.16 ± 0.04	0.29 ± 0.06	16.63 ± 2.99
LDL native+$NaBH_4$	3.48 ± 0.11	0.16 ± 0.03	0.29 ± 0.05	19.5 ± 3.62
LDL oxidised	3.52 ± 0.06	0.17 ± 0.03	7.6 ± 0.68	351.12 ± 49.67
LDL oxidised+$NaBH_4$	3.53 ± 0.07	0.17 ± 0.04	1.22 ± 0.24**	338.21 ± 30.77
HDL native	0.59 ± 0.06	0.047 ± 0.01	0.19 ± 0.06	6.48 ± 1.13
HDL native+$NaBH_4$	0.61 ± 0.08	0.049 ± 0.01	0.19 ± 0.06	6.81 ± 2.13
HDL oxidised	0.64 ± 0.06	0.059 ± 0.01	3.77 ± 0.18	99.14 ± 13.25
HDL oxidised+$NaBH_4$	0.61 ± 0.07	0.05 ± 0.004	1.21 ± 0.15**	88.68 ± 18.23

Data are presented as mean ± S.E.M. and are expressed relative to protein content ($n = 3$). *$P < 0.05$, **$P < 0.02$.

ated Aβ fibrillogenesis. The fluorescence values for ascorbate- and trolox-treated samples were within 10% of those obtained with samples that had not been treated with antioxidants at all the incubation time points examined. Fluorescence values for native LDL/Aβ incubated in the presence and absence of trolox (96 h of incubation) were, for example, 118.9 ± 10.9 arbitrary units (AU) and 126 ± 10.8 AU, respectively. Similarly, the values for oxidised LDL/Aβ with or without trolox were 175.8 ± 15.5 AU and 181.1 ± 12.8 AU.

3.2. Sodium borohydride

The possible role of LPDA in promoting $A\beta_{1-40}$ fibrillogenesis was first investigated using the selective aldehyde reductant $NaBH_4$. When incubated with oxidised lipoprotein for 1 h $NaBH_4$ markedly reduced aldehyde formation, as indicated by significantly decreased TBARS production. Thus, treatment of oxidised VLDL, LDL and HDL with $NaBH_4$ reduced TBARS concentrations by 77.4% ($P < 0.05$), 83.9% ($P < 0.05$) and 67.9% ($P < 0.02$), respectively (Table 1). The exposure of oxidised lipoproteins to $NaBH_4$ for longer periods (i.e. 24 h) failed to reduce TBARS concentrations further. The protein, triglyceride, cholesterol and conjugated diene contents of oxidised lipoproteins were unaffected by $NaBH_4$ treatment (Table 1). The electrophoretic mobilities of oxidised lipoproteins on agarose plates were also unaltered by $NaBH_4$ treatment, indicating that the reductant does not affect the oxidative state of the protein moieties of the lipoproteins.

Having established that $NaBH_4$ reduced the aldehyde groups on lipoproteins effectively the influence of aldehyde-free and aldehyde-containing lipoproteins on $A\beta_{1-40}$ fibrillogenesis was next examined. Hence, $NaBH_4$ treatment was found not to influence native or oxidised lipoprotein-potentiated Aβ polymerisation. Fluorescence values for $NaBH_4$-treated and untreated oxidised VLDL, LDL and HDL, for example, did not differ by more than 9% at any of the time points examined.

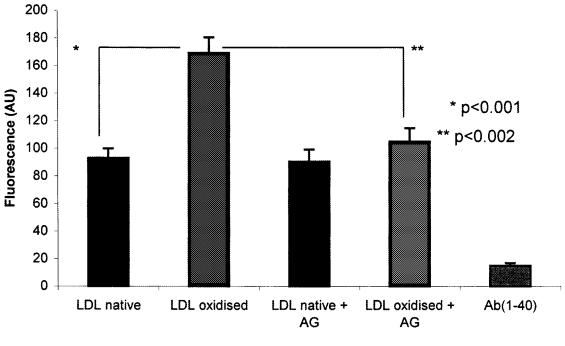

Fig. 1. The effect of AG treatment upon native and oxidised LDL-induced $A\beta_{1-40}$ polymerisation. Data are mean ± S.E.M. ($n = 3$).

3.3. Aminoguanidine

To investigate the possibility that LPDA influence Aβ polymerisation indirectly via modification of the protein components (apoproteins) of plasma lipoproteins, experiments were conducted with the proposed LPDA scavenger AG.

The oxidation of lipoproteins in the presence of 25 mM AG resulted in fractions with markedly reduced effects on Aβ fibrillogenesis as compared with lipoproteins oxidised in its absence. Extents of fibril formation observed with AG-treated oxidised lipoprotein fractions were similar to those seen with native lipoproteins (Fig. 1). Differences were evident after 6 h of incubation and remained throughout the incubation period. For example, after 24 h of incubation fibril formation induced by oxidised LDL prepared in the presence of AG was 38% lower than that induced by oxidised LDL alone ($P < 0.002$) (Fig. 1). If the influence of native LDL on Aβ fibrillogenesis was taken into account (i.e. the values obtained with native LDL were subtracted from those obtained with oxidised LDL), then the decrease in Aβ fibril formation was found to be of the order of 81%. Extents of Aβ polymerisation

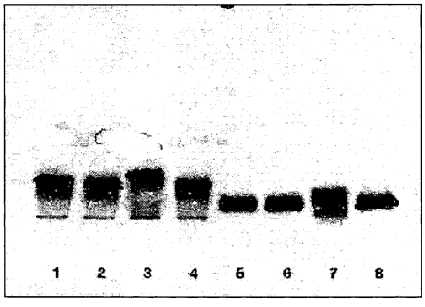

Fig. 2. The effect of AG on the agarose gel electrophoretic mobility of native/oxidised VLDL and LDL. Lane 1, native VLDL; lane 2, native VLDL+AG; lane 3, oxidised VLDL; lane 4, oxidised VLDL+AG; lane 5, native LDL; lane 6, native LDL+AG; lane 7, oxidised LDL; lane 8, oxidised LDL+AG.

Table 2
Characterisation of lipoproteins oxidised in the presence or absence of AG

Lipoprotein class	Cholesterol (μmol mg^{-1})	Triglyceride (μmol mg^{-1})	TBARS (nmol mg^{-1})	Conjugated dienes (nmol mg^{-1})
VLDL native	2.48 ± 0.15	5.14 ± 0.72	0.63 ± 0.16	80.1 ± 32.48
VLDL native+AG	2.46 ± 0.11	5.11 ± 0.76	0.59 ± 0.12	81.55 ± 27.9
VLDL oxidised	2.56 ± 0.13	5.13 ± 0.70	15.8 ± 3.64	440.6 ± 7.75
VLDL oxidised+AG	2.49 ± 0.17	5.10 ± 0.51	1.01 ± 0.3*	112.54 ± 20.7***
LDL native	3.43 ± 0.16	0.19 ± 0.03	0.442 ± 0.07	8.98 ± 5.39
LDL native+AG	3.39 ± 0.12	0.19 ± 0.04	0.546 ± 0.06	8.0 ± 4.53
LDL oxidised	3.44 ± 0.13	0.18 ± 0.02	10.01 ± 0.84	385.9 ± 44.78
LDL oxidised+AG	3.47 ± 0.14	0.19 ± 0.03	0.733 ± 0.08***	32.49 ± 7.6**

Data are presented as mean ± S.E.M. and are expressed relative to protein content ($n = 3$). *$P < 0.05$, **$P < 0.02$, ***$P < 0.01$.

induced by native LDL coincubated with AG were identical to those obtained with untreated lipoprotein.

Coincubation of VLDL or LDL with AG did not alter their triglyceride or cholesterol contents. AG treatment of lipoproteins during oxidation, however, not only decreased TBARS formation and protein oxidation, as indicated by reduced electrophoretic mobility (Table 2, Fig. 2), but also decreased conjugated diene generation (Table 2). These data are consistent with AG exerting its inhibitory action on the oxidative modification of lipoproteins via a potent antioxidant effect, rather than through the scavenging of reactive aldehydes. Hence, the reductions in Aβ fibril formation observed after AG treatment of lipoproteins may merely reflect the diminished oxidative state of the lipoprotein particle. It was observed that measures of oxidation for LDL coincubated with AG were approximately 10% of the values obtained for fully oxidised LDL but were significantly higher than those for native LDL. This was mirrored by the finding that the extents of fibril formation observed with these samples exceeded the values obtained with native LDL by 10% of that induced by the fully oxidised lipoprotein.

3.4. 4-Hydroxynonenal

The data obtained with $NaBH_4$ may be interpreted as indicating that LPDA do not influence Aβ polymerisation directly. When, however, native lipoproteins were treated with a highly purified preparation of the reactive aldehyde HNE, marked effects on lipoprotein-induced Aβ polymerisation were observed.

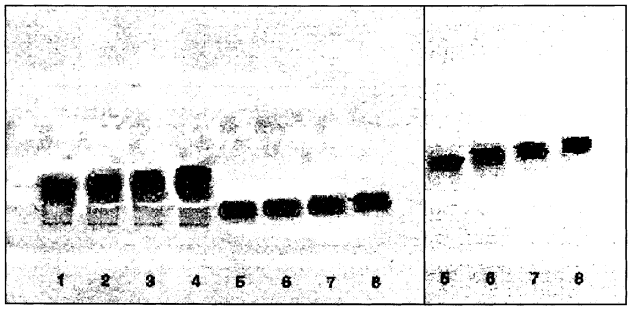

Fig. 3. Effect of HNE modification upon the agarose gel electrophoretic mobility of plasma lipoproteins. a: Lane 1, VLDL native; lane 2, VLDL+1 mM HNE; lane 3, VLDL+2 mM HNE; lane 4, VLDL+3 mM HNE; lane 5, LDL native; lane 6, LDL+1 mM HNE; lane 7, LDL+2 mM HNE; lane 8, LDL+3 mM HNE. b: Lane 5, HDL native; lane 6, HDL+1 mM HNE; lane 7, HDL+2 mM HNE; lane 8, HDL+3 mM HNE.

The incubation of VLDL, LDL and HDL with HNE resulted in concentration-dependent increases in electrophoretic mobility (Fig. 3). This was accompanied by sample band broadening on the agarose gels, a phenomenon frequently observed with lipoproteins on oxidation. Protein, cholesterol, triglyceride, TBARS and conjugated diene concentrations were all unaffected by HNE treatment.

Prior treatment of plasma lipoproteins with HNE resulted in concentration-dependent enhancement of lipoprotein-induced Aβ polymerisation over a 24 h period (Fig. 4). The increases were particularly pronounced for VLDL and LDL with less marked increases being observed with HDL. Thus, at 3 h of incubation, treatment of VLDL and LDL with 3 mM HNE resulted in increases in Aβ fibrillogenesis which exceeded the increase induced by the respective untreated lipoproteins by 154% and 185%, whilst HNE treatment of HDL caused increases of only 55%. HNE itself did not influence Aβ fibrillogenesis and increases in fluorescence were not seen when HNE-treated lipoproteins were incubated alone.

3.5. Lipoprotein deproteinisation

To investigate the influence of the lipid components of the lipoproteins alone on Aβ polymerisation, and the significance of interactions between the apoproteins and Aβ in this process, experiments were conducted with deproteinised lipoprotein extracts.

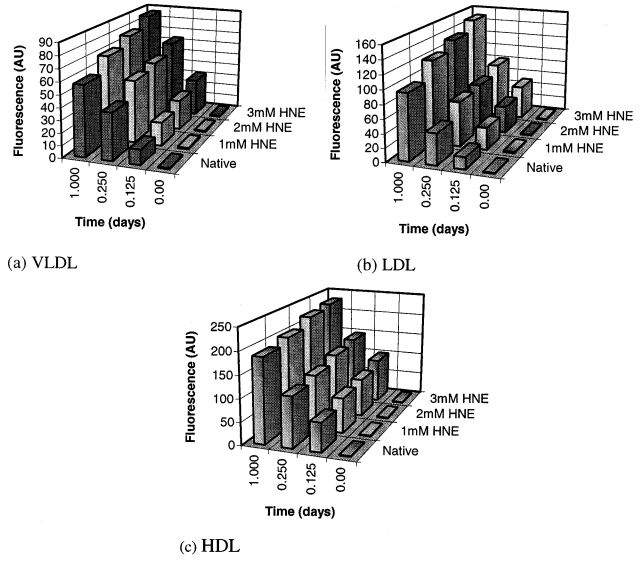

Fig. 4. Effect of HNE modification of plasma lipoproteins on $A\beta_{1-40}$ fibrillogenesis. Data are expressed as mean fluorescence ($n = 4$).

Table 3a
Characterisation of native/oxidised lipoproteins and lipid-extracted samples

Lipoprotein class	Protein (mg μmol^{-1})	Cholesterol (μmol μmol $^{-1}$)	Free cholesterol (μmol μmol $^{-1}$)	Triglyceride (μmol μmol $^{-1}$)
VLDL native	0.66 ± 0.07	1.26 ± 0.18	0.676 ± 0.06	3.96 ± 0.34
VLDL native–lipid only	0.0 ± 0.0*	1.24 ± 0.14	0.66 ± 0.05	3.68 ± 0.49
VLDL oxidised	0.60 ± 0.05	1.16 ± 0.19	0.65 ± 0.04	3.65 ± 0.24
VLDL oxidised–lipid only	0.0 ± 0.0*	1.23 ± 0.14	0.67 ± 0.03	3.71 ± 0.55
LDL native	0.86 ± 0.05	2.42 ± 0.22	0.59 ± 0.07	0.14 ± 0.01
LDL native–lipid only	0.0 ± 0.0*	2.05 ± 0.06	0.57 ± 0.08	0.13 ± 0.01
LDL oxidised	0.82 ± 0.05	2.31 ± 0.25	0.57 ± 0.07	0.13 ± 0.01
LDL oxidised–lipid only	0.0 ± 0.0*	2.27 ± 0.06	0.59 ± 0.09	0.12 ± 0.01
HDL native	1.51 ± 0.03	1.06 ± 0.08	0.23 ± 0.02	0.08 ± 0.01
HDL native–lipid only	0.0 ± 0.0*	0.81 ± 0.13	0.24 ± 0.03	0.07 ± 0.02
HDL oxidised	1.72 ± 0.10	1.05 ± 0.11	0.28 ± 0.02	0.08 ± 0.01
HDL oxidised–lipid only	0.0 ± 0.0*	0.96 ± 0.14	0.33 ± 0.03	0.07 ± 0.01

Data are presented as mean ± S.E.M. and are expressed relative to phospholipid content ($n = 4$). *$P < 0.001$.

Characterisation of the lipoproteins which had been chloroform–methanol-extracted indicated that all traces of protein had been successfully removed (Table 3). Comparison of extracted and unextracted lipoprotein fractions, however, revealed that no differences occurred in relation to total cholesterol, free cholesterol, triglyceride and phospholipid concentrations (Table 3). Indices of oxidation, i.e. conjugated dienes and TBARS, also were not different (Table 3).

Chloroform–methanol lipid extracts had a significantly reduced influence on Aβ fibril formation, as compared with the intact lipoprotein fractions ($P < 0.001$ for all lipoproteins, ANOVA). For example, after 24 h of incubation, native VLDL increased Aβ fibril formation by 310% as compared to Aβ incubated alone ($P < 0.04$), whereas VLDL lipid extracts increased fibril formation by only 23.2% (non-significant) (Fig. 5a). Differences were evident after only 6 h of incubation and continued over the entire period of incubation (5 days). Oxidised VLDL increased Aβ polymerisation by 500% as compared with Aβ alone ($P < 0.02$; 24 h of incubation), whereas the corresponding chloroform–methanol extracts increased it by 34% (non-significant) (Fig. 5a). Again, significant differences occurred throughout the 5 day incubation period. As reported previously, intact oxidised VLDL enhanced Aβ fibril formation to a significantly greater extent than its native counterpart. Significant differences were, however, not observed when Aβ was incubated with the corresponding native and oxidised lipid extracts.

Deproteinisation of native and oxidised LDL also resulted in decreased rates and extents of Aβ polymerisation as compared with intact LDL fractions (Fig. 5b). Fluorescence values for coincubates of LDL lipid extracts and Aβ were equivalent to the values obtained with Aβ alone. Meanwhile, after 24 h of incubation intact native and oxidised LDL were found to increase Aβ fibrillogenesis by 457% and 991% respectively, compared with Aβ incubated alone.

With HDL similar results to those observed with VLDL and LDL were obtained (Fig. 5c). Thus, deproteinisation of native and oxidised HDL negated the potentiating effects of the intact species. Extents of polymerisation after 5 days of incubation for HDL lipid extracts/Aβ coincubates compared to those obtained with intact HDL/Aβ were reduced by 76% for native HDL ($P < 0.001$) and 81% for oxidised HDL ($P < 0.03$).

4. Discussion

It is generally accepted that Aβ cytotoxicity relates to the polymerisation state of the peptide. Various chemically unrelated substances promote Aβ fibrillogenesis and, one might therefore infer, toxicity [1–5]. With respect to the vasculature it is possible that molecules present in the blood may influence the polymerisation and toxicity of Aβ released by platelets [12]. We have previously shown that VLDL, LDL and HDL promote $A\beta_{1-40}$ fibrillogenesis, with VLDL and LDL producing especially marked effects when in their oxidised state [13]. In the present study potential mechanisms whereby lipoproteins potentiate Aβ fibrillogenesis were investigated.

Oxidative stress has been implicated in the pathogenesis of AD and hypercholesterolaemia, which is associated with raised plasma LDL [22,23]. In hypercholesterolaemia, however, it is not only plasma LDL that is raised but also the oxidised form of this species. Oxidised LDL is particularly

Table 3b
Measures of oxidation for complete lipoproteins and lipid-extracted samples

Lipoprotein class	TBARS (nmol μmol $^{-1}$)	Conjugated dienes (nmol μmol $^{-1}$)
VLDL native	0.47 ± 0.03	59.95 ± 5.74
VLDL native–lipid only	0.77 ± 0.12	42.45 ± 6.25
VLDL oxidised	7.73 ± 1.36	333.68 ± 36.73
VLDL oxidised–lipid only	6.08 ± 0.39	312.12 ± 41.45
LDL native	0.17 ± 0.04	9.52 ± 3.44
LDL native–lipid only	0.17 ± 0.06	7.2 ± 1.5
LDL oxidised	2.83 ± 0.13	272.94 ± 37.07
LDL oxidised–lipid only	3.64 ± 0.59	272.94 ± 32.54
HDL native	0.48 ± 0.13	14.4 ± 1.04
HDL native–lipid only	0.52 ± 0.19	24.81 ± 9.89
HDL oxidised	3.45 ± 0.28	232.63 ± 35.81
HDL oxidised–lipid only	3.78 ± 0.34	225.26 ± 41.92

Data are presented as mean ± S.E.M. and are expressed relative to phospholipid ($n = 4$).

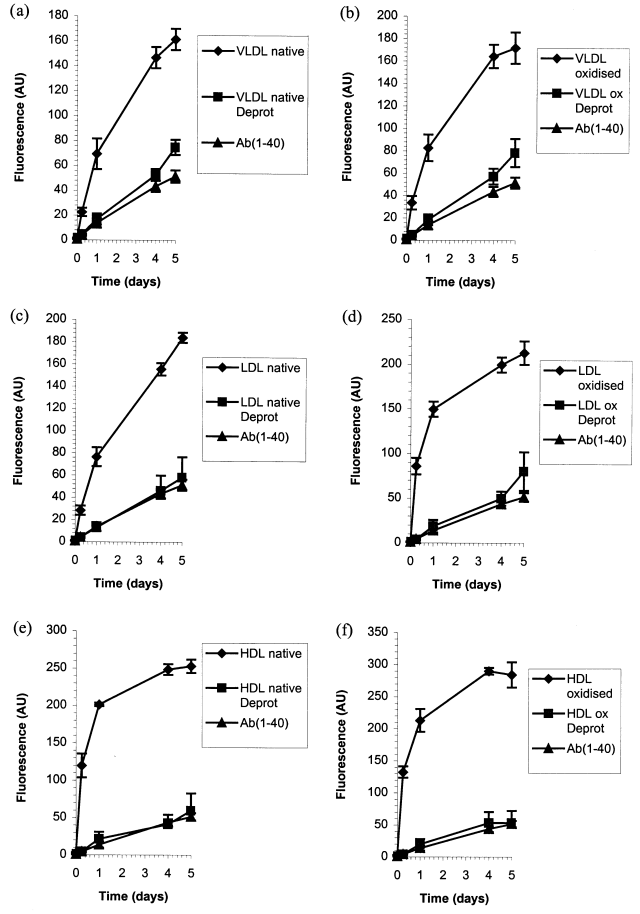

Fig. 5. Effect of lipoprotein protein removal upon lipoprotein-mediated $A\beta_{1-40}$ fibril formation. a: Native VLDL/VLDL lipid extract $+A\beta_{1-40}$. b: Oxidised VLDL/VLDL lipid extract+$A\beta_{1-40}$. c: Native LDL/LDL lipid extract+$A\beta_{1-40}$. d: Oxidised LDL/LDL lipid extract $+A\beta_{1-40}$. e: Native HDL/HDL lipid extract+$A\beta_{1-40}$. f: Oxidised HDL/HDL lipid extract+$A\beta_{1-40}$. Data are mean ± S.E.M. Complete lipoprotein fractions were all significantly different from lipid extracts with regard to their influence upon $A\beta_{1-40}$ polymerisation by a significance value of at least $P < 0.0001$ (ANOVA), whereas lipid extracts were found to have no significant influence upon peptide fibrillogenesis. $n = 3$.

damaging to the blood vessel wall, causing endothelial damage, which is seen in AD [24]. Various potentially pathogenic molecular species are generated as a consequence of oxidative mechanisms, some of which could influence the development of AD. These products of oxidation include free radicals, LPDA and oxidised apoproteins [14,15,25,26]. The presence of LPDA and oxidised apoproteins in oxidised lipoprotein fractions was confirmed in the present study by the increases recorded for TBARS and electrophoretic mobility, respectively. We did not, however, confirm the presence of free radicals in the lipoprotein fractions as these were not determined. But given their short half-life and the fact that the divalent cation chelator EDTA was used to halt lipoprotein oxidation, their presence seems unlikely. It has been reported that Aβ preparations spontaneously generate protein free radicals [27], a possibility which we investigated employing the antioxidants ascorbic acid and trolox. Neither compound influenced Aβ fibrillogenesis indicating, perhaps, that free radicals are not directly involved in the polymerisation process. Clearly, however, more detailed studies are required before this question can be answered unequivocally.

Apoproteins are significantly altered during oxidation, as reflected by increases in the electronegative charge on lipoprotein particles and changes in their chromatographic behaviour. These alterations occur primarily as a result of interactions between LPDA and specific amino acid residues on the apoproteins [26,28–31], although direct oxidation of the apoproteins cannot be discounted. Free radical-driven lipid peroxidation is essentially responsible for triggering the process that begins with the removal of a hydrogen atom, by an initiating radical, from one of the polyunsaturated fatty acids on the lipoprotein particle. These molecules are particularly susceptible to free radical attack, and hydrogen abstraction under these circumstances, in the presence of molecular oxygen, initiates a reaction that results in the formation of lipid hydroperoxides. Lipid hydroperoxide decomposition, a process accelerated by transition metal ions, may then lead to the formation of aldehydes, which react readily with amino acids, particularly histidine, lysine and cysteine. This may result in the production of Michael-type adducts, which, in turn, may undergo secondary reactions to form inter- and intramolecular cross-links [26,31]. Thus, one could suggest that the initiating event in lipoprotein-induced Aβ fibrillogenesis involves molecular cross-linking, either via lipoprotein–Aβ interaction or intramolecular cross-linking of apoproteins resulting in conformational changes which are conducive to the promotion of Aβ polymerisation. The data obtained with $NaBH_4$, however, would appear to preclude the involvement of LPDA in promoting Aβ fibril formation. Although $NaBH_4$ treatment of oxidised lipoprotein fractions reduced TBARS generation, an indicator of LPDA production, Aβ polymerisation promoted by plasma lipoproteins was unaffected. By contrast, treatment with the putative LPDA scavenger AG blocked Aβ fibrillogenesis potentiated by oxidised VLDL and LDL. Initially, this was thought to provide evidence for LPDA playing a key role in Aβ polymerisation potentiated by plasma lipoproteins. But close examination of the data obtained following lipoprotein analysis revealed that AG treatment not only prevented TBARS formation and protein oxidation, as previously reported [16], but also prevented conjugated diene formation. The inhibitory action of AG upon the oxidative modification of lipoproteins therefore may not occur as a result of it acting as a scavenger of reactive aldehydes but rather as a potent antioxidant. Consequently, the decrease in lipoprotein-potentiated Aβ fibril formation observed with AG may merely reflect the diminished oxidative state of the lipoproteins.

The modification of lipoproteins by free radicals and aldehydes has been investigated intensively [26,32] and the products of these modifications, i.e. protein carbonyls, have been reported to be elevated in both hypercholesterolaemia and AD [15,33,34]. Despite the data obtained with $NaBH_4$ and AG, the possibility that LPDA influence Aβ polymerisation via the modification of apoproteins was investigated further adopting an alternative approach. Hence, pre-treatment of VLDL and LDL with HNE was found to enhance their stimulatory effects on fibrillogenesis, indicating that reactive aldehydes may play an important role in Aβ polymerisation promoted by lipoproteins. The fact that modification of lipoproteins by HNE increased their electrophoretic mobility but did not increase markers of lipoprotein oxidation (i.e.

TBARS and conjugated dienes) indicates that its effects are specific to the apoproteins (in the case of LDL, apo B_{100}, as it is the sole apoprotein present in this lipoprotein) and does not involve its incorporation into the lipid regions of the lipoprotein particle. The results obtained with deproteinised (lipid-only containing) lipoprotein fractions appear to underline the importance of the protein components of the lipoprotein particle in promoting Aβ polymerisation.

To summarise our findings, these studies indicate that reactive aldehydes and the apoprotein components of lipoprotein particles may be important with respect to their potentiating effects on Aβ fibrillogenesis.

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