2012

Selective reduction of hydroperoxyeicosatetraenoic acids to their hydroxy derivatives by apolipoprotein D: implications for lipid antioxidant activity and Alzheimer's disease

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**Publication Details**
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Abstract
ApoD (apolipoprotein D) is up-regulated in AD (Alzheimer’s disease) and upon oxidative stress. ApoD inhibits brain lipid peroxidation in vivo, but the mechanism is unknown. Specific methionine residues may inhibit lipid peroxidation by reducing radical-propagating L-OOHs (lipid hydroperoxides) to non-reactive hydroxides via a reaction that generates MetSO (methionine sulfoxide). Since apoD has three conserved methionine residues (Met49, Met93 and Met157), we generated recombinant proteins with either one or all methionine residues replaced by alanine and assessed their capacity to reduce HpETEs (hydroperoxyeicosatetraenoic acids) to their HETE (hydroxyeicosatetraenoic acid) derivatives. ApoD, apoDM49-A and apoDM157-A all catalysed the reduction of HpETEs to their corresponding HETEs. Amino acid analysis of HpETE-treated apoD revealed a loss of one third of the methionine residues accompanied by the formation of MetSO. Additional studies using apoD(M93-A) indicated that Met93 was required for HpETE reduction. We also assessed the impact that apoD MetSO formation has on protein aggregation by Western blotting of HpETE-treated apoD and human brain samples. ApoD methionine oxidation was associated with formation of apoD aggregates that were also detected in the hippocampus of AD patients. In conclusion, conversion of HpETE into HETE is mediated by apoD Met93, a process that may contribute to apoD antioxidant function.

Keywords
hydroperoxyeicosatetraenoic, alzheimer, reduction, selective, activity, antioxidant, lipid, implications, apolipoprotein, derivatives, hydroxy, their, acids, disease, CMMB

Disciplines
Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

Publication Details

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This journal article is available at Research Online: http://ro.uow.edu.au/scipapers/4729
Selective reduction of hydroperoxyeicosatetraenoic acids to their hydroxy derivatives by apolipoprotein D: implications for lipid antioxidant activity and Alzheimer’s disease

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INTRODUCTION

ApoD (apolipoprotein D) is up-regulated in AD (Alzheimer’s disease) and upon oxidative stress. ApoD inhibits brain lipid peroxidation in vivo, but the mechanism is unknown. Specific methionine residues may inhibit lipid peroxidation by reducing radical-propagating L-OOHs (lipid hydroperoxides) to non-reactive hydroxides via a reaction that generates MetSO (methionine sulfoxide). Since apoD has three conserved methionine residues (Met49, Met93 and Met157), we generated MetSO (methionine sulfoxide). Since apoD has three conserved methionine residues (Met49, Met93 and Met157), we generated MetSO (methionine sulfoxide). Since apoD has three conserved methionine residues (Met49, Met93 and Met157), we generated MetSO (methionine sulfoxide). Since apoD has three conserved methionine residues (Met49, Met93 and Met157), we generated MetSO (methionine sulfoxide). Since apoD has three conserved methionine residues (Met49, Met93 and Met157), we generated MetSO (methionine sulfoxide).

Studies in mice showed that cerebral apoD expression is increased in response to Paraquat-induced oxidative stress, that cerebral lipid peroxidation is increased in apoD-null compared with wild-type animals and that overexpression of human apoD prevents cerebral lipid peroxidation [8]. In addition, expression of human apoD reduced the accumulation of aldehydic end-products of lipid peroxidation in an aged Drosophila model [9]. Even though several lines of evidence demonstrate that apoD protects against lipid peroxidation in the brain, the mechanisms underlying this action remain to be defined.

Previous studies have firmly established the antioxidant function of protein methionine residues [10]. The pathway involves the selective oxidation of methionine residues by a variety of reactive species to generate MetSO (methionine sulfoxide) [11]. MetSO may be reduced back to methionine by MSRs (MetSO reductases) that are in turn redox-cycled back to their reduced state by thioredoxin [12,13]. In the case of lipid peroxidation, it is known that specific methionine residues of plasma apolipoproteins apoA-I and apoA-II interact with L-OOHs (lipid hydroperoxides) to yield non-reactive L-OOHs (lipid hydroxides) and MetSO [14]:

\[
L-\text{OOH} + \text{Met} \rightarrow L-\text{OH} + \text{MetSO}
\] (1)

This two-electron reduction of L-OOH to L-OH competes with transition metal-catalysed Fenton-type reactions (e.g. one-electron transfers generating lipid alkoxyl radicals, L-O•) that accelerate lipid peroxidation [15]:

\[
L-\text{OOH} + \text{Fe}^{2+}/\text{Cu}^{2+} \rightarrow L-\text{O}^* + \text{OH} + \text{Fe}^{3+}/\text{Cu}^{2+}
\] (2)

The methionine/L-OOH reaction [see eqn (1)] thereby prevents the propagation of lipid peroxidation that would otherwise be caused by L-O•-mediated abstraction of bis-allylic hydrogen atoms from proximal lipid acyl chains. This reaction pathway explains a significant proportion of the lipid antioxidant capacity associated with plasma HDL (high-density lipoprotein) apolipoproteins [14,15]. On the basis of these studies and the previously discovered in vivo role of apoD as a lipid antioxidant [8,9], we were prompted to examine the role that apoD methionine

Abbreviations used: AD, Alzheimer’s disease; apo, apolipoprotein; AQC, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate; CHO, Chinese-hamster ovary; CHO-apoD, CHO-K1 cells stably expressing apoD; GdnHCl, guanidinium chloride; HDL, high-density lipoprotein; HEK, human embryonic kidney; HETE, hydroperoxyeicosatetraenoic acid; HMW, high molecular mass; HpETE, hydroperoxyeicosatetraenoic acid; L-OOH, lipid hydroperoxide; MetSO, methionine sulfoxide; MSR, MetSO reductase; PC, phosphatidylcholine; PLA2, phospholipase A2; RT, retention time; t-BOOH, t-butyl hydroperoxide; TBS, Tris-buffered saline; THF, tetrahydrofuran.

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residues may play in the reduction of L-OOH to L-OH [see eqn (1)]. The results of the present study indicate that apoD Met<sup>93</sup> reduces a variety of fatty acid L-OOHs to their corresponding L-OHs and that this is associated with lipid antioxidant capacity. The concomitant formation of MetSO results in an increased propensity for apoD self-association to yield aggregates that are similar to those detected in the insoluble fraction of human hippocampal homogenates from AD patients. The present study provides important insights into the mechanisms by which apoD may protect against lipid peroxidation in the brain.

**MATERIALS AND METHODS**

**Materials**

All HETEs (hydroxyeicosatetraenoic acids) and HpETEs (hydroperoxyeicosatetraenoic acids) were purchased from Cayman Chemicals. Analytical grade ethanol, TFA (trifluoroacetic acid), THF (tetrahydrofuran), acetic acid, urea and GdnHCl (guanidinium chloride) were purchased from Sigma. HPLC-grade acetonitrile and propan-2-ol were purchased from Ajax Chemicals. Cell culture media and additives were purchased from Invitrogen.

**Protein expression and purification**

Human apoA-I (GenBank<sup>®</sup> accession number NM_000039.1) and apoD (GenBank<sup>®</sup> accession number NM_001647.3) were expressed in human HEK (human embryonic kidney)-293 cells using the pcDNA3.1(+) vector. A linker, FLAG-tag and poly-His tail (SGGGGSDYKDDDDKHHHHHH) was included at the C-terminus and the secreted proteins were purified using a Ni-HiTrap column with a linear gradient from 20–500 mM imidazol at 35–55 C. The ethanol fraction was removed and the protein fraction precipitated with 9 vol. of 5 M GdnHCl. Analysis was achieved using a 5 μl aliquot of 6 M GdnHCl. The MetSO peak derived from the samples was contaminated with MetSO and methionine sulfone standards were clearly separated using this method [RTs (retention times) of 5.55 min and 5.85 min for MetSO and methionine sulfone standards were clearly separated].

**Protein/HpETE incubation conditions**

Apolipoproteins (0.5 mg/ml) were incubated in PBS with H<sub>2</sub>O<sub>2</sub> (0.1 or 1.0 M), or the indicated HpETEs (0.05 mg/ml) for periods of up to 8 h. At the indicated time points aliquots of the samples were removed and the protein fraction precipitated with 9 vol. of ice-cold ethanol at −20°C. The samples were then centrifuged at 16000 g for 5 min at 4°C. The ethanol fraction was removed (and analysed for lipid where appropriate), whereas the apolipoprotein pellet was dried under vacuum and re-suspended in 20 μl of PBS in preparation for HPLC analysis.

**HPLC analysis of lipids and proteins**

Eicosatetraenoic acid-derived L-OOHs (40 μl aliquots) were analysed using a 5 μm, 25 cm×0.46 cm, C18 reversed-phase column at a flow rate of 1 ml/min at 22°C. The mobile phase used to achieve separation of hydroxy and hydroperoxy derivatives of eicosatetraenoic acid was 0.1% acetic acid in H<sub>2</sub>O/acetoniitrile/THF [45:45:10, by vol.] with UV236 nm absorbance detection. Ethanol precipitated apoD (18 μl in PBS) was analysed by reversed-phase HPLC after the addition of 54 μl of 6 M GdnHCl. Analysis was achieved using a 5 μm, 25 cm×0.46 cm, C18 protein and peptide column (Vydac) and 35–55% acetonitrile gradient containing 0.1% tetrafluoroacetic acid, over 20 min, at 1 ml/min at 22°C with UV214 nm absorbance detection.

**Protein extraction from brain tissues**

Human brain tissue was obtained from the Sydney Brain Bank and the New South Wales Tissue Resource Centre with ethics approval from the University of New South Wales Human Research Ethics Committee. This research was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. Tissue samples were obtained from three normal (control) and three cases of AD brain clinically and pathologically defined using National Institute on Aging-Reagan criteria as described previously [16]. The demographic and basic clinical data for these samples is provided as supplementary data (Supplementary Table S1 at http://www.BiochemJ.org/bj/442/bj4420713add.htm). The age, PMIs (post-mortem intervals) and tissue pH were not significantly different between the groups: 80 ± 7 years compared with 83 ± 0.3 years; 15 ± 3 h compared with 14 ± 6 h; and pH 6.7 ± 0.2 compared with pH 6.3 ± 0.03, for control compared with AD samples respectively (all data means ± S.E.M.). Fractions of homogenized human hippocampus that were insoluble in TBS (Tris-buffered saline) and TBS containing 1% (v/v) Triton X-100 detergent were extracted from control (n = 3) and AD (n = 3) cases using GdnHCl as described previously [17]. GdnHCl was removed from tissue samples by ethanol precipitation as described previously [17]. Since the total protein levels in the GdnHCl fraction can vary when comparing control and AD samples, protein concentrations were determined by BCA (bicinchoninic acid) assay and equal amounts of protein were re-suspended in loading dye, separated on 8% PAGE gels and transferred on to 0.45 μm nitrocellulose membranes at 100 V for 30 min. The membranes were blocked in 5% non-fat dried milk in PBS for 2 h at 22°C then probed with an anti-apoD mouse monoclonal antibody (1:1000 dilution; Sapphire Biosciences) overnight at 4°C followed by incubation with horseradish peroxidase-conjugated rabbit anti-mouse (1:5000 dilution) polyclonal antibody for 1 h at 22°C. The membranes were washed and protein was detected using enhanced chemiluminescence and X-ray film. The membranes were stripped and re-probed for β-actin (1:2000 dilution; Sigma) to confirm equal protein loading.

**ApoD amino acid analysis using enzymatic hydrolysis**

Methionine and MetSO were determined using enzymatic hydrolysis to release the amino acids from the protein following quantification using a pre-column derivatization reversed-phase HPLC procedure. The amino acids were derivatized with AQC (6-aminoquinolinyl-N-hydroxysuccinimidyl carbamate) reagent [18] and analysed using an Acquity UPLC system (Waters Corporation) with a 2.1 mm×150 mm, 1.7 μm, reversed-phase C18 column (Acquity UPLC BEH130, Waters). The enzyme digestion was based on a procedure developed to analyse MetSO in milk proteins [19]. In brief, protein samples (20 μg) were dissolved in 50 μl of Hepes buffer (0.1 M, pH 7.5) and subjected to a single-pot three-enzyme digestion using pronase E (2.5 μl), leucine aminopeptidase M (1 μl) and prolidase (0.5 μl) for 20 h at 37°C. Each of the enzymes was prepared at 2 mg/ml and an enzyme blank was included. After the addition of an internal standard (norvaline), the digest mix was derivatized with AQC reagent using the AccQTag Ultra derivatization kit (Waters). MetSO and methionine sulfone standards were clearly separated using this method [RTs (retention times) of 5.55 min and 5.85 min respectively] and this was sufficient to exclude the possibility that the MetSO peak derived from the samples was contaminated with methionine sulfone.

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Liposome antioxidation

The liposome system consisted of 1 mg/ml total L-α PC (phosphatidylcholine) (95%) derived from soy (Avanti Polar Lipids). The phospholipid was prepared from a 10 mg/ml stock solution [in 2:1 (v/v) chloroform/methanol], and evaporated under nitrogen at 37 °C for 1 h until dry. The resulting film was hydrated in 140 mM NaCl and mixed by vortexing for 10 min at 37 °C. A Sanyo Soniprep 150 probe-type sonicator was then used (amplitude of 10 microns for three lots of 45 s on ice) to form small unilamellar vesicles. After liposome preparation, 10 μg/ml (final) of apoD (wild-type or all methionine-to-alanine mutants) was added to the liposome preparation. These additives were made up in PBS and diluted 1:10 into the liposome preparations. The control liposome conditions contained an equivalent amount (0.1 vol.) of PBS. The liposomal preparations were incubated at 37 °C for 24 h. Note that an exogenous radical initiator was not added in this assay to avoid direct inhibition/scavenging by the added proteins.

L-OOHs in the liposomes were measured by Fe2+ oxidation using the xylene orange assay in samples collected at 0 h and 24 h. A 50 μl aliquot of sample was taken from the liposomal preparation at each time point and 900 μl of xylene orange reagent (1 mM butylated hydroxytolulene, 0.25 mM ammonium ferrous sulfate, 0.25 mM sulfuric acid and 100 μM xylene orange) was added. The samples were incubated with the reagent for 50 min, and the absorbance was read at 560 nm. A standard curve was generated using t-BOOH (t-butyl hydroperoxide).

ApoD stable cell lines

CHO (Chinese-hamster ovary)-K1 cell lines expressing wild-type apoD or apoD<sub>M–A</sub> mutant were generated as described in Supplementary Online Data (at http://www.BiochemJ.org/bj/442/bj4420713add.htm). For continuous culture, CHO cells stably expressing wild-type or mutant apoD were cultured in CHO-K1 growth medium supplemented with 400 μg/ml G418. To determine if apoD could act as a cellular lipid antioxidant, non-transfected CHO cells and wild-type apoD and apoD<sub>M–A</sub> mutant cells were seeded in 96-well plates at a density of 1 × 10<sup>4</sup> cells/well. After 24 h the cells were incubated with vehicle (0.1% ethanol) or 50 μM t-BOOH for 24 h to induce cellular lipid peroxidation. After treatment, the culture medium was removed and cell monolayers were washed twice with PBS and assayed directly for L-OOH using the xylene orange PeroxiDetect<sup>TM</sup> kit (Sigma, catalogue number PD1) according to the manufacturer’s instructions.  

Statistics

Unless otherwise noted, experiments were routinely repeated at least twice in either duplicate or triplicate conditions. Statistical significance was assessed using the two-tailed Student’s t test for unpaired data with P < 0.05 considered significant. For multiple comparisons, differences between groups were analysed using a one-way ANOVA with post-hoc analysis using Tukey’s test.

RESULTS

Reversed-phase HPLC RT of recombinant apoA-I and apoD (but not methionine-to-alanine mutants) is reduced by exposure to H<sub>2</sub>O<sub>2</sub>

In order to assess the role that apoD methionine residues may play in L-OOH reduction, we utilized a human HEK-293 cell expression system to generate recombinant proteins. Owing to the introduction of an oxygen atom in the methionine side chain, formation of MetSO in native apolipoproteins can be measured by a reduction in reversed-phase HPLC RTs [15,20]. To confirm that conversion of methionine into MetSO in proteins purified using this method (based on nickel purification via a C-terminal histidine tag) exhibit changes in HPLC RT that are comparable with changes observed in native proteins, we first synthesized human wild-type apoA-I and a mutant form of the protein in which the three methionine residues (Met<sup>66</sup>, Met<sup>112</sup> and Met<sup>140</sup>) were replaced by alanine (apoA-I<sub>M–A</sub>). Both the wild-type and mutant forms of the protein were secreted and detected at the predicted molecular mass of ~29 kDa (Figure 1A). Treatment of apoA-I with either 1.0 M or 0.1 M H<sub>2</sub>O<sub>2</sub> for 1 h resulted in a dose-dependent reduction in RT (Figure 1B) that was in very close agreement to previous results obtained using human plasma-derived apoA-I [15,20]. In contrast, the RT for apoA-I<sub>M–A</sub> was not changed by H<sub>2</sub>O<sub>2</sub> treatment, thereby confirming the specificity of this change to oxidation of methionine residues (Figure 1B). In agreement with earlier studies using HDL-derived apoA-I [14], the metal chelator EDTA did not inhibit the shift in RT associated with recombinant apoA-I methionine oxidation (Supplementary Figure S1A).
Apod (but not an apoD all methionine-to-alanine mutant) catalyses the reduction of 5s-, 12s- and 15s-HpETEs to their respective HETEs

Products of lipoxygenase-mediated arachidonic acid oxidation (5s-, 12s- and 15s-HpETEs) were investigated as these L-OOHs are highly relevant to brain oxidative stress and inflammation [23]. Incubation of apoD with 15s-HpETE resulted in a decrease in apoD HPLC RT, whereas there was no impact on apoDM–A treated under identical conditions (Figure 2A). ApoD accelerated the conversion of 15s-HpETE into the corresponding L-OH (15s-HETE) as assessed by HPLC (Figure 2B), whereas there was very little conversion of 15s-HpETE into 15s-HETE in the presence of apoDM–A (Figure 2B). The rate of L-OOH reduction by apoD declined after the 1 h time point and this may be due to the consumption of methionine residues in the early phase of the incubation (Figure 2C).

To examine the specificity of apoD for HpETE reduction, a series of experiments were conducted using 15s-, 12s- and 5s-HpETE. ApoD efficiently converted all HpETEs into their corresponding HETEs (Figure 3). There were no significant differences in the extent of HpETE reduction observed when comparing the different substrates (although a trend for accelerated reduction of 5s-HpETE was noted). Incubation of 15s-HpETE with apoD did not result in a significant change in 15s-HETE concentration compared with the PBS 15s-HETE control incubation (Figure 3). These data indicate that apoD can reduce the major lipoxygenase-derived reactive L-OOHs to relatively inert L-OHs and that the reaction is dependent on one or more apoD methionine residues.

ApoD (but not an apoD all methionine-to-alanine mutant) inhibits liposome autoxidation and cell-associated lipid peroxidation induced by t-BOOH

To further investigate the potential antioxidant activity of apoD, we used a soy PC liposome autoxidation system. Autoxidation of soy PC liposomes is a reliable means for investigating antioxidant action [24,25]. Soy PC liposomes have a fatty acid content of approximately 15% C16:0 (palmitic), 4% C18:2 (stearic), 11% C18:1 (oleic), 63% C18:2 (linoleic), 6% C18:1 (linolenic) and 1% other fatty acids. The major target for autoxidation is therefore C18:2. In agreement with the data derived from the HpETE reduction experiments, apoD inhibited liposome lipid peroxidation more effectively than did apoDM–A (Figure 4A). We also assessed the capacity for apoD to act as a cellular lipid antioxidant. When CHO cells were treated with 50 μM t-BOOH for 24 h, total cellular L-OOH levels were increased by 17% (P < 0.05) compared with non-treated cells, whereas there were no significant changes in cellular L-OOH levels induced by t-BOOH in CHO-apoD (CHO cells that stably express apoD) compared with non-treated CHO-apoD cells (Figure 4B). This protective effect of apoD was not detected in CHO cells that stably expressed apoDM–A (CHO-apoDM–A) where t-BOOH treatment resulted in a 22% increase (P < 0.01) in cellular L-OOH compared with non-treated CHO-apoDM–A cells (Figure 4B). The basal levels of cellular L-OOH were not reduced in the CHO-apoD cells compared with...
apoDM–A). All mutant proteins were correctly synthesized and L-OOH-reducing activity (in comparison with both apoD and L-OOH reducing activity (similar to the apoD M–A) and its RT was not significant by incubation with 5s-HpETE to 5s-HETE and in both cases this was associated with a decrease in mutant apoD RT consistent with MetSO formation (Figure 5B). In stark contrast, apoD M93–A was deficient in L-OOH-reducing activity (similar to the apoD M49–A and apoD M157–A, mutants efficiently reduced 5s-HpETE to 5s-HETE and in both cases this was associated with the induction of MetSO (Figure 6). This further indicates that Met93 is the primary methionine residue involved in L-OOH reduction.

To confirm that the reduction of L-OOH by apoD was associated with methionine conversion into MetSO, we used a sensitive and specific amino acid analysis method that utilises a protease mixture for full protein hydrolysis. This approach overcomes problems associated with artefactual methionine oxidation that may occur as a result of gas-phase chemical protein hydrolysis and electrospray ionization MS methods [19,26]. The amino acid analysis indicated that after a 1 h incubation period, reduction of either 5s- or 15s-HpETE to their respective HETEs was associated with an increase in MetSO levels to account for approximately one-third (37%) of the total methionine (MetSO + Met) in wild-type apoD (Figure 6). When apoD M93–A and apoD M157–A were assessed under the same conditions, approximately half of the methionine was converted into MetSO (48% and 45% respectively; Figure 6). In contrast, the interaction of apoD M93–A with these HpETEs resulted in conversion of only 10% of methionine into MetSO (Figure 6). This further indicates that Met93 is the primary methionine residue involved in L-OOH reduction and that as a result of this reaction MetSO is generated. It is noteworthy that methionine sulfone (a further oxidation product of MetSO) was well separated from MetSO in our HPLC method and was not detected in any of the samples (results not shown). This indicates that a two-electron transfer is the most likely mechanism by which apoD reduces L-OOH to L-OH. Standard gas-phase HCl hydrolysis of the proteins indicated that the stable amino acids (i.e. excludes tryptophan, cysteine and methionine)

non-transfected CHO cells (Figure 4B). This may be due to the fact that the vast majority of apoD is secreted and thereby unable to protect against endogenous intracellular oxidative reactions.

**ApoD Met93 is required to reduce HpETEs to their respective HETEs via a processes that generates MetSO**

We next investigated which of the three apoD methionine residues may be involved in L-OOH reduction. To address this issue we generated recombinant proteins in which each of the three apoD methionine residues were individually replaced by alanine (apoD M49–A, apoD M93–A and apoD M157–A) and assessed these for L-OOH-reducing activity (in comparison with both apoD and apoD M–A). All mutant proteins were correctly synthesized and secreted at the predicted molecular mass (Figure 5A). Both the apoD M49–A and apoD M157–A, mutants efficiently reduced 5s-HpETE to 5s-HETE and in both cases this was associated with a decrease in mutant apoD RT consistent with MetSO formation (Figure 5B). In stark contrast, apoD M93–A was deficient in L-OOH-reducing activity (similar to the apoD M–A) and its RT was not significantly altered by incubation with 5s-HpETE (Figure 5B). These experiments indicate that apoD Met93 is the primary methionine residue involved in L-OOH reduction.

To confirm that the reduction of L-OOH by apoD was associated with methionine conversion into MetSO, we used a sensitive and specific amino acid analysis method that utilises a protease
and all yielded similar results. The experiments using apoD methionine-to-alanine single mutants were repeated four times and all yielded similar results. The x-axis labels are identical for all plots.

A recent study revealed that oxidation of apoA-I methionine to MetSO leads to aggregation of the protein into fibrils [28]. We also provided evidence that apoD-mediated reduction of L-OOH to L-OH is specific to methionine residues.

**ApoD MetSO generated as a consequence of HpETE reduction induces apoD dimerization and aggregation**

A recent study revealed that oxidation of apoA-I methionine to MetSO leads to aggregation of the protein into fibrils [28]. We also provided evidence that apoD-mediated reduction of L-OOH to L-OH is specific to methionine residues.

**ApoD dimers are present in the hippocampus of AD patients**

Previous studies of healthy control post-mortem brain samples (across a broad range of ages) as well as patients with AD and schizophrenia did not detect aggregated apoD [3–5]. These studies all used simple phosphate or Tris buffers during homogenization which may not be sufficient to extract less-soluble protein aggregates. We therefore used an established GdnHCl extraction method that extracts less-soluble proteins from aged brain tissue [17] to probe for the presence of apoD aggregates in human hippocampal samples taken from both control and AD cases.

This analysis indicated that apoD was predominantly detected as a dimerized aggregate in the GdnHCl-extracted fraction of the AD hippocampal samples taken from both control and AD cases. We therefore used an established GdnHCl extraction method that extracts less-soluble proteins from aged brain tissue [17] to probe for the presence of apoD aggregates in human hippocampal samples taken from both control and AD cases. This analysis indicated that apoD was predominantly detected as a dimerized aggregate in the GdnHCl-extracted fraction of the AD tissues (Figure 7C). On the basis of the known increase in brain lipid peroxidation that occurs in AD [30–33], and the results in the present paper, it is plausible that L-OOH interacts with apoD.

**Figure 5** ApoD MetSO is required for HpETE reduction

(A) Recombinant human single methionine-to-alanine apoD mutants were analysed by Western blotting. Samples were loaded at 1.0 μg and 0.5 μg per lane as indicated. Positions of molecular mass markers are indicated on the left-hand side. (B) Wild-type apoD and the indicated apoD mutants (0.5 mg/ml) were incubated with 15s-HpETE (0.05 mg/ml) for 4 h in PBS at 37 °C (grey line) and compared with incubation in PBS alone (black line) by HPLC. The pie chart inserts illustrate the proportion of 15s-HpETE (dark grey) and 15s-HETE (light grey) present at the 4 h time point. A PBS control that contained 15s-HpETE, but no apoD, is also included (PBS). HPLC data are from single samples representative of triplicates. 15s-HpETE and 15s-HETE data are mean values derived from triplicates. The S.E.M. for all HpETE and HETE samples was <1.0%. The experiments using apoD methionine-to-alanine single mutants were repeated four times and all yielded similar results. The experiments using apoD and apoDM-A were repeated six times and all yielded similar results. The x- and y-axis labels are identical for all plots.

**Figure 6** ApoD MetSO content assessed by enzymatic hydrolysis and HPLC

ApoD (0.5 mg/ml) was incubated at 37 °C for 4 h in PBS or either 15s- or 5s-HpETE (0.05 mg/ml) in PBS as indicated. Protein was isolated from lipid by ethanol precipitation and methionine and MetSO were determined using enzymatic hydrolysis and reversed-phase HPLC. The amount of MetSO in each sample is expressed as a percentage of total methionine (Met + MetSO). The number of samples analysed in each experiment (equal for both + HpETE) was wild-type (WT), n = 4, M49-A, n = 3; M93-A, n = 6; and M157-A, n = 3. Data are means with S.E.M. shown by the error bars. Statistical difference comparing each of the mutants to wild-type was determined by Student’s t test for the HpETE conditions only. **P < 0.01, n.d., not detected.

were recovered in the expected amounts with no changes induced by incubation with HpETE (Supplementary Table S2 at http://www.BiochemJ.org/bj/442/bj4420713add.htm). Other amino acid side chains (e.g. valine, tyrosine, threonine and phenylalanine) that may be oxidized by free radicals [27] were therefore not affected by HpETE treatment. This provides further evidence that apoD-mediated reduction of L-OOH to L-OH is specific to methionine residues.

**ApoD dimers are present in the hippocampus of AD patients**

Previous studies of healthy control post-mortem brain samples (across a broad range of ages) as well as patients with AD and schizophrenia did not detect aggregated apoD [3–5]. These studies all used simple phosphate or Tris buffers during homogenization which may not be sufficient to extract less-soluble protein aggregates. We therefore used an established GdnHCl extraction method that extracts less-soluble proteins from aged brain tissue [17] to probe for the presence of apoD aggregates in human hippocampal samples taken from both control and AD cases. This analysis indicated that apoD was predominantly detected as a dimerized aggregate in the GdnHCl-extracted fraction of the AD tissues (Figure 7C). On the basis of the known increase in brain lipid peroxidation that occurs in AD [30–33], and the results in the present paper, it is plausible that L-OOH interacts with apoD.

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in the AD brain and this results in apoD methionine oxidation that may contribute to the formation of apoD aggregates and their accumulation in amyloid plaques.

**ApoD dimers generated as a consequence of HpETE reduction are dissociated by 5 M urea**

The precise nature of the apoD aggregates we have detected is unknown, but the fact that apoD aggregates in AD brain were stable in 5 M GdnHCl could suggest covalent intermolecular cross-linking. In a final set of experiments, apoD dimers that were generated in vitro after 4 h incubation with 15s-HpETE were analysed by Western blotting both in the absence of strong denaturants and after treatment with either 5 M GdnHCl or 5 M urea. The apoD dimer was stable in 5 M GdnHCl, but almost completely dissociated by 5 M urea. This indicates that the apoD dimer generated in vitro is not due to covalent intermolecular cross-linking. We acknowledge the possibility that the formation of HMW oligomers resulting from 72 h incubation of apoD with HpETEs (Figure 7B) may result in additional types of intermolecular association that could include covalent cross-linking.

**DISCUSSION**

The present study demonstrates that apoD catalyses the reduction of HpETEs to their corresponding HETEs and that this reaction is dependent on a single methionine residue at position 93. We suggest that this L-OOH-reducing activity may contribute to the lipid antioxidant function of apoD in the brain and, as is the case with other antioxidant proteins [3,34], this would help to explain why apoD expression is up-regulated under conditions associated with increased cerebral lipid peroxidation such as aging and AD [3,5,6]. Although the precise mechanism involved in the reduction of HpETEs to HETEs by apoD Met93 is not known, we suggest that it may involve a direct two-electron reduction of L-OOH to L-OH; as has been reported previously for the interaction of specific methionine residues of plasma apoA-I and apoA-II with HDL-associated L-OOH [15]. One caveat in this hypothesis is that the amount of L-OOH converted into L-OH typically exceeds the protein methionine concentration and

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Figure 7  Formation of apoD aggregates in vitro and their identification in AD hippocampus

(A) Wild-type (WT) and mutant (M-A) apoD (0.5 mg/ml) was incubated with 5s-HpETE (0.05 mg/ml) in PBS at 37°C for up to 8 h. At the indicated times the samples were assessed by Western blotting. (B) Using the same initial conditions described in (A), lipid fractions were removed from apoD/ apoD mutants (or mock-extracted where no 5s-HpETE was added) after 1 h incubation. The proteins were then redissolved in PBS and incubated for a further 72 h at 37°C. After 72 h the samples were assessed by Western blotting. (C) GdnHCl-extracted proteins from control (Con) and AD hippocampal homogenates were assessed for apoD by Western blotting after controlling for equal total protein loading. Similar levels of β-actin were detected across all fractions after stripping and reprobing the blots. Positions of molecular mass markers are indicated on the left-hand side. Positions of apparent monomer, dimer, trimer and HMW oligomer (HMW Olig.) forms of apoD are indicated on the right-hand side.

Figure 8  ApoD dimerization can be reversed by treatment with 5 M urea, but not 5 M GdnHCl

ApoD (0.5 mg/ml) was incubated with PBS or 15s-HpETE (0.05 mg/ml) in PBS at 37°C for 4 h. The samples were collected and the protein separated from the lipid fractions and incubated with either 5 M GdnHCl (A) or 5 M urea (B) for 1 h at 37°C, as indicated, before Western blot analysis. Positions of molecular mass markers are indicated on the left-hand side.

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it is thus possible that additional mechanisms may play a role. Regardless of this issue, our data clearly indicate that apoD Met93 selectively catalyses the reduction of HpETEs to their HETE derivatives. The interaction of HpETEs with apoD Met93 may be particularly relevant in the AD brain where the activity of the group IVA PLA2 (phospholipase A2) that cleaves arachidonic acid from the sn-2 position of membrane phospholipids is increased along with up-regulation of 5-, 12- and 15-lipoxygenase activity [5,6,31,35,36].

Non-enzymatic free radical-mediated lipid peroxidation may also play a role in AD and there is evidence that the amyloid-β peptide itself promotes neuronal membrane lipid peroxidation [32,37]. Even though such oxidized phospholipid fatty acids are preferred substrates for cleavage by PLA2 [38] (and would therefore be likely to be released from cellular membranes as free fatty acid hydroperoxides relatively soon after they are generated), our data indicating that apoD also inhibits liposome phospholipid peroxidation suggests that docking of the released L-OOH within the binding pocket is not strictly required for apoD antioxidant activity. It is possible that the hydrophobic surface that contains apoD Met93 [39] may interact with cellular lipid membrane surfaces (from which L-OOH may protrude [40]) in order to convert potential radical-generating L-OOH into relatively inert L-OH and thereby inhibit membrane lipid peroxidation chain reactions.

In addition to the role that apoD may play as an antioxidant in the brain, the apoD-mediated modulation of eicosanoid metabolism through conversion of HpETEs into HETEs may also influence inflammatory pathways. For example, the modulation of the 5-HpETE to 5-HETE ratio regulates the synthesis of downstream inflammatory leukotrienes LTC4 and LTE4, [23,41]. It is also clear that many HpETEs and HETEs exhibit distinct biological activities in the brain, including regulation of synaptic function and cerebrovascular permeability [23]. For example, 5s-HpETE potently inhibits (IC50 = 10-8 M) neuronal Na+/K+-ATPase activity (important for the maintenance of neuronal excitability and synaptic transmission), whereas the reduced product, 5s-HETE, has no impact [42].

Similar to solvent exposed methionine residues in other proteins, apoD Met93SO is probably rapidly reduced to methionine by MSR expressed in the brain. This would be predicted to maintain the antioxidant function of apoD [12,13]. Under conditions associated with depletion of MSR activity or high levels of lipid peroxidation, the failure of apoD Met93SO to be reduced back to methionine may lead to apoD aggregation. In AD, enzymatic (lipoxygenase) and radical-mediated lipid peroxidation is increased and this is reported to be associated with decreased MSR activity [43]. This may explain why SDS-stable apoD aggregates were detected in the GdnHCl-soluble fractions of the hippocampus of AD patients in the present study. Alternatively, the fact that amyloid plaques contain both oxidized lipids [44] and apoD [45] might indicate that the aggregates are formed within the plaques in a region that is devoid of active MSR.

The mechanism underlying the increase in apoD aggregation that is associated with MetSO formation is not entirely clear. Previous research indicates that even though the introduction of an oxygen atom in the methionine side chain would be expected to decrease the hydrophobicity of the protein (consistent with the decrease in reversed-phase HPLC RT we have reported in the present paper), this modification can induce structural changes in the protein that increase the exposure of hydrophobic residues [46,47]. On the basis of a previous research [29], this would be predicted to induce local unfolding of the protein structure and increase the propensity for apoD to self-associate.

The fact that apoD dimers that were formed concomitantly with Met93 oxidation could be dissociated with 5 M urea indicates that the aggregates are non-covalent. Although GdnHCl is often considered to be a stronger denaturant than urea [48–51], it is also known that the modes of denaturation action for GdnHCl and urea are quite distinct. For example, GdnHCl (a salt) and urea (uncharged) may be used to selectively assess hydrophobic compared with electrostatic interactions. This difference has been used previously to assess hydrophobic and electrostatic interactions in coiled-coil analogues where, depending on the relative number and type of electrostatic interactions present, either compound can be observed to exhibit stronger denaturation properties [52]. The fact that apoD dimerization is reversed by 5 M urea raises the possibility that apoD aggregation resulting from MetSO formation is at least partly dependent on changes in inter- or intra-molecular electrostatic interactions. The latter interactions could lead to protein destabilization, whereas the former could directly contribute to protein–protein interactions.

In summary, our present studies reveal that apoD Met93 selectively reduces HpETEs to their corresponding HETEs and that this is associated with conversion of methionine into MetSO. This activity represents a previously unknown function of apoD that may contribute to its neuroprotective and lipid antioxidant functions in the brain. In age-related pathological settings such as AD, high levels of lipid peroxidation and/or decreased capacity for MSR-mediated apoD MetSO reduction back to methionine may contribute to the formation of apoD aggregates and their deposition in amyloid plaques.

**AUTHOR CONTRIBUTION**

Surabhi Bhatia, Bianca Knoch, Jenny Wong and Woonjin Kim conducted the experimental work; Surabhi Bhatia, Paul Else and Brett Garner developed the methods; Surabhi Bhatia, Bianca Knoch, Jenny Wong, Woonjin Kim, Paul Else, Aaron Oakley and Brett Garner contributed to experimental design, and data analysis and interpretation. Surabhi Bhatia, Aaron Oakley and Brett Garner wrote the paper with contributions from Bianca Knoch, Jenny Wong, Woonjin Kim and Paul Else.

**ACKNOWLEDGEMENTS**

We thank Dr Marijka Batterham (Director, Statistical Consulting Service, University of Wollongong, Australia) for statistical advice. Tissues were received from the New South Wales Tissue Resource Centre at the University of Sydney and the Sydney Brain Bank which is supported by the National Health and Medical Research Council of Australia, The University of New South Wales, Neuroscience Research Australia, Schizophrenia Research Institute and the National Institute of Alcohol Abuse and Alcoholism. Professor Glenda Halliday organized the human tissue and its sampling (tissue collection and diagnosis, ethics and tissue request).

**FUNDING**

This research was supported by the Australian National Health and Medical Research Council [grant number APP1003886] and the Australian Research Council [grant numbers FT0991986 and FT0996267].
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38015–164

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SUPPLEMENTARY ONLINE DATA

Selective reduction of hydroperoxyeicosatetraenoic acids to their hydroxy derivatives by apolipoprotein D: implications for lipid antioxidant activity and Alzheimer’s disease

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MATERIALS AND METHODS

ApoD amino acid analysis using gas-phase HCl hydrolysis

ApoD amino acids that are stable under acid hydrolysis (excludes cysteine, tryptophan and methionine) were analysed after 24 h gas-phase hydrolysis in 6 M HCl at 110°C, AccQTag labelling and subsequent separation using an Acquity UPLC system (Waters) with a 2.1 mm × 150 mm, 1.7 μm, reversed-phase C18 column (Acquity UPLC BEH130, Waters). As asparagine and glutamine are converted into aspartic acid and glutamic acid respectively, during acid hydrolysis the sum of these respective amino acids is presented. All samples were analysed in duplicate and results expressed as means. The coefficient of variation (CV) for this amino acid analysis method was <2.0%.

Deglycosylation of apoD

ApoD N-glycans were removed using the Flavobacterium meningosepticum PNGase F (peptide N-glycosidase F) provided in a deglycosylation kit (New England BioLabs, catalogue number P0705L) and according to the manufacturer’s instructions.

Generation of apoD and apoDm-A mutant stable CHO cell lines

CHO-K1 cells were obtained from the A.T.C.C. (Manassas, VA, U.S.A.), cultured at 37°C in a 5% CO2 atmosphere in Dulbecco’s modified Eagle’s medium/Ham’s F12 medium (1:1 mixture) containing 10% (v/v) fetal bovine serum-supplemented glutamax (2 mM). For the generation of apoD and apoDm-A mutant over-expressing stable cell lines, CHO-K1 cells were transfected with pcDNA3.1 wild-type apoD or apoDm-A mutant (1 μg) for 24 h using LipofectamineTM 2000 transfection reagent (4 μl/well in a six-well plate), and stable transfectants were selected for G418 (800 μg/ml) resistance by limiting dilution and screened for apoD protein expression by Western blotting.

Figure S1 Characterization of recombinant apoA-I and apoD

(A) Recombinant human wild-type apoA-I was incubated in PBS (black line) or with 0.1 M H2O2 (grey line) or 1.0 M H2O2 (broken line) in PBS for 1 h at 22°C in the presence of 10 mM EDTA. ApoA-I was then assessed by HPLC. (B) N-linked oligosaccharides were removed from recombinant apoD using PNGase F with 1 h incubation at 37°C (unless stated otherwise). Con, control condition contains reaction buffer without incubation; HI PNG, heat-inactivated (5 min at 95°C) PNGase F; PNG, active PNGase F; 37°C Con, reaction buffer only. Molecular mass is given in kDa on the left-hand side.

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**Figure S2**  Generation of apoD CHO-K1 cell lines

CHO-K1 cells were transfected with pcDNA3.1 wild-type apoD or apoD_M–A mutant (1 μg) and stable transfectants were selected using G418 resistance. (A) Phase-contrast microscopy of CHO-K1 (Con), wild-type apoD (apoD WT) and mutant apoD_M–A (apoD_M–A) cell lines. Scale bar = 20 μm. (B) Western blot analysis of cellular and secreted apoD. Molecular mass is given in kDa on the left-hand side.

**Table S1**  Clinical details of control and AD hippocampal samples

Frozen post-mortem hippocampal tissues were received from three control and three AD cases that were clinically and pathologically defined using National Institute on Aging-Reagan criteria. The demographic and basic clinical data for these samples is provided. Con, control; PMI, post-mortem interval.

<table>
<thead>
<tr>
<th>Case ID</th>
<th>Gender</th>
<th>Age (years)</th>
<th>PMI (h)</th>
<th>Brain pH</th>
<th>Clinical cause of death</th>
<th>AD duration (years)</th>
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<td>11</td>
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<td>Respiratory failure</td>
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<td>-</td>
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**Table S2**  ApoD amino acid composition using gas-phase HCl hydrolysis

ApoD (0.5 mg/ml in PBS) was kept at 4 °C for 1 h (Con), or incubated at 37 °C for 1 h in the absence (PBS) or presence (L-OOH) of 15s-HpETE (0.05 mg/ml). Protein was isolated from lipid by ethanol precipitation and amino acids (excluding cysteine, tryptophan and methionine) were determined using gas-phase acid hydrolysis and reversed-phase HPLC. Amino acid values are provided as percentages.

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<th>Observed PBS</th>
<th>Observed L-OOH</th>
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Received 30 June 2011/12 December 2011; accepted 12 December 2011
Published as BJ Immediate Publication 12 December 2011, doi:10.1042/BJ20111166