Native and oxidized low-density lipoproteins modulate the vasoactive actions of soluble β-amyloid peptides in rat aorta

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ABSTRACT
Cerebrovascular accumulation of Aβ (β-amyloid) occurs in aging and AD (Alzheimer’s disease). Hypercholesterolaemia, which is associated with raised plasma LDL (low-density lipoprotein), may predispose to AD. Soluble Aβ is found in the circulation and enhances vasoconstriction. Under conditions that may favour the formation of short Aβ oligomers, as opposed to more severe polymerization leading to Aβ fibrillogenesis, we investigated the influence of LDLS on the vasoactive actions of soluble Aβ. Thus the actions of Aβ40 and Aβ42 in combination with native or oxidized LDL on vasoconstriction to NA (noradrenaline) and vasodilatation to ACh (acetylcholine) were examined in rat aortic rings. LDL, particularly when oxidized, potentiated NA-induced constriction when combined with soluble Aβ40 and, especially, Aβ42. Soluble Aβ40 reduced relaxation induced by ACh, but Aβ42 was ineffective. Native and oxidized LDL also attenuated relaxation. Synergism occurred between oxidized LDL and Aβ with respect to ACh-induced relaxation, but not between native LDL and Aβ. We have shown for the first time that, under conditions that may result in Aβ oligomer formation, LDL, particularly when oxidized, modulates the vascular actions of soluble Aβ to extents greater than those reported previously for fibrillar Aβ preparations. Mechanisms whereby a treatable condition, namely hypercholesterolaemia, might contribute to the development of the cerebrovascular component of AD are indicated.

INTRODUCTION
Aβ (β-amyloid) is a 39–43-amino-acid cytotoxic peptide that is derived from a 695–770-amino-acid transmembrane amyloid precursor protein [1]. It is deposited as insoluble fibrils in both the brain parenchyma and in the walls of cerebral blood vessels in normal aging and, to an exaggerated extent, in AD (Alzheimer’s disease). Aβ occurs in a soluble form throughout the body, including in the circulation, where it has been detected in plasma [2,3] and platelets [4], primarily as the Aβ40 and Aβ42 isoforms [3]. Platelets have been shown to release significant quantities of Aβ on aggregation, prompting the suggestion that these particles may represent a source of the Aβ deposited in the tissues, including the cerebrovasculature [4–7].

It has been suggested that risk factors for coronary heart disease may also constitute risk factors for AD [8]. These factors include, hypercholesterolaemia, a common condition associated with elevated plasma levels of the atherogenic species LDL (low-density lipoprotein) and, significantly, of oxidized LDL [9]. We have reported

Key words: Alzheimer’s disease, aortic ring, β-amyloid, hypercholesterolaemia, low-density lipoprotein (LDL), vasoconstriction, vasorelaxation.

Abbreviations: Aβ, β-amyloid; ACh, acetylcholine; AD, Alzheimer’s disease; BBB, blood–brain barrier; LDL, low-density lipoprotein; NA, noradrenaline; TBARS, thiobarbituric acid-reactive substances; Th-T, thioflavin T.

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previously [7] that platelet Aβ efflux is increased in hypercholesterolaemia. Subsequently, we demonstrated [10,11] that native and, particularly, oxidized LDL promoted Aβ polymerization in a process involving β-sheet formation. Mullan and co-workers [12–14] have shown that soluble Aβ peptides enhance constriction of rat aortic rings stimulated by phenylephrine and endothelin-1. We have obtained results indicating that fibrillar Aβ40 generated in the presence of native LDL and, to a greater extent, oxidized LDL enhance NA (noradrenaline)-induced contraction [11]. However, results have been presented indicating that Aβ oligomers, rather than large Aβ polymers (i.e. fibrils), may be particularly potent with respect to the toxicity of the Aβ peptide and its cellular responses [15]. The aim of the present study, therefore, was to establish whether Aβ peptides, under conditions that may exist in vivo and could favour Aβ oligomer formation, produce more marked effects on vessel tone than demonstrated previously for Aβ40 fibrils [11]. To this end, the influence of soluble Aβ40 and Aβ42 on contraction induced by NA and relaxation stimulated by ACh (acetylcholine) in the absence or presence of native and oxidized LDL was examined. For comparison, studies were also conducted with fibrillar (polymeric) Aβ preparations. Thus it was hoped that potential pathological biochemical mechanisms mediating the actions of Aβ might be identified.

MATERIALS AND METHODS

Animals
Aortae from adult male Sprague–Dawley rats (n = 72; 300–400 g; Charles River) were used. The animals were kept under conditions and the experiments were conducted in accordance with the Animals (Scientific Procedures) Act 1986 and the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

Chemicals
Synthetic Aβ40 and Aβ42 peptides were purchased from Biosource International and were stored at −85 °C. All other chemicals were supplied by Sigma.

Plasma lipoprotein isolation
LDL was isolated from normal human plasma by sequential density gradient ultracentrifugation [16]. EDTA (240 µmol/l) was included in all solutions used during the preparation of LDL as an antioxidant. After isolation, lipoproteins were dialysed extensively against saline [0.9 % (w/v) NaCl] at 4 °C.

Lipoprotein oxidation
Oxidation of LDL was carried out by dialysis against 1000 vol. of saline containing 1 µmol/l FeSO4 at 4 °C for 72 h with a change of buffer every 24 h. Oxidation was halted by dialysis against 1 litre of saline with 240 µmol/l EDTA [10,11]. Native (non-oxidized) lipoproteins were subjected to a similar dialysis procedure in which the FeSO4 was replaced by EDTA. Oxidized LDL exhibited enhanced migration on agarose electrophoresis (mobility relative to native LDL = 1.5), indicating that oxidative modification of both the lipid and apoB (apolipoprotein B) components of LDL had occurred.

Lipoprotein characterization
The protein content of lipoprotein fractions was determined as described by Wang and Smith [17]. Confirmation that successful oxidation of LDL had occurred was achieved by measuring lipoprotein peroxidation products. Total LDL peroxidation products were determined by measuring TBARS (thiobarbituric acid-reacting substances) [18], and conjugated dienes, a specific lipid peroxidation product, were measured by employing second-derivative UV spectroscopy [19]. Comparing native LDL and oxidized LDL, TBARS values were increased approx. 13-fold, whereas conjugated dienes were increased approx. 20-fold.

Aβ42 and Aβ40 fibril formation
Aβ42 fibrillogenesis was assessed by Th-T (thioflavin-T) fluorescence spectroscopy [10,11,20]. Briefly, Aβ42 peptide (final concentration, 1 mg/ml) dissolved in PBS was incubated at 37 °C for up to 1 h. At specific time intervals (0, 2.5, 5, 10, 15, 30, 45 and 60 min), aliquots (20 µl) were removed, combined with Th-T reagent [2 µmol/l Th-T in 5 mmol/l glycine (pH 8.75)] and the fluorescence measured (excitation and emission wavelengths, 425 and 480 nm respectively; band pass slits, 5 and 10 nm respectively). Aβ40 fibrils were prepared as described previously [10,11].

Native and oxidized LDL and Aβ42 and Aβ40 fibrillogenesis
The actions of native and oxidized LDL on Aβ42 polymerization were determined by co-incubating the lipoproteins (final concentration, 2 mg/ml) with soluble peptide (final concentration, 1 mg/ml). Samples were incubated at 37 °C for up to 1 h, and Th-T fluorescence was measured as outlined above.

Aβ40 fibrils generated in the presence of LDL were obtained as described previously, i.e. Aβ40 was incubated with native or oxidized LDL for 120 h at 37 °C [10,11].

Aβ peptides and contraction and relaxation of rat aortic rings
The influence of Aβ40 and Aβ42 peptides together with LDL on arterial contraction and relaxation was studied using the rat aortic ring preparation [11,21]. Ring segments 4 mm in length were prepared from aortae removed from 3-month-old Sprague–Dawley rats. The rings were mounted in 10 ml organ baths containing Krebs bicarbonate buffer maintained at 37 °C and gassed with 95% O2/5% CO2. Changes in ring tension, as
measured via a Grass FT03 C pressure transducer, were recorded using a MacLab system (AD Instruments). Aortic rings were equilibrated for 1 h at a resting tension of 1.5 g. The rings were then treated with either saline or Aβ peptides (final peptide concentration in the organ bath, routinely 1 µmol/l; i.e. 4.3 and 4.5 µg/ml Aβ40 and Aβ42 respectively) for 15 min before contraction with NA, at a range of concentrations between 1 × 10^{-9} and 1 × 10^{-6} mol/l, was assessed.

In experiments in which the effects of Aβ peptides on ACh-induced relaxation were examined, contraction to a full range of NA concentrations was first tested in order to establish the NA EC50 dose. Then, following extensive washing (three changes of buffer), aortic rings were challenged with the EC50 dose of NA followed by increasing concentrations of ACh (1 × 10^{-9}−1 × 10^{-5} mol/l). When the influence of native and oxidized LDL on the contractile and relaxant responses to Aβ peptides were tested, lipoproteins were added so as to yield a final concentration in the organ bath of 2 µg of protein/ml.

In the interests of clarity, it should be noted that in experiments in which the actions of soluble Aβ peptides together with LDL were examined, the components were added to the organ bath separately, whereas in experiments in which fibrillar Aβ preparations were investigated the components had been co-incubated (120 h) prior to their addition to the organ bath.

**Data analysis**

Values are means ± S.E.M., and were evaluated statistically by Student’s t test and/or ANOVA with Bonferroni post-hoc analysis, where appropriate.

**RESULTS**

**Aβ42 fibrillogenesis**

The incubation of solubilized Aβ42 at 37°C resulted in a time-dependent increase in Th-T fluorescence, which peaked at 15–30 min (Table 1). Maximal values for fluorescence obtained at 30 min of incubation exceeded basal values 3.7-fold (P < 0.001). Native and oxidized LDL did not influence the rates or extents of Aβ42 polymerization significantly as determined by statistical analysis, although an apparent trend towards an increase was seen with native LDL.

**Soluble Aβ40, LDL and NA-induced vasoconstriction**

Neither native nor oxidized LDL (final concentration in the organ bath, 2 µg/ml) altered NA-induced contraction of aortic rings significantly (Figure 1).

As reported previously [11], 1 µmol/l (4.3 µg/ml) soluble (and fibrillar) Aβ40 failed to influence vasoconstriction, although increasing the concentration to 5 µmol/l (21.6 µg/ml) enhanced constriction (P < 0.01 and P < 0.05 with 1 × 10^{-9} and 5 × 10^{-9} mol/l NA respectively; Figure 2, left-hand panel). In the present study, however, 1 µmol/l soluble Aβ40 when combined with native LDL and, particularly, oxidized LDL (2 µg/ml) enhanced NA-induced constriction over the entire agonist concentration range (Figure 3). The increases in contraction were particularly marked and statistically significant for the lower agonist concentrations. Thus with 1 × 10^{-9}, 5 × 10^{-9} and 1 × 10^{-8} mol/l NA, soluble Aβ40 + native LDL increased constriction 407% (P < 0.001), 104% (P < 0.001) and 51% (P < 0.05) respectively. Meanwhile, with soluble Aβ40 + oxidized LDL,
contraction was increased 886% ($P < 0.01$), 185% ($P < 0.001$) and 122% ($P < 0.01$) respectively, at these NA concentrations.

**Aβ42, LDL and vasoconstriction**

Soluble and fibrillar Aβ42 at a concentration of 1 μmol/l (4.5 μg/ml) failed to enhance contraction stimulated by NA (Figure 2, right-hand panel). However, when soluble Aβ42 concentrations were increased to 5 μmol/l (22.6 μg/ml), NA-induced responses were potentiated, although not to the extents observed for 5 μmol/l soluble Aβ40 (Figure 2). As seen with Aβ40, but to a more marked degree, when soluble Aβ42 was applied to aortic rings together with native LDL and, to a greater extent, oxidized LDL potentiation of the vasoconstrictive responses to NA occurred (Figure 4). Hence, with $1 \times 10^{-9}$, $5 \times 10^{-9}$, $1 \times 10^{-8}$, $5 \times 10^{-8}$, $1 \times 10^{-7}$, $5 \times 10^{-7}$ and $1 \times 10^{-6}$ mol/l NA, soluble Aβ42 + native LDL enhanced contraction 1583% ($P < 0.05$), 334% ($P < 0.05$), 198% ($P < 0.01$), 81% ($P < 0.01$), 88% ($P < 0.001$) and 83% ($P < 0.001$) respectively. With soluble Aβ42 + oxidized LDL, contraction was increased $2250\% \ (P < 0.001), 514\% \ (P < 0.001), 327\% \ (P < 0.01), 148\% \ (P < 0.01), 147\% \ (P < 0.01), 134\% \ (P < 0.01)$ and 139% ($P < 0.01$) respectively.

Aβ42 fibrils generated in the presence of oxidized LDL yielded responses (across the complete NA concentration range) comparable with those obtained with soluble Aβ42 + oxidized LDL ($P < 0.001$; Figure 5). With Aβ42
Figure 4 NA-induced constriction of aortic rings in the absence and presence of soluble Aβ42 with native or oxidized LDL

(◇) Control; (■) 1 μmol/l soluble Aβ42 + 2 μg/ml native LDL; (▲) 1 μmol/l soluble Aβ42 + 2 μg/ml oxidized LDL. Results are means ± S.E.M. (n = 4–5). Soluble Aβ42 + native or oxidized LDL significantly enhanced contraction induced by 1 × 10⁻⁹, 5 × 10⁻⁹, 1 × 10⁻⁸, 5 × 10⁻⁸, 1 × 10⁻⁷, 5 × 10⁻⁷ and 1 × 10⁻⁶ mol/l NA (∗P < 0.05, **P < 0.01 and †P < 0.001) compared with control.

Figure 5 NA-induced constriction of aortic rings in the absence and presence of fibrillar Aβ42 generated with native or oxidized LDL

(◇) Control; (■) 1 μmol/l fibrillar Aβ42 + 2 μg/ml native LDL; (▲) 1 μmol/l fibrillar Aβ42 + 2 μg/ml oxidized LDL. Results are means ± S.E.M. (n = 3–10). Contraction in the presence of Aβ42 + native LDL fibrils and Aβ42 + oxidized LDL fibrils was significantly increased with all NA concentrations (**P < 0.01 and †P < 0.001) compared with control.

Figure 6 ACh-induced relaxation of aortic rings in the absence and presence of Aβ40

(◇) Control; (■) 1 μmol/l Aβ40; (▲) 5 μmol/l Aβ40. Results are means ± S.E.M. (n = 4–7). Differences between control and Aβ40-treated aortic rings occurred with all ACh concentrations (∗P < 0.05, **P < 0.01 and †P < 0.001).

fibrils prepared together with native LDL, however, the effects produced were similar to those observed with oxidized LDL + Aβ42 fibrils (P values between < 0.01 and < 0.001), and greatly exceeded the responses seen with soluble Aβ42 + native LDL.

Aβ peptides, LDL and ACh-induced vasorelaxation

Soluble Aβ40 at 1 and 5 μmol/l inhibited relaxation induced by all ACh concentrations (P values ranged between < 0.05 and < 0.001) to similar extents, although at lower ACh concentrations 5 μmol/l Aβ40 was more potent (Figure 6). Fibrillar Aβ40 and fibrillar Aβ42 produced only marginal effects, whereas soluble Aβ42 was without effect. Native and oxidized LDL produced marked inhibition of relaxation: with ACh concentrations between 5 × 10⁻⁹ and 1 × 10⁻⁶ mol/l, native LDL reduced relaxation 33–46 % (P values ranged between < 0.01 and < 0.001) and oxidized LDL reduced relaxation 20–34 % (P values ranged between < 0.05 and < 0.01) (Figure 7A). Synergy between soluble Aβ40 and native LDL was not evident. With oxidized LDL + soluble Aβ40 or oxidized LDL + Aβ42, however, results were obtained indicative of synergistic interactions between the two factors. Thus, with oxidized LDL + soluble Aβ42 and ACh concentrations ranging between 1 × 10⁻⁹ and 1 × 10⁻⁶ mol/l, relaxation was reduced 43–80 %, whereas with oxidized LDL + soluble Aβ42 and 5 × 10⁻⁸ – 1 × 10⁻⁶ mol/l ACh relaxation was reduced 36–44 % (P values ranged between < 0.05 and < 0.01;
ACh-induced relaxation of aortic rings in the absence and presence of Aβ40 or Aβ42 in combination with native or oxidized LDL

(A) Control (LDL absent); (■) 2 μg/ml native LDL; (▲) 2 μg/ml oxidized LDL. (B) Control (Aβ40 and LDL absent); (■) 1 μmol/l Aβ40 + 2 μg/ml native LDL; (▲) 1 μmol/l Aβ40 + 2 μg/ml oxidized LDL. (C) Control (Aβ42 and LDL absent); (■) 1 μmol/l Aβ42 + 2 μg/ml native LDL; (▲) 1 μmol/l Aβ42 + 2 μg/ml oxidized LDL. Results are means ± S.E.M. (n = 3–8). Native and oxidized LDL with ACh concentrations between 5 × 10⁻⁹ and 1 × 10⁻⁵ mmol/l, oxidized LDL + Aβ40 with ACh concentrations between 1 × 10⁻⁹ and 1 × 10⁻⁵ mol/l, and oxidized LDL + soluble Aβ42 with ACh concentrations between 5 × 10⁻⁹ and 1 × 10⁻⁵ mol/l yielded values which differed from those obtained under control conditions (*P < 0.05, **P < 0.01 and †P < 0.001).

Figures 7B and 7C. A similar picture was revealed when the results from the experiments conducted with Aβ42 fibrils generated in the presence of oxidized LDL were examined (results not shown). On the other hand, Aβ40 fibrils generated in the presence of native or oxidized LDL only reduced relaxation to ACh marginally.

DISCUSSION

In the present study, we have obtained results indicating that native and oxidized LDL interact with soluble Aβ40 and Aβ42 to enhance NA-induced vasoconstriction and reduce ACh-induced vasorelaxation. We suggest that such a mechanism could represent a means whereby hypercholesterolaemia might contribute to the development of AD, given that hypercholesterolaemia has been suggested as a risk factor for AD [8].

The accumulation of Aβ in the walls of cerebral blood vessels associated with cerebrovascular degeneration is a recognized phenomenon in AD [1,22]. Animal studies have shown that the administration of Aβ leads to cerebrovascular breakdown and impairment of the BBB (blood–brain barrier), and Aβ traversing the BBB [23,24]. It has been suggested that the Aβ that accumulates in the cerebrovasculature in AD may originate in the circulation [4,6,7]. We are still, however, lacking important information concerning the mechanisms that mediate Aβ-induced vascular actions. Previously, it was proposed that the formation of Aβ polymers, in the form of fibrils, may be a key factor in enhancing the toxicity of the peptide and that other factors present in the blood may promote this process [25]. Indeed, we have reported that LDL enhances Aβ40 fibrillogenesis and that this leads to increased vascular constriction and toxicity [10,11]. The conformational state of Aβ has been identified as being a key element in influencing its vasoactivity, with peptide preparations containing moderate amounts of β-pleated sheet structure being the most effective [13]. More recently, it was proposed that Aβ oligomers may be particularly potent with respect to the biological actions of the peptide [15]. We have speculated that Aβ40 when incubated with LDL may yield peptide preparations enriched in the moderately β-pleated sheet conformation [11]. Results arising from the present study, particularly that relating to Aβ40, can be interpreted as being consistent with the formation of oligomers. We would also suggest that under the conditions employed Aβ oligomer formation may have been enhanced and that this may have been reflected by altered vascular responses. Thus soluble Aβ40 in combination with native or oxidized LDL produced vasoconstrictive effects that greatly exceeded those reported previously for Aβ40 fibrils generated in the presence of LDL and which will possess a greater degree of β-pleated sheet structure [11]. In vivo, Aβ40 is the form of the Aβ peptide that predominates in the circulation and is deposited primarily in the vasculature [4,5,26,27]. Given the relative slowness of Aβ40 polymerization compared with Aβ42 it can be inferred that oligomer formation in the early stages of the polymerization process would be favoured in preference to fibril formation, with important consequences for vascular pathology and function. It is possible that Aβ40 fibril formation proceeds via the generation of intermediate oligomers and that a ‘seeding
process’, as proposed for Aβ fibrillogenesis, may be involved in the formation of toxic forms of Aβ [13]. The Th-T assay routinely employed to assess Aβ polymer formation, however, lacks the acuity necessary to distinguish between different Aβ conformations and more sensitive analytical techniques will need to be utilized in order to establish unequivocally which forms of the peptide predominate under different experimental conditions, including those used in the present study.

The responses obtained with Aβ42 were not as straightforward as those seen with Aβ40. As reported for Aβ40, soluble and fibrillar Aβ42 when added to the organ bath, so as to achieve a final concentration of 1 µmol/l, failed to influence NA-induced aortic contraction [11,21]. Similar to soluble Aβ40 [11,21], raising the concentration of soluble Aβ42 to 5 µmol/l enhanced NA-stimulated constriction, albeit to a lesser extent than for Aβ40. We have proposed that, at higher Aβ concentrations and under the influence of the law of mass action, the conditions in situ (i.e. in the organ bath) may be conducive to oligomer formation [21]. Clear differences between Aβ42 and Aβ40 with respect to the vascular responses were, however, observed when the peptides were tested in combination with LDL. Thus, as with Aβ40, soluble Aβ42 in combination with native LDL and, to a greater extent, oxidized LDL significantly enhanced constriction. However, unlike Aβ40, Aβ42 fibrils prepared with native and oxidized LDL yielded responses that, in the case of the former, exceeded those seen with the soluble peptide + native LDL, and the latter were similar to those obtained with soluble peptide + oxidized LDL. The apparent discrepancy between the findings obtained with Aβ40 and Aβ42 may reflect differences with regard to the kinetics of the polymerization processes exhibited by the two peptides, the relative quantities of peptide oligomers generated or differences regarding the proportions of oligomers and polymers produced. Our results confirm that fibril formation in Aβ42 preparations occurs at a much greater rate than for Aβ40 [10,11], and it is possible that this may result in a greater yield of oligomers compared with Aβ40.

The concentrations of Aβ used in the present study were greater than those reported for plasma [2]; however, higher concentrations of Aβ peptide could occur locally, e.g. at the sites of platelet aggregation, and approach those that were employed. It cannot be ruled out that the results obtained with Aβ40 or Aβ42 in combination with LDL may reflect synergistic interactions between the two species with the activation of their relevant receptors. In the case of native LDL + Aβ, activation of LDL receptors coupled with the activation of, for example, RAGE (receptor for advanced glycation end-products) by Aβ [28], may have occurred, resulting in the threshold for activation of the individual receptors being exceeded and hence enhancement of NA-induced constriction. A similar mechanism may also have operated with oxidized LDL and Aβ, although, unlike native LDL, oxidized LDL is not a ligand for the LDL receptor, but instead is taken up by the scavenger receptor [29].

The results of experiments in which the actions of Aβ peptides in combination with LDL on ACh-induced vasorelaxation were assessed reflected, to some extent, those obtained for vasoconstriction. Thus, as seen with vasoconstriction, the effects on ACh-induced relaxation produced by Aβ40 or Aβ42 together with LDL (i.e. attenuated vasorelaxation) were particularly marked when LDL was in an oxidized form.

In conclusion, we have shown for the first time that soluble Aβ peptides when combined with LDL under conditions that may lead to the generation of Aβ oligomers produced effects on vessel tone that exceeded those reported previously by this laboratory for fibrillar Aβ preparations generated by incubating Aβ for extended periods in the presence of LDL [10]. Given studies that Aβ oligomers exhibit greater degrees of cytotoxicity than their polymeric counterparts [15], our findings are of particular relevance for the pathophysiological processes occurring in AD, especially in relation to the cerebrovasculature. Our results also add further weight to the theory that cardiovascular risk factors and, in the case of the present study, hypercholesterolaemia, which is prevalent in the general population, may be involved in the aetiology of AD.

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